VILNIAUS UNIVERSITETO

MEDICINOS FAKULTETO

DISERTACIJOS

2019

VILNIUS UNIVERSITY

FACULTY OF MEDICINE

DISSERTATIONS

VILNIAUS UNIVERSITETO MEDICINOS FAKULTETO DISERTACIJOS

2019

VILNIUS UNIVERSITY FACULTY OF MEDICINE DISSERTATIONS

VILNIAUS UNIVERSITETO LEIDYKLA

2020

Apsvarstė ir rekomendavo spausdinti
Vilniaus universiteto Medicinos fakulteto taryba

Leidinio iniciatorius
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Leidinio sudarytoja
Lina KOCIENĖ

ISSN 2538-8495 (spausdinta knyga)
ISSN 2538-8517 (skaitmeninis PDF)

Leidinio bibliografinė informacija pateikiama
Lietuvos nacionalinės Martyno Mažvydo bibliotekos
Nacionalinės bibliografijos duomenų banke (NBDB).

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Pratarmė

Kiekvieno mokslo raktas yra paprastas klaustukas, kirbantis mokslininko galvoje. Jau tuomet kai žmogus pradeda kelti klausimus ir ieškoti į juos atsakymų, juos pagrindžiančių tyrimų, duomenų – jis žengia svarų žingsnį mokslo link. Džiaugiamės kasmet sulaukdami didžiulio susidomėjimo doktorantūros studijomis ir didžiuojamės Vilniaus universiteto Medicinos fakulteto doktorantų pasiekimais!

Mokslas visada reikalauja kantrybės, kruopštumo, užsispyrimo ir nuoširdaus darbo – to tikimasi iš kiekvieno pasirenkančio doktorantūros studijas. Vienas žymiausių visų laikų mokslininkų Albertas Einšteinas yra pasakęs – „Diena be mokslo – yra tuščiai praleista diena, Tiek daug yra ko išmokti, o laiko turime tiek nedaug". Būtent tokį mąstymą puoselėjame ugdydami jaunuosius mokslininkus. Dažnu atveju doktorantūros studijos persipina tarpdisciplininiais ryšiais, reikalauja inovacijų, plataus akiračio ir didelės erudicijos. Kiekviena diena atneša kažką naujo ir netikėto.

Penktus metus iš eilės leidžiamas „Disertacijų santraukų rinkinys" jau tapo gražia ir prasminga tradicija. Šiame leidinyje rasite 2019 metais Medicinos fakultete apgintų disertacijų santraukas. Ši knyga skirta Medicinos fakultete vykdomų doktorantūros darbų ir tyrimų reprezentacijai. Leidiniu siekiama skatinti mokslo sklaidą, tarpdisciplininį ir tarptautinį bendradarbiavimą, raginti doktorantus rengti aukštos kokybės disertacijas, didinti jaunųjų mokslininkų motyvaciją ir populiarinti mokslą visuomenėje.

Medicinos fakulteto dekanas
prof. (HP) dr. Algirdas Utkus

Foreword

The key to any science is a simple question mark buzzing in the mind of a scientist. As soon as a person starts asking questions and looking for answers supported by research, he or she takes an important step towards science. We are happy to receive a great amount of interest in doctoral studies every year, and we are proud of the achievements of the doctoral students at the Faculty of Medicine of Vilnius University!

Science always requires patience, diligence, perseverance, and sincere work; this is expected of anyone who chooses doctoral studies. One of the most famous scientists of all time, Albert Einstein, said, "A day without science is a day spent in vain. There is so much to learn and so little time." This is the kind of thinking we nurture when educating young scientists. Often, doctoral studies intertwine with interdisciplinary connections and require innovation, a broad horizon, and extraordinary erudition. Every day brings something new and unexpected.

Publishing a collection of dissertation summaries each year has become a beautiful and meaningful tradition. In this publication, our fifth in a row, you will find summaries of dissertations defended at the Faculty of Medicine in 2019. This book represents doctoral theses and research carried out at the Faculty of Medicine. The aim of the publication is to promote the dissemination of science and interdisciplinary and international cooperation, to encourage doctoral students to prepare high-quality dissertations, to increase the motivation of young researchers, and to popularise science in society.

Dean of the Faculty of Medicine
Prof. Dr. Algirdas Utkus

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THE INFLUENCE OF *CYP2D6* AND *CYP2C19* GENE POLYMORPHISMS ON THE CLINICAL EFFICIENCY OF ANTIDEPRESSANTS IN TREATING MODERATE AND SEVERE DEPRESSION

CYP2D6 IR *CYP2C19* GENŲ POLIMORFIZMŲ ĮTAKA KLINIKINIAM ANTIDEPRESANTŲ EFEKTYVUMUI GYDANT VIDUTINIO SUNKUMO IR SUNKIĄ DEPRESIJĄ

Medicine and health Sciences
Medicinos ir sveikatos mokslai

1. Introduction

Research problem, work rele vance, and clinical value

In psychiatry, more than one hundred different medicines are used. Psychopharmacotherapy – treatment with medication – is an important and effective therapy method in treating many mental health disorders and symptoms. However, patient response to standard psychotropic medication doses is different and individual. Only 25% to 60% of cases of medical treatment are successful when using standard doses of medication [1]. This is a serious medical problem – an individual response to the treatment may occur in insufficient treatment efficiency, serious side-effects or unexpected interactions with other drugs [2]. It is believed that this may be due to various factors: age, sex, morbidity, a poor doctor-patient relationship, as well as due to medications interactions, nutrition and smoking habits. However, one of the essential reasons is the CYP450 enzyme system, responsible for the metabolism of many drugs and gene polymorphisms. It is found that the effect of medication occurs when a sufficient concentration in the body is achieved. The CYP450 enzyme activity determines the metabolism of drugs and is one of the most important determinants of the concentration of drug in the plasma. The activity of the enzymes depends on the genotype and its polymorphisms. Due to the differences in genetic variation of enzyme activity – drugs' plasma concentration may vary even up to 1000 times. According to the meta-analysis published in the United States, a frequency of severe side effects sometimes reaches 6.7% and is responsible for 100 000 deaths every year [3]. For the healthcare system, this is an extra cost of more than

100 billion dollars per year [4]. It is known that about 10–20% of the Europids' *CYP* superfamily genes are changed. We do not have data on the situation in Lithuania, so exact figures are unknown. Statistically, it should be about 300 000 people who would have a non-standard treatment response. For them, the usual drug doses would cause side effects or would not be effective.

Various properties of the cytochrome P450 enzymes are well investigated, and the influence of these enzymes on the metabolism of many drugs is also known. On the basis of this knowledge, it would be possible to understand and anticipate the individual treatment response of patients, which can be determined by drug-drug interaction and genetic variability. The most important enzymes in the psychiatric practice of treating depression are CYP2D6 and CYP2C19.

Pharmacogenetics is a rapidly developing branch of genetics; its results are directly applicable in everyday clinical practice. Pharmacogenetic tests may reduce the excess medical costs of adverse drug reactions and enhance the patient's treatment efficiency. In Lithuania, molecular genetic tests of drug metabolism are already being done, which allows for selecting the drug and it's initial dose more precisely.

The practical value of pharmacogenetic tests is increasing each year. Pharmacogenetic tests have been successfully adapted for the individualization of treatment in the areas of cardiology, endocrinology, and oncology [5, 6].

Recent studies [7, 8] show that the CYP450 enzyme genotype determination may provide significant information about psychotropic medication metabolism rates and could help to choose the medicines and their doses purposefully.

The goal of this thesis is to determine how *CYP450* gene (*CYP2D6* and *CYP2C19*) polymorphisms lead to the clinical effectiveness of antidepressant response in moderate and severe depression. One must bear in mind that *CYP* gene polymorphism frequencies in depressed people are significantly different from healthy individuals and other mentally ill patients [9].

Academia is becoming more and more focused on pharmacogenetic research and drug metabolism, but any detailed work linking genetics and clinical outcomes is not plentiful. At the present moment, pharmacogenetic studies are mostly used retrospectively – when attempting to explain the atypical (inefficient or toxic) response to treatment cases.

This study was conducted prospectively and, for the first time in Lithuanian history, linked genetics with clinical psychiatry.

Psychiatrists in clinical practice lack recommendations for drug dosages adjustments according to CYP450 pharmacogenetic testing. Currently, in clinical practice, the selection of psychotropic medication, including antidepressants, is performed empirically, based on clinical response or the lack thereof, and the presence of side effects. The trial and error method is applied due to the individual response when taking psychotropic drugs [10], which, until this day, affects not only the often occurring treatment mistakes but also the lack of a patient's confidence in the necessity of treatment.

It is also important that when prescribing antidepressants in clinical practice, the titration method from the lowest dose is used, and these medications begin to work only after 2 weeks. When changing the drug due to ineffective treatment, the doctors must wait again at least 2 weeks to be able to determine whether the drug has worked this time. Therefore, proper and effective treatment with antidepressant medication takes even longer than treating with other psychotropic drugs (e.g., benzodiazepines, antipsychotics). Due to the lack of rapid clinical symptom relief, the depressed person can have an increasing feeling of hopelessness. This not only gives additional suffering to the patient but also increases the suicide risk.

The treatment is effective when applying drugs in usual dosages only within a range of 30–60% of all cases. When selecting the treatment, the individual genetic and biochemical properties of the person are usually ignored, so patients may be falsely attributed to having treatment-resistant depression or be exposed to danger-

ous side effects of the prescribed drugs. With the emergence of new opportunities to analyze the human genome, as well as the introduction of new prescribing possibilities according to genetic selection criteria, there is a necessity for more specific *CYP* genotyping tests that could advantage and the clinical benefits of genotyping and phenotyping even before the prescribing of treatment. The aim of this study is to help to individualize treatment, objectify the selection of drugs and their doses, and seek that with the help of genetic tests, every patient could be able to receive individualized treatment.

2. The objective of the study

The objective of this study is to determine the influence of *CYP2D6* and *CYP2C19* gene polymorphisms on the clinical response to antidepressant drugs when treating moderate and severe depression.

3. The aims of the study

- To collect and analyze the demographic data of Lithuanian patients with moderate and severe depression.
- To determine the *CYP2D6* and *CYP2C19* genes polymorphisms in people with moderate and severe depression.
- To asses *CYP2D6* gene polymorphisms in healthy individuals and compare it with those who have depression.
- To assess the clinical symptoms of depression using validated depression questionnaires upon arrival and after treatment with antidepressants.
- To evaluate the influence that *CYP2D6* and *CYP2C19* gene polymorphisms have on the clinical efficacy of antidepressants.

4. Statements th at are being defended

1. The polymorphisms rates of *CYP2C19* and *CYP2D6* in the Lithuanian population do not differ from the European populations.
2. *CYP2C19* and *CYP2D6* gene polymorphism frequencies in patients and the control group are different.
3. *CYP2D6* and (or) *CYP2C19* polymorphisms can affect the clinical efficacy of antidepressants.

5. The scientific novelty of the study

Researchers have only recently started to discuss the relationship between genotype-induced phenotypes and drug concentrations in the plasma. Other authors are trying to find a correlation between drug concentration and clinical efficiency. The purpose of this study was to determine the *CYP2D6* and *CYP2C19* polymorphisms and the influence of these genomic factors on the clinical antidepressant efficacy. So far in scientific literature, there were no data about most frequent alleles of *CYP2D6* and *CYP2C19* and the influence of their polymorphisms when empirically administering the antidepressant drugs.

6. Practical value of the study

BENEFIT FOR THE PATIENTS: in this study, patients with moderate and severe depression had their *CYP2D6* and *CYP2C19* genomic polymorphisms, which could have influenced the pharmacokinetics of antidepressants, which are metabolized by these enzymes, determined. Additionally, regular objective evaluation of depression was performed using validated scales. Determined polymorphisms and the prognosed phenotypic expression, which may affect pharmacotherapy, were noted in the electronic health records of the patient. Participation in this study provided to the patients information about their genotype, which remains unchanged throughout a lifetime. Such knowledge will, in the future, help to adapt to faster-acting, personalized treatment with fewer side effects and will provide additional information about any potential drug-drug interaction.

SCIENTIFIC VALUE: after performing molecular genetic tests, gene polymorphism frequencies were determined in the investigated groups; the groups were then compared with each other, and the frequencies of gene polymorphisms were compared with the Europid population. The statistical data analysis determined the correlation between the polymorphism of *CYP* genes and the predicted phenotypic expression and treatment response in the investigational group treated with antidepressant medication. Also, the possibility of performing pharmacogenetic research in clinical practice was evaluated.

PRACTICAL BENEFITS: this study was prospective and, for the first time in Lithuanian history, linked genetics with clinical psychiatry. Scientific cooperation between psychiatrists and geneticists was initiated, new laboratory research methods were mastered. *CYP2D6* and *CYP2C19* gene polymorphism frequencies in the Lithuanian population were performed – so far this has not been done on such a

scale. After determining the predicted phenotype that could affect pharmaceutical effectiveness, the patients were recommended to change treatment. This elucidated the necessity of missing research – the determination of the drug concentration in the blood and improved psychiatrists' knowledge about pharmacogenetic application problems. The study showed the advantages and the clinical benefit of genotyping and phenotyping even before the administration of treatment.

7. Methodology

7.1. Course of the study

Schemes of the study flowchart are provided in Figures 1 and 2.

7.2. Variables evaluated

Demographic, epidemiological and clinical

- Gender.
- Age.
- Treatment – outpatient or inpatient.
- Hospital stay length in days (inpatients).
- Antidepressants and their dosage.
- Results of depression questionnaires (The Beck Depression Inventory – BDI; The Hamilton Depression Rating Scale – HAM-D and The Montgomery-Åsberg Depression Rating Scale – MADRS) on arrival, after 2 and 4 weeks.
- Molecular genetic testing for *CYP* genes polymorphisms. (Figure 1 and 2)

7.3. Statistical analysis

Data statistical analysis was performed using the open source program R, version 3.4.3.

When assessing quantitative data, the arithmetic mean was used. To compare means, criteria were calculated and evaluated.

When there were a small number of observations, or when at least one observation had less than five cases, an additional Fisher exact criterion was calculated.

To test the normality of variable distribution, a *Shapiro-Wilk* test was used, as well as a histogram and rectangular diagram.

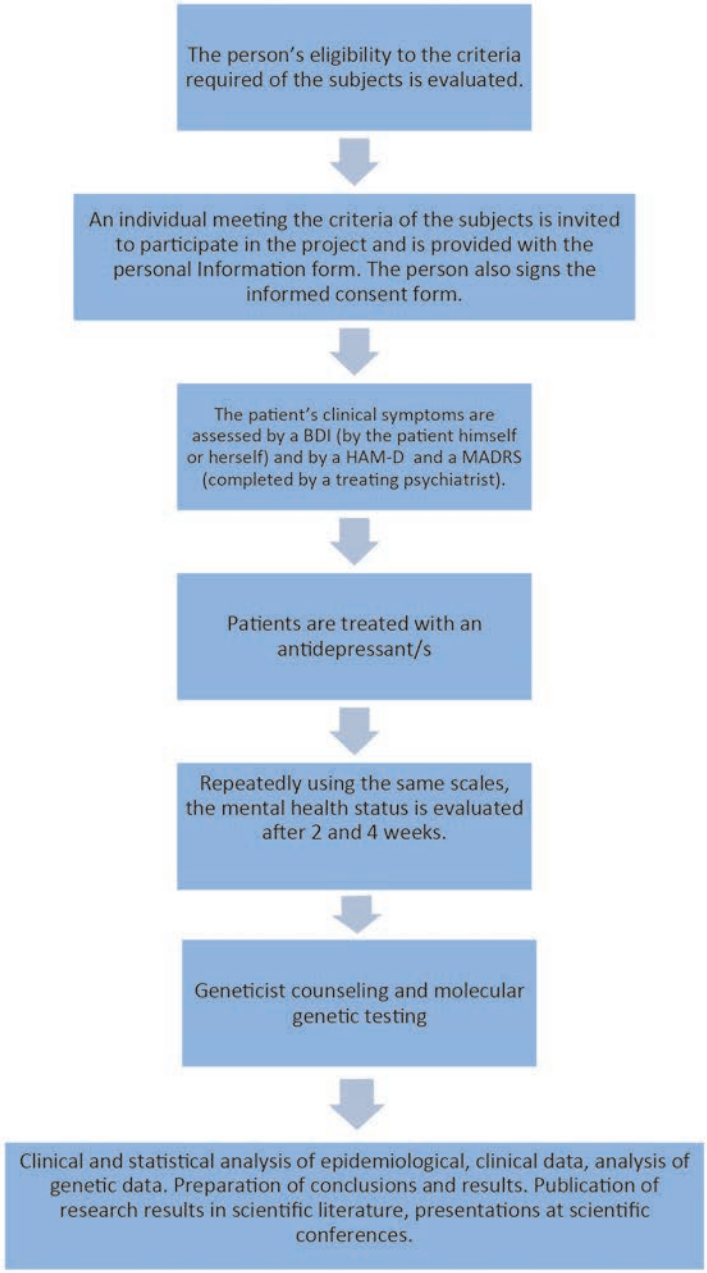

FIGURE 1. Recruitment flowchart of the patient group.

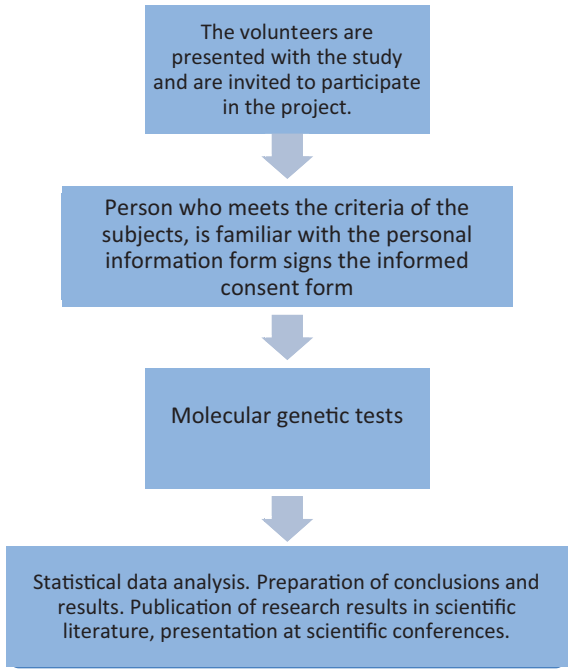

FIGURE 2. Recruitment flowchart of the control group.

Two independent sample tests were compared using *t*-test or *Mann-Whitney-Wilcoxon* sum of range criteria regarding the condition of data normality.

To evaluate linear dependence of two samples Pearson (r_p) and Spearman (r_p) correlation coefficients, taking into account the condition of data normality.

8. Results

The control group

Control group consisted of 108 people who never had depression. In the study, 104 people who were included were sequenced for their *CYP2D6* gene polymorphisms. No other data on the control group were collected; no other tests were performed.

The patient group

This group consisted of all people who were treated or consulted in the Department of Psychiatry at the Vilnius University Hospital Santaros Klinikos Neurology Center (VUH SC NCPD) from March

2016 to July 2018 and who were diagnosed by an experienced psychiatrist with at least 5 years of clinical experience with a depression episode (F32) or a recurrent depressive episode (F33) according to the International Statistical Classification of Diseases and Related Health Problems, Tenth Revision, Australian Modification (ICD-10AM). Of the 85 individuals who fulfilled the inclusion criteria, 78 people were included in the final analysis. Seven patients were excluded for the following reasons: three because of the inversion of affect that had manifested during the treatment of manic symptoms, and for whom the diagnoses were changed to bipolar affective disorder, the other three who did not show up for consultation of geneticists, and one patient refused to further participate in the study (Figure 3).

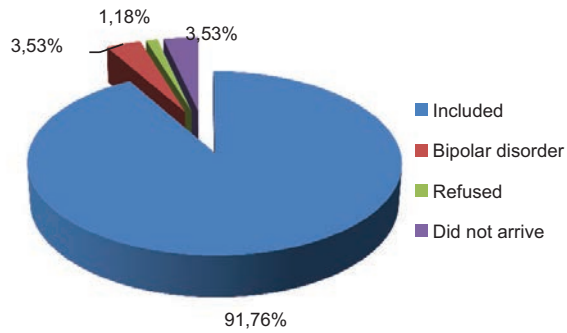

FIGURE 3. The distribution of included and not included patients in the study.

Variables evaluated in the patient group

The study consisted of 58 women and 17 men. The percentage distribution is presented in Figure 4.

Patient distribution according to age was even, large deviations were not observed. The average age between the genders did not differ. The average age of men was 48.94 ± 16.50 (median 47), women 51.09 ± 18.94 (median 57) years (Figure 5).

In the intervention group, 17 (relative frequency – 22.67%) patients were diagnosed with a moderate depressive episode unspecified as arising during the postnatal period (code F32.10 according to ICD-10-AM); 9 (12.00%) – with a severe depres-

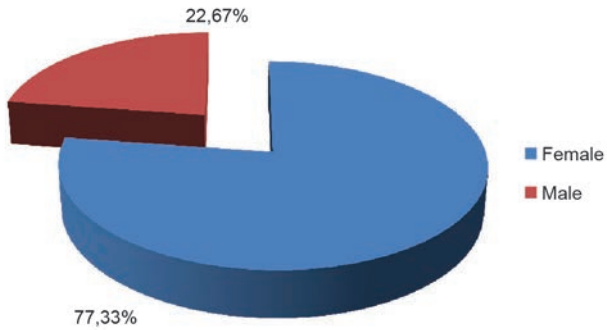

FIGURE 4. Patient distribution by gender.

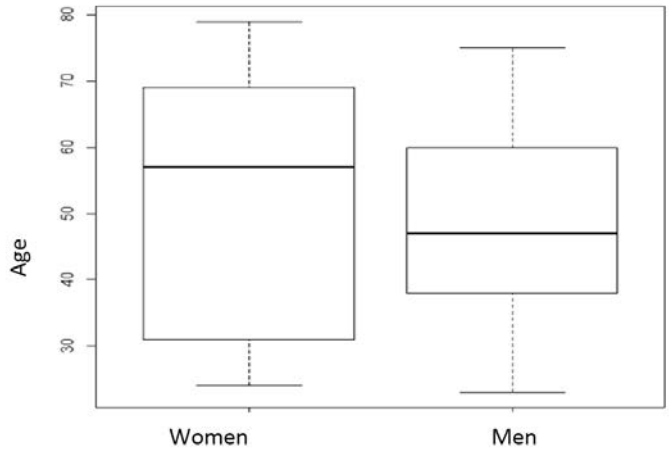

FIGURE 5. The distribution of men and women by age.

sive episode without psychotic symptoms, unspecified as arising during the postnatal period (F32.20); 1 (1.33%) – with a severe depressive episode without psychotic symptoms as arising during the postnatal period (F32.21); 25 (33.33%) – with a recurrent depressive disorder, a moderate depressive episode (F33.1); 23 (30.67%) – a recurrent depressive disorder, severe depressive episode without psychotic symptoms (F33.2). Most of the patients (n = 48; 64.00%,) consisted of individuals with a recurrent depressive disorder. Patient distribution is presented in Table 1.

In the intervention group, 62 (82.67%) patients were treated in inpatient service VUH SC NCPD. The average inpatient stay in this group were 24.56 days (Standard deviation ± 8.43) (median 24).

TABLE 1. Patients' distribution by gender and diagnosis.

Diagnosis Gender	F32.10	F32.20	F32.21	F33.1	F33.2	Total
Men	29.41%	33.33%	0.00%	24.00%	13.04%	22.67%
Women	70.59%	66.67%	100.00%	76.00%	86.96%	77.33%

Thirteen (17.33%) patients were treated on an outpatient basis and were consulted in the VUH SC NCPD. All the patients with the depressive symptoms were empirically treated with antidepressants and with other medications (antipsychotics, benzodiazepines). Thirty- nine (50.00%) patients were treated with one antidepressant, while the other 39 (50.00%) – with two antidepressants.

The most commonly prescribed drug was mirtazapine (n = 30; 25.64%). The second most frequent antidepressant was paroxetine (n = 19; 16.23%), followed by sertraline and venlafaxine (both n = 15; 12.82%). Fifty-eight patients (49.56%) were treated with the SSRI class of antidepressants (Table 2).

TABLE 2. The distribution of antidepressants according to frequency.

Class of antidepressants	Antidepressant	Frequency	Frequency, %
SSRI	Paroxetine	19	16.23
	Sertraline	15	12.82
	Escitalopram	11	9.40
	Citalopram	10	8.55
	Fluoxetine	3	2.56
NaSSA	Mirtazapine	30	25.64
SNRI	Venlafaxine	15	12.82
	Duloxetine	2	1.71
Melatoninergic receptor agonist and 5HT2C receptor antagonist	Agomelatine	7	5.98
SARI	Trazodone	4	3.42
Tricyclic antidepressants	Clomipramine	1	0.85

The most commonly prescribed combination of two antidepressants was mirtazapine with an SSRI. Eight patients (20.51%) were treated with a paroxetine and mirtazapine combination, 7 patients (17.95%) with a sertraline and mirtazapine combination, 6 patients (15.38%) with a citalopram and mirtazapine combination.

The assessment of depressive symptoms with scales

Depressive symptoms in the patients' group were evaluated with standardized measures – validated depression questionnaires in the Lithuanian language. When evaluating depressive symptoms with BDI, after two weeks of treatment, it was discovered that 25 (32.05%) patients had a positive response to the treatment. The threshold value of the treatment response was the diminishment of the initial score by ≥ 50%. After four weeks of treatment with antidepressants, positive responses were seen in 46 (58.97%) patients.

When using the questionnaires filled out by a psychiatrist, instead of self-reported questionnaires like the BDI, a positive response was seen in a higher number of patients. According to HAM-D, after 2 and 4 weeks of treatment, a positive response was seen in 32 (41.03%) and 68 (87.18%) patients respectively, and with MADRS – in 25 (32.05%) and 62 (79.49%). The dynamics of the treatment are provided in Table 3. After 4 weeks treatment, evaluations were performed and, according to both the psychiatrist and the patient, it was concluded that treatment was effective for most of the patients.

TABLE 3. Dynamics according to depression scales after 2 and 4 weeks.

Scales	According to BDI[1]	According to BDI[2]	According to HAM-D[1]	According to HAM-D[2]	According to MADRS[1]	According to MADRS[2]
Frequency	25	46	32	68	25	62
Frequency, %	32.05	58.97	41.03	87.18	32.05	79.49

[1] – number of patients who reached ≥ 50% improvement after 2 weeks from arrival,
[2] – number of patients who reached ≥ 50% improvement after 4 weeks from arrival.

TABLE 4. The score of the scales estimates and changes in the patient group.

Scales	Upon arrival	After 2 weeks	p-value	After 4 weeks	p-value
	$\bar{X} \pm S$	$\bar{X} \pm S$		$\bar{X} \pm S$	
BDI	27.64 ± 10.95	17.46 ± 9.74	**< 0.001***	12.38 ± 9.59	**< 0.001***
HAM-D	29.27 ± 7.05	15.32 ± 7.18	**< 0.001***	8.24 ± 5.32	**< 0.001***
MADRS	32.67 ± 6.72	18.13 ± 8.18	**< 0.001***	10.49 ± 7.01	**< 0.001***

* – statistically significant change; $\bar{X} \pm S$ – data are presented as arithmetic averages ± the average standard deviation of the sample

The statistical analysis of the BDI estimates found that according to the assessment upon arrival, after 2, and after 4 weeks, the patients' conditions had significantly ($p < 0.001$) improved. The assessment of the status by the HAM-D and

MADRS also found a statistically significant ($p < 0.001$) improvement in all cases. The comparison of scales results is presented in Table 4.

CYP2C19 genotype frequency

The frequency of *CYP2C19* polymorphisms in the patient group

After the analysis of 75 patients' *CYP2C19* gene, it was discovered that when assessing the overall allele frequency (sum of the first and second alleles), the most common alleles were **1, *2* and **17* alleles (respectively 64.00%, 11.33% and 23.33%). More information about the alleles' distribution is provided in Tables 5, 6 and 7.

TABLE 5. The frequency of the first *CYP2C19* allele.

Patient group	Fist allele			Total
	*1	*2	*17	
Frequency	66	6	3	75
Frequency, %	88.00	8.00	4.00	100

TABLE 6. The frequency of the second *CYP2C19* allele.

The patient group	Second allele					Total
	*1	*2	*4	*8	*17	
Frequency	30	11	1	1	32	75
Frequency, %	40.00	14.67	1.33	1.33	42.67	100

TABLE 7. The frequency and functional activity of most common *CYP2C19* alleles.

The patient group	Alleles					Total
	*1	*2	*4	*8	*17	
Frequency	96	17	1	1	35	150
Frequency, %	64.00	11.33	0.67	0.67	23.33	100
Functional activity	F	NF	NF	NF	IF	-

F – functioning allele; DF – decreased activity allele; NF –non-functioning allele;
IF – allele with increased functional activity

CYP2C19 gene polymorphism frequency comparison

With the help of the genetic database in which the frequency of *CYP2C19* alleles in the Europid population is provided (Myrand et al. 2008) [11], intervention

group alleles were compared with those of the Europids – North American and European inhabitants.

Statistically significant differences were not found. A comparison of the frequency of *CYP2C19* alleles in Lithuanian patients with those of the Europids is presented in Table 8.

TABLE 8. *CYP2C19* gene allele differences.

Alleles Groups	*1	*2	*4	*8	*17	Total
The patient group	96	17	1	1	35	150
Europids	189	39	1	0	57	286
p-value	0.199	0.495	1'	0.344	0.408	-

'– Fisher exact test

CYP2C19 phenotype frequency

In the intervention group, 40% of the people had the *CYP2C19*1/*1* gene; others had a gene responsible for a fast or slow metabolism phenotype.

With the help of a genetic database in which the frequency of the *CYP2C19* alleles in the Europid population is provided (Myrand et al. 2008) [11], intervention group alleles were compared with those of the Europids – North American and European inhabitants. Statistically significant differences were not found. Genotype frequencies and the predicted phenotype comparison are presented in Table 9.

TABLE 9. *CYP2C19* genotype and predicted phenotype frequency comparison.

CYP2C19 genotype	*1/*1	*1/*2	*1/*4	*1/*8	*1/*17	*2/*17	*17/*17	*2/*2	Total
The patient group	30	10	1	1	24	5	3	1	75
Europids	58	27	0	0	46	9	1	2	143
p-value	0.936	0.299	0.344[1]	0.344[1]	0.980	1[1]	0.119[1]	1[1]	-
Phenotype	NM	IM	IM	IM	FM	IM	UF	SM	-

NM – normal metabolizer; FM – fast metabolizer; UF – ultrafast metabolizer; IM – intermediate metabolizer; SM – slow metabolizer; [1] Fischer exact test.

CYP2D6 genotype frequency

The frequency of *CYP2D6* gene polymorphisms in the patient group

The distribution of 75 patients' *CYP2D6* gene's first and second allele is presented in Tables 10 and 11.

TABLE 10. A frequency of *CYP2D6* first allele.

The patient group	Fist allele						Total
	*1	*2	*3	*4	*5	*41	
Frequency	55	15	0	3	1	1	75
Frequency, %	73.33	20.00	0	4.00	1.33	1.33	100

TABLE 11. A frequency of the *CYP2D6* second allele.

The patient group	Second allele									Total
	*1	*2	*3	*4	*6	*9	*10	*41	*2x2	
Frequency	14	16	1	27	1	1	3	11	1	75
Frequency, %	18.67	21.33	1.33	36.00	1.33	1.33	4.00	14.67	1.33	100

After adding the first and the second alleles together, it was found that the most common alleles were **1, *2*, and**4*- respectively 46.00%, 20.67% and 20.00% (table 12).

TABLE 12. The *CYP2D6* allele frequencies.

The patient group	*1	*2	*3	*4	*5	*6	*9	*10	*41	*2x2	Total
Frequency	69	31	1	30	1	1	1	3	12	1	150
Frequency, %	46.00	20.67	0.67	20.00	0.67	0.67	0.67	2.00	8.00	0.67	100

The frequency of *CYP2D6* gene polymorphisms in the control group

The *CYP2D6* gene results of the control group (n = 104) are presented in Tables 13 and 14.

TABLE 13. The frequency of the *CYP2D6* first allele.

The control group	Fist allele				Total
	*1	*2	*3	*4	
Frequency	67	30	2	5	104
Frequency, %	64.42	28.85	1.92	4.81	100

TABLE 14. The frequency of the *CYP2D6* second allele.

Control group	Second allele						Total
	*1	*2	*4	*6	*10	*41	
Frequency	25	42	15	1	2	19	104
Frequency, %	24.04	40.38	14.42	0.96	1.92	18.27	100

After adding first and second alleles in the control group together, it was discovered that the most common alleles were ***1, *2, *4*** and*41* – respectively 44.23%, 34.62%, 9.62% and 9.13%. The allele frequencies are presented in Table 15.

TABLE 15. *CYP2D6* allele frequencies.

The control group	*1	*2	*3	*4	*6	*10	*41	Total
Frequency	92	72	2	20	1	2	19	208
Frequency, %	44.23	34.62	0.96	9.62	0.48	0.96	9.13	100

A comparison of the *CYP2D6* gene polymorphism frequency

After assessing the allele frequency in patients and the control group, it was discovered that the prevalence of alleles **2* and **4* were different. The patients' group

TABLE 16. Differences between the patient and control group of the *CYP2D6* allele frequencies.

Groups	Alleles									
	*1	*2	*3	*4	*5	*6	*10	*41	*2x2	Total
The patient group	69	31	1	30	1	1	3	12	1	150
The control group	92	72	2	20	0	1	2	19	0	208
p-value	0.740	**0.004**[1]	[1]2	**0.005**[1]	0.419[2]	[1]2	0.653[2]	0.706	0.419[2]	0.740

[1] Bonferroni correction, P < 0.0056; [2] Fisher's exact test

had a statistically significantly ($p < 0.004$) less active functional **2* allele and a statistically significantly ($p < 0.005$) more nonfunctional **4* allele. Other differences between the allele prevalence were not statistically significant (Table 16).

After combining both the control and patient group, the common frequency of the study participants' *CYP2D6* alleles was obtained (sample size n = 179). The results are presented in Table 17.

TABLE 17. *CYP2D6* allele frequency.

Study subjects	Alleles										
	*1	*2	*3	*4	*5	*6	*9	*10	*41	*2x2	Total
Frequency	161	103	3	50	1	2	1	5	31	1	358
Frequency, %	44.97	28.77	0.84	13.97	0.28	0.56	0.28	1.40	8.66	0.28	100

With the help of the genetic database in which the frequency of the *CYP2D6* alleles in the Europid population is provided according to the 2010 data of Manet et al. [12] (Table 18), the allele distribution between the Lithuanian inhabitants and the Europids – the North Americans and Europeans – was compared (n = 454).

TABLE 18. *CYP2D6* allele frequency in the Europid population.

Europid	Alleles										
	*1	*2	*3	*4	*5	*6	*7	*9	*10	*17	*34
Frequency	380	228	20	171	30	15	1	15	25	1	5
Frequency, %	41.90	25.10	2.20	18.80	3.30	1.70	0.10	1.70	2.80	0.10	0.50

The statistical analysis found that the prevalence of the **5* allele differed statistically significantly ($p <0.002$) between the data provided about the North American and European population and the Lithuanian subjects (patients and control groups). The other allele prevalence between populations did not differ significantly. The results are presented in Table 19.

TABLE 19. Differences of the *CYP2D6* allele.

Population	Alleles							
	*1	*2	*3	*4	*5	*6	*9	*10
Lithuanian	161	103	3	50	1	2	1	5
Europides	380	228	20	171	30	15	15	25
P value	0.312	0.181	0.101	0.040	**0.002**[2]	0.176 [1]	0.052[1]	0.152

[1] Fisher's exact test, [2] Bonferroni correction, $P < 0.00625$

CYP2D6-predicted phenotype frequency

After assessing the genotype-predicted phenotype, a common distribution of alleles was determined according to the activity score. The results are presented in Table 20.

TABLE 20. *CYP2D6* allele distribution according to phenotype activity scores.

Characteristics	Alleles									
	*1	*2	*3	*4	*5	*6	*9	*10	*41	*2x2
Frequency, n	161	103	3	50	1	2	1	5	26	1
Frequency, %	44.97	28.77	0.84	13.97	0.28	0.56	0.28	1.40	8.66	0.28
Activity score	1	1	0	0	0	0	0,5	0,5	0,5	> 2
Functioning	F	F	NF	NF	NF	NF	DF	DF	DF	IF

n – number; F – functioning allele; DF – decreased activity allele; NF – non- functioning allele; IF – allele with increased functional activity.

After combining all functional, decreased functionality and nonfunctional alleles and after comparing the patient group with the control group, it was discovered that nonfunctional alleles were found statistically significantly more often in the patient group: 33 vs. 23 ($p < 0.017$). Also, functional alleles were found statistically significantly less often in the patient than in the control group (101 *vs.* 164 $p < 0.017$). The results are presented in Table 21.

TABLE 21. The difference between the functional, of decreased functionality and nonfunctional alleles between the patients and the control group.

The Lithuanian population	Functional alleles (1, 2 and 2x2)	Nonfunctional alleles (3–8; 11–15; 19–21; 31; 38)	Reduced activity alleles (9; 10; 17; 29; 41)	Total
Patients	101	33	16	150
Control	164	23	21	208
P value	**0.014**[1]	**0.005**[1]	0.861	-

[1] Bonferroni correction, $P < 0.0167$

Out of 75 investigated patients, 5 had a genetic polymorphism that may have affected drug metabolism; 4 (5.33%) patients according to their genotype had a decline in prognostic score in the phenotypic value of the drug metabolism, and 1 (1.33%) patient had a score of ultrarapid metabolizer phenotype. Five people (4.81%) out of the 104 in the control group had a reduction in the score of the prognosed phenotype, 2 (1.92%) – a slow metabolizer phenotype predicted by their genotype.

The distribution of patient and control groups according to from the genotype predicted phenotype is presented in Table 22.

TABLE 22. The predicted phenotype according to the *CYP2D6* genotype.

Groups	n	Phenotype			
		SM %	UM %	IM %	NM%
The patient group	75	0 (0%)	1 (1.33%)	4 (5.33%)	70 (93.33%)
The control group	104	2 (1.92%)	0 (0%)	5 (4.81%)	97 (93.27%)
Total	179	2 (1.12%)	1 (0.56%)	8(4.47%)	167 (93.85%)

n – number of individuals. SM – slow metabolizer phenotype; UM – ultrarapid metabolizer phenotype; IM – intermediate metabolizer phenotype; NM – normal metabolizer phenotype

With the help of a genetic database in which the frequency of the *CYP2D6* alleles in the Europid population is provided (Myrand et al. 2008) [11], intervention group alleles were compared with those of the Europids – North American and European inhabitants. Results of the comparison are presented in Table 23. It does not include the genotypes, which the groups had only one case of.

TABLE 23. Comparison of the *CYP2D6* genotype.

CYP2D6 Genotypes	*1/*1	*1/*2	*1/*4	*1/*41	*1/*10	*2/*2	*2/*4	*2/*41	*4/*41
The patient group	14	14	19	3	3	2	8	3	3
Europids	27	39	16	0	1	11	17	0	0
p value	0.969	0.159	**0.006**	**0.039**	0.118	0.227	0.788	**0.039**	**0.039**
Activity score	2	2	1	1,5	1,5	2	1	1,5	0,5

Influence of from the *CYP2D6* genotype predicted phenotype on the clinical efficacy of antidepressants when treating empirically

based on from the *CYP2D6* genotype predicted phenotype, it is not possible to evaluate depression scales' scores, since the sample sizes of different groups are very unevenly distributed – 4, 70, 1.

Based on the activity scores of the enzyme predicted from the genotype, the patients were divided into two groups:

- Group I: phenotypically normal metabolizers, according to *CYP2D6* genotype having activity score of 1.5 and 2;
- Group II – consisting of slow metabolizer and ultrarapid metabolizer phenotype patients, having activity scores of 0.5; 1; > 2.

On the basis of this distribution, the first group consisted of 39 patients, the second – 36. These two groups were compared with each other (a normal metabolizer phenotype compared with an altered metabolizer phenotype).

After the statistical analysis of the BDI results, it was concluded that depression scores in the first group and in the second group were not different upon arrival, after 2 weeks and after 4 weeks. BDI means and standard deviations of both groups are presented in Table 24.

TABLE 24. BDI scores comparison between the investigational groups.

Scale	Group I (n = 39)	Group II (n = 36)	p-value
	$\bar{X} \pm S$	$\bar{X} \pm S$	
B I	28.18 ± 11.59	26.55 ± 10.1	0.520
B II	17.64 ± 10.46	16.61 ± 8.23	0.639
B III	12.36 ± 9.84	12.11 ± 9.44	0.912

B I – BDI score on arrival; B II – BDI score after 2 weeks; B III – BDI score after 4 weeks; $\bar{X} \pm S$ – data are presented as arithmetic averages ± average standard deviation of the sample size; n – the number of the subjects.

The two groups were compared with each other according to the HAM-D scores. Scores of both the first and second group diminished greatly after 2 and 4 weeks of treatment compared with the results upon arrival. However, in all cases, statistically significant differences between the groups were not found. The means and standard deviations of HAM – D of both groups are presented in Table 25.

TABLE 25. A comparison of HAM-D scores between the investigated groups.

Scale	Group I (n = 39)	Group II (n = 36)	p-value
	$\bar{X} \pm S$	$\bar{X} \pm S$	
H I	29.33 ± 6.99	29.47 ± 7.44	0.934
H II	15.44 ± 6.85	15.11 ± 7.67	0.847
H III	8.56 ± 5.92	7.75 ± 4.38	0.503

H I – score of HAM-D upon arrival; H II – score of HAM-D after 2 weeks; H III – score of HAM-D after 4 weeks; $\bar{X} \pm S$ – data are presented as arithmetic averages ± mean sample standard deviation; n – the numbers of participants.

After evaluating both groups scores of MADRS upon arrival, after 2 and 4 weeks, statistically significant differences were not found. MADRS arithmetical means and standard deviations of both groups are presented in Table 26.

TABLE 26. A comparison of MADRS scores between the investigated groups.

Scale	Group I (n = 39)	Group II (n = 36)	p-value
	$\bar{X} \pm S$	$\bar{X} \pm S$	
M I	32.28 ± 6.87	33.17 ± 6.69	0.575
M II	19.08 ± 8.02	16.94 ± 7.73	0.245
M III	11.25 ± 7.67	9.36 ± 5.69	0.231

M I – MADRS score upon arrival; M II – MADRS score after 2 weeks; M III – MADRS score after 4 weeks; $\bar{X} \pm S$ – data are presented as arithmetic averages ± average standard deviation of the sample size; n – the number of the subjects.

Influence of from the *CYP2C19* genotype predicted phenotype on the clinical efficacy of antidepressants when treating empirically

When evaluating the influence of the phenotype predicted from the *CYP2C19* genotype on the clinical effectiveness of drugs, all patients with normal *CYP2D6* predicted phenotype were divided into two groups according to *CYP2C19* polymorphisms:

- Group I – normal metabolizers according to the CYP2D6 genotype and normal metabolizers according to the CYP2C19 genotype (n = 28);
- Group II – normal metabolizers according to CYP2D6 and impaired metabolizers (slow and ultrarapid) metabolizers according to CYP2C19 (n = 40).

After the statistical analysis, it was discovered that the first group BDI score upon arrival was statistically significantly lower ($p < 0.043$) than that of the second group. This means that the score indicating the severity of depression was higher in the second group. After 2 weeks, the difference between groups remained statistically significant ($p < 0.030$), and again, scores were higher in the second group. After 4 weeks, the differences between the groups remained (Table 27). The median scores varied considerably: I gr. – 6 points and II gr. – 11 points, but the differences were not statistically significant.

TABLE 27. BDI scores' comparison between the investigated groups.

Scale	Group I (n = 28)	Group II (n = 40)	p-value
	$\bar{X} \pm S$	$\bar{X} \pm S$	
B I	24.57 ± 12.08	30.10 ± 9.92	**0.043***
B II	14.18 ± 7.87	19.15 ± 9.82	**0.030***
B III	9.82 ± 8.74	13.90 ± 10.04	0.087

B I – BDI score on arrival; B II – BDI score after 2 weeks; B III – BDI score after 4 weeks; $\bar{X} \pm S$ – data are presented as arithmetic averages ± average standard deviation of the sample, * – statistically significant differences; n – the number of the subjects.

The two groups were compared with each other according to the HAM-D scores. The second group score averages of the patients on arrival and after 2 weeks were statistically significantly higher ($p < 0.006$ and $p < 0.005$). Respectively, this group had stronger depressive symptoms than the first group (Table 28). When evaluating after the 4 weeks, the difference between groups remained; however, because of the small sample sizes in both groups, the difference was not statistically significant.

TABLE 28. Comparison of HAM-D scores between the investigated groups.

Scale	Group I (n = 28)	Group II (n = 40)	p-value
	$\bar{X} \pm S$	$\bar{X} \pm S$	
H I	26.68 ± 7.92	31.65 ± 6.37	**0.006***
H II	12.18 ± 8.05	17.18 ± 6.18	**0.005***
H III	6.71 ± 4.91	8.88 ± 5.14	0.087

H I – score of HAM-D upon arrival; H II – score of HAM-D after 2 weeks; H III – score of HAM-D after 4 weeks; $\bar{X} \pm S$ – data are presented as arithmetic averages ± mean sample standard deviation; * – statistically significant differences; n – the numbers of participants.

After the statistical analysis, it was discovered that the first group's MADRS score after 2 weeks was statistically significantly lower ($p < 0.005$) than that of the second group (Figure 14). After the evaluation of the score difference between groups after 4 weeks, differences were distinct. However, they were statistically insignificant (table 29).

TABLE 29. A comparison of MADRS scores between the investigated groups.

Scale	Group I (n = 28)	Group II (n = 40)	p-value
	$\bar{X} \pm S$	$\bar{X} \pm S$	
M I	31.07 ± 7.44	34.10 ± 6.37	0.077
M II	14.50 ± 7.96	20.08 ± 7.57	**0.005***
M III	8.61 ± 6.62	11.30 ± 6.94	0.114

M I – MADRS score upon arrival; M II – MADRS score after 2 weeks; M III – MADRS score after 4 weeks; $\bar{X} \pm S$ – ata are presented as arithmetic averages ± average standard deviation of the sample size; * – statistically significant differences; n – the number of the subjects.

The influence of the phenotype predicted from the *CYP2C19* and *CYP2D6* genotypes on the clinical efficacy of antidepressants when treating empirically

When assessing the influence of *CYP2D6* and *CYP2C19* genotype polymorphisms on the predicted phenotype and the clinical efficacy of drugs, all patients were divided into two groups according to the predicted phenotype:

- Group I – normal metabolizers according to *CYP2D6* and *CYP2C19* genotypes (n = 28);
- Group II – individuals with impaired metabolism (slow and ultrarapid) phenotypic variants according to *CYP2D6* and *CYP2C19* genotypes (n = 45).

BDI scores upon arrival, after 2 and after 4 weeks were higher in the second group, but statistically significant differences were not found. The questionnaire arithmetic means standard deviations and p values of both groups are presented in Table 30.

TABLE 30. BDI scores comparison between the investigated groups.

Scale	Group I (n = 28)	Group II (n = 45)	p-value
	Mean ± Sd	Mean ± Sd	
B I	24.57 ± 12.08	29.28 ± 9.82	0.722
B II	14.18 ± 7.87	19.22 ± 9.65	0.072
B III	9.82 ± 8.74	13.89 ± 9.85	0.078

B I – BDI score on arrival; B II – BDI score after 2 weeks; B III – BDI score after 4 weeks; $\bar{X} \pm S$ – data are presented as arithmetic averages ± average standard deviation of the sample, Md – median; n – the number of the subjects.

Group I and II were compared with each other according to the HAM-D scores. The mean HAM-D scores of the first group upon arrival and after 2 weeks were statistically significantly lower ($p < 0.006$ and $p < 0.003$) than that of the second group. After 4 weeks, the difference between the groups became statistically irrelevant. The arithmetic means, standard deviations, medians and p values of both groups are provided in Table 31.

TABLE 31. A comparison of HAM-D scores between the investigated groups.

Scale	Group I (n = 28)	Group II (n = 45)	p-value
	$\bar{X} \pm S$	$\bar{X} \pm S$	
H I	26.68 ± 7.92	31.33 ± 6.13	**0.006***
H II	12.18 ± 8.05	17.20 ± 5.93	**0.003***
H III	6.71 ± 4.91	9.02 ± 4.97	0.057

H I – score of HAM-D upon arrival; H II – score of HAM-D after 2 weeks; H III – score of HAM-D after 4 weeks; $\bar{X} \pm S$ – data are presented as arithmetic averages ± mean sample standard deviation; * – statistically significant differences; n – the numbers of participants.

The MADRS score after 2 weeks from the beginning of the treatment was statistically significantly ($p < 0.003$) lower in the first group than in the second. The difference between score upon arrival and after 4 weeks between groups was not statistically significant.

The arithmetic means standard deviations and p values of both groups are provided in Table 32.

TABLE 32. A comparison of MADRS scores between the investigated groups.

Scale	Group I (n = 28)	Group II (n = 45)	p value
	$\bar{X} \pm S$	$\bar{X} \pm S$	
M I	31.07 ± 7.44	33.84 ± 6.27	0.092
M II	14.50 ± 7.96	20.16 ± 7.26	**0.003***
M III	8.61 ± 6.62	11.49 ± 6.70	0.077

M I – MADRS score upon arrival; M II – MADRS score after 2 weeks; M III – MADRS score after 4 weeks; $\bar{X} \pm S$ – data are presented as arithmetic averages ± average standard deviation of the sample size; * – statistically significant differences; n – the number of the subjects.

Patients from both groups who had statistically significant improvements were evaluated according to all scales used in this study. It was discovered that patients from group two felt statistically significantly better after 4 weeks of treatment according to the BDI ($p < 0.001$) and the MADRS ($p < 0.014$) and after 2 weeks of treatment according to the HAM-D ($p < 0.004$). Data are presented in Table 33.

TABLE 33. A comparison of clinically significantly improved patients and their questionnaires' scores between phenotypically different groups.

Scales	Group I (n = 28)		Group II (n = 45)		p-value
	Frequency (%)	$\bar{X} \pm S$	Frequency n (%)	$\bar{X} \pm S$	
B I	12 (42.86%)	9.25 ± 5.77	12 (26.67%)	10.17 ± 5.11	0.684
B II	16 (57.14%)	4.00 ± 1.75	27 (60.00%)	8.26 ± 5.16	**< 0.001***
H I	14 (50.00%)	6.79 ± 4.19	16 (35.56%)	11.88 ± 4.60	**0.004***
H II	26 (92.86%)	6.23 ± 4.45	39 (86.67%)	7.82 ± 3.97	0.137
M I	15 (53.57%)	9.07 ± 6.05	9 (20.00%)	9.89 ± 2.93	0.660
M II	22 (78.57%)	6.18 ± 4.84	37 (82.22%)	9.24 ± 4.27	**0.014***

B I – BDI score after 2 weeks; B II – BDI score after 4 weeks; H I – HAM-D scores after 2 weeks; H II - HAM-D score after 4 weeks; M I – MADRS score after 2 weeks; M II – MADRS score after 4 weeks; Frequency n (%) – the number of patients who had significant (≥ 50% scale estimate) decrease from the initial level; $\bar{X} \pm S$ – arithmetic means ± mean standard deviation of the scales scores; p value of the comparison between two groups; * – statistically significant differences; n – number of participants.

The influence of the phenotype predicted from the *CYP2C19* and *CYP2D6* genotype on the clinical efficacy of antidepressants when the administered drug is metabolized by a phenotypically altered enzyme

When assessing the influence of *CYP2D6* and *CYP2C19* genotype polymorphisms on phenotypical expression and the clinical efficacy of drugs with the help of the genetical data and knowing what antidepressant was empirically administered for the treatment, patients, according to by *CYP2C19* and *CYP2D6* polymorphisms' prognostic phenotypical expression, were divided into two groups:

- Group I – normal metabolizers according to *CYP2D6* and *CYP2C19* genotypes (n = 28). The group remained the same, as described above;
- Group II – patients who, according to the phenotype predicted from the genotype, were slow or ultrarapid metabolizers according to *CYP2D6* and (or) slow, decreased or ultrarapid metabolizers according to *CYP2C19*. Retrospectively, it has been estimated that these patients were treated with an antidepressant, metabolized by the *CYP2C19* or *CYP2D6* (n = 22).

BDI scores were statistically significantly ($p < 0.015$) higher in the second group after 2 weeks of treatment. Although the differences between groups were seen between upon arrival and 4 weeks of treatment - they were not statistically significantly different. The questionnaire arithmetic means standard deviations and p values of both groups are presented in Table 34.

TABLE 34. BDI scores comparison.

Scale	Group I (n = 28)	Group II (n = 22)	p value
	Mean ± Sd	Mean ± Sd	
B I	24.57 ± 12.08	29.59 ± 9.76	0.120
B II	14.18 ± 7.87	20.40 ± 9.65	**0.015***
B III	9.82 ± 8.74	13.77 ± 8.57	0.116

B I – BDI score upon arrival; B II – BDI score after 2 weeks; B III – BDI score after 4 weeks; Mn ± Sd – data are presented as arithmetic means ± mean standard deviation of the sample; * – statistically significant differences; n – number of the participants.

The two groups were compared with each other according to the HAM-D. The HAM-D scores of the first group upon arrival ($p < 0.024$) and after 2 weeks ($p < 0.012$) of treatment were statistically significantly different than that of the second group. After 4 weeks, the difference between the groups remained; however, it was not statistically significantly different. The arithmetic means of the standard deviations and p values of both groups are provided in Table 35.

TABLE 35. A comparison of the HAM-D scores between the investigated groups.

Scale	Group I (n = 28)	Group II (n = 22)	p value
	$\bar{X} \pm S$	$\bar{X} \pm S$	
H I	26.68 ± 7.92	31.36 ± 5.74	**0.024***
H II	12.18 ± 8.05	17.55 ± 5.84	**0.012***
H III	6.71 ± 4.91	9.41 ± 5.86	0.083

H I - score of HAM-D upon arrival; H II - score of HAM-D after 2 weeks; H III - score of HAM-D after 4 weeks; $\bar{X} \pm S$ – data are presented as arithmetic averages ± mean sample standard deviation; * – statistically significant differences; n – numbers of participants

The first group's MADRS score after 2 weeks was statistically significantly lower ($p < 0.008$) than that of the second group. The score difference upon arrival and after 4 weeks between groups was not statistically significant. The arithmetic means standard deviations and p values of both groups are provided in Table 36.

TABLE 36. A comparison of MADRS scores between the investigated groups.

Scale	Group I (n = 28)	Group II (n = 22)	p value
	$\bar{X} \pm S$	$\bar{X} \pm S$	
M I	31.07 ± 7.44	32.90 ± 5.62	0.341
M II	14.50 ± 7.96	20.41 ± 6.97	**0.008***
M III	8.61 ± 6.62	12.19 ± 6.70	0,075

M I – MADRS score upon arrival; M II – MADRS score after 2 weeks; M III – MADRS score after 4 weeks; $\bar{X} \pm S$ – data are presented as arithmetic averages ± average standard deviation of the sample size; * – statistically significant differences; n – the number of the subjects.

After comparing patients from both groups who had improved clinically according to the scales, a significant difference between the groups was found. The scores of the second group patients were statistically significantly lower after 4 weeks of treatment according to BDI ($p < 0.004$), MADRS ($p < 0.048$) and after 2 weeks of treatment according to HAM-D ($p < 0.006$), Table 37.

TABLE 37. A comparison of the clinically significantly improved patients and their questionnaires' scores between the phenotypically different groups.

Scales	Group I (n = 28)		Group II (n = 22)		p-value
	Frequency n (%)	$\bar{X} \pm S$	Frequency n (%)	$\bar{X} \pm S$	
B I	12 (42.86%)	9.25 ± 5.77	6 (27.27%)	11.67 ± 6.77	0.473
B II	16 (57.14%)	4.00 ± 1.75	13 (59.09%)	8.62 ± 4.70	**0.004***
H I	14 (50.00%)	6.79 ± 4.19	9 (40.91%)	12.89 ± 4.70	**0.006***

H II	26 (92.86%)	6.23 ± 4.45	19 (86.36%)	8.00 ± 4.67	0.207
M I	15 (53.57%)	9.07 ± 6.05	5 (22.72%)	11.40 ± 2.07	0.216
M II	22 (78.57%)	6.18 ± 4.84	17 (77.28%)	9.12 ± 4.12	**0.048***

B I – BDI score after 2 weeks; B II – BDI score after 4 weeks; H I – HAM-D scores after 2 weeks; H II – HAM-D score after 4 weeks; M I – MADRS score after 2 weeks; M II – MADRS score after 4 weeks; Frequency n (%) – the number of patients who had a significant (≥ 50% scale estimate) decrease from the initial level; $\bar{X} \pm S$ – arithmetic means ± mean standard deviation of the scales scores; p value of the comparison between two groups; * – statistically significant differences; n – number of participants.

9. Discussion of the results

Demographic and epidemiological data

The results of the study showed that women made up a higher proportion of patients with depression, and this is in line with global and European trends. The study included three times more women than men. According to meta-analyses performed in Europe, women make up to two times more people with depression than men [13]. This is partly due to the lower morbidity of men, less frequent attempts to seek help from healthcare professionals, especially psychiatrists, as well as other social and cultural aspects. In this study, an equal number of women and men refused to participate. Therefore, the number of people included in the study mirrors the distribution of patients that are being treated for depression in VUHSC NCPD.

By the arithmetic average and the average of the sample standard deviation, it was found that the greater part of the patients were of mature age. The age median for males was 47 years, for females – 57 years. This is explained and related to the biological factors (older age depression due to the neurotransmitter metabolism disorder), chronic somatic diseases, as well as the psychological, existential and social changes occurring at this age. Similar data were published by foreign scientists as well [14, 15].

The greater part of the subjects consisted of patients with recurring depression. Moderate and severe depression cases were almost equally distributed. It is known that the depressive disorder tends to recur and often becomes chronic [16, 17]. The distribution of patients with first- episode depression and recurrent episodes by gender has remained the same.

A higher proportion of patients consisted of those who were treated in the psychiatric inpatient department, while depression is more often treated on an outpatient basis and only when there is resistance to medication, an increased suicide risk or co-morbidities – a patient is then being referred for inpatient treatment. Since the principal investigator works in the VUH SC NCPD, the greater part of the patients

included in the study were treated in inpatient settings. The results better reflect the inclusion in the study circumstances than the demographic situation.

It can be said that all the patients were treated with the latest, state of the art antidepressants registered in the European Union, which are characterized by the same efficiency as the previously widely used tricyclic antidepressants but cause a lot fewer side effects. Half of all the patients in the study were treated with the group of antidepressants belonging to the selective serotonin reuptake inhibitors (SSRI). Half of the patients were treated with one antidepressant, the other half – with two. This can be explained by the fact that the greater part of the patients were hospitalized, and it was known from their medical records that treatment was unsuccessful, so the conditions were severe and required treatment with two antidepressants. According to their medical status, patients were treated because of co-morbid conditions with various classes of drugs: benzodiazepines, antipsychotics, and drugs for physical problems. The possible clinical effectiveness of any additional classes of medications to clinical antidepressant response was not evaluated.

The assessment of depressive symptoms with scales

The treatment has been given empirically – according to the clinical status, medical history, complaints, and it was adjusted if needed. After evaluating the patients status with depression severity scales, it was found that for the greater part, the patients' treatments were effective, and that their mental health had improved. According to the BDI, which was completed by patients, more than half of the patients (59%) had a clinical improvement: a score decrease of ≥ 50% of the initial level. According to the HAM-D assessment and MADRS, which are completed by psychiatrists, this number was even higher –87% and 79% respectively. After the evaluation of the mean changes in the scales of all patients after 2 and after 4 weeks of treatment, in all cases, the improvement was statistically significant ($p < 0.001$). Such a high level of efficiency can be explained by the fact that patients were usually monitored in an inpatient unit, had received extra attention as research participants, their treatments, according to clinical status and outpatient experience, were adjusted more rapidly, and they had not received any previously ineffective medications. For treating co-occurring conditions like anxiety, racing thoughts, as well as to potentiate the antidepressant efficacy – other classes of medication were given (benzodiazepines, antipsychotics, mood stabilizers). A higher percentage of the patients had seen improvements in their conditions according to psychiatrists than according to the patients themselves. The differences in assessing depression symptom improvement could have arisen because different attention was given for the somatic and psychological symptoms by doctors and by the patients themselves.

Scales that are completed by psychiatrists highlight somatic and vegetative symptoms more than psychological ones, since these symptoms decrease faster with antidepressant treatment. For example, in the HAM-D, 3 of the 17 criteria assess sleep disorders (insomnia), and this amounts to 11.5% of the total estimate. The other scales (BDI, for example) are less sensitive for physical factors [18]. Subjectively even treated patients felt lack of self-confidence, anxiety about the future, guilt, dissatisfaction with their appearance. So psychologically they could feel the need to show to the doctor their status of "incomplete improvement".

The *CYP2C19* genotype and the predicted phenotype frequency of the patient group

The analysis of 75 patients with *CYP2C19* polymorphisms showed that allele frequency corresponds to the frequency between the Europids from the gene database. Statistically significant differences were not found.

After evaluating the *CYP2C19* genotype it was discovered, that from genotype predicted phenotype in patients group corresponded to the frequency in the Europid population [11]. Phenotypically, only 40% of patients had normal metabolism, and for them, the treatment response could have been foreseen. Of the examined patients, 36% had fast metabolism according to the CYP2C19 phenotype, and they could have felt a lack of treatment effects because of the fast metabolism of the drug. Other patients' metabolism was medium or slow (1.33%), and this could have led to an increase in side effects risks [19].

CYP2D6 genotype polymorphism and projected phenotype frequency

After determining *CYP2D6* polymorphisms in patients and the control group and comparing them with each other, it was discovered that the frequencies of alleles are different. After applying the Bonferroni correction, the patient group had statistically significantly ($p < 0.004$) less functional *2 alleles and statistically significantly more *4 dysfunctional alleles ($p < 0.005$). Other differences were not statistically significant.

The patients' group had statistically significantly ($p < 0.005$) more all dysfunctional alleles when combined and statistically significantly less ($p < 0.014$) functional alleles than the control group.

When comparing all *CYP2D6* alleles (both the Lithuanian patients and controls) with the frequency of the Europid alleles from the genetic database [11], it was discovered that the Lithuanian population had statistically a significantly ($p < 0.002$) less dysfunctional *5 allele than in Europid population [20]. There were only 6% of

people with phenotypically ultra-rapid or slow metabolism according to *CYP2D6*, so statistical analysis was not possible.

After a comparison of the patients' group's *CYP2D6* genotype with the prevalence among Europids, four statistically significant differences were established. The patients' group more often had **1/*4, *1/*41, *2/*41* and **4/*41* genotypes. However, only **4/*41* (slow-metabolism phenotype) is clinically significant, which could affect medication metabolism. Only three patients had it, so a statistical analysis was not possible.

The *CYP2D6* phenotype's influence on the clinical efficacy of antidepressants when treating empirically

Two groups, according to *CYP2D6*-predicted phenotype activity scores, were compared – those having normal metabolic speed and those having altered metabolic speed (ultrarapid and slow metabolizer); after empirically adapting the treatment, a clinical response was evaluated. According to the scores of the depression scales, the groups did not differ significantly among each other.

It is important that patients were not specially selected to be treated with antidepressants that are metabolized by the CYP2D6 enzyme. The greater part of the patients was treated with the SSRI group of antidepressants that are metabolized mostly by CYP2C19.

The results obtained may have been influenced by the inclusion of patients with marginal predicted phenotypic activity values in the changed metabolic group. Also, if needed, all patients were treated with the other groups of drugs; treatment with antidepressants has been adjusted in accordance with the patients' clinical status, and the clinical condition of most patients had improved. Therefore, the difference between the groups was not significant.

CYP2C19-predicted phenotype influence on the clinical efficacy of antidepressants when treating empirically

When examining the *CYP2C19* phenotype's influence on drug metabolism, the possible influence of the *CYP2D6* polymorphism was rejected. Only those subjects with normal *CYP2D6*-predicted phenotype metabolism were analyzed, and they were further divided into two groups according to the *CYP2C19* metabolism: normal metabolizers and impaired metabolizers (either rapid or slow).

On arrival and after 2 weeks of treatment, patients who had an altered metabolism felt worse. This means that the outpatient and inpatient treatment was less effective, and that the patients were recovering slower. The differences between the results of the different scales could be explained by the different emphasis given to somatic and psychological depressive symptoms by different instruments.

It should be noted that after 2 weeks, the difference between groups for all scales scores was statistically significant (according to BDI, $p < 0.030$, according to HAM-D and MADRS, $p < 0.005$), and antidepressants began to work precisely after 2 weeks.

This confirms the assumption that patients with CYP gene polymorphisms that determines other than the normal phenotype feel worse as they react to the medication differently than anticipated. Their medical condition/mental status improves later on due to the correction of pharmacotherapy with clinical observation and applied polypharmacotherapy.

CYP2C19- and *CYP2D6*-predicted phenotype influence on the clinical efficacy of antidepressants when treating empirically

The assessment of CYP2D6 polymorphisms together with the previously described CYP2C19 polymorphisms shows that the first group with normal metabolism has not changed, while into the second group, five patients were additionally assigned, with changes in the predicted phenotype by CYP2D6. When creating groups, it was not taken into account whether people were treated with antidepressants metabolized by enzymes with altered activity. Such a situation is a replication of real empirical drug administration.

According to psychiatrists, the difference between groups remained identical, as it has been described by analyzing the influence of the *CYP2C19* phenotype. Although patients who had altered polymorphism subjectively evaluated their condition as being worse, the difference between groups was not statistically significant.

Those patients who had clinical improvements were more often in the normal metabolism group. Their depression scales' averages were statistically significantly lower (by HAM-D $p < 0.004$ after 2 weeks of treatment, by BDI $p < 0.001$ and by MADRS $p < 0.014$ after 4 weeks of treatment) than those of patients with polymorphisms, which predicted an accelerated or slowed metabolizer phenotype. This means that patients with predicted normal phenotypes had fewer and less pronounced symptoms of depression after the treatment.

A small number of patients with phenotypically important CYP2D6 polymorphisms, explains the persisting differences between the groups. When combining CYP2C19 and CYP2D6 polymorphisms and assessing their expected phenotypes, – the trend remains the same, likewise the assessment of only CYP2C19 polymorphisms. Even when adding a group of patients whose metabolism has changed, some significant differences (according to BDI) disappear. This indicates that the CYP2D6 polymorphisms are not significant for the clinical efficiency of the newer antidepressants.

CYP2C19 and *CYP2D6* phenotype influence on the clinical efficiency of antidepressants when treating with drugs that are metabolized by these enzymes

The purpose was to determine what importance a phenotypically altered, accelerated or slowed down enzyme system has for the clinical efficacy of antidepressant drugs.

The first group has remained unchanged – it consisted of patients with normal metabolism by *CYP2C19* and *CYP2D6*. The second group consisted of patients who had an altered predicted phenotype and received treatment with antidepressant drugs that are metabolized by enzymes with altered activity.

Even though the second group consisted of almost twice as less of selected patients than previously compared with *CYP2C19* polymorphisms, the results were almost identical. Significant differences were found on all the scales after 2 weeks of treatment: according to the BDI ($p < 0.015$), according to HAM-D ($p < 0.012$) and MADRS ($p < 0.008$). This reaffirms the importance of *CYP2C19* polymorphisms on clinical antidepressant efficacy.

After evaluating patients from both groups, who have had clinical improvement – decreases in scales scores of 50% or more – it was discovered that these patients were less numerous in the second group, and that their scores, according to BDI ($p < 0.004$) and MADRS ($p < 0.048$), were statistically significantly higher than the first group, which had a normal predicted phenotype. It means that patients with a normal predicted phenotype had recovered faster and experienced less severe symptoms.

The obtained results completely coincide with the results when it was not taken into account, whether the patient is being treated by the antidepressants that are metabolized by the enzymes with altered activity. It can be assumed that when treating empirically, what corresponds to the real clinical situation, the influence of the polymorphisms on the clinical antidepressant efficacy remains the same as when artificially choosing treatment with antidepressants that are metabolized by enzymes with altered activity.

Such an important result indicates that the polymorphisms of the CYP enzymes when treating empirically (in real-world conditions) have significant impact on clinical efficiency.

According to the obtained results of the study, it can be concluded that *CYP2D6* polymorphisms did not have any clinical impact on the efficacy of antidepressants, and that *CYP2C19* polymorphisms did have clinical impact on the efficacy of antidepressants.

Factors that could have affected the results obtained could be divided into several groups.

Factors associated with genetics:

- a small number of individuals with altered *CYP2D6* metabolism, who could not have been evaluated statistically because of the very uneven sample sizes;
- it is known that genetic variations are responsible only for a part of an individual's response to the treatment [21];
- more often the effects of a drug are determined by more than one genetic factor [22];
- regardless of genotype, many other factors can contribute to gene expression, including promoter and regulator sequences, transcriptions, mRNA stability, post-transcriptional regulation, epigenetic DNA methylation factors, histone modification or non-coding RNA. It should be noted that the activity of many CYP enzymes is changed by the transforming of DNA by methylation in the promoter region [21];
- there is no significant evidence that allele polymorphisms could have been related to clinical efficacy and safety of the treatment. There is little research to suggest that such a link exists, and all such studies are small in scale [23];
- the mechanism of many psychotropic drugs is not entirely clear, and there is a lack of evidence of how genetic factors influence the clinical response of a new generation of antidepressants [24].

Factors associated with the metabolism and action of antidepressants:

- the CYP2D6 enzyme mainly metabolizes the tricyclic antidepressants, which the subjects had not received, except for one case;
- drugs, like all materials, are not only metabolized by the CYP system –they can be its substrate, but they may also be inhibitors or inductors of this system;
- pharmaceutically active drug metabolites with their own metabolic pathways may change the predicted therapeutic effect of the main drug [25];
- additional metabolic pathways (phase II enzymes, drug transport) as well are changing the effect of the polymorphism on the final effect of the drug [25];
- medications, including antidepressants, are often metabolized by several enzymes. The reduction in the activity of the principal metabolic pathway of the drug can be corrected by an increase in activity of the alternative metabolic pathway;
- indirect drug effects, such as antidepressant-induced reduction of inflammatory cytokines, may alter CYP activity in the opposite direction than the antidepressant itself [25];

- diet, alcohol, and smoking have been shown to induce CYP enzymes, which metabolizes some antidepressants in a way that their concentration can be reduced up to 50% [25] – this was not taken into account in this study;
- CYP enzymes are found not only in the liver but also the brain. Therefore, factors acting on the liver enzymes, but not crossing the hematoencephalic barrier, may not have any clinical impact.

Features of the depressed patients included in the study:

- very high levels of increased mental health status were achieved in all of the patients;
- depressed people are characterized by sensitivity to placebo, so differences between groups may be less clear;
- although only about 35–45% of the patients treated with antidepressants achieve remission with first-choice drugs, a large proportion of subjects enrolled had recurrent depression, so the treatment was chosen earlier;
- the study did not assess the impact of factors such as education, economic status, age, marital status, chronic illness, concomitant disorders, such as an anxiety disorder etc., on the clinical response [26];
- chronic diseases, comorbidities, and concomitantly used medications affect the distribution of antidepressants and their amount in the blood as well as the response of the targeted organs [23];
- the obtained estimates of scales are different – a subjective assessment of the improved status was inferior compared to the objective status by doctors for the reasons discussed above.

Pharmacogenetic antidepressant research results are contradictory. Kirchheiner et al., back in 2004, described the recommendations of how to adjust doses of several antidepressants depending on the genotype [27]. However, in 2007, because of a lack of clear evidence, the international working group EGAPP's (The Evaluation of Genomic Applications in Practice and Prevention) [28] recommendations guided to refrain from using CYP450 routine tests before starting the treatment with the SSRI group of antidepressants, until further research is carried out.

The medical society is having a debate on whether pharmacogenetic tests should be carried out for all patients or only for groups with higher risks.

The arguments for the second option would be as follows: about 90% of people have moderate and normal metabolism phenotypically, so the response to medication can be predicted in these patients. Due to the relatively small number of patients who need to have adjusted treatment after a pharmacogenetic screening,

an unfavorable cost- benefit balance may be expected. The benefits and efficacy of the pharmacogenetic tests are significantly increased in patients with inadequate clinical response or adverse reactions to medication. The benefits and efficacy of the pharmacogenetic tests are significantly increased in patients with inadequate clinical response or adverse reactions to medication.

It can be concluded that pharmacogenetic studies do not yet explain all the differences in antidepressant response and are only an important part of the big, complex picture [29]. Based on the most prominent pharmacogenetic studies – *STAR*D* in the United States and *GenDep* [30] conducted in Europe, which evaluate the relationship between *CYP2C19* polymorphisms and antidepressant response, Mrazek et al. identified the effect of the *CYP2C19* genotype on the clinical efficacy of citalopram treatment [31].

However, in a study conducted in Europe, only the association with the concentration of the drug in the blood was found, not with the clinical efficacy.

However, the results of this research, as well as the global trends, indicate that individual psychopharmacotherapeutic treatment, based on a patient's metabolic profile, will result in a faster therapeutic effect, while also lowering the cost of treatment, the number of side effects and the number of drugs used [32]. Pharmacogenetic tests will help to develop a better therapeutic relationship between the psychiatrist and patient [33], reduce self-treatment, improve drug adherence [34], and increase medical authority [35].

Although the primary objective of pharmacogenetic tests is individualized treatment – a better selection of drugs and their doses – the secondary benefit is increased patient confidence in the objectivity of psychiatric treatment [36]. The fact of the genetic test itself may reduce the anxiety about the suitability of the medicine. This increases the likelihood that the patient will adhere better to the treatment regimen and the treatment will be successful [37].

This will help to reduce the stigmatization of psychiatric treatment and will encourage people to seek professional help earlier. With an increasing interest in these studies, the cost of pharmacogenetic tests is decreasing because of an economy of scale. So, it is likely that the price in the future will continue to decrease [26, 38, 39].

The pharmacoeconomic benefits of these tests are increasing, and the results can be used by doctors of various specialties during the whole lifetime of the patient with the help of the development of an electronic medical health records system.

10. Conclusions

- The distribution of people with depression based on gender, age, and disease severity did not differ between the Lithuanian and European epidemiological data.
- based on the predicted phenotype, the Lithuanian patients' group had more
  - normal metabolizers with the genotypes *CYP2D6*1/*4*, *CYP2D6*1/*41*, and *CYP2D6*2/*4*,
  - and slow metabolizers with the genotype *CYP2D6*4/*41*, compared with the Europid population. In patients with moderate and severe depression, the rate of *CYP2C19* polymorphisms and the prognosed phenotype frequency did not differ from the rate of polymorphisms in the Europid population discovered by other researchers.
- The patient group, compared with the control group of healthy individuals, had statistically significant
  - less active *CYP2D6 *2* alleles ($p < 0.004$),
  - less of all functional *CYP2D6* alleles ($p < 0.014$),
  - more nonfunctional *4 alleles ($p < 0.005$), o more all nonfunctional alleles ($p < 0.005$).

The Lithuanian subjects had a statistically significantly less nonfunctional *5 allele than had been shown by other researchers in the Europid population ($p < 0.002$).

- After evaluating the clinical symptoms of depression with the help of scales and questionnaires, it has been found that patients' mental health conditions after 2- and 4-week treatments with antidepressants had improved significantly. A clinically significant improvement, where scores had diminished more than 50% compared with an initial visit, has been achieved with 46 (58.97%) patients according to BDI, 68 (87.18%) patients according to HAM-D, and 62 (79.49%) patients according to MADRS.
- After evaluating, from the *CYP2D6* genotype, the prognosed phenotype influence, it was found that the *CYP2D6* gene polymorphisms did not affect the efficacy of the antidepressants, or the impact could not have been assessed statistically due to the very unequal sample sizes. After evaluating the prognosed phenotype influence from the *CYP2C19* genotype, it was found that the *CYP2C19* gene polymorphisms did have an influence on the response to clinical antidepressants: those patients who had an altered enzyme speed (slow, decreased or ultrarapid metabolism) recovered slower and experienced statistically significantly more severe symptoms of depression.

11. Practical recommend ations

Based on the results of the study, it can be said that the detection of polymorphisms of *CYP* genes as part of the pharmacogenetic tests is a significant and important tool for personalized medicine.

The pharmacogenetic tests have greater practical benefit for patients who:

- did not have the expected therapeutic effect with the prescribed drugs;
- are receiving poly-pharmacy – that is, to whom different medicines are given simultaneously;
- need to avoid dangerous medication interactions;
- ill have to take long-term medical treatment with different groups of drugs (those who have chronic somatic or oncological diseases, those after organ transplantation etc.).

It should be noted that the results of the genetic tests do not change during the lifetime of the person. Therefore, the received pharmacogenetic information remains relevant not only in the retrospective assessment of any unsuccessful treatment cases but also in the future, when choosing individualized treatment.

It is important to familiarize as many specialists-doctors as possible with pharmacogenetic studies and their possible applications.

Due to the extensive and ever-renewing data, it is advisable for doctors to use licensed computer programs or websites to assess the clinical relevance of a particular polymorphism.

Health policy-makers and economists should calculate for which group of patients to compensate the cost of pharmacogenetic tests in order for the public to be able to save money on healthcare spending, which would offset the cost of treatment failure.

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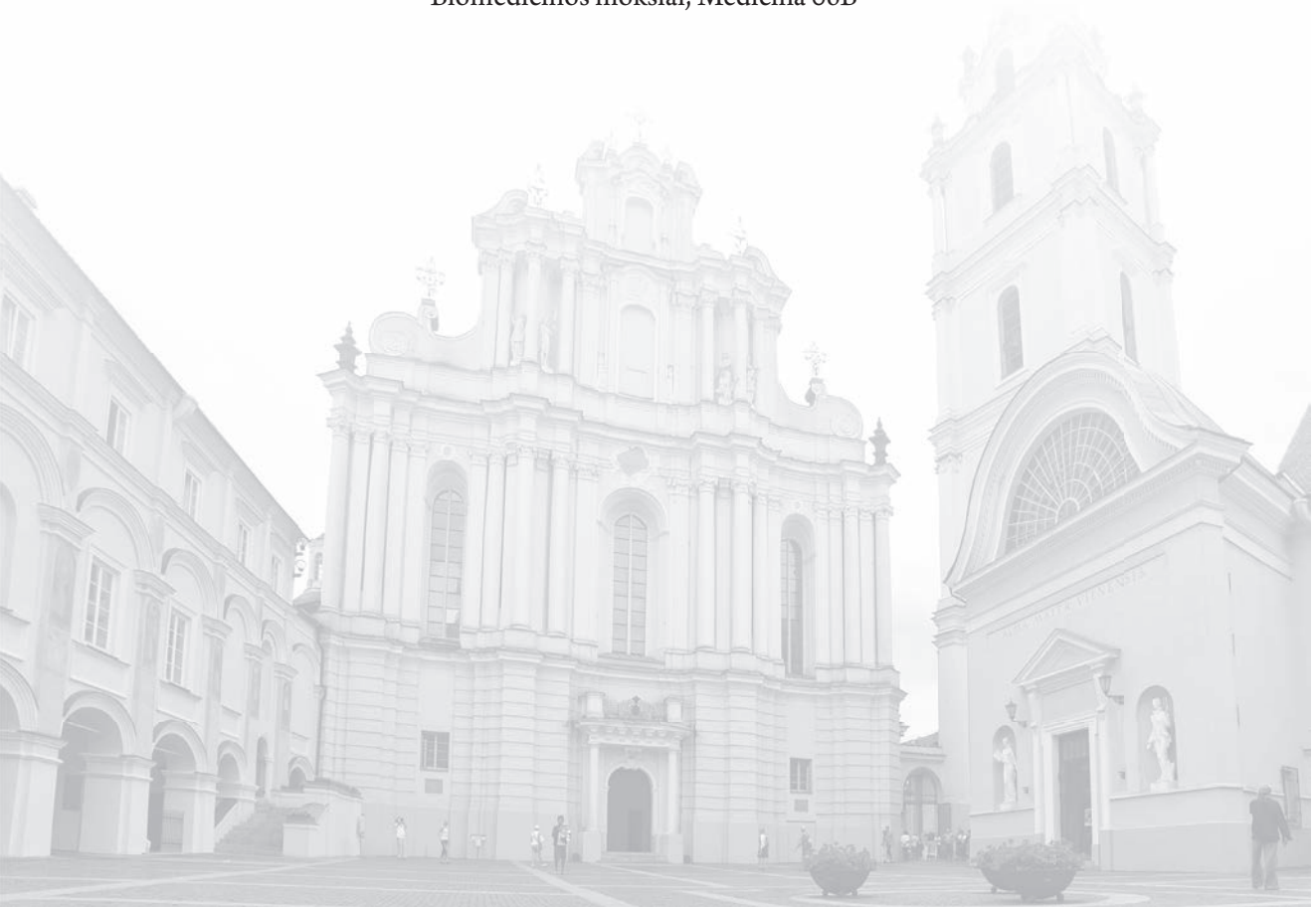
Beata Aleksiūnienė

AN EVALUATION OF GENOMIC FACTORS AND THEIR IMPORTANCE IN THE ETIOPATHOGENESIS OF CONGENITAL HEART DISEASES

GENOMINIŲ VEIKSNIŲ ĮVERTINIMAS IR REIKŠMĖ ĮGIMTŲ ŠIRDIES YDŲ ETIOPATOGENEZĖJE

Biomedical Sciences, Medicine 06B
Biomedicinos mokslai, Medicina 06B



Abbreviations

ACMG – American College of Medical Genetics and Genomics
Akt – V-akt murine thymoma viral oncogene homolog signaling
bp – base pair
CGH – comparative genome hybridization
CHD – congenital heart disease
CNV – copy number variation
DECIPHER – the Database of Chromosomal Imbalance and Phenotype in Humans using Ensembl Resources database
ECARUCA – European Cytogeneticists Association Register of Unbalanced Chromosome Aberrations
FISH – fluorescence *in situ* hybridization
GO – gene ontology
HI – haploinsufficiency score
ISCN – International System for Human Cytogenetic Nomenclature
kb – kilobase
LITGEN – acronym of the national project "Genetic Diversity of the Population of Lithuania and Changes of its Genetic Structure Related with Evolution and Common Diseases"
MGI – Mouse Genome Informatics
MLPA – multiplex ligation – dependent probe amplification
NCBI – National Center for Biotechnology Information
OMIM – Online Mendelian Inheritance in Man
RefSeq – Reference Sequences
SNP – single nucleotide polymorphism
SNP-CGH – SNP comparative genomic hybridization
TL-PGR – real time – polymerase chain reaction
UCSC – University of California santa Cruz
Wnt – Wingless-related integration site signaling

Abbreviations of proteins

ACP6 – acid phosphatase 6, lysophosphatidic
ADAMTS3 – ADAM metallopeptidase with thrombospondin type 1 motif 3
ALDH1A2 – aldehyde dehydrogenase 1 family member A2
ANKRD1 – ankyrin repeat domain 1
ANKRD17 – ankyrin repeat domain 17
APLN – apelin
BCL9 – B cell CLL/lymphoma 9
BMP – bone morphogenetic protein
BMPR1A – bone morphogenetic protein receptor type 1A
BMPR2 – bone morphogenetic protein receptor type 2
BRAF – B-Raf proto-oncogene, serine/threonine kinase
CASP3 – caspase 3
CHD1L – chromodomain helicase DNA binding protein 1 like
CHRM3 – cholinergic receptor muscarinic 3
CHRNA3 – cholinergic receptor nicotinic alpha 3 subunit
CLCN6 – chloride voltage-gated channel 6
CRKL – CRK like proto-oncogene, adaptor protein
DVL3 – dishevelled segment polarity protein 3
ECE2 – endothelin converting enzyme 2
ELN – elastin
ERBB4 – erb-b2 receptor tyrosine kinase 4
ERMARD – ER membrane associated RNA degradation
EVC – EvC ciliary complex subunit 1
EVC2 – EvC ciliary complex subunit 2
FBN1 – fibrillin 1
FGF – fibroblast growth factor
FGF9 – fibroblast growth factor 9
FKBP6 – FK506 binding protein 6
FLNA – filamin A
FMO5 – flavin containing monooxygenase 5
GJA5 – gap junction protein alpha 5
GPC3 – glypican 3
HAND2 – heart- and neural crest derivatives-expressed 2
HRAS – HRas proto-oncogene, GTPase
IFT88 – intraflagellar transport 88
IGF1R – insulin like growth factor 1 receptor

IRX4 – iroquois homeobox 4
KCNG2 – potassium voltage-gated channel modifier subfamily G member 2
KCNH2 – potassium voltage-gated channel subfamily H member 2
KRIT1 – ankyrin repeat containing
LMNA – lamin A/C
MAPK12 – mitogen-activated protein kinase 12
MEF2A – myocyte enhancer factor 2A
MEF2C – myocyte enhancer factor 2C
MESP1 – mesoderm posterior basic helix-loop-helix transcription factor 1
MTHFR – methylenetetrahydrofolate reductase
MTR – 5-methyltetrahydrofolate-homocysteine methyltransferase
MTRR – 5-methyltetrahydrofolate-homocysteine methyltransferase reductase
NFATC1 – nuclear factor of activated T cells 1
NIPA1 – NIPA magnesium transporter 1
NIPA2 – NIPA magnesium transporter 2
NOS3 – nitric oxide synthase 3
NPPA – natriuretic peptide A
NPPB – natriuretic peptide B
NR2F2 – nuclear receptor subfamily 2 group F member 2
NRG4 – neuregulin 4
PER2 – period circadian regulator 2
PRKAB2 – protein kinase AMP-activated non-catalytic subunit beta 2
PRKAG2 – protein kinase AMP-activated non-catalytic subunit gamma 2
PRKCI – protein kinase C iota
PRKD1 – protein kinase D1
PTEN – phosphatase and tensin homolog
SEMA3C – semaphorin 3C
SEMA3D – semaphorin 3D
SMAD6 – SMAD family member 6
SMARCD3 – SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily d, member 3
TBX1 – T-box 1
TBX5 – T-box 5
TERT – telomerase reverse transcriptase
TRNAU1AP – tRNA selenocysteine 1 associated protein 1
TRPM8 – transient receptor potential cation channel subfamily M member 8
TXNL4A – thioredoxin like 4A
ZIC3 – Zic family member 3

1. Introduction

1.1. Research Problem and Its Significance

Congenital heart diseases (CHDs) are the most frequent congenital developmental anomalies during neonatancy and are diagnosed in 8 of 1 000 liveborn infants (van der Linde et al. 2011; Mozaffarian et al. 2016). In accordance with the information gathered by the Institute of Hygiene, 412 infants with CHDs were born in Lithuania in 2016 (http://www.hi.lt/medical-data-of-births). Some of the CHDs have poor survival predictions and are one of the main causes of non-infectious death in infancy (Billett et al. 2008; Connor et al. 2014). The aetiology of many CHDs is still unclear.

Congenital heart diseases are a group of genetically heterogenous diseases. Heart diseases can manifest themselves in conjunction with additional extracardiac anomalies or may be one of the features of a syndrome; however, they most frequently manifest themselves as isolated defects. Both single nucleotide variants and multiple genome rearrangements may lead to the etiopathogenesis of genetic CHDs.

Comparative genome hybridization (CGH) technologies, which were applied in studying CHDs, proved that a copy number variations (CNVs) are significant factors in the aetiology of CHDs, which result in approximately 3–28% of CHDs with extracardiac anomalies and 3–4.3% of isolated CHDs (Geng et al. 2014; Wu et al. 2017). The latest research has determined that rare, large and genic CNVs manifest themselves significantly more frequently in individuals suffering from CHDs. This, in turn, demonstrates that the overall CNV burden may also be an important factor in CHD (Lander and Ware 2014).

The determination of the aetiology of CHDs is important for a patient suffering from a CHD and to that individual's family not only on the psychosocial point of view, but also in terms of family planning. Due to successful surgical treatment and a higher survival rate, the majority of children suffering from CHDs become adults and reach reproductive age; therefore, the population of adults suffering from CHDs is increasing, and the information about the aetiology of the disease and the risk of recurrence becomes particularly important.

An early identified genetic diagnosis of CHDs is important in a number of clinical aspects. Determining causative genomic variants and the disease they cause, e.g., a genetic syndrome or the neurological diseases that accompany CHDs, fundamentally changes the treatment strategy for the patient and opens more possibilities for multidisciplinary, optimized healthcare. The obtained information about the inheritance of a disease, as well as the recurrence risk that is known due to determining the genomic variants and the genes they contain, enables exact genetic counselling and prenatal diagnostics (Chaix et al. 2016). The discovery of new candidate genes and molecular pathways is important for scientific reasons. New knowledge on the pathophysiological processes of CHDs enables creating modern diagnostic markers for the early detection of CHDs and for exact therapeutic targets.

The latest scientific research has revealed that some genomic variants are important modifying factors, linked with postoperative complications, and that they increase the risk of death after heart surgeries and transplantations by a few times (Kim et al. 2016; Ramroop et al. 2017). From this point of view, the assessment of individual genetic as well as non-genetic factors (such as the age, sex, health and treatment history), which all have an effect on the individual's state of health and on response to treatment, are important (Russell et al. 2018).

1.2. Aim of the Study

To determine and evaluate the importance of genomic variants and candidate genes in the etiopathogenesis of congenital heart diseases.

1.3. Main Tasks of the Study

1. To compose a group of subjects who suffer from congenital heart diseases and to evaluate the group's aetiological structure;
2. To determine the pathogenic copy number variations in the group of subjects who suffer from congenital heart diseases and to evaluate the importance of these variations in the manifestation of congenital heart diseases;
3. To use bioinformatics gene prioritization methods in order to determine candidate genes, which are linked to congenital heart diseases, in pathogenic copy number variations;
4. To evaluate the effectiveness of determining a diagnostic and potentially pathogenic copy number variations of a single-nucleotide polymorphism – the comparative genomic hybridization method – in the group of congenital heart diseases;
5. To carry out an analysis of the prevalence of rare genetic copy number variations in subject and control groups and to evaluate their link with congenital heart diseases.

1.4. Relevance and Novelty of the Research

In this piece of research, an evaluation of genomic variants was carried out on individuals suffering from CHDs. It is the first piece of scientific research wherein modern technologies, used for genomic analysis, were applied for CHD research in Lithuania. The phenotypic and genetic diversity of CHDs and their heterogeneity were evaluated in this piece of research. Unique pathogenic genomic variants and the potential gene candidates of CHDs were determined by carrying out comparative genome hybridization analysis. Bioinformatics tools were used for carrying out a functional analysis of pathogenic genomic variants. A link between major genetic changes and congenital cardiac anomalies was determined.

Diagnoses of genetic diseases were determined, which would enable clinicians to provide information on the risk of disease recurrence in the family and the descendants to the subjects and their family members in the course of genetic counselling.

The results obtained supplement previously carried out research on copy number variations with new data and are valuable for further scientific research. Studies of this type are particularly useful for determining new genomic variants, which are of importance to heart development and the pathogenesis of cardiovascular diseases.

1.5. Statements to be Defended

1. Large genetic and phenotypic heterogeneity is typical to congenital heart diseases;
2. Pathogenic copy number variations are important causal factors in the groups of syndromic congenital heart defects and of congenital heart diseases with additional extracardiac anomalies;
3. Unique copy number variations, localized in various parts of the genome, include specific genes linked with different types of congenital heart diseases;
4. Wide-ranging single-nucleotide polymorphism – comparative genomic hybridization is an effective method for determining pathogenic genetic changes in the group of congenital heart diseases;
5. Rare genic copy number variations manifest themselves in greater frequency in the group of congenital heart diseases and may be of importance to their manifestation.

2. Materials and methods

The Vilnius Regional Biomedical Research Ethics Committee issued an approval (No. 158200–15–782–296) to conduct this piece of scientific research and approved the consent forms of informing a individual and those of an informed individual.

2.1. Selection of Subjects

In order to have a group of subjects, the following selection criteria were considered:

- A congenital heart disease that was diagnosed at any age;
- A complex congenital heart disease that was diagnosed at any age;
- A congenital heart disease with other congenital developmental anomalies and/or dysmorphic features that were diagnosed at any age;
- A congenital heart disease that is a feature of one of any clinically recognizable syndromes and that is not confirmed by a genetic cause.

The individuals below were not included in the biomedical research: 1) Individuals who had a chromosome aneuploidy, i.e., *Down*, *Edwards*, *Patau*, *Turner* or *Klinefelter* syndromes; 2) Individuals who were diagnosed with a simple CHD – patent foramen ovale; 3) Individuals who were diagnosed with an arrhythmia or a cardiomyopathy.

One hundred thirty-two individuals were included in the group of subjects, 57 of whom were male and 75 were female. The Electronic Illness History (Eli) of the Body, governed by public law at Vilnius University Hospital Santaros Klinikos, was used for collecting the information on the medical history and on the clinical data from medical patient documents.

A group of healthy individuals of ethnic Lithuanian population of LITGEN biomedical research made up a control group for this biomedical study. A control group of 355 healthy individuals was used for comparison of frequency for determining CNV. It was made up of 170 male and 185 female individuals. The individuals from the control group were not diagnosed with CHD or with any other congenital developmental anomaly.

2.2. Genetic Research in the Subject Group

A SNP-CGH test was carried out on the subjects suffering from CHD in order to determine copy number variations and to assess their pathogenesis in CHD etiopathogenesis (Figure 1).

HumanCytoSNP–12 BeadChip v2.1 chips (Illumina Inc., USA) were used for carrying out a SNP genotyping in accordance with the protocol provided by the

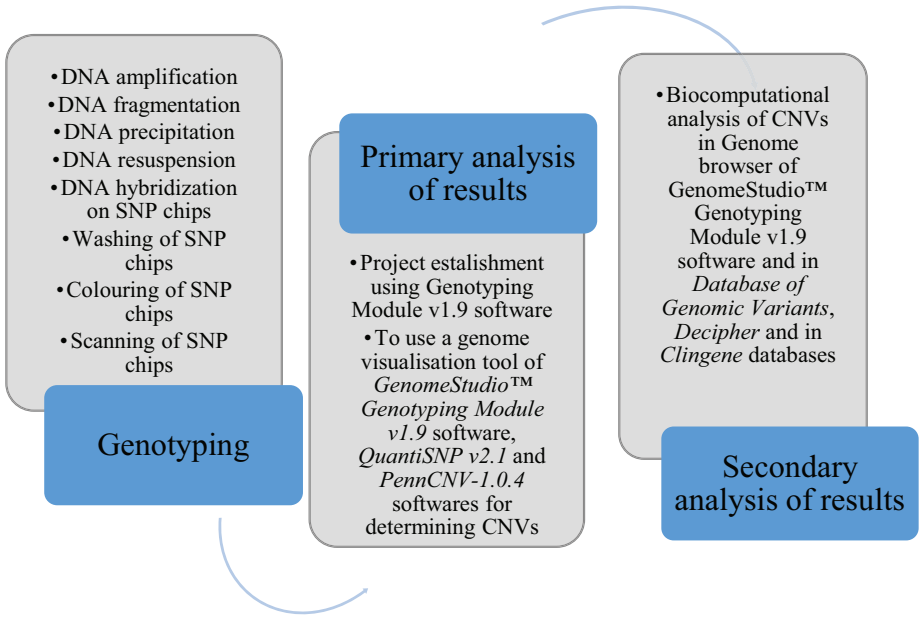


FIGURE 1. Scheme of the SNP-CGH test.

manufacturer, Infinium® HD Assay Ultra Manual Experienced User Card. The characteristics of the HumanCytoSNP–12 BeadChip v2.1 chip are as follows: covering 299140 SNP across the genome, a medium resolution of 9,7 kb. The next generation, high-performance genome analysis system Illumina HiScan™SQ (Illumina Inc., USA) was used for genotyping. GenomeStudio v2011.1 software (Illumina® GenomeStudio 2011, Illumina Inc., USA) and GenomeStudio™ Genotyping Module v1.9 were used for carrying out a primary analysis of the genotyping data and CNV visualization in accordance with the protocol provided by the manufacturer. Data analysis was carried out by assessing the SNP diagrams and the numerical values of the logarithmic estimation ratio (*LogR ratio*, LogRR) of the fluorescence intensity (R) of their parameters (threshold values: – 0.1 and 0.1) and of the B allele frequency (BAF).

Two software packages, QuantiSNP v2.1 and PennCNV–1.0.4 (Colella et al. 2007; Wang et al. 2007) were used for carrying out CNVs calling from genotyping data and CNVs analysis. These software packages use a statistical Objective Bayes–Hidden Markov Model (HMM) algorithm for determining CNVs. *NCBI* human genome GRCh37/hg19 assembly was used for determining the Reference Sequence (RefSeq) genes that can be found in the CNV domain.

Pathogenic/potentially pathogenic genomic rearrangements were approved, and the SNP-CGH method, routine karyotyping, FISH, RT-PCR and MLPA methods were used for determining their origin. A number of bioinformatics tools and online data sources were used for searching for new CHD gene candidates in the pathogenic CNVs determined. ToppGene software (Chen et al. 2009) was used for carrying out gene prioritization. The Gene Expression Database (GXD) in Mouse Genome Informatics (MGI) (http://www.informatics.jax.org/expression.shtml/) was used for analyzing the models of gene expression in mice heart embryogenesis.

2.3. Interpretation of Results and Biocomputational Analysis

The Database of Genomic Variants (DGV) (MacDonald et al. 2014) was used for carrying out biocomputational data analysis in order to assess the clinical significance of the determined CNVs. In accordance with the ACMG recommendations, the determined CNVs were divided into the following categories (Kearney et al. 2011): 1) Benign CNVs; 2) Pathogenic CNVs; 3) CNVs of unclear clinical significance, potentially pathogenic CNVs. The test result is described in accordance with the ISCN recommendations (McGowan–Jordan et al. 2016).

In order to compare the features and the frequency of CNVs, the CNVs determined in the research and in the control groups were divided into common CNVs and rare CNVs based on the CNVs overlapping with the common CNVs in a nor-

mal population. The CNVs, which overlapped by >70% with at least a single CNV, which was provided in the DGV, were interpreted as **common CNVs**. The CNVs that had partly overlapped (<30%) or failed to overlap with the CNVs, which were provided in the DGV, were interpreted as **rare CNVs**. Rare CNVs were selected for further analysis.

The databases Decipher (Bragin et al. 2014), *Clingene* (https://www.clinicalgenome.org/) and ClinGen Dosage Sensitivity Map (http://www.ncbi.nlm.nih.gov/projects/ dbvar/clingen/) were used for assessing the pathogenicity of pathogenic CNVs, of CNVs of unclear clinical significance, potentially pathogenic CNVs and rare CNVs. The Databases NCBI OMIM (Amberger et al. 2015), ECARUCA (Feenstra et al. 2006) and the genome browsers ENSEMBL (Ruffier et al. 2017), UCSC (Tyner et al. 2017), as well as scientific literature, were used for assessing the pathogenicity in the determined changes in the genome. The genes, which had been encompassed by a pathogenic or a potentially pathogenic CNV region, were analyzed. The function of the genes encompassed by rearrangements and which take part in the development of the heart was assessed.

2.4. Collection of Data of Genetic Research of the Control Group

From 2011 to 2013, staff members at the Department of Human and Medical Genetics, Faculty of Medicine, Vilnius University carried out the SNP-CGH method in order to genotype the control group, which consisted of a group of healthy individuals of ethnic Lithuanian population of LITGEN biomedical research.

A phenol-chloroform extraction method or an automated nucleic acid purification system (TECAN Freedom EVO® 200, Tecan Schweiz AG, Switzerland) was used for extracting the genomic DNA of the control group subjects from 10ml of venous blood. The 770K HumanOmniExpress–12 v1.0 and HumanOmniExpress–12 v1.1 chips (Illumina Inc., USA) with Infinium® HD technology were used for carrying out CHD genotyping in accordance with the protocol provided with the Infinium® HD Assay Ultra Manual Experienced User Card. The characteristics of HumanOmniExpress–12 v1.0 and HumanOmniExpress–12 v1.1 chips are as follows: covering 730525 SNP across the genome, a medium resolution of 4 kb. The next-generation, high-performance genome analysis system Illumina HiScan™SQ (Illumina Inc., USA) was used for genotyping.

2.5. Biostatistical Analysis

Open source software R, version 3.4.3 and MS Excel 2016 calculator were used for carrying out statistical data analysis. When assessing the quantitative indicators, an arithmetic mean $\bar{x}$ was calculated. The χ^2 criterion was used for comparing the

data of the CHD subjects with normal cardiac segmental connections with that of the subjects with abnormal cardiac segmental connections. An exact Fisher criterion was additionally calculated when the number of observations was low or when at least a single number of observations was lower than five. A Shapiro-Wilk test and certain graphic methods, i.e., a histogram and a rectangular diagram, were used for testing the hypotheses about the normality of variable distribution. The Wilcoxon criterion was used for comparing two dependent samples, whereas the Mann-Whitney-Wilcoxon rank-sum criterion was used for comparing two independent samples. The Friedman criterion was used for comparing three dependent samples, whereas the Kruskal-Wallis rank criterion was used for comparing independent samples.

3. Results and discussion

3.1. Distribution of Congenital Heart Defects in the Study Group

A study group consisting of 132 subjects was divided into three groups: 1) Subjects with isolated CHDs; 2) Subjects with CHDs with other congenital developmental anomalies and/or dysmorphic symptoms; 3) Subjects with CHDs and a genetic syndrome-specific phenotype. The term "isolated CHD" was used in cases where congenital heart disease was the main phenotypic feature of the patient during diagnosis. Accordingly, the term "CHDs with other congenital developmental anomalies and/or dysmorphic symptoms" was used in cases where the patient was diagnosed with CHD and other congenital developmental anomalies and/or dysmorphic symptoms, but not diagnosed with a genetic syndrome. The term "CHDs and a genetic syndrome-specific phenotype" was used to characterize the cases where a patient with a CHD with other congenital anomalies was suspected of having a genetic syndrome based solely on his or her phenotype.

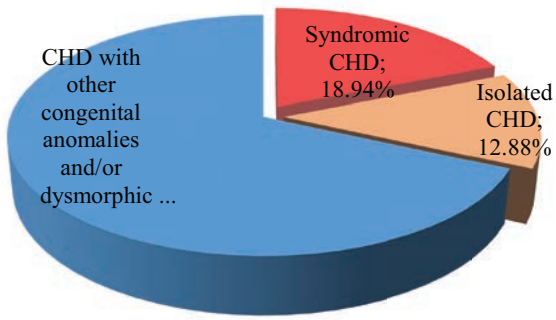


FIGURE 2. The distribution of subjects in the study group by congenital heart defect.

The group of subjects with isolated CHDs consisted of 17/132 (12.88%) individuals; the group of subjects who had CHDs with other congenital developmental anomalies and/or dysmorphic symptoms consisted of 90/132 (68.18%) individuals; the group of subjects who had CHDs and a genetic syndrome-specific phenotype consisted of 25/132 (18.94%) individuals (Figure 2).

The phenotype assessment of the subjects with CHDs and other congenital developmental anomalies and/or dysmorphic symptoms revealed that the most common clinical manifestations included delays in physical and psychomotor development, microanomalies and congenital developmental defects of other organ systems. Meanwhile, in the group where the CHDs were combined with a genetic syndrome-specific phenotype, intellectual disability was observed in addition to the aforementioned clinical symptoms.

Of the 132 subjects, 57 (43.18%) were male and 75 (56.82%) were female. Upon comparing the sex distribution in the group with isolated CHDs, the group where the CHDs occur with other congenital anomalies, and the group with syndromic CHDs, the incidence of men and women in each group was not found to be statistically significant (p=0.690) (Figure 3).

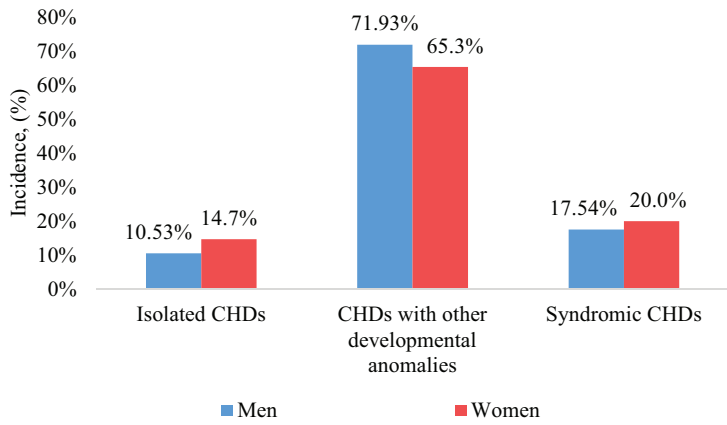


FIGURE 3. Sex distribution among the subjects in the different CHD groups.

Based on the pathomorphological classification of CHDs (Anderson et al. 2013), the CHDs observed in the study group were divided into four groups by cardiological phenotype, two of which were further divided into smaller subgroups. In total, 189 cases of congenital heart defects were detected in the study group (39 subjects had complex CHDs) (Table 1). The largest group, 135/189 (71.43%) of all the congenital heart defect cases, had CHDs with normal cardiac segmental con-

nections; CHDs with abnormal cardiac segmental connections comprised 15/189 (7.94%) congenital heart defect cases, major vascular anomalies were seen in 36/189 (19.05%) congenital heart defect cases, and in 3/189 (1.59%) congenital heart defect cases, the congenital heart defect was accompanied by situs anomalies (abnormal cardiac position). Septal defects were the most common cardiac anomaly and were seen in 66/189 cases (34.92%).

TABLE 1. Incidence distribution among the cardiological phenotypes.

Morphological CHD groups	Cardiological phenotypes: subgroups	Incidence	Relative incidence (%)
CHDs with normal cardiac segmental connections	Septal defects	66	34.92
	Atrioventricular septal defects	6	3.17
	Atrioventricular valve anomalies	21	11.11
	Arterial valve anomalies/Outflow tract obstructions	26	13.76
	Tetralogy of Fallot	16	8.46
	Total:	135	–
CHDs with abnormal cardiac segmental connections	Functional univentricular heart	8	4.23
	Transposition of the great arteries	3	1.60
	Double-outlet ventricle	1	0.50
	Truncus arteriosus	3	1.60
	Total:	15	–
Anomalies of great vessels		36	19.05
Situs anomalies		3	1.60
	Total:	**189**	**100**

An analysis of the connection between sex and CHD manifestation revealed a statistically significant relationship between the incidence of major vascular anomalies and sex ($p=0.029$, $p<\alpha$, $\alpha=0.05$). No other statistically significant dependence on sex was found in the other morphological groups (Figure 3). In comparison with the male group, major vascular anomalies were more frequent in the female group, with 26 (72.22%) cases detected in females as opposed to 10 (27.78%) cases in males.

The incidence of each cardiological phenotype was calculated in order to evaluate the distribution of the cardiological phenotypes among the subjects, divided into groups by CHD type (Table 2). The highest incidence of CHDs was found in the group where the CHDs were with other congenital anomalies and/or dysmorphic symptoms and accounted for 133/189 (70.37%) cardiac anomalies; 24/189 (12.7%) cardiac anomalies were identified in the group with isolated CHDs, and 32/189 (16.93%) cardiac anomalies were identified in the group with syndromic CHDs (Table 2).

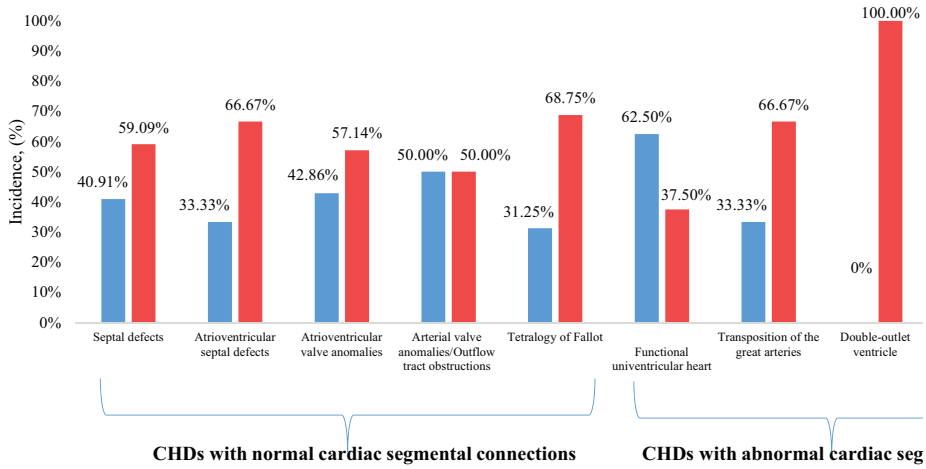


FIGURE 3. The distribution of cardiological phenotypes by sex.

TABLE 2. Incidence distribution of the cardiological phenotypes in the study group divided by CHD type.

Morphological CHD groups	Cardiological phenotypes: subgroups	Isolated CHDs	CHDs with other developmental anomalies and/or dysmorphic features	Syndromic CHDs	p value
CHDs with normal cardiac segmental connections	Septal defects	8	46	12	0.931
	Atrioventricular septal defects	0	5	1	–
	Atrioventricular valve anomalies	3	14	4	0.937
	Arterial valve anomalies/ Outflow tract obstructions	5	15	6	0.350
	Tetralogy of Fallot	6	7	3	**0.006**
CHDs with abnormal cardiac segmental connections	Functional univentricular heart	0	6	2	0.734
	Transposition of the great arteries	0	3	0	–
	Double-outlet ventricle	0	1	0	–
	Truncus arteriosus	0	3	0	–
Anomalies of great vessels		1	31	4	**0.020**
Situs anomalies		1	2	0	0.419
	Total:	24	133	32	

Statistically significant p values are highlighted in bold, $p<\alpha$, $\alpha=0.05$.

Depending on their complexity, the CHDs were divided into two groups: 1) Simple CHDs, defined as anatomically isolated (e.g., ASD, VSD and others); this group includes the compound cardiac defect known as the Tetralogy of Fallot; 2) Complex CHDs, defined as complexes of several different heart defects; this group includes the functional univentricular heart (Botto et al. 2007). Complex CHDs may be unique, i.e., seen in a single CHD patient. Upon an assessment of cardiological phenotype distribution among the subjects depending on the complexity of their HDs, simple CHDs were found to account for 93/189 (49.21%) cardiac anomalies, as opposed to 96/189 (50.79%) cardiac anomalies for complex CHDs (Table 3). Septal defects were the most common in both the simple CHD and the complex CHD groups and comprised, respectively, 42/93 (45.16%) and 24/96 (25.00%) cases.

TABLE 3. Incidence distribution among the cardiological phenotypes by CHD complexity.

Morphological CHD groups	Cardiological phenotypes: subgroups	Simple CHDs	Complex CHDs	p value
CHDs with normal cardiac segmental connections	Septal defects	42	24	**0.011**
	Atrioventricular septal defects	0	6	–
	Atrioventricular valve anomalies	5	16	**<0.001**
	Arterial valve anomalies/ Outflow tract obstructions	14	12	0.247
	Tetralogy of Fallot	13	3	**0.012**
CHDs with abnormal cardiac segmental connections	Functional univentricular heart	3	5	**0.011**
	Transposition of the great arteries	1	2	–
	Double-outlet ventricle	0	1	–
	Truncus arteriosus	1	2	–
Anomalies of great vessels		14	22	**<0.001**
Situs anomalies		0	3	–
	Total:	**93**	**96**	

Statistically significant p values are highlighted in bold, $p<\alpha$, $\alpha=0.05$.

3.2. The Detection of Copy Number Variants in the Study Group

Extensive genotyping using the SNP-CGH method was carried out for the 132 subjects. A total of 253 CNVs that meet the CNV criteria were identified. In keeping with the ACMG recommendations (Kearney et al. 2011), 202/253 (79.84%) CNVs

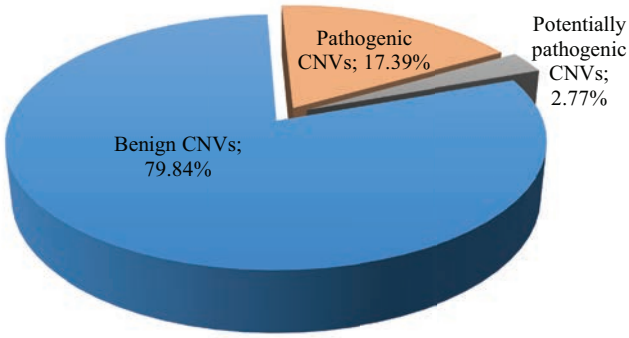


FIGURE 4. Results of the CNV study in the study group.

were classified as benign, 44/253 (17.39%) as pathogenic and 7/253 (2.77%) as potentially pathogenic with an unclear clinical significance (Figure 4).

Pathogenic CNVs were detected in 39/132 (29.55%) subjects, of whom five (3.79%) were found to have a genome with two pathogenic CNVs (Table 4). A total of 44/253 (17.39%) pathogenic CNVs were identified. Pathogenic CNVs included deletions (34/44, 77.27%) and duplications (10/44, 22.73%).

TABLE 4. Pathogenic CNVs detected in the study group.

No.	Subject IN	Sex	CNV	Chromosomal locus	Start of alteration*	End of alteration*	CNV size (kb)
1.	CG-007	F	Del	22q11.21	19068642	21339751	2271
2.	CG-010	F	Del	10q22.3q23.33	81388196	95542677	14154
3.	CG-017	F	Dup	2q34	212159787	212766614	607
4.	CG-018	F	Del	4q13.3	72647749	74208199	1560
5.	CG-019	F	Del	5p15.33q13.3	1176601	31541142	30365
6.	CG-037	F	Del	1p36.22p36.32	4236982	12602979	8366
7.	CG-039	M	Del	15q11.2	22754322	23066736	312
8.	CG-040	M	Del	22q11.21	19204210	21312429	2108
9.	CG-044	F	Del	18q22.1q23	65094544	78014582	12920
10.	CG-051	F	Del	4p16.3p16.1	85800	6523395	6438
11.	CG-052	M	Del	2q37.1q37.3	234819199	243029573	8210
12.	CG-061	F	Del	16q24.3	89243584	89596063	352
13.	CG-062	F	Del	7q11.23	73172999	74050694	878
14.	CG-067	F	Del	1q22q23.1	155630752	157193893	1563
15.	CG-070	F	Dup	14q23.1	60652103	61536933	885
16.	CG-072	F	Del	1p36.11p35.3	27862451	29004746	1142
17.	CG-074	F	Del	22q13.31q13.33	44286531	51105556	6819
18.	CG-084	F	Del	1q42.3q44	235827443	249202755	13375

No.	Subject IN	Sex	CNV	Chromosomal locus	Start of alteration*	End of alteration*	CNV size (kb)
19.	CG-089	F	Del	6q27	167615010	171115067	3500
20.	CG-090	M	Del	13q12.11 q12.12	20079051	25441945	5363
21.	CG-092	F	Del	7q11.23	72722981	74138121	1415
22.	CG-093	F	Del	22q11.21	18844632	21462353	2618
23.	CG-097	F	Dup	1q21.1q21.2	146476526	147820342	1344
24.	CG-099	M	Del	15q11.2	22754322	23080702	326
25.	CG-107	M	Dup	7q11.23	72773966	74138121	1364
26.	CG-109	M	Dup	11p15.5	419167	900809	482
			Dup	14q12	29887615	30208568	321
27.	CG-112	F	Del	18q23	76881798	78014582	1133
28.	CG-113	M	Del	4p16.3p16.1	85800	8728783	8643
29.	CG-116	M	Del	7q11.22q11.23	72722981	74138121	1415
30.	CG-117	F	Del	4p16.3p15.2	85800	21866646	21781
31.	CG-118	M	Del	15q11.2	22754322	23140114	386
32.	CG-120	F	Del	1q25.1q31.1	175629207	189971441	14342
33.	CG-124	M	Del	22q11.21	18938367	21462353	2252
34.	CG-126	M	Del	22q11.21	18967371	21462353	2495
35.	CG-132	F	Del	7q34q36.3	141245033	159119486	17874
36.	CG-008	F	Dup	15q21.2q26.3	52649116	99980473	47710
			Del	15q26.3	100052234	102397836	2346
37.	CG-078	F	Dup	3q26.2q29	168175261	197845233	29670
			Del	8p23.3p23.2	176818	3276617	3100
38.	CG-085	F	Del	4q32.3q35.2	167263766	3276617	23617
			Dup	5p15.33p15.2	38139	12392815	12355
39.	CG-106	F	Dup	7q11.21q36.3	64679561	159119486	94440
			Del	Xq25q28	128325352	154880326	25555

IN – identification number; Del – deletion; Dup – duplication; F – female; M – male; * – according to the characteristics of the chip used.

In the **syndromic CHD group**, 25/253 (9.88 %) pathogenic CNVs were identified in 25/132 (18.94 %) subjects who had a CHD and a genetic syndrome-specific phenotype, along with a confirmed one of the following genetic syndromes: DiGeorge syndrome (5/25), Williams–Beuren syndrome (3/25), 15q11.2 microdeletion syndrome (3/25), Wolf-Hirschhorn syndrome (3/25), Cri du chat syndrome (1/25), 1p36 microdeletion syndrome (1/25), 18q deletion syndrome (1/25), 2q37 monosomy syndrome (1/25), 16q24.3 microdeletion syndrome (1/25), Xia-Gibbs syndrome (1/25), Phelan-Mcdermid syndrome (1/25), 1q microdeletion syndrome (1/25), 1q21.1 recurrent multiduplication syndrome (1/25), 7q11.23 duplication syndrome (1/25), Burn-McKeown syndrome (1/25).

In the group where the **CHDs** were with **other congenital developmental anomalies and dysmorphic symptoms**, 19/253 (7.5%) pathogenic CNVs were identified in 14/132 (10.61%) subjects (one subject had two pathogenic CNVs, four had unbalanced translocations consisting of deletion and duplication). The pathogenic alterations included 11/19 deletions and 8/19 duplications. The deletions encompassed the 1q22q23.1, 1q25.1q31.1, 4q13.3, 4q32.3q35.2, 6q27, 7q34q36.3, 8p23.3p23.2, 10q22.3q23.33, 13q12.11 q12.12, 15q26.3 and Xq25q28 genomic loci, whereas the duplications encompassed 2q34, 3q26.2q29, 5p15.33p15.2, 7q11.21q36.3, 11p15.5, 14q12, 14q23.1 and 15q21.2q26.3 genomic loci.

In this group, 5/132 (3.79%) subjects were found to have 6/253 (2.37%) CNVs classified as potentially pathogenic, with an unclear clinical significance. The CNVs whose clinical significance was uncertain included 15q11.1q11.2 duplications (4/6), 10q21.3 deletion (1/6) and 1p36.32p36.31 deletion (1/6).

No pathogenic CNVs were detected in the **isolated CHD group**. One female subject (1/132, 0.76 %) was found to have a 1/253 (0.4%) CNV (15q11.1q11.2 deletion), which was classified as having uncertain clinical significance.

3.3. Diagnostic Efficiency of the Detection of Copy Number Variants in the Study Group

The incidence was 29.55% for pathogenic CNVs and 4.55% for potentially pathogenic CNVs with an unclear clinical significance. The overall diagnostic efficiency of the CNV detection in the study group was 34.10%, with the pathogenic CNVs and the potentially pathogenic CNVs with an unknown clinical significance interpreted as positive findings (Figure 5). After the exclusion of the genetic syndromes where known CHD genes had been identified, the diagnostic efficiency, with regard to the detection of new pathogenic CNVs, was found to be as high as 18.94%.

Tests were carried out to detect the established pathogenic CNVs and the potentially pathogenic CNVs with an uncertain clinical significance in the subjects' parents in order to determine whether the CNVs in question were inherited from one of the parents or arose *de novo*. The origin of the pathogenic CNVs and the potentially pathogenic CNVs with uncertain clinical significance was established for 16 subjects: 11 CNVs arose *de novo*, 2 CNVs were maternal and 3 were paternal in origin.

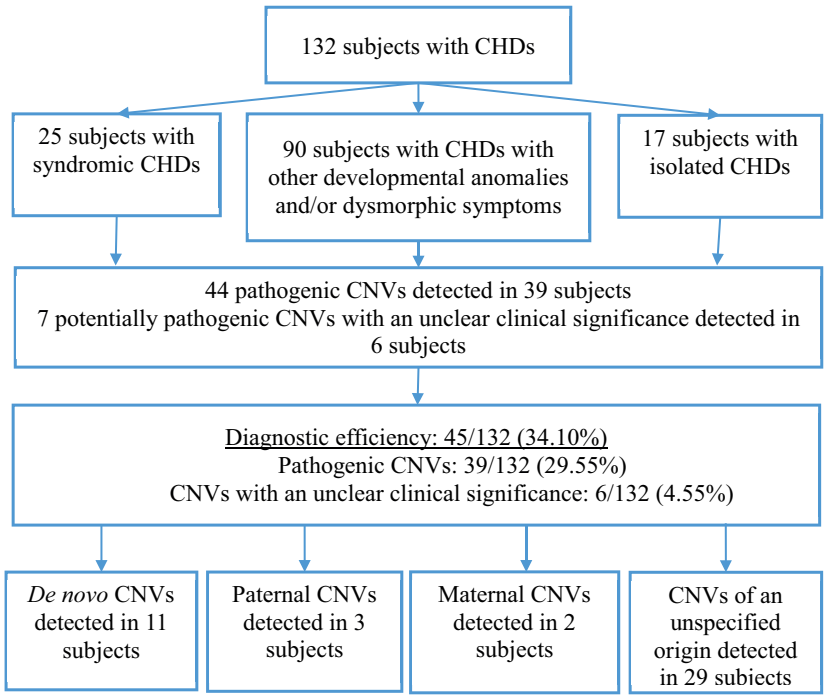


FIGURE 5. The diagnostic efficiency of the pathogenic CNV detection process.

3.4. Characterization of the CNVs Associated with Known Genetic Syndromes and CHDs

The group of subjects who had a CHD in combination with a known genetic syndrome consisted of 25/132 (18.94%) individuals. Across the entire group, 44/253 pathogenic CNVs were detected, of which 25/253 (9.88%) were found in subjects who had syndromic CHDs; 23/253 (9.1%) pathogenic CNVs overlapped with loci in the genome affected by the following microdeletion syndromes: DiGeorge syndrome (5/25), Williams-Beuren syndrome (3/25), 15q11.2 microdeletion syndrome (3/25), Wolf-Hirschhorn syndrome (3/25), Cri du chat syndrome (1/25), 1p36 microdeletion syndrome (1/25), 18q deletion syndrome (1/25), 2q37 monosomy syndrome (1/25), 16q24.3 microdeletion syndrome (1/25), Xia-Gibbs syndrome (1/25), Phelan-Mcdermid syndrome (1/25), 1q microdeletion syndrome (1/25), Burn-McKeown syndrome (1/25). The remaining 2/253 (0.78%) pathogenic CNVs overlapped with the genomic loci characteristic for the recurrent microduplication syndromes: 1q21.1 recurrent multiduplication syndrome (1/25), 7q11.23 duplication syndrome (1/25).

TABLE 5. Sixteen CNVs that caused genetic syndromes and involved CHD genes.

CNV	Chromosomal locus	CNV size (kb)	Genetic syndrome	Congenital heart defect	Known CHD genes/candidate genes
Del	22q11.21	2271	*DiGeorge*	TF	***TBX,*** *CRKL**
Del	5p15.33q13.3	30365	*Cri du chat*	ASD	***MEF2C***
Del	15q11.2	312	15q11.2 microdeletions	TV atresia, RV hypoplasy	***NIPA1***, *NIPA2*
Del	22q11.21	2108	*DiGeorge*	TF	***TBX,*** *CRKL**
Del	4p16.3p16.1	6438	*Wolf-Hirschhorn*	AoS, ASD	***EVC, EVC2***
Del	7q11.23	878	*Williams-Beuren*	Supravalvular AoS	***ELN***
Del	7q11.23	1415	*Williams-Beuren*	PAS	***ELN***
Del	22q11.21	2618	*DiGeorge*	TF	***TBX,*** *CRKL**
Del	15q11.2	326	15q11.2 microdeletions	ASD	***NIPA1***, *NIPA2*
Dup	7q11.23	1364	7q11.23 duplications	Ao regurgitation, Ao aneurysm	***ELN***
Del	4p16.3p16.1	8643	*Wolf-Hirschhorn*	ASD	***EVC, EVC2***
Del	7q11.22q11.23	1415	*Williams-Beuren*	Ao regurgitation	***ELN***
Del	4p16.3p15.2	21781	*Wolf-Hirschhorn*	ASD, PVS	***EVC, EVC2***
Del	15q11.2	386	15q11.2 microdeletions	MVP	***NIPA1***, *NIPA2*
Del	22q11.21	2252	*DiGeorge*	ASD, VSD	***TBX,*** *CRKL**
Del	22q11.21	2495	*DiGeorge*	ASD, VSD	***TBX,*** *CRKL**

Known CHD genes are highlighted in bold, * – genes identified through animal studies.
Del – deletion; Dup – duplication; Ao – aorta, AoS – aortic stenosis, RV – right ventricle, MVP – mitral valve prolapse, ASD – atrial septal defect, PVS – pulmonary valve stenosis, VSD – ventricular septal defect, TF – tetralogy of Fallot.

Of the 25 CNVs overlapping with the loci for genetic syndromes, 16 CNVs involved the genes responsible for syndromic CHDs: *TBX1*, *CRKL* (DiGeorge syndrome), *ELN* (Williams-Beuren syndrome and 7q11.23 duplication syndrome), *EVC2* and *EVC* (Wolf-Hirschhorn syndrome), *MEF2C* (Cri du chat syndrome), *NIPA1* and *NIPA2* (15q11.2 microdeletion syndrome) (Table 5). The other 9 syndromic CNVs did not involve any known CHD genes in the relevant genomic locus and were selected for further analysis and search for candidate genes.

3.4.1. Characterization of the CNVs that Include Known CHD Genes/Candidate Genes

Four (4/39) subjects were diagnosed with unbalanced translocations that include known CHD genes; therefore, these CNVs were not included in the subsequent search for CHD genes (Table 6). An unbalanced *de novo* translocation was detected in a female subject (CG-008) with an atrioventricular septal defect accompanied by a massive primary ASD, atrioventricular valve regurgitation and PDA: duplication in the 15q21.2q26.3 locus of 47710 kb in size and deletion in the 15q26.3 locus of 2346 kb in size. The duplication involved the *NR2F2* gene (MIM# 10773)

TABLE 6. Characterization of the subjects whose CNVs were found to involve known CHD genes.

Subject IN	Congenital heart defect	CNV	Chromosomal locus	CNV size (kb)	Known CHD genes/ candidate genes
CG-008	AVSD, atrioventricular valve regurgitation, PDA	Dup	15q21.2q26.3	47710	***SMAD6***, *STRA6, NRG4, CHRNA3, MESP1,* ***NR2F2***, *IGF1R, MEF2A*
		Del	15q26.3	2346	MAP2K1, ALDH1A2, FBN1
CG-078	VSD	Dup	3q26.2q29	29670	PRKCI, DVL3, ECE2
		Del	8p23.3p23.2	3100	-
CG-085	PDA, ASD	Del	4q32.3q35.2	23617	***HAND2***, *CASP3*
		Dup	5p15.33p15.2	12355	***TERT, IRX4***, *MTRR*
CG-106	ASD, VSD. Dilated pulmonary artery. Hypoplastic Ao arch and descending Ao	Dup	7q11.21q36.3	94440	***ELN***, *FKBP6, SEMA3C, SEMA3D, SRI, KRIT1, CAV1, BRAF, NOS3, SMARCD3, SHH*
		Del	Xq25q28	25555	***FLNA, GPC3, ZIC3***, *APLN, TA*

CHD genes are highlighted in bold.
IN – identification number; Del – deletion; Dup – duplication; Ao – aorta, ASD – atrial septal defect, VSD – ventricular septal defect, AVSD – atrioventricular septal defect, PDA – patent ductus arteriosus.

associated with an atrioventricular septal defect (Al Turki et al. 2014) and the CHD gene *SMAD6* (MIM# 602931); three CHD genes, *MAP2K1/MEK* (MIM# 176872), *ALDH1A2* (MIM# 603687) and *FBN1* (MIM# 134797), were detected in the locus where the deletion was observed.

An unbalanced translocation of paternal origin was detected in a female subject with VSD (CG-078). A deletion in the 8p23.3p23.2 genomic locus of 3100 kb in size and a duplication in the 3q26.3q29 genomic locus of 29670 kb in size, which involved the CHD candidate genes *PRKCI* (MIM# 600539), *DVL3* (MIM# 601368) and *ECE2* (MIM# 610145), were detected.

An unbalanced translocation was detected in a female subject diagnosed with ASD and PDA (CG–085): a 4q32.3q35.2 deletion of 23617 kb in size involving the CHD gene *HAND2* (MIM# 602407), a duplication of 12355 kb in size involving the CHD candidate genes *TERT* (MIM# 187270) and *IRX4* (MIM# 606199) and localized in the affected locus.

An unbalanced *de novo* translocation was detected in a female subject with ASD, VSD, a dilated pulmonary artery, a hypoplastic aortic arch and a descending aorta (CG–106): a duplication in the q11.21q36.3 genomic locus of the 7 chromosome of 94440 kb in size and a deletion in the q25q28 genomic locus of the X chromosome of 25555 kb in size. The duplication involved the CHD gene *ELN* (MIM# 130160), whereas the deletion involved the non-syndromic CHD gene *ZIC3* (MIM# 300265), the *FLNA* gene (MIM# 300017), which is responsible for cardiac valvular dysplasia, and another CHD gene, *GPC3* (MIM# 30037).

3.5. Identification and Characterization of the New CHD Genes

During the initial analysis, 20/253 pathogenic CNV variants with no known CHD genes involved were identified and selected for further analysis and search for CHD genes. These pathogenic variants were not found in the control group (355 control subjects with 545 CNVs) and did not overlap with the polymorphic CNV loci described in the DGV database. The scheme for the detection of CNVs and the search for candidate CHD genes is presented in Figure 6.

Out of 44/253 pathogenic CNVs, 16/44 CNVs overlapped with the genomic sequences corresponding to known syndromes and included known CHD genes. 8/44 CNVs consisted of unbalanced translocations and included known CHD genes. Therefore, an analysis of the genes involved in 20 CNVs was performed to detect new candidate genes for CHD (Table 7).

TABLE 7. CNVs selected for the search for CNV candidate genes and for further analysis.

CNV	Chromosomal locus	CNV size (kb)	Genic syndrome	Origin	Congenital heart defect
Del	10q22.3q23.33	14154	-	*de novo*	ASD
Dup	2q34	607	-	n/a	PDA, TV regurgitation
Del	4q13.3	1560	-	maternal	Ao valve anomaly
Del	1p36.22p36.32	8366	1p36 microdeletion	*de novo*	VSD, PDA, TV atresia, RV hypoplasy
Del	18q22.1q23	12920	18q deletion	*de novo*	PVS
Del	2q37.1q37.3	8210	2q37 monosomy	n/a	VSD
Del	16q24.3	352	16q24.3 microdeletion	n/a	AVSD
Del	1q22q23.1	1563	-	*de novo*	ASD, PDA
Dup	14q23.1	885	-	n/a	ASD, PVS
Del	1p36.11p35.3	1142	*Xia-Gibbs*	n/a	ASD, PAS
Del	22q13.31q13.33	6819	*Phelan-Mcdermid*	n/a	VSD
Del	1q42.3q44	13375	1qter microdeletion	n/a	VSD, ASD
Del	6q27	3500	-	*de novo*	AoS
Del	13q12.11q12.12	5363	-	*de novo*	MVP
Dup	1q21.1q21.2	1344	1q21.1 recurrent microdupli-cation	maternal	VSD, CA
Dup Dup	11p15.5 14q12	482 321	-	n/a	Ao valve regurgitation
Del	18q23	1133	*Burn-McKeown*	n/a	MVP, aortic and mitral valve defects
Del	1q25.1q31.1	14342	-	n/a	VSD, ASD, PDA
Del	7q34q36.3	17874	-	n/a	VSD

Del-deletion; Dup-duplication; n/a – no data available.
Ao – aorta, AoS – aortic stenosis, ASD – atrial septal defect; AVSD – atrioventricular septal defect, CA – coarctation of the aorta, MVP – mitral valve prolapse, PAS – pulmonary artery stenosis, PDA – patent ductus arteriosus, PVS – pulmonary valve stenosis, RV – right ventricle, VSD – ventricular septal defect, TF – tetralogy of Fallot, TV – tricuspid valve.

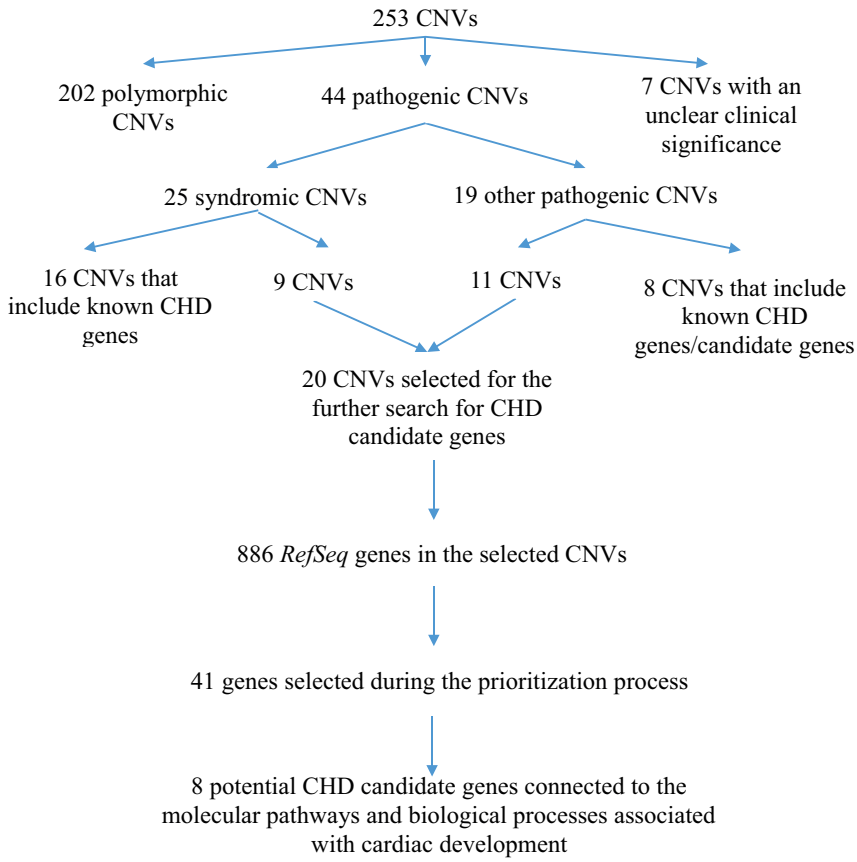


FIGURE 6. Scheme for the detection of CNVs and search for new CHD candidate genes.

3.5.1. Analysis of the Genes Rncompassed by Pathogenic CNVs

The molecular pathways and networks, as well as the candidate genes involved in the deletions and duplications, were analyzed separately. There were 840 genes involved in 15 deletions and 46 genes involved in 5 duplications.

During the study, 20/253 genes found in the loci of the pathogenic CNVs were compared to the CHD candidate genes from published listings (Tomita-Mitchell et al. 2012; Sanchez-Castro et al. 2016). Forty-one CHD candidate gene, mentioned in at least two other bioinformation sources, was selected (Table 8). Eight of the selected genes, which are known to be CHD genes (*ANKRD1, GJA5, ACP6, BCL9, CHD1L, FMO5, PRKAB2,* and *HRAS*), were not included in the further screening for CHD candidate genes.

TABLE 8. 41 CHD candidate genes selected in accordance with published CHD gene listings.

CNV	Chromosomal locus	Known CHD genes/candidate genes
Del	10q22.3q23.33	*BMPR1A*, PTEN*,* ***ANKRD1****
Dup	2q34	*ERBB4*•
Del	4q13.3	*ADAMTS3*▲*, ANKRD17*▲
Del	1p36.22p36.32	*MTHFR*, CLCN6*, NPPA*, NPPB**
Del	18q22.1q23	*NFATC1*, KCNG2**
Del	2q37.1q37.3	*TRPM8, PER2*
Del	16q24.3	-
Del	1q22q23.1	*LMNA**
Dup	14q23.1	*MNAT1*
Del	1p36.11p35.3	*TRNAU1AP*
Del	22q13.31q13.33	*CELSR1, MAPK12*
Del	1q42.3q44	*MTR, CHRM3*
Del	6q27	*ERMARD, TCTE3*
Del	13q12.11q12.12	*IFT88*, FGF9**
Dup	1q21.1q21.2	***GJA5***▲***, ACP6***▲***, BCL9***▲***, CHD1L***▲***, FMO5***▲***, PRKAB2***▲
Dup	11p15.5	***HRAS***•***,*** *TALDO1*•
Dup	14q12	*PRKD1*•
Del	18q23	*NFATC1, TXNL4A*
Del	1q25.1q31.1	-
Del	7q34q36.3	*PRKAG2*•*, KCNH2*•*, NOS3*•*, SMARCD3*•*, SHH*•

Del – deletion; Dup – duplication; Known CHD genes are highlighted in bold; *– genes detected *de novo* CNV; ▲ – genes detected in maternal CNV; • – genes detected in CNV of unknown origin.

The next stage in the gene prioritization process was carried out using the ToppGene network (Chen et al. 2009). The study revealed that 18 of the genes associated with GO biological processes were annotated in the program: "GO:0072358 *Cardiovascular system development*," "GO:*0072359 Circulatory system development*," "GO:0007507 *Heart development*," "GO:0048738 *Cardiac muscle tissue development*," "GO:0060043 *Regulation of cardiac muscle cell proliferation,*" "GO:0003007 *Heart morphogenesis*" (Table 9).

During the following stage, the Gene Expression Database (GXD) in the Mouse Genome Informatics (MGI) (http://www.informatics.jax.org/expression.shtml/) database was used to analyze the gene expression patterns peculiar to cardiac embryogenesis in mice. The list of CHD candidate genes was further prioritized depending on gene expressions in the endothelium, the heart and the valves during embryogenesis. It was established that 14 genes were annonated in the MGI database and asso-

TABLE 9. Statistically significant ontological (GO) and *MGI* terms of genes encompassed by rare CNVs

Identification number	Definition	Genes	Gene number	Annotated genes	p value
Molecular function					
GO:0004672	Protein kinase activity	*FGF9, BMPR1A, ERBB4, PRKD1, MAPK12, PRKAG2, MNAT1, KCNH2*	8	649	7.956E-6
GO:0016772	Transferase activity, transferring phosphorus-containing groups	*FGF9, BMPR1A, ERBB4, PRKD1, MAPK12, PRKAG2, MNAT1,KCNH2*	8	1006	1.801E-4
GO:0070851	Growth factor receptor binding	*FGF9, ERBB4, PTEN*	3	143	1.692E-3
Biological process					
GO:0072358	Cardiovascular system development	*ANKRD17, NPPA, FGF9, NPPB, BMPR1A, ERBB4, PRKD1, PTEN, LMNA, NFATC1, IFT88, MNAT1, SHH, SMARCD3, NOS3*	15	1058	2.393E-11
GO:0072359	Circulatory system development	*ANKRD17, NPPA, FGF9, NPPB, BMPR1A, ERBB4, PRKD1, PTEN, LMNA, NFATC1, IFT88, MNAT1, SHH, SMARCD3, NOS3*	15	1058	2.393E-11
GO:0007507	Heart development	*NPPA, FGF9, BMPR1A, ERBB4, PTEN, LMNA, NFATC1, IFT88, MNAT1, SHH, SMARCD3*	11	593	1.477E-9
GO:0048738	Cardiac muscle tissue development	*NPPA, FGF9, ERBB4, PTEN, LMNA, IFT88*	6	215	1.278E-6
GO:0060043	Regulation of cardiac muscle cell proliferation	*FGF9, ERBB4, PTEN*	3	34	2.413E-5
GO:0003007	Heart morphogenesis	*FGF9, BMPR1A, NFATC1, IFT88, SHH, SMARCD3*	6	252	2.116E-6
Mouse phenotypes					
MP:0005294	Abnormal heart ventricle morphology	*ANKRD17, NPPA, FGF9, ERBB4, PRKD1, PTEN, LMNA, NFATC1, IFT88, NOS3*	10	745	1.479E-5
MP:0000266	Abnormal heart morphology	*ANKRD17, NPPA, FGF9, BMPR1A, ERBB4, PRKD1, PRKAG2, PTEN, LMNA, NFATC1, IFT88, KCNH2, SHH, NOS3*	14	1465	6.869E-6
MP:0000267	Abnormal heart development	*ANKRD17, FGF9, BMPR1A, ERBB4, PTEN, LMNA, NFATC1, IFT88, KCNH2, SHH*	10	388	4.036E-8
MP:0003921	Abnormal heart left ventricle morphology	*NPPA, FGF9, ERBB4, PRKD1, PTEN, LMNA, NFATC1, NOS3*	8	343	2.492E-6
MP:0000288	Abnormal pericardium morphology	*ANKRD17, BMPR1A, PTEN, NFATC1, IFT88, KCNH2, SHH*	7	259	4.586E-6

ciated with the manifestation of CHDs in mice, with the following *MGI* terms used: "MP:0005294 *Abnormal heart ventricle morphology*," "MP:0000266 *Abnormal heart morphology*," "MP:0000267 *Abnormal heart development*," "MP:0003921 *Abnormal heart left ventricle morphology*," "MP:0000288 *Abnormal pericardium morphology*" (Table 9).

3.5.2. Characterization of the New CHD Candidate Genes

Eight new CHD candidate genes were identified during the prioritization process: *FGF9, BMPR1A, PTEN, ANKRD17, NPPA, LMNA, NFATC1* and *IFT88*. The CHD candidate genes were detected in pathogenic CNVs, of which five genomic alterations arose *de novo* and one genomic alteration was of maternal origin. These genes are involved in important embryonic development processes, such as Akt, BMP, and Wnt on signaling pathways and chromatin remodelling.

Two *de novo* deletions were detected in chromosome 1. One *de novo* deletion in the 1p36.22p36.32 locus of 8366 kb in size, which involved the CHD candidate gene *NPPA* (MIM#108780), was detected in a female subject (CG-037) with an RV hypoplasy, VSD, TV atresia and PDA. Another *de novo* interstitial deletion in the 1q22q23.1 locus of 1563 kb in size, which involved the CHD candidate gene *LMNA* (MIM#150330), was detected in a female patient with an ASD and PDA (CG-067).

The CHD candidate gene *ANKRD17* (MIM#615929) was localized in the genomic locus of 4q13.3. A female patient with an aortic valve anomaly (CG-018) was found to have a deletion in the 4q13.3 locus of 1560 kb in size, which she inherited from her mother, who has hypertrophic cardiomyopathy.

Two genes associated with CHDs, *BMPR1A* (MIM#601299) and *PTEN* (MIM#601728), were found to be involved in a *de novo* deletion in the genomic locus of 10q22.3q23.33 of 14154 kb in size. The cardiological phenotype of the female subject in question (CG-010) is ASD.

The *de novo* deletion in the genomic locus of 13q12.11q12.12 of 5363 kb in size involved the CHD candidate genes *IFT88* (MIM#600595) and *FGF9* (MIM#176943). The deletion was detected in a male subject with MVP (CG-090).

The CHD candidate gene *NFATC1* (MIM#600489) was identified in the genomic locus of 18q23. Two female patients with abnormal heart valve formations were discovered to have several deletions in the long arm of chromosome 18. A female patient with pulmonary valve stenosis (CG-044) was found to have a *de novo* deletion in the 18q22.1q23 locus of 12920 kb in size. Another female patient with MVP and abnormal aortic formation (CG-112) had a deletion in the 18q23 locus of 1133 kb in size, whose origin could not be determined.

3.6. Comparison of CNVs in the Study and Control Groups

3.6.1. Comparison of CNV Incidence in the Study and Control Groups

Based on the initial results of this study and the results of similar studies published in the past (Kaminsky et al. 2011; Xie et al. 2017), the hypothesis that rare genic CNVs may be relevant for the manifestation of CHDs was formulated. In order to evaluate the incidence distribution of the rare CNVs in the study and control groups, CNVs were categorized according to the type of chromosomal changes (deletions or duplications) and grouped into five categories: genic CNVs, common CNVs, common genic CNVs, rare CNVs and rare genic CNVs. The CNVs that occurred with a frequency of less than 1% and overlapped with the CNVs provided in the DGV database to a minimal extent (less than 30%) or not at all were regarded as rare. The CNVs that encompassed a *RefSeq* gene sequences were defined as genic. The CNVs that overlapped with at least one of the CNVs provided in the DGV database with a frequency of over 70% were regarded as common. CNV distribution was compared between the study group (132 individuals), where 253 CNVs were detected, and the control group (355 individuals), where 545 CNVs were detected.

TABLE 10. CNV incidence in the study group consisting of subjects with CHDs and in the control group.

CNV category	Study group 253 CNVs	Control group 545 CNVs	Ratio	p value
	CNV (%)	CNV (%)		
Rare genic	64 (25.30)	52 (9.54)	**2.65**	<0.0001
Rare	69 (27.27)	70 (12.84)	**2.12**	<0.0001
Common genic	133 (52.57)	274 (50.28)	**1.05**	0.0086
Common	184 (72.73)	475 (87.16)	**0.83**	0.0003
Genic	197 (77.87)	326 (59.82)	**1.30**	<0.0001

Significant differences in the CNV categories are highlighted in bold.

Insofar as the incidence distribution of genic CNVs in the study group and control group (Table 10) is concerned, all genic CNVs were 1.3 times more common in subjects with CHDs (77.87% in the study group and 59.82% in the control group), and the difference was statistically significant ($p<0.0001$). Common genic CNVs were also significantly more prevalent in subjects with CHDs ($p=0.0086$). Rare genic CNVs were 2.65 times more common in subjects with CHDs (25.30% in the study group, 9.54% in the control group), and the difference was statistically significant ($p<0.0001$).

A comparative analysis of the common CNV distribution in the study and control groups revealed that the incidence of common CNVs in the control group was statistically higher (p=0.0003). There was no significant difference between the groups when it came to common genic CNVs. However, all rare CNVs manifested 2.12 times more frequently in the study group (27.27% in the study group, 12.84% in the control group) and the difference was significant (p<0.0001).

TABLE 11. Deletion incidence in the study group consisting of subjects with CHDs and in the control group.

Deletions	Study group 253 CNVs	Control group 545 CNVs	Ratio	p value
	CNV (%)	CNV (%)		
Rare genic	36 (43.37)	27 (10.80)	**4.02**	<0.0001
Rare	39 (46.99)	36 (14.40)	**3.26**	<0.0001
Common genic	29 (34.94)	112 (44.80)	0.78	0.1961
Common	44 (53.01)	214 (85.60)	0.62	0.3845
Genic	65 (78.31)	139 (55.60)	**1.41**	<0.0001

The significant differences in deletion distribution are highlighted in bold.

Upon analyzing the incidence of rare CNVs in the deletion category, rare deletions were found to be significantl more common (3.26-fold) among subjects with CHDs (46.99% in the study group and 14.40% in the control group) (p<0.0001). Meanwhile, the incidence of common deletions was higher in the control group than in subjects with CHDs, but the difference was not significant. A significant difference (p<0.0001) in the distribution of all genic deletions in the study and control groups was observed, with these deletions being 1.41 times more common in the study group (78.31% in the study group and 55.60% in the control group). There was no significant difference between the two groups as far as the incidence of common genic deletions was concerned (p=0.1961). However, rare genic deletions were significantly (4.02-fold) more common (p <0.0001) in the study group (43.37% in the study group and 10.80% in the control group) (Table 11).

Upon comparing the incidence distribution of duplications in the two groups, a significant difference was found in the category of rare duplications, which were 1.53 times more common in the study group than in the control group (p=0.0076) (Table 12). The incidence of common duplications was significantly higher in the control group (p=0.0015), but common genic duplications were significantly (1.11-fold) more prevalent in the study group (61.18% in the study group, 54.2% in the control group). Upon assessing the distribution of all genic duplications in the study and control groups, it was discovered that genic duplications were 1.22 times more common in subjects with CHDs (77.65% in the study group and 63.39% in the con-

trol group) and the difference was statistically significant (p=0.0001). Rare genic duplications were also significantly (1.94-fold) more common in the study group (16.47% in the study group and 8.47% in the control group) (p=0.0037). There was a statistically significant difference between the study and control groups with regard to the incidence of common genic duplications (p=0.0205) (Table 12).

TABLE 12. Incidence of duplications among subjects treated for CHDs as compared to the control group.

Duplications	Study group 253 CNVs	Control group 545 CNVs	Ratio	p value
	CNV (%)	CNV (%)		
Rare genic	28 (16.47)	25 (8.47)	**1.94**	0.0037
Rare	30 (17.65)	34 (11.53)	**1.53**	0.0076
Common genic	104 (61.18)	162 (54.92)	**1.11**	0.0205
Common	140 (82.35)	261 (88.47)	**0.93**	0.0015
Genic	132 (77.65)	187 (63.39)	**1.22**	0.0001

The significant differences in duplication distribution are highlighted in bold.

3.6.2. Comparison of CNV Incidence between the Groups according to CNV Size (≤500kb ir ≥501kb)

To evaluate the incidence distribution of CNVs in the study and control groups, the CNVs were categorized according to the type of chromosomal changes (deletions or duplications) and classified into five categories: genic CNVs, common CNVs, common genic CNVs, rare CNVs, and rare genic CNVs. In light of the evidence regarding the mechanisms behind large CNV formation, which has been presented in the relevant scientific literature (Redon et al. 2006), the CNVs examined in the study were additionally divided into two groups: ≤500kb and ≥501kb.

Upon evaluating the distribution of all CNVs the size of which was less than or equal to 500 kb in the study and control groups, statistically significant differences were found in the categories of genic and common genic CNVs: genic CNVs had manifested 1.27 times more frequently (75.15% in the study group, 59.35% in the control group) (p=0,0002), while common genic CNVs were 1.25 times more common in subjects with CNVs than in the control group (62.13% in the study group, 49.59% in the control group) (p=0.0049). A 1.33-fold higher incidence was also observed in the category of rare genic CNVs, but the difference was insignificant (p=0.2473) (Table 13).

TABLE 13. CNV incidence in the study and control groups according to size.

CNV size	CNV category	Relative incidence (%)		Ratio	p value
		Study group	Control group		
≤500 kb	Rare genic	13.02	9.76	1.33	0.2473
	Rare	14.20	12.80	1.11	0.6925
	Common genic	62.13	49.59	**1.25**	0.0049
	Common	85.80	87.20	0.98	0.6432
	Genic	75.15	59.35	**1.27**	0.0002
≥501 kb	Rare genic	50.00	7.55	**6.63**	<0.0001
	Rare	53.57	13.21	**4.06**	<0.0001
	Common genic	33.33	56.60	**0.59**	0.0073
	Common	46.43	86.79	**0.53**	<0.0001
	Genic	83.33	64.15	**1.30**	0.0106

The significant differences in CNV distribution are highlighted in bold.

A similar significant difference in incidence was found among deletions whose size was equal to or exceeded 500 kb: genic deletions were 1.29 times more common in subjects with CHDs (70.00% in the study group and 54.47% in the control group) (p=0.0439). No significant difference was found upon comparing the incidence of common and common genic deletions in the study and control groups (p=0.7202 and p=0.1169, respectively). Rare and rare genic deletions the size of which was equal to or less than 500 kb were more common in subjects with CNVs than in members of the control group, with the difference being insignificant (p=0.7202 and p=0.4942, respectively) (Table 14).

TABLE 14. Incidence of deletions in the study and control groups according to size.

CNV size	Deletions	Relative incidence (%)		Ratio	p value
		Study group	Control group		
≤500 kb	Rare genic	14.00	10.64	1.32	0.4942
	Rare	16.00	14.04	1.14	0.7202
	Common genic	56.00	43.83	1.28	0.1169
	Common	84.00	85.96	0.98	0.7202
	Genic	70.00	54.47	**1.29**	0.0439
≥501 kb	Rare genic	87.88	13.33	**6.59**	<0.0001
	Rare	93.94	20.00	**4.70**	<0.0001
	Common genic	3.03	60.00	**0.05**	<0.0001
	Common	6.06	80.00	**0.08**	<0.0001
	Genic	90.91	73.33	1.24	0.1830

The significant differences in deletion distribution are highlighted in bold.

The incidence distribution of the rare genic duplications the size of which was less than or equal to 500 kb was 1.41 times higher in the study group, but the difference was not significant (p=0.2740) (Table 15). No significant difference was found upon comparing the incidence of rare duplications the size of which was less than or equal to 500 kb in the study and control groups (p=0.6257). Common genic duplications of the same size were 1.18 times more common in the study group, but the difference was statistically negligible (p=0.0721). As far as the incidence of common duplications whose size was less than or equal to 500 kb were more common in the control group, with the difference being insignificant (p=0.6257). Genic duplications were significantly more common in the control group in comparison with the study group (p<0.0001) (Table 15).

Below, we shall present a comparative analysis of CNVs that encompassed large genomic changes, i.e., that exceeded or were equal to 501 kb in size, in the study and control groups. Genic CNVs whose size exceeded or was equal to 501 kb were found to be 1.3 times more common in subjects from the study group (83.33% in the study group, 64.15% in the control group), and the difference was statistically significant (p=0.0106) (Table 13).

TABLE 15. Incidence of duplications in the study and control groups according to size.

CNV size	Duplications	Relative incidence (%)		Ratio	p value
		Study group	Control group		
≤500 kb	Rare genic	12.61	8.95	1.41	0.2740
	Rare	13.45	11.67	1.15	0.6257
	Common genic	64.71	54.86	1.18	0.0721
	Common	86.55	88.33	0.98	0.6257
	Genic	77.31	97.28	**0.79**	<0.0001
≥501 kb	Rare genic	25.49	5.26	**4.84**	0.0117
	Rare	27.45	10.53	**2.61**	0.0493
	Common genic	52.94	55.26	0.96	0.8279
	Common	72.55	89.47	**0.81**	0.0492
	Genic	78.43	60.52	**1.30**	0.0020

The significant differences in duplication distribution are highlighted in bold.

Rare CNVs the size of which exceeded or was equal to 501 kb differed significantly between the study and control groups, and manifested 4.06 times more frequently in subjects from the study group (53.57% in the study group, 13.21% in the control group) (p<0.0001). The difference was even greater in the category of rare deletions the size of which exceeded or was equal to 501 kb, which were significantly

(4.7-fold) more common (p<0.0001) in the study group (93.94% in the study group, 20.00% in the control group) (Table 14). Meanwhile, rare duplications the size of which exceeded or was equal to 501 kb were 2.61 times more common in the study group (27.45% in the study group, 10.53% in the control group), and this difference was significant (p=0.0493) (Table 15).

The results of the study demonstrated that the greatest significant differences between the study and control groups with respect to CNVs equal to or over 501 kb in size were to be found in the category of rare genic CNVs, which were 6.63 times more common (p<0.0001) in subjects from the study group treated for CHDs (50.00% in the study group, 7.55% in the control group) (Table 13). It is obvious that the aforementioned significant difference (p<0.0001) was determined by the high incidence of rare genic deletions, which were 6.59 times more common in the study group (87.88% in the control group, 13.33% in the control group) (Table 14). Rare genic duplications also manifested 4.84 times more frequently (p=0.0117) in the study group as opposed to the control group (Table 15).

3.7. Comparison of the CNV Properties and the Functional Impact Thereof in the Study and Control Groups

3.7.1. Comparison of CNV Size in the Study and Control Groups

A comparison of CNV properties in the study and control groups was carried out (Table 16). Two hundred fifty-three CNVs were detected in the study group, which consisted of 132 subjects. The number of CNVs per subject was 1.92. The size of the CNVs in the study group varied from 21 kb to 94440 kb, the average CNV size was 2019 ± 7794.43 kb, the median CNV size was 286 kb.

Five hundred forty-five CNVs were detected in the control group, which consisted of 355 subjects. The number of CNVs per subject was 1.54. The size of the

TABLE 16. Comparison between the properties of the CNVs in the study and control groups.

CNV properties	Study group 253 CNVs	Control group 545 CNVs
Average number of CNVs per individual	1.92	1.54
Average number of SNPs corresponding to one CNV	210	56
Average CNV size (kb)	2019	240
Median CNV size (kb)	286	141
CNV size range	21–94440	28–3941
Proporion of CNVs ≤500 kb	0.67	0.90
Proporion of large (≥501 kb) CNVs	0.33	0.10

CNVs varied from 28 kb to 3941 kb, with an average CNV size of 240 ± 364.75 kb and a median CNV size of 140 kb.

Upon comparing the median size of the CNVs in the study and control groups, the median CNV size was found to be greater in the study group, and the difference was statistically significant ($p<0.0001$).

In order to compare and characterize the properties of CNVs in the study and control groups, the CNVs were categorized according to the type of chromosomal alteration (deletions and duplications). The 253 CNVs found in the study group included 84 deletions (33.20%) and 169 duplications (66.80%) (Table 17). Deletion sizes varied from 21 kb to 30.365 kb, the average deletion size was 3080 ± 6310.63 kb, the median size was 319 kb. Duplication sizes varied from 46 to 94440 kb, the average duplication size was 1492 ± 8394.98 kb, the median size was 283 kb (Table 17).

TABLE 17. Comparison between the properties of the deletions and duplications in the study and control groups.

CNV type	Study group 253 CNVs	Control group 545 CNVs
Deletions		
Total number	84	250
Proportion of the total CNV number	0.33	0.46
Average size (kb)	3080	166
Median size (kb)	319	123
Size range	21–30365	28–1335
Duplications		
Total number	169	295
Proportion of the total CNV number	0.67	0.54
Average size (kb)	1492	304
Median size (kb)	283	151
Size range	46–94440	41–3941

Deletions in the control group accounted for 45.87% of the CNVs (250 deletions), while duplications accounted for 54.13% (295 duplications). The size of the deletions varied from 28 kb to 1335 kb, the average deletion size was 166 ± 178.26 kb, the median size was 123 kb. The size of the duplications varied from 41 kb to 3941 kb, the average duplication size was 304 ± 458.78 kb, the median size was 151 kb (Table 17).

A comparison of the median deletion size between the study and control groups revealed that the median size in the study group exceeded that in the control group 2.59-fold, and that the difference was statistically significant ($p<0.0001$). The median size of the duplications also differed significantly between the study and control groups and was 1.87 times greater in the study group ($p <0.0001$) (Table 18).

TABLE 18. Comparison between the median size of the deletions and duplications in the study and control groups.

CNV type	Group	Median size (kb)	Study group as opposed to control group	
			Ratio	p value
Deletions	Study group	319	**2.59**	<0.0001
	Control group	123		
Duplications	Study group	283	**1.87**	<0.0001
	Control group	151		

Significant differences in median size are highlighted in bold.

3.7.2. Comparison between the Number of Genes Encompassed by CNVs in the Study and Control Groups

An analysis of genic CNVs revealed a significant difference between the mean number of CNV-encompassed genes in the study and the control groups (Table 19). In the study group, the mean number of genes encompassed by CNVs was 3.41 times higher (9.38 ± 18.71 in the study group and 2.75 ± 2.76 in the control group) in all CNV categories, with the difference being statistically significant (p<0.0001). Common CNVs encompassed 1.75 times more genes in the study group as opposed to the control group (4.64 ± 5.98 in the study group and 2.65 ± 2.68 in the control group) (p=0.0071). As far as rare CNVs were concerned, the average number of the encompassed genes was even greater, and was as much as 6.96 times higher in the study group than in the control group (23.05 ± 31.95 in the study group and 3.31 ± 3.12 in the control group). The statistical difference in this regard between the study and control groups was significant (p <0.0001).

TABLE 19. Comparison between the average number of CNV-encompassed genes in the study and control groups.

CNV category	Study group	Control group	Study group as opposed to control group	
	$\bar{x} \pm s$	$\bar{x} \pm s$	Ratio	p value
Rare	23.05 ± 31.95	3.31 ± 3.12	**6.96**	<0.0001
Common	4.64 ± 5.98	2.65 ± 2.68	**1.75**	0.0071
All	9.38 ± 18.71	2.75 ± 2.76	**3.41**	<0.0001

$\bar{x} \pm s$ – mean value. The significant differences in mean value are highlighted in bold.

Upon analyzing the differences in the average number of genes encompassed by CNVs in the duplication category, it was found that common duplications en-

compassed 2.27 times significantly more genes (5.54 ± 6.41 in the study group and 2.44 ± 2.65 in the control group) and the difference was statistically significant (p <0.001); rare duplications encompassed 3-times more genes, and the difference was likewise statistically significant (p=0.0020) (8.27 ± 7.83 in the study group and 2.76 ± 3.65 in the control group) (Table 20). An even greater difference in the average number of genes between the study and control groups was observed in the deletion category. In the case of all deletions, the mean number of genes was 5.8 times higher in the study group than in the control group (18.11 ± 32.56 in the study group and 3.12 ± 2.67 in the control group), and the difference was statistically significant (p=0.0035). In the case of rare deletions, the mean number of genes in the study group was 10.31 times higher (39.30 ± 40.05 in the study group and 3.81 ± 2.48 in the control group) compared to the control group, with the difference being statistically significant (p=0.0010). The mean number of genes encompassed by common deletions differed significantly between the control and control groups (p <0.0001) (Table 20).

TABLE 20. Comparison between the average number of genes involved in deletions/duplications in the study and control groups.

CNV type	CNV category	Study group	Control group	Study group as opposed to control group	
		$\bar{x} \pm s$	$\bar{x} \pm s$	Ratio	p value
Deletions	Rare	39.30 ± 40.05	3.81 ± 2.48	**10.31**	0.001
	Common	1.16 ± 0.55	2.96 ± 2.70	**0.39**	<0.0001
	All	18.11 ± 32.56	3.12 ± 2.67	**5.80**	0.0035
Duplications	Rare	8.27 ± 7.83	2.76 ± 3.65	**3.00**	0.002
	Common	5.54 ± 6.41	2.44 ± 2.65	**2.27**	<0.0001
	All	6.05 ± 6.74	2.48 ± 2.79	**2.44**	<0.0001

$\bar{x} \pm s$ – mean value. The significant differences in mean value are highlighted in bold.

2.7.3. Comparison between the Haploinsufficiency (HI) Score of Genes Encountered in Rare CNVs in the Study and Control Groups

The results of the study demonstrated that the genes encompassed by rare CNVs were associated with a lower genes haploinsufficiency (HI) score, which indicates the dose sensitivity of the gene (Huang et al. 2010). Upon comparing the mean value for the number of all CNV-related genes in the study and control groups, it was found that in the study group, the CNVs encompassed 1.94 times more HI genes, with the difference being statistically significant (p <0.0001) (Table 21). Rare CNVs encompassed 2.45 times more HI genes in subjects with CHDs than in members of the control group, and the difference was statistically significant (p=0.0187). With

regard to common CNVs, there was no significant difference between the average number of CNV genes in the study and control groups (p=0.6010) (Table 21).

TABLE 21. Comparison between the average number of HI genes encompassed by CNVs in the study and control groups.

CNV category	Study group	Control group	Study group as opposed to control group	
	$\bar{x} + s$	$\bar{x} + s$	Ratio	p value
Rare	245 ± 2.52	1.00 ± 0.00	**2.45**	0.0187
Common	1.00 ± 0.00	1.04 ± 0.20	0.96	0.6010
All	2.00 ± 2.19	1.03 ± 0.18	**1.94**	<0.0001

$\bar{x} + s$ – mean value. The significant differences in mean value are highlighted in bold.

In the case of deletions, the average number of HI genes was significantly higher in the study group than in the control group (p=0.002). Meanwhile, in the case of duplications, a slight 1.15-fold difference was observed between the average number of HI genes in the study and control groups, but the difference had no statistical significance (p=0.7180) (Table 22). The average number of HI genes did not differ between the study and control groups in the case of common deletions and common duplications.

TABLE 22. Comparison of the average number of HI genes involved in deletions/duplications in the study and control groups.

CNV type	Study group	Control group	Study group as opposed to control group	
	$\bar{x} + s$	$\bar{x} + s$	Ratio	p value
Deletions	3.08 ± 3.03	1.00 ± 0.00	**3.08**	0.0020
Duplications	1.24 ± 0.75	1.08 ± 0.28	1.15	0.7180

$\bar{x} + s$ – mean value. The significant differences in mean value are highlighted in bold.

Rare duplications, which accounted for 56.25% (9/16) of all HI gene deletions in the study group, included 1.4 times more HI genes in comparison with the control group, but the difference was statistically negligible. Rare deletions, which accounted for 84.62% (11/13) of all HI gene deletions in the study group, included 3.5 times more HI genes in comparison with the control group, but the small sample size made it impossible to tell whether the difference was significant (Table 23).

TABLE 23. Comparison between the incidence of CNVs involving HI genes in the study and control groups.

CNV type	CNV category	Study group		Control group	
		Incidence	Relative incidence (%)	Incidence	Relative incidence (%)
Deletions	Rare	11	84.62	1	5.88
	Common	2	15.38	16	94.12
	All	**13**	**100**	**17**	**100**
Duplications	Rare	9	56.25	3	23.08
	Common	7	43.75	10	76.92
	All	**16**	**100**	**13**	**100**

3.8. Discussion of the Results

Since the majority of CHDs are of unknown etiology, the main objective of this piece of research was to evaluate new, clinically important, significant genomic variants in the population of subjects with CHDs. Modern extensive research revealed that CNVs are not only a source of human genetic variability, but that these genomic variants are important in the manifestation of multifactorial diseases, including heart diseases.

One hundred thirty-two subjects with CHD were examined in this piece of research. The heart diseases of the subjects typically manifested themselves as heterogeneous diseases: 12.88% were isolated CHDs, 18.94% were syndromic CHDs, and 68.18% manifested with other congenital developmental anomalies and/or dysmorphic features. The CHDs of the groups of subjects included four groups of cardiological phenotypes, the biggest group of which (71.43%) were CHDs with normal cardiac segment connections. In terms of complexity, there were two groups of CHDs – anatomically separate CHDs (49.21%) and complex CHDs (50.79%). Such a diverse manifestation of CHDs shows considerable phenotypic heterogeneity and complexity, which is typical for heart diseases. The frequency distribution of the subjects in terms of gender in all the aforementioned groups, the frequency of male and female were not statistically significantly different in all the groups ($p=0.690$). The assessment of the distribution of phenotype frequency between CHDs determined in a group of subjects, the most frequent CHDs were septal defects, which made up 48.89% of all the cases of CHDs. It coincides with the frequencies of congenital heart diseases in populations, which were described in the literature (Wang et al. 2011; Yang et al. 2012). In accordance with the literature, in terms of the manifestation of CHDs between genders, CHDs of all types manifest more frequently in females (4.55 CHDs in 1 000 females and 3.61 CHDs in 1 000 males; $p<0.0001$) (Marelli et al. 2007). When assessing the dependency of manifestation of CHDs on the gender,

no significant dependence was determined ($p>\alpha$, $\alpha=0.05$). A statistically significant dependence was determined only between the anomalies of great vessels and gender ($p=0.029$, $p>\alpha$, $\alpha=0.05$). Women had a larger number of anomalies of great vessels. In accordance with the epidemiological study data, certain types of CHDs manifest themselves by a different manifestation frequency between genders. It was determined that a coarctation of the aorta and the transposition of the great vessels, which are considered as anomalies of great vessels, manifest themselves in males more frequently than in females (Samánek 1994; Digilio et al. 2001b). Females in this group of subjects were diagnosed with anomalies of great vessels 2.6 times more frequently than males. This difference was a consequence of the structure of CHDs in the group of subjects selected.

Large-scale SNP-CGH genotyping was used and 39 (29.55%) subjects with CHDs were diagnosed with 44 pathogenic CNVs, which made up 17.39% of all CNV determined; 6 (4.55%) subjects were diagnosed with potentially pathogenic CNVs of uncertain clinical significance. The diagnostic efficiency of this piece of research was 34.1% and was higher than that in other studies described in literature. In accordance with the literature, pathogenic and/or potentially pathogenic CNVs are determined in 10–20% of all the cases of CHDs (Carey et al. 2013; Kim et al. 2016). Pathogenic CNVs are most frequently determined in patients with clinically recognizable syndromes, such as DiGeorge syndrome or Williams-Beuren syndrome, and in patients with multiple developmental abnormalities and dysmorphic features. But pathogenic CNVs are significantly more determined in the cases of isolated CHDs than in the population of healthy individuals. Deletions (77.27%) made up a major part of pathogenic CNVs. The results of this piece of research reflect the results of the studies that were published by other authors and confirm that deletions are more linked with clinical phenotypes. The genes, which take part in the processes of cardiac embryogenesis and which are located in the deletion regions, result in CHDs due to an altered expression.

Twenty-five pathogenic CNVs were determined in the group of syndromic CHDs of the group of subjects in the piece of research, 16 pathogenic CNVs of which had a clear cause of pathogenicity because they included the following CHD genes – *TBX1*, *CRKL*, *ELN*, *EVC2*, *EVC*, *MEF2C*, *NIPA1* and *NIPA2*. In accordance with the literature, CNVs result in 17–18% of cases of syndromic CHDs (Thienpont et al. 2007; Breckpot et al. 2010; Geng et al. 2014). But in some distinct studies, the diagnostic efficiency of pathogenic CNVs was 25% and even 52.7% (Richards et al. 2008; Syrmou et al. 2013). At least a single pathogenic change was determined in all the subjects of this study, who were suffering from syndromic CHDs. Including subjects with clinically recognizable genetic syndromes in this study resulted in such a high diagnostic efficiency. The most frequent genomic variant was the 22q11.2 deletion

(DiGeorge syndrome), which manifests itself in 0.5–1.9% of all cases of CHDs. In this study, 22q11.21 deletions in the genomic region, which include the *TBX1* gene in all of the cases, were determined in five patients. They made up 3.79% of all the cases of CHDs in the subject group.

Nineteen pathogenic CNVs were determined in 14 subjects in the group of CHDs with other congenital developmental anomalies and/or dysmorphic features, 8 CNVs of which included the following known genes of heart diseases: *SMAD6*, *MAP2K1/MEK1*, *FBN1*, *HAND2*, *IRX4*, *ELN*, *FLNA* and *ZIC3*. The diagnostic efficiency for determining CNVs in this group was 10.61% and was in accordance with the research data provided in the literature. In accordance with the literature, CNVs result in 3–28% of the cases of CHDs with other congenital developmental anomalies (Lalani et al. 2013; Syrmou et al. 2013; Wu et al. 2017).

No pathogenic CNVs were determined in the group of isolated CHDs, which makes up 12.88% of all the group of subjects. A potentially pathogenic 15q11.1q11.2 deletion of an uncertain clinical significance, which did not include the candidate genes of CHDs, *NIPA1* and *NIPA2*, was determined in a female patient, who had an aortic valve regurgitation and muscular VSD. Potentially pathogenic CNVs are more frequently determined in the subjects with isolated CHDs than in the subjects who do not have CHDs (Kim et al. 2016). A comparison of diagnostic efficiency in the group of isolated CHDs with syndromic CHDs and in the groups of CHDs with other congenital developmental anomalies yields that the diagnostic efficiency of CNVs is lower and accounts for 3–4.3% (Erdogan et al. 2008; Breckpot et al. 2011; Soemedi et al. 2012; Geng et al. 2014). Erdogan et al. determined that 3% of *de novo* pathogenic CNVs are related to isolated CHDs (Erdogan et al. 2008). Other pieces of research also revealed that the frequency of causal CNVs in the group of non-syndromic CHDs is lower than that in the group of syndromic CHDs – 3.6% and 19%, respectively (Breckpot et al. 2011). Geng et al. determined 4.3% of pathogenic CNVs in the group of isolated CHDs (Geng et al. 2014). Other pieces of research revealed that 4% of rare deletions are linked to the risk of sporadic CHDs (Soemedi et al. 2012). Clinically significant *de novo* CNVs were determined in 1.8% of the subjects in a study of isolated, left-sided heart failure (Hanchard et al. 2017). Although no pathogenic CNV changes were determined in the subjects with isolated CHDs in other studies, the authors say that it is possible that very small CNVs may be found in the genome of the subjects (Richards et al. 2008).

The high diagnostic efficiency in the common group of CHDs and in individual groups of CHDs proves that CGH genotyping is a genetic diagnostic method that should be chosen first for the genetic testing of patients with CHDs. This method is recommended for early genetic testing of neonates with CHDs (Bachman et al. 2015) and of children with CHDs, especially with CHDs with other developmental anoma-

lies and/or dysmorphic features (Wu et al. 2017). The diagnostic efficiency of pathogenic CNVs determined in this piece of research confirms the results of other studies.

Determining CNVs in individuals with CHDs provide a unique source for determining candidate genes of CHDs. Since pathogenic CNVs, associated with CHDs, are located in various regions of the genome and include hundreds of genes, it is important to determine the specific genes and signaling pathways that are linked with different CHDs. This, in turn, will enable the development of therapeutic strategies and the improvement of how well the risk of CHDs is assessed.

After pathogenic CNVs, which included the known CHD genes, were excluded, 20 unique rare pathogenic CNVs, which were selected for functional analysis, were determined. All of these CNVs, together made 41 genes, were linked with molecular pathways and the biological processes of heart development. Gene prioritization methods were used, and the following 8 candidate genes of CHDs were determined – *FGF9*, *BMPR1A*, *PTEN*, *ANKRD17*, *NPPA*, *LMNA*, *NFATC1* and *IFT88*, which were made up of five deletions of *de novo* origin and one deletion of maternal origin. The aforementioned genes were related to GO biological processes, annotated in the MGI database and associated with CHD mouse models in accordance with MGI terms.

This study determined a *de novo* 10q22.3q23.33 deletion of 14154 kb in size, which included candidate genes of CHDs, namely *BMPR1A* and *PTEN*. Large 10q22q23 deletions are linked with manifestation of CHDs. Literature describes patients with large 10q deletions with typical cardiological phenotypes – AVSD, ASD, VSD and tricuspid valve regurgitation (Alliman et al. 2010; Breckpot et al. 2011; van Bon et al. 2011). *BMPR1A* encodes the bone morphogenetic receptor type 1A and participates in the BMP signaling pathway. The BMP signaling pathway regulates cell proliferation, migration, differentiation and apoptosis. *Bmpr1a* gene deletion in mouse models damages heart morphogenesis in mice and manifests itself with defects in the interventricular septum and in the endocardial cushions (Gaussin et al. 2002). The *PTEN* gene, which is located more distally to the *BMPR1A* gene, is important for tumor suppression. Hamada et al. demonstrated that *PTEN*, which regulates the expression of vascular signaling molecules, is necessary for normal cardiovascular morphogenesis and for postnatal angiogenesis (Hamada et al. 2005).

Deletions in the *de novo* 18q23 region, which were determined in two patients with heart valve development diseases, included another CHD candidate gene. The NFATC1 protein is a part of the Rel/NF–kB family of transcription factors, the members of which regulate the proliferation, differentiation and homeostasis of the majority of cells (Crabtree et al. 2002). The NFATC1 protein is important for the development of heart valves. The expression of *NFATC1* takes part in the primary heart tube of endocardial endothelial cells, and the expression of this gene in the early stages of the formation of endocardial cushions specifically manifests itself in the

endothelial cells of the atrioventricular canal and of the outflow tract (de la Pompa et al. 1998). An abnormal expression of the *NFATC1* gene results in atrioventricular and semilunar valve anomalies in mice (de la Pompa et al. 1998; Ranger et al. 1998). Abdul-Sater et al. proved that *NFATC1* gene mutations result in tricuspid valve atresia (Abdul-Sater et al. 2012).

ANKRD17 is another candidate gene, which is located in genomic region 4q13.3. *ANKRD17* encodes a protein, which has two clusters of ankyrin repeats. A mice model research revealed that the mutations of the *Ankrd17* gene in mouse embryos lead to cardiovascular defects (Hou et al. 2009). The 4q13.3 deletion, which was determined in a female patient with an aortic valve abnormality, was inherited from her mother, who had hypertrophic cardiomyopathy.

A *De novo* 13q12.11q12.12 deletion in a genomic region included the candidate genes of CHDs – *IFT88* and *FGF9.* Both of the genes in mouse models are linked to impaired cardiac morphogenesis. The FGF9 protein belongs to the family of fibroblast growth factors. FGF proteins act as paracrine or endocrine signals during heart development. It was determined that the *FGF9* expression took place during embryonic development in mouse heart cells. Cardiomyocyte proliferation in *FGF9* mice in *knockout* mouse models was significantly weakened. This revealed that *FGF9* was a growth factor of embryonic cardiomyocytes (Itoh et al. 2016). The *IFT88* gene takes part in the formation of primary cilia and is linked to the regulation of the *hedgehog* signalling pathway and early cardiogenesis. It was revealed that *Ift88–null* mouse E11.5 embryos, which failed to form cilia, had an impaired development of the outflow tract of the heart. It shows that the primary cardiac cilia play an important role in the early embryogenesis of the heart by participating in the coordination of the *hedgehog* signalling pathway (Clement et al. 2009).

A *De novo* 1p36.22p36.32 deletion included a CHD candidate gene *NPPA*. *NPPA* is an evolutionarily conservative gene encoding atrial natriuretic peptide (ANP). During the embryonic development, the expression of the *NPPA* gene intensively takes place in the atrial and ventricular myocardium (Houweling et al. 2005). It was established that due to the fact that the *NPPA* and the gene paralog *NPPB* were located close to one another, they formed an evolutionarily conserved cluster of genes, which interacted with NKX2-5, GATA, TBX5 and other transcription factors, which were functionally important in heart development (Houweling et al. 2005; Man et al. 2018). The damage of these transcriptional regulatory mechanisms leads to CHDs (Bruneau 2011).

Dosage-sensitive genes may be linked with the manifestation of CHDs in the subjects. Deletions cause a disease phenotype mainly as a result of dosage-sensitive gene haploinsufficiency, whereas duplications may lead to a development of the disease due to triplosensitivity of a gene, a termination of the gene sequence or gene fu-

sion through breakpoints. Another *de novo* interstitial 1q22q23.1 deletion included *LMNA*, which is a candidate gene of CHDs. A 1q22q23.1 microdeletion, which was determined in a patient, is the smallest deletion to be described so far, including the *LMNA* gene (Aleksiūnienė et al. 2018). The *LMNA* gene encodes lamins A and C, which form due to the alternative splicing of transcripts (Depreux et al. 2015). Lamins A and C, which are located in the inner nuclear membrane, interact with the proteins in the nuclear envelope, chromatin and transcription factors (Gupta et al. 2010). Mutations and deletions of the function loss of the *LMNA* gene lead to gene haploinsufficiency and are linked to dilated cardiomyopathy (Zaragoza et al. 2016). The subject of this piece of research (CG-067) and the sibs with 1q21.3q23.3 deletion, described by Quinonez et al., had an identical cardiological phenotype, ASD, PDA, and limb contractures (Quinonez et al. 2012). This suggests that the dosage-sensitive gene haploinsufficiency of the *LMNA* gene could have led to the formation of structural heart defects.

A link between major genomic changes and congenital heart defects was determined in this piece of research. The rare frequency of CNVs (and rare of ≥501 kb) in the subject group, which includes both syndromic CHDs, CHDs with other congenital developmental anomalies and/or dysmorphic features, and isolated CHDs, was significantly higher than the frequency of CNVs in the control group. Rare genetic CNVs manifested themselves in the group of subjects with CHD 2.65 times more frequently, whereas large (of ≥501 kb), rare genetic CNVs were 6.63 times significantly more frequent in subjects with heart diseases.

Despite the differences between the design of phenotypic research of CHDs and the resolution of genotyping chips, the majority of research determined a significantly higher frequency in the population of rare, large and/or *de novo* CNVs congenital heart diseases. Silversides et al. found that large, rare CNVs were more frequent in the group of subjects with the Tetralogy of Fallot than in the control group (8.8% in the subject group and 4.33% in the control group, p=0.0117) (Silversides et al. 2012). Other pieces of research revealed 12.7% of *de novo* CNVs, 5.6% of which were significantly linked with CHDs, in comparison with just 2% in the control group (Warburton et al. 2014). Greenway et al. found that *de novo* CNVs resulted in 10% of isolated sporadic TOF in comparison with 4% in the control group (Greenway et al. 2009).

The results of this study revealed that rare genic CNVs, as well as extremely rare genic deletions, which manifested themselves 4.02-times more frequently in the group of subjects with CHDs, are linked to heart diseases. A significant difference between the average value of a number of genes, which include rare genic CNVs, was determined. It may be concluded that rare deletions and duplications, including a few times genes more on average, lead to a higher risk of CHDs.

Haploinsufficiency is a phenomenal feature of diploid organisms, which is defined as the loss of function of a functional gene copy, which results in a gene product deficiency and an altered phenotype, which is, most of the time, pathological. Point mutation or chromosomal rearrangements, including CNVs, most frequently inactivate one of the functional gene copies (Huang et al. 2010). The score of gene haploinsufficiency (HI) varies between 0–100% and demonstrates a high degree of gene haploinsufficiency when the estimated score of haploinsufficiency HI=0–10%, and a low degree of gene haploinsufficiency, when HI=90–100%. The results of this study revealed that rare genic CNVs included 2.45 HI genes more in the subject group of subjects with CHDs than in the control group when this difference was statistically significant (p=0.0187). A high degree of the haploinsufficiency of the genes, which were included in genic CNVs, shows that rare genic CNVs, which were determined in this study, are possibly functionally significant.

This study revealed that a large-scale CGH genotyping is an effective method for determining the genetic causes of CHDs, and a bioinformatics analysis of biological processes and of molecular pathways shows a possible mutual interaction between these pathways during cardiogenesis. Pathogenic CNVs, which include the genes known/related to CHDs, make an important part of genomic variants which lead to the pathogenesis of CHDs. The results obtained demonstrate a high phenotypic and genetic heterogeneity, which are typical for CHDs, and reveal the possible candidate genes in this group of congenital developmental defects. However, further and more comprehensive research is necessary in order to confirm that these genes participate in the pathogenesis of CHDs.

3. Conclusions

1. Phenotypic heterogeneity and complexity are typical for congenital heart diseases of the subjects:
   - Congenital heart diseases, which manifest themselves with other congenital developmental anomalies and/or dysmorphic features made 68.18%; 18.94% of congenital heart diseases were one of the clinical features of a genetic syndrome, and 12.88% of congenital heart diseases were isolated;
   - Congenital heart diseases with normal cardiac segmental connections, i.e., 71.43% were most frequent, with the biggest part, 34.92%, of septal defects;
   - Anatomically isolated congenital heart diseases made up 49.21%, whereas complex congenital heart diseases made up 50.79% of all congenital heart anomalies;

- A significant dependence between the manifestation of major vascular anomalies and gender in the female group ($p=0.029$) was determined, but no significant results were determined in separate groups of congenital heart diseases ($p>\alpha$, $\alpha=0.05$).

2. Pathogenic copy number variations are frequent causal variants in the etiology of syndromic congenital heart diseases and of congenital heart diseases, which manifest themselves with other congenital developmental anomalies:
   - Of pathogenic copy number variations, 17.39% were determined, 56.82% of which were in the group of syndromic congenital heart diseases, and 43.18% of which were in the group of congenital heart diseases that had manifested themselves with other congenital developmental anomalies and/or dysmorphic features;
   - Of pathogenic copy number variations, 38.64% included known genes of congenital heart diseases and led to syndromic congenital heart diseases.
3. Eight candidate genes of congenital heart diseases were determined, which were as follows: *FGF9, BMPR1A, PTEN, ANKRD17, NPPA, LMNA, NFATC1* and *IFT88*. Due to an altered expression, the candidate genes that participate in heart development may cause congenital heart diseases.
4. The diagnostic efficiency of the method of large-scale, single-nucleotide polymorphism – a comparative genomic hybridization in the group of congenital heart diseases – was 34.4%. After excluding the cases of the genetic syndromes, where the genes of congenital heart diseases were knowingly determined, the diagnostic efficiency of determining new pathogenic copy number variations amounted to 18.94%.
5. Rare, large copy number variations (rare deletions and duplications), which include a few times more genes on average, may possibly result in a higher risk of congenital heart diseases:
   - Rare genic copy number variations manifested 2.65 times more often in the group of subjects with congenital heart diseases than in the control group ($p<0.0001$);
   - Rare genic copy number variations included 2.45 times more genes with a high degree of haploinsufficiency in the groups of subjects with congenital heart diseases than in the control group ($p=0.0187$).

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List of publications

Published articles

1. **Aleksiūnienė B**, Matulevičiūtė R, Matulevičienė A, Burnytė B, Krasovskaja N, Ambrozaitytė L, Mikštienė V, Dirsė V, Utkus A, Kučinskas V. Opposite chromosome constitutions due to a familial translocation t(1;21)(q43;q22) in 2 cousins with development delay and congenital anomalies: A case report. *Medicine* (Baltimore). 2017 Apr; 96(16):e6521.

2. **Aleksiūnienė B**, Preiksaitiene E, Morkūnienė A, Ambrozaitytė L, Utkus A. A *de novo* 1q22q23.1 interstitial microdeletion in a girl with intellectual disability and multiple congenital anomalies including congenital heart defect. *Cytogenet Genome Re*s. 2018; 154; 6-11.

3. Vaisvilas M, Dirse V, **Aleksiuniene B**, Tamuliene I, Cimbalistiene L, Utkus A, Rascon J. Acute pre-B lymphoblastic leukemia and congenital anomalies in a child with a de novo 22q11.1q11.22 duplication. *Balkan Journal of Medical Genetics*. 2018; 21(1):87-92.

Spoken presentations

1. Matulevičienė A, **Aleksiūnienė B**, Mikštienė V, Krasovskaja N, Griškevičius L, Utkus A, Kučinskas V. Dup(1)(q43–q44)&del(21)(q22.2–q22.3) characterized by facial dysmorphism, congenital heart defect and mental retardation. The 25th European Dysmorphology Meeting, 2014, Strasbourg, France.

2. **Aleksiūnienė B**, Matulevičiūtė R, Čiuladaitė Ž, Matulevičienė A, Utkus A, Kučinskas V. Coarctation of the aorta with dysmorphic features in a patient with triplication of 15q26.1-q26.3: clinical and molecular analysis. The 26th European Dysmorphology Meeting, 2015, Strasbourg, France.

3. Matulevičienė A, **Aleksiūnienė B**, Tamulienė L, Liubšys A, Čiuladaitė Ž, Utkus A, Kučinskas V. A novel *de novo* del (4)(q28.2-qter)&del(8)(pter-p23.1) due to unbalanced translocation in a girl: clinical and molecular analysis. The 26th European Dysmorphology Meeting, 2015, Strasbourg, France.

4. **Aleksiūnienė B**, Ambrozaitytė L, Matulevičienė A, Krasovskaja N, Utkus A. Comparative whole genome hybridization methods in molecular diagnostics. The XIII Baltic Congress in Laboratory Medicine, 2016, Tartu, Estonia.

5. **Aleksiūnienė B,** Ambrozaitytė L, Dagytė E, Laimutė R, Molytė A, Utkus A. Identification of rare copy number variants in patients with congenital heart diseases. The XIV Baltic Congress Of Laboratory Medicine, 2018, Vilnius, Lithuania.

6. Braždžiūnaitė D, Laimutė R, **Aleksiūnienė B**, Dagytė E, Ambrozaitytė L, Benušienė E, Cimbalistienė L, Utkus A. Molecular karyotyping: clinical utility and practice. The IX International Scientific Conference *Retų ligų gydymo inovacijos* ("Innovations in Treating Rare Diseases"), 2018, Vilnius, Lithuania.

Poster presentations

1. **Aleksiūnienė B**, Matulevičienė B, Benušienė E, Tarutis V. Hypoplastic left heart syndrome in a case with partial 18p monosomy and partial 20q trisomy. European Human Genetics Conference 2015, Glasgow, United Kingdom.

2. Cimbalistienė L, **Aleksiūnienė B**, Dirsė V, Tamulienė I, Utkus A. 6,6 Mb *de novo* 22q11.1-q11.22 duplication in a patient with anomalous pulmonary venous drainage, intestinal malrotation and pre B acute lymphoblastic leukemia. European Human Genetics Conference 2016, Barcelona, Spain.

3. Preikšaitienė E, Maldžienė Ž, **Aleksiūnienė B,** Kučinskas V. Mild intellectual disability, congenital heart defect and skeletal abnormalities in three patients with 4q13.3 microdeletion. European Human Genetics Conference 2016, Barcelona, Spain.

4. **Aleksiūnienė B**, Cimbalistienė L, Dirsė V, Gineikienė E, Marcinkutė R, Utkus A. *De novo* 15q26.2q26.3 duplication and 15q26.3 deletion in a patient with an anomalous parietal sutures. European Human genetics Conference 2016, Barcelona, Spain.

5. **Aleksiūnienė B**, Matulevičienė A, Ambrozaitytė L, Jakutis V, Utkus A. Hypoplastic right ventricle, dysmorphic features and brain structural anomalies in a patient with a *de novo* 1p36.33p36.32 deletion. European Human Genetics Conference 2017, Copenhagen, Denmark.

6. **Aleksiūnienė B**, Tumienė B, Utkus A. *AHDC1* gene truncating 1p36.11p35.3 microdeletion in a patient with developmental delay, dysmorphic features and congenital heart defects. European Human Genetics Conference 2018, Milan, Italy.

7. Blažytė EM, Samsonė VG, Matulevičienė A, **Aleksiūnienė B**, Burnytė B, Dagytė E, Tumienė B, Utkus A. The spectrum of associated congenital malformations in Down syndrome: a retrospective Lithuanian cohort study. European Human Genetics Conference 2018, Milan, Italy.

Laura Pranckėnienė

DE NOVO MUTACIJŲ INTENSYVUMO ĮVERTINIMAS BENDROSIOS LIETUVOS POPULIACIJOS IR ASMENŲ SU INTELEKTINE NEGALIA EGZOMUOSE

AN EVALUATION OF THE INTENSITY OF *DE NOVO* MUTATIONS IN THE EXOMES OF THE GENERAL OPULATION OF LITHUANIA AND OF INDIVIDUALS WITH INTELLECTUAL DISABILITIES

Medicinos ir sveikatos mokslai, Medicina M 001
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Introduction

The analysis of *de novo* mutations, which is generalized on the Lithuanian population scale and not based on individual case descriptions, is important because the research strategy of trios (the father, mother, and at least one child) allows us to determine the mutation rate of different genome variants as well as the mutation rate of individual genomic regions in the studied Lithuanian and heterogeneous genetic disease model – in the groups of individuals with intellectual disability (ID) and their siblings. The analysis of whole-exome sequencing data in trios allows for a direct identification of different *de novo* mutations unaffected by natural selection that may be associated with both common and rare diseases or adaptive regions of the genome [1–7]. A functional and contextual analysis of all the identified genomic variants make it possible to identify the possible mechanisms of mutation formation and mechanisms of intellectual disabilty pathogenesis in the group of individuals with intellectual disability.

Aim of the Study

To analyze the distribution, mechanisms of formation, and effects of exome *de novo* mutations in the Lithuanian population and in groups of individuals with intellectual disability.

Tasks of the Research

1. To select the optimal algorithm for detecting *de novo* mutations.
2. To identify the *de novo* mutation spectrum specific to the Lithuanian population, a group of individuals with intellectual disability, and a group of individuals with intellectual disability sibs; to compare it with the composition of the spectrum of Utah from Northern and Western Europe (CEU), Ibadan Yoruba (YRI), Icelandic, Scottish, and South Dakota populations; to identify the potential mechanisms of *de novo* mutation formation.
3. To calculate the intensity rate of *de novo* mutations in the genome and compare it with those identified in Icelandic, Danish, Dutch, Canadian, and American general populations as well as the Autism Spectrum Disease group.
4. To determine the influence of parental age, genomic and epigenetic factors on the intensity and formation of *de novo* mutation emergence.
5. To determine *in silico* the influence of the function of *de novo* mutations on the phenotype of the Lithuanian population and individuals with intellectual disabilities; to identify the potential mechanisms of the pathogenesis of intellectual disability;

6. To evaluate whether *de novo* mutations affect the relative fitness of amino acids, and, if affected, what patterns of relative fitness describe the Lithuanian population.

Statements to be Defended

1. The Lithuanian population is characterized by a distinctive spectrum, intensity, and mechanisms of *de novo* mutation patterns.
2. The *de novo* mutations that occur in the Lithuanian population have an overall relative fitness that does not adversely affect an individual's phenotype and health, and may therefore persist in the genome for many generations.
3. The exomes of individuals with intellectual disabilities have significantly more harmful *de novo* mutations than in the other groups studied, and thus may be the cause of intellectual disability.

Materials and methods

1.1 Samples

Data from two research studies (LITGEN and UNIGENE) were used to fulfill the tasks and purpose of the research.

The data of 147 individuals (49 trios, the first group) participating in the LITGEN project were used to complete the study. This study is part of the LITGEN project, which was approved by the Vilnius Regional Research Ethics Committee 235 No. 158200-05-329-79 on May 3, 2011.

The individuals involved in the project UNIGENE were divided into two groups (the second and the third group). The second group consisted of 37 trios participating in the UNIGENE project, where the proband has an intellectual disability or autism spectrum disease (111 individuals in total). In this paper, intellectual disability was chosen as a model for genetic disease to investigate *de novo* mutations. It is a highly heterogeneous genetic disease the causes and consequences of which are different in each analyzed case, while the number of individuals with ID is constantly increasing [8]. This study consisted of subjects meeting the main selection criterion, that of intellectual disabilities, when the intelligence quotient on the Wecksler scale is <70, or when a developmental delay was observed (in children under 6 years of age).

The third group consisted of 17 trios, the proband being the healthy sibs of a person with intellectual disability (51 individuals in total). During the study, only the individuals whose whole exome had been sequenced were analyzed. This study is part of the UNIGENE project, which was approved by the Vilnius Regional Research Ethics Committee 235 No. 158200-12-563-164 on November 29, 2012.

In accordance with the Declaration of Helsinki, forms of written informed consent were received from all of the study participants. Genomic DNA was extracted from venous blood using either the phenol-chloroform extraction method or the automated DNA extraction platform TECAN Freedom EVO® (Tecan Schweiz AG, Switzerland) based on the paramagnetic particle method.

1.2 Whole Exome Sequencing and Data Analysis

The proband and his healthy parents were assessed using whole exome sequencing (WES). The exomes of the LITGEN project were sequenced using the *SOLiD 5500* sequencing system (75 bp reads). Sequencing data were processed and prepared using *Lifescope* software. Exomes were mapped according to the human reference genome build 19. An average read depth of sequencing was 38.5. BAM-formatted files of the mother, father, and child generated by *Lifescope* were combined using *Samtools* software for each trio.

To analyze the UNIGENE project data set, the exomes were captured using the Agilent SureSelect Human All Exon V5 enrichment kit and multiplex sequenced (6-plex) using the *Illumina HiSeq 2500* platform to reach an about 100-fold coverage on average and were mapped, using the *Burrows-Wheeler Aligner* algorithm, according to the human reference genome build 38. BAM-formatted files of the mother, father, and child, generated by the GATK, were combined using *Samtools* software for each trio.

1.3 Identification and Verification of *De Novo* Mutations

An initial identification of *de novo* mutations was performed using the open-access *VarScan* v.2.4.2 software [9] in accordance with protocol and an alternative commercial *VarSeq*™ software. A potential *de novo* mutation was identified if the child has a genomic variant when neither parent has it in the same genome position. The results are provided in a generated *.vcf* format data file. *SnpSift v.4* software was used for the initial rejection of false positive results derived from *VarScan* software. The following conservative filtering criteria were applied: 1) a genotype quality of the individual ≥ 50; 2) the number of reads at each site > 20. The same filtering criteria were applied to the *dn*M list obtained by *VarSeq*™ software in the *Trio Workflow* part.

Furthermore, in order to discard the remaining variants that were somatic (only present in a fraction of the sequenced blood cells) with low allele balance or sequencing artefacts, *dn*Ms were filtered by setting a threshold for the observed fraction of the reads in individuals with the alternative allele (the allele balance) for the trios [0.3;0.7]. In addition, all possible identified and filtered *de novo* single nu-

cleotide variants were manually reviewed using *Integrative Genomics Viewer* [10]. In cases where *dn*M was detected significantly more frequently than in all other trios, biological paternity verification using the *AmpFLSTR™ Identifiler™* kit (Applied Biosystems™, USA) was performed according to the manufacturer's recommendations.

Due to the large number of identified *dn*Ms, for the validation of variants by *Sanger* sequencing, 50 *de novo* single nucleotide variants were randomly selected for the first group, and 50 – for the second and third groups. Sanger sequencing was performed using an ABI PRISM 3130xl Genetic Analyzer.

Verificated *dn*M annotation was performed using ANNOVAR (v.20170601 hg19 and v.20170912 hg38 reference genome) software [11]. The annotation tool package consisted of *SIFT*, *PolyPhen*, *LRT*, *MutationTaster*, *MutationAssessor*, *FATHMM*, *PROVEAN*, *CADD*, *GERP++*, *PhyloP*, *SiPhy*, and *COSMIC* algorithm annotation tools. Databases providing information on the gene identifying the *dn*M and the *dbSNP* database were also connected, thus assigning the *rs* code to each genome variant, evaluating the genomic variant pathogenicity in *ClinVar*, and evaluating the *1000 genome* project and ExAC database genome variant frequencies in different populations.

1.4 Calculating the *De Novo* Mutation Rate

The probability that a calling position was a *dn*M in the trio was calculated independently for each trio. The *de novo* rate per position per generation (PPPG) was calculated as follows:

$$De\ novo\ rate_{PPPG} = \frac{\sum_{i=1}^{f} n_i}{2\sum_{i=1}^{f}\sum_{j=1}^{N} P_j^i(de\ novo)}$$

where *f* is the number of trios and *N* is the number of callable sites that may potentially be identified as *de novo* sites for each trio separately, regardless of the sequencing depth. This number varies depending on the trio. n_i is the number of identified *dn*Ms for trio *i*. The probability $P_j^i(de\ novo)$ (*de novo* single nucleotide) for the called single nucleotide site *j* and trio *i* to be mutated was calculated as follows:

$$P_j^i(dnM) = P_j^i\left(C_{Hetero}\middle|M_{HomR,}T_{HomR}\right) + P_j^i\left(C_{Hetero}\middle|M_{HomA,}T_{HomA}\right)$$

The probability $P_j^i(de\ novo\ indel)$ for the called indel site *j* and trio *i* to be mutated was calculated as:

$$P_j^i(de\ novo\ indel) = P_j^i\left(C_{HomR}\middle|M_{HomA,}F_{HomA}\right)$$

where *C*, *M*, and *F* stand for offspring, mother, and father, respectively, and Hetero, HomR, and HomA denote heterozygous, homozygous for reference, and homozygous for alternative allele, respectively. The probability $P_j^i(de\ novo)$ was calculated with respect to the sequencing coverage. Confidence intervals for rate estimates were calculated as for binomial proportions. For the estimation of the *dn*M rate and for further calculations, R package (version 3.4.3) was used [12].

1.5 Analysis of Parental Age, Genomic and Epigenetic Factors That May Influence *dn*M Intensity

In order to test the hypothesis that variations in the *dn*M rate across different regions of the genome could be explained by intrinsic characteristics of the genomic region itself and parent age, a linear regression analysis was performed, for which the "secondary" annotation of each *dn*M was carried out using data from the ENCODE [13], LITGEN, and UNIGENE projects. First, according to a previous study [14], in order to collect records regarding the genomic landscape of the identified *dn*Ms, lymphoblastoid cell lines (LCL and GM12878) [15] were chosen. Data were collected for: (1) expression rates (eQTL) [13, 16, 17] in different tissues. According to the expression of regions with *de novo* mutations, these were divided into positions with specific and nonspecific expression; (2) measurements of DNase1 hypersensitivity sites (DHS) [16, 17]. The DHS status was assigned to 0 if outside the DHS peak and 1 if within; (3) measurements of context of CpG islands [16, 17]. If *dn*M was within the CpG islands, a status of position was assigned 1, and if outside – 0; (4) three histone marks (H3K27ac, H3K4me1, and H3K4me3 [16]) from the ENCODE project. If *dn*M was in a position marked with histone, it was assigned with 1, and if not – 0; 5) GERPP++ conservation values were collected using the ANNOVAR annotation tool. According to conservation values, positions with *de novo* mutations were assigned to conservative (GERP++ score >12) and nonconservative positions (GERP++ score < 12) [11]. Based on questionnaire records from the LITGEN project, data on parental age were collected. After the collection of parameters, a number of positions with each parameter was calculated for each trio. A correlation analysis, followed by linear regression modeling of the *dn*M rate and parameters, was then performed.

1.6 Identification and Evaluation of the Composition and Patterns of *De novo* Mutation Spectrum in the Lithuanian Population, Individuals with ID, and Individuals with ID Sibs Groups

To analyze the mutational spectrum and templates, reference mutational templates from the https://cancer.sanger.ac.uk/cosmic/signatures/SBS/ database were

used. During the study, each triplet, having a unique *dn*M in the middle position and nucleotides at the 3‘ and 5‘ ends of the reference genome sequence, were respectively assigned to one of the 96 possible triplets. Using the Spearman's correlation coefficient, a correlation analysis was performed between the identified triplet frequencies in the Lithuanian population, the group of individuals with ID, the group of individuals with ID sibs, and the mutation templates from the COSMIC database. The identified templates with *dn*Ms were further compared to the catalog of 6570 *de novo* mutation-based templates compiled by Rahbari et al. for the Ibadan Yoruba (Nigeria) (YRI) population, the population of Utah with Northern and Western European Ancestry (CEU), and Icelandic, Scottish, and South Dakota (USA) populations [29].

1.7 Evaluation of the Relative Fitness of Sequences with *de novo* Mutations

To analyze whether *dn*M affects the relative fitness of individual amino acid types, and, if so, what relative fitness patterns characterize the Lithuanian population, an evaluation of the relative fitness of amino acid sequences with *dn*M was conducted.

Calculations of the selection coefficients or the otherwise relative fitness effect distribution of protein coding amino acid sequences in which the *dn*M had occurred were performed using the *TdG12 site-wise mutation-selection calculation model* (swMutSel0) [18]. This model was chosen because of the highest specificity of the models developed to evaluate the mutability process, where the evolution of each amino acid under study is characterized by an individual fitness value for the amino acid position and a mutation model at the nucleotide level. In this model, it was assumed that the sequences of the genes were formed independently of each other and the effect of natural selection is very small, therefore insignificant. The evolution process has been evaluated over a long period of time (millions of years), and the time from the formation of new sequences with *dn*Ms to their capture is instantaneous. According to the sequences being analyzed, κ (transition and transversion bias), π (base nucleotide composition), and μ (branch scaling) were used. Variants with the relative fitness value <0 were considered as a damaging variant, while those of >0 as a beneficial variant. Using the parameters described above for TdG12, the relative fitness of the amino acid sequences before and after the mutations was evaluated. To analyze whether *dn*M affects the relative fitness of individual amino acid types, and, if so, what relative fitness patterns characterize the Lithuanian population, an evaluation of the relative fitness of amino acid sequences with *dn*M was conducted.

1.8 *In silico* Identification of the Pathogenicity Mechanisms of *de novo* Mutations

Possible pathogenicity mechanisms of pathogenic genome variants with identified *dn*Ms were *in silico* investigated using the following bioinformatic tools and databases:

- *SWISS-MODEL* sofware [19];
- *STRING* sofware [20];
- *OMIM* (*Online Mendelian Inheritance in Man*);
- *GeneCards®*;
- *Ensembl*;
- *ClinVar*;
- *Human Splicing Finder*;
- *denovo-db*;
- *IDGenetics* (http://www.ccgenomics.cn/IDGenetics/);
- *SFARI gene* (https://gene.sfari.org/database/human-gene/);
- *AutDB* (http://autism.mindspec.org/autdb/Welcome.do).

In one case, when a probably pathogenic variant of the *ARID1B* gene was identified, all proband RNA was isolated from venous blood to determine its pathogenicity mechanism using *Tempus™ Blood RNA Tube* and *Tempus™ Spin RNA Isolation* kits according to the manufacturer's recommendations. Copy DNA from RNA was synthesized using the High-Capacity RNA-to-cDNA kit according to the manufacturer's recommendations. A PCR reaction of the cDNA cutting sequence was performed using specific oligonucleotide primers designed with the Primer Blast (NCBI) tool. PCR products were scanned using the standard Sanger sequencing method. Mutation Taster (http://www.mutationtaster.org/) and Human Splicing Finder (http://www.umd.be/HSF3/) databases were used to evaluate the pathogenicity of the change in the splicing site. Possible effects of gene variants with *dn*M were evaluated using the ExPASy Bioinformatics (https://www.expasy.org/) and Pfam 32.0 (https://pfam.xfam.org/) databases.

1.9 Data Analysis and Statistical Methods

The Shapiro-Wilk test was used to test the distribution of samples in the normal distribution. Pearson or Spearman coefficients were used to evaluate correlation. Linear regression analysis, calculations of Mann-Whitney *U* test, Shapiro-Wilk Test, and the Fisher exact criterion were performed using the Open Access *R* package, version 3.4.3, and *R studio*, version 1.2.1335 [12]. A *p-value* of less than 0.05 was considered statistically significant in the analysis. All other calculations were performed using *Microsoft Office Professional Plus 2013* Excel.

Results and discussion

Almost all common variants of the genome are already known. Thousands of genetic variants and/or genes are associated with relevant human traits and diseases. Meanwhile, the identification of rare genetic variants is still progressing rapidly. The need for this research is based on the fact that the diversity of rare variants directly depends on the regions of origin of the individuals, so the more of these regions, the more rare variants are found. For this reason, the analysis of rare variants, most of which are unique *de novo* variants, requires different populations, which requires considerable effort. This work involved a detailed analysis of the distribution and rate of *de novo* mutations in the whole human exome regions of the Lithuanian population (hereafter referred to as the first group), the trio when the proband has an intellectual disability (hereafter referred to as the second group), and the trio when the proband is a sibling of a person with an intellectual disability (hereafter referred to as the third group) (275 individuals studied in total).

2.1 Selection of the Algorithm for Detecting *de novo* Mutations

When investigating the first group, it was aimed at selecting the optimal *dn*M detection software for further work. It should be noted that *VarScan* software was selected for *dn*M detection, because the exome sequencing of the Lithuanian population group was performed using the *5500 SOLiD*™ Sequencer genetic analyzer, and the primary sequencing files in *.XSQ* format were immediately converted to *.BAM* format files using *LifeScope*™ Genomic Analysis Software 2.5.1. In these files, there is either no information about the software used or it is formatted differently than in the *.BAM* files obtained by sequencing exomes with the now widely used Illumina genetic analyzer and processed by the GATK software according to best practice guidelines. Regarding the missing or improperly formatted information about sequenced fragments using LifeScope™ software, the *.BAM* files have resulted from similarly invalid *.vcf* files to the widely used, sensitive, and specific *dn*M identification algorithm, *PhaseByTransmission* [21]. Meanwhile, *VarScan* software is appropriate for analyzing the *.BAM* files obtained by sequencing with the use of both the *5500 SOLiD*™ Sequencer and *Illumina HiSeq 2500* genetic analyzer regardless of the software used to generate the *.BAM* files. Based on the data of Warden CD et al. (2014) [22], this algorithm provides the results that are >97% consistent with the high quality data obtained using *GATK UnifiedGenotyper* and *HaplotypeCaller* softwares. *VarSeq*™ software has been chosen as an analogue of the open-access *VarScan* software, as it is commercial, more visually appealing, and user-friendly for Windows users, used for both scientific and clinical research. *VarScan* runs on Linux.

Thus, according to the study data, a total of 95 *dn*M was set for 34 trios in the first group using *VarScan* software, while using *VarSeq*™ – 84 *dn*M for 31 trios. Although the two algorithms used are designed to identify *dn*M in the proband exome when those variants are not present in the parent exomes, only 5.37% of all *dn*Ms detected by both softwares have matched. After the *dn*M filtration stage, it was determined that the *VarScan* algorithm has a higher sensitivity (5.42%) than the *VarSeq*™ algorithm (1.76%), while the efficiency of mutation detection, as verified by the *Sanger* sequencing method, varied by only 4%. Thus, it can be concluded that none of the tools could correctly detect true *dn*M due to its extremely high specificity (> 99%) and low sensitivity. For this reason, it is important to emphasize the need for additional rigorous data filtering methods when detecting *dn*M. When applied, the number of true *dn*Ms identified ranged from 1752 to 95 (by *VarScan* data) and from 4756 to 84 (by *VarSeq*™ data). Since it is known that softwares with higher (≥100) horizontal sequence overlaps should also have a higher sensitivity, the *VarScan* algorithm was chosen due to its higher sensitivity and open access for the further identification of *dn*M in other groups of subjects. When applied in the second and third groups, 78% of the mutations were confirmed and verified by *dn*M *Sanger* sequencing.

2.2 Rate of *de novo* Mutations in the Lithuanian Population, in Groups of Individuals with Intellectual Disability, and Individuals with Intellectual Disability Sibs

Germ cell mutations represent a fundamental force of evolution that shapes phenotypic diversity; therefore, the accurate detection of the *de novo* mutation rate is extremely necessary and can be applied in a wide variety of ways: interpretations of disease-causing mutations [23–25], dates from natural selection studies [25, 26], and demographic events based on genetic analysis [27–30] to human mutagenesis studies [31]. In addition, the *de novo* mutation rate is a key and central biological parameter that helps us understand evolutionary phenomena. One of the most important is the "molecular clock": the continuous process by which genomic changes accumulate during evolution, and the neutrality theory that explains the causes of the effect of neutral genetic changes, such as changes without effect on protein fit, on the genome.

Many different methods have been developed to determine the rate of *dn*M based on population genetics models or fossil dating data, which require many additional implicit estimates to be realized. Some methods require demographic population models (based on the time to first common ancestor of the subjects), rate of recombination of a genome divided into short segments, or the approximate duration of one generation in years to estimate the annual *dn*M rates. Using this

additional data, the rate of *dn*M is determined from the autozygous regions of the genome that originate from the closest common ancestor that can be reliably identified. This technique is advantageous in that the potential contribution of somatic mutations is minimal when estimating the rate of *dn*M, but only the *dn*M average of both genders is determined, and for several generations at once.

Another approach is to determine the average rate of *dn*M at different periods of evolution from fossil DNA dating data. In this way, the divergence time of the analyzed object, such as the surviving human DNA, and the outer object – for example, chimpanzee DNA – are compared with the fossil genomes, thus estimating the rate of *dn*M according to their differences [32, 33].

In this thesis, the rate of *dn*M was calculated and evaluated using one of the most accurate methods – based on a directly determined *dn*M throughout whole exome sequencing. Based on the obtained data, the estimated *de novo* single nucleotide mutation rate in the Lithuanian population is 2.74×10^{-8}, and the *de novo* indels – 1.77×10^{-8} per position per generation (PPPG). In the second group, the rate of a single *de novo* nucleotide is 3.05×10^{-8} PPPG, and in the third group – 7.19×10^{-8} PPPG. The incidence of *de novo* indel mutations in the genome of the second group is 1.74×10^{-7} PPPG, and in the third group – 1.08×10^{-6} PPPG. In all three groups, the estimated rate of *dn*M is significantly higher (*p-value* = 4.49×10^{-9} for the first group, *p-value* = 5.15×10^{-11} for the second group, and *p-value* = 4.8×10^{-6} for the third group, respectively) than that found in previously published sustainable studies of the Icelandic, Danish, Dutch, Canadian, and American general populations and the group of autists [32, 33], where *de novo* single-nucleotide mutations have a rate between 0.96×10^{-8} and 1.28×10^{-8} PPPG, and *de novo* indels – 1.1×10^{-9} PPPG. The higher rate of *dn*M found in this study can be based on the fact that the study was performed using only exome sequencing data. It is worth noting that exomes exhibit a significantly higher (about 30%) mutation rate than the whole genome. This is explained by the fact that the composition of heterocyclic bases throughout the genome differs from that of the exome: in the exome, the regions with repeats of guanine and cytosine dinucleotides account for about 50% of the total size of the exome, whereas for the whole genome, they account for about 40% [34]. Cytosine and guanine dinucleotide-rich regions methylated in human genomes are known to be highly mutable due to a spontaneous deamination of cytosine bases [34]. Based on comparative genomics studies, higher mutation rates in CpG dinucleotide-rich regions are thought to have occurred approximately during the increase of taxonomic diversity in mammals (also known as mammalian radiation) [35]. This hypothesis was raised based on the knowledge that, as species diverged, CpG dinucleotide-rich coding regions had a higher mutation rate than the noncoding DNA regions [36]. During evolution, as the mutations progressed, the coding regions turned into non-

coding regions, so the context effect of the CpG dinucleotide genome, like the average mutation rate, declined over time until it reached the mutation rate detected in the noncoding DNA surrounding the coding sequences. However, although there was sufficient time in the genomic regions with a different CpG dinucleotide context to create neutral genomic regions and thus achieve equilibrium, purifying natural selection in functional genomic regions retained the share of hypermutable CpG dinucleotides [35–38]. This study identified a higher rate of *dn*M than the genomic studies of other researchers, presumably because of the specific context of the CpG exome sequences and whether natural selection affects the exome. Based on linear regression models, it was found that ~68–94% of the total rate of *dn*M in the genome of the Lithuanian population is explained by DNAse1 hypersensitivity, CpG context, and regional conservatism (based on GERPP++ conservatism estimates) regional expression levels (TABLE 1).

TABLE 1. Influence of the number of DNase1 highly sensitive regions, regions abundant with CpG dinucleotides, number of conservative regions, and number of genomic regions with different expression on the intensity of the *dnM* rate in Lithuanian populations and the group of individuals with intellectual disability.

Response variable	Independent variable	β*±SE	p-value	R^2
Lithuanian population				
***dn*M rate**	DNase1 hypersensitivity sites	$2{,}83\times10^{-6}\pm1{,}96\times10^{-7}$	$2{,}31\times10^{-13}$	0,89
	Conservative positions	$5{,}28\times10^{-6}\pm5{,}67\times10^{-7}$	$1{,}49\times10^{-6}$	0,88
	Nonconservative positions	$2{,}56\times10^{-6}\pm2{,}67\times10^{-7}$	$2{,}45\times10^{-9}$	0,8
	Context of CpG islands	$2{,}76\times10^{-6}\pm4{,}94\times10^{-7}$	$8{,}93\times10^{-6}$	0,68
	Positions with nonspecific expression	$2{,}05\times10^{-6}\pm1{,}144\times10^{-7}$	$8{,}34\times10^{-14}$	0,94
The group of individuals with intellectual disability				
***dn*M rate**	Positions with nonspecific expression	$8{,}56\times10^{-7}\pm3{,}52\times10^{-7}$	0,02	0,13
β – regression coefficient showing how much the rate of *dn*M increases as the independent variable increases by one (one position or region, respectively), **SE** – standard error, **R^2** – coefficient of determination.				

The rate of *dn*M in the group of individuals with ID was also moderately (13%, *p-value* = 0,02) explained by the expression levels of the exome regions (Table 1). Based on these data, it was concluded that the *dn*M exome is formed in promoter-like, transcriptionally active regions of the genome, regardless of DNA sequence conservatism, although the rate of *dn*M was higher in genes with nonspecific expression. This coincides with the location of gene expression, transcription factor binding, and histone modifications in many species of organisms and their tissue

map data [13, 17, 39]. They have revealed that the expression of identical or similar tissue genes in different species is conservative [40], but that the enhancer and promoter sequences are characterized by rapid changes in DNA structure [41–43]. For example, a recent study of hepatocyte promoters and enhancers in 20 mammalian species revealed that 25% of enhancers and 10% of promoters were unique to all species, even when sequences were particularly conservative [41, 44]. Similar results were obtained in human, macaque, and mouse limb tissues [45].

Contrary to other research work, this study found that fathers' ages did not correlate with the *dn*M rate in the Lithuanian population (fathers $R_S = 0.2$, *p-value* = 0.09, mothers $R_S = 0.08$, *p-value* = 0.98) (FIGURE 1).

This can be explained by the fact that the age of the fathers of the trios analyzed in the study is too similar, and that the power of age and *dn*M rate analysis is therefore not sufficient. Also, the study analyzed only a small (~1.5%) part of the entire genome – in the exome, therefore, on average, 1.9 (*VarScan*) and 1.7 (*VarSeq*™) *de novo* single-nucleotide mutations were detected in each of the analyzed exomes, while approximately 40 to 82 *de novo* single-nucleotide substitutions were identified throughout the genome [34, 35, 46–49]. However, in the Lithuanian population, the number of *de novo* indels (on average 0.0625 *de novo* of indel per person) was similar to that found in Danish and Dutch studies [14, 49]. The *de novo* SNP was higher in the group of individuals with intellectual disability – an average of 4.2 *de novo* single nucleotide and 1.3 *de novo* indel, ranging in size from one to 30 nucleotides. Meanwhile, in the group of individuals with intellectual disability sibs, on average, each proband studied had 2.4 *de novo* SNP and 0.92 *de novo* indels between three and 31 nucleotides in size. In the third group, only one *dn*M was detected in the coding sequence (8%) among the *de novo* indels, whereas ten (24%) were found in the

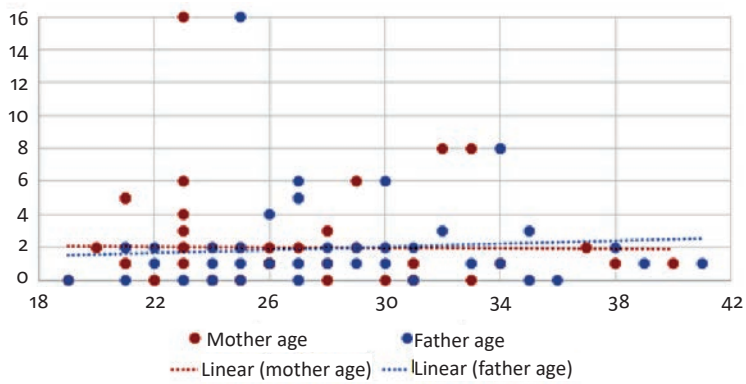

FIGURE 1. Graph of correlation between the dnM number and fathers' ages.

second group, of which as many as four *dn*M are of the frame shift type. Hence, the higher number of *dn*M detected in the second group suggested that the *dn*M may have been the cause of intellectual disability in this group.

Lithuanian populations and groups of individuals with intellectual disability sibs were similar in functional regions where *dn*Ms are detected. In these, the majority (54–64%) consisted of *dn*M present in introns and only 21–24% were in exons. Meanwhile, in the group of individuals with intellectual disability, *dn*M was 48% in introns and 34% in exons. Analogous group similarities and differences can be seen in the analysis of groups by *dn*M types: an abundance of mutations leading to altered protein composition due to altered amino acids was evident in the group of individuals with intellectual disability: two stop codon *dn*M, five frameshift *dn*M, 13 synonymous, 28 nonsynonymous *dn*M, and five *de novo* deletions of three to 31 nucleotides in size, when the basis for *dn*M in the Lithuanian population and in the groups of individuals with intellectual disability sibs were 13 synonymous and 18 nonsynonymous *dn*M.

Based on the analysis of the *de novo* mutation spectrum composition, there is no difference in the composition of the *dn*M spectrum between the groups of the Lithuanian population, individuals with ID, and those with ID sibs (*p-value* = 0.575, $\chi 2$ criterion). Furthermore, the composition of the *dn*M spectrum is close to populations of Utah Yoruba, originating from Northern and Western Europe (CEU), Nigeria (YRI), Iceland, Scotland, and South Dakota (USA) [29]. For the most part of all groups, C> T (33% in the first group, 35% in the second group, and 59% in the third group) and T> C (28% in the first group, 27% in the second group, and 18.5% in the third group) substitutions were identified.

However, an analysis of the correlation of the triplets with a *dn*M frequency in the Lithuanian population with the 49 exome templates of the COSMIC database revealed that all groups analyzed in this work differ in their composition from the *de novo* template catalog based on the research conducted by Rahbari et al. [50] and are unique in the following way: the Lithuanian population is characterized in almost equal proportions (42–49%) by six mutation templates: 26, 10a, 15, 41, and 7a. The 44, sixth, fifth, 20, and seventh b mutation templates describe the group of individuals with ID, and the fifth, 33, and 23 mutation templates describe the group of siblings for individuals with ID (FIGURE 2). Meanwhile, in the CEU, YRI, Icelandic, Scots, and South Dakota populations, the fifth template is assigned for ~ 75% of all *dn*Ms and the first for ~ 25%.

The *dn*M templates identified in the Lithuanian population are thought to result from errors in the DNA strand mismatch repair mechanism (template 26, 15), ε polymerase exonuclease domain mutations (template 10a), and ultraviolet-induced cyclobutane pyrimidine dimers (template 7a). The etiology of the 41 mutation tem-

plate is unknown. Mutation patterns identified in individuals with the ID group are thought to result from errors in the repair mechanism of DNA strand mismatches (templates 44, 6, and 20), also mutations in the *POLD1* gene (Template 20), and UV radiation (Template 7b). The etiology of the fifth mutation template is unknown, but the expression of this template was found to be characterized by bladder cancer cells with *ERCC2* mutations. In the group of siblings for individuals with ID, the etiology of any of the identified mutation templates is unknown.

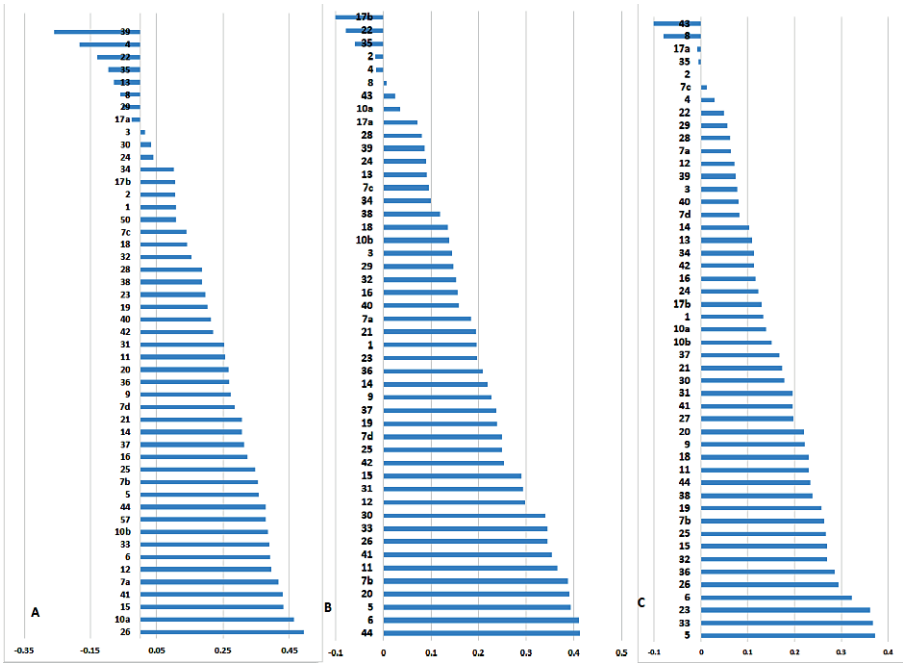

FIGURE 2. Correlation of the frequency of triplets with *dn*M detected in (A) the Lithuanian population, (B) the group of individuals with ID, and (C) the group of siblings for individuals with ID with 49 templates of exomes in the COSMIC database. The horizontal axis represents the Spearman correlation coefficient, the vertical indicates the number of the mutation template.

2.3 Discussion of the Results of the Analysis of the Relative Suitability of Protein Sequences in which *de novo* Mutations are Detected

In population genetics studies, the rate distribution of individuals with different types of *de novo* mutations and their associated selection coefficients is an essential subject of population genetics research [51, 52]. The mutation rate and relative fitness affect a wide range of evolutionary and biological phenomena, such as the maintenance of genetic variation, alteration of quantitative traits, recombination, sex evolution, evolution of aging, persistence of gene copies, and so on [53]. When a

de novo mutation occurs in the genome, it can have one of three effects on the fit of an individual's phenotype (hereafter $w = s + 1$, where w is the relative fit and s is the selection factor): mutation can be harmful ($s < 0$), resulting in the reduced survival or fertility of individuals, neutral ($s \approx 0$), where the mutation has such a small effect on the fitness of individuals that its rate in the population is directly dependent on gene drift, or beneficial ($s > 0$), when individuals with such a mutation have a longer survival time or greater reproductive success than others. The final fate of *dn*M, whether captured or removed from the population genome, depends on the effect of natural selection on the strength and gene drift on the effective population size; therefore, fitness is described by a fitness coefficient S equal to the product of the effective population size N_e and the natural selection s ($S = 2 N_e \times s$). In this work, the suitability of each amino acid with *dn*M to factor S prior to natural selection was analyzed, as *dn*M was evaluated instantaneously. Using the *swMutSel0* model, it was found that in the Lithuanian population, neutral ($-2 < S < 0$) mutations accounted for only ~ 9%, while harmful ($S < -2$) did up to 91%, of which the highly harmful ($S < -10$) were ~ 73% (FIGURE 3). In the group of individuals with intellectual disability, neutral mutations accounted for about 27.5%, harmful ~72.5%, of which highly harmful accounted for ~60%, and in the group of individuals with intellectual disability sibs, neutral mutations were similar to those in the first group – 12.5 %, and harmful – 87.5 %. The part of neutral and pathogenic mutations in the group of individuals with intellectual disability was similar to that found by Eyre-Walker A and Fay JC [52, 54] – 20% and 80%, respectively. Adaptive mutations have not been detected in any of the groups, although recent data indicate that the rate of adaptive mutations in genome $\hat{\alpha}$ is as high as 13.5% [55].

Paradoxically, although this study is based on a study conducted in 2016 by Lelieveld SH et al. [56], it was hypothesized that, in Lithuania, the proportion of harmful mutations in the group of individuals with intellectual disability should be significantly higher than in the other studied groups; based on Fisher's exact criterion, there was no statistically significant difference in the rate distribution of amino acids with different relative fitness between all three groups (*p-value* = 0,63). Moreover, during the study, it was found that in Lithuania and in the group of individuals with intellectual disability sibs, the harmful mutations accounted for a ~18% higher *dn*M share than in the group of individuals with intellectual disability. Assuming that this result was potentially influenced by the fact that in the first and third groups of all of the detected *dn*M, relative fitness was assessed only for the respective 11% and 17% of the detected *dn*M located in the coding regions of the genome; the part of the harmful *dn*M for each group was re-evaluated by including in the analysis those *dn*Ms that could not be included in the relative fitness analysis due to their positions in non-coding genomic sequences. Considering that additionally

included *dn*M are potentially neutral because they do not significantly influence the health of the subjects (in the questionnaire, survey subjects assessed themselves as "healthy" although they may become ill in the future), the above hypothesis was confirmed, in which case the part of harmful *dn*M in the group of individuals with intellectual disability reaches ~20%, and in the first and third groups – ~10% and ~13%, respectively.

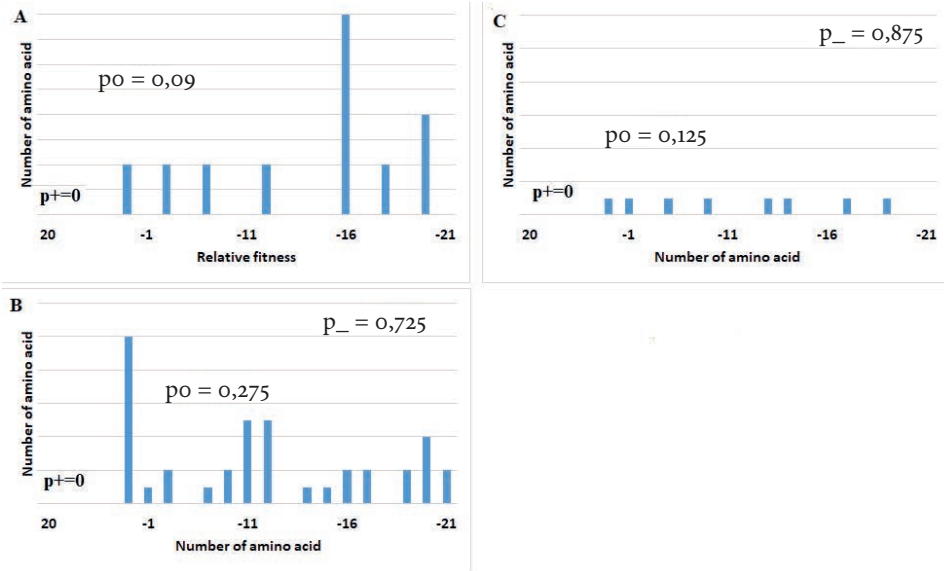

FIGURE 3. Distribution of relative fitness coefficient *S* in the genes for which the determined *dn*M. *p* corresponds to the harmful (*S* < -10) *dn*M part, *p*+ beneficial (*S* >0) *dn*M part, and p_o – neutral (-2 < *S* < 0) *dn*M part. Data of the analysis of (A) the Lithuanian population, (B) the group of individuals with intellectual disability, and (C) the group of individuals with intellectual disability sibs.

At the same time, the data of the relative fitness analysis reveal that before the effects of natural selection on the number of *dn*M (and at the same time intensity) in the genome gives a direct indication of the possible effects *de novo* mutations on the genome and on the individual's phenotype. Since a significantly higher number of *dn*M increases the likelihood that unique mutations (especially in the coding regions of the genome) may have a harmful effect on an individual's phenotype, and thus may be the cause of intellectual disability [57]. Meanwhile, when exposed to natural selection, the effects of harmful mutations and the rate of *dn*M in the genome that individuals can tolerate depend only on whether it affects the absolute or relative fitness of the person's phenotype with intellectual disability [58].

The theory of neutrality is evidenced by the "mirror reflection effect" observed in the first group, where most of the amino acids with high negative relative fitness (S < -16) turned into neutral (0) or low (up to -5) relative amino acids after *dn*M (FIGURE 4). All non-polar amino acids had less negative relative fitness values after *dn*M, whereas the relative fitness of polar amino acids changed slightly after *dn*M. The number of amino acids by type remained unchanged, and *dn*M had no significant effect on the structure and function of the amino acids and the proteins they formed. Meanwhile, in the second group, in the group of individuals with intellectual disability, *dn*M significantly changed the amino acid composition – non-polar amino acids increased by 1.9 times, while charge-loaded amino acids had,

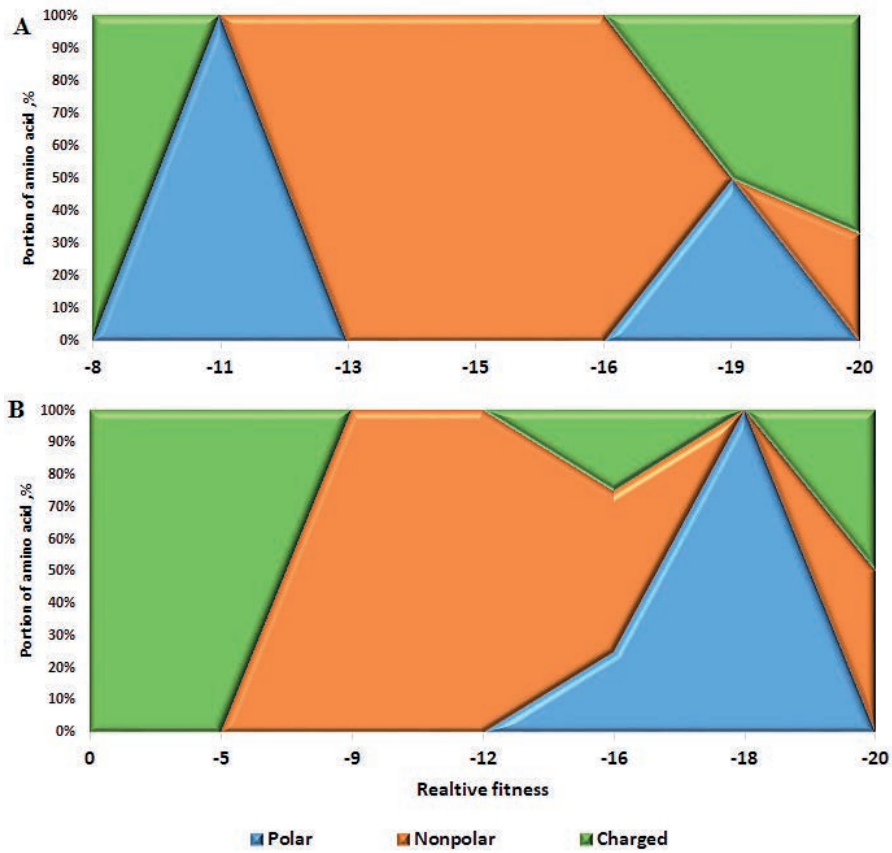

FIGURE 4. Summarized estimates of relative fitness in the group of general population of Lithuania. **A** – estimates of the relative fitness of amino acids before mutations, **B** – following *de novo* mutations.

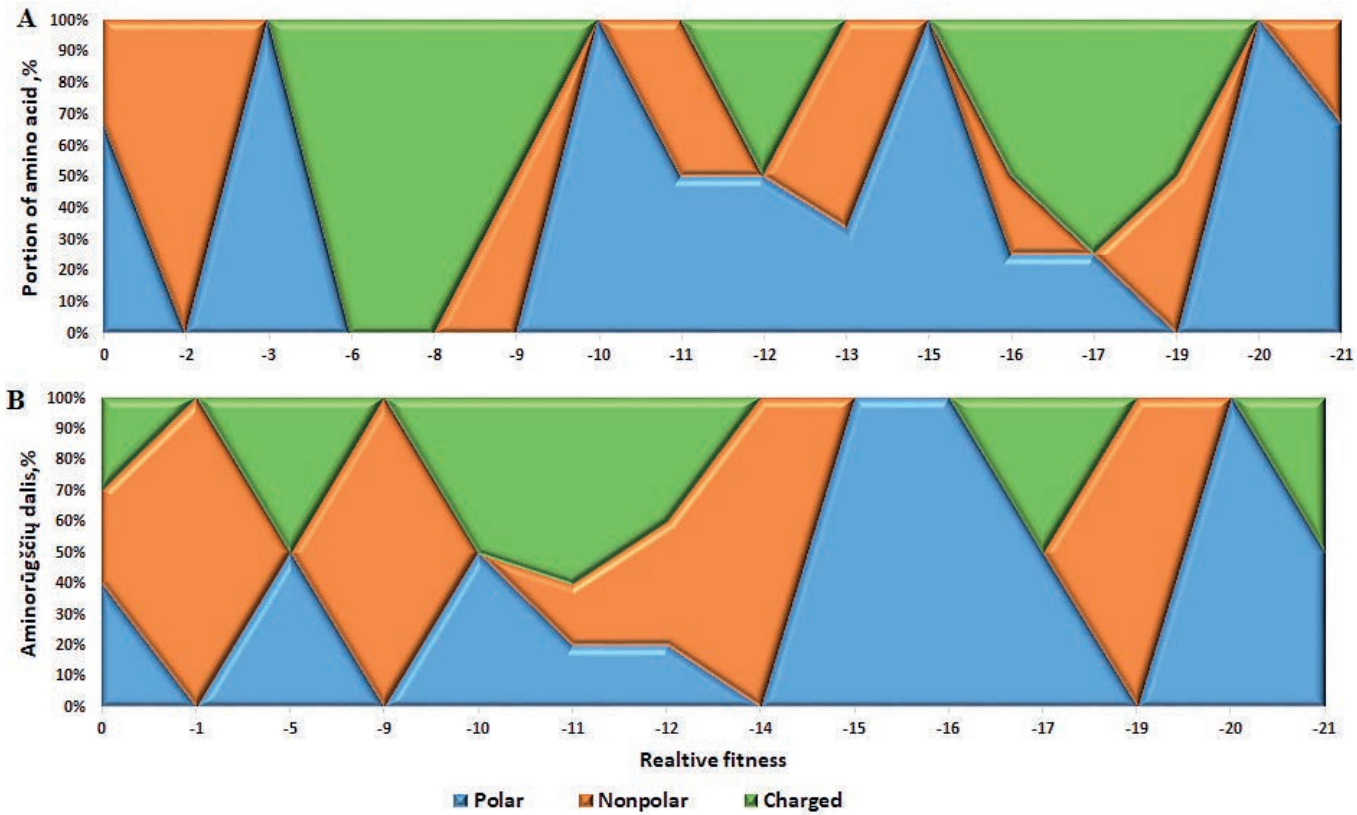

FIGURE 5. Summarized estimates of relative fitness in the group of individuals with intellectual disability. **A** – figure shows the estimates of the relative fitness of amino acids before mutations, **B** – following *de novo* mutations.

on the contrary, decreased by 1.7 times. Also, the relative fitness of only the three polar amino acids increased from -3, -10, and -21 to 0 and -16, the relative fitness of the resulting non-polar amino acids increased for six of the 11 (54.5%) amino acids, and for the charged amino acids, the relative fitness increased for four (33%) and decreased for five (42%) of the 12 amino acids with charge (FIGURE 5). Thus, when summarized, both in terms of the number of amino acids and changes in their relative fitness, it can be predicted that the structure of the proteins encoded by the studied amino acids should undergo significant changes following *dn*M, leading to possible changes in protein function associated with phenotypic traits.

The relative fitness data in the third group replicates the first group, because the relative fitness effect values of all types (except for one amino acid with charge) of amino acids decreased with *dn*M, while their number by type did not change. This indicates that all types of amino acids have become more neutral following *dn*M than before, suggesting that the analyzed *dn*M has no significant effect on changes in the structure or function of the amino acids and thus the protein.

2.4 A Discussion of the Functional *in silico* Analysis of *de novo* Mutation Results

The results of an extended functional *in silico* analysis indicated that in the Lithuanian population group, among all the identified *dn*M, 4 *dn*M detected by *VarScan* software and 48 by *VarSeq*™ software were potentially pathogenic. The large difference in the number of pathogenic *dn*M can be explained by the fact that, depending on the algorithm used to detect *dn*M, the number of detected *dn*M in the coding sequences differed significantly. For example, the *dn*M detected with the *VarScan* algorithm was only 21.05%, and 95.24% with the *VarSeq*™ algorithm. Based on the knowledge of protein structure and function algorithms for assessing the effects of mutations in the coding part of the genome are abundant, there is little knowledge of regulatory regions of the genome, and thus of algorithms to evaluate their structural changes, so mutations in coding sequences are more often assessed as pathogenic. Meanwhile, regulatory regions of the genome that affect protein structure and function indirectly make up a large part of the non-coding genome, accounting for ~11% of the total genome size per protein. Their scattering in the genome ensures minimal impact of pathogenic genomic variants [15]. The pathogenic *dn*M detected in the first group were in genes the encoding proteins of which were important for DNA repair, chromatin remodeling, ribosome biogenesis, immune response, lipid biosynthesis, post-translational labeling of proteins in the endoplasmic network, signaling pathways in the cytoplasm, transport of molecules across membranes, regulation of cell cytoskeleton, cell growth and survival, and the initiation of neuronal responses that influence odor perception. However, despite the fact that these *dn*Ms were found to

be pathogenic, all individuals considered themselves "healthy" during the LITGEN survey. Thus, it demonstrates that, despite the possible pathogenicity of *dn*M, such changes are apparently tolerated by the genomes containing these *dn*Ms, so that the phenotypic effects of these *dn*Ms do not occur. This demonstrates that the pathogenic *dn*Ms detected in this study are not harmful enough to diminish the overall fitness of the encoded proteins and therefore have the ability to persist for many generations and avoid the effects of natural selection. According to Szamecz's study, the more frequently *dn*M occurs in conservative regions of the genome, the stronger the effect of natural selection is through genetic compensatory mechanisms on genetic changes [59]. The harmful effects of change can be mitigated in four ways. Some genes may tolerate truncated protein variants because their functional effects may be masked by (1) incomplete gene expression, (2) compensatory protein variants, or (3) functional significance due to low protein truncation [60]. Other cases of gene changes associated with non-synonymous *dn*M can be compensated (4) by the accumulation of the required number of useful mutations in the genome [59].

Functional *dn*M analysis in the group of individuals with ID (second group) allowed 13.5% of the individuals to identify the genetic cause of ID and to explain the etiopathogenesis of the disease. Five pathogenic *dn*Ms in the *ARID1B*, *PACS1*, *TCF4*, *CHD7*, and *MECP2* genes have been identified and confirmed in the group of individuals with ID, possibly pathogenic mutations, three of which have so far not been described in the literature. For a pathogenic *de novo* mutation in the *CREBBP* gene that is not described in all evaluation estimates, the patient's phenotype will be reassessed to determine whether the mutation detected results in a phenotype specific to the *Rubinstein-Taybi* syndrome. According to the OMIM database, ~800 genes are now known to cause intellectual disability, accounting for ~19% of the known genetic disease genes, so in many cases, as in this study, the causes of intellectual disability remain unclear.

All of the detected *dn*M were in the heterozygous condition. DNA and RNA analysis revealed that NC_000006.12 (NM_020732.3): c.5025+2T>C *dn*M in the *ARID1B* gene induces the autosomal dominantly inherited Coffin-Siris syndrome (MIM #135900). Proband cDNR sequencing by the *Sanger* sequencing method revealed that *dnM* disrupts the cleavage center at the 5' end of intron 19, causing exon 19 to be omitted. Missing one exon during splicing is a frequent result of pathogenic pre-mRNA, resulting in open DNA reading frame shifts, the formation of premature termination codons, or shorter protein synthesis [61]. Due to the presence of both wild-type and mutant transcripts in the cells, the mutant transcripts are not completely disintegrated, and instead the truncated protein NP_065783.3:p.(Thr1633Valfs*11) is synthesized. Truncated ARID1B results in the loss of the BAF250 domain, which is part of the ATP-dependent SWI/SNF-like chromatin

modification complex that regulates gene expression. BAF250 may act similarly to the E3 ubiquitin ligase targeting H2B histones [62]. In addition, previous studies of mouse embryonic cells with an inactive BAF250b complex have shown that BAF250 is particularly important for early embryogenesis, as modified cell regeneration is impaired and differentiation is increased [63–65]. ARID1B-associated BAF complexes are involved in neuronal differentiation and mammalian brain development. In particular, their expression is important for the development of pyramidal neurons during the formation of complex dendritic architecture [62, 63]. The haploinsufficiency of ARID1B is also known to cause a decrease in GABAergic interneurons in the cerebral cortex. In this way, excitatory and neurotransmission-inhibiting processes are affected during brain development. Furthermore, due to the interaction of the BAF complex with histones, ARID1B haploinsufficiency may also function through epigenetic regulation.

NM_018026.4:c.607C>T (Arg203Trp) in the *dn*M PACS1 gene induces autosomal dominantly inherited intellectual disability with a disease-specific phenotype (MIM #615009). In 2015, Gadzicki D et al. [66]. identified this mutation and explained the etiopathogenesis of intellectual disability [67]. PACS1 is a membrane regulator of the Gold's apparatus that directs proteins in the required direction. During embryogenesis, high levels of expression occur in the brain and are also regulated in the postnatal period [68]. To investigate this change, it was hypothesized that c.607C> T *dn*M near the proximal CK2-binding site motif alters the binding properties of PACS1 [66]. Studies on zebrafish and antisense DNA sequences have confirmed this hypothesis. These genomic variants were found to have a dominant negative effect on the survival of zebrafish neurons with SOX10 as a result of the formation of facial dysmorphia [69].

De novo duplication of 18 nucleotide was detected in the *CHD7* gene (c.6341_6358dup, p.Asp2119_Pro2120ins6); NM_017780; NP_060250; MIM# 214800). The mutation was found to be in the evolutionarily conservative region, and protein modeling revealed that the conformation of the DNA sequence containing *dn*M changed from a linear strand to a short α helix. CHD7 is known to encode the chromodomain DNA-binding helicase, which is important in early embryogenesis and controls gene expression through chromatin modification during the cell cycle [70]; therefore, even in three out of four cases, mutations in individuals with the phenotypic features of CHARGE (MIM #214800) syndrome are specifically identified by the *CHD7* gene. The subject was also confirmed to have CHARGE syndrome, and the mutation was not only *de novo*, but also unique, so far not described. CHARGE syndrome is characterized by a combination of symptoms of coloboma, heart defect, growth or developmental malformation, genital and ear abnormalities, deafness, and chuan atresia (occlusal nasal passages).

NM_001243234:c.1259G>A:p.420R>Q (rs121909121) *dn*M was detected in the *TCF4* gene leading to autosomal dominantly inherited *Pitt-Hopkins* syndrome (MIM #610954). The *TCF4* gene encodes a helix-loop-helix structure transcription factor (bHLH), also known as a member of the E protein family. It is evolutionarily conservative and is particularly important for neuronal determination and differentiation because of its ability to bind DNA as homodimers or heterodimers [71, 72]. When the resulting mutation does not allow TCF4 to interact properly with ASCL1, the tissue-specific HLH protein, the development of individual brain structures becomes impaired in the PHOX-RET pathway [73]. In addition, mouse studies have shown that embryo development is directly dependent on the number of copies of the TCF4 protein and, when their number is less than 3 – the mouse embryos did not survive [74]. The expression of this protein is high in the human brain, lungs, heart, and other muscles. Individuals with *Pitt-Hopkins* syndrome have intellectual disabilities, a wide mouth, distinctive facial features, and intermittent hyperventilation followed by apnea.

*Dn*M NM_001316337:c.194C>T :p.65T>M (rs28934906) in the *MECP2* gene eads to *Rett* syndrome (MIM #312750). MECP2 is a multifunctional protein that can alter gene expression and metabolism in a number of ways: it can activate the AKT/mTOR signaling pathway, is involved in alternative gene cutting, and is involved in microRNA and long non-coding RNA expression, as well as in epigenetic chromatin modification [75–79]. Its main function is to recognize and bind specifically to methylated and A/T-based cytosine residues (5MeCyt) in the DNA strand. Mutations in the *MECP2* gene result in the loss of specificity for binding to 5MeCyt and are at the same time the cause of the *Rett* syndrome. The *Rett* syndrome defines the phenotype as the expression of genes that alter neurobiological activity, network formation, and function through epigenetic regulation of MECP2. Individuals with *Rett* syndrome (predominantly females as it is a predominantly hereditary disease of the X chromosome) have impaired development between the ages of 6 and 18 months, a regression of acquired skills, loss of speech, stereotypical (usually hand) movements, microcephaly, seizures, and, of course, intellectual disability. The *Rett* syndrome is classified as a neurological and autistic spectrum disease [75, 77, 78]. Differences in dose compensation of the *MECP2* gene on the X chromosome may result in different phenotypic expression of the syndrome.

In all five cases of the confirmed pathogenic *dn*M, the phenotypes of the subjects studied were very similar to the phenotypes of published individuals with identical intellectual disability syndromes.

In conclusion, the analysis of the distribution of *dn*M, spectrum and their genetic and epigenetic context in this work provided knowledge and insights into the genetic diversity of the Lithuanian genome and its possible causes. These data and

results enhance researchers' capabilities and at the same time facilitate the separation of true pathogenic *dn*M from a tolerable *dn*M background and help identify potential causal *dn*M for intellectual disability.

Conclusions

1. For the detection of *de novo* single nucleotide and small indels in the genome, when it is sequenced by different technologies, the *VarScan* open access algorithm is more appropriate because it has a higher degree of sensitivity, is suitable for working with target sequences, exome, or the whole genome, analyzes data from various genetic analyzers (Illumina, *SOLID*, *Life/PGM*), is based on a heuristic / statistical method for identifying genomic variants that meet the desired estimates of horizontal region coverage, base quality, variant allele rate, and statistical significance.
2. a) The Lithuanian population, a group of individuals with an intellectual disability, and group of siblings of a person with an intellectual disability did not differ from each other in the *de novo* mutations spectrum (*p-value* = 0.575) and are close to the population of Utah from Northern and Western Europe (CEU), Ibadan Yoruba (Nigeria), Icelandic, Scottish, and South Dakota (USA) *dn*M spectra of: C> T (33% in the first, 35% in the second, 59% in the third group) and T> C (28% in the first, 27% in the second and 18.5% in the third group).
   b) Based on the mechanisms of the *dn*M spectrum formation, all studied groups are unique:
   - The *dn*M detected in the Lithuanian population is thought to be due to errors in the repair mechanism of DNA strand mismatches, mutations in the exonuclease domain of ε polymerase, and cyclobutane pyrimidine dimers formed by UV radiation.
   - The *dn*Ms detected in the goup of individuals with an intellectual disability are believed to be due to errors in the repair mechanism of DNA strand mismatches, mutations in the *POLD1* gene, and UV radiation.
   - The etiology of any identified *dn*M in the group group of sibs of a person with an intellectual disability is unknown.
3. The estimated *de novo* mutation rate in all the studied groups is significantly higher (*p-value* = 4.49×10^{-9} for the first group, *p-value* = 5.15×10^{-6} for the second group, and *p-value* = 4.8×10^{-6} for the third group) than in the Icelandic, Danish, Dutch, Canadian, and American general populations and the

group of autists, and this was determined by the genetic structure of the analyzed genome, the exome.

- The rate of *de novo* single nucleotide and *de novo* indels in the Lithuanian population is 2.74×10^{-8} and 1.77×10^{-8} per position per generation, respectively; for the group of individuals with intellectual disability – 3.05×10^{-8} and 1.74×10^{-7} per position per generation, respectively, and for the individuals with intellectual disability sibs – 7.19×10^{-8} and 1.08×10^{-6} per position per generation, respectively.

4. *De novo* mutations in the exome are generated in promoter-like, transcriptionally active regions of the genome, regardless of the conservatism of the DNA sequence.
   - The intensity of *de novo* mutations (~68–94%) in the exomes of the Lithuanian population is influenced by regions particularly sensitive to DNAse1, CpG context, regional expression levels.
   - In the group of individuals with intellectual disabilities, the intensity of *de novo* mutations was slightly (13%) influenced by the expression levels of exome regions.
5. There are significantly more pathogenic mutations (*p-value* = 0.02) in the exomes of individuals with intellectual disabilities than in the Lithuanian population and the groups of individuals with intellectual disabilities sibs; therefore, unique *de novo* mutations (especially in the coding regions of the genome) are likely to have harmful effects on a person's phenotype and to cause intellectual disability.
   - Five pathogenic *de novo* mutations in the *ARID1B*, *PACS1*, *TCF4*, *CHD7*, and *MECP2* genes were detected in 13.5% of subjects with intellectual disabilities, three of which (in the *ARID1B*, *CHD7*, and *PACS1* genes) have not yet been described in the literature previously, causing *Coffin-Siris*, CHARGE, and autosomal dominantly inherited intellectual disability, respectively.
6. The Lithuanian population is characterized by a "mirror reflection effect," where high negative relative fitness (S < -16) amino acids are converted to neutral (0) or low (up to -5) relative fitness amino acids after *dn*M, thus confirming the theory of neutrality.

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List of publications

Published articles:

1. **Pranckėnienė** L, Kučinskas V. Trumpų *de novo* iškritų ir intarpų intesnyvumo įvertinimas skirtinguose žmogaus genomo regionuose. *Laboratorinė medicina*, 2016; 18, No. 3 (71), p. 118–122.

2. **Pranckėnienė L**, Jakaitienė A, Ambrozaitytė L, Kavaliauskienė I, Kučinskas V. Insights Into *de novo* Mutation Variation in Lithuanian Exome. *Frontiers in Genetics*. 2018;9:315. doi:10.3389/fgene.2018.00315. Impact factor – 4,151.

3. **Pranckėnienė L**, Preikšaitienė E, Gueneau L, Reymond A, Kučinskas V. *De novo* duplication in the CHD7 gene associated with severe CHARGE syndrome. *Genomics Insights*. 2019. Factor H – 5.

4. **Pranckėnienė L**, Bumbulienė Ž, Dasevičius D, Utkus A, Kučinskas V, Preikšaitienė E. Novel Androgen Receptor gene variant containing a premature termination codon in a patient with androgen insensitivity syndrome. *J Pediatr Adolesc Gynecol*. 2019 Aug 8. pii: S1083-3188(19)30257-8. doi: 10.1016/j.jpag.2019.08.001.

Poster presentations:

1. **Pranckėnienė L**, Kučinskas V. Trumpų *de novo* iškritų ir intarpų intensyvumo įvertinimas skirtinguose žmogaus genomo regionuose. *Laboratorinė medicina*, 2016; 18, No. 3 (71), p. 118–122.

2. **Pranckėnienė L**, Jakaitienė A, Ambrozaitytė L, Kavaliauskienė I, Kučinskas V. Insights Into *de novo* Mutation Variation in Lithuanian Exome. *Frontiers in Genetics*. 2018;9:315. doi:10.3389/fgene.2018.00315. Impact factor – 4,151.

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Oral presentations:

1. Pranckėnienė L, Kučinskas V. An evaluation of *de novo* mutation process intensity in the different regions of human genome. International Conference "Evolutionary Medicine: Pre-Existing Mechanisms and Patterns of Current Health Issues," 14–17 06 2016, Vilnius, Lithuania.

2. **Pranckėnienė L**, Jakaitienė A, Ambrozaitytė L, Kučinskas V. *De novo* mutacijų ir jų intensyvumo analizė lietuvių triosų grupėje. 07 12 2017, LSA Conference of Young Scientists "Bioateitis." Diploma for Best Oral presentation, 3rd place. Vilnius, Lithuania.

3. **Pranckėnienė L.** Distribution and rates of exonic *de novo* mutations in patients with intellectual disability. 10–12 05 2018, Baltic Congress in Laboratory Medicine (BALM), Vilnius, Lithuania.

4. **Pranckėnienė L**, Jakaitienė A, Kučinskas V. *De novo* mutacijų ir jų intensyvumo analizė lietuvių triosų grupėje. 14 12 2018, LSA Conference of Young Scientists "Bioateitis." Diploma for Best Oral presentation, 3rd place. Vilnius, Lithuania.

Traineeship:

21–31 01 2017. The University Medical Center Groningen (UMCG), the Netherlands.

Sigitas Chmieliauskas

EVALUATING HOMICIDE IN LITHUANIA IN TERMS OF FORENSIC, ETHICAL, SOCIAL, AND LEGAL ASPECTS

ŽMOGŽUDYSČIŲ LIETUVOJE VERTINIMAS TEISMO MEDICININIU, ETINIU, SOCIALINIU IR TEISINIU POŽIŪRIU

Medical and Health Sciences, Medicine (M 001)
Medicinos ir sveikatos mokslai, Medicina (M 001)

1. Introduction

1.1 Relevance of the Work

Homicide, not only in the context of the criminal offences, but also according to its nature and caused consequences, is the most serious and dangerous crime, which not only violates the subject and other individual rights, but generally negates the possibility to use any other rights and freedoms in the future.

There are not many long-term studies on homicide. The legal definitions of homicide differing across countries and the differences of the data sources hinder any possibility to study and compare homicides as a phenomenon internationally. There is no common homicide definition in the European Union, because each country uses different homicide definitions and applies various selection criteria. That is why uneven violent occurrences are included. Furthermore, separate homicide data sources contain different homicide information. Some sources make a detailed accident characterization, while others evaluate the patients if the attention is paid only to the guilty individuals and (or) to the appointed court judgement.

Another homicide evaluation factor is that homicides in Europe are evaluated based on different economic, geographic, social, or even political aspects. Ethnical dependency, political and economic differences are observed. After the founding of the European Union, countries, both from the political and economical perspective, became more equal; as a result, the possibility that joint researches will be executed at the European level rose.

In this project, the performed review tried to achieve a homicide comparison in different European countries.

The definition of homicide used in practice is not so simple – due to the evaluation of the act of killing, the criminal offence, and the responsibility. Also, the evaluation of the motive is different in various countries. Objectively, homicides may be associated with an active and intentional activity or carelessness, but they may also be determined due to inaction. Furthermore, some authors emphasize some specific homicide types, which include abortions, aiding suicide, euthanasia, infanticide (infant homicides), assaults, postulating death, or dangerous driving.

In 1994, the intentional homicide rate in Lithuania was 14.2 cases for 100 000 residents, while in 2017 this rate amounted to 5.2 cases for 100 000 residents, which shows that the homicide rate has decreased twice during the indicated period; however, it still stays huge while comparing with the homicide rate in other European Union countries, where the intentional homicide average is 2.0 ± 1.9 cases in 100 000 residents.

Research of homicides as a "phenomenon" requires more detailed analysis than a simple, common homicide statistic evaluation. One of the present study's tasks is to

identify the variables that are associated with the research in order to create important data categories. These data may be classified into various groups – for example, suffered and guilty of homicide individual, social, demographical characteristics, suffered and guilty of homicide individual relationships, offence location peculiarities, injury mechanisms, as well as alcohol and other psychotropic substance use during the crime. Generally, the analysis of all those variables would let us define the homicide as a psychological and sociological phenomenon and would let us have a better understanding of which social groups are the most vulnerable. Independently from the crime tendencies, the homicide rate research and the search of prevention decisions are still considered to be important questions. Generally, all the studies indicate crime prevention as the main obligation of law enforcement institutions.

1.2 Project Aim

To evaluate the peculiarities of homicides in Lithuania for the period 2004–2017 in terms of forensic and social aspects; to identify the stipulating factors of homicide spreading in Lithuania; to identify the victim and guilty of homicide individual profiles; to analyze the injuries made during the crimes and their leading variables.

1.3 Project Objectives

1. To evaluate the stipulating factors of homicide spreading and dynamics in Lithuania.
2. To evaluate injuries made during homicide and the leading patterns of injuries.
3. To evaluate the victim's social and demographic characteristics (social profile).
4. To evaluate the offender's social and demographic characteristics (social profile).

1.4 Project Novelty and Meaning

The novelty of the present study is the performed complex analysis of homicides regarding their peculiarities in Lithuania, the individual characteristics of victims and those guilty of homicide, according to gender, employment during the time of crime, education, alcohol, spread of drugs and strong psychotropic substance use during the time of crime, characteristics of the offence location, nature of the injuries, traumatic mechanisms that were performed during the crime time, and their evaluation in different groups. While executing the criminal investigation in homicide cases, this data may help to identify the victims and those guilty of homicide at the level of individual profiles.

The most informative variables of the homicide survey were provided with the help of statistical methods. These variables can form the basis for developing a unified homicide evaluation system (register) in Europe.

1.5 Statements to be Defended

1. The spread of homicide in Lithuania is determined by social and economic changes.
2. More often the murdered victims are considered to be younger men and older women who have a secondary education, are unemployed, and are intoxicated from ethyl alcohol.
3. Individuals who have committed homicide are often younger than the victims they have murdered, have a secondary education, are unemployed, are intoxicated from ethyl alcohol, and happen to be in a close environment with their victims.
4. The dominating homicide mechanism in Lithuania is that of the injuries being made with hard, blunt objects and punctured-stab wounds.
5. Victims who had lived a longer period of time, would be sober, experienced injuries with hard, blunt objects, and had 3 or less traumatic experiences.
6. Women more often murder men with objects that possess cutting or stabbing features.

2. Project methodology

2.1 Project Stages

2.1.1 Literature Review

Homicide literature was collected using issued publications on the homicide topics, *medline*, *embase* databases, by using the following keywords: intentional homicide, killed victim, killer, homicide mechanism, injuries. According to the collected literature, the homicide peculiarities in Europe and Lithuania were summarized.

2.1.2 Characteristics of the Study Group

In order to evaluate the homicides in Lithuania, The State Forensic Medicine Service (SFMS) anonymized (with regard to homicides) forensic medicine archive data (n = 2202) of 2004–2017 were collected. Data for the years 2013–2017 from the Information Technology and Communications Department near the Ministry of the Interior, when the crime was identified according to the Criminal Procedure Code's Articles 129–134 (n = 463), were retrospectively analyzed.

2.1.3 Retrospective Data Analysis

The data was collected at the Lithuania State Forensic Service from the Forensic Expert's autopsy reports. Furthermore, different variables, such as, the victim's sex, age, the place where the body was found, the relationship with the killer, the victim's and the killer's employment and education, the injuries, the number of traumatic afflictions, the time the victim survived after the assault, the number of tests done during the autopsy, the investigator's questions, and the duration of the investigation were collected at the Ministry Of Interior Affairs at the Department of Informatics and Public Relations. After collecting all of the information, a database was formed.

Forensic researches, autopsies were performed for all the homicide victims. For the identifying alcohol concentration in the internal medium, toxicological tests were performed for each case. In all the cases, the information regarding the offence place, possible death time, and other incident circumstances were received from the legal institutions.

Exclusion criteria of this research – identified accidents, suicides, suicides from faling, suicides in cases of mechanical asphyxia, traffic accidents, and deaths when advanced victim decomposition was identified. Cases when the advanced victim decomposition was identified were removed from the investigative scope due to the inability to identify the precise death cause. Data about registered homicide cases, country gross domestic product, number of residents, emigration, number of performed autopsies, used alcohol amount, and other resident variables were received from the statistical databases.

2.1.4 Statistical Data Analysis

By indicating the determining homicide variable evaluation, the method of a descriptive statistical analysis was applied. From the collected data, a database was compiled where all the data were statistically analyzed using the *R Commander* program. In order to check whether the variables are distributed according to the normal distribution law, the test of *Shapiro-Wilk* was used. *Spearman* correlation coefficient was applied. A weak correlation was identified when the r value was < 0.39; a medium intensity correlation – when r values were between 0.40 and 0.69; and a strong correlation – when the r value was > 0.70. In order to identify between which variable, the significant differences were observed, the *T-test* was used. The *Chi-square* test was used to compare the differences between groups. Confidence intervals of 95% were calculated. Data differences were considered significant when the value of p was smaller than 0.05.

2.1.5 Authorization by the Biomedical Research Ethics Committee

The research authorization was issued by the meeting (protocol No. 158200-2017/12) decision of Vilnius University, Faculty of Medicine, Vilnius Regional Biomedical Research Ethics Committee on December 5, 2017.

3. Investigative part

3.1 Determinants of Homicide

A strong and significant negative correlation between the Lithuanian gross domestic product (GDP) and the rate of intentional homicides (r = -0.85, p = 0.003) was identified. After the application of the *Poisson* regression model between the GDP, emigration rate, number of residents, alcohol use, and the number of performed autopsies, the variable of GDP was significantly related to the homicide rate (p = 0.01) (Fig. 1–5).

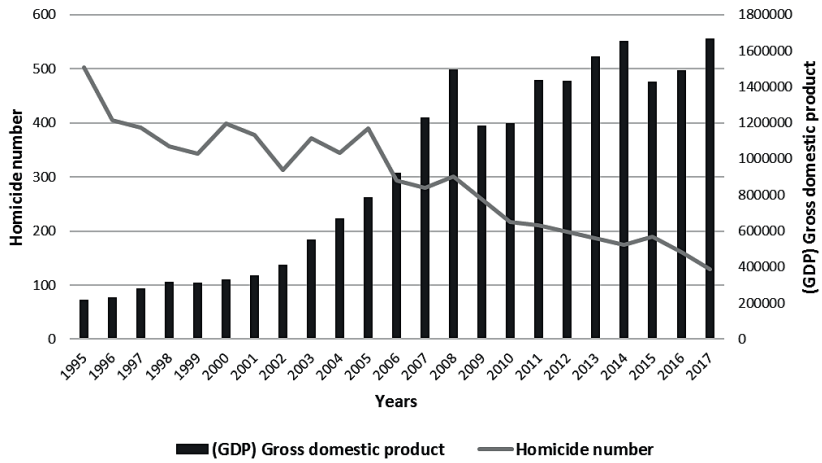

FIG. 1. Lithuanian gross domestic product for one resident and one homicide in Lithuania.

A weak correlation between the homicide rate and the emigration variable (r = 0.06, p > 0.05) was identified (Fig. 2).

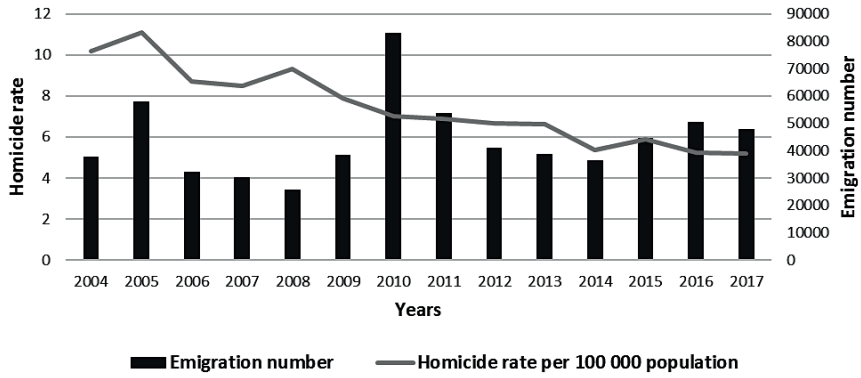

FIG. 2 Emigration and homicide rate in Lithuania.

Medium intensive negative correlation between the alcohol consumption countries (L, for one resident) and homicide rate (r = -0.49, p > 0.05) (Fig. 3).

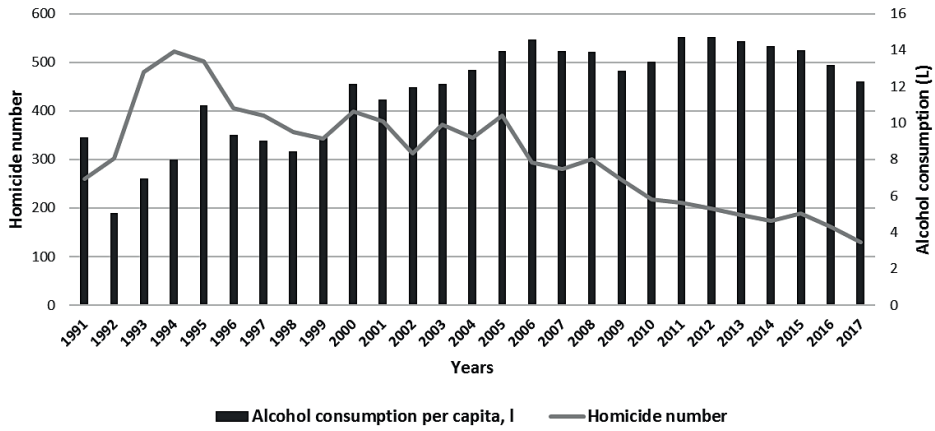

FIG. 3. Homicide number in Lithuania and alcohol consumption for one resident.

Observed was a strong correlation between the homicide rate and the number of performed forensic autopsies (r = 0.86, p = 0.002) (Fig. 4).

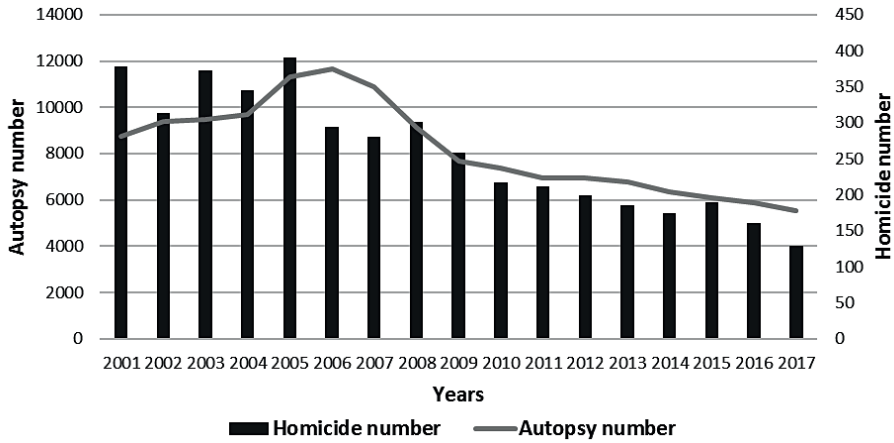

FIG. 4. Number of homicides and performed autopsies in Lithuania.

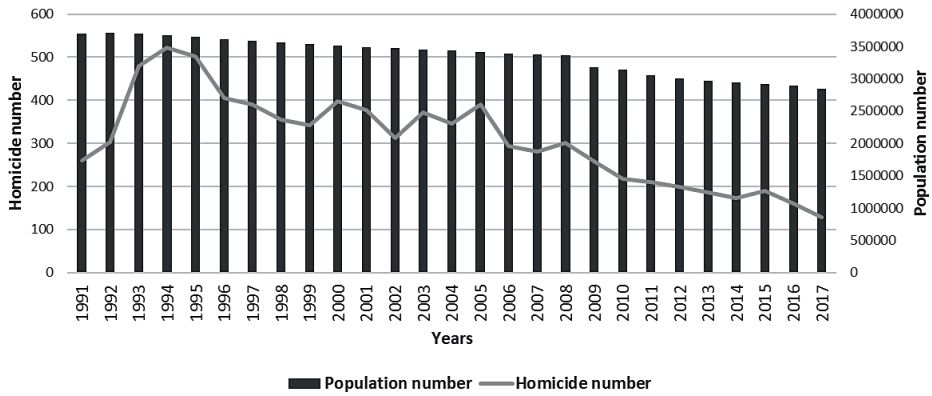

FIG. 5. Number of residents and homicide spread in Lithuania.

The descending trend of population and the prevalence of homicide were observed (Fig. 5).

There is a significant correlation between the Lithuanian GDP and the frequency of homicide, so it can be assumed that this variable is most relevant to the homicide rate.

3.2 Evaluation of Injury

From all investigated homicide cases, the most often used (52.2%) homicide mechanism was the operation of (a) hard, blunt object(s), which includes 28% of homicides made without any object, that is by punching or kicking; 33.2% of victims

suffered from an item of cutting or stabbing features. When a death cause was considered to be a gunshot, of all the homicides 5.6% were such cases; in 5.4% of cases the homicide mechanism was asphyxia. In 2.4% of all the investigative scope, an object considered to have chopping features was used. The most rarely used homicide method (1.2%) were deaths determined by the physical factors, such as leaving a victim in a helpless state, leaving them in a cold environment, burning the victim, or others (Fig. 6).

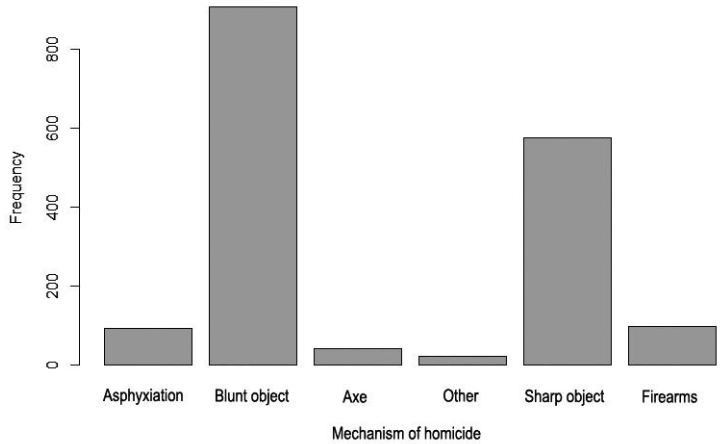

FIG. 6. Homicide method allocation.

Between the male homicides, in 53.2% of cases the homicide mechanism was bodily contact with (a) hard, blunt object(s); 35.0% were killed by using (an) object(s) with cutting or stabbing features; 5.8% were killed with a firearm; in 2.7% of cases the homicide mechanism was asphyxia; in 2.5% homicide cases, an object with chopping features was used; and in 0.8% of cases the reasons of death were other physical factors.

Between the female homicides, the homicide mechanism in 49.9% of cases was bodily contact with (a) hard blunt object(s); in 28.2% of cases the victims were killed using (an) object(s) that have cutting or stabbing features; in 5.1% of cases the victims were killed with a firearm; in 12.4% of cases, the homicide mechanism was asphyxia; in 2.3% of cases, an object of chopping features was used; and in 2.1% of cases the reasons of death were other physical factors. In evaluating the mechanism of homicide across the different gender groups, the homicide mechanism of asphyxia is more often found among women than men (Fig. 7).

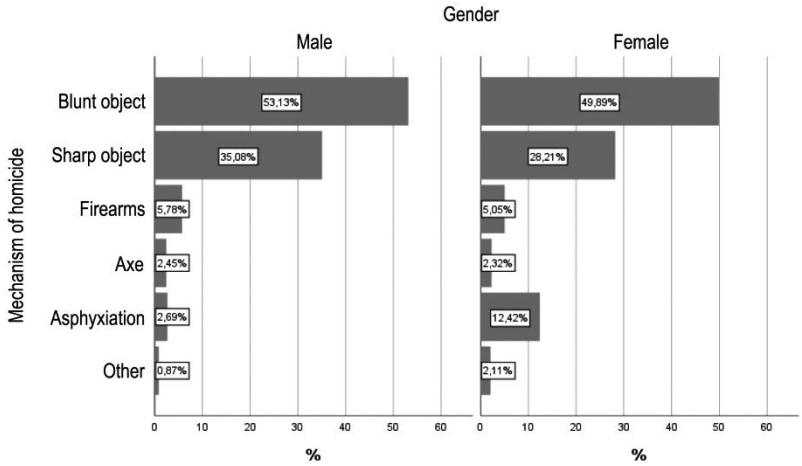

FIG. 7. The homicide mechanism in different gender groups.

The mean male age in cases of bodily contact with (a) hard, blunt object(s) was 48.4 ± 14.4 years. For women who were killed by such mechanism, the mean age was 52.8 ± 15.9 years. For men who were killed with (an) object(s) contain-

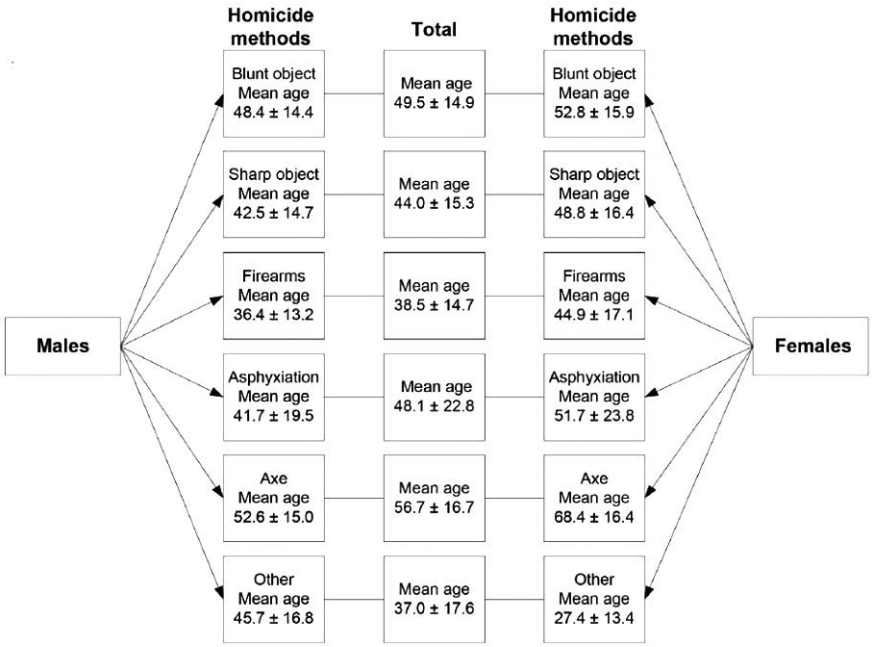

FIG. 8. Homicide mechanism according to the homicide victims' age and gender.

ing the features of cutting or stabbing, the mean age was 42.5 ± 14.7 years. In this group, women's mean age was 48.8 ± 16.4 years. For men who were killed with a firearm, the mean age was 36.4 ± 13.2 years. The women's mean age in this group was 44.9 ± 17.1 years. According to this data, it can be stated that older women tend to became homicide victims more often (Fig. 8).

In order to identify whether the homicide mechanisms significantly differ across the gender groups, the *Pearson Chi-square* criterion was applied. Proportions were compared with a *Bonferroni* correction. It was identified that in gender groups ($p = 0.0001$), the homicide mechanism statistically significantly differs. The men's homicides were more often executed with (a) cutting or stabbing object(s), while in the women's group, more often than in the men's group, the identified homicide mechanism was asphyxia or other mechanisms like deaths determined by such physical factors as leaving the victim in a helpless state, leaving them a cold environment, burning the victim, and others.

The reason of the death for the majority of homicides (68%) was due to the injuries of one type injury object. Homicides when two, three, or four injury object types were used accordingly match 30%, 1.7%, and 0.3% of all cases. While comparing with the men's group, women were more often killed using more than one injury type object ($p < 0.001$). Homicide victim identified with a higher ethyl alcohol concentration in their blood have experienced more traumatic inflictions ($p < 0.001$). Those homicide victims who had experienced the homicide mechanism of bodily contact with (a) hard, blunt object(s) also happen to have had experienced more traumatic inflictions ($p < 0.001$) (Table 1).

TABLE 1. Homicide mechanism allocation in the different gender groups.

		Gender			
		Male		Female	
		%	Mean	%	Mean
Mechanism of homicide	Blunt object	53,1		49,9	
	Sharp object	35,1		28,2	
	Firearms	5,8		5,1	
	Axe	2,5		2,3	
	Asphyxiation	2,7		12,4	
	Other	0,9		2,1	
Number of traumatic inflict			8,01		10,36
Different traumatic mechanisms			1,32		1,43

The mean men's age of 48.19 years and the mean women's age of 53.21 years were seen in the homicides of one type injury factor of (a) hard and blunt object(s). Age in this group differs significantly ($p < 0.05$). The number of traumatic inflictions when the homicide of (a) hard, blunt object(s) was identified has many abstractions;

however, in cases of traumatic inflicts of one type, the committed male homicides resulted in (mean) 10 traumatic inflictions, and for women – 14 (mean) traumatic inflictions, which reveals that in cases of women's homicides, a significantly higher number of traumatic inflictions have occurred ($p = 0.0002$).

When a homicide of one type of injury factor of (a) cutting or stabbing object(s) was identified, the mean men's age was 43.34 years, and women's – 46.58. Age in this group does not differ so significantly ($p = 0.21$). The number of traumatic inflicts when a homicide made using (an) object(s) of cutting or stabbing features was identified also has many abstractions; in cases of one type traumatic inflicts, the committed men's homicides were made with (mean) 2.8 traumatic inflictions, and in the cases of women – 6.8 (mean) traumatic inflicts, which reveals that there is a significantly higher number of traumatic inflicts in cases of women's homicides ($p = 0.002$).

When a homicide of one type injury factor – a gunshot wound – was identified, the mean men's age was 36.85 years, while the mean women's age was 45.93 years. Age in this group differs significantly ($p = 0.03$). The number of traumatic inflicts when a homicide with a firearm was identified also has many abstractions; however, in cases of one type traumatic inflicts, the committed men's homicides were made (mean) with 2 traumatic inflictions, and for women – 1.8 (mean) traumatic inflictions; this reveals that the traumatic infliction number of men's and women's homicides that has been committed with a firearm does not differ ($p = 0.56$).

The mean men's age of 57.14 years and that of women's 62.0 years were ascertained in cases when a homicide of one type injury factor using an object with chopping features (cutting injuries) was identified. The ages in this group do not differ significantly ($p = 0.83$). The number of traumatic inflictions of homicides using objects of chopping features had one abstraction (when the cutting injuries were made by 21 traumatic infliction); however, after removing this abstraction, cases of one type traumatic inflicts, when men's homicides were committed, were made (mean) with 4.7 traumatic inflictions, and 7.5 (mean) traumatic inflictions for women, which reveals that the number of traumatic inflictions of women's and men's homicides executed using objects with chopping features does not significantly differ ($p = 0.64$).

The distribution of variable (number of traumatic inflictions) is asymmetric, where the data are allocated according to the non-normal distribution law, *Shapiro-Wilk normality test* $p < 0.05$, with a large amount of abstractions, which meaning ranges from 1 to 164, its mean 8.65 ± 14.53, median is equal to 3 (Fig. 9).

Therefore, the variable differences between the various homicide ways were identified using a non-parametric *Kruskal-Wallis* test, which allows to compare the qualitative variable rage means in several groups.

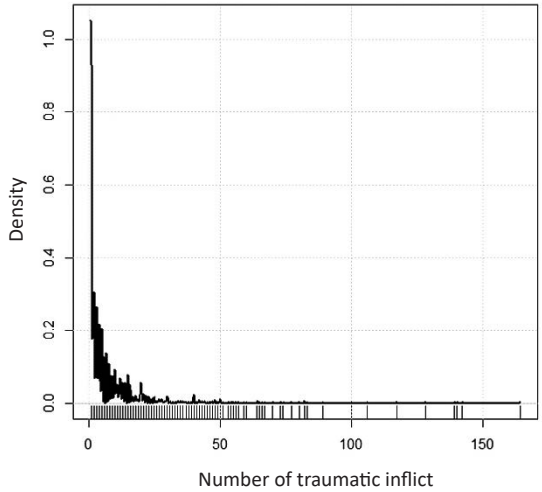

FIG. 9. Allocation of the scope of the traumatic inflictions number.

The amount of traumatic inflictions does not statistically significantly differ between the victim homicide ways ($p = 0.0001$); however, it is not clear if a statistically significant difference occurs in all homicide ways. Therefore, as an alternative, the number of traumatic inflictions was logged in order to set it closer to the normal data allocation and to reduce the abstraction; here the *ANOVA* test was applied with an original as well as a logged variable by applying several post-hoc criteria according to the group size and dispersion difference. The used criteria were *Games-Howell, Tamhane* and *Dunnett T3*. All these *post-hoc* criteria have revealed that the number of traumatic inflictions does not statistically significantly differ between the homicides committed with hard, blunt objects and with objects with chopping features; as for the means – both contain 12 ± 16 and 12 ± 15 traumatic inflictions. After variable logging, for homicides committed with objects with cutting or stabbing features, the number of traumatic inflictions statistically significantly differs from all other traumatic inflictions numbers of all homicide methods. The mean of the traumatic infliction number of homicides executed with objects of cutting or stabbing features is 5.74 ± 13.14. Meanwhile, the number of traumatic inflictions of gunshot wounds, asphyxia, and other homicide methods does not statistically significantly differ. Their traumatic infliction number means are similar – 1.17 ± 1 and 1.91 ± 1 traumatic inflictions.

The homicide victim survival time after experiencing injuries is coded in a range scale from 1 to 4. In order to evaluate for which homicide mechanisms the victim survival time (time from injury to death) belongs, the binary logistic regres-

sion was applied. Also, for each way separately, separate binary logistic regression models were created. The *Enter* method was used, statistically not significant independent variables were mechanically eliminated, and the model was repeated until only the statistically significant variables remained. Victims who have suddenly died had been of older age, females found indoors, identified with severe intoxication, and have had experienced more injury mechanisms and traumatic inflictions.

For the majority of homicide victims (71%), medics did not manage to provide first aid and, shortly after sustaining injuries, they died (sudden deaths) (Fig. 10). While for the 29% of victims, medics had provided first aid measures; however, they died shortly after sustaining their injuries (Table 2).

TABLE 2. Homicide mechanism and time of death after experiencing an injury (%).

	Mechanism of homicide					
	Blunt object	**Sharp object**	**Firearms**	**Axe**	**Asphyxiation**	**Other**
Sudden deaths	62,1	80,1	80,4	83,3	95,7	76,2
Emergency Medical Assistance	3,0	6,1	2,1		1,1	9,5
Survived less than 24 h	9,8	10,9	14,4	2,4	2,2	
Survived over 24 h	25,1	2,9	3,1	14,3	1,1	14,3
	100%	100%	100%	100%	100%	100%

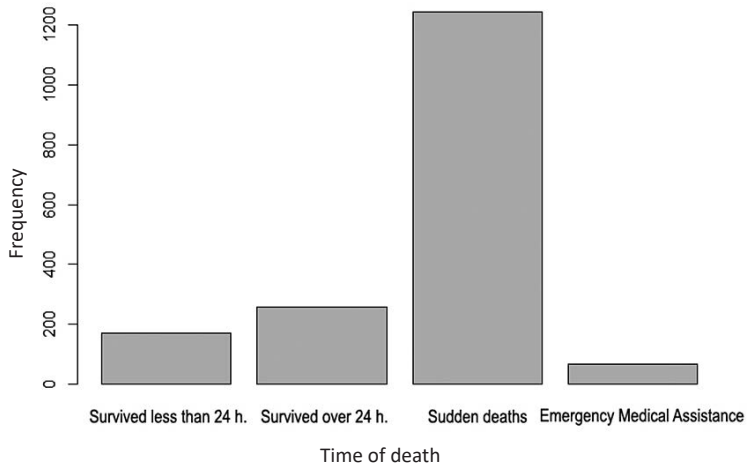

FIG. 10. Death time allocation after experiencing an injury.

Cases when the emergency medical service (EMP) was provided but victims died before hospitalization amount only to 3.8%, and that is why the prognostication of those cases using the binary logistic regression is harder. As an alternative, the *CHAID* classification method was applied and the following conditions identified:

EMS help (when victim did not manage to live until they were taken into a care institution) was more often provided only for those victims who had experienced a cutting or stabbing injury and had 3 or less injuries. From all the data, those cases include 47.8%. EMS help was provided only for those victims who were identified as having experienced injuries from hard, blunt objects and were moderately intoxicated. Those cases make up 29.9%. EMS help was also provided when other homicide mechanisms were identified, and when the victim had a light level of intoxication in their bloodstream or were sober. There were 16.4% of those cases.

Victims who, after experiencing their injuries, have survived more than 24 hours in a care institution, were identified with a light, moderate level of intoxication or were sober and younger than 34 years, the percentage of these cases – 27.8%. Cases where the identified victim was older than 33 years old and the place of their discovery was outdoors include 27.8%. If the victim's place of discovery was indoors, such cases make up 31.4%.

Victims who, after experiencing their injuries, have survived more than 24 hours make up 14.84% of all the victims that were taken into a care institution. Using a binary logistic regression, it was identified that between the victims who have survived longer, men who have been identified with a light level of intoxication were more often found outdoors, and in cases of experiencing injuries from hard, blunt (objects), they had experienced less traumatic inflictions.

For the victims found outdoors, emergency medical help was provided more often than for the victims found indoors ($p < 0.001$). Medical help for men was provided more often than for women ($p < 0.001$). More often sober victims would survived more than 24 hours in a hospital than the victims with a severe levels of intoxication ($p < 0.001$). Victims who have survived more than 24 hours in a hospital would have more often experienced injuries from hard, blunt objects ($p < 0.001$). The injured who had been identified with less traumatic inflictions would more often, after experiencing their injuries, survive more than 24 hours in a hospital ($p < 0.001$).

While comparing the victims' age across the different survival groups (applied one *Anova* factor with a *Games-Howell* criterion*)*, these statistically significant differences were identified: the mean age of victims who had survived less than 24 hours in a care institution was 4.12 years younger than the age of victims who had died suddenly, and it was 4.8 years younger than the age of victims who had survived more than 24 hours in a care institution. The mean age of all scope victims who had died shortly after experiencing injuries – 47.45 ± 16 years; the mean age of those who had been provided only with an EMS – 44.2 ± 15.39; the mean age of victims who, after experiencing their injuries, had survived less than 24 hours in a care institution – 43.33 ± 16.1 years; victims who, after experiencing their injuries, had survived more than 24 hours – 48.12 ± 16.11 years (Fig. 11).

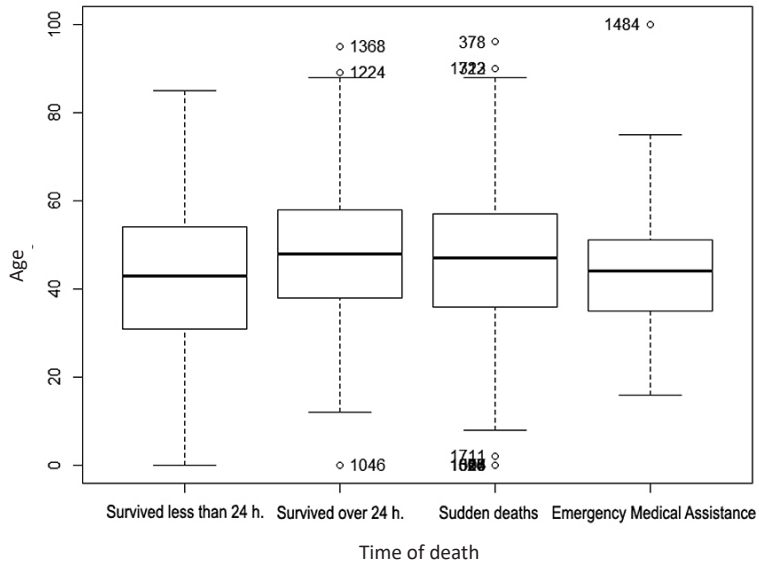

FIG. 11. Age allocation according to the different times of death after experiencing injuries.

Comparing the time of death in the different gender groups (*Chi-square* test), it was identified that, males are dominant in these variable categories – that is, a sudden death was identified more often in the women's group than in the men's group ($p = 0.0001$). Also, men, more often than women, would have survived more than 24 hours in a care institution ($p = 0.0001$) (Table 3).

In order to evaluate on which variables does the survival of a homicide victim depend, the method of the General Finite Line Source Model was applied. The dependent model variable is ranges from 4 categories, where a higher category means a better chance of survival after experiencing injuries. Independent factorial vari-

TABLE 3. Time of death allocation in different gender groups.

		Gender			
		Male		Female	
		Number	%	Number	%
Sudden deaths	No	405	32,07	89	18,74
	Yes	858	67,93	386	81,26
Emergency Medical Assistance	No	1208	95,65	463	97,47
	Yes	55	4,35	12	2,53
Survived less than 24 h	No	1128	89,31	441	92,84
	Yes	135	10,69	34	7,16
Survived over 24 h	No	1048	82,98	432	90,95
	Yes	215	17,02	43	9,05

ables are injury mechanism and recoded dichotomy – how many traumatic mechanisms were applied (one or more than one).

The quantitative trauma variable was set as a covariate. The received model is statistically significant ($p = 0.0001$), and all independent variables show the impact for survival ($p < 0.05$, B = 1.106). A higher possibility to survive longer would be present if the victim had experienced injuries from a hard, blunt object ($p = 0.037$); however, if the cause of death were asphyxia, it would more often identify with the occurrence of sudden death ($p = 0.015$, B = -1.775). In cases of a victim being injured from one type of trauma, a survival possibility of more than 24 hours would be higher than for the victims, who had experienced more than one trauma type ($p = 0.002$, B = 0.475). The higher traumatic inflict number is experienced, the lower possibility of survival ($p = 0.0001$, B = -0.062) (Table 4).

As an alternative for the General Finite Line Source Model, the *CHAID* classification tree was applied. The traumatic mechanism makes the highest impact on victim survival. If an injury is made from contact with a hard, blunt object, there is an increase in the time of death of victims, who have lived longer than 24 hours. It was identified that from the 258 homicide victims who have survived more than 24 hours after experiencing their injuries, 228 have experienced the injuries of a hard, blunt object, which is over than 88% of cases. If an injury from a hard, blunt object was identified, the time of death also depends on how many traumas the homicide victim has experienced – the lesser the number of traumas, the longer the time of survival. Homicide victims who had survived more than 24 hours after experiencing their injuries and who experienced 3 or less traumatic inflictions with a hard, blunt object make up 107 (46.9%) cases; in cases of 4–8 traumatic inflictions of a hard and blunt object – 67 (29.3%); if 9–20 traumatic inflictions of a hard, blunt object – 49 (21.5%).

TABLE 4. Variable allocation according to the different time of death intervals.

		Number (n)	%
Time of death	Sudden deaths	1244	71,6
	Emergency Medical Assistance	67	3,9
	Survived less than 24 h	169	9,7
	Survived over 24 h	258	14,8
Mechanism of killing	Blunt object	908	52,2
	Sharp object	577	33,2
	Firearms	97	5,6
	Axe	42	2,4
	Asphyxiation	93	5,4
	Other	21	1,2
Number of traumatic inflict	1	1183	68,1
	2–4	555	31,9

3.3 Characteristics of Homicide Victims

Victim allocation, according the gender, analysis shows that men contain the major part, 73% of all cases, while women contain only 27% of all investigates.

The mean age of the all investigated murder victims was 47 ± 16 years. Victims younger than 18 years old included 4.2% of all investigated cases. The mean age of the murdered women is defined as 50.9 ± 17 years. A statistically significant fact is that the major part of the murder victims were younger men and older women ($p < 0.001$). From all the murdered victims, 72% were unemployed, and 64% of them had only a secondary education; 73% of the murder victims are men; 66% are homicides made indoors, and 57% were committed within the urban territory (Table 5).

Victims whose blood tests came positive to the ethyl alcohol concentration contains a larger part (58.6%) of all the homicide cases; 9.4% of them were identified with light intoxication (0.4 ‰ – 1.5 ‰); 18.1% identified with moderate intoxication (1.51 ‰ – 2.5 ‰); and the most often – 31.1 – those identified with severe intoxication in the bloodstream (more than 2.5 ‰). Finally, only 41.4% of the murder victims were sober.

Evaluating the places where the victims were found, the major part (57%) of persons were murdered in urban places, while 43% were murdered in rural areas. Men: 57% of them were found in urban places, while 43% in rural areas. Women: 56% were murdered in urban places, while 44% in rural areas. The mean age of a victim found in an urban place was 46.28 ± 15.97 years, in a rural area – 48.02 ± 16.13 years. In order to compare the victims who were found in the urban and rural areas, the independent scope *T-test* was applied. An evaluation of the place of victim discovery yielded that the victims of rural areas were younger by 1.74 year than the victims murdered in urban places; in other words, older victims were more often murdered in rural areas ($p = 0.025$) (Fig. 12).

Though, the difference is not so big, its statistical significance is observed due to the large scope of investigatives. Therefore, according to *Cohen's d* formula, the effect size of the found differences was additionally calculated. The effect size is evaluated as low when its value is from 0.3 till 0.5, and as high if its value is less than 0.8. In this group, *Cohen's d* value is equal to 0.11, which identifies very low effect: 57.9% of victims when the homicide mechanism was bodily contact with hard, blunt objects, 60.3% of victims murdered using a cutting or stabbing features-containing object, 50.5% of victims murdered with a firearm, and 55.9% of victims whose homide mechanism was asphyxia, were found in urban places; 64.3% of victims who have been killed with objects containing chopping features and 66.7% of victims whose reasons of death were other physical factors were found in rural areas.

TABLE 5. Characteristics of homicide victims.

Years	Male (%)	Female (%)	Male age		Female age		Outdoor killings	Indoor killings	Intoxicated	Rural area	Urban area
			Mean	SD	Mean	SD					
2004	66%	34%	42.8	14.5	49.8	18.8	38.2%	61.8%	57%	44.8%	55.2%
2005	72.1%	27.9%	44.6	15.7	51.1	16.5	32%	68%	58.6%	39.7%	60.2%
2006	71.9%	28.1%	43.9	14.6	56.6	19	32.2%	67.8%	60.2%	45.4%	54.6%
2007	76.9%	23.1%	45.7	13.9	50.9	16.2	35.7%	64.3%	55.8%	37.2%	62.8%
2008	75.1%	24.9%	48.7	15.6	51.2	15	38.7%	61.3%	57.8%	43.1%	56.9%
2009	75.9%	24.1%	43.8	15.8	51.4	18.4	36.9%	63.1%	58.1%	43%	57%
2010	65.9%	34.1%	47.9	16.5	48.7	18	27.1%	72.9%	60.5%	45%	55%
2011	66.2%	33.8%	46.7	12.5	46.6	17.8	31.5%	68.5%	59.2%	43.8%	56.2%
2012	76.7%	23.3%	47	13.8	50.1	19	26.2%	73.8%	67%	41.8%	58.2%
2013	79.5%	20.5%	45.9	15.9	52.2	20.5	29.5%	70.5%	57.4%	45.1%	54.9%
2014	66.1%	33.9%	42.9	15.6	47.6	17.1	37.8%	62.2%	55.1%	45.4%	54.6%
2015	61.5%	38.5%	41.7	13.6	48.8	19.6	33.8%	66.2%	61.2%	35.4%	64.6%
2016	69.7%	30.3%	45.3	15.2	54.4	19.2	35.6%	64.4%	57.9%	41.7%	58.3%
2017	66.2%	33.8%	43.4	16.3	48.1	23.6	33.3%	66.7%	56.8%	32%	68%

SD – standard deviation

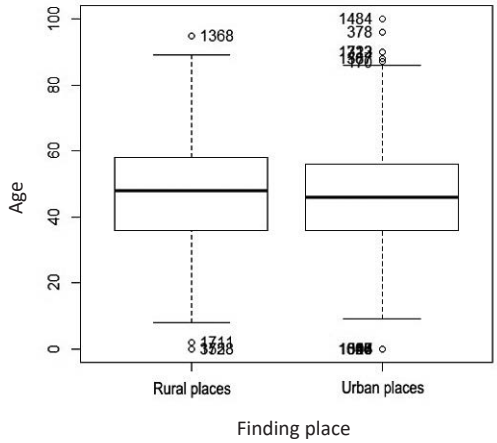

FIG. 12. The age distribution of homicide victims found in urban and rural areas.

The major part (66%) of murdered individuals were found indoors (in a flat, house, stairwell, basement, etc.), while 34% of victims were found outdoors (on a street, forest, etc.). Men: 61% of them were found indoors, 39% – outdoors. Women: 82% of them were found in indoors, 18% – outdoors. The mean age of murdered women found indoors was 49.45 ± 15.81 years, and found outdoors – 42.23 ± 15.47 years (Fig. 13). In order to compare the mean ages of victims found indoors and outdoors, the independent scope *T-test* was applied. Individuals murdered indoors were older (by 7.23 years) than those who were killed outdoors ($p < 0.001$). According to *Cohen's d* formula, the effect size of the found differences was additionally

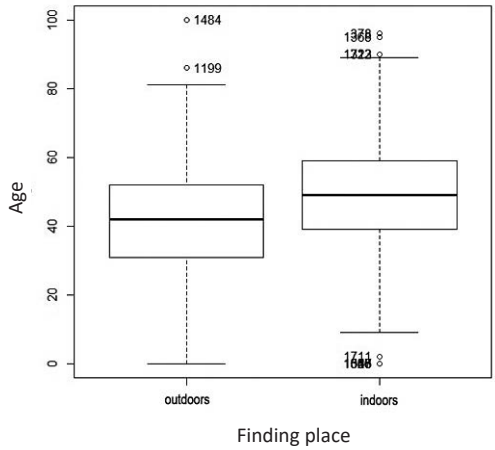

FIG. 13. The allocation of homicide victims found outdoors and indoors.

calculated, and its value in this group is 0.45, which shows an average effect. Women were more often killed indoors than outdoors (*Chi – squared test,* $p < 0.001$). According to these variables, it can be stated that the bigger the homicide rate is indoors, the higher the homicide risk is for the group of older women.

In 39.1% of the investigated cases, the murdered men were sober. Light intoxication in a group of men's homicides, was identified as 9.2%; in 18.8% of cases, moderate intoxication was identified, and severe intoxication was in 32.9% of cases. Sober women were killed in 47.1% of cases. Light intoxication was identified in 10.1% of the women's group; moderate intoxication – 16.4%, and severe intoxication – 26.5% of cases. Severe intoxication was identified in the group of men more often than in the group of women ($p = 0.001$) (Table 6). Severe intoxication in the homicide victims found outdoors was identified more often than in the victims found indoors ($p < 0.001$). More often, severe levels of intoxication were identified for victims, killed with objects with cutting or stabbing features ($p < 0.001$).

TABLE 6. Age and gender allocation according to the alcohol level.

	Sober	0,41–1,5‰	1,51–2,5‰	> 2,51‰
Mean age of male	47,5 ± 17,2	43,2 ± 17,5	44,4 ± 14,5	44,4 ± 11,9
Mean age of female	54,4 ± 21,2	48,9 ± 17,6	49,7 ± 14,1	46,4 ± 11,2
Mean age of all	49,7 ± 18,8	44,9 ± 17,7	45,7 ± 14,9	44,9 ± 11,1

3.4 Characteristics of Homicide Suspects

In the period of 2013–2017, in 463 of cases the persons who have committed the homicides was identified. The men's group, is not only dominant in the homicide victim group (71%) but also in the homicide suspect group, where, according to the gender allocation (n = 402), men convicted for murder make up 87% of cases, while women amount to 13% (n = 61).

In evaluating the murderer age groups, it can be seen that the majority (73%) of them are middle ages, 15% – older aged, and 8% are younger than 18 years old. The mean age of persons who have been convicted for murder is 34.9 ± 15 years. This shows that younger individuals tend commit homicides more often ($p < 0.05$) (Fig. 14).

The mean age of men convicted for homicide was 34.5 ± 14, while for women, the mean age was 38.5 ± 14 years (Table 7). A statistically significant difference is observed between the mean age of men and women (independent samples *T-test,* $p = 0.004$). In all the samples, the age allocation of persons who have committed a homicide is asymmetric where the data are allocated according to the normal dis-

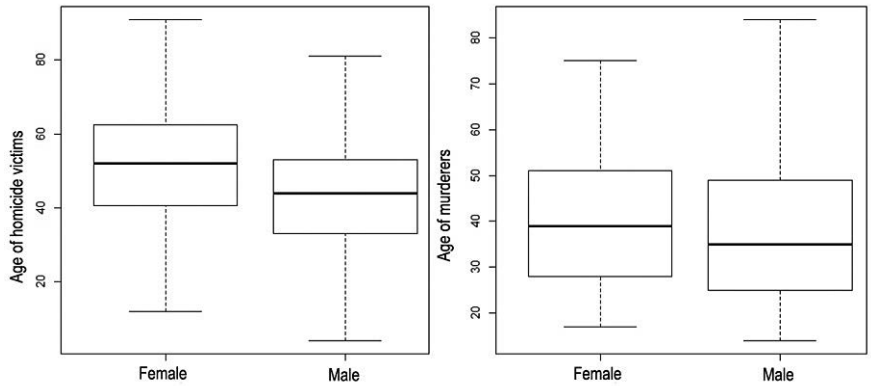

FIG. 14. the allocation of homicide victims and murderers according to their gender and age.

tribution law, *Shapiro-Wilk normality test* $p < 0.05$, with abstractions where the age varies from 14 to 84 years and the median is equal to 32 years (Fig. 20). From 2013 to 2017 years, a percentage of an increase of women commiting homicides was observed.

TABLE 7. Individuals who have commited homicides; allocation according to gender and age.

				Mean of age	
Years	**Male (%)**	**Female (%)**	**Intoxicated**	**Male**	**Female**
2013	98%	2%	63,5%	34,5 ± 15,1	38,2 ± 12,7
2014	89,7%	10,3%	65,4%	33,7 ± 14,4	39,8 ± 15,6
2015	89%	11%	70,7%	34,2 ± 14,1	41,6 ± 12,4
2016	88,5%	11,5%	53,6%	36,7 ± 13,9	36,1 ± 12,4
2017	86,2%	13,8%	56,7%	33,8 ± 12,9	37,3 ± 15,2

3.5 Relationship between Variables

Comparing the homicide victim and murderer ages in a victim-suspect gender matrix, a *Pearson* correlation coefficient and dispersion chart was used. Found correlations: if the victim and suspect are men, there is no relation between the victim and the suspect; a wide data dispersion is observed. The correlation coefficient is not statistically significant ($r = 0.07$, $p = 0.273$); if a victim is a woman and the suspect a man, a weak ($r = 0.231$), statistically significant correlation ($p = 0.01$) would be identified; the dispersion chart shows that the relation could be stronger, and that the age of men and women could synchronize; however, there is a major part of younger men who were accused of murdering women much more older than themselves; if a victim is a man and the suspect a woman, the relation is average ($r = 0.680$) and sta-

tistically significant ($p = 0.0001$), where the majority of the accused women's age is equal to the age of the murdered men; however, there are cases of women who have been suspected of murdering infants, and these abstractions weaken the relation; if a victim is a woman and the suspect a woman, the relation is strong ($r = 0.757$) and statistically significant ($p = 0.003$), even though, only 13 of these cases were observed – including some these cases that include infant homicide (Fig. 15).

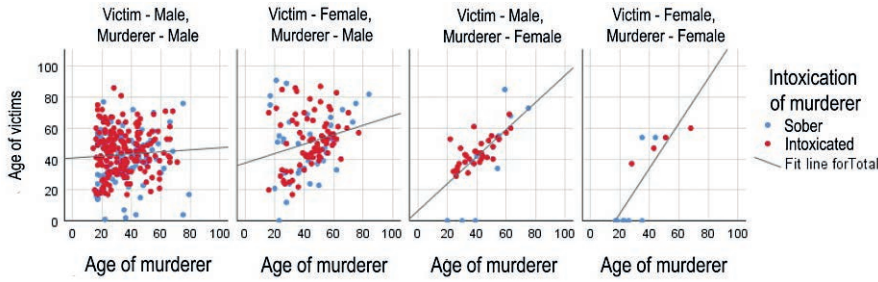

FIG. 15. Homicide victim and murderer age allocation.

In these charts, the intoxication of murderers is marked using the different colors. It is noted that most often the suspects of children and infant murder were sober both in men and women's groups. In groups where women were suspected of murdering men, a major part was identified as having been intoxicated.

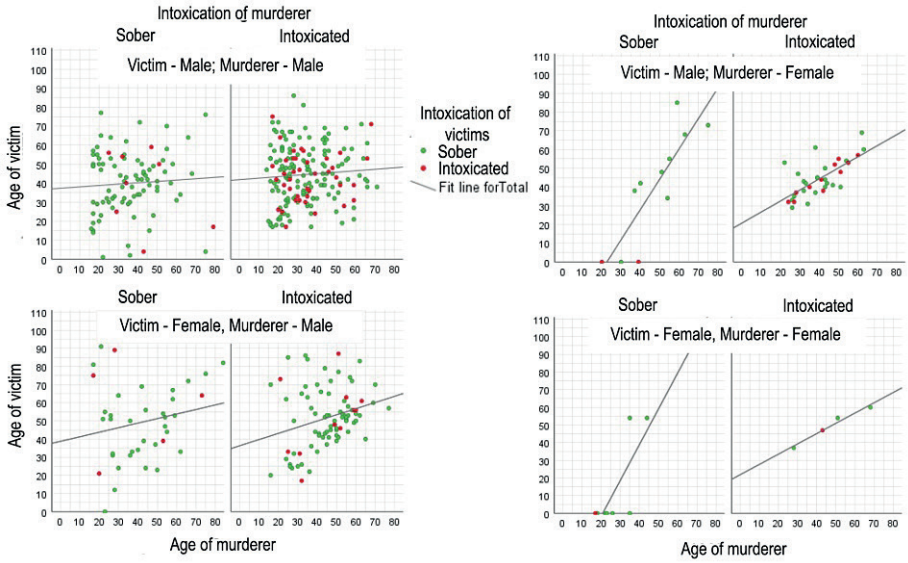

FIG. 16. Intoxication relation of homicide suspects and victims.

The relation of intoxication between homicide suspects and homicide victims is analyzed in detail using dispersion charts, the *Chi-square* and *Fisher exact test*, and by additionally allocating the data according to the suspect and victim gender combinations (Table 8), wherein the diagrams also reflect the age of homicide suspects and victims (Fig. 16).

A statistically significant relation is observed only in the group where the homicide victims were men and the persons to have committed the homicides were also men. By using the proportion comparison with a *Bonferoni* correction, it was identified that this groups' proportion is bigger than it should be, as in cases of sober homicide victims and homicide suspects, as in cases of intoxicated homicide victims and homicide suspects, while it is also less believed that the intoxication was identified only for one of the parties. In other groups, any relation of the sobriety/intoxication between the homicide victim and murderers is not found, even though the number of observations is too small in cases where women were both homicide suspects and victims (the scope is considered to be insufficient).

TABLE 8. Data allocation according to the homicide victim and murderer gender combinations.

		The intoxication of male who executed of homicide					
		Sober	Intoxicated	Total	Sober	Intoxicated	Total
Intoxication of male which was killed	Sober	83	147	230	29,9%	52,9%	82,7%
	Intoxicated	8	40	48	2,9%	14,4%	17,3%
	Total	91	187	278	32,7%	67,3%	100%

Pearson Chi-square test = 6,620, Fisher exact test p = 0,01

		The intoxication of female who executed of homicide					
		Sober	Intoxication	Total	Sober	Intoxicated	Total
Intoxication of male which was killed	Sober	10	23	33	21,7%	50,0%	71,7%
	Intoxicated	2	11	13	4,3%	23,9%	28,3%
	Total	12	34	46	26,1%	73,9%	100%

Pearson Chi-square test = 0,973, Fisher exact test p = 0,464

		The intoxication of male who executed of homicide					
		Sober	Intoxicated	Total	Sober	Intoxicated	Total
Intoxication of female which was killed	Sober	33	71	230	27,5%	59,2%	86,7%
	Intoxicated	5	11	48	4,2%	9,2%	13,3%
	Total	38	82	278	31,7%	68,3%	100%

Pearson Chi-square test = 0,001, Fisher exact test p = 0,969

		The intoxication of female who executed of homicide					
		Sober	Intoxicated	Total	Sober	Intoxicated	Total
Intoxication of female which was killed	Sober	8	3	11	61,5%	23,1%	84,6%
	Intoxicated	1	1	3	7,7%	7,7%	15,4%
	Total	9	4	13	69,2%	30,8%	100%

Pearson Chi-square test = 1,593, Fisher exact test p = 0,505

A major part of murderers (66%, n = 307) were intoxicated, while 32% (n = 150) of murderers were sober. Meanwhile, 2% were under the effect of drugs. Among the sober men murderers, the mean age was 38.1 ± 16 years, among women – 37.7 ± 16 years. In this group a statistically significant difference, between the mean age of women and men ($p = 0.91$) is not observed. When the person who had committed a homicide were not intoxicated from ethyl alcohol (the common mean age was 43.8 ± 18 years), the mean age of the homicide victim was 37.7 ± 16 years. This group observes a significant difference between the mean age of murderers and homicide victims ($p < 0.008$).

Between the murderers who were intoxicated with ethyl alcohol, the mean men's age was 37.5 ± 14 years, and the mean women's age – 41.3 ± 12 years. In this group, no significant difference between the mean age of men and women ($p = 0.07$) is observed. When the murderers were intoxicated with ethyl alcohol (common mean age 37.9 ± 14 years), their homicide victims' mean age was 46.5 ± 14 years. This group observes a significant difference between the mean ages of murderers and homicide victims ($p < 0.05$).

In comparing the intoxication of homicide victims and murderers in two groups – when the identified homicide resulted from a hard, blunt object and and an object of cutting or stabbing features – the *Chi-square* criterion was used; however, there were no statistically significant differences between the homicide mechanisms (*Chi-square* = 2.970, $p = 0.227$).

A murderer employment analysis reveals that the majority (73%, n = 338) of murderers were unemployed, their mean age was 38.1 ± 13 years, while during the homicides only 15% (n = 70) of them had a job, their mean age being 36.0 ± 11 years. There is no statistically significant difference between the mean ages of employed and unemployed persons ($p = 0.33$). During the homicide time, only 6% (n = 26) of the investigated persons were in the process of acquiring an education, their mean age being 17.4 ± 2 years. In the group of young people (who had been studying) convicted for murder, the homicide victim mean age was 41.1 ± 20 years. This group observes a statistically significant difference between the mean age of victims and their murderers ($p < 0.05$).

In evaluating the homicide victim and murderer employment proportions, a group of unemployed suspects was distinguished to be the riskiest, while the victims and the proportion of the unemployed persons consists of even 37.6% of all cases. When, during the homicide, both parties (homicide victim and murderer) were employed, the incident proportion was equal to 6.2% (Fig. 17).

An education analysis of individuals convicted for homicide revealed that the majority (56%, n = 257) had a primary or professional education. The mean age of individuals convicted for homicide was 34.8 ± 15 years; 37% (n = 172) of persons

FIG. 17. The employment allocation of individuals who have been convicted for homicide and homicide victims.

convicted for homicide had a secondary education, their mean age being 40.4 ± 14 years. Only 3% (n = 16) of persons convicted for homicide had acquired a university education, their mean age being 44.5 ± 12 years. A statistically significant difference was observed between the mean age of individuals convicted for murder who had a primary or professional education and those who had a secondary education (p = 0.001). There is no statistically significant difference between the mean age of

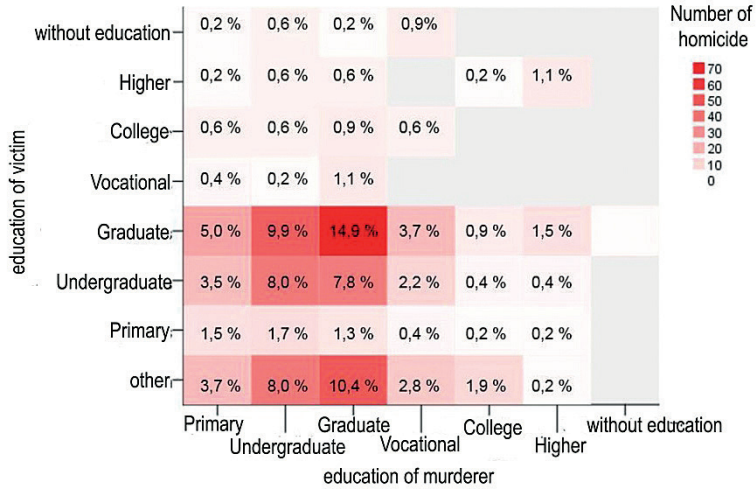

FIG. 18. The allocation of persons convicted for homicide and homicide victim education.

individuals convicted for murder who had a secondary education and those who had finished university studies ($p = 0.48$).

In valuating the education proportions of the murderers and homicide victims, the group of people with primary and secondary education became distinguished. The proportion of the homicide victims who had a primary education and suspects with a secondary education consists of 14.9% of all cases (Fig. 18).

No statistically significant difference between the mean age of individuals who had committed a homicide in urban and rural places ($p = 0.54$) is observed. However, victims murdered in the rural areas were older than the victims murdered in urban places ($p = 0.041$). A statistically significant difference between the mean age of convicted murderers who had committed a homicide indoors (mean age 39.6 ± 15 years) and outdoors (mean age 31.1 ± 13 years) is observed ($p < 0.05$), while victims who had been found indoors (mean age 47.7 ± 15 years) were also older than the victims found outdoors (mean age 39.1 ± 15 years) ($p < 0.001$). A statistically significant difference was observed between the mean age of individuals who had committed a homicide indoors and the homicide victims who had been found indoors ($p < 0.05$); also, a significant difference was observed between the mean age of individuals who had committed a homicide outdoors and homicide victims who had been found outside ($p = 0.0004$). When a homicide was committed with hard, blunt objects or with objects of cutting or stabbing features, the crimes significantly differ according to the location of offence ($p = 0.028$). Homicides with hard, blunt objects were committed more often outdoors than indoors, while homicides committed with object of cutting or stabbing features were committed more often indoors than outdoors.

Relations between the homicide victims and convicted murderers: 10% of all homicide cases, the victim was a murderer's spouse; in 9% – brother or sister, in 2% of cases – the murderer's child, and 5% – mother or father. In more than half of the cases the victims knew their murderer, even though the victims were not family members. And only in 28% of cases the murderers did not know their victims.

In the 52.2% of homicide cases when a homicide was committed with hard, blunt objects, the mean age of individuals who had committed a crime was 33.6 ± 14 years. In homicide cases made with objects of cutting or stabbing features, which constitute 33.2%, the mean murderer age was 38.5 ± 14 years. In homicide cases made with a firearm (5.6%), the mean murderer age was 43.6 ± 21 years. In the 2.4% of cases when the homicides were commited using objects with chopping features, the mean murderer age was 65.7 ± 12 years. Other homicide methods were executed by individuals with the mean age of 32.3 ± 4 (Fig. 19). A statistically significant difference was observed between the murderer mean age when the homicide was commited with a hard, blunt object and the murderer mean age when a homicide was

made with an object of cutting or stabbing features ($p = 0.02$). While investigating cases of whether the mean victim and suspect ages differ across the different homicide categories, the *independent samples T-test* was used.

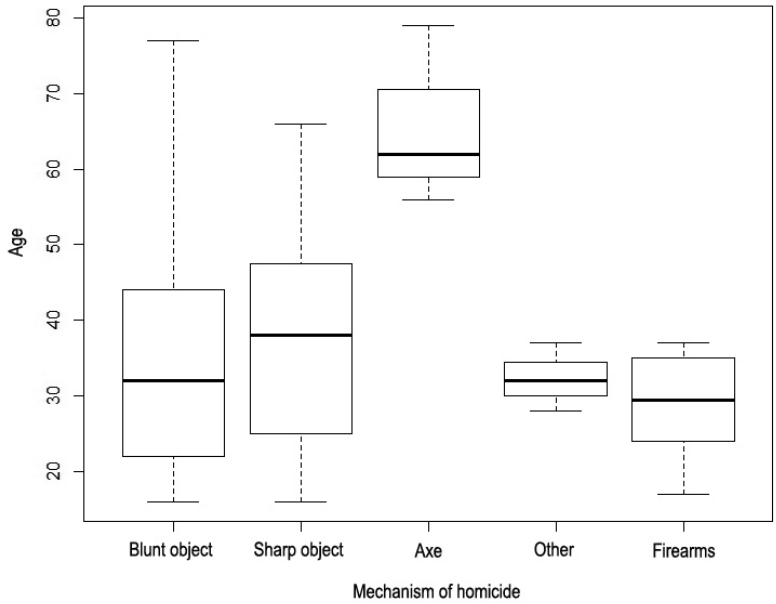

FIG. 19. Murderer age allocation according to the different homicide mechanism cases.

A reliable age difference was observed between the groups of homicide victims and murderers when homicides were committed with hard, blunt objects and when an objects of cutting or stabbing features were used. In both groups, the homicide victim was older than the murderer. In order to investigate whether a relation between the homicide victim and a murderer differs, age was evaluated with the *Paired Samples T-test* with correlations. In crimes where the homicide mechanism was a hard, blunt object, the mean age difference was 14 years, and in 95% of all cases the mean age difference of the homicide victim and murderer variated between 9.9 and 18.8 years. The difference is statistically significant ($p = 0.0001$); however, the correlation is weak ($r = 0.202$) and not statistically significant (Table 9).

If a homicide method is cutting or stabbing injuries, the age difference between the homicide victim and murderer was not high – 4.7 years – and variated from 0.6 to 8.8 years. This difference is statistically significant ($p = 0.024$), and there exists a statistically significant positive medium intensity correlation between the age of the homicide victim and murderer (Table 10). So, the older the victim is, the older the murderer is as well. The correlation was also reflected in the dispersion charts; how-

TABLE 9. Homicide victim and murderer age allocation according to the different homicide mechanisms.

	Blunt object	Sharp object	Firearms	Axe	Other
Age of the homicide victims	49,5 ± 14,9	44,0 ± 15,3	38,5 ± 14,7	56,7 ± 16,7	37,0 ± 17,6
Age of the murderer	33,6 ± 14	38,5 ± 14	28,6 ± 7,6	65,7 ± 12	32,3 ± 4
Independent samples T-test	$p < 0{,}05$	$p = 0{,}03$	$p = 0{,}11$	$p = 0{,}28$	$p = 0{,}64$

ever, it may be additionally seen that there exists a group different from the common graph, where young individuals, aged less than 25 years, have murdered persons older than 50 years (their parents).

TABLE 10. Age differences in both homicide mechanism groups.

	Mechanism of homicide	Difference of mean age (95%)
Age of the homicide victim and the murders (paired cases)	Blunt object	14,384 (9,970 – 18,797)
	Sharp object	4,745 (0,659 – 8,831)

The gender allocation of the homicide victims and murderers, according to the homicide mechanism, is also observed in the dispersion charts (Fig. 20). The group of men is much more often not only in the group of homicide victims but also in the

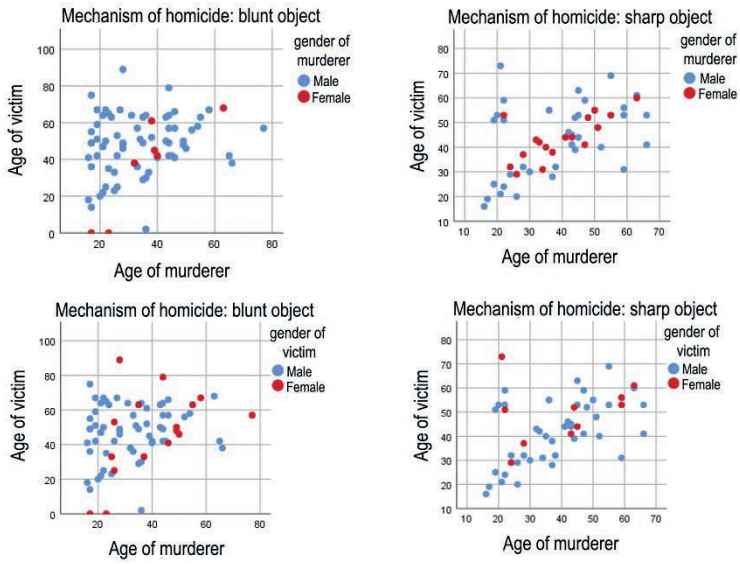

FIG. 20. Allocation of homicide victims and murderers.

group of murderers. While evaluating the gender proportions of homicide victims and murderers in the different groups according to the homicide mechanism, no statistically significant difference was found. *Fisher exact test p* > 0.05 (Tables 11–12).

However, in the group of homicides committed with objects of cutting or stabbing features, women had murdered men almost twice as much as men had murdered women.

TABLE 11. Homicides committed with hard, blunt objects.

		Murderer			Murderer		
		Male	Female	Total	Male	Female	Total
Homicide victims	Male	54	5	59	72,0%	6,7%	78,7%
	Female	14	2	16	18,7%	2,7%	21,3%
		68	7	75	90,7%	9,3%	100,0%

Fisher exact test p = 0,637

TABLE 12. Homicides committed with objects of cutting or stabbing features.

		Murderer			Murderer		
		Male	Female	Total	Male	Female	Total
Homicide victims	Male	25	16	41	49,0%	31,4%	80,4%
	Female	9	1	10	17,6%	2,0%	19,6%
		34	17	51	66,7%	33,3%	100,0%

Fisher exact test p = 0,135

However, if the gender matrix proportions would be compared in two homicide groups – blunt traumas and cases of cutting or stabbing injuries – then the proportions could be observed as statistically significant ($p = 0.003$, *Chi-square* = 13.75). In comparing the proportions, a statistically significant difference is observed only when both the homicide victim and the murderer were men. In this case, the blunt trauma proportion is higher (68.4%) than the cutting or stabbing injuries (31.6%). When the homicide victim is a man and the murderer a woman, the proportion of cutting or stabbing injuries (76.2%) is higher than the blunt traumas (23.8%) (Table 13).

TABLE 13. Homicide victim and murderer allocation according to gender.

	Blunt trauma		Stabbing - cutting		Total	
	N	%	N	%	N	%
Victim – male, murderer – male	54	72,0%	25	49,0%	79	62,7%
Victim – female, murderer – male	14	18,7%	9	17,6%	23	18,3%
Victim – male, murderer – female	5	6,7%	16	31,4%	21	16,7%
Victim – female, murderer – female	2	2,7%	1	2,0%	3	2,4%
Total	75	100,0%	51	100,0%	126	100,0%

In comparisons of the homicide victims and murderers, victims were placed in the suspect matrix, and the *Pearson* correlation coefficient and dispersion charts were used. Found correlations: if a homicide victim was a man and the person who had committed the homicide also a man, there was no relation between the ages of homicide victims and murderers. The correlation coefficient is not statistically significant ($p = 0.273$). If a homicide victim is a woman and the person who had committed the homicide was a man, the relation was weak ($r = 0.231$); a statistically significant ($p = 0.01$) correlation was observed in such cases. In the dispersion chart, it is seen that the relation between the homicide victim and murderer could be stronger, and even their age could synchronize, but it would consist mainly of younger men who had murdered much older women than themselves. If a homicide victim is a man and the murderer a woman, the relation is of average intensity ($r = 0.680$) and statistically significant ($p = 0.0001$). The majority of women's ages coincide with the ages of murdered men. However, there is a segment of women who had killed infants, and these cases would only weaken this relation. If both the homicide victim and murderer were women, relation is strong ($r = 0.757$) and statistically significant ($p = 0.003$), although only 13 of these cases were identified.

Conclusions

1. A significantly strong correlation is observed between the homicide rate and the Lithuanian gross domestic product, which shows that homicide rates are increasing due to poverty.
2. Among all the investigated cases, the dominating cause of death was performed using a hard, blunt object, which was used in 52.2% of cases, while a stabbing or cutting object was used in 33.2% of cases. The dominating cause of death (68%) was observed to have been from injuries performed by one type of object. Comparing with the male group, women had experienced a higher number of traumatic afflictions. Homicides that were committed using objects with cutting or stabbing features were used more often in the men's group. In the female group, asphyxia was the most common mechanism of death. The offenders who had committed homicide using sharp, stabbing or cutting objects were more likely to be severely drunk. The victims who were sober, experienced 3 or less traumatic afflictions, and were attacked with hard, blunt objects during the crimes were more likely to have survived more than 24 hours.
3. Male victims contributed to the majority of homicide cases and made up 73% of cases. Younger men (45.5 ± 15 years) and older women (50.9 ± 17

years) made the majority of homicide victims. Of all cases, 58.6% homicide victims were intoxicated with ethyl alcohol. The majority of victims were unemployed and had only acquired primary or secondary education. In more than half of the cases (53%) the victims were acquainted with the murderers, but the murderers were not the victims' family members.

4. The male group contributed to the majority (87%) of cases as the murderers. The mean age of the murderers was 34.5 ± 14 years, and the mean age of female murderers – 38.5 ± 14 years. The majority (66%) of the offenders were intoxicated with ethyl alcohol. An occupational analysis revealed that the majority (73%) of them were unemployed and had achieved only primary or secondary education. The most likely scenario of a homicide in Lithuania is when the murderer and the victim are both male.

Recommendations

The number of homicides committed by individuals younger than 18 years old comprise a quite low percentage of 10% of all cases. However, it is observed that the number of homicides committed by young people (younger than 18 years old) has been increasing. Only a few of the authors did observe these tendencies; however, the increase of underage crime, due to its peculiar nature, clearly deserves the attention of scientific study.

The maturity of younger, under 18-year-old individuals allows them to understand the consequences of homicide quite early, and that is why it would be appropriate to consider the question of whether a criminal liability for homicide should be applied to individuals younger than 14 years old.

During primary prevention, it is very important to form the following values in the individual: understanding of life, respect for life, respect for the individual and their rights, respect for family. Also, any violent statements (physical, psychological, sexual) the final stages of which could evolve into a homicide should be considered from the early days.

Secondary prevention is directed at individuals who have already committed a homicide and are carrying the sentence out; that is why recidivist prevention is important in this group, where complex means are essential – it is not only important to educate murderers; the duty of social workers in noticing families where latent violence manifests is also important.

While evaluating the criminal offences, including homicides, that were committed by individuals who had already been convicted for crimes by administering the penalties for homicide, it would be appropriate to consider administering a

stricter criminal liability, also considering the gravity of the offence and the suspect's personality.

It is recommended to unify the rules/regulations of court medical examinations – autopsies. The homicide investigation variables can serve as a basis for the development of a unified homicide rating system (register) in Europe. Such databases may include information regarding the method of homicide, the victim's and the offender's social profile, the place of the homicide, and the victim's relationship with the offender.

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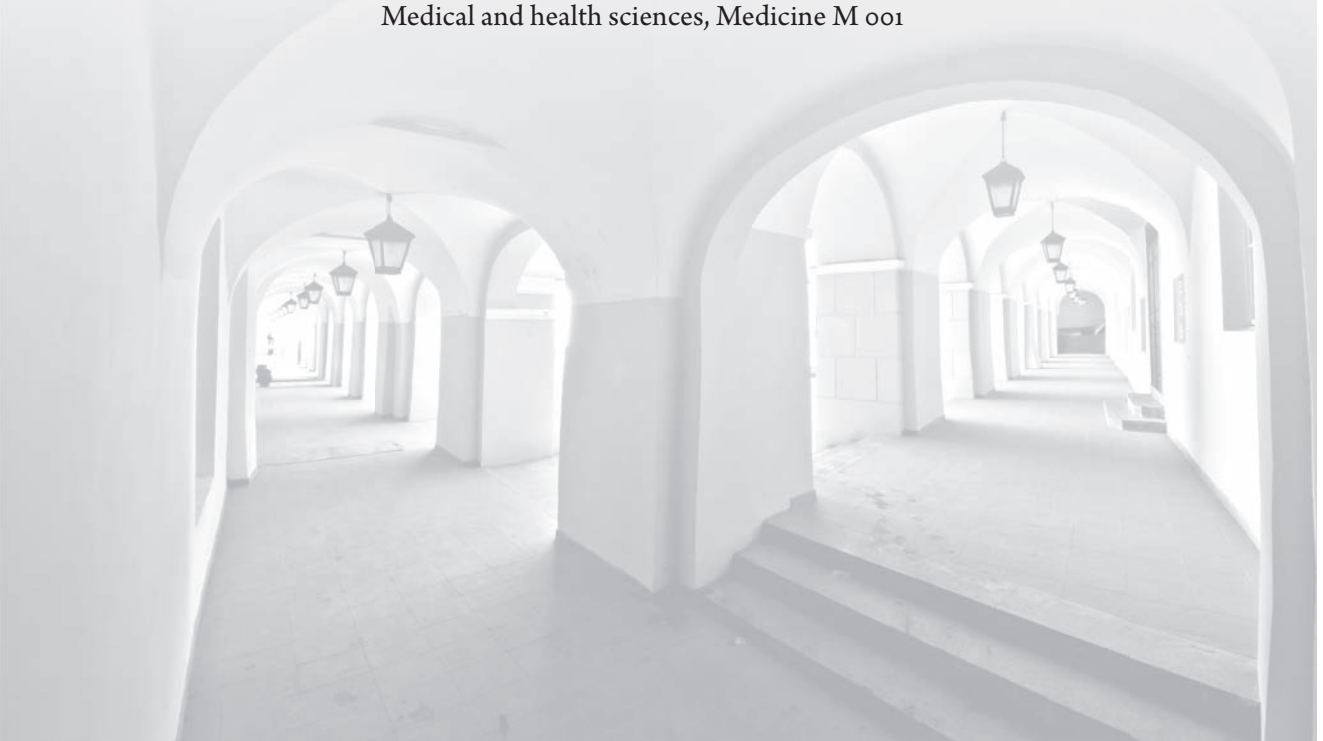
Vytautas Žėkas

LĄSTELIŲ *MIKRODALELĖS* KAIP NAUJAS ANKSTYVOS ATEROSKLEROZĖS BIOŽYMUO SVEIKŲ IR IŠEMINE ŠIRDIES LIGA SERGANČIŲ VYRŲ POPULIACIJOSE

EXTRACELLULAR VESICLES AS A NEW BIOMARKER OF EARLY ATHEROSCLEROSIS IN HEALTHY AND ISCHEMIC HEART DISEASE-AFFECTED MALE POPULATIONS

Medicinos ir sveikatos mokslai, medicina M 001
Medical and health sciences, Medicine M 001



Santrumpos

BCh – bendrasis cholesterolis
CD105 – endoglinas
CD144 – VE (vazoendotelinis)-kadherinas
CD31 (PECAM) – trombocitų ir endoteliocitų adhezijos molekulė
CD42a – glikoproteinas IX
CD61 – integrinas β 3
CD62e – E-selektinas
CRB – C reaktyvusis baltymas
DTL-C – didelio tankio lipoproteinų cholesterolis
ELM – ekstraląstelinės mikrodalelės
ELISA – imunofermentinė analizė (angl. *Enzyme linked immunosorbent assay)*
EMD – endotelinės mikrodalelės
KŠL – koronarinė širdies liga
MTL-C – mažo tankio lipoproteinų cholesterolis
MDA – malondialdehidas
NS – nėra patikimumo
PECAM – trombocitų ir endoteliocitų adhezijos molekulė
TMD – trombocitinės mikrodalelės

1. Įvadas

Praėjusį dešimtmetį buvo išsiaiškinta, kad ekstraląstelinės mikrodalelės (ELM) gali dalyvauti ląstelių tarpusavio bendravime. Šiuo metu yra intensyviai nagrinėjami šių veiksnių ryšiai su patologiniais procesais siekiant panaudoti juos klinikinėje ligų diagnostikoje. Aterosklerozė ir jos klinikinės formos, kaip antai koronarinė širdies liga (KŠL), yra puikus to pavyzdys. ELM gali atspindėti ląstelės funkcijas ir parodyti pažeistą audinį geriau ir greičiau nei bet kuris kitas tradicinis rodiklis. Taip pat šių mikrodalelių nešamas krovinys dalyvauja įvairiuose tiek teigiamuose, tiek neigiamuose ląstelės vidaus procesuose. Nors apie šių dalelių funkcijas žmogaus organizme jau yra nemažai žinoma, kol kas jos nėra visiškai aiškios. Šiame darbe mikrodalelės buvo nagrinėjamos kaip aterosklerozės biožymenys. Siekiant geriau išsiaiškinti jų reikšmę, buvo taikomi keli metodai, kuriuos pasitelkus buvo galima detaliau apibrėžti didelių endotelinių (EMD) ir trombocitinių mikrodalelių (TMD) bei egzosomų (mažųjų mikrodalelių) skaičius ir sąsajas su rizikos veiksniais bei oksidacinio streso žymeniu – malondialdehidu.

1.1. Problema

Endotelinės ir trombocitinės mikrodalelės bei egzosomos būtų geras endotelio pažeidimo rodiklis, tačiau dar nėra pakankamai duomenų, kurie leistų naudoti šiuos žymenis klinikinėje praktikoje.

1.2. Tyrimo pranašumas ir naujumas

Šiame darbe pateikti duomenys gali padėti geriau suprasti ryšį tarp oksidacinio bei lėtinio streso ir endotelinių mikrodalelių susidarymo ankstyvose aterosklerozės stadijose. Galutinis tokių tyrimų tikslas būtų šių žymenų naudojimas ankstyvai aterosklerozės ir jos klinikinių komplikacijų diagnostikai.

1.3. Tyrimo tikslas

Darbo tikslas buvo ištirti endotelines ir trombocitines mikrodaleles bei egzosomas įvertinant jų kiekio ryšį su aterosklerozės rizikos veiksniais ir oksidaciniu stresu jaunų (25–39 metų), vyresnių (40–60 metų) ir patyrusių miokardo infarktą (40–60 metų) vyrų populiacijose.

1.4.Tyrimo uždaviniai

1.4.1. Ištirti ir įvertinti endotelinių ir trombocitinių mikrodalelių kiekį sergančių ir sveikų žmonių kraujo plazmoje.

1.1.2. Ištirti ir įvertinti visas endotelines mikrodaleles, kurios turi CD62e (nustatyti skirtingų mikrodalelių aktyvacijos lygį).

1.4.3. Surasti egzosomas ir nustatyti bei įvertinti jų kiekį sveikų ir sergančių asmenų kraujyje.

1.4.4. Ištirti malondialdehido koncentraciją ir įvertinti jos ryšį su mikrodalelių kiekiais sveikų ir sergančių miokardo infarktu kraujyje.

1.4.5. Ištirti ir įvertinti lipidų žymenų (bendrojo cholesterolio, didelio ir mažo tankio lipoproteinų cholesterolio), C reaktyviojo baltymo ir gliukozės koncentracijas sveikų ir sergančių asmenų kraujyje, įvertinti šių veiksnių ryšį su mikrodalelių kiekiu bei jų aktyvacija.

1.5. Ginamieji teiginiai

1.5.1. Skirtingų ekstraląstelinių mikrodalelių populiacijų kiekiai organizme ir šių kiekių tarpusavio santykiai sveikų ir sergančių asmenų skiriasi.

1.5.2. Skirtingų endotelinių mikrodalelių populiacijų aktyvacijos lygiai sveikų ir sergančių asmenų skiriasi ir yra svarbūs aterosklerozės ir endotelio pažaidos patogenezėje.

1.5.3. Egzosomų kiekiai sveikų ir sergančių asmenų skiriasi ir šie kiekiai siejasi su oksidacinio streso žymens malondialdehido koncentracija kraujyje.

1.5.4. Ekstraląstelinių mikrodalelių ir egzosomų kiekiai bei mikrodalelių aktyvacijos lygis yra susiję su oksidacinio ir streso žymens malondialdehido koncetracija kraujyje.

1.5.5. Aterosklerozės rizikos veiksniai (dislipidemija, sistolinis kraujospūdis ir kt.) siejasi su mikrodalelių ir egzosomų kiekiais sveikų ir sergančių asmenų populiacijose.

2. Metodai

2.1. Tyrimo dalyvių parinkimas.

Sveiki žmonės buvo kviečiami į tyrimą nuo 2015 metų gruodžio iki 2017 metų liepos.

Pacientai, sergantys koronarine širdies liga, buvo kviečiami į tyrimą praėjus trims mėnesiams po miokardo infarkto. Jie buvo ištirti vėliau nei sveiki žmonės. Visi pacientai buvo gydyti 2018 metų sausį–vasarį Vilniaus universiteto ligoninės Santaros klinikų Intensyvios kardiologijos, reanimacijos ir intensyvios terapijos ir pirmajame kardiologijos skyriuose.

2.2. Kraujo mėginių paėmimas.

EMD ir TMD kiekiui nustatyti iš alkūninės venos buvo paimta 4 ml kraujo į „Vacutainer“ tipo vakuuminius mėgintuvėlius su natrio heparinu. Pakopinė centrifugacija buvo pritaikyta siekiant išgauti betrombocitinę plazmą.

C reaktyviojo baltymo (CRB), malondialdehido (MDA), trigliceridų ir bendrojo cholesterolio (BCh) bei didelio ir mažo tankio lipoproteinų cholesterolio (DTL-C ir MTL-C) koncentracijų matavimai buvo atlikti iš kito 4 ml „Vacutainer“ tipo mėgintuvėlio, turinčio gelio, atskiriančio serumą nuo ląstelių. Mėginiai buvo paimti naktį nevalgius. Serumo mėgintuvėlis visada buvo imamas pirmas.

2.3. Derivatizacija ir didelės gebos skysčių chromotografijos metodo taikymas nustatant malondialdehido koncentraciją kraujo serume.

MDA yra lipidų peroksidacijos žymuo ir dažnai naudojamas nustatant oksidacinio streso lygį [1]. Mėginiai buvo paruošti taikant Khoschsorur ir kitų. metodą. Serumo malondialdehido koncentracija buvo ištirta *Shimadzu Nexera X2 UHPLC* aparatu [2]. Duomenys buvo apdoroti naudojant *LabSolutions* programinę įrangą.

2.4. Tėkmės citometrija

Tyrime buvo naudojami fluorescenciniai polistireno rutuliukai Megamix-Plus FSC (*Bio-Cytex*, Prancūzija). Šis mišinys buvo sudarytas iš žaliai fluorescuojančių 100, 300, 500 ir 900 nm dydžio rutuliukų. Pagal šiuos rutuliukus buvo nustatyta mikrodalelių, kurių spindis yra mažesnis nei vienas mikrometras, vieta taškiniame grafike. EMD ir TMD koncentracija buvo išreikšta kaip mikrodalelės/µl. CD62e ekspresija buvo išreikšta kaip procentinė mikrodalelių, ekspresuojančių šį žymenį, dalis nuo bendro endotelinių mikrodalelių kiekio kiekvienoje EMD populiacijoje.

Nustatant CD9 ant egzosomų buvo taikomas metodas, aprašytas Mellisho ir kitų [3]. Mėginiai buvo tiriami LSRII tėkmės citometru (BD, San Jose, Kalifornija, JAV).

2.5. Tyrimai *in vitro* ląstelių kultūroje

Siekiant įsitikinti, kad aptiktos dalelelės iš tikrųjų yra endotelinės mikrodalelės, buvo atliktas *in vitro* tyrimas su HUVEC endotelinių ląstelių linija (*Lonza*). Siekiant palyginti aktyvintų ir neaktyvintų ląstelių išskirtų mikrodalelių kiekių skirtumus, endotelinės ląstelės buvo augintos su ciklofilinu A (*Abcam*, Vokietija) ir be jo augimo terpėje. Mikrodalelės buvo išskirtos ir ištirtos tuo pačiu metodu kaip ir *in vivo* eksperimentuose. Tokios pačios keturios skirtingos EMD populiacijos buvo stebimos *in vitro* ir *in vivo.* Paveiktos ciklofilinu A endotelinės ląstelės generavo du kartus daugiau mikrodalelių nei tos, kurios augo ramybės sąlygomis. Reikėtų pažymėti, kad tos ląstelės, kurios augo aukštesnės nei 10 ng/ml ciklofilino A koncentracijos terpėje, generavo mažiau EMD negu tos, kurios augo nepaveiktos ciklofilino A. Tikėtina, kad taip įvyko dėl to, kad ciklofilinas A yra stiprus apoptozės sukėlėjas [4].

2.6. Western blotas

Atliekant Western bloto analizę, mėginiai buvo tirti naudojant 10 % arba 12 % poliakrilamido gelius. Buvo naudojami pirminiai antikūnai: prieš vėžinio jautrumo geną (angl. *tumour susceptibility gene 101-TSG101*), prieš flotiliną-1 (angl. *anti-flotillin-1*), prieš karščio šoko baltymą 70 (angl. *antiheat shock protein 70* (HSP70)).

2.7. Bradfordo baltymų koncentracijos kiekio analizė

Buvo naudojama skaidri 96 šulinėlių plokštelė. Į neigiamos kontrolės šulinėlį buvo dedama tik buferio. Šviesos absorbcija buvo nustatyta ties 595 nm.

2.8. Nanodalelių sekimo analizė

Dalelių dydis ir jų koncentracija mėginiuose buvo nustatyti nanodalelių sekimo analizės metodu naudojant *ZetaView* platformą (*ParticleMetrix*, Vokietija). Gauti duomenys analizuojami su *ZetaView* analizės programa (versija 8.03.08.02).

3. Rezultatai

3.1. Klinikinės charakteristikos

Demografinės ir klinikinės charakteristikos yra pateiktos 1 lentelėje.

1 LENTELĖ. Tirtos populiacijos demografinės ir klinikinės charakteristikos. Duomenys pateikti kaip medianos ir interkvartiliniai skirtumai arba kaip vidurkiai (tai priklauso nuo rodiklio pasiskirstymo). NS reiškia, kad jokio patikimumo neaptikta.

Rodikliai	Mediana (IQR) arba vidurkiai (SD)			P
	Jaunų sveikų asmenų grupė (25–40 metų) (n = 52)	Vyresnių sveikų asmenų grupė (40–60 metų) (n = 29)	MI patyrusių asmenų grupė (40–60 metų) (n = 15)	
Amžius, metais	Vidurkis (SD) 30,31 (0,49)	Vidurkis (SD) 47,28 (0,84)	Vidurkis (SD) 51,93 (1,54)	< 0,001
Sistolinis kraujospūdis	Mediana (IQR) 131,50 (12)	Mediana (IQR) 131 (18,50)	Mediana (IQR) 120 (15)	0,021
Diastolinis kraujospūdis	Mediana (IQR) 78,50 (8)	Mediana (IQR) 71 (17,50)	Mediana (IQR) 80 (6)	NS
Širdies susitraukimų dažnis per minutę	Vidurkis (SD) 71,35 (1,84)	Vidurkis (SD) 71,55 (2,28)	Vidurkis (SD) 67,07 (9,09)	NS
CRB koncentracija (mg/l)	Mediana (IQR) 0,46 (0,70)	Mediana (IQR) 0,60 (1,70)	Mediana (IQR) 1,78 (1,90)	0,002
MTL-C koncentracija (mmol/l)	Mediana (IQR) 2,92 (0,98)	Mediana(IQR) 3,14 (0,97)	Mediana (IQR) 2,02 (0,9)	< 0,001
DTL-C koncentracija (mmol/l)	Mediana (IQR) 1,20 (0,30)	Mediana (IQR) 1,18 (0,35)	Mediana (IQR) 1 (0,36)	NS
Trigliceridų koncentracija (mmol/l)	Mediana (IQR) 1,12 (0,59)	Mediana (IQR) 1,29 (1)	Mediana (IQR) 1,44 (0,52)	NS
BCh koncentracija (mmol/l)	Mediana (IQR) 4,79 (1,31)	Mediana (IQR) 5,08 (1,13)	Mediana (IQR) 3,73 (1,31)	< 0,001
Gliukozės koncentracija (mmol/l)	Mediana (IQR) 5,13 (0,57)	Mediana (IQR) 5,32 (0,66)	Mediana (IQR) 5,82 (3,29)	0,03
MDA koncentracija	Mediana (IQR) 92,17 (30,98)	Mediana (IQR) 101,61 (34,85)	Mediana (IQR) 154,72 (8,21)	< 0,001
Kairiojo skilvelio išmetimo frakcija	–	–	Vidurkis (SD) 45,7 (7,50)	–

3.2. Tėkmės citometrija

Visų keturių EMD populiacijų charakteristikos pateiktos 2 ir 3 lentelėse.

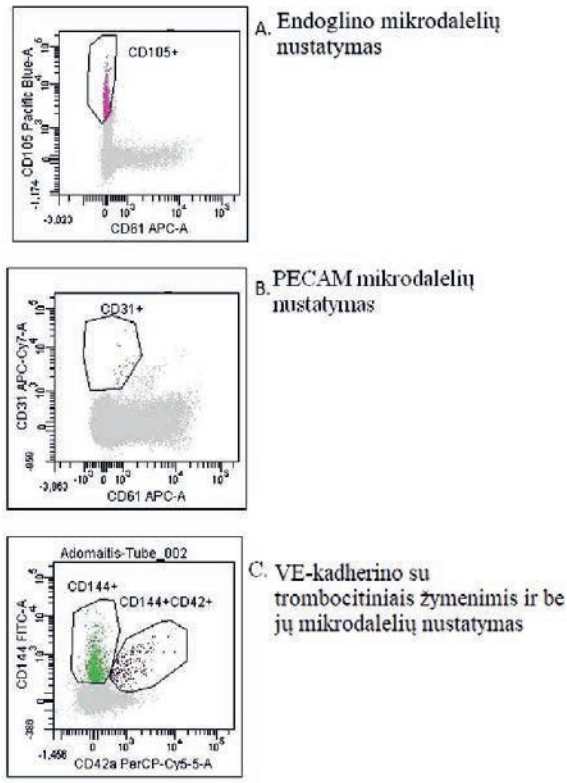
2 LENTELĖ. EMD charakteristika, išreikšta medianomis ir interkvartiliniais skirtumais. Pateiktos P (statistinio patikimumo) reikšmės.

	Mikrodalelių kiekis (mikrodalelės/μ) (mediana ± IQR)			
Mikrodalelių populiacija	Jaunų sveikų asmenų grupė (25–40 metų)	Vyresnių sveikų asmenų grupė (40–60 metų)	Miokardo infarktą patyrusių asmenų grupė (40–60 metų)	P
Endoglininių EMD kiekiai	22,89 ± 75,20	10,93 ± 53,81	33,68 ± 205,47	NS
VE-kadherininių be trombocitinių žymenų EMD kiekiai	322,79 ± 232,64	173,37 ± 426,84	215,56 ± 133,67	NS
VE-kadherininių su trombocitiniais žymenimis EMD kiekiai	254,68 ± 185,15	304,73 ± 210,64	73,29 ± 49,23	< 0,001
PECAM EMD kiekiai	0,63 ± 2,16	1,94 ± 2,62	1,38 ± 2,02	0,04
TMD kiekiai	257,14 ± 353,43	186,92 ± 307,86	338,99 ± 375	NS

3 LENTELĖ. EMD charakteristika, išreikšta medianomis ir interkvartiliniais skirtumais. CD62e ekspresija yra skaičiuojama kaip procentinis santykis nuo bendro mikrodalelių kiekio. Pateiktos P (statistinio patikimumo) reikšmės.

	CD62e ekspresija (%) (mediana ± IQR)			
Mikrodalelių populiacija	Jaunų sveikų asmenų grupė (25–40 metų)	Vyresnių sveikų asmenų grupė (40–60 metų)	Miokardo infarktą patyrusių asmenų grupė (40–60 metų)	P
Endoglininių EMD kiekiai	1,75 ± 4,58	1,40 ± 5,05	14,80 ± 17,60	< 0,001
VE-kadherininių be trombocitinių žymenų EMD kiekiai	5,55 ± 6,13	7 ± 5,95	1,80 ± 6	0,03
VE-kadherininių su trombocitiniais žymenimis EMD kiekiai	41,25 ± 87,80	63,70 ± 79,45	98,90 ± 5,80	0,002

Buvo rastos keturios skirtingos mikrodalelių populiacijos (1 pav.): 1) CD105+, CD42a– ir CD61– arba endoglino EMD; 2) CD31+, CD42a– ir CD61– arba PECAM (trombocitų ir endoteliocitų adhezijos molekulė) EMD; 3) CD144+, CD42a– ir CD61– arba VE-kadherino EMD be trombocitinių žymenų; 4) CD144+, CD42a+ ir CD61– arba VE-kadherino EMD su trombocitiniais žymenimis. Kiekviena subpopuliacija turėjo vieną endotelinį žymenį: CD105 (endoglinas), CD144 (VE-kadherinas) arba CD31 (PECAM), kuris galėjo atspindėti jų funkciją. CD144+ EMD populiacijos išsiskyrimas į dvi grupes pagal trombocitinių žymenų ekspresiją ant membranos atitiko tėkmės citometrijos duomenis ir abiejų populiacijų kiekiai skirtingai koreliavo su klinikiniais žymenimis bei oksidacinio streso žymeniu – malondialdehidu.



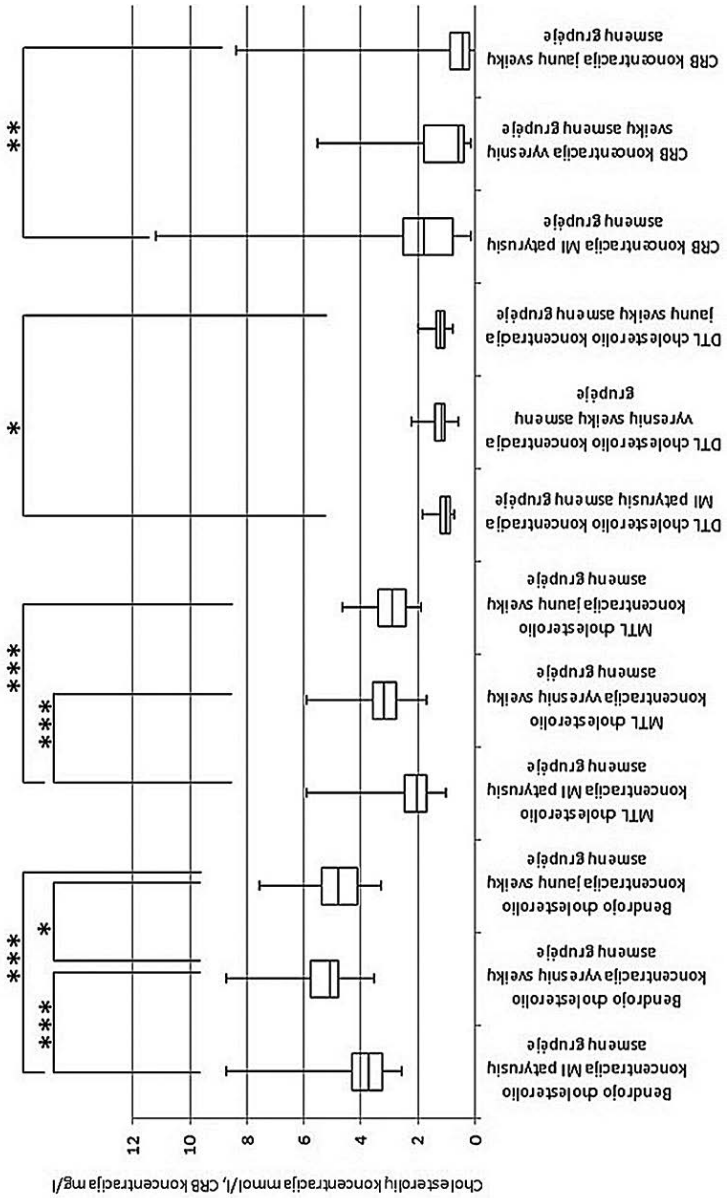
1 PAV. Skirtingų endotelinių mikrodalelių populiacijų nustatymas. A. Endoglino mikrodalelių; B. PECAM mikrodalelių; C. VE-kadherino su trombocitiniais žymenimis ir be jų mikrodalelių.

3.3. Aterosklerozės rizikos veiksnių skirtumai tarp grupių

Sergantiems asmenims buvo labiau padidėjusi MDA koncentracija (154,72 ± 8,21) nei vyresniems sveikiems asmenims (101,61 ± 34,85) ar jauniems sveikiems asmenims (92,17 ± 30,98, P < 0,001). Sergančių asmenų taip pat buvo mažesnė mažo tankio lipoproteino cholesterolio koncentracija (2,02 ± 0,9) nei jaunų sveikų asmenų (2,92 ± 0,98) ar vyresnių sveikų asmenų (3,14 ± 0,97, P < 0,001) ir mažesnė bendrojo cholesterolio koncentracija, palyginti su abiejų grupių sveikais asmenimis – patyrusių miokardo infarktą asmenų grupė (3,73 ± 1,31), jaunų sveikų asmenų grupė (4,79 ± 1,31), vyresnių sveikų asmenų grupė (5,08 ± 1,13, P < 0,001), (1 lentelė, 2 ir 3 pav.). Buvo fiksuotas statistinis amžiaus skirtumas tarp miokardo infarktą patyrusių asmenų grupės ir vyresnių sveikų asmenų grupės. Šį skirtumą galima paaiškinti tuo, kad miokardo infarktą patyrusių asmenų grupėje buvo daugiau 50–60 metų asmenų, palyginti su vyresnių sveikų asmenų grupe (d = –4,83 ± 1,62, P = 0,005).

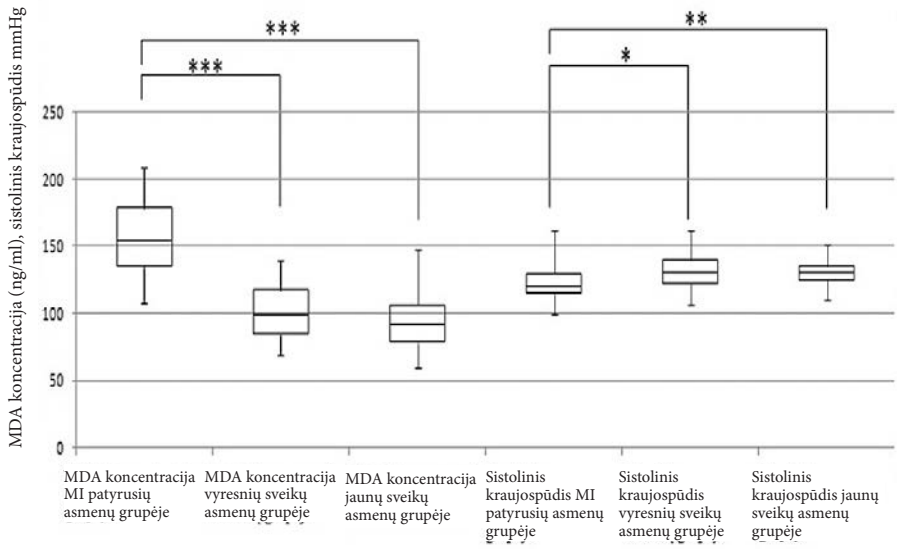
Lyginant jaunų sveikų asmenų grupę su miokardo infarktą patyrusių asmenų grupe, statistiškai reikšmingas skirtumas buvo aptiktas ištyrus MDA koncentraciją: jaunų sveikų asmenų grupė (92,17 ± 30,98), miokardo infarktą patyrusių asmenų grupė (154,72 ± 8,21, P < 0,001); MTL-C koncentraciją: jaunų sveikų asmenų grupė (2,92 ± 0,98), miokardo infarktą patyrusių asmenų grupė (2,02 ± 0,9, P < 0,001); DTL-C koncentraciją: jaunų sveikų asmenų grupė (1,2 ± 0,3), miokardo infarktą patyrusių asmenų grupė (1 ± 0,36, P = 0,023); CRB koncentraciją: jaunų sveikų asmenų grupė (0,46 ± 0,7), miokardo infarktą patyrusių asmenų grupė (1,78 ± 1,9, P = 0,001); BCh koncentraciją: jaunų sveikų asmenų grupė (4,79 ± 1,31), miokardo infarktą patyrusių asmenų grupė (3,73 ± 1,31, P < 0,001); gliukozės koncentraciją: jaunų sveikų asmenų grupė (5,13 ± 0,57, miokardo infarktą patyrusių asmenų grupė (5,82 ± 3,29, P = 0,029) ir sistolinį kraujospūdį: jaunų sveikų asmenų grupė (131,27 ± 1,64), miokardo infarktą patyrusių asmenų grupė (120 ± 15, P = 0,005) (2 ir 3 pav.).

Lyginant vyresnių sveikų asmenų grupę su miokardo infarktą patyrusių asmenų grupe, statistiškai reikšmingų skirtumų buvo aptikta įvertinus MDA koncentraciją: miokardo infarktą patyrusiųjų grupės asmenims (154,72 ± 8,21) buvo nustatyta didesnė koncentracija, nei vyresnių sveikųjų grupės asmenims (101,61 ± 34,85, P < 0,001). BCh koncentracija buvo didesnė vyresnių sveikųjų grupės asmenims (5,08 ± 1,13), nei miokardo infarktą patyrusiųjų grupės asmenims (3,73 ± 1,31, P < 0,001). MTL-C koncentracija buvo didesnė vyresnių sveikųjų grupės asmenims (3,14 ± 0,97), nei miokardo infarktą patyrusiųjų grupės asmenims (2,02 ± 0,9, P < 0,001). Sistolinis kraujospūdis taip pat buvo aukštesnis vyresnių sveikų asmenų grupės asmenims (131,41 ± 2,51), nei miokardo infarktą patyrusiųjų grupės asmenims (120 ± 15, P = 0,029) (2 ir 3 pav.). Malondialdehido, BCh, MTL-C koncentracijos ir



2 PAV. Sveikų ir sergančių asmenų CRB ir lipidų koncentracijų skirtumai.
* P < 0,05, ** P < 0,01, *** P < 0,001

sistolinis kraujospūdis abiejų sveikų asmenų grupių ir pacientų reikšmingai skyrėsi (2 ir 3 pav.). Sveikų ir sergančių žmonių lipidų koncentracijos ir sistolinio kraujospūdžio skirtumai susidarė dėl statinų ir antihipertenzinio gydymo, kuris buvo taikomas sergantiesiems.



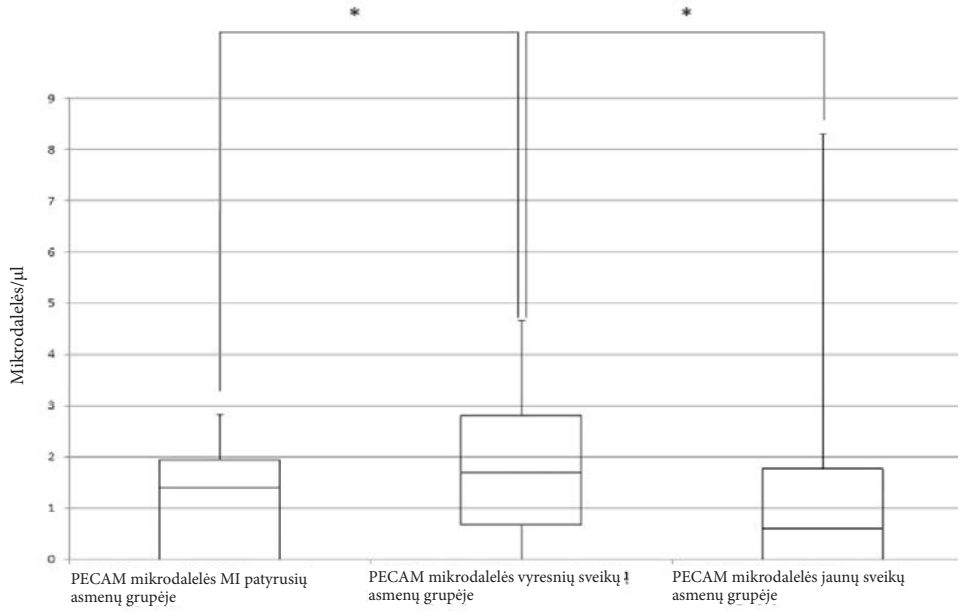
3 PAV. Sveikų ir sergančių asmenų malondialdehido koncentracijos ir sistolinio kraujospūdžio palyginimas. Ūsai rodo kiekvieno rodiklio medianą ir interkvartilinį skirtumą. * P < 0,05, ** P < 0,01, *** P < 0,001

Lyginant abi sveikų asmenų grupes tarpusavyje, tik bendrojo cholesterolio (4,79 ± 1,31 (jaunų sveikų asmenų grupė), 5,08 ± 1,13 (vyresnių sveikų asmenų grupė), P = 0,021) ir gliukozės (5,13 ± 0,57 (jaunų sveikų asmenų grupė), 5,32 ± 0,66 (vyresnių sveikų asmenų grupė), P = 0,05) koncentracijos buvo statistiškai reikšmingai skirtingos.

3.4. Cirkuliuojančių endotelinių ir trombocitinių mikrodalelių kiekio skirtumai

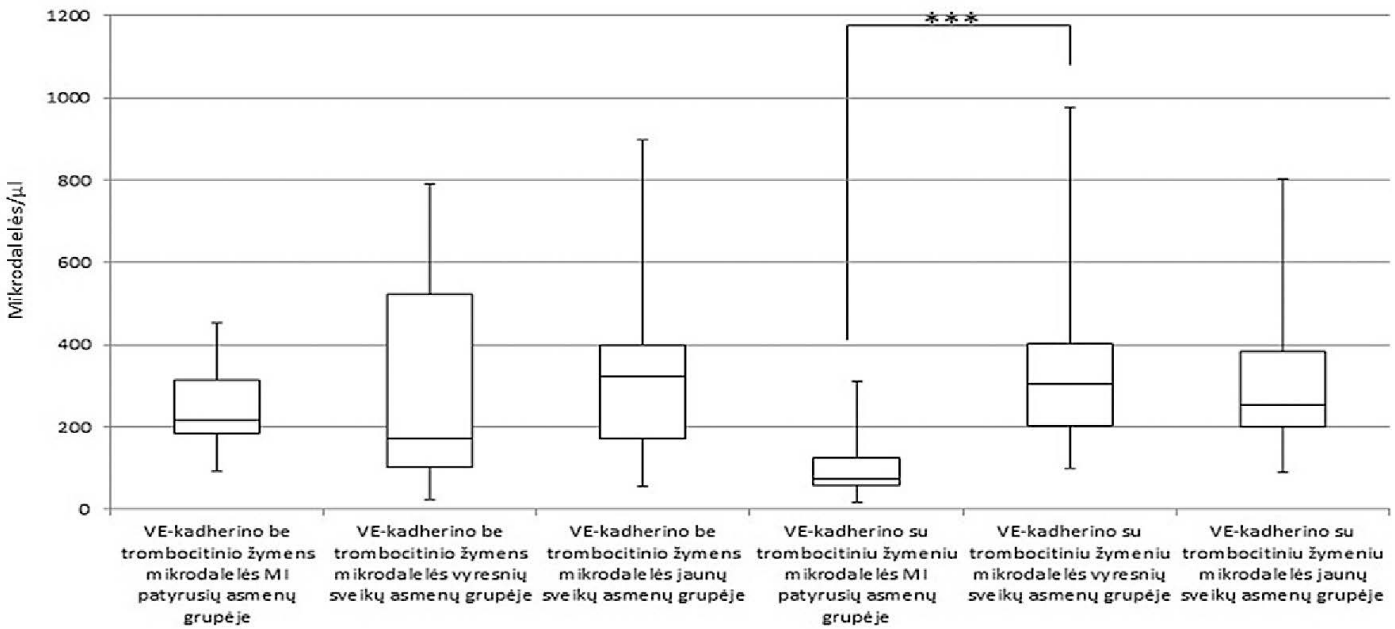
Lyginant tiriamų asmenų grupes buvo aptiktas VE-kadherino EMD su trombocitiniais žymenimis kiekio medianos skirtumas: jaunų sveikų asmenų grupė (254,68 ± 185,15), vyresnių sveikų asmenų grupė (304,73 ± 210,64) ir miokardo infarktą patyrusių asmenų grupė (73,29 ± 49,23, P < 0,001) (2 lentelė, 5 pav.), taip pat PECAM EMD bendro kiekio medianos skirtumas: jaunų sveikų asmenų grupė

(0,63 ± 2,16), vyresnių sveikų asmenų grupė (1,94 ± 2,62) ir miokardo infarktą patyrusių asmenų grupė (1,38 ± 2,02, P = 0,04) (2 lentelė, 4 pav.).

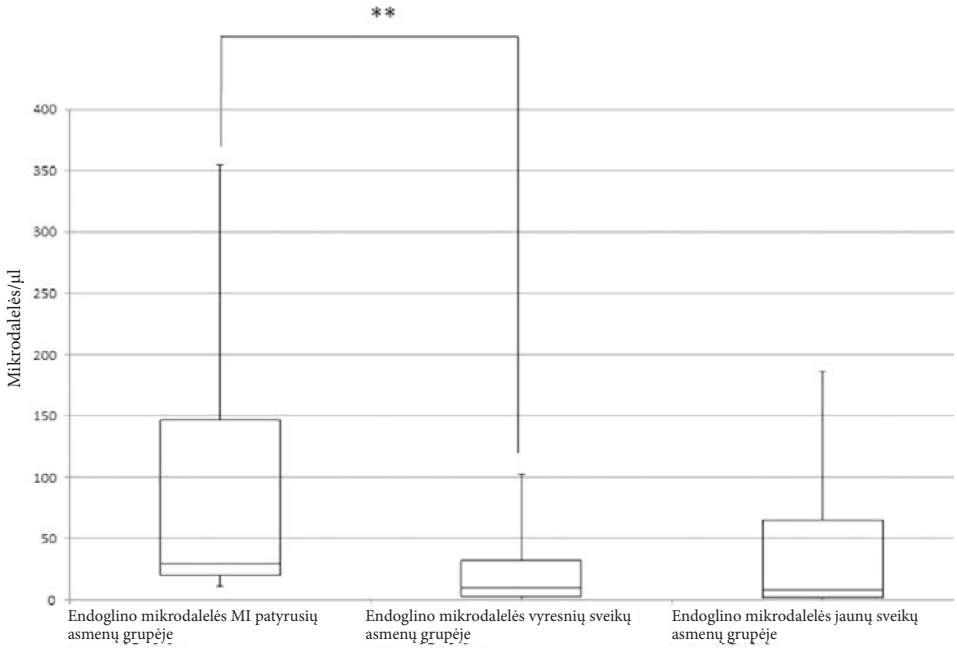


4 PAV. PECAM mikrodalelių kiekio skirtumai tarp grupių.
* P < 0,05, ** P < 0,01, *** P < 0,001

Lyginant jaunų sveikų asmenų grupę (254,68 ± 185,15) su miokardo infarktą patyrusių asmenų grupe (73,29 ± 49,23, P < 0,001), buvo aptiktas tik VE-kadherino mikrodalelių su trombocitiniais žymenimis kiekio medianos skirtumas. Lyginant vyresnių sveikų asmenų grupę ir miokardo infarktą patyrusių asmenų grupę, buvo aptiktas mikrodalelių kiekio medianos skirtumas visose EMD populiacijose, išskyrus VE-kadherino be trombocitinių žymenų: 1) VE kadherino mikrodalelių su trombocitiniais žymenimis populiacijoje – vyresnių sveikų asmenų grupė (304,73 ± 210,64), miokardo infarktą patyrusių asmenų grupė (73,29 ± 49,23, P < 0,001) (5 pav.); 2) endoglino mikrodalelių populiacijoje – vyresnių sveikų asmenų grupė (10,93 ± 53,81), miokardo infarktą patyrusių asmenų grupė (33,68 ± 205,47, P = 0,009) (6 pav.); 3) PECAM mikrodalelių populiacijoje – vyresnių sveikų asmenų grupė (1,94 ± 2,62), miokardo infarktą patyrusių asmenų grupė (1,38 ± 2,02, P = 0,032) (4 pav.). Lyginant abi sveikų asmenų grupes, kiekio medianos skirtumas buvo rastas PECAM mikrodalelių populiacijoje – jaunų sveikų asmenų grupė (0,63 ± 2,16), vyresnių sveikų asmenų grupė (1,94 ± 2,62, P = 0,025) (4 pav.).

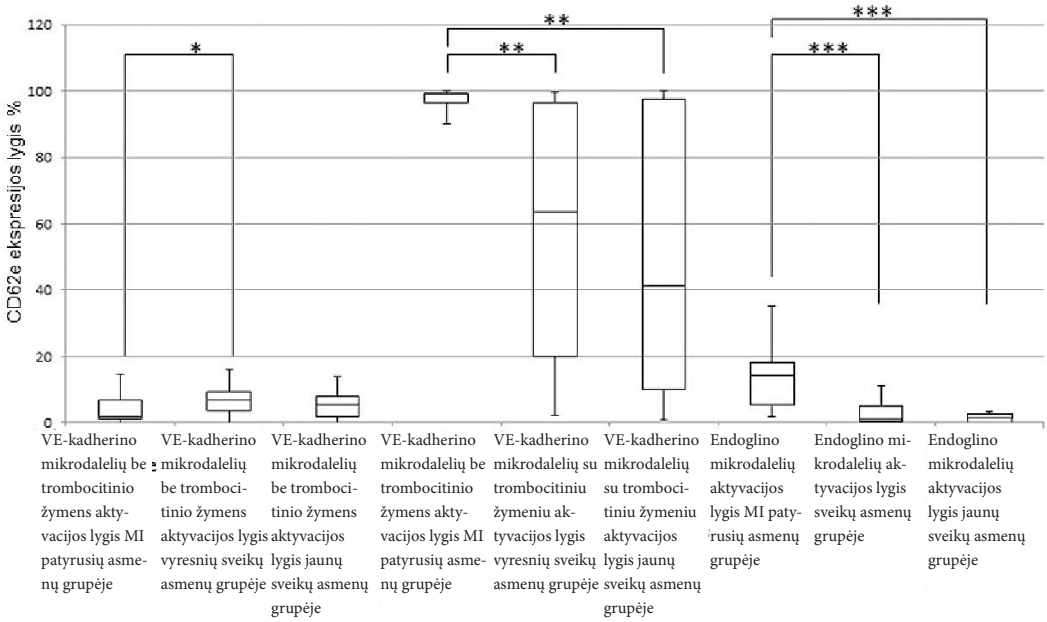


5 PAV. VE-kadherino mikrodalelių su trombocitiniais žymenimis ir be jų kiekio skirtumai tarp grupių.
* P < 0,05, ** P < 0,01, *** P < 0,001



6 PAV. Endoglino mikrodalelių kiekio skirtumai tarp grupių.
* P < 0,05, ** P < 0,01, *** P < 0,001

Lyginant sveikų ir sergančių asmenų grupių CD62e ekspresiją, visų mikrodalelių populiacijose, išskyrus PECAM mikrodaleles, buvo aptikta ekspresijos skirtumų (3 lentelė, 7 pav.). Lyginant sveikų asmenų grupes, buvo rasta endoglino (jaunų sveikų asmenų grupė (1,75 ± 4,58), vyresnių sveikų asmenų grupė (1,4 ± 5,05), P < 0,001) ir VE-kadherino be trombocitinių žymenų mikrodalelėse (jaunų sveikų asmenų grupė (5,55 ± 6,13) ir vyresnių sveikų asmenų grupė (7 ± 5,95), P = 0,002) medianos skirtumų. Lyginant vyresnių sveikų asmenų grupę su patyrusių miokardo infarktą asmenų grupe, CD62e ekspresija buvo statistiškai reikšmingai skirtinga visose mikrodalelių populiacijose, išskyrus PECAM mikrodaleles, – endoglino mikrodalelių populiacijoje (vyresnių sveikų asmenų grupė (1,4 ± 5,05), miokardo infarktą patyrusių asmenų grupė (14,8 ± 1,76), P < 0,001), VE-kadherino mikrodalelių be trombocitinių žymenų populiacijoje (vyresnių sveikų asmenų grupė (7 ± 5,95), miokardo infarktą patyrusių asmenų grupė) (1,8 ± 6), P = 0,013) ir VE-kadherino su trombocitiniais žymenimis populiacijoje (vyresnių sveikų asmenų grupė (63,7 ± 79,45), miokardo infarktą patyrusių asmenų grupė (98,9 ± 5,8), P = 0,001). Tarp jaunų sveikų asmenų grupės ir miokardo infarktą patyrusių asmenų grupės buvo rastas aktyvacijos lygio statistiškai reikšmingas endoglino (jaunų sveikų asmenų grupė (1,75 ± 4,58) ir miokardo infarktą patyrusių asmenų grupė (14,8 ± 17,6),



7 PAV. Aktyvacijos lygio (CD62e raiška ant mikrodalelių membranos) skirtumai tarp grupių.
* P < 0,05, ** P < 0,01, *** P < 0,001

P < 0,001) ir VE-kadherino su trombocitiniais žymenimis mikrodalelių populiacijų (jaunų sveikų asmenų grupė (41,25 ± 87,8) ir miokardo infarktą patyrusių asmenų grupė (98,9 ± 5,8), P = 0,002) skirtumas (7 pav.). Sveikų asmenų grupių mikrodalelių aktyvacijos lygio medianos skirtumų nebuvo rasta. Ant PECAM mikrodalelių membranos CD62e nebuvo aptiktas.

3.5. Tradicinių žymenų ir endotelinių mikrodalelių kiekio bei jų aktyvacijos lygio sąsajos

Jaunų sveikų asmenų grupėje CD62e+ ekspresijos lygis endoglino populiacijoje neigiamai koreliavo su amžiumi. Vyresnių sveikų asmenų grupėje taip pat buvo rastos koreliacijos su mikrodalelių kiekiais: 1) bendras kiekis VE-kadherino mikrodalelių be trombocitinių žymenų neigiamai koreliavo su DTL-C koncentracija (R = –0,37, P = 0,046); 2) TMD kiekis teigiamai koreliavo su amžiumi (R = –0,39, P = 0,036); 3) endoglino mikrodalelių bendras kiekis neigiamai koreliavo su DTL-C koncentracija (R = –0,47, P = 0,011); 4) CD62e+ ekspresijos lygis endoglino mikrodalelėse neigiamai koreliavo su DTL-C koncentracija (R = –0,64, P < 0,001).

Miokardo infarktą patyrusių asmenų grupėje buvo rastos šios koreliacijos: 1) bendras VE-kadherino mikrodalelių be trombocitinių žymenų kiekis neigiamai koreliavo su BCh koncentracija (R = –0,63, P = 0,011) ir MTL-C koncentracija (R = –0,68, P = 0,005); 2) bendras endoglino mikrodalelių kiekis neigiamai koreliavo su BCh koncentracija (R = –0,64, P = 0,01) ir MTL-C koncentracija (R = –0,69, P = 0,004) ir teigiamai koreliavo su sistoliniu kraujospūdžiu (R = 0,54, P = 0,038); 3) CD62e ekspresijos lygis VE-kadherino be trombocitinių žymenų mikrodalelėse teigiamai koreliavo su DTL-C koncentracija (R = 0,78, P = 0,001) ir neigiamai – su sistoliniu kraujo spaudimu (R = –0,54, P = 0,037); 4) CD62e ekspresijos lygis VE-kadherino mikrodalelėse su trombocitiniais žymenimis neigiamai koreliavo su gliukozės koncentracija (R = –0,65, P = 0,009) ir sistoliniu kraujo spaudimu (R = –0,53, P = 0,04). Jokių koreliacijų su aktyvacijos lygiu endoglino mikrodalelėse nebuvo aptikta. Tai rodo galimus funkcinius skirtumus tarp VE-kadheriną ir endogliną ekspresuojančių mikrodalelių.

Širdies dažnis ir diastolinis kraujospūdis neturėjo jokio ryšio su EMD visose trijose grupėse.

3.6. Mikrodalelių kiekio ir malondialdehido koncentracijos kraujo serume ryšys

Jaunų sveikų asmenų grupėje tik VE-kadherino mikrodalelių be trombocitinių žymenų koncentracija teigiamai koreliavo su MDA koncentracija (R = 0,28, P = 0,04). Vyresnių sveikų asmenų grupėje MDA koncentracija teigiamai koreliavo su TMD koncentracija (R = 0,64, P < 0,001), VE-kadherino mikrodalelių be trombocitinių žymenų koncentracija (R = 0,49, P = 0,007), endoglino mikrodalelių koncentracija (R = 0,38, P = 0,04) ir su aktyvacijos lygiu endoglino mikrodalelėse (R = 0,49, P = 0,008).

Miokardo infarktą patyrusių asmenų grupėje nebuvo rasta jokio ryšio tarp MDA koncentracijos ir mikrodalelių kiekio ar jų aktyvacijos lygio.

3.7. Ryšiai tarp atskirų endotelinių mikrodalelių populiacijų

Jaunų sveikų asmenų grupėje VE-kadherino mikrodalelių be trombocitinių žymenų koncentracija teigiamai koreliavo su bendra endoglino mikrodalelių koncentracija (R = 0,41, P = 0,003) ir aktyvacijos lygiu endoglino mikrodalelėse (R = 0,37, P = 0,007). Endoglino mikrodalelių koncentracija teigiamai koreliavo su aktyvacijos lygiu VE-kadherino be trombocitinių žymenų mikrodalelių populiacijoje (R = 0,42, P = 0,002) ir aktyvacijos lygiu VE-kadherino su trombocitiniais žymenimis mikrodalelių populiacijoje (R = 0,66, P < 0,001).

Vyresnių sveikų asmenų grupėje endoglino mikrodalelių koncentracija teigiamai koreliavo su VE-kadherino be trombocitinių žymenų mikrodalelių koncentracija (R = 0,69, P < 0,001) ir su VE-kadherino su trombocitiniais žymenimis mikrodalelių koncentracija (R = 0,47, P = 0,01). Taip pat toje pačioje grupėje aktyvacijos lygis endoglino mikrodalelių populiacijoje turėjo neigiamą ryšį su TMD koncentracija (R = 0,47, P = 0,018) ir teigiamai koreliavo su VE-kadherino mikrodalelių be trombocitinių žymenų populiacijos koncentracija (R = 0,48, P = 0,009). Aktyvacijos lygis VE-kadherino mikrodalelėse su trombocitiniais žymenimis teigiamai koreliavo su endoglino mikrodalelių koncentracija (R = 0,53, P = 0,003).

Miokardo infarktą patyrusių asmenų grupėje VE-kadherino be trombocitinių žymenų mikrodalelių populiacija teigiamai koreliavo su endoglino mikrodalelių populiacijos koncentracija (R = 0,64, P = 0,01) ir neigiamai su CD62e ekspresija VE-kadherino mikrodalelėse su trombocitiniais žymenimis (R = –0,76, P = 0,001). CD62e ekspresija endoglino mikrodalelėse neigiamai koreliavo su PECAM mikrodalelių bendru kiekiu (R = –0,57, P =0 ,027). Aktyvacijos lygis VE-kadherino mikrodalelėse su trombocitiniais žymenimis neigiamai koreliavo su TMD (R = –0,56, P = 0,031) ir endoglino mikrodalelių koncentracijomis (R = –0,76, P = 0,001).

3.8. Santykiai tarp skirtingų endotelinių ir trombocitinių mikrodalelių koncentracijų ir jų aktyvacijos lygio

Nustačius ryšį tarp mikrodalelių populiacijų kiekių, buvo nuspręsta apskaičiuoti santykius tarp šių mikrodalelių populiacijų ir juos įvertinti. EMD aktyvacijos lygis taip pat buvo įtrauktas į analizę, tačiau santykis tarp mikrodalelių kiekių ir jų aktyvacijos lygio nebuvo skaičiuojamas dėl nesutampančių matavimo dimensijų. Tiek santykis tarp endoglino mikrodalelių ir VE-kadherino mikrodalelių su trombocitiniais žymenimis koncentracijų (P < 0,001), tiek santykis tarp endoglino mikrodalelių ir VE-kadherino be trombocitinių žymenų mikrodalelių koncentracijų (P = 0,011) buvo statistiškai reikšmingi. Santykis tarp aktyvintų endoglino ir aktyvintų VE-kadherino su trombocitiniais žymenimis mikrodalelių lygių taip pat reikšmingai skyrėsi (P = 0,002). TMD koncentracija taip pat buvo įtraukta į skaičiavimus ir jos santykis su VE-kadherino mikrodalelių su trombocitiniais žymenimis koncentracija buvo reikšmingas (P = 0,001).

3.9. Cirkuliuojančių egzosomų (mažųjų mikrodalelių) skaičius

Remiantis nanodalelių sekimo analize (*Zeta View*, *Particle metrix*, Vokietija) buvo rastas dalelių koncentracijos medianos skirtumas tarp miokardo infarktą patyrusių asmenų grupės ($3{,}1*10^{10}$/ml) ir vyresnių sveikų asmenų grupės ($7{,}07*10^{10}$/ml, P < 0,001). Sveikiems žmonėms koncentracija buvo didesnė nei asmenims, patyru-

siems miokardo infarktą. Nagrinėjant dalelių dydį ar tūrį, tarp grupių nebuvo rasta jokio skirtumo.

Tetraspanino CD9 ekspresija ant mažųjų mikrodalelių membranos buvo nustatoma naudojant tėkmės citometriją. Vidutinio fluorescencijos intensyvumo ir procentinio santykio nuo visų įvykių skaičiaus analizė parodė, kad sveiki žmonės turėjo didesnį kiekį mažųjų mikrodalelių nei miokardo infarktą patyrusiųjų grupės asmenys (MFI – 275 ± 39,5 (vyresnių sveikų asmenų grupė), 252 ± 13 (miokardo infarktą patyrusių asmenų grupė), P < 0,001, įvykių procentinė dalis – 23,01 % ± 17,4 (vyresnių sveikų asmenų grupė) 1,3 % ± 0,6 (miokardo infarktą patyrusių asmenų grupė), P < 0,001). Analizuojant bendrą baltymų koncentraciją, didesnis jų kiekis buvo aptiktas pacientų mėginiuose (1,01 μg/ml ± 0,49 (vyresnių sveikų asmenų grupė) 1,95 μg/ml ± 1,07 (miokardo infarktą patyrusių asmenų grupė), P = 0,001). Mėginio priemaišos gali būti šio fenomeno priežastis, nes nanodalelių sekimo analizės duomenys koreliavo su Bradfordo tyrimo rezultatais sveikų asmenų grupėje, bet nekoreliavo miokardo infarktą patyrusių asmenų grupėje.

3.10. Egzosomų (mažųjų mikrodalelių) fenotipavimas

TSG101, flotilinas-1 ir karščio ir šoko baltymas 70 ant egzosomų membranos nustatyti taikant Western bloto metodą, bet buvo matomas tik TSG101. Flotilino 1 buvimas ant membranos buvo patvirtintas tėkmės citometrijos metodu, naudojant po 4 mėginius iš kiekvienos grupės.

3.11. Ryšiai tarp tradicinių aterosklerozės žymenų, malondialdehido koncentracijos ir mažųjų mikrodalelių koncentracijos bei CD9 mažųjų mikrodalelių kiekio

Sveikų vyresnių asmenų grupėje MDA koncentracija koreliavo su mažųjų mikrodalelių koncentracija, nustatyta nanodalelių sekimo analizės metodu (R = 0,4, P = 0,003), vidutinu CD9 ekspresuojančių mažųjų mikrodalelių fluorescencijos intensyvumu (R = 0,49, P = 0,008) ir CD9 ekspresuojančių mažųjų mikrodalelių procentine dalimi nuo bendro įvykio skaičiaus (R = 0,55, P = 0,002). Miokardo infarktą patyrusių asmenų grupėje jokių ryšių neaptikta.

Sveikų vyresnių asmenų grupėje taip pat buvo rasta koreliacija tarp didelio tankio lipoproteinų cholesterolio koncentracijos ir mažųjų mikrodalelių koncentracijos (R = 0,46, P = 0,01).

3.12. Tioredoksino nustatymas Western bloto metodu

Tioredoksino, taikant Western bloto metodą, buvo rasta visuose mėginiuose, tačiau atliekant ELISA analizę jokio tioredoksino aktyvumo nebuvo aptikta. Galima teigti, kad tioredoksinas yra lokalizuotas labiau mažųjų mikrodalelių viduje, bet ne ant membranos.

4. Aptarimas

Šio darbo tikslas buvo nustatyti mikrodalelių kiekio skirtumus tarp sveikų asmenų (skirtinguose laiko intervaluose) ir pacientų, kuriems nuo miokardo infarkto yra praėję trys mėnesiai. Buvo nustatytos keturios skirtingos endotelinių mikrodalelių grupės ir rastas ryšys tarp šių dalelių kiekio ir oksidacinį stresą parodančio veiksnio – malondialdehido koncentracijos kraujo serume. Taip pat CD62e (e-selektino) ekspresijos ant mikrodalelių membranų lygis buvo aukštesnis asmenims, kurie turėjo širdies ir kraujagyslių rizikos veiksnių.

MDA koncentracija buvo susijusi su padidėjusiu mikrodalelių kiekiu abiejose sveikų asmenų grupėse, bet ne miokardo infarktą patyrusių asmenų grupėje. Taip pat buvo aiškiai matyti, kad VE-kadherino mikrodalelių kiekiai skirtingai koreliavo su aterosklerozės rizikos veiksniais – tai priklausė nuo CD42a buvimo ant jų membranos. Tos, kurios turėjo CD42a, buvo labiau susijusios su aterosklerozės rizikos veiksniais, o neturinčios CD42a – su oksidaciniu stresu. Tikėtina, kad toks rezultatas buvo gautas dėl šių mikrodalelių skirtingų funkcijų organizme, kurios, greičiausiai, susijusios su šių mikrodalelių nešamomis adhezijos molekulėmis.

Analizuojant rizikos veiksnių ryšį su mikrodalelių kiekiais ar jų aktyvacijos lygiu, buvo aiškiai matomas skirtumas tarp endogliną ar VE-kadheriną nešančių endotelinių mikrodalelių. Tai aiškiai matoma lyginant DTL-C koncentracijos sąsajas su endotelinių mikrodalelių populiacijų kiekiu ir aktyvacijos lygiu. Skirtingos studijos rodo, kad padidėjęs VE-kadherininą ir E-selektiną nešančių mikrodalelių kiekis yra siejamas su dislipidemija [5]. Tačiau yra gaunama ir prieštaraujančių duomenų. Vienoje studijoje buvo nustatyta, kad endoglino ir VE-kadherino mikrodalelių kiekiai nesiskyrė sveikų asmenų ir asmenų, sergančių KŠL [6].

Mikrodalelių kiekiai dažniau skyrėsi tarp pacientų ir vyresnių sveikų asmenų: VE-kadherino mikrodalelių daugiau buvo sveikų asmenų grupėje, o endoglino mikrodalelių daugiau miokardo infarktą patyrusių asmenų grupėje. Tas pats matyti ir nagrinėjant šių populiacijų aktyvacijos lygius. Abu žymenys dalyvauja endotelio pažaidos ir reparacijos mechanizmuose. VE-kadherinas yra reikalingas siekiant palaikyti endotelio barjerą, taip pat atlieka ląstelės jungties molekulės funkciją [7]. Ši molekulė yra aprašoma kaip tikėtinai susijusi su endotelio pažaida [8]. Jos fosforilinimo ir degradacijos procesai yra glaudžiai susiję su limfocitų ir neutrofilų migracija per endotelio sluoksnį [9]. Endoglino molekulė yra kaip papildomas transformuojančio augimo faktoriaus β (TGFβ) receptorius ir yra reikalinga endotelio aktyvacijos procese [10]. Endoglinas paprastai yra siejamas su sveiku endoteliu ir atvirkščiai koreliuoja su koronarinės aterosklerozės sunkumu [11]. Tikėtina, kad VE-kadherino mikrodalelės labiau susijusios su uždegimu, o endoglino – su sveiko endotelio regeneracijos procesais. Reikėtų pažymėti, kad, lygindami jaunesnių sveikų asmenų

grupę su miokardo infarktą patyrusių asmenų grupe, tokio skirtumo nematome, tačiau tikėtina, kad tam turėjo įtakos taikomas gydymas. Statinai yra aprašyti literatūroje kaip galintys mažinti EMD sintezę [12], tačiau yra ir tam prieštaraujančių rezultatų [13]. Kitas galimas paaiškinimas yra, kad mikrodalelių išskyrimas didėjant amžiui skiriasi dėl atsirandančių pokyčių endotelio sluoksnyje. Taikomas gydymas galėjo sumažinti pažeidžiamą tirtų žymenų poveikį endotelio ląstelėmis ir taip pakeisti endotelinių mikrodalelių kiekius ir tarpusavio santykius. Tačiau reikėtų nepamiršti ryšio su DTL-C koncentracija, nes didelio tankio lipoproteinų cholesterolis yra žinomas kaip atliekantis antioksidacinę protekcinę funkciją [14], todėl dalis aptariamų mikrodalelių gali turėti protekcinę funkciją.

Taip pat buvo atliktas egzosomų arba mažųjų mikrodalelių tyrimas, tačiau į jį buvo įtraukta tik vyresnių sveikų asmenų grupė ir pacientai. Taip buvo pasielgta nustačius, kad, tiriant didesnes mikrodaleles, tarp šių grupių buvo rasta ryškesnių skirtumų. Vertinant mažųjų mikrodalelių tyrime gautus duomenis matyti, kad skirtumai tarp grupių buvo panašūs į gautus mikrodalelių tyrime, ir tai rodo ryšį tarp mažų ir didelių mikrodalelių. Egzosomų mėginiuose buvo nustatytas tioredoksinas, kuris yra žinomas kaip antioksidantas [15]. Taip pat buvo nustatytas mažųjų mikrodalelių kiekio ir CD9 ekspresijos ryšys su malondialdehido koncentracija. Tai rodo jų dalyvavimą oksidacinio streso procese ir vėliau atsirandančioje endotelio pažaidoje.

Apibendrinant rezultatus yra aiškiai matomas CD62e arba e-selektino vaidmuo pažeidžiant endotelį. Mikrodalelių, nešančių šį žymenį, kiekių skirtumai tarp grupių, ryšiai su aterosklerozės rizikos faktoriais ar malondialdehido koncentracija aiškiai rodo, kad yra svarbu ne tik įvertinti patį mikrodalelių kiekį, bet ir nustatyti, kokia jų dalis neša CD62e. Tai gali būti reikšmingas ankstyvo endotelio pažeidimo žymuo.

5. Išvados

1. Sveikų ir sergančių (patyrusių miokardo infarktą) asmenų kraujyje buvo rastos keturios skirtingos endotelinių mikrodalelių populiacijos:
   a) VE-kadherino žymenį turinčiose mikrodalelių populiacijose:
      - VE-kadherino mikrodalelių, nešančių trombocitinį žymenį, kiekis buvo didžiausias vyresnių sveikų asmenų grupėje (304,73 ± 210,64 mikrodalelės/µl), mažiausias miokardo infarktą patyrusių asmenų grupėje (73,29 ± 49,23 mikrodalelės/µl).
      - VE-kadherino mikrodalelių, kurios neneša trombocitinio žymens, kiekis buvo didžiausias jaunų sveikų asmenų grupėje (322,79 ± 232,15 mikrodalelės/µl), mažiausias vyresnių sveikų asmenų grupėje (173,37 ± 426,84 mikrodalelės/µl).

b) PECAM žymenį turinčių mikrodalelių kiekis didžiausias vyresnių sveikų asmenų grupėje (1,94 ± 2,62 mikrodalelės/µl), mažiausias jaunų sveikų asmenų grupėje (0,63 ± 2,16 mikrodalelės/µl).
c) Endoglininių mikrodalelių kiekiai buvo gerokai mažesni sveikų asmenų grupėse: mažiausi vyresnių sveikų asmenų grupėje (10,93 ± 53,81 mikrodalelės/µl) ir didžiausi miokardo infarktą patyrusių asmenų grupėje (33,68 ± 205,47 mikrodalelės/µl).
d) Trombocitinių mikrodalelių kiekių skirtumų tarp grupių nebuvo aptikta.

2. Aktyvacijos lygis (CD62e ekspresija) buvo aukštesnis miokardo infarktą patyrusių asmenų grupėje visose endotelinių mikrodalelių populiacijose, išskyrus VE-kadherino mikrodaleles, kurios neturėjo trombocitinio žymens (endoglino mikrodalelių populiacijoje (14,8 ± 17,6 %), VE-kadherino be trombocitinio žymens mikrodalelių populiacijoje (1,8 ± 6 %); VE-kadherino su trombocitiniu žymeniu mikrodalelių populiacijoje (98,9 ± 5,8 %). Tai rodo, kad mikrodalelių kiekiai ir aktyvacijos lygis yra vienodai svarbūs.
3. Egzosomų (mažųjų mikrodalelių) koncentracija, nustatyta nanodalelių sekimo analizės metodu, buvo didesnė vyresnių sveikų asmenų grupėje ($7{,}07*10^{10}$/ml ± 3), nei MI patyrusių asmenų grupėje ($3{,}1*10^{10}$/ml ± 1,9).
4. Malondialdehido koncentracija koreliavo su VE-kadherino mikrodalelių (neturinčių trombocitinio žymens) kiekiu jaunų ir vyresnių sveikų asmenų grupėse, su endoglino mikrodalelių kiekiu ir su jų aktyvacijos lygiu – vyresnių sveikų asmenų grupėje, taip pat su trombocitinių mikrodalelių kiekiu vyresnių sveikų asmenų grupėje. Buvo aptiktas malondialdehido koncentracijos ryšys su mažųjų mikrodalelių koncentracija vyresnių sveikų asmenų grupėje, o tioredoksino buvo rasta mažųjų mikrodalelių mėginiuose. Tai rodo ryšį tarp mikrodalelių, egzosomų (mažųjų mikrodalelių) ir oksidacinio streso.
5. Ryšiai su aterosklerozės rizikos faktoriais:
   5.1. VE-kadherino be trombocitinio žymens ir endoglino mikrodalelių kiekiai koreliavo su bendro, mažo ir didelio tankio lipoproteinų cholesterolio koncentracijomis ir pacientų, ir sveikų asmenų kraujo mėginiuose. Endoglino mikrodalelių e-selektino ekspresija sveikiems asmenims buvo susijusi su didelio tankio cholesterolio koncentracija.
   5.2. E-selektino ekspresija VE-kadherino mikrodalelėse, kurios neturėjo trombocitinio žymens, buvo susijusi su sistoliniu kraujospūdžiu. Ryšiai rodo, kad ligos procese dalyvauja endotelinės, bet ne trombocitinės mikrodalelės.

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Stažuotė

Stažuotė Tartu universiteto Technologijos institute, Tartu, Estija (2018 m. gegužę ir 2019 m. sausį–vasarį). Priėmėjas – instituto direktorė dr. Reet Kurg. Stažuotės metu buvo tirtos mažosios mikrodalelės ir jų ryšys su oksidaciniu stresu. Tam buvo taikomi Western bloto, tėkmės citometrijos, nanodalelių sekimo analizės, Bradfordo baltymų koncentracijos tyrimo ir ELISA metodai.

Andrius Rybakovas

THE ROLE OF INTRAOPERATIVE NEURO-STIMULATION USING LARYNGEAL PALPATION AND INTRAOPERATIVE LARYNGEAL ULTRASOUND IN OPTIMIZING THE SAFETY OF THYROIDECTOMY

INTRAOPERACINĖS PALPACINĖS NEURO-STIMULIACIJOS IR INTRAOPERACINĖS GERKLŲ SONOSKOPIJOS REIKŠMĖ OPTIMIZUOJANT TIROIDEKTOMIJOS SAUGUMĄ

Biomedical Sciences, Medicine (06B)
Biomedicinos mokslai, medicina (06B)

Abbreviations

AR – autoregression
IONM – intraoperative neuromonitoring
LP – laryngeal palpation
LU – laryngeal ultrasound
RLN – recurrent laryngeal nerve
VC – vocal cord

Introduction

According to Loyo et al., the number of thyroid surgical cases in the US is steadily increasing [1]. The annual average of thyroid operations performed in Lithuania is 1500 [2]. Recurrent laryngeal nerve (RLN) palsy is a widely acknowledged complication in thyroid surgery. Incidence rates vary significantly in published literature and are generally accepted as underreported. The reason for this disparity could be attributed to the fact that many studies do not incorporate pre- and postoperative laryngeal examinations in their patient assessments and rely only on voice changes. According to a systematic review published in 2009 by Jeanon et al., which included 27 articles and 25 000 patients, the average incidence of temporary RLN palsy was 9.8% and ranged from 1.4% to 38.4%, whilst the incidence of a permanent RLN palsy ranged from 0% to 18.6% with an average value of 2.3% [3]. Another, more recent French study, published in 2017 by Lifante et al., included 3605 patients and 22 surgeons and showed similar results. The immediate RLN palsy rate demonstrated in this study was 9.3% (ranging from 3.8% to 21%), and the permanent RLN palsy rate was 3.1% (ranging from 0% to 9.1%) [4]. Though voice and speech have significant roles in human social life and professional performance, bilateral RLN injury is a particularly pressing concern for a surgeon, as it can inflict such serious ill effects as breathing difficulties, reintubation, tracheostomy or even death. In the case of bilateral RLN injury after thyroid surgery, symptoms can be acute with a need for critical airway management or may worsen after several weeks due to synkinesis caused by aberrant reinnervation [5]. Tracheostomy, as a common procedure in bilateral RLN palsy cases, diminishes the quality of life and even after the removal of a tracheostomy tube – only an incomplete psychosocial recovery is observed [6]. According to the Scandinavian Quality Registry, 0.36% of patients with bilateral thyroid surgery experienced bilateral RLN palsy [7]. A systematic review and meta-analysis published in 2016 by Pardal-Refoyo et al., which included 30 922 patients, demonstrated an incidence of bilateral RLN palsy in the series with neuromonitoring (IONM) to be 0.243% (0.155–0.35%) and without IONM to be 0.518% (0.253–0.87%) [8]. Most surgeons agree that an RLN should be identified in all cases. The superiority of this approach, introduced by Bier from Berlin in 1911

[9], Lahey from Boston in 1938 [10] and Riddell from London in 1956 [11], has been proven by many studies [12-14]. Identifying the functional status of an RLN during thyroidectomy is of a paramount significance, as in most cases, an injured nerve seems visually intact at surgery. Two-staged thyroidectomy remains a topic under discussion, and according to a survey of the French Association of Endocrine Surgeons (AFCE), the majority of surgeons continued the operation on the second side despite a loss of an intraoperative IONM signal [15]. Nevertheless, the majority of authors suggest that according to the IONM results, the surgical strategy should be reconsidered in patients with loss of signal: the operation on the second side can be cancelled, and bilateral nerve injury, with its devastating consequences, avoided [16-22]. Several different techniques, such as finger palpation of the cricoarytenoid muscle or the so-called laryngeal palpation (LP) [23-29], rigid or flexible fiberoptic laryngoscopy [30, 31], intramuscular vocal cord (VC) electrodes and others were used to confirm the response of the RLN to low-voltage stimulation [32, 33]. Electromyographic methods, brought into practice by Flisberg and Lindholm in 1970 [33], are adopted in modern thyroid surgery. IONM has gained general acceptance among thyroid surgeons, with the most common method in use to be a special endotracheal tube with embedded electrodes for electromyographic recordings. Despite the widespread popularity of this technique, disadvantages, such as increased cost, additional setup time, equipment dysfunctions and low sensitivity, as well as positive predictive value, are repeatedly mentioned; also, the possibility of a reliable intraoperative prediction of the RLN functional status or of reducing the RLN injury rate are sometimes seriously doubted [34-36]. A variety of different techniques without electromyographic documentation are less popular. A particular method – VC visualization using laryngeal mask anesthesia with flexible fiberoptic laryngoscopy – causes concern because of the potential loss of control of the airway [30, 31]. An alternative, simple and inexpensive technique of VC mobility evaluation is previously mentioned as the LP method. According to published data, the LP method's sensitivity and specificity for nerve injury detection varies between 33–100% and 92–100%, respectively [23-29], and are comparable to the values of IONM. According to Cavicchi et al., this method has very similar predictive values to IONM [26]. Cha et al. conclude that the LP method might be used to predict VC mobility when IONM is unavailable [29]. Most authors recommend this method for clinical use (Table 1).

The LP method is recommended by the International Neural Monitoring Study Group in the case of EMG activity being either not present or at an unusually low amplitude when using IONM systems [37]. However, it is not still clear if this method is specific and sensitive enough.

TABLE 1. The sensitivity and specificity of the LP method.

Publication year	Authors	Country of the study's origin	Study type	Number of patients	Sensitivity	Specificity	Recommendations for application to clinical practice
2018	Cavicchi et al.	Italy	Retrospective cohort	716	90%	99.1%	Recommended
2016	Cha et al.	South Korea	Prospective cohort	293	81.82%	100%	Recommended
2012	Cavicchi et al.	Italy	Prospective double blind RCT	250	33.4%	97.5%	Recommended
2009	Cavicchi et al.	Italy	Retrospective case – control	993	55.3%	95.2%	Recommended
2006	Tomoda et al.	Japan	Prospective cohort	1376	69.3%	99.7%	Questionable
2004	Randolph et al.	USA	Prospective cohort	449	100%	100%	Recommended
2002	Otto et al.	USA	Retrospective cohort	55	75%	92.2%	Recommended

Another simple, inexpensive and noninvasive technique of VC mobility evaluation gaining popularity in the perioperative setting is the laryngeal ultrasound (LU) [38-48]. This method was proven as practical and accurate in preoperative and postoperative patient evaluation, but to our knowledge, there was no attempt to assess the feasibility of LU as an intraoperative technique. Keeping in mind such important points as its short learning curve [43], as well as the less invasive and more objective character of the intraoperative LU technique, it could be considered as a good alternative to the LP method. The sensitivity of LU in demonstrating VC palsy ranges from 62% to 93.3%, and specificity – from 97% to 97.8% [39, 44, 45]. Our study was designed to clarify the relationship between real-time VC mobility demonstrated by LU during intraoperative electrical stimulation of the RLN and vagal nerve and postoperative laryngoscopy. We hypothesized that nerve fibers capable of conducting a signal and producing visible muscle contraction on intraoperative LU at the suprathreshold stimulation of the RLN are sufficient for generating volitional VC movement postoperatively. The main disadvantage of LU method is a VC visualization rate. According to recently published data, VC cannot be assessed perioperatively by LU in approximately 20% of cases in general and less so in males [39, 40, 42, 48-50]. Different solutions are proposed to improve the rate of visualization. Woo

et al. introduced a lateral approach [41]. A lateral vertical approach was suggested by Fukuhara et al. [47]. According to data published by Wong et al., the identification of all three sonographic landmarks (true cords, false cords and arytenoids) is not mandatory, as the visualization of normal movement in one of the sonographic landmarks is sufficient to exclude VC palsy [38]. Another objective of this study was to compare the predictive values of the intraoperative LU and LP methods. In both methods, responses can be elicited using a nerve stimulator, avoiding the costs of the disposable special endotracheal tubes used as a recording part of IONM systems.

Different factors for the increased VC palsy rate, such as malignancy, thyroiditis, recurrent disease, RLN identification and the extent of surgery are frequently suggested [14, 51-53]. Another goal of our study was to evaluate such factors as gender, age under or over 55 years, malignant histology, thyroiditis, the weight of a thyroid lobe under or over 100g and dysphonia as predictors for VC palsy.

Acoustic Speech Analysis for Voice Disorders

The literature shows that an altered voice is a common problem after thyroid surgery. The voice changes were reported in 25% to almost 90% of patients within the first few weeks after thyroidectomy [54-58]. It is very important to realize that VC palsy may occur without any voice changes [59, 60]. The majority of endocrine surgeons agree that pre- and particularly postoperative laryngoscopies should be mandatory in all patients undergoing thyroid surgery, as it is the most trustworthy method for determining VC palsy. Despite the reliability of this method, it could be uncomfortable and unpleasant for the patient, adds extra costs, needs special instruments and trained personnel, causes logistic problems [61]. As an alternative to invasive techniques, acoustic signal analysis-based non-invasive techniques were being explored extensively during the last two decades. The idea of applying an acoustical analysis of speech for voice disorder detection and evaluation is not new. Similar ideas were proposed 50–60 years ago [62, 63]. Various acoustic parameters were proposed and employed for this purpose[64]. These include, but are not limited to, the perturbations of fundamental tone [65], various noise estimation techniques [66], cepstral features [67, 68] and others. During the last decade, the task of acoustic signal analysis-based detection and the evaluation of pathological voices was studied intensively. A vast majority of studies focus on combining various features without any physiological reasoning.

The speech signal is generated in two stages. First, the so-called source signal is induced. The air flow generated by the lung causes the vibration of the VC. This vibration is known as the phonation process, and the fundamental frequency value describes its intensity. In the next step, the glottal flow is modulated by the voice tract. The result of this modulation is the speech signal, transmitting information of both

the VC and the voice tract resonant properties. The disorders of VC (palsy among them) affect the speech inevitably. The effect depends on the dysfunction degree of the VC and can vary from inaudible changes up to severe changes of voice – for example, it becomes breathy, harsh, and weak. An acoustical analysis of the speech signal is considered to be an objective evaluation of the vocal tract's functionality rather than a perceptual analysis of the speech. Acoustic parameters represent the generative and articulatory properties of the voice and thus could be applied for detecting and evaluating pathologies. Different acoustic parameters describe different stages of the speech signal's production, thus should be chosen reasonably. To estimate the functionality (or immobility) of VC, we have to analyze the glottal flow.

Inverse Filtering Technique

The most common technique for estimating the glottal flow is to employ a source-filter production model. This model describes the speech signal as the convolution of the source signal (glottal flow) and a filter (vocal tract). Both the source signal and the vocal tract can be modelled using various joint estimation models, or separately, by ignoring or considering the close phase of the glottal cycle [69]. If we consider the glottal flow and the vocal tract as independent, the glottal flow can be extracted with an inverse filtering of the speech signal [68, 69]. The inverse filter eliminates the effect of the vocal tract, thus giving the estimate of the glottal flow. The process of inverse filtering can be simplified using linear modelling of the vocal tract. In this study, we applied the autoregression (AR) model for modelling the vocal tract. The choice was due to the following reasons: (1) the AR model (also known as the Linear Predictive Coding model) is an all-pole filter and had great success in speech applications [68, 70, 71]; (2) the linearity of the filter enables us to obtain an inverse version of the filter very easy; (3) the chosen parameter estimation technique enables us to obtain a variable model order, which is adequate to the individual characteristics of human vocal properties. Therefore, we can expect a more accurate estimation of the glottal flow.

Aim of the Study

The main objective of the prospective cohort study *The Role of Intraoperative Neurostimulation using Laryngeal Palpation and Intraoperative Laryngeal Ultrasound in Optimizing the Safety of Thyroidectomy* was to determine the reliability of LP and intraoperative LU methods for evaluating the RLN functional status during thyroid surgery.

The Goals of the Prospective Cohort Study

1. To determine the sensitivity, specificity, accuracy, positive predictive and negative predictive value of the LP method;
2. To assess the validity of the intraoperative LU method; determine the visualization rate, sensitivity, specificity, accuracy, positive predictive and negative predictive value of the intraoperative LU method;
3. To compare the prognostic values of the LP method with the intraoperative LU method;
4. To determine the influence of such factors as gender, age under or over 55 years, malignant histology, the weight of a thyroid lobe under or over 100g and thyroiditis for VC palsy after thyroidectomy. To determine the significance of dysphonia as a predictor of VC palsy after thyroidectomy;
5. To apply acoustic speech analysis based on the inverse filtering technique to determine the prediction error-based glottal flow assessment criteria for detecting VC palsy.

Novelty of the Study

1. The LU method has never been tested in the intraoperative setting, and the validity of the method was not documented before our study was conducted;
2. As the acoustic speech analysis was applied for the same patients before and after thyroid surgery, in contrast to other studies, the same voices with and without VC palsies were evaluated. The autoregressive, model-based digital inverse filtering technique is presented for the estimation of the glottal flow. The novelty of the proposed method is the objective and adequate selection of a variable model order, which enables us to obtain a more accurate evaluation of individual articulation properties than a fixed-order modelling. This postulates the more accurate estimation of the glottal flow, disturbances of which are direct evidence of VC palsy.

Application in Clinical Practice

Nerve identification alone is insufficient as visual integrity does not guarantee a normal RLN functional status. One of the most significant attributes of neural testing is a prognostication of the postoperative neural function of RLN and its impact on surgical strategy in bilateral thyroid disease. An accurate identification of a unilateral RLN injury might postpone the second lobe surgery, and a bilateral nerve injury can be avoided. We designed this study to evaluate the efficacy of LP and intraoperative LU for the verification of the RLN functional status.

As an alternative to the invasive technique of laryngoscopy, non-invasive, acoustic signal analysis-based techniques were assessed to diagnose postoperative VC palsy.

Statements to be Defended

The following statements stand as those to be defended by this study:

1. LP is an accurate method for evaluating the intraoperative RLN functional status;
2. Intraoperative LU is a feasible and accurate method for evaluating the intraoperative RLN functional status;
3. There is no statistically significant difference between the results obtained by the LP method and the results obtained by the intraoperative LU method;
4. Such factors as gender, age under or over 55 years, malignant histology, the weight of a thyroid lobe under or over 100g and thyroiditis have no statistically proven predictive value for postoperative VC palsy. Postoperative dysphonia is not an accurate predictor of postoperative VC palsy;
5. Prediction error-based glottal flow assessment criteria, developed applying acoustic speech analysis based on the inverse filtering technique, could be used to detect pathological voices in the VC palsy group.

Methods

Patient selection

All patients who underwent thyroid surgery between March 2016 and December 2017 in Vilnius University Hospital Santaros Klinikos were screened for inclusion in this prospective cohort study. The protocol of the study was approved by the Vilnius Regional Bioethics Committee. All patients aged 18 years or older who had had thyroid pathologies and were scheduled for thyroid surgery were eligible. Exclusion criteria were as follows: pregnancy, refusal to participate in the study or a presence of RLN palsy before the surgery.

Study Protocol

All patients before the inclusion into the study underwent preoperative laryngeal examination by indirect or fiberoptic laryngoscopy for VC mobility evaluation. Those patients who had no evidence of RLN palsy were enrolled in the study and underwent thyroid surgery. In all cases, surgeries and all intraoperative tests for RLN functional status evaluation were performed by a single surgeon (A.R.). In

order to reduce errors in LU interpretation, the surgeon was additionally trained in the Instructional Course of Transcutaneous Laryngeal Ultrasonography at the first World Congress of Neural Monitoring in Thyroid and Parathyroid Surgery (September 2015, Kraków) held by the International Neural Monitoring Study Group. Intraoperative LP and LU were performed as described below. A voice recording was performed a day before surgery and on the first postoperative day. Laryngoscopy was performed in all patients on the first postoperative day. Follow-up laryngoscopy was reserved for those patients who were diagnosed with VC palsy on the first postoperative laryngeal examination. Follow-up checkups were performed once in every 3 months during the first year after the surgery. If VC palsy was present for more than 1 year, it was considered as permanent RLN palsy.

RLN and Vagus Nerve Stimulation

Both the vagus nerve and RLN were stimulated during LP and LU at the end of the surgery of each side. The nerve stimulator Stimuplex HNS 12 (BBraun, Melsungen, Germany) was used for stimulation with a stimulus amplitude as suggested by Phelan et al. [72], 1 mA and a stimulus frequency of 2Hz. No neuromuscular blocking agents were used after the induction of anesthesia. The proper functioning of the neural stimulator was tested in each case on the muscular tissue of the surgical field before direct nerve stimulation. As the international neural monitoring study group has emphasized the importance of vagal nerve stimulation for accurate nerve prognostication, the vagus nerve was dissected and visually identified in all cases [37].

Laryngeal Palpation Technique

For laryngeal palpation, intraoperative neurostimulation was performed as described above, and the functional status of RLN was assessed by palpating the postcricoid region of the larynx, as well as sensing posterior cricoarytenoid muscle contraction, the so-called "laryngeal twitch response" [23] (Fig. 1).

Laryngeal Ultrasound Technique

Ultrasound system BK Flex Focus 800 8815, 4–10 MHz was used for ultrasound evaluation. The transcutaneous laryngeal ultrasonography technique was adopted for intraoperative use, applying acoustic gel and covering the probe with a sterile sleeve. To improve surface contact, warm, sterile saline was used on the outer surface of the probe cover. Anterior-approach and lateral approach LU were used to increase the assessability rate of VC. The techniques were performed as described elsewhere [40, 41]. Using the anterior-approach, a transducer was placed on the skin above the surgical wound at the middle of the thyroid cartilage (Fig. 2).

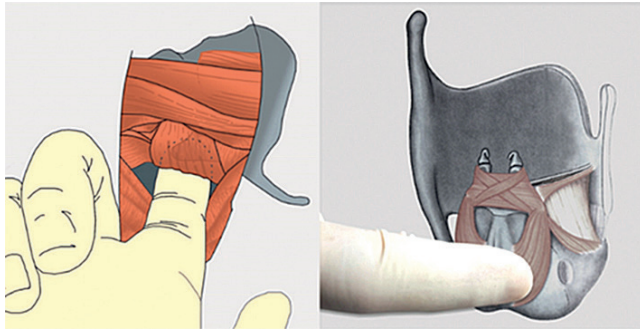

FIGURE 1. Laryngeal palpation technique.

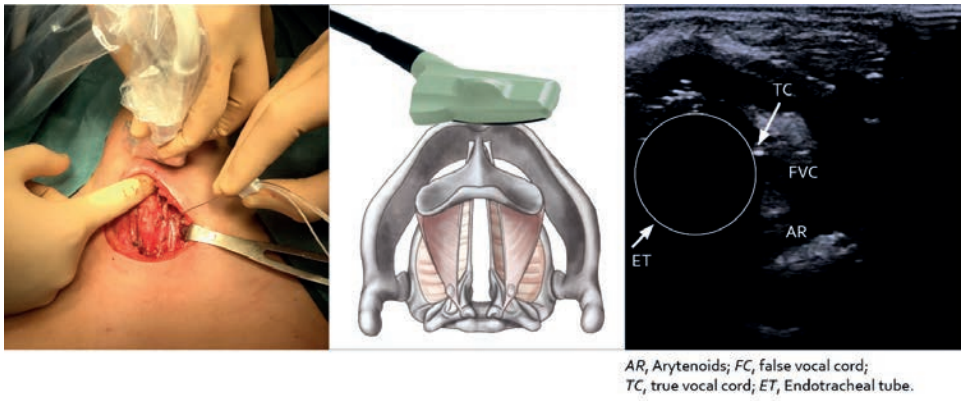

FIGURE 2. Anterior approach laryngeal ultrasound.

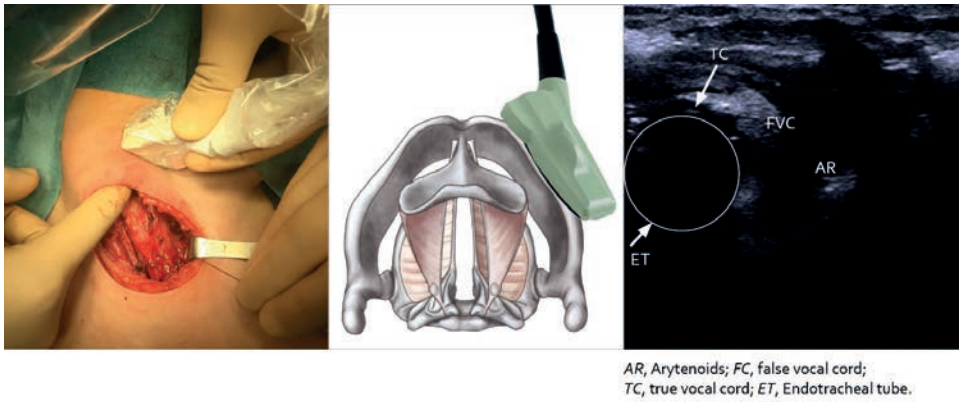

FIGURE 3. Lateral approach laryngeal ultrasound.

In the case of a lateral-approach, the transducer was placed on the lateral surface of the thyroid cartilage lamina (Fig. 3).

The three most commonly described sonographic landmarks were chosen for ultrasonographic visualization: false cords, true cords, and arytenoids (Fig. 2, Fig. 3). An intraoperative LU examination was defined as assessable if 1 or more landmarks were identified. The anterior-approach LU was performed first, whereas the lateral-approach LU was performed only when a visualization using anterior-approach technique proved unsuccessful. The observed movement in any of 3 landmarks was considered as sufficient proof of a normal RLN function [38].

Voice Recording and Acoustic Signal Analysis

All the patient voices were recorded using a headset microphone in a quiet clinician's room environment. There were two recording sessions organized: one day before surgery and one day after surgery. The control group consisted of healthy people with no voice complaints. The voices of ten female and ten male speakers were recorded using the voice recorder with an external microphone in a quiet room environment. The control group voice recordings were used in previous studies of healthy voice analysis. All the recorded persons were asked to pronounce the vowels Y, A, O and U in a sustained manner for 3–4 seconds and cough for three times. The vowel A was chosen for voice acoustic signal analysis, as this vowel is characterized by a minimal lip restriction during the radiation phase and a fully expressed phonation level. Besides, the vowel A is typical for most languages, what makes it universal for comparison purposes. The estimated signal of glottal flow and its spectral density function were analyzed to estimate the qualities of the pathological and healthy voices. The glottal flow was obtained from the voiced speech segments. Considering the source filter approach, the speech signal $s(t)$ was expressed as the convolution of the glottal flow $g(t)$ and the vocal tract filter $h(t)$

$$s(t) = g(t) * h(t).$$

Here, the lip radiation effect (modelled as a first-order differentiating filter) was included in the vocal tract processing and was not considered separately. We obtained an estimate of the inverse vocal tract filter $\hat{h}^{-1}(t)$ and applied it to the analyzed speech signal $s(t)$; in this way, we eliminated the effect of the vocal tract and thus obtained the estimate of the glottal flow.

$$\hat{g}(t) = s(t) * \hat{h}^{-1}(t)$$

In this study, we applied the AR model for the modelling of the vocal tract.

The quality of the estimated glottal flow was assessed on the basis of the ratio of estimated squared prediction error and estimated signal variance $\hat{b}^2/\hat{D}$. The value of

$\hat{b}^2/\hat{D}$ indicates the relative part of the unmodelled signal: the higher ratio value we obtain, the higher signal prediction error is. Therefore, we can expect a low $\hat{b}^2/\hat{D}$ value for normal glottal flow and high values for pathological voices (with VC palsy). In this study, we expressed this ratio in percentages and called it the estimated error of glottal flow. For healthy and normal voices, this ratio would approach toward the level of zero, and for pathological voices, it would converge to 100% (in case of the paralysis of VC).

Definitions and Statistical Analysis

The study enrolled 112 patients and 200 nerves at risk.

If both the LU and postoperative laryngoscopy confirmed VC palsy, the case was defined as a true-positive. If both tests refuted VC palsy, the case was defined as a true negative. If movement was observed in any of the three chosen sonographic landmarks on intraoperative LU, but postoperative laryngoscopy had revealed a VC palsy, the case was interpreted as a false negative, and vice versa – if no movement was observed on intraoperative LU, but if postoperative laryngoscopy demonstrated a normal movement of the VC, the case was attributed to the false-positive category. The same principles were equally applied for the LP method interpretation.

Statistical analysis was performed using SPSS v.23 For Windows (SPSS 23, IBM, Armonk, NY, United States of America). The RLN injury incidence calculations were based on the number of the nerves at risk and per patient. The McNemar's test was applied to compare tests sensitivity and specificity. P values indicated the levels of significance. All p values of less than 0.05 were considered to indicate statistical significance. Sensitivity, specificity, positive and negative predictive values, and accuracy were calculated for LU, LP for vagal and RLN stimulation, considering VC palsy diagnosed postoperatively as the target outcome. Predictive values of both methods were calculated based on nerves at risk.

For comparing the difference in the sensitivity and specificity of the LU and LP examinations with postoperative laryngoscopy, the McNemar test was used. The McNemar test is the best test for dichotomous variables with two dependent sample studies. In our case, the McNemar test evaluates the probability that the nerves, damaged during surgery, will be recognized as damaged nerves and normal nerves will be recognized as the normal nerves. To more accurately evaluate the ability of the LU and LP methods to correctly diagnose the state of RLN, we calculated the degrees of dependence between the real state of the nerves (determined by laryngoscopy) and the results of the state of nerves, obtained using the appropriate techniques of LU and LP, respectively (the value equal to zero corresponds to the complete independence of the variables, and the value equal to one – to maximum dependence).

The degrees of dependences were determined by Cramer's V and Cohen's kappa. The dependency ratio of Cramer's V is the most commonly used ratio of nominal variables, calculated on the basis of $\chi 2$. Cohen's kappa (also calculated on the basis of $\chi 2$) is used to determine the compatibility of the two methods that evaluate the same object or phenomenon. The Cohen's kappa value 1 indicates a complete overlap between the method estimates. The Cohen's kappa applies only if both variables have the same category values and the same number of categories.

To establish the influence of different factors for VC palsy after thyroidectomy, a statistically significant relationship between the related variables was determined using Fisher's Exact Test. The degree of dependence of variables for the nominal variables was determined using Cramer's V, the Contingency Coefficient and Spearman's rho. All p values of less than 0.05 were considered to indicate statistical significance. The confidence interval (CI) was calculated for a 95% confidence level.

Results

Clinicopathological characteristics

During the research period, we prospectively studied 112 patients with 200 nerves at risk. The statistical used tests confirmed that there were no missing values and all 200 nerves at risk were analyzed. Gender distribution was 20 (18%)/92 (82%), with the male to female ratio being 1:4.6. The age median was 58 years, with a range of 21–81 years and the mean 56.22 years and a standard deviation of 13.57

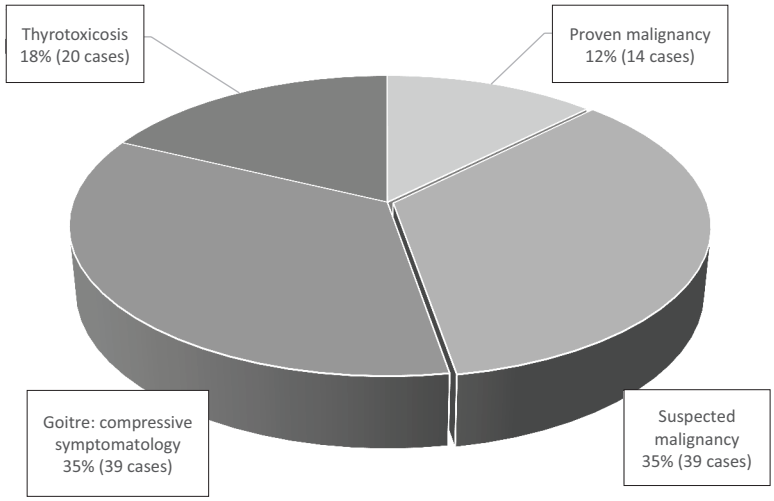

FIG. 4 Indications for surgery.

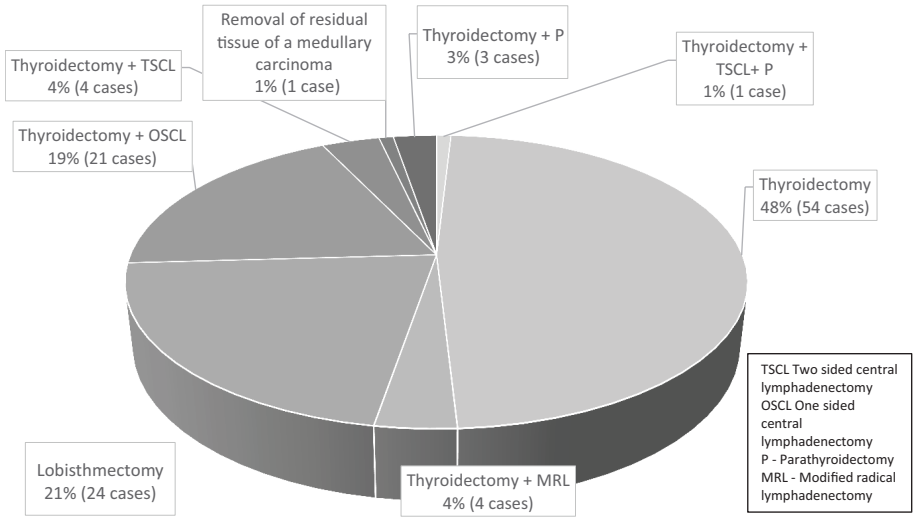

FIG. 5 Types of surgery performed.

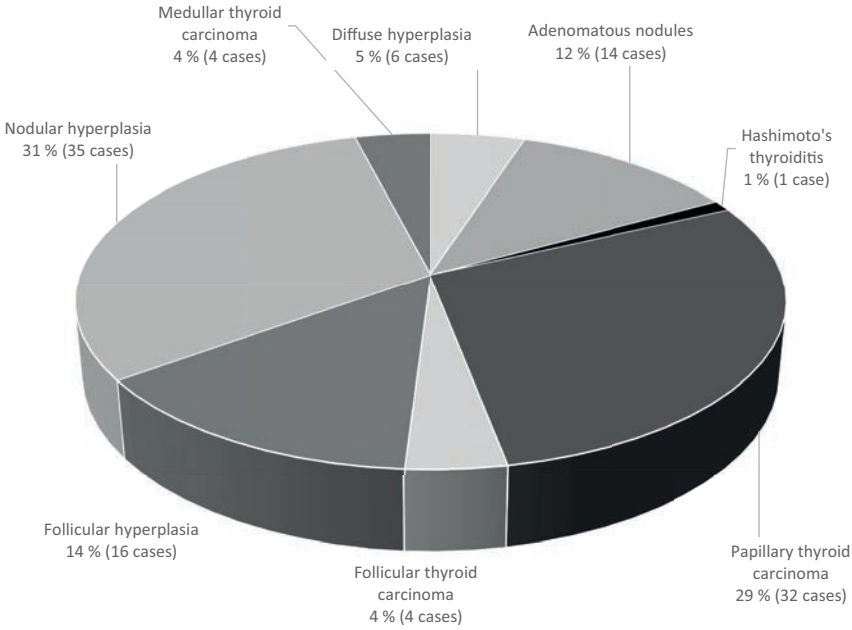

FIG. 6 Pathological diagnosis.

years. The two most common indications for surgery were suspected malignancy and goiter with compressive symptoms, both of them making up equally 39 cases (35%). Proven malignancy made up 14 cases (12%) (Fig. 4).

The most common operation was a total thyroidectomy, which made up 54 cases (48%); lobisthmectomies made 24 cases (21%) (Fig. 5). There were no subtotal thyroidectomies which could underestimate the RLN palsy rate. Only two operations were reoperative cases, with a removal of residual medullar cancer tissue from the RLN in one of them.

The maximal weight for the right lobe was 371.21g and for the left lobe 409.91 g. Postoperative histological specimen evaluation revealed that 72 (64%) cases were benign and 40 (36%) malignant, and a papillary carcinoma accounted for 32 (29%) cases (Fig. 6).

RLN Functional Status Assessment

The RLN and the vagal nerve were successfully dissected and visualized in all cases. Also, in all cases, the RLN and vagal nerve stimulation resulted in the same response. A visualization of at least one of the laryngeal sonographic landmarks by one of the two intraoperative LU techniques was successful in all cases (200 nerves at risk).

In order to meet entry criteria, VC palsy before enrollment into the study was excluded in all 112 patients. A high rate of compliance with the protocol for postoperative laryngoscopy was observed. Day 1 laryngoscopy was performed and documented in all 112 cases (100%). The measurement of the RLN palsy rate based on the number of nerves at risk was 3% and based on the number of patients was 5.4%. (6 cases). Based on consecutive documented laryngoscopic examinations, all 6 VCP patients recovered in 2–9 months during the follow-up period. None of the patients were lost to follow-up.

Using the LP method, the following results were obtained:

The 6 out of 6 RLN damaged during the surgery were diagnosed correctly;

The 6 normal RLN were classified as damaged;

There were no damaged RLN attributed to the normal nerve category.

Using the LU method, the following results were obtained:

The 5 out of 6 RLN damaged during the surgery were diagnosed correctly;

The 3 normal RLN were classified as damaged;

The 1 damaged RLN was attributed to the normal nerve category.

The LP method's sensitivity counted per nerve at risk both in the vagus nerve and the RLN group was 100%, specificity – 96.9%, accuracy – 97%, positive predictive value – 50% and negative predictive value – 100%.

LU method sensitivity counted per nerve at risk both in the vagus nerve and the RLN group was 83.3%, specificity – 98.5%, accuracy – 98%, positive predictive value – 62.5%, negative predictive value – 99.5%.

In the case of LP, the McNemar test showed a statistically significant difference between the quantity of the nerves actually damaged during thyroid surgery and the result obtained using the LP method ($p = 0.031$). There is a significant discrepancy between the two proportions. In other words, there is a large discrepancy between the real number of actually normal and damaged nerves and the number obtained by the LP method. Nevertheless, both the Cramer's V (association coefficient = 0.669) and Cohen's kappa (extent of agreement = 0.653) indicate a strong association between the actual functional state of the nerves and their estimation with the LP method. In both cases, the relationship shown is statistically significant, since the significance level is $p = 0.000$ ($p < 0.05$).

In the case of LU, the McNemar test showed that there was no statistically significant difference between the number of normal and damaged RLN (identified during postoperative laryngoscopic examination) and the normal and damaged nerves determined using intraoperative LU ($p = 0.625$) (there is no significant discrepancy between the two proportions). In other words, the LU method recognizes both damaged and normal nerves reasonably well. Both the Cramer's V (associa-

TABLE 2. Thee predictive values of the LP and LU methods.

	McNemar						Cramer's V		Cohen's kappa	
Methods	**p-value (2-sided)**	**Sensitivity**	**Specificity**	**Accuracy**	**Positive predictive value**	**Negative predictive value**	**Value**	**p-value**	**Value**	**p-value**
LU method, RLN stimulation	0.625	83.3%	98.5%	98%	62.5%	99.5%	0.712	0.00	0.704	0.00
LU method, Vagal stimulation	0.625	83.3%	98.5%	98%	62.5%	99.5%	0.712	0.00	0.704	0.00
LP method, RLN stimulation	0.031	100%	96.9%	97%	50%	100%	0.696	0.00	0.653	0.00
LP method, Vagal stimulation	0.031	100%	96.9%	97%	50%	100%	0.696	0.00	0.653	0.00

tion coefficient = 0.712) and Cohen's kappa (extent of agreement = 0.704) indicate a strong correlation between the number of actually normal and damaged nerves and the normal and damaged nerves identified by LU method. In both cases, the shown relationship is statistically significant, since the significance level is $p = 0.000$ ($p < 0.05$) (Table 2).

Comparing both the LU and the LP methods, the McNemar test showed that there is no statistically significant difference between the result obtained using the LU method and the result obtained using the LP method ($p = 0.289$). In other words, the results obtained using the LU method are statistically not different from the results obtained using the LP method. However, both the Cramer's V (association coefficient = 0.593) and the Cohen's kappa (extent of agreement = 0.580) shows only the mean correlation between the results obtained using the LU method and the results obtained using the LP method. In both cases, the relationship shown is statistically significant, since the significance level is $p = 0.000$ ($p < 0.05$) (Table 3).

TABLE 3. A comparison of the LU and LP methods.

	McNemar	Cramer's V		Cohen's kappa	
Methods	**p-value (2-sided)**	**Value**	**p-value**	**Value**	**p-value**
LU method VS. LP method	0.289	0.593	0.00	0.580	0.00

Such factors as gender, age under or over 55 years, malignant histology, the weight of a thyroid lobe under or over 100g and thyroiditis had no statistically proven predictive value for the VC palsy. The statistically significant dependence was detected only in the analysis of the dependence of the VC palsy from the dysphonia on the first postoperative day ($p = 0.004 < 0.05$). However, dependence, as shown in dependency coefficients, is weak. The symmetric dependence, calculated by the Cramer's V, is equal to 0.211, $p = 0.003$. Similar coefficients of symmetric dependence are captured and by other variables dependency criteria:

Contingency Coefficient = 0.206, $p = 0.003$. Spearman's rho = 0.211, $p = 0.003$. An odds ratio is calculated for the cohort "Dysphonia = VC palsy." It is equal to 0.392. This shows that when having a dysphonia, the risk to have a VC palsy is 39.2% higher than in the absence of a dysphonia (Table 3).

TABLE 4. The correlation of different factors with postoperative VC palsy.

Factors	VC palsy Yes (Cases, %) *N=6 (3.0%)*	VC palsy No (Cases, %) *N=194 (97.0%)*	P – value, two-tailed	Coefficient of dependancy	Odds Ratio	Confidence interval 95% Lower; Upper
Gender: *Male* *Female*	 1 (2.9%) 5 (3.0%)	 33 (97.1%) 161 (97.0%)	1.00 (Fisher's Exact test)	0.002 (Cramer's V)	$p \geq 0.05$. There is no statistically significant dependence	
Age group: *<55 years* *≥55 years*	 3 (3.6%) 3 (2.6%)	 81 (96.4%) 113 (97.4%)	0.697 (Fisher's Exact test)	0.029 (Cramer's V)	$p \geq 0.05$. There is no statistically significant dependence	
Weight of a lobe: *<100 g* *≥100 g*	 5 (2.8%) 1 (5.3%)	 174 (97.2%) 18 (94.7%)	0.459 (Fisher's Exact test)	0.042 (Cramer's V)	$p \geq 0.05$. There is no statistically significant dependence	
Thyroiditis: *Yes* *No*	 2 (5.6%) 4 (2.5%)	 34 (94.4%) 159 (92.5%)	0.297 (Fisher's Exact test)	0.070 (Cramer's V)	$p \geq 0.05$. There is no statistically significant dependence	
Cancer: *Yes* *No*	 1 (1.3%) 5 (4.1%)	 76 (98.7%) 117 (95.9%)	0.408 (Fisher's Exact test)	0.080 (Cramer's V)	$p \geq 0.05$. There is no statistically significant dependence	
Dysphonia: *Yes* *No*	 6 (7.3%) 0 (0.0%)	 76 (92.7%) 118 (100.0%)	0.004 (Fisher's Exact test)	0.211 (Cramer's V)	0.392	0.329; 0.467

Voice acoustic signal analysis

Case Analysis

For the analysis of pathological and healthy voices, we have selected two voices for the inverse filtering procedure and the estimation of glottal flow. The estimated signal of glottal flow and its spectral density function were analyzed to estimate the qualities of the pathological and healthy voices. Figure 7 presents the results obtained for the healthy female's voice. The estimated order of the vocal tract filter was 11 (i.e., the vocal tract had 6 resonant frequency values). The estimated glottal flow can be evaluated as periodic and normal (Fig. 7(b)). Spectral density function (Fig. 7(c)) is also periodic, and the harmonic components are vivid through the entire frequency range of the signal. The results of pathological male voice analysis are given in Fig. 8. Here, we can see the distorted waveform of the utterance (Fig. 8(a)). The vocal tract was modelled by the 20th order model, which means ten resonant frequencies of the tract. The estimated glottal (Fig. 8(b)) flow is noisy with no sign of

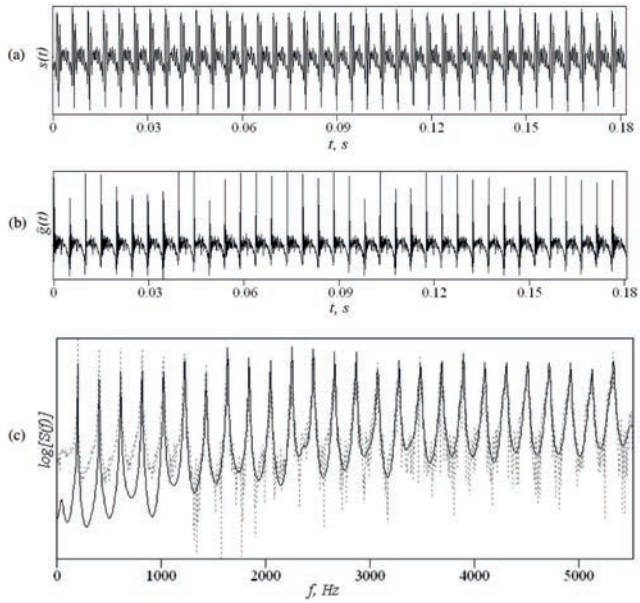

FIG. 7. The healthy voice: (a) the waveform of the vowel A; (b) the estimated glottal flow; (c) the spectral density of the glottal flow (an AR model-based spectral density is given in a solid line, a Fourier transform-based spectral density is given in a dotted line).

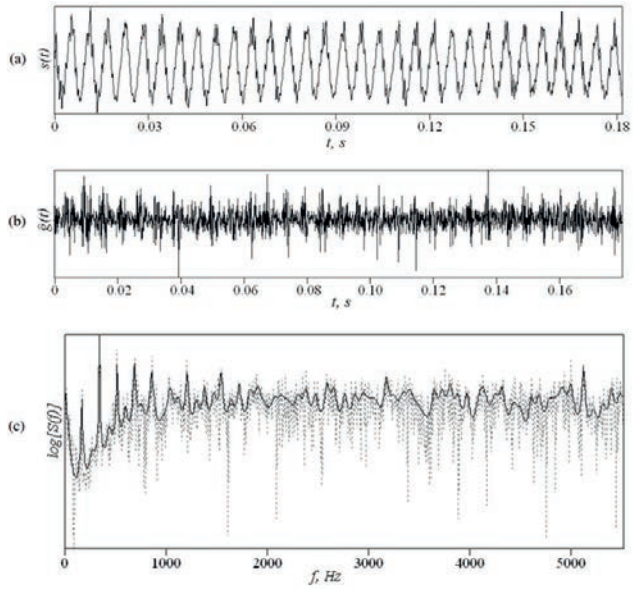

FIG. 8. The pathological voice: (a) the waveform of the vowel A; (b) the estimated glottal flow; (c) the spectral density of the glottal flow (the AR model-based spectral density is given in solid line, Fourier transform-based spectral density is given in dotted line).

periodicity (what is characteristic for the vocalized vowel). The spectral density (Fig. 8(c)) of the flow is noise-like; here, we can see only 4–5 harmonic components. This is the evidence of VC immobility, which can be the result of the VC palsy. Similar results were obtained for all pathological voices: non-periodicity of the estimated glottal flow, noise-like spectral density function. The degree of non-periodicity was different for the individual voices. This difference may be an individual characteristic of the voices and require a more detailed study with larger datasets.

We evaluated the error level of glottal flow for healthy and pathological voices. The averaged results are given in Fig. 9. We can see the clear difference between healthy and pathological voices. The patients' voices (even before thyroidectomy) have, on average, a 21.7% higher error level than the healthy ones.

The thyroidectomy procedure with the output of the immobility of the VC increased the error level in the same patient on average by 13.5% and by 35.2% in comparison with the control group of normal voices. In the case of the VC palsy in the male patient (1 case assessed in our study), the error level in comparison to preoperative estimate increased by 43.9%. Therefore, the prediction error level of the glottal flow enables us to identify the case of VC palsy.

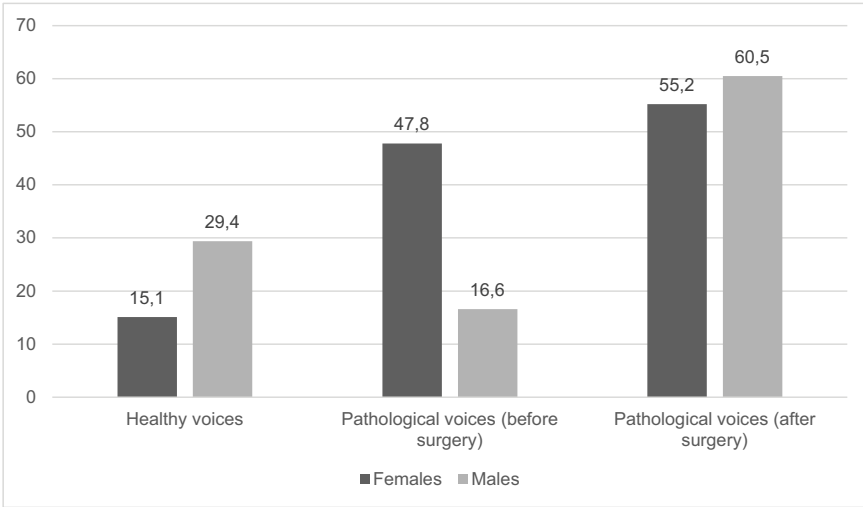

FIGURE 9. The averaged results for the evaluated error level of glottal flow for healthy and pathological voices.

The individual error levels of glottal flow are given in Table 5.

TABLE 5. Individual levels of glottal flow.

Patient	Error levels of glottal flow before operation %	Error levels of glottal flow after operation %
Pathological (parethic) voices		
Female 1	38.1	15.6
Female 2	25.7	42.1
Female 3	41.2	60.1
Female 4	69.2	82.1
Female 5	64.8	76.2
Male 1	16.6	60.5
Males (average)	16.6	60.5
Females (average)	47.8	55.2
All (average)	42.6	56.1
Healthy voices		
Males (average)	29.4	-
Females (average)	15.1	-
All (average)	20.9	-

Discussion

Our study demonstrated that LP, as well as the new method – LU, are both effective for evaluating the intraoperative RLN functional status. Nerve identification alone is insufficient because visual integrity does not guarantee a normal RLN functional status [7, 73]. Even when the nerve is visually intact, as few as 11 (3%) of unilaterally and 16 (7%) bilaterally damaged nerves are recognized intraoperatively [7]. In our study, in all 6 cases of VC palsy, RLN seemed visually intact during an operation. In general, only some kind of intraoperative neural testing can be relied on to indicate the function of the RLN, as information on neuropraxic nerve injury is missing through visual assessment alone. Despite different categories of benefit such as RLN identification, tracing the nerve and its branches in a surgical field and even nerve preservation are referred to in the growing body of literature; probably the most significant attribute of neural testing is a prognostication of postoperative neural function and its impact on surgical strategy in bilateral thyroid disease [17-19, 28]. An accurate identification of unilateral RLN injury might prevent second lobe surgery and bilateral nerve injury. Currently, IONM has received considerable attention and has gained popularity, but controversy remains as to whether electromyographic monitoring has any clear advantage in preventing nerve injury and

whether it precisely predicts VC function postoperatively [32, 34, 36, 74]. Also, it is clear that the need for specialized neural monitoring equipment increases the surgery costs, and IONM is not used for this reason in some centers [32, 75, 76]. While the LP and LU methods require only a fixed cost of a neural stimulator and an ultrasound machine, electromyography demands additional costs associated with specialized endotracheal tubes. According to Gremillion et al., the use of IONM increases the cost of each surgery by $387 [75]. A cost-utility analysis performed by Rocke et al. suggests that only visual RLN identification in comparison to universal IONM application, can save $683 per patient [76]. Data provided by Dionigi et al. suggest that costs are 5–7% higher using IONM in comparison to traditional thyroidectomy [77]. The precise placement of the endotracheal tube electrodes over the VC requires fiberoptic laryngoscopy. That may add costs of equipment and increase operating time. The application of LP and LU methods adds no extra costs in our experience. According to a systematic appraisal of the literature presented by Dralle et al., the reported sensitivity of IONM for RLN injury is very variable and ranges from 23.5% to 100%. The revealed values of specificity ranged from 94% to 99.7% [78]. More recent data published by Mirallie et al. demonstrated low IONM sensitivity (29%) and high specificity (98%) values for detecting postoperative VC palsy [79]. Randolph et al. presented similar data: the sensitivity of IONM's loss of signal for postoperative VC palsy detection was 33%, and specificity was 99.9% [80]. The results published by Calo et al., on the contrary, showed very high predictive values of IONM: sensitivity was 93.6%, and specificity was 99.4% [22]. In general, the predictive values of IONM are comparable to the same values of the LP method with similar inconsistency in sensitivity. Our study demonstrated high sensitivity values of the LP method. This could be partially explained by the fact of the previous experience of LP method application. Some studies indicate that the LP method is as reliable and sensitive as IONM in predicting RLN functional status or can be used when IONM is unavailable [26, 27, 29]. Dionigi et al. assessed the learning curve of standardized IONM and determined that a safe application of the technique requires the surgeon to perform 50 cases [81]. Pragacz et al. suggest the same number of procedures to achieve comparable outcomes published by referral centers [82]. According to Jonas et al., the learning curve of IONM can last several years [83]. Recent studies described a new technique – the LU – to assess VC mobility and RLN functional status in a preoperative and postoperative setting [38-42, 49, 50]. The learning curve of the transcutaneous laryngeal ultrasound is shorter in comparison to IONM. According to data published by Wong et al., surgeons could master the skill in transcutaneous laryngeal ultrasound after seven examinations and assess VC function consistently and accurately after 40 procedures [43]. With regard to advantages as simplicity, the possibility of use in combination with minimally invasive surgery and

the documentation of the real-time VC mobility, we considered that application of LU as an intraoperative technique would be valuable in improving thyroid surgery quality. However, it was never tested in the intraoperative setting, and the validity of the method was not documented before our study was conducted. The results of our study confirmed that intraoperative LU could be used for the intraoperative functional status assessment of RLN with a slightly higher accuracy than the technique of LP. Moreover, intraoperative LU can be a good alternative to LP in confined surgical fields, such as mini-invasive thyroidectomy or parathyroidectomy, when LP is complicated and probably dangerous [26]. The main disadvantage of LU may be the poor VC visualization rate. According to recently published studies, in a perioperative setting, VC cannot be visualized and assessed in approximately 20% of cases [40, 42, 48, 49]. However, we did not face this problem in our study, where the visualization rate was 100%. To increase the VC visualization rate, we used a modified LU technique – a combination of the traditional anterior-approach and the novel lateral approach LU as described by Woo et al. [41]. To achieve further increase in VC visualization rate, the LU examination was defined as assessable even if one out of the three laryngeal sonographic landmarks were identified, as was suggested by Wong et al. [38] Another potential limitation of the new intraoperative LU method is the distortion of normal laryngeal anatomy due to an endotracheal tube position, but our study achieved a 100% visualization rate. Our experience showed that in one case, there was no sensible contraction of the cricoarytenoid muscle, despite this a weak movement was observed on intraoperative LU. We can speculate that remaining nerve fibers after RLN damage would produce only muscular movement on suprathreshold stimulation, and too few of them have been left intact to produce volitional movement on postoperative laryngeal examination. We can presume that any movement observed on intraoperative LU could be a reliable prognostic factor for temporary palsy with a reasonable prospect of recovery. A single surgeon performed all operations in our study, and this fact gives rise to uncertainty as to whether the same results can be reproduced in other studies.

Our study established the dependence of the VC palsy from the dysphonia on the first postoperative day ($p = 0.004$); however, dependence, as shown in dependency coefficients, is weak. We conclude, in agreement with published data, that dysphonia is not a reliable predictor of VC palsy [60, 84].

It is difficult to draw conclusions when dealing with small numbers, and the low rates of RLN palsy may preclude the achievement of the most reliable evidence of LU predictive values.

Prediction error-based global and universal glottal flow assessment criteria for palsy detection cannot be formulated so far. The voice production system is very specific to each speaker, the impact of the surgery is also very specific. Thus, VC

mobility should be estimated individually, taking into account individual qualities, comparing preoperative and postoperative voice qualities. The employed AR model parameter estimation technique is capable of describing these individual properties and using a prediction error to monitor the dynamics of VC functionality before and after thyroidectomy; however, the limited case number warrants a more comprehensive study to confirm these findings.

Conclusions

1. The LP method is an accurate method for evaluating the intraoperative RLN functional status. The LP method sensitivity counted per nerve at risk is 100%, specificity – 96.9%, accuracy – 97%, positive predictive value – 50% and negative predictive value – 100%;
2. The intraoperative LU is a feasible and accurate method for evaluating the intraoperative RLN functional status. The visualization rate of the intraoperative LU method is 100%, sensitivity counted per nerve at risk – 83.3%, specificity – 98.5%, accuracy – 98%, positive predictive value – 62.5%, and negative predictive value – 99.5%;
3. Comparing both the LU and the LP methods, the McNemar test showed that there is no statistically significant difference between the results obtained by the LU method and the results obtained by the LP method ($p = 0.289$);
4. Such factors as gender, age under or over 55 years, malignant histology, the weight of a thyroid lobe under or over 100g and thyroiditis had no statistically proven predictive value for VC palsy. The only significant predictor was dysphonia ($p = 0.004$) present on the first postoperative day; however, its dependence, as shown by the dependency coefficients, is weak.
5. The prediction error level of the glottal flow increased, on average, by 13.5% and up to 43.9% in the case of VC palsy; therefore, the prediction error level of the glottal flow enables us to identify the case of VC palsy. Prediction error-based global and universal glottal flow assessment criteria for palsy detection cannot be formulated so far;

Practical recommendations

1. Intraoperative neurostimulation should be performed in all bilateral cases;
2. Only after the confirmation of the RLN functional integrity by the presence of muscle response to the nerve stimulation on the first side, an operation should be continued contralaterally;

3. Both the LP and intraoperative LU methods can be used based on the individual preference when IONM is unavailable. In regard to the higher sensitivity of the LP method despite its lower accuracy, LP could be the method of first choice.

Presentations

1. G. Tamulevičius, J. Kaukėnas, A. Rybakovas, "The Evaluation of a Speaker's Individual Qualities Using a High Order Autoregressive Model," Data Analysis Methods for Software Systems, Druskininkai, Lithuania, December 1–3, 2016.

2. A. Rybakovas, V. Beiša, G. Žaldokas, K. Strupas, „Could the Safety of Thyroid Operations be Improved? Assessing the Feasibility of Intraoperative Vocal Cord Ultrasonography in Identifying Vocal Cords Movement and Reducing the Risk of Bilateral Palsy. Data from a Prospective Trial," The 9th Congress of Baltic Association of Surgeons, Klaipėda, Lithuania, May 10–12, 2018.

3. G. Žaldokas, A. Rybakovas, E. Lesinskas, K. Strupas, "The Effectivness of Intraoperative Recurrent Laryngeal Nerve Neurostimulation with Laryngeal Palpation and Vocal Cord Ultrasonography for Predicting Vocal Cord Palsy after Thyroid Surgery," The 7th Baltic Otorhinolaryngology Congress, Riga, Latvia, June 7–9, 2018.

4. A. Rybakovas, V. Beiša, G. Tamulevičius K. Strupas, "Could We Use Intraoperative Vocal Cord Ultrasonography in Identifying Vocal Cord Movements and Reducing the Risk of Bilateral Palsy? Prospective Trial Data," Evoliucinė medicina: sveikata ir ligos besikeičiančioje aplinkoje, Vilnius, Lithuania, June 5–10, 2018.

Publications

1. Andrius Rybakovas, Augustinas Bausys, Andrius Matulevicius, Gytis Zaldokas, Mindaugas Kvietkauskas, Gintautas Tamulevicius, Virgilijus Beisa, Kestutis Strupas. Recurrent laryngeal nerve injury assessment by intraoperative laryngeal ultrasonography: a prospective diagnostic test accuracy study. *Videosurgery and Other Miniinvasive Techniques*, 2018/12/10. DOI: https://doi.org/10.5114/wiitm.2018.80066.

2. Andrius Rybakovas, Virgilijus Beiša, Kestutis Strupas, Jonas Kaukėnas, Gintautas Tamulevičius. Inverse Filtering of Speech Signal for Detection of Vocal Fold Paralysis After Thyroidectomy. *INFORMATICA*, 2018, Vol. 29, No. 1, 91–105. DOI: http://dx.doi.org/10.15388/Informatica.2018.159.

3. Andrius Rybakovas, Andrius Matulevičius, Viktorija Belogorceva, Virgilijus Beiša, Kęstutis Strupas. Laringinė palpacija skydliaukės chirurgijoje: praktinės rekomendacijos, remiantis išsamia literatūros apžvalga ir perspektyviojo kohortinio tyrimo rezultatais (Laryngeal palpation in thyroid surgery: practical recommendations for method integration into daily clinical practice according to a comprehensive literature review and prospective cohort study results). *Lietuvos chirurgija*, Vol. 17 No. 3–4 (2018).

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1. Samalavičius NE, Zdanytė E, Stanaitis J, Radžiūnas G, Rybakovas A, Kairevičiūtė D, Civilka R. Familial adenomatous polyposis in Lithuania : an update. 2-nd Baltic congress of oncology and radiology . 10-12 September, 1998, Riga, Latvia. Abstract book, p. 93.

2. Andrius Rybakovas, Augustinas Bausys, Andrius Matulevicius, Gytis Zaldokas, Mindaugas Kvietkauskas, Gintautas Tamulevicius, Virgilijus Beisa, Kestutis Strupas. Recurrent laryngeal nerve injury assessment by intraoperative laryngeal ultrasonography: a prospective diagnostic test accuracy study. Videosurgery and Other Miniinvasive Techniques, 2018/12/10. DOI: https://doi.org/10.5114/wiitm.2018.80066.

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4. Andrius Rybakovas, Andrius Matulevičius, Viktorija Belogorceva, Virgilijus Beiša, Kęstutis Strupas. Laringinė palpacija skydliaukės chirurgijoje: praktinės rekomendacijos, remiantis išsamia literatūros apžvalga ir perspektyviojo kohortinio tyrimo rezultatais (Laryngeal palpation in thyroid surgery: practical recommendations for method integration into daily clinical practice according to a comprehensive literature review and prospective cohort study results). Lietuvos chirurgija, Vol. 17 No. 3–4 (2018).

Presentations in International Conferences:

1. G. Tamulevičius, J. Kaukėnas, A. Rybakovas, "The Evaluation of a Speaker's Individual Qualities Using a High Order Autoregressive Model," Data Analysis Methods for Software Systems, Druskininkai, Lithuania, December 1–3, 2016.

2. A. Rybakovas, V. Beiša, G. Žaldokas, K. Strupas, „Could the Safety of Thyroid Operations be Improved? Assessing the Feasibility of Intraoperative Vocal Cord Ultrasonography in Identifying Vocal Cords Movement and Reducing the Risk of Bilateral Palsy. Data from a Prospective Trial," The 9th Congress of Baltic Association of Surgeons, Klaipėda, Lithuania, May 10–12, 2018.

3. G. Žaldokas, A. Rybakovas, E. Lesinskas, K. Strupas, "The Effectivness of Intraoperative Recurrent Laryngeal Nerve Neurostimulation with Laryngeal Palpation and Vocal Cord Ultrasonography for Predicting Vocal Cord Palsy after Thyroid Surgery," The 7th Baltic Otorhinolaryngology Congress, Riga, Latvia, June 7–9, 2018.

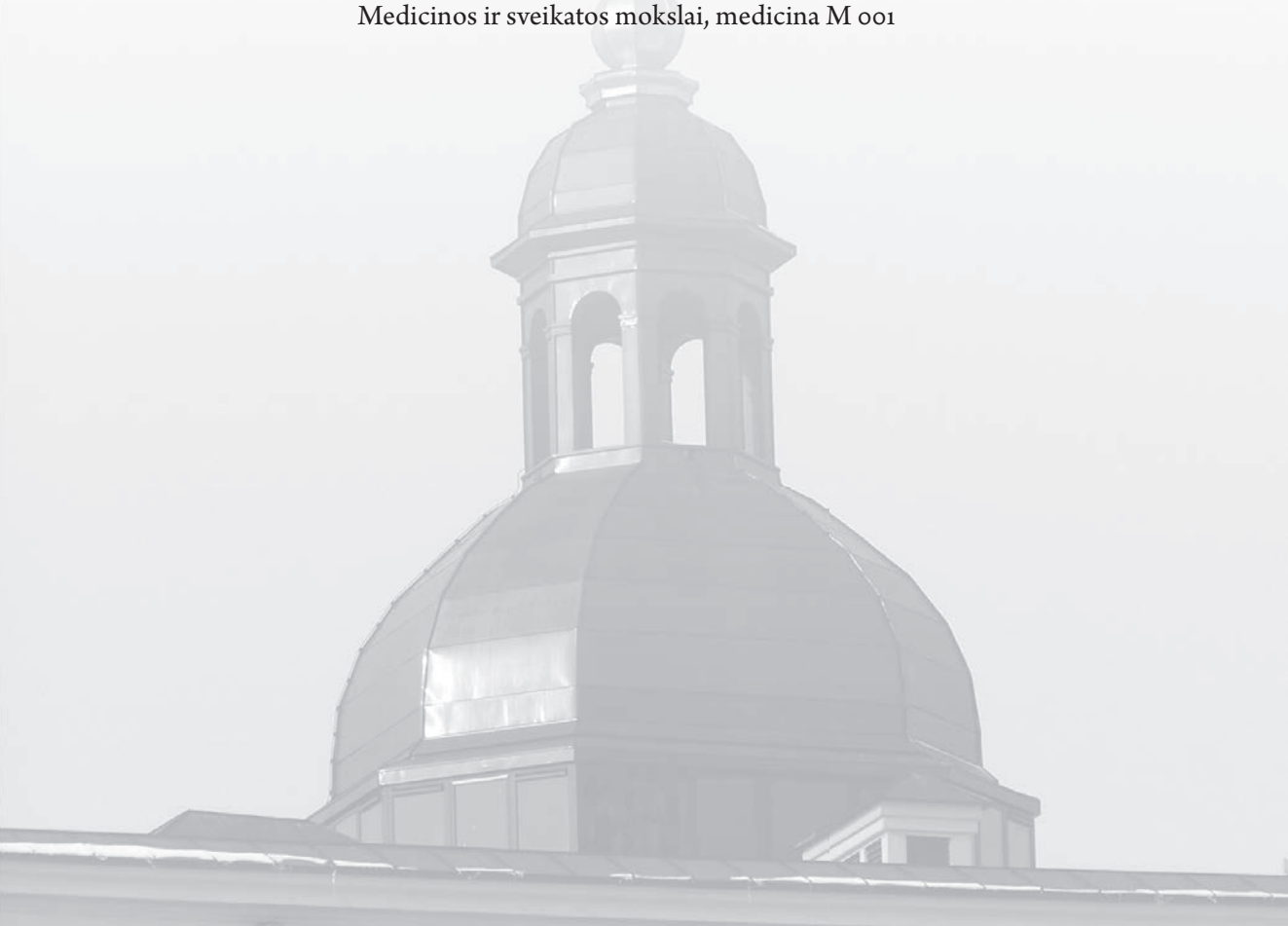
4. A. Rybakovas, V. Beiša, G. Tamulevičius K. Strupas, "Could We Use Intraoperative Vocal Cord Ultrasonography in Identifying Vocal Cord Movements and Reducing the Risk of Bilateral Palsy? Prospective Trial Data," Evoliucinė medicina: sveikata ir ligos besikeičiančioje aplinkoje, Vilnius, Lithuania, June 5–10, 2018.

Jonas Jarašūnas

ANALYSIS OF THE LINK BETWEEN VARIOUS CARDIOVASCULAR FACTORS AND ATRIAL FIBRILLATION

KARDIOVASKULINĖS SISTEMOS RODIKLIŲ SĄSAJOS SU PRIEŠIRDŽIŲ VIRPĖJIMU TYRIMAS

Medicine and Health Sciences, Medicine M 001
Medicinos ir sveikatos mokslai, medicina M 001



Abbreviations

ABP – arterial blood pressure
AF – atrial fibrillation
BMI – body mass index
BPM – beats per minute
BSA – body surface area
CRP – C reactive protein
DD – diastolic dysfunction
ECG – electrocardiogram
EDd – end diastolic diameter
EF – ejection fraction
HR – heart rate
IVS – interventricular septum
LA – left atrial
LV – left ventricular
MI – mass index
NT pro BNP – N-terminal prohormone of brain natriuretic peptide
PAH – primary arterial hypertension
PTD – percent time dropped
PTE – percent time elevated
PWd – posterior wall diameter
RAAS – renin-angiotensin-aldosterone system
TSH – Thyroid-Stimulating Hormone

1. Introduction

1.1 The Problem and Relevance of the Study

Atrial fibrillation (AF) is the most common long-standing form of atrial tachyarrhythmia, the prevalence of which is ever-increasing [1–4]. In a meta-analysis published in 2014, it was estimated that there are 33.5 million patients in the world suffering from this form of arrhythmia [2]. Having in mind the growth of the global population and the increasing prevalence of AF, it is likely that this number is even larger by now and will double in the coming 50 years [5]. AF increases the risk of heart failure, embolic complications, and cardiovascular mortality [6]. Patients with this arrhythmia have not only an increased risk of a stroke but also worse results of treatment and a larger residual neurologic deficiency [7]. The most effective way to prevent embolic complications is the use of anticoagulants. Anticoagulants are proven to reduce the risk of stroke by approximately two thirds regardless of the baseline risk [8, 9]. In order to prevent AF complications, it is vital to diagnose the arrhythmia in time, which is sometimes difficult with paroxysmal AF. Approximately a third of patients with paroxysmal AF are either asymptomatic or have nonspecific complaints [10]. When AF episodes are short-lasting, the ECG, and even a 24-hour ambulatory ECG monitoring, are sometimes not able to detect the arrhythmia and lead to the correct diagnosis [11]. Devices that can monitor a patient's ECG for at least 7 days in an ambulatory setting can increase the probability of detecting AF [11], but their availability is limited. In order to use these devices with maximum efficiency, it is essential to identify patients with the largest risk of AF and its com-

plications. Though primary arterial hypertension (PAH) increases the risk of AF by a mere 1.42–1.9 fold [12, 13], it is the high prevalence of the disease that makes PAH the leading cause of AF in the general population [6, 12]. A study is needed to determine the cardiovascular factors linked to the increased risk of paroxysmal AF in a group of patients with PAH. By selecting patients with an increased risk of arrhythmia, it would be possible to employ diagnostic methods that last longer but are more effective in establishing the correct diagnosis, such as 7-day ambulatory ECG monitoring. By treating these patients with anticoagulants, we could prevent embolic complications. There is data showing that renin-angiotensin-aldosterone system (RAAS) blockers are effective not only in secondary but also in the primary prevention of AF. The positive effect is more pronounced in the group of patients with PAH [14]. By identifying patients with an increased risk of AF, we could prevent it with RAAS blockers.

There are many articles published that prove both AF and PAH to worsen quality of life [15–18], but in the majority of the studies patients with these pathologies were compared to a healthy population. The absolute majority of data on life quality in patients with AF are derived from randomized interventional trials, and that is why the data might be biased toward picking out patients that are the most symptomatic and have the worse quality of life [19]. A study is needed to define what influence on quality of life, anxiety, and depression does paroxysmal AF caused by PAH have by comparing these patients to the general PAH population in the absence of other comorbidities.

1.2 Research Hypothesis

Patients with paroxysmal AF can be identified by a specific blood biomarker, a heart ultrasound, a 24-hour ambulatory ECG and arterial blood pressure (ABP) monitoring changes within the population of patients with PAH.

Paroxysmal AF is related to a worse quality of life as well as increased levels of anxiety and depression in patients with PAH.

1.3 Research Objectives

1. To define the cardiovascular factors and their changes that would allow to identify patients with paroxysmal atrial fibrillation or an increased risk of the arrhythmia in the population of patients with primary arterial hypertension.
2. To define the influence of paroxysmal atrial fibrillation on quality of life, anxiety, and depression in patients with primary arterial hypertension.

1.4 Research Tasks

1. To define the blood biomarkers and their changes that are linked to paroxysmal atrial fibrillation in patients with primary arterial hypertension.
2. To define the heart ultrasound parameters and their changes that are linked to paroxysmal atrial fibrillation in patients with primary arterial hypertension.
3. To define the 24-hour ambulatory ECG and ABP monitoring parameters and their changes that are linked to paroxysmal atrial fibrillation in patients with primary arterial hypertension.
4. To define the cardiovascular parameters with the best performance in identifying patients with paroxysmal atrial fibrillation and ability to predict the risk of the arrhythmia in patients with primary arterial hypertension.
5. To examine the differences in the parameters of the Short Form 36 quality of life questionnaire (SF-36) and Hospital Anxiety and Depression Scale (HADS) linked to paroxysmal atrial fibrillation in the population of patients with primary arterial hypertension.

1.5 Statements to be Defended

1. Paroxysmal atrial fibrillation is linked to increased concentrations of NT pro BNP and troponin I in patients with primary arterial hypertension.
2. Paroxysmal atrial fibrillation is linked to a decreased left atrial reservoir strain, reservoir strain rate, and ejection fraction values in patients with primary arterial hypertension.
3. Paroxysmal atrial fibrillation is linked to an increased number of short-run (<30 s) atrial tachyarrhythmia episodes during 24-hour ambulatory ECG monitoring in patients with primary arterial hypertension.
4. In the population of patients with primary arterial hypertension, heart ultrasound parameters of the left atrium (reservoir strain, reservoir strain rate, and ejection fraction) and the number of short-run atrial tachyarrhythmia episodes during 24-hour ambulatory ECG monitoring are able to predict paroxysmal atrial fibrillation.
5. Paroxysmal atrial fibrillation leads to a worse quality of life in patients with primary arterial hypertension.

1.6 Novelty of the Study

Even though it is agreed upon that patients with AF have a lower quality of life, the majority of data we have is derived from interventional randomized trials; that is why there might be a bias toward more symptomatic and therefore lower quality

of life patients [19]. Quality of life research regarding AF in general and especially AF caused by primary arterial hypertension is lacking. It is difficult to clarify paroxysmal AF's contribution to the reduction of life quality, as the arrhythmia is often associated with other cardiovascular disease also leading to a worse quality of life. By excluding patients with other cardiovascular pathologies except for PAH and comparing AF patients not with healthy ones but with those that have PAH, we could clearly evaluate the influence of paroxysmal AF on quality of life, anxiety, and depression.

In the literature regarding AF, the greatest attention is paid to predicting the risk of complications and not the arrhythmia itself. Nevertheless, there are some schemes to evaluate the risk of atrial fibrillation [20], the most important being FHS [21], ARIC [22], WHS [23], and CHARGE-AF [24]. These models are able to predict the risk of AF in the general population, but they include mostly clinical factors without paying enough attention to novel ultrasound methods used to assess LA function, such as 2D speckle tracking. There is data showing that LA strain and the strain rate are linked to the risk of AF recurrence after pulmonary vein isolation [25, 26], cardioversion success or failure [27], embolic complications [27–30], and LA appendage function in patients after stroke [31]. To our knowledge, there are no studies including the LA strain and strain rate parameters with their cut-off values in the models defining the risk of paroxysmal AF among patients with primary arterial hypertension. As the LA strain and strain rate parameters can be easily determined during a routine ultrasound of the heart, they can be used in predicting the risk of AF. A study is needed to define their performance in predicting the risk of arrhythmia. Another important aspect of novelty is the population of the study. A lone AF is a rare pathology that has a prevalence of 1.6%–30%, depending on the definition used [32–34]. The most common cardiovascular pathology causing AF in the general population is PAH [35]. Though the association of AF and PAH is the most common, there is a lack of studies in this numerous population. At the start of our study there were no published studies comparing AF caused by PAH patients with the ones with PAH. In August 2019, a study by I. Petre et al. was published that compared these two populations. Though very similar in design, the study had a lower number of included patients, and the group with AF was significantly older, which made the results questionable [36]. Matching subjects by age and gender allowed us to determine the influence of solely paroxysmal AF on the studied cardiovascular factors in this abundant though little-studied population of patients with PAH.

2. Methodology

2.1 Overview

The study was conducted in Klaipėda Seamen's Hospital. An approval by the Lithuanian Bioethics Committee was obtained before starting the study (No. L-16-05/1). All the patients signed an informed consent form. A prospective case-control study was conducted over the period July 20, 2016 to December 31, 2018, which enrolled patients with paroxysmal atrial fibrillation and primary arterial hypertension. The patients filled out a dedicated study questionnaire, and instrumental tests were performed. Data without any possibility of identifying the patients were saved in a dedicated study database.

The protocol of the study was created by Prof. Audrius Aidietis and the author of this work, Jonas Jarašūnas. Patient screening, enrollment, questioning, and all the instrumental tests were performed by Jonas Jarašūnas. The overview of the study design is given in Figure 1.

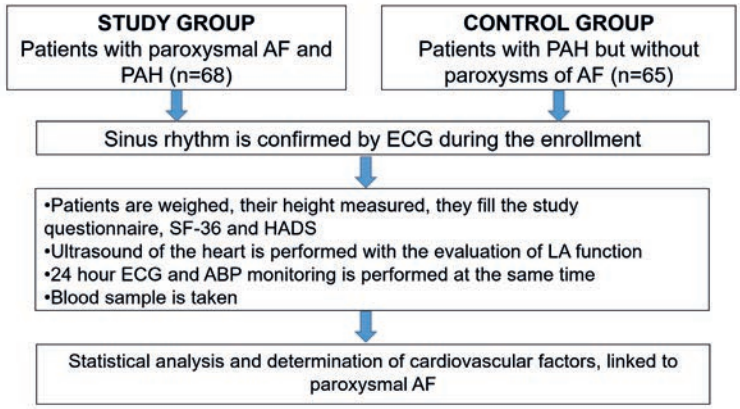


FIGURE 1. Overview of the study design.

The patients were divided into two groups:

- Study group – patients with PAH and at least one ECG documented AF episode within one year prior to inclusion in the study.
- Control group – patients with PAH but without any AF symptoms or ECG with the arrhythmia.

To enroll only the patients with PAH as the most likely reason for AF, we excluded patients with any other pathology known to cause AF.

2.2 Inclusion and Exclusion Criteria

Inclusion criteria to the study group:

1. Established diagnosis of PAH for more than a year prior to enrollment;
2. Paroxysmal AF in the anamnesis according to the definition given in the 2016 ESC guidelines (AF is considered paroxysmal if the duration of the episode is less than 7 days) [6];
3. At least one ECG-documented episode of AF during one year prior to enrollment;
4. ECG-confirmed sinus rhythm at the moment of enrollment;
5. 18–80 years of age;
6. Agreement to participate in the study;
7. Ability to read, understand, and fill out the questionnaires.

Inclusion criteria for the control group had only one difference – only the patients without any symptoms of arrhythmia were included in this group. Inclusion criteria to the control group:

1. Established diagnosis of PAH for more than a year prior to enrollment;
2. No symptoms and documented ECG of AF;
3. ECG-confirmed sinus rhythm at the moment of enrollment;
4. 18–80 years of age;
5. Agreement to participate in the study;
6. Ability to read, understand, and fill out the questionnaires.

Exclusion criteria to both the study and control groups:

1. Verified coronary heart disease or its symptoms;
2. Heart surgery;
3. Heart failure signs or symptoms;
4. Left ventricular (LV) ejection fraction (EF) <55 %;
5. Glomerular filtration rate calculated using MDRD formula <60 ml/min/1,73 m^2;
6. Clinically relevant valvular heart disease or congenital heart disease;
7. TSH concentration outside normal range;
8. Chronic obstructive pulmonary disease;

Resistant hypertension according to the definition given in the 2013 ESC hypertension guidelines (when the combination of a diuretic and two other antihypertensive class agents fail to reduce blood pressure to <140/90 mm Hg) [37].

2.3 Study Questionnaire, HADS, and SF-36

All the patients completed a questionnaire that was specifically designed for the study and included the following information:

- Age;
- Gender;
- Education;
- Level of physical activity during the last month in minutes per week;
- Alcohol and tobacco consumption, other addictions;
- Medications taken regularly during the last month;
- Factors provoking AF;
- Number of AF episodes during the last year;
- Known duration of PAH in years;
- CHA_2DS_2-VASc score.

All the patients filled out the Hospital Anxiety and Depression Scale (HADS), which had been translated and adapted to the Lithuanian language [38]. The HADS consists of 14 questions. Seven of those are dedicated to the evaluation of depression, and the other 7 – to the evaluation of anxiety. The patients were instructed to choose the answer that is the closest to how they were feeling during the last week and to provide the answers that are the first to come to their mind and are the most intuitive ones rather than ones appearing after careful consideration. The levels of depression and anxiety are calculated in scores from 0 to 21. Scores from 0 to 7 represent normal levels of anxiety and depression, from 8 to 10 – light depression or anxiety, 11 to 14 – moderate, and 15 to 21 – severe levels of depression or anxiety.

All the patients completed the Short Form 36 quality of life questionnaire (SF-36), which was translated and adapted to the Lithuanian language [39]. The questions in the SF-36 represent eight sections of health: energy/fatigue, physical functioning, pain, general health perceptions, physical role functioning, emotional role functioning, social role functioning, and emotional well-being. These eight sections are combined into two categories of health: mental and physical. Mental health is represented by energy/fatigue, social role functioning, emotional role functioning, and emotional well-being. Physical health is represented by physical functioning, bodily pain, general health perceptions, and physical role functioning. There is also one separate section dedicated to the perception of health change. Using a special algorithm, each of the section was estimated in scores from 0 to 100. Higher scores indicate a better quality of life.

Twelve derivation ECGs were recorded in the supine position. The weight and height of the patients were recorded using dedicated calibrated devices.

2.4 Laboratory Tests

Venous blood samples were taken from all the patients, and concentrations of the following substances and cells were measured in a certified laboratory of the Klaipėda Seamen's Hospital: hemoglobin (g/l), leukocyte count (10^9/l), K^+ (mmol/l), Na^+ (mmol/l), Cl^-(mmol/l), Mg^{2+} (mmol/l), Ca^{2+} (mmol/l), creatinine (μmol/l), C reactive protein (CRP) (mg/l), troponin I (ng/l), N-terminal prohormone of brain natriuretic peptide (NT pro BNP) (pg/ml), and Thyroid-Stimulating Hormone (TSH) (mlU/l).

2.5 Ultrasound of the Heart

All the patients underwent ultrasound testing of the heart, which was carried out by Jonas Jarašūnas using the GE Vivid E9 (*General Electrical-Vingmed Ultrasound AS*, Horten, Norway) ultrasound system with a M5S-D (*General Electrical-Vingmed Ultrasound AS*, Horten, Norway) probe. Only patients with views of acceptable quality were included in the final analysis. During the ultrasound of the heart, patients were in the supine position on the left side, with the left hand placed under the head. All the limb leads of the ECG were recorded during the test, but only the one with the best distinguishable P waves and QRS complexes were used for analysis. The views were saved for further analysis. Ultrasounds of the heart and standard measurements were carried out according to the recommendations of the 2005 American Society of Echocardiography and the European Association of Cardiovascular Imaging and their 2015 update [40–42].

The diameter of the ascending aorta and aortic sinuses, LA diameter, interventricular septum diameter (IVSd), LV posterior wall diameter (PWd), end diastolic (ED), and end systolic (ES) LV diameters were measured from the parasternal long LV axis ultrasound views.

LV myocardial mass (MM) was calculated using Cube's formula, which is recommended by the American Society of Echocardiography and the European Association of Cardiovascular Imaging [41]:

$$\text{LV MM} = 1{,}04\ [(\text{IVSd}+\text{PWd}+\text{LV EDd})^3 - \text{LV EDd}^3] \times 0{,}8 + 0{,}6$$

LV myocardial mass index (LVMMI) (g/m^2) was calculated using the following formula:

$$\text{LVMMI} = \text{MM/body surface area (BSA)}$$

BSA (m^2) was calculated using the *Du Bois* formula:

$$\text{BSA} = 0{,}007184 \times \text{weight}^{0{,}425} \times \text{height}^{0{,}725}$$

LV volume at the end of systole before the opening of the mitral valve (MV) and at the end of diastole before the closure of the mitral valve (minimal and maximal LV volume (ml), accordingly) was calculated using Simpson's method of discs from two and four chamber apical views. The LV ejection fraction (EF) (%) was calculated using the following formula:

LV EF = (LV max. vol. – LV min. vol.)/ LV max. vol. × 100

LA volume (ml) was also calculated using Simpson's method of discs from two and four chamber apical views. The following volumes of LA were measured: minimal, taken at the end of LV diastole before the closure of MV, maximal, at the end of LV systole before the opening of MV, and LA volume before the P wave on the ECG. LA EF (%) was calculated using the following formula:

LA EF = (LA max. vol. – LA min. vol.)/ LA max. vol. × 100

LA volume index (VI) was calculated using the following formula:

LA VI = LA max. vol. / BSA

MV inflow and MV annular velocity were analyzed using a pulse wave Doppler and tissue Doppler from four chamber apical views. A pulse wave Doppler was used to measure the MV inflow velocities: E – maximal early LV filling velocity through MV (m/s); A – maximal LV filling velocity through MV during the contraction of the LA (m/s); EdecT – the duration of the deceleration of the E wave (ms). A tissue Doppler was used to measure the medial and lateral MV annular velocities: e' – velocity during early LV diastolic filling (cm/s), and a' – velocity during late LV diastolic filling. Mean medial and lateral e' and a' velocities were calculated.

In the presence of a tricuspid regurgitation, a continuous wave Doppler was used to measure the peak velocity of the regurgitant jet (m/s).

The LV diastolic function was evaluated using the 2016 recommendations of the American Society of Echocardiography and the European Association of Cardiovascular Imaging [43]. The following factors of LV diastolic dysfunction (DD) were evaluated:

- MV annular velocity – e' medial <7 cm/s or e' lateral <10 cm/s);
- E/e' ratio >14. Parameter was calculated using the average of e' medial and e' lateral;
- LA VI >34 ml/m^2;
- Peak regurgitant jet velocity through tricuspid valve >2.8 m/s.

In order to evaluate the link between the LV DD factors and the LA strain and strain rate parameters the patients were grouped by the number of LV DD factors present. LA strain and strain rate parameters were compared between the groups of patients with a different number of factors showing LV DD.

2.5.1 Left Atrial Strain and Strain Rate

The LA strain and LA strain rate were evaluated using a 2D speckle tracking technology and the GE EchoPAC (version 112) software. Views used for the analysis were saved according to the expert recommendations published in the *European Journal of Echocardiography* [44]. The LA strain and strain rate were calculated from two and four chamber apical views, carefully avoiding foreshortening. In order to achieve the best quality, the focus was set to the middle of LA, while the width and depth were adjusted to achieve > 80 frames per second. The patients were asked to hold their breath, and three consecutive heart cycles were recorded. The walls of the LA were marked manually. The software then automatically picked speckles and tracked them throughout the heart cycle. The walls of the LA were automatically divided into 6 segments, and the tracking quality of each segment was shown. If the tracking quality was not satisfying, the walls were adjusted to achieve better quality. The starting point of the analysis in the ECG was then changed manually from preset, which was the peak of the QRS complex to the start of the P wave. The software then generated separate curves for all the 6 segments, and one averaged, representing the global longitudinal strain of the LA that was used for further analysis. Three deformation points were identified in each curve, which represented different functions of the LA (%):

- First negative deflection peak at maximal contraction of the LA corresponding to the contractile function of the LA – the contractile strain.
- First positive deflection peak at MV opening corresponding to conduit function of the LA – the conduit strain.
- The difference between the first two peaks corresponding to the reservoir function of the LA – the reservoir strain.

LA 2D global longitudinal strain curve with certain points corresponding to the contractile, conduit, and reservoir strains is given in Figure 2. Identical calculations were performed for two and four chamber apical views, and the mean was used for final analysis.

After the strain values were calculated using the same settings and views, the strain rate measurement setting was selected. The software automatically generated 6 strain rate curves for each segment of the LA wall and one averaged global longitudinal strain rate curve, which was used for further analysis. Three deformation points were identified in each curve, which represented the different functions of the LA (1/s):

- First negative peak in the curve representing the rate of the LA contractile strain – the contractile strain rate.
- Second negative peak in the curve representing the rate of the LA conduit strain – the conduit strain rate.

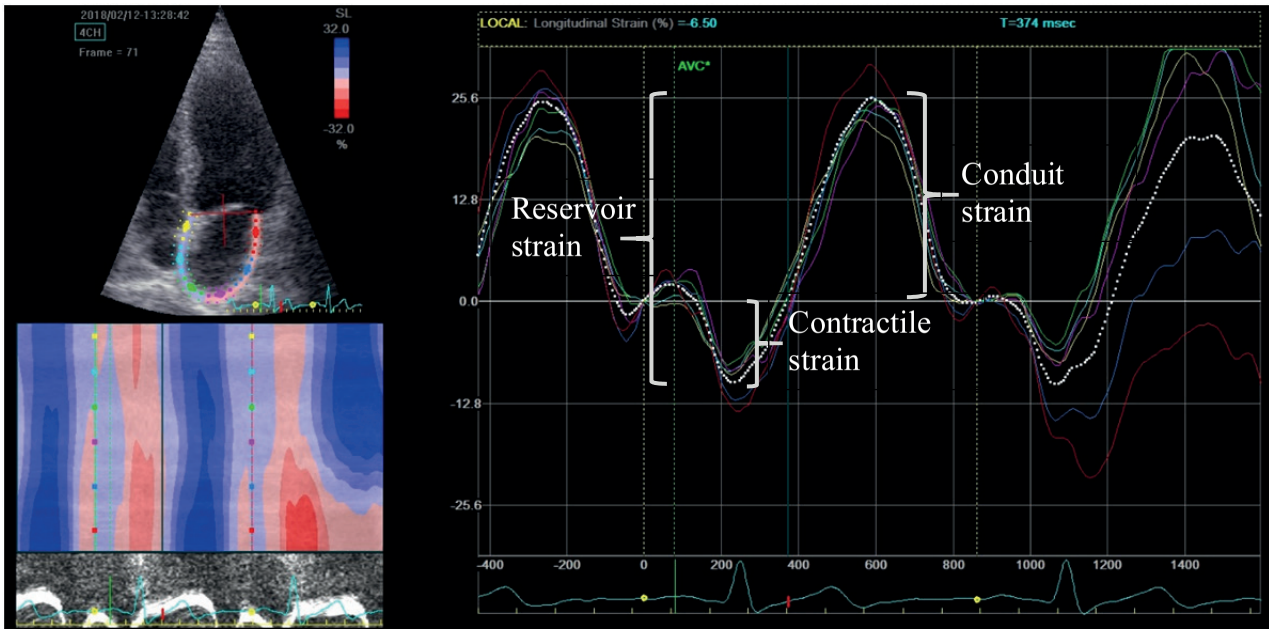


FIGURE 2. Contractile, conduit, and reservoir strain determination using a four chamber apical view and using the P wave as a starting point in the ECG.

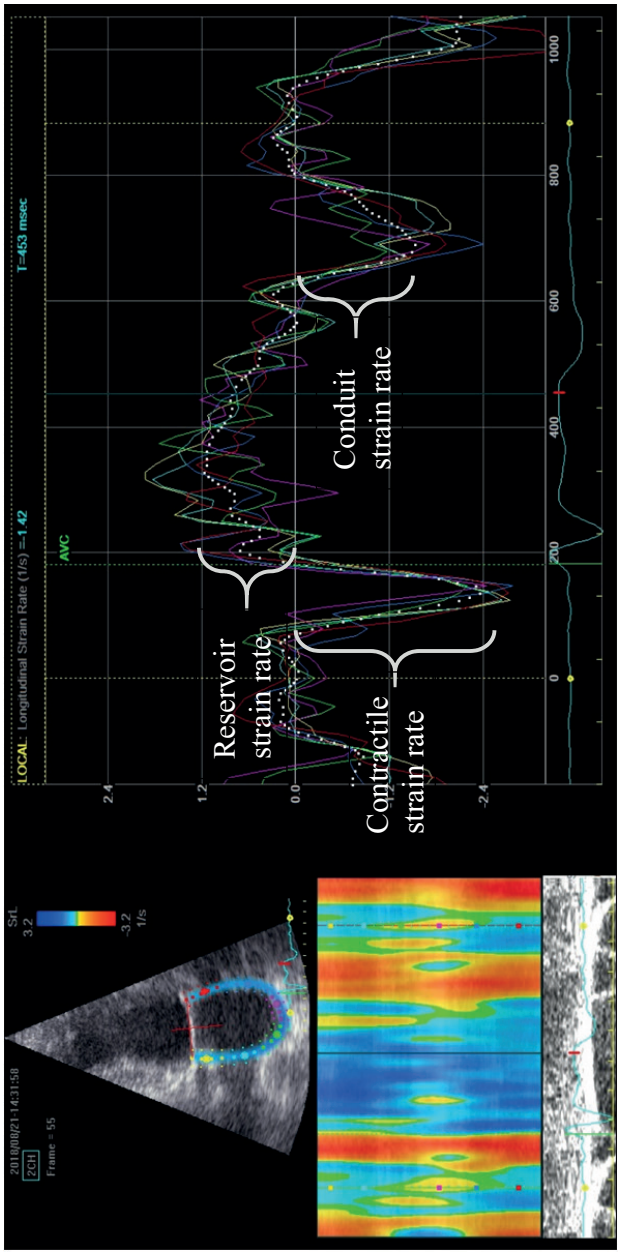


FIGURE 3. Contractile, conduit, and reservoir strain rate determination using a two chamber apical view using the P wave as a starting point in the ECG.

- First positive peak in the curve representing the rate of the LA reservoir strain – the reservoir strain rate.

The 2D global longitudinal strain rate curve, together with certain points corresponding to the contractile, conduit, and reservoir strain rates, is given in Figure 3. Identical calculations were performed using two and four chamber apical views, and the mean was used for the final analysis.

2.5.2 Reproducibility of Left Atrial Strain Parameters

The intra- and interobserver variability of contractile, conduit, and reservoir LA strains was assessed in 20 randomly selected patients. Intraobserver variability was performed by the same echocardiographer, blind to the previous measurements, and interobserver variability was performed by a second experienced echocardiographer also blind to the previous measurements.

2.6 24-Hour Ambulatory ECG and Arterial Blood Pressure Monitoring

The 24-hour ambulatory ECG and ABP monitoring was done at the same time for all the patients using the *Meditech card(X)plore* device (Meditech Ltd., Budapest, Hungary). The device recorded 3 independent bipolar channels of electrograms and measured ABP at a predefined intervals using the upper arm cuff (oscillometric tonometry method, stepwise deflation). The analysis of the data was carried out using the CardioVisions version 1.23.0 software.

A 24-hour ambulatory ABP monitoring was performed according to the recommendations of the European Society of Hypertension [45]. Target time for ABP monitoring was 24 hours. The patients with a monitoring time of less than 22 hours had to repeat the test until more than 22 hours of consecutive monitoring was achieved. When programming the device, the patients were asked to specify the approximate time of when they go to sleep and wake up – this information was used for setting daytime and nighttime modes. During the daytime, ABP was measured at 20-minute intervals, while during the nighttime – 40-minute intervals. Measurements were repeated in case a failed attempt was activated. The cuff was put on the right upper arm, aiming for the level of the right atrium, and the device itself was attached to the waist with the help of a special belt. The patients were instructed to live their normal daily lives but to temporarily stop physical activity during the measurements. The monitoring was stopped the next day at the same time. The data were transferred to the computer for further analysis.

The minimal requirements for 24-hour ABP monitoring were:

- ≥70 % successful measurements;
- ≥ 20 successful measurements during daytime;
- ≥ 7 successful measurements during nighttime.

The monitoring was repeated if these requirements were not met.

For the calculation of the mean ABP parameters, a weighted average was used, which was done automatically using the software.

ABP parameters assessed:

- 24-hour systolic ABP mean (mm Hg);
- 24-hour systolic ABP mean standard deviation (SD);
- 24-hour diastolic ABP mean (mm Hg);
- 24-hour diastolic ABP mean SD;
- Daytime systolic ABP mean (mm Hg);
- Daytime systolic ABP mean SD;
- Daytime diastolic ABP mean (mm Hg);
- Daytime diastolic ABP mean SD;
- Nighttime systolic ABP mean (mm Hg);
- Nighttime systolic ABP mean SD;
- Nighttime diastolic ABP mean (mm Hg);
- Nighttime diastolic ABP mean SD;
- Systolic ABP drop d uring the night (%);
- Diastolic ABP drop during the night (%).
- Morning surge – the difference between the mean systolic ABP during the 2 hours after waking and rising minus the mean systolic ABP of the lowest blood pressure during sleep and its nearby neighbors (3 values). The software interpreted the last night measurement as the time of waking.
- Systolic ABP Percent Time Elevation (PTE) – percent of the total analyzed time the sABP was above cut-off lines (%).
- Diastolic ABP PTE – percent of the total analyzed time the sABP was above cut-off lines (%).
- Systolic ABP Percent Time Drop (PTD) – percent of the total analyzed time the sABP was below cut-off lines (%). Hypotensive PTE.
- Diastolic ABP PTD – percent of the total analyzed time the sABP was below cut-off lines (%). Hypotensive PTE.

A 24-hour ECG monitoring was performed at the same time as the ABP monitoring. At the sites recommended by the manufacturer, 7 electrode stickers were applied and connected to the device itself via provided cables. After the end of monitoring, the data were transferred to the computer, analyzed by the software, and

verified by the author of this dissertation. In case of inaccuracies, the data were adjusted manually. The following parameters were assessed:

- 24-hour mean heart rate (HR) in beats per minute (bpm);
- Minimal HR (lowest mean HR for a 30 s time interval) (bmp);
- Maximal HR (highest mean HR for a 30 s time interval) (bmp);
- Ventricular premature beats (calculated as percentage in all QRS complexes) (%);
- Atrial premature beats (calculated as percentage in all QRS complexes) (%);
- Short-run atrial tachyarrhythmia (AT) episodes (calculated during the monitoring period of 24 hours). The episode was diagnosed if all the following criteria were present: more than 3 consecutive premature beats with a narrow QRS, RR intervals of > 600 ms, and a duration of less than 30 s. An example of the episode is given in the Figure 3. All the episodes were inspected and verified manually by the author of this dissertation.

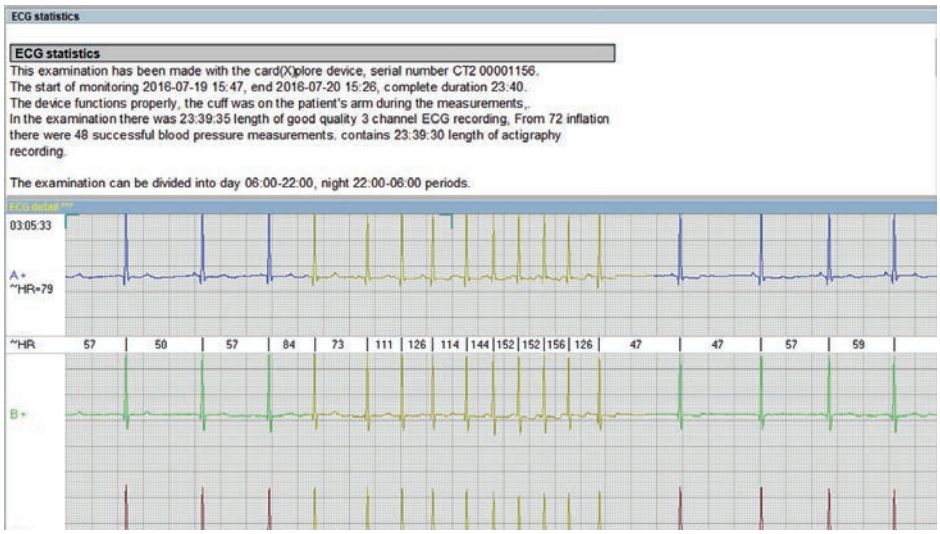


FIGURE 3. An example of a short-run atrial tachyarrhythmia of a duration less than 30 s.

2.7 Statistical Analysis

Statistical analysis of the data was performed using the IBM SPSS Statistics 23 (IBM, Armonk, New York, United States of America) software. First of all, mean values of the analyzed parameters were calculated, and the numbers were compared between the groups. Mean values are given together with a standard deviation. The normality of the data distribution was checked using the Shapiro-Wilk test. If the data were distributed normally, the means were compared using Student's t-test;

the Mann Whitney U test was used otherwise. Nonparametric data were compared using the Chi square. The equality of variances between the groups was compared using Levene's test. The difference was considered statistically significant when the p value was <0.05. When the parameters with a statistically significant difference between the groups were identified, a binary logistic regression analysis method was applied to determine the factors that are independently linked to paroxysmal AF. Age, gender, and body mass index were also included in the regression analysis. The results are given as an odds ratio with 95% confidence intervals and a p value. To characterize the regression model and its performance, R^2 was calculated using the Cox and Snell as well as Nagelkerke formulas. Sensitivity, specificity, and positive and negative prognostic values, together with an ROC curve and calculated area under the curve values, are given. A binary logistic regression analysis was repeated including just the factors that were statistically significant in the primary analysis. The same parameters characterizing the first model are given for the second one. In order to determine a decision-making algorithm, the most important parameters, and their cut-off values in predicting paroxysmal AF, a logistic regression tree analysis was performed. The parameters of the tree were: growing method CRT (Classification and Regression Tree), depth ≤ 5 levels, and a minimum number of cases for child node – 10. The importance of the parameters in the model is shown graphically. In order to characterize the tree and its performance, a classification table, specificity, sensitivity, and positive and negative prognostic values, together with an ROC curve and a calculated area under the curve's values were all given.

To compare the LA strain parameter values between the groups of patients with different number of LV DD factors, an ANOVA analysis with an LSD post hoc test were used. Box and whisker charts are used to show the differences between the groups graphically. The difference was considered significant when the p value was <0.05.

To evaluate the reproducibility of the LA strain, measurements of the intraclass correlation coefficient, together with the absolute difference (divided by the mean of two measurements and given as a percentage), were calculated for both intra- and interobserver variability.

3. Results

3.1 Overview of the Study and Control Group Patients

The final analysis included 133 patients – 68 patients in the study group (with PAH and paroxysmal AF) and 65 patients in the control group (with PAH but without paroxysmal AF). The general characteristics of the patients are given in Table 1.

TABLE 1. General characteristics of the study and control groups.

Parameter	Study group (n=68)	Control group (n=65)	P value
Age (±SD)	62.9 (11.61)	59.15 (11.77)	0.067
Male %	44.11	43.07	0.862
Higher education %	**35.29**	**61.54**	**0.012**
Body mass index (±SD)	29.58 (4.27)	29.03 (4.79)	0.484
Diabetes %	7.35	7.69	1
24-hour sABP mean (±SD)	129.4 (10.58)	133.57 (14.67)	0.053
24-hour dABP mean (±SD)	**74.4 (8.59)**	**77.89 (8.44)**	**0.015**
Physical activity level (min./week) (±SD)	**58.75 (105.21)**	**118.77 (199.17)**	**0.015**
Number of antihypertensives taken daily (±SD)	1.59 (1.14)	1.59 (1.24)	0.986
ACEI %	**55.88**	**32.81**	**0.009**
ARB %	16.2	26.56	0.201
CCB %	26.47	34.38	0.349
β-blockers %	29.41	32.81	0.71
Diuretics %	17.65	21.88	0.662
α-blockers %	1.47	0	1
Centally acting agents %	4.41	9.36	0.314
Spironolactone %	7.35	3.12	0.442

SD – standard deviation; sABP – systolic arterial blood pressure; dABP – diastolic arterial blood pressure; ACEI – angiotensin converting enzyme inhibitors; ARB – angiotensin II receptor blockers; CCB – calcium channel blockers.

3.2 Quality of Life, Anxiety, and Depression Comparison Between the Study Groups

A comparison between scores of anxiety and depression evaluated by the HADS, together with the SF-36 evaluated scores of life quality sections, are given in Table 2.

TABLE 2. HADS and SF-36 data comparison between the groups.

Parameter	Study group (±SD)	Control group (±SN)	Difference of means	P value
HADS				
Anxiety	7.57 (±4.43)	6.35 (±3.75)	1.22	0.126
Depression	5.06 (±3.59)	3.91 (±2.58)	1.15	0.104
SF-36				
Physical functioning	**68.31 (±22.92)**	**78.77 (±18.71)**	**-10.46**	**0.004**
Physical role functioning	**39.71 (±42.59)**	**60 (±41.41)**	**-20.29**	**0.007**
Emotional role functioning	60.29 (±42.85)	68.71 (±40.38)	-8.42	0.343
Energy/fatigue	56.47 (±18.73)	61.85 (±18.64)	-5.38	0.138
Emotional well-being	65.77 (±17.83)	67.02 (±14.44)	-1.25	0.805
Social role functioning	69.37 (±21.94)	73.86 (±23.27)	-4.49	0.237
Pain	65.91 (±24)	67.32 (±25.46)	-1.41	0.838
General health	**46.03 (±18.92)**	**53.92 (±17.33)**	**-7.89**	**0.016**
Health change	36.03 (±28.45)	39.23 (±23.37)	-3.2	0.466

SD – standard deviation; HADS – hospital anxiety and depression scale; SF-36 – short form 36.

Patients with paroxysmal AF had limited physical functioning, physical role functioning, and estimated their general health as worse compared to the control group.

3.3 Laboratory Tests

Mean values of laboratory tests are given in Table 3.

TABLE 3. Mean values of laboratory tests compared between the groups.

Parameter	Study group (±SD)	Control group (±SD)	Difference of means	P value
Hemoglobin(g/l)	141.34 (±13.59)	142.87 (±13.5)	-1.53	0.53
Leukocyte count (10^9/l)	6.2 (±1.28)	6.72 (±2.1)	-0.52	0.451
K^+(mmol/l)	4.52 (±0.39)	4.63 (±0.36)	-0.08	0.091
Na^+ (mmol/l)	141.45 (±1.84)	139.92 (±3.29)	1.53	0.06
Cl^- (mmol/l)	105.73 (±2.96)	104.63 (±3.57)	1.1	0.126
Mg^{2+} (mmol/l)	0.87 (±0.076)	0.89 (±0.08)	-0.02	0.248
Ca^{2+}(mmol/l)	2.38 (±0.1)	2.41 (±0.14)	-0.03	0.136
Creatinine (μmol/l)	77.87 (±17.18)	74.5 (±17.26)	3.37	0.22
CRP (mg/l)	1.87 (±2.26)	2.76 (±3.1)	-0.89	0.087
Troponin I (ng/l)	**4.16 (±4.8)**	**2.66 (±2.7)**	**1.5**	**0.045**
NT pro BNP (pg/ml)	**287.68 (±325.59)**	**124.54 (±105.11)**	163.14	<0.001
TSH (mlU/l)	1.68 (±1.05)	1.07 (±0.46)	0.61	<0.001

SD – standard deviation; CRP – C reactive protein; NT pro BNP – N-terminal prohormone of brain natriuretic; TSH – Thyroid-Stimulating Hormone.

Patients with paroxysmal AF had higher values of NT pro BNP, troponin I and TSH. Though the difference of TSH concentrations between the groups was statistically significant, according to the exclusion criteria, none of the test values were outside the normal range.

3.4 Ultrasound of the Heart

General ultrasound of the heart parameters are given in Table 4.

TABLE 4. Mean values of ultrasound parameters compared between the groups.

Parameter	Study group (±SD)	Control group (±SD)	Difference of means	P value
Ascending aorta diameter (cm)	3.44 (±0.48)	3.5 (±0.46)	-0.06	0.455
Aortic sinus diameter (cm)	3.46 (±0.4)	3.44 (±0.43)	0.22	0.764
IVSd (cm)	1.06 (±0.13)	1.09 (±0.12)	-0.03	0.186
LV EDd (cm)	5.07 (±0.5)	4.95 (±0.43)	0.12	0.171
LV PWd (cm)	0.91 (±0.1)	0.89 (±0.12)	0.02	0.347
LV MI (g/m²)	92.46 (±19.21)	90.25 (±16)	2.21	0.473
LV EF (%)	61.43 (±4.94)	61.25 (±5.05)	0.18	0.835
E (m/s)	0.74 (±0.16)	0.73 (±0.16)	0.01	0.723
A (m/s)	**0.72 (±0.19)**	**0.81 (±0.16)**	**-0.09**	**0.004**
LAd (cm)	4.09 (±0.62)	4.14 (±0.44)	-0.57	0.549
LA VI (ml/m²)	35.81 (±9.48)	33.55 (±6.98)	2.26	0.119
LA max. volume (ml)	71.09 (±19.52)	66.05 (±15.64)	5.04	0.104
LA pre P volume (ml)	53.68 (±14.77)	50.52 (±11.47)	3.16	0.178
LA min volume (ml)	**38.09 (±13.87)**	**31.35 (±9.11)**	**6.74**	**0.002**
LA EF (%)	**46.95 (±9.76)**	**53.72 (±8.53)**	**-6.77**	**<0.001**
e'lat. (m/s)	0.099 (±0.029)	0.101 (±0.029)	-0.002	0.698
e'med. (m/s)	0.079 (±0.022)	0.078 (±0.017)	0.001	0.745
e' mean (m/s)	0.089 (±0.023)	0.09 (±0.02)	-0.001	0.926
E/e' mean	8.72 (±2.83)	8.4 (±2.35)	0.32	0.476
E deceleration time (ms)	226.7 (±55.1)	237.5 (±61.7)	-10.8	0.3

SD – standard deviation; IVSd – interventricular septum diameter; LV – left ventricular; EDd – end diastolic diameter; PWd – posterior wall diameter; MI – mass index; EF – ejection fraction; LA – left atrium; VI – volume index

Patients with paroxysmal AF had a significantly lower A wave velocity, a higher minimal volume of LA, and a lower LA EF.

All the patients were grouped according to the number of LV DD factors present and compared between the study and control groups. The numbers are given in Table 5.

TABLE 5. Comparison of LV DD factors between the groups. P=0.362.

LV DD factors present	Study group	Control group
0 factors	17	24
1 factor	26	23
2 factors	23	15
3 factors	2	3
4 factors	0	0

LV DD – left ventricular diastolic dysfunction.

There was no statistically significant difference between the groups.

3.5 Left Atrial Strain and Strain Rate

The LA strain and strain rate parameters are compared between the groups in Table 6.

TABLE 6. Mean values of the LA strain and strain rate compared between the groups.

Parameter	Study group (±SD)	Control group (±SD)	Difference of means	P value
Contractile strain (%)	14.25 (±3.92)	15.51 (±3.05)	-1.26	0.041
Conduit strain (%)	15.48 (±4.22)	16.42 (±3.97)	-0.94	0.192
Reservoir strain (%)	**29.74 (±6.6)**	**31.93 (±5.41)**	**-2.19**	**0.038**
Contractile strain rate (1/s)	1.72 (±0.63)	1.87 (±0.49)	-0.15	0.121
Conduit strain rate (1/s)	1.17 (±0.42)	1.25 (±0.37)	-0.08	0.266
Reservoir strain rate (1/s)	1.04 (±0.29)	1.23 (±0.27)	-0.19	<0.001

SD – standard deviation.

Patients with paroxysmal AF had significantly lower values of contractile strain, reservoir strain, and reservoir strain rate.

3.6 Reproducibility of Left Atrial Strain Measurements

Intraclass correlation coefficients (95 % CI) for intraobserver variability of the LA contractile, conduit and reservoir strains were 0.91 (0.79—0.96), 0.92 (0.81—0.97) and 0.94 (0.86—0.98) respectively. The absolute difference divided by the mean of two measurements for intraobserver variability of the LA contractile, conduit, and reservoir strains was 5.7 %, 5.5 %, and 4.9 % respectively. Intraclass correlation coefficients (9 5% CI) for interobserver variability of the LA contractile, conduit, and reservoir strains were 0.89 (0.75—0.96), 0.91 (0.79—0.96), and 0.93 (0.82—0.97), respectively. The absolute difference divided by the mean of two measurements for interobserver variability of the LA contractile, conduit, and reservoir strains was 8.6%, 6.0%, and 5.5%, respectively.

3.7 The Link Between Left Atrial Strain/Strain Rate Parameters and the Left Ventricular Diastolic Dysfunction Factors

All patients of the study and control groups were regrouped according to the number of LV DD factors present, and the LA strain and strain rate mean values were compared between the groups. The data are shown in Table 7.

TABLE 7. LA strain and strain rate comparison between the groups of patients with different number of LV DD factors present.

Parameter		Mean (±SD)	P value for difference between groups (ANOVA)
Contractile strain (%)	0 LV DD factors	16.52 (3.37)	0.001
	1 LV DD factor	14.55 (2.77)	
	2 LV DD factors	13.63 (4.11)	
Conduit strain (%)	0 LV DD factors	17.9 (4.11)	<0.001
	1 LV DD factor	15.96 (3.84)	
	2 LV DD factors	13.94 (3.29)	
Reservoir strain (%)	0 LV DD factors	34.42 (5.63)	<0.001
	1 LV DD factor	30.51 (4.76)	
	2 LV DD factors	27.56 (6.02)	
Contractile strain rate (1/s)	0 LV DD factors	2.14 (0.52)	<0.001
	1 LV DD factor	1.71 (0.55)	
	2 LV DD factors	1.57 (0.5)	
Conduit strain rate (1/s)	0 LV DD factors	1.45 (0.36)	<0.001
	1 LV DD factor	1.23 (0.39)	
	2 LV DD factors	0.94 (0.27)	
Reservoir strain rate (1/s)	0 LV DD factors	1.34 (0.3)	<0.001
	1 LV DD factor	1.12 (0.25)	
	2 LV DD factors	0.95 (0.21)	

SD – standard deviation; LV DD – left ventricular diastolic dysfunction.

All the parameters of the LA strain and strain rate had statistically significant differences between the groups of patients with a different number of LV DD factors present. With the increasing number of LV DD factors, there was a statistically significant drop in all LA strain and strain rate values. LA reservoir strain and strain rate differences are shown in Figures 3 and 4, the LA conduit strain and strain rate – Figures 5 and 6, and the LA contractile strain and strain rate – Figures 7 and 8.

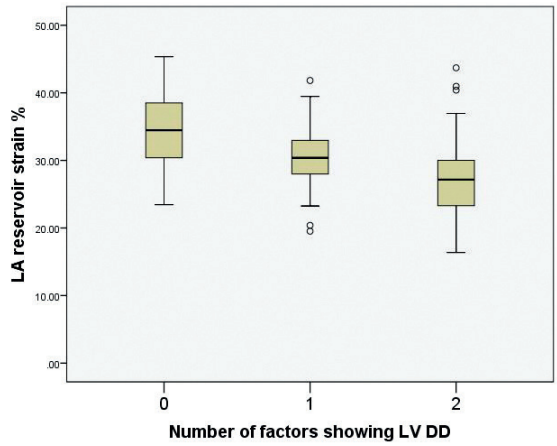


FIGURE 3. LA reservoir strain values between the groups of patients with a different number of factors showing LV DD. P<0.05 between all the groups.

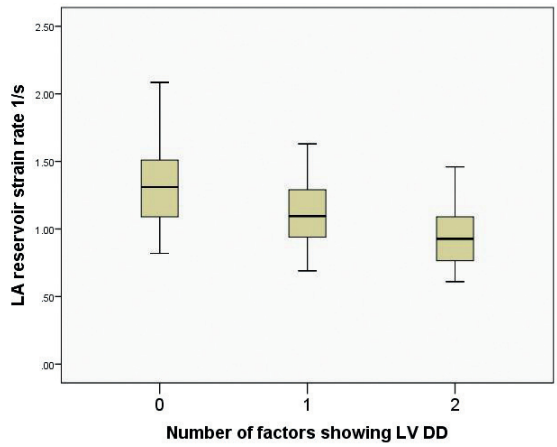


FIGURE 4. LA reservoir strain rate values between the groups of patients with a different number of factors showing LV DD. P<0.05 between all the groups.

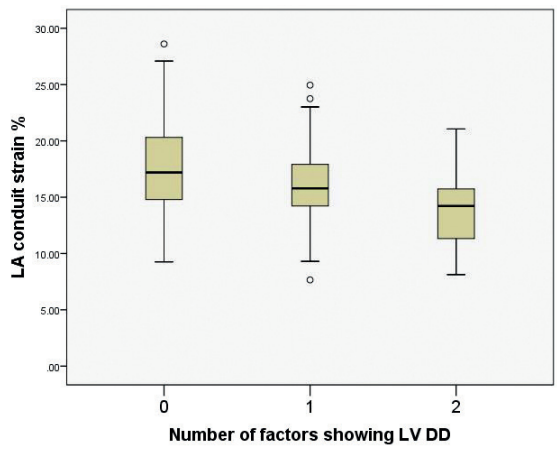


FIGURE 5. LA conduit strain values between the groups of patients with a different number of factors showing LV DD. P<0.05 between all the groups.

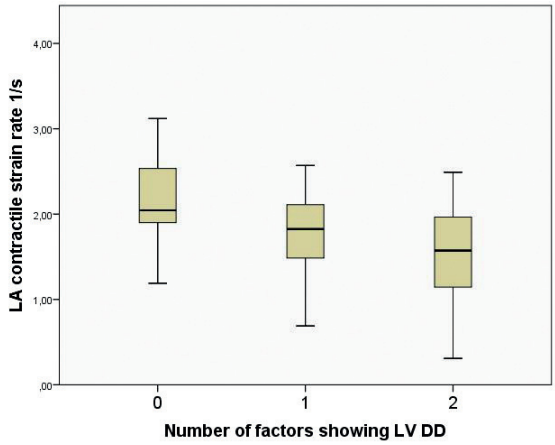


FIGURE 6. LA conduit strain rate values between the groups of patients with a different number of factors showing LV DD. P<0.05 between all the groups.

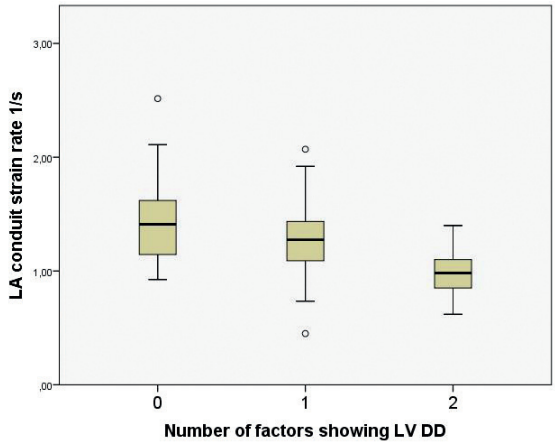


FIGURE 7. LA contractile strain values between the groups of patients with a different number of factors showing LV DD. The difference between the groups of 1 and 2 LV DD factors is not statistically significant (p=0.212); other differences are statistically significant (p<0.05).

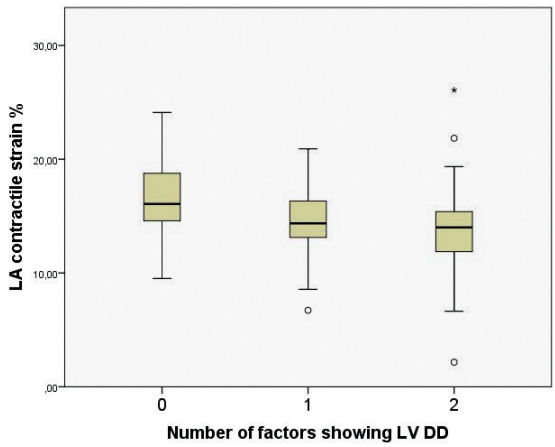


FIGURE 8. LA contractile strain rate values between the groups of patients with a different number of factors showing LV DD. The difference between the groups of 1 and 2 LV DD factors is not statistically significant (p=0.202); other differences are statistically significant (p<0.05).

LA reservoir strain and strain rate association with E/e' rate is graphically displayed in Figures 9 and 10.

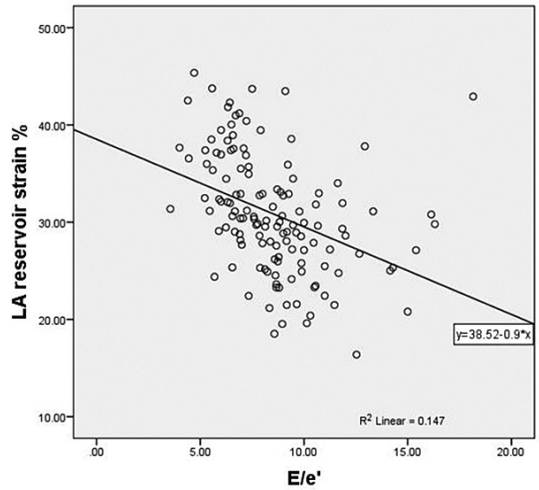


FIGURE 9. LA reservoir strain and E/e' association. Correlation coefficient -0.459, p<0.001.

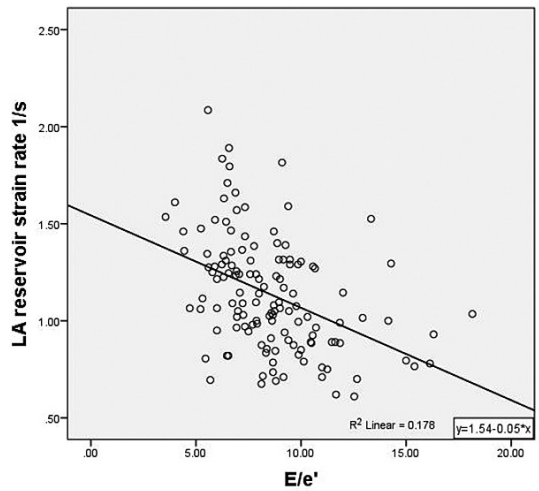


FIGURE 10. LA reservoir strain rate and E/e' association. Correlation coefficient -0.451, p < 0.001.

3.8 24-Hour Ambulatory Arterial Blood Pressure Monitoring

A comparison of 24-hour ambulatory ABP monitoring parameters is shown in Table 8.

TABLE 8. 24-hour ambulatory ABP monitoring parameters compared between the groups.

Parameter	Study group (±SD)	Control group (±SD)	Difference of means	P value
24-hour systolic ABP mean (mm Hg)	129.63 (±10.67)	133.57 (±14.67)	-3.94	0.08
24-hour systolic ABP mean SN	14.97 (±3.24)	16.17 (±4.11)	-1.2	0.065
24-hour diastolic ABP mean (mm Hg)	**74.4 (±8.56)**	**77.9 (±8.44)**	**-3.5**	**0.026**
24-hour diastolic ABP mean SD	11.04 (±2.96)	11.91 (±3.63)	-0.87	0.135
Daytime systolic ABP mean (mm Hg)	133.75 (±10.76)	138.15 (±14.48)	-4.4	0.05
Daytime systolic ABP mean SD	14.11 (±3.51)	15.37 (±3.86)	-1.26	0.052
Daytime diastolic ABP mean (mm Hg)	**77.88 (±8.58)**	**81.89 (±8.65)**	**-4.01**	**0.008**
Daytime diastolic ABP mean SD	10.54 (±3.42)	11.15 (±3.68)	-0.61	0.323
Nighttime systolic ABP mean (mm Hg)	121.41 (±13.12)	125.12 (±17.3)	-3.71	0.165
Nighttime systolic ABP mean SD	12.35 (±8.42)	12.88 (±5.56)	-0.53	0.674
Nighttime diastolic ABP mean (mm Hg)	67.91 (±9.43)	70.95 (±10.28)	-3.04	0.077
Nighttime diastolic ABP mean SD	**7.79 (±2.76)**	**8.97 (±3.34)**	**-1.18**	**0.029**
Systolic ABP drop during the night (%)	9.3 (±7.34)	9.45 (±7.76)	-0.15	0.917
Diastolic ABP drop during the night (%)	12.68 (±8.3)	13.22 (±10.06)	-0.54	0.736
Morning surge (%)	18.15 (±12)	19.43 (±12.62)	-1.28	0.549
systolic ABP PTE (%)	47.03 (±26.94)	53.35 (±29.97)	-6.32	0.174
diastolic ABP PTE (%)	**30.94 (±25.91)**	**42.12 (±26.95)**	**-11.18**	**0.013**
systolic ABP PTD (%)	0.79 (±1.84)	1.4 (±2.57)	-0.61	0.103
diastolic ABP PTD (%)	15.22 (±18.3)	10.65 (±13.34)	4.57	0.297

SD – standard deviation; ABP – arterial blood pressure; PTE – percent time elevation; PTD – percent time drop.

Patients with paroxysmal AF had significantly lower values of the 24-hour diastolic ABP mean, daytime diastolic ABP mean, nighttime diastolic ABP mean SD, and diastolic ABP PTE. Even though the difference was statistically significant, we cannot reject the influence of medication on the ABP parameters. Patients in the study group were more frequently on ACEI as compared to the control group, which could have contributed to lower diastolic blood pressure. We therefore did not include the blood pressure parameters in the further analysis.

The percentage of dippers (nighttime drop >10%) and non-dippers (nighttime drop <10%) was compared between the groups. There were no statistically significant differences. The results are shown in Tables 9 and 10.

TABLE 9. Systolic ABP drop comparison between the groups. P=0.853.

Systolic ABP drop during the night	Study group	Control group
>10 %	37	33
0-10 %	24	26
<0	7	6

TABLE 10. Diastolic ABP drop comparison between the groups. P=0.927.

Diastolic ABP drop during the night	Study group	Control group
>10 %	42	42
0-10 %	22	19
<0	4	4

ABP – arterial blood pressure.

3.9 24-Hour Ambulatory ECG Monitoring

The parameters of the 24-hour ambulatory ECG monitoring are compared in Table 11.

TABLE 11. 24-hour ambulatory ECG monitoring parameters compared between the groups.

Parameter	Study group (±SN)	Control group (±SN)	Difference of means	P value
24-hour mean HR (bpm)	**64.73 (9.96)**	**69.12 (8.85)**	**-4.39**	**0.008**
Minimal HR (bpm)	**48.21 (5.75)**	**50.48 (8.02)**	**-2.27**	**0.063**
Maximal HR (bpm)	110.25 (23.68)	113.23 (17.52)	-2.98	0.26
Ventricular premature beats (%)	0.3 (0.87)	0.32 (0.95)	-0.02	0.918
Atrial premature beats (%)	**1.23 (3.36)**	**0.52 (2.16)**	**0.71**	**<0.001**
Short-run atrial tachyarrhythmia episodes	23 (77.25)	1 (2.07)	22	<0.001

SD – standard deviation; HR – heart rate; bpm – beats per minute.

Patients with atrial fibrillation had a significantly lower 24-hour mean HR, minimal HR, a higher percentage of atrial premature beats, and a higher number of short-run atrial tachyarrhythmia episodes. In order to exclude the influence of taken medications, we repeated the analysis, excluding the patients that were on antiarrhythmics in both groups. The results are shown in Table 12.

TABLE 12. 24-hour ambulatory ECG monitoring parameters compared between the groups. Patients on antiarrhythmics excluded.

Parameter	Study group (±SD) (n = 39)	Control group (±SD) (n = 43)	Difference of means	P value
24-hour mean HR (bpm)	67.74 (9.94)	69.02 (7.73)	-1.28	0.515
Minimal HR (bpm)	49.23 (5.75)	48.67 (7.5)	0.56	0.705
Maximal HR (bpm)	116.9 (23.97)	116.42 (18.21)	0.48	0.919
Ventricular premature beats (%)	0.41 (1.12)	0.29 (1.03)	0.12	0.638
Atrial premature beats (%)	1.12 (2.83)	0.56 (2.51)	0.56	0.345
Short-run atrial tachyarrhythmia episodes	**11.55 (25.52)**	**0.93 (2.04)**	**10.62**	**0.015**

SD – standard deviation; HR – heart rate; bpm – beats per minute.

When the influence of antiarrhythmics was excluded, only one statistically significant parameter remained: the number of short-run atrial tachyarrhythmia episodes.

3.10 Binary Logistic Regression Analysis

After identifying the potentially useful parameters in predicting paroxysmal AF, we performed a binary logistic regression analysis. Parameters included in the

TABLE 13. Results of a binary logistic regression analysis with potentially useful parameters in predicting paroxysmal AF.

Parameter	Odds ratio Exp(B)	95% confidence interval		P value
Age	0.983	0.924	1.046	0.591
BMI	0.923	0.809	1.053	0.232
Gender	0.759	0.247	2.332	0.63
NT pro BNP	1.002	0.998	1.006	0.255
Troponin I	0.984	0.84	1.154	0.844
A wave velocity	0.047	0.002	1.182	0.063
LA EF	**0.925**	**0.864**	**0.990**	**0.025**
Reservoir strain	**1.184**	**1.042**	**1.346**	**0.01**
Reservoir strain rate	**0.003**	**0**	**0.079**	**<0.001**
Short-run atrial tachyarrhythmia episodes	**1.268**	**1.051**	**1.53**	**0.013**

BMI – body mass index; LA EF – left atrial ejection fraction.

analysis were age, gender, BMI, NT pro BNP, troponin I, A wave velocity, LA EF, reservoir strain, and reservoir strain rate. Although we found differences in minimal LA volume and the contractile strain, these parameters were excluded from the regression analysis because of their close connection with already included parameters: the LA EF and reservoir strain. Data of the model are shown in Tables 13, 14, and 15.

TABLE 14. R^2 values.

Cox and Snell R^2	Nagelkerke R^2
0.415	0.556

Model characteristics: sensitivity 79.7%, specificity 73.6%, positive prognostic value 78.5%, negative prognostic value 75%. The ROC curve, together with its characteristics, is shown in Figure 11 and Table 15.

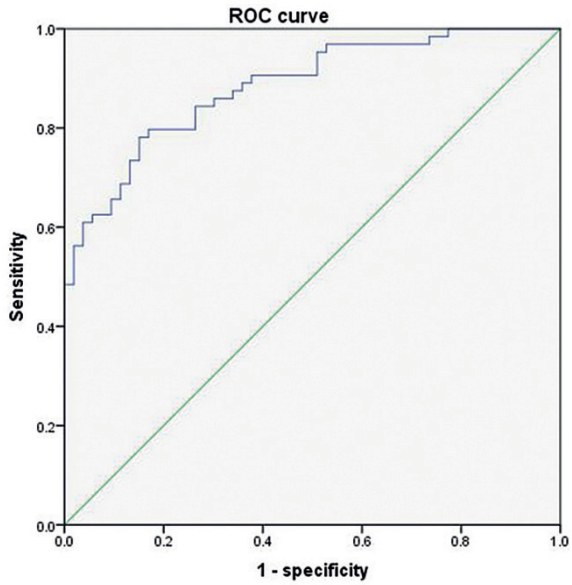


FIGURE 11. ROC curve for binary logistic regression model.

TABLE 15. Characteristics of the ROC curve.

Area under the curve	Standard error	P value	95% confidence intervals	
0.884	0.03	<0.001	0.826	0.942

The binary logistic regression analysis showed that independent and statistically significant parameters linked to paroxysmal AF are the LA EF, LA reservoir strain, LA reservoir strain rate, and short-run atrial tachyarrhythmia episodes during a 24-hour ambulatory ECG monitoring cycle. A new model of binary logistic regression was formed including only the parameters that were statistically significant in the previous one. The results are shown in Tables 16 and 17.

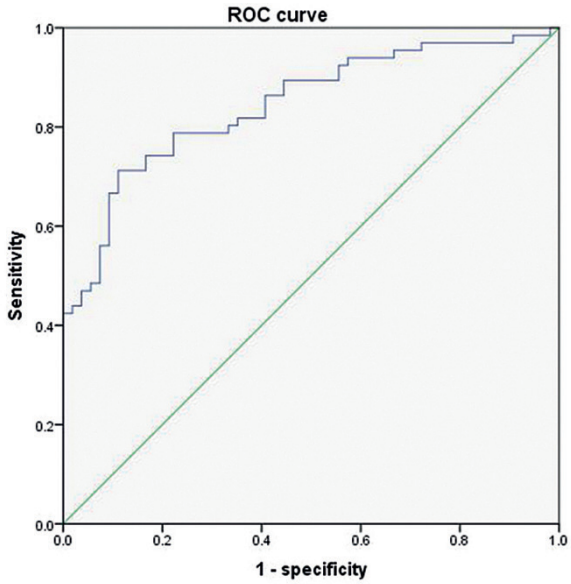
TABLE 16. Results of the binary logistic regression analysis with the parameters that were statistically significant in the previous model.

Parameter	Odds ratio Exp(B)	95% confidence interval		P value
LA EF	**0.922**	**0.872**	**0.975**	**0.005**
Reservoir strain	**1.164**	**1.037**	**1.307**	**0.01**
Reservoir strain rate	**0.013**	**0.001**	**0.133**	**<0.001**
short-run atrial tachyarrhythmia episodes	**1.217**	**1.037**	**1.429**	**0.016**

LA EF – left atrial ejection fraction.

TABLE 17. R^2 values.

Cox and Snell R^2	Nagelkerke R^2
0.328	0.438



Model characteristics: sensitivity 78.8%, specificity 70.4%, positive prognostic value 76.5%, negative prognostic value 73.1%. The ROC curve and its characteristics are shown in Figure 12 and Table 18.

FIGURE 12. ROC curve for the binary logistic regression model.

TABLE 18. Characteristics of the ROC curve.

Area under the curve	Standard error	P value	95% confidence intervals	
0.845	0.035	<0.001	0.775	0.914

3.11 Classification and Regression Tree

A classification and regression tree analysis was carried out to identify the most useful parameters, their cut-off values, and the decision algorithm for predicting paroxysmal AF. The following factors were included in the analysis: NT pro BNP, troponin I, LA EF, LA reservoir strain, LA reservoir strain rate, and short-run atrial tachyarrhythmia episodes. The classification and regression tree is shown in Figure 13.

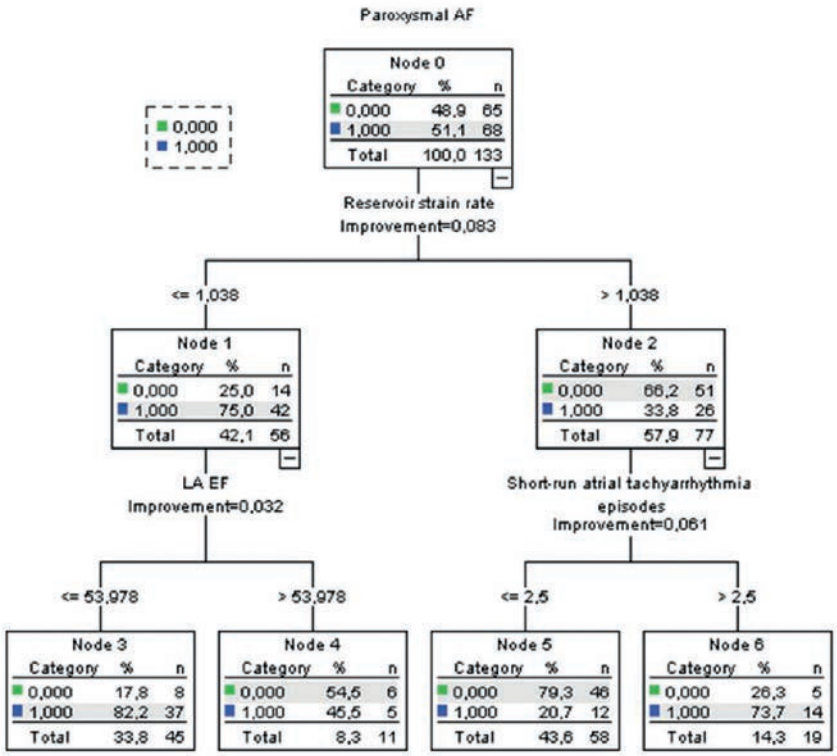


FIGURE 13. Classification and regression tree. The green color represents the control group (without AF), and blue – the study group patients (with paroxysmal AF).

The normalized importance of the variables is shown in Figure 14.

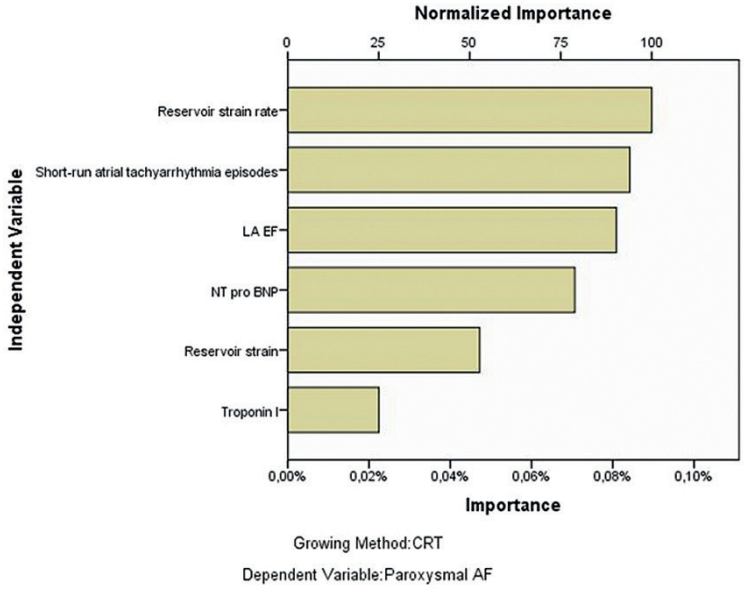


FIGURE 14. Normalized importance of the independent variables in the model for predicting paroxysmal AF in patients with PAH.

A classification and regression tree revealed that the most important cardiovascular factors in predicting paroxysmal AF are the LA reservoir strain rate, short-run atrial tachyarrhythmia episodes during a 24-hour ambulatory blood pressure monitoring, and LA EF. Cut-off values of the variables were also determined: 1.038 for the LA reservoir strain rate, 2.5 for the short-run atrial tachyarrhythmia episodes during a 24-hour ambulatory blood pressure monitoring, and 53.978 % for the LA EF.

The classification table of the model is shown in Table 19.

TABLE 19. Classification table of the classification and regression tree model.

Observed		Predicted		
		Paroxysmal AF		Correct %
		No	Yes	
Paroxysmal AF	No	52	13	80
	Yes	17	51	75
Total %				77,4

AF – atrial fibrillation.

Characteristics of the model: sensitivity 75%, specificity 80%, positive prognostic value 79.7%, negative prognostic value 75.4%.

The ROC curve and its characteristics are shown Figure 15 and Table 20.

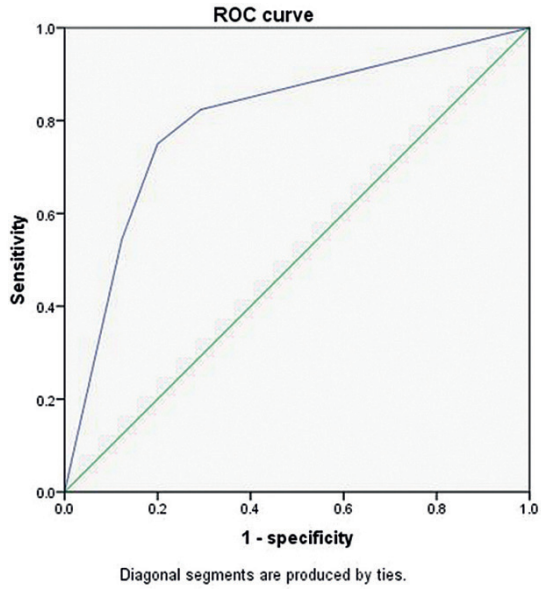


FIGURE 15. ROC curve for the classification and regression tree model.

TABLE 20. Characteristics of the ROC curve for the classification and regression tree model.

Area under the curve	Standard error	P value	95% confidence intervals	
0.801	0.04	<0.001	0.723	0.879

4. Discussion

4.1 General Characteristics of Study and Control Group Patients

Before discussing the results, it is of paramount importance to describe the characteristics of the patients included in the study. The patients included in both groups were mostly middle-aged (the mean age of the study and control groups is 62.9±11.61 and 59.15±11.77, respectively) and a little overweight (the mean BMI of the study and control group is 29.58±4.27 and 29.03±4.79, respectively). Type II diabetes was not an exclusion criteria, but there was the same number of patients

with this pathology in both groups (5 patients in each group), so we do not expect this to influence the results in any way. Gender-wise, the groups were almost identical (44 % and 43 % males in the study and control groups, accordingly). All in all, the patients were well-matched, as there were no statistically significant differences in the general chracteristics of the patients.

The patients in the study mostly had stage I or II hypertension, which was well-controlled by taking on average just 1.59 antihypertensive medications. This number was identical between the groups. To diminish the possibility of secondary hypertension, the ones with resistant PAH were excluded. The fact that hypertension was well-controlled is proved by the 24-hour ambulatory ABP monitoring: systolic ABP values were 129.4±10.58 and 133.57±14.67 mm Hg, and the diastolic values were 74.4±8.59 and 77.89±8.44 in the study and control group patients, respectively. Although a small one (3.49 mm Hg), the difference of diastolic blood pressure values between the groups was statistically significant (p=0,015). We think that this might be due to the differences of the antihypertensive medications taken: the patients with AF were significantly more frequently on ACEI as compared to the control group. There were no other significant differences of the medications taken. As the majority of the patients were on ACEI, ARB, CCB, β-blockers, and diuretics, we can conclude that the patients were mostly treated according to the valid ESC guidelines.

4.2 Quality of Life, Anxiety and Depression

We have not found any studies of life quality, anxiety, and depression on patients with paroxysmal AF caused by PAH comparing them with hypertensive patients. Most of the studies in the field are interventional, aimed to find the effect of treatment on quality of life in patients with AF. The fact that paroxysmal AF reduces quality of life in the general population is somehow taken for granted without much strong evidence. Our study allowed us to exclusively evaluate the influence of paroxysmal AF on quality of life, anxiety, and depression, as we excluded all other pathologies. Paroxysmal AF was the single factor differentiating the study and control groups.

Though the difference of anxiety and depression levels between the groups did not meet the cutoff for statistical significance, patients with AF had higher levels of both. The level of anxiety in the study group was higher by 19.2 %, and the level of depression – by 29 %. In our opinion, the cutoff for statistical significance was not met because of the low number of patients included in the study. It is worth mentioning that patients with AF and PAH had anxiety levels above the cutoff where an anxiety disorder can be suspected. In comparing our results with similar studies, one aspect must be highlighted: there is more evidence about the increased level of

anxiety and not depression in patients with AF. A study published in 2018 found that patients with AF had higher levels of anxiety than those of depression (HADS values 8.9 and 5.8, respectively). These levels of anxiety were also significantly higher in patients with AF as compared to the control group, which was not the case with depression [17]. Other studies also highlight increased levels of anxiety and not depression in patients with AF [46]. Our results support the fact that patients with paroxysmal AF have high levels of anxiety.

Patients with AF had worse quality of life in all sections evaluated by SF-36, but statistical significance was reached in sections corresponding to physical health. Patients with paroxysmal AF had lower levels of physical functioning, physical role functioning, and general health. We used the SF-36 questionnaire to evaluate quality of life, and this allowed us to compare the results with other studies, as it is the most popular tool. Other studies, which did not have perfectly matched groups, show similar results [19, 47, 48]. Paroxysmal AF caused by PAH, with the influence of other pathologies set aside, leads to a worse quality of life, mostly limiting physical functioning. The results are further validated by our questionnaire of physical activity, which revealed that patients with paroxysmal AF were indeed less physically active if compared with the control group.

Discussing quality of life questions cannot be done without taking into consideration the age of the patients. Perret-Guillaume et al. published a study in 2010 that evaluated the quality of life in elderly patients with AF. The mean age in both groups was approximately 72 years. The study proved that in elderly patients, AF primarily alters the mental quality of life [49]. Having in mind these data, we can deduce that the influence that AF has on quality of life is dependent on the age of the patients: physical health is affected more in younger patients, while the older ones tend to suffer more from a decline in mental health. All in all, we can undoubtedly say that paroxysmal AF leads to a worse quality of life in patients with hypertension.

4.3 Blood Biomarkers

Comparing the means of laboratory parameters revealed significant differences of three biomarkers: troponin I, NT pro BNP, and TSH. We decided not to include TSH into further analysis because of two things. First, we had excluded all the patients having TSH concentration outside the normal range, therefore creating a biased population regarding this aspect. Second, large scale prospective Framingham population studies revealed no influence of increased TSH on AF [50], whereas decreased TSH was clearly linked to arrhythmia [51].

Though there was not a single patient with troponin I concentrations outside the normal range, the study group patients had significantly higher concentrations of this biomarker. From the perspective of coronary heart disease diagnostics, the

values of troponin I were very little (in the study and control groups – 4.16±4.8 and 2.66±2.7 ng/l, respectively), but the difference revealed the potential usefulness of the biomarker in predicting paroxysmal AF. Other studies also show that troponin I might be linked with AF even when the concentrations are low [52]. The mechanism of troponin I release during the episodes of paroxysmal AF is proved in animal studies. At high frequency pacing of the heart, conditions similar to AF were created, and this led to increased concentrations of troponin I [53].

The values of NT pro BNP had a substantial difference between the groups. Patients with paroxysmal AF had a 2.3-fold increased concentrations of NT pro BNP (287.68±325.59 in the study group and 124.54 ±105.11 pg/ml in the control group). Though the patients with heart failure symptoms and LV EF < 55% were excluded, NT pro BNP values were surprisingly high in the paroxysmal AF group. At the moment, the cutoff value of NT pro BNP in diagnosing heart failure is 125 pg/ml [54]. Our results show not only the fact that increased concentrations of NT pro BNP are linked with paroxysmal AF, but also raise the question of the cutoff for diagnosing heart failure in patients with AF. Other studies revealing high concentrations of NT pro BNP in patients with AF also discussed the valid cutoff values. [55].

The facts that NT pro BNP is linked with early LV DD, LA stretch, and remodeling, as well as fibrosis, confirm the close link between this biomarker and paroxysmal AF in patients with PAH.

Although higher concentrations of troponin I and NT pro BNP should raise suspicion of paroxysmal AF in certain situations, a binary logistic regression analysis revealed that the studied biomarkers were not among the best AF predicting factors.

4.4 Ultrasound of the Heart

Our study revealed that paroxysmal AF is particularly related to the parameters of LA function. Of all the routine ultrasound parameters, a significant difference was obtained in three: A wave velocity, minimal LA volume, and LA EF. These variables surpassed LA volume index, the E/e' ratio, and mitral annular velocity. Our findings unequivocally answer the question of the importance of LA size or function parameter in the early detection of paroxysmal AF. The LA function is far more sensitive than size in predicting paroxysmal AF. Mean LA volume index values were on the cutoff of being increased in both groups (34 ml/m^2) with a minor and not statistically significant difference. This shows that patients with PAH can have increased LA size, but it is the function that can answer the question whether the patient has paroxysms of AF or not. A logistic regression showed that LA EF, differently from A wave velocity and minimal LA volume, remained an independent and significant

prognostic factor of paroxysmal AF. This did not surprise us, as LA EF corresponds to the reservoir function of LA and, subsequently, LA fibrosis [56, 57].

Among the LA strain and strain rate parameters, a significant difference was obtained in mean LA contractile and reservoir strains as well as the reservoir strain rate. A regression analysis showed that the best independent predictors were the LA reservoir strain and reservoir strain rate. All in all, we can conclude that the reservoir function of the LA is essential in predicting the risk of paroxysmal AF in patients with PAH.

4.5 Left Atrial Strain – An Early Marker of Left Ventricular Diastolic Dysfunction

Though it is well-defined that the decline in the LA reservoir function is linked with the remodeling of LV, there is also data showing that the changes in the LA cannot be explained solely by LV DD [58]. The remodeling of the LV and LA is possibly occurring in parallel with each other, and this brings up the idea that the parameters of the LA function can be early markers of LV DD. When we grouped the patients according to the number of traditional ultrasound variables present showing LV DD, the groups had significantly different values of the LA strain and strain rate parameters. That means that we could group the patients into the same groups without using the traditional parameters of LV DD. This leads us to two conclusions. First, the LA strain and strain rate are undoubtedly related with LV DD. This proves the fact that LA and LV diastolic functions are closely related with each other. Second, our results show that LA remodeling occurs very early in the course of the disease. The LA strain and strain rate variables are among the earliest markers of LV DD, certainly preceding traditional ones. The results of other studies also prove this hypothesis. In 2016 a study published by A. Brecht et al., ultrasound data of 473 patients was analyzed. Of them, 153 had LV DD according to the traditional parameters recommended by the guidelines. All the patients were asymptomatic, which means the disease was in its early stages, when the changes are subtle and hard to find. When the LA strain parameters were compared among different LV DD grade patients, the LV reservoir and conduit strains appeared to be the most sensitive and specific markers of the disease surpassing LA VI [59]. This topic is also discussed in the 2019 publication by L. Thomas et al. in *JACC* [60]. Studies analyzing the link between different LA ultrasound parameters and LV DD are reviewed. The conclusions of the publication are on par with the ones of our study. First, the parameters of the LA reservoir function are important in diagnosing and grading LV DD as well as evaluating the effect of treatment. Second, parameters of LA size are not sensitive enough in the early stages of the disease. Increased LA size is already a late consequence of ongoing processes in the LA and LV. This is also proved in our study.

4.6 24-Hour Ambulatory Blood Pressure Monitoring

All the statistically significant differences between the groups are in the values of diastolic blood pressure. The patients with paroxysmal AF had mildly though significantly lower values of 24-hour mean and daytime diastolic blood pressure, a higher variability of nighttime diastolic blood pressure, and their diastolic blood pressure exceeded the nighttime cutoff value less often as compared with the control group. This all leads to a conclusion that the patients with paroxysmal AF were treated better by prescribing ACEI. The reason that there were statistically significant differences in the antihypertensive medication profile between the groups made us refrain from any further analysis of these differences.

4.7 24-Hour Ambulatory ECG Monitoring

There were statistically significant differences in 24-hour mean and minimal HR, atrial premature beat percentage, and short-run atrial tachyarrhythmia episodes between the groups. The fact that a substantial part of the patients in both groups were on antiarrhythmic drugs made us repeat the analysis, including only those that were not on antiarrhythmic medications. This revealed a single relevant variable: short-run atrial tachyarrhythmia episodes. A regression analysis confirmed that this factor is independent and one of the most important in predicting the risk of paroxysmal AF. According to the ESC guidelines, we can diagnose AF when the episode without apparent P waves and irregular RR intervals on the ECG is longer than 30 s [6]. However, the duration of 30 s is not proven by any trials, and the results of ours as well as others' studies raise questions regarding its validity. This is taken further by a 2017 publication in *Stroke,* which analyzed the risk of stroke in patients with short-run atrial tachyarrhythmia episodes. The results revealed that these episodes, as well as atrial premature beats, were related to an increased risk of stroke [61]. The most important question regarding short-run atrial tachyarrhythmia episodes is whether we can reduce the risk of stroke in these patients with anticoagulation. Although we are not aware of any published studies dealing with this question, if this was proven, the 30 s cutoff for the diagnosis of AF would be compromised.

4.8 Limitations of the Study

Firstly, the study is limited by a rather small number of patients in both groups. This possibly did not allow us to reach statistical significance when the changes were subtle. Ideally, the patients should have been divided into two groups and, after the useful variables were identified in one part of the patients, their performance in predicting AF should have been tested in another part of the patients. The small number of patients did not allow us to perform such an analysis. Another limitation is

the design of the study. In order to fully answer the question, a prospective longitudinal study design would have been a better choice, when the patients are included with hypertension only and observed until they develop paroxysmal AF. However, this would have required huge resources and a very long observation period, as the annual percentage of patients with PAH developing AF is not that high and would have probably not exceeded a few percent a year.

5. Conclusions

1. Paroxysmal atrial fibrillation is linked to increased concentrations of NT pro BNP and troponin I in patients with primary arterial hypertension.
2. Paroxysmal atrial fibrillation is linked to a decreased left atrial reservoir strain, reservoir strain rate, and ejection fraction values in patients with primary arterial hypertension.
3. Paroxysmal atrial fibrillation is linked to an increased number of short-run (<30 s) atrial tachyarrhythmia episodes during 24-hour ambulatory ECG monitoring in patients with primary arterial hypertension.
4. In the population of patients with primary arterial hypertension, heart ultrasound parameters of the left atrium (reservoir strain, reservoir strain rate, and ejection fraction) and the number of short-run atrial tachyarrhythmia episodes during 24-hour ambulatory ECG monitoring are able to predict paroxysmal atrial fibrillation.
5. Paroxysmal atrial fibrillation leads to a worse quality of life in patients with primary arterial hypertension.

6. Recommendations for clinical practice

1. In order to differentiate the risk of paroxysmal AF among patients with primary arterial hypertension, it is useful to perform laboratory tests of NT pro BNP and troponin I, a 24-hour ambulatory ECG monitoring searching for short-run atrial tachyarrhythmia episodes, and an ultrasound of the heart determining the parameters of the LA function.
2. The most important variables in predicting paroxysmal atrial fibrillation in patients with primary arterial hypertension are the LA strain rate, LA EF, and the number of short-run atrial tachyarrhythmia episodes during 24-hour ambulatory ECG monitoring.
3. The parameters of the LA function are better than the ones of LA size in predicting the risk of paroxysmal atrial fibrillation in patients with primary arterial hypertension.

4. LA strain and strain rate parameters are useful in detecting and grading the left ventricular diastolic dysfunction in patients with primary arterial hypertension.

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Annexes

Authorization Provided by the Lithuanian Bioethics Committee

PATVIRTINTA
Lietuvos bioetikos komiteto direktoriaus
2016 m. birželio 10 d. įsakymu Nr. V-14

LIETUVOS BIOETIKOS KOMITETAS

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LEIDIMAS ATLIKTI BIOMEDICININĮ TYRIMĄ

2016-07-26 Nr.: L-16-05/1

Vilnius

Tyrimo pavadinimas: **„Arterinio kraujospūdžio bei kitų kardiovaskulinių veiksnių įtaka prieširdžių virpėjimo išsivystymui pacientams, sergantiems pirmine arterine hipertenzija“**	
Protokolo Nr.:	**AKS-PV-1**
Versija:	**02**
Data:	**2016 m. liepos 17 d.**
Tiriamiesiems skirti dokumentai: Asmens informavimo ir informuoto asmens sutikimo forma (lietuvių kalba) : Versija: **02** Data: **2016 m. balandžio 18 d.**	
Pagrindinis tyrėjas:	**Gyd., med. m. dr., prof. Audrius Aidetis**
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Leidimas atlikti biomedicininį tyrimą išduotas Lietuvos bioetikos komiteto Biomedicininių tyrimų ekspertų grupės posėdžio, įvykusio **2016 m. liepos 19 d.**, sprendimu.

Direktorius Eugenijus Gefenas

LIETUVOS RESPUBLIKA
Lietuvos bioetikos komitetas
VILNIUS

The List of Publications

1. **Jarasunas J**, Aidietis A, Aidietiene S. Left atrial strain – an early marker of left ventricular diastolic dysfunction in patients with hypertension and paroxysmal atrial fibrillation. *Cardiovasc Ultrasound*. 2018 Oct 31;16(1):29. DOI: 10.1186/s12947-018-0147-6.

2. Berukstis A, **Jarasunas J**, Daskeviciute A, Ryliskyte L, Baranauskas A, Steponeniene R, Laucevicius A. How to interpret 24-h arterial stiffness markers: comparison of 24-h ambulatory Mobil-O-Graph with SphygmoCor office values. *Blood Press Monit*. 2019 Apr;24(2):93–98. DOI: 10.1097/MBP.0000000000000369.

The List of Presentations

1. **Jarasunas J**, Berukstis A, Daskeviciute A, Misonis N, Ryliskyte L, Laucevicius A. Evaluation and comparison of ambulatory Mobil-O-Graph 24-hour mean arterial stiffness parameter values with Sphygmocor office values in high cardiovascular risk patients. Cardiology Update Conference 17, February 11–15, 2017, Davos, Switzerland.

2. **Jarasunas J**, Jakovlevaitė G, Aidietis A. Anxiety and depression in patients with paroxysmal atrial fibrillation. The 4th International Conference "Evolutionary Medicine: Health and Diseases in a Changing Environment," June 5–10, 2018, Vilnius University, Vilnius, Lithuania.

3. **Jarasunas J**, Aidietis A. LA function assessed by 2D speckle tracking in patients with hypertension and paroxysmal atrial fibrillation. The IX International Congress "Cardiology at the Crossroads of Sciences," May 23–25, 2018, Tyumen, Russia.

SANTRAUKA

Įvadas

Prieširdžių virpėjimu (PV) serga 5,5 % populiacijos > 55 metų, tai labiausiai paplitusi ilgos trukmės prieširdinė aritmija. PV susijęs su didele embolinių komplikacijų rizika, kurią galima ženkliai sumažinti skiriant antikoaguliantus. Pirminė arterinė hipertenzija – dažniausia PV sukelianti patologija bendroje populiacijoje. Kadangi esant paroksizminiam PV aritmijos epizodai gali būti retesni arba asimptomiai, ligą nustatyti dažnai nėra lengva. Tikslinga išaiškinti lengvai nustatomus rodiklius, kurie leistų identifikuoti didelę PV riziką turinčius pacientus, bei jiems taikyti ilgesnę ambulatorinę EKG stebėseną, siekiant diagnozuoti PV bei paskirti gydymą. Taip pat yra duomenų, jog renino-angiotenzino-aldosterono sistemos blokatoriai efektyvūs tiek pirminėje, tiek antrinėje PV prevencijoje. Nustačius didelę PV riziką būtų tikslingas gydymas šiais medikamentais.

Nors visuotinai sutariama, jog PV sergančių pacientų gyvenimo kokybė yra prastesnė, absoliuti dauguma duomenų šioje srityje gauti iš randomizuotų intervencinių PV gydymo tyrimų, todėl galimai į tyrimą įtraukti tik labiausiai simptomus jaučiantys, o tuo pačiu metu ir prasčiausią gyvenimo kokybę turintys pacientai. Gyvenimo kokybės tyrimų bendroje PV, o ypač PV nulemto pirminės arterinės hipertenzijos, populiacijoje yra nepakankamai.

Tyrimo hipotezė

Paroksizminiu PV ir pirmine arterine hipertenzija sergantys pacientai turi būdingų kraujo biožymenų, širdies ultragarsinio tyrimo, 24 val. EKG ir arterinio kraujo spaudimo (AKS) stebėsenos rodiklių pokyčių, kurie leidžia identifikuoti paroksizminiu PV sergančius pacientus tarp pirmine arterine hipertenzija sergančių pacientų.

PV susijęs su blogesne pacientų, sergančių pirmine arterine hipertenzija, gyvenimo kokybe, didesniu nerimo ir depresijos lygiu.

Tyrimo tikslas

1. Nustatyti kardiovaskulinės sistemos rodiklius ir jų pokyčius, kurie leistų identifikuoti paroksizminiu prieširdžių virpėjimu sergančius ir didesnę aritmijos riziką turinčius pacientus tarp pirmine arterine hipertenzija sergančių pacientų.
2. Nustatyti, kokį poveikį paroksizminis prieširdžių virpėjimas turi pacientų, sergančių pirmine arterine hipertenzija, gyvenimo kokybei, nerimo ir depresijos lygiui.

Tyrimo uždaviniai

1. Nustatyti su paroksizminiu prieširdžių virpėjimu susijusius kraujo biožymenis, tiriant pacientus, sergančius pirmine arterine hipertenzija.
2. Nustatyti su paroksizminiu prieširdžių virpėjimu susijusius širdies ultragarsinio tyrimo rodiklius, tiriant pacientus, sergančius pirmine arterine hipertenzija.
3. Nustatyti su paroksizminiu prieširdžių virpėjimu susijusius 24 val. EKG ir AKS stebėsenos rodiklius, tiriant pacientus, sergančius pirmine arterine hipertenzija.
4. Nustatyti kardiovaskulinės sistemos rodiklius, kurie labiausiai susiję su paroksizminiu prieširdžių virpėjimu ir geriausiai leidžia prognozuoti, ar pacientas turi šios aritmijos paroksizmų, tiriant pacientus, sergančius pirmine arterine hipertenzija.
5. Nustatyti pagal gyvenimo kokybės klausimyną (SF-36) ir ligoninės nerimo ir depresijos skalę (HADS) vertinamų pacientų, sergančių pirmine arterine hipertenzija, gyvenimo kokybės, nerimo ir depresijos rodiklių skirtumus, susijusius su paroksizminiu prieširdžių virpėjimu.

Tyrimo naujumas

Šiuo metu literatūroje aprašyti PV rizikos vertinimo modeliai geba nusakyti prieširdžių virpėjimo riziką bendroje populiacijoje, tačiau į juos daugiausia įtraukti klinikiniai faktoriai, neskiriant pakankamo dėmesio naujiems kairiojo prieširdžio (KP) funkcijos vertinimo metodams, tokiems kaip ultragarsinis deformacijos bei deformacijos greičio matavimas. Yra nemažai duomenų, jog KP deformacijos rodikliai susiję su PV rizika po plaučių venų izoliacijos procedūros, kardioversijos sėkme arba nesėkme, tromboembolinėmis komplikacijomis bei insultą patyrusių pacientų KP ausytės funkcija. Tyrimų, įtraukiančių KP deformacijos bei deformacijos greičio rodiklius į PV riziką nusakančias schemas tarp pirmine arterine hipertenzija sergančių pacientų bei nustatančių šių parametrų ribines vertes nėra publikuota. Kitas svarbus tyrimo naujumo aspektas yra tiriamoji populiacija. Nesusijęs su kita kardiovaskuline patologija PV yra reta liga, kurios paplitimas įvairių tyrimų duomenimis priklausomai nuo naudojamo apibrėžimo svyruoja nuo 1,6 % iki 30 %. Dažniausia kardiovaskulinė patologija, nulemianti PV išsivystymą bendroje populiacijoje yra pirminė arterinė hipertenzija. Nors šių dviejų patologijų sąsaja pasitaiko dažniausiai, tačiau tyrimų šioje pacientų grupėje yra nepakankamai.

Kadangi PV dažniausiai susijęs su kita gyvenimo kokybę bloginančia kardiovaskuline patologija, išskirti vien PV įtaką gyvenimo kokybei sudėtinga. Neįtraukdami pacientų, turinčių kitų reikšmingų patologijų, išskyrus hipertenziją, ir lygindami ne su sveikais, o hipertenzija sergančiais pacientais, mes galėjome įvertinti, kaip (ar) PV prisideda prie gyvenimo kokybės bloginimo bei didesnio nerimo ir depresijos lygio.

Metodika

Į tyrimą iš viso įtraukti 133 pacientai, sergantys pirmine arterine hipertenzija. Jie suskirstyti į 2 grupes. Tiriamąją grupę (n = 68) sudarė pacientai, sergantys paroksizminiu PV, o kontrolinę grupę (n = 65) pacientai, niekada neturėję PV paroksizmų. Siekiant įtraukti tik pirminės arterinės hipertenzijos nulemtus paroksizminio PV atvejus, į tyrimą neįtraukti pacientai, sergantys kitomis aritmiją galinčiomis sukelti ligomis: koronarine širdies liga, vožtuvine širdies liga, širdies nepakankamumu, inkstų nepakankamumu, lėtine obstrukcine plaučių liga, turintys skydliaukės patologiją bei patyrę širdies operaciją.

Ištyrimas:

- gyvenimo kokybės klausimynas SF-36, HADS
- širdies ultragarsinis tyrimas su išplėstiniu KS diastolinės funkcijos vertinimu
- ultragarsinis KP rezervuarinės, konduitinės bei kontraktilinės funkcijos vertinimas taikant 2D taškelių žymėjimo metodiką
- 24 val. EKG stebėsena
- 24 val. AKS stebėsena
- laboratoriniai tyrimai (troponinas I, NT pro BNP, TSH, CRB, elektrolitų konc.)

Rezultatai

Tiriamąją ir kontrolinę grupę sudarė panašių charakteristikų pacientai: vidutinis amžius buvo atitinkamai 62,9 ± 11,61 ir 59,15 ± 11,77, vyrai sudarė 44,11 ir 43,07 %, kūno masės indeksas 29,58 ± 4,27 ir 29,03 ± 4,79, pacientai vartojo vidutiniškai po 1,59 antihipertenzinio medikamento abiejose grupėse.

Lyginant gyvenimo kokybės parametrus, paroksizminiu PV sergantys pacientai statistiškai patikimai ($p < 0,05$) blogiau vertino savo bendrą sveikatą, fizinį aktyvumą bei veiklos apribojimą dėl fizinių problemų (įvertinimai tiriamosios bei kontrolinės grupės atitinkamai 46,03 ± 18,92 ir 53,92 ± 17,33, 68,31 ± 22,92 ir 78,77 ± 18,71, 39,71 ± 42,59 ir 60 ± 41,41). Nors paroksizminiu PV sirgę pacientai pasižymėjo didesniu nerimo bei depresijos lygiu (įvertinimai atitinkamai 7,57 ir 6,35, 5,06 ir 3,91), statistinis reikšmingumas nebuvo pasiektas (atitinkamai $p = 0,126$ bei 0,104).

Lyginant tiriamąją ir kontrolinę grupes reikšmingiausi skirtumai rasti tarp šių rodiklių:

Rodiklis	Tiriamoji grupė n = 68 (±SN)	Kontrolinė grupė n = 65 (±SN)	Vidurkių skirtumas	P reikšmė
Troponino I koncentracija (ng/l)	4,16 (±4,8)	2,66 (±2,7)	1,5	0,045
NT pro BNP koncentracija (pg/ml)	287,68 (±325,59)	124,54 (±105,11)	163,14	<0,001
KP išstūmio frakcija (%)	46,95 (±9,76)	53,72 (±8,53)	-6,77	<0,001
Rezervuarinė deformacija (%)	29,74 (±6,6)	31,93 (±5,41)	-2,19	0,038
Rezervuarinės deformacijos greitis (1/s)	1,04 (±0,29)	1,23 (±0,27)	-0,19	<0,001
< 30 s trukmės prieširdinės tachiaritmijos epizodų skaičius	23 (77,25)	1 (2,07)	22	<0,001

Taikant binarinės logistinės regresijos analizę nustatyti nepriklausomai su paroksizminiu PV susiję rodikliai: KP IF (p = 0,025), rezervuarinė deformacija (p = 0,001), rezervuarinės deformacijos greitis (p < 0,001), < 30 s trukmės prieširdinės tachiaritmijos epizodų skaičius (p = 0,013). Modelio jautrumas 78,8 %, specifiškumas 70,4 %, teigiama prognostinė vertė 76,5 %, neigiama prognostinė vertė 73,1 %, plotas po ROC kreive 0,845.

Siekiant nustatyti didžiausią prognostinę vertę turinčius rodiklius bei jų ribines vertes, sudarytas klasifikacijos ir regresijos medis. Jame didžiausia verte pasižymėjo rezervuarinės deformacijos greitis (ribinė vertė 1,038), KP IF (ribinė vertė 53,98) bei < 30 s trukmės prieširdinės tachiaritmijos epizodai (ribinė vertė 2,5). Sudaryto prognozavimo modelio jautrumas 75 %, specifiškumas 80 %, teigiama prognostinė vertė 79,7 %, neigiama prognostinė vertė 75,4 %, plotas po ROC kreive 0,801.

Išvados

1. Paroksizminis prieširdžių virpėjimas susijęs su padidėjusia NT pro BNP bei troponino I koncentracija kraujyje tarp pacientų, sergančių pirmine arterine hipertenzija.
2. Paroksizminis prieširdžių virpėjimas susijęs su širdies ultragarsinio tyrimo metu nustatytomis mažesnėmis kairiojo prieširdžio rezervuarinės deformacijos, rezervuarinės deformacijos greičio ir kairiojo prieširdžio išstūmio frakcijos reikšmėmis tarp pacientų, sergančių pirmine arterine hipertenzija.
3. Paroksizminis prieširdžių virpėjimas susijęs su didesniu < 30 s trukmės prieširdinės tachiaritmijos epizodų kiekiu 24 val. EKG stebėsenos metu tarp pacientų, sergančių pirmine arterine hipertenzija.
4. Pirmine arterine hipertenzija sergančių pacientų grupėje ultragarsiniai kairiojo prieširdžio funkcijos rodikliai (rezervuarinė deformacija, rezervuarinės deformacijos greitis ir išstūmio frakcija) bei < 30 s trukmės prieširdinės tachiaritmijos epizodų kiekis 24 val. EKG stebėsenos metu leidžia prognozuoti, ar pacientas serga paroksizminiu prieširdžių virpėjimu.
5. Paroksizminis prieširdžių virpėjimas susijęs su blogesne pacientų, sergančių pirmine arterine hipertenzija, gyvenimo kokybe.

Arturas Lipnevičius

A COMPARISON OF POSTOPERATIVE OUTCOMES BETWEEN TRANSAPICAL AND CONVENTIONAL MITRAL VALVE REPAIR

TRANSAPIKALINĖS IR KLASIKINĖS MITRALINIO VOŽTUVO PLASTIKOS POOPERACINIŲ REZULTATŲ PALYGINIMAS

Biomedical sciences, Medicine (06 B)
Biomedicinos mokslai, Medicina (06 B)

Abbreviations

ACC/AHA – The American College of Cardiology and American Heart Association
AF – atrial fibrillation
ACT – activated clotting time
AMVL – anterior mitral valve leaflet
BMI – body mass index
BSA – body surface area
CMVR – conventional mitral valve repair
CMV – controlled mechanical ventilation
COPD – chronic obstructive pulmonary disease
CPB – cardiopulmonary bypass
ECMO – extracorporeal membrane oxygenation
ePTFE – expanded polytetrafluoroethylene
FED – fibroelastic deficiency
GFR – glomerular filtration rate
IABP – intra-aortic balloon pump
ICU – intensive care unit
IQR – interquartile range
LA – left atrium
LV – left ventricle
LVEDD – left ventricular end-diastolic diameter
LVEF – left ventricular ejection fraction
LVESD – left ventricular end-systolic diameter
MI – myocardial infarction
MIMV repair – minimally invasive mitral valve repair
MR – mitral regurgitation
MV – mitral valve
MVARC - Mitral Valve Academic Research Consortium
MVR – mitral valve replacement
MVr. – mitral valve repair
NYHA – New York Heart Association
PMVL – posterior mitral valve leaflet
PPM – permanent pacemaker
RA – right atrium
RF – renal failure
RV – right ventricle
SAM – systolic anterior motion
TAMVR – transapical mitral valve repair
TR – tricuspid regurgitation
TV – tricuspid valve
VUHSK – Vilnius University Hospital Santaros Klinikos

Introduction

1. Relevance of the problem. Importance and novelty of the research.

Mitral regurgitation (MR) caused by degenerative mitral valve (MV) disease is the most common valvular heart disease in developed countries and affects 2-3% of the population. The main cause of MR is a prolapse of one or both leaflets due to changes in the morphology of MV leaflets and subvalvular apparatus. Left untreated, MR often causes arrhythmia and heart failure, and worsens the quality of the patient's life.

Reconstructive MV surgery became an established gold standard treatment of degenerative MR. The aim of MV repair is a restoration of the normal anatomy and function. Compared with MV replacement, MV repair is associated with better

long-term survival and more preserved LV function, it restores normal life quality and reduces the risk of infective endocarditis and thromboembolic events.

The traditional and most popular method of MV repair is conventional MV repair through median sternotomy. This procedure is always performed using cardiopulmonary bypass (CPB) and cardioplegia; therefore, the patient is exposed to the risks related to CPB and cardioplegia, i.e. stroke, systemic inflammatory response, postoperative cardiac and multiorgan failure, arrhythmias, infection and increased postoperative bleeding.

In the 1980s, ePTFE artificial chords were introduced for use in MV surgery. The implantation of artificial chords allowed the valve to be repaired without resecting the prolapsing segment and can be used to correct prolapse of both anterior and posterior leaflets. Over the next few decades, artificial chords have become very popular and developed into a standard tool in modern MV surgery.

Over the past 20 years, several less-invasive MV repair approaches have emerged. They were designed to decrease the morbidity of MV surgery, whilst maintaining an acceptable level of safety and quality of repair. These techniques also aimed to minimise surgical trauma, enhance recovery and reduce the length of in-hospital stays. Although these techniques undoubtedly have certain advantages, they still carry risks associated with the use of CPB.

One of the recently presented innovative minimally invasive techniques is transapical off-pump implantation of artificial chords using the NeoChord DS1000 system. The system allows transoesophageal echocardiography (TEE)–guided delivery of the ePTFE chordae to the prolapsing or flailing mitral leaflet and restores the normal function of the valve. This procedure is performed through a small left thoracotomy under beating-heart conditions without CPB, so the patient is not exposed to the complications related to CPB. Transapical MV repairs have been performed at Vilnius University Hospital Santaros Klinikos since 2011. Within the same time frame, conventional MV repairs were also performed.

Transapical MV repair is a novel procedure which is not common in the world, therefore, there is an insufficient amount of data on this method of treatment. Publications describing postoperative results are also rare. There has only been one publication (Colli et al., 2018) presenting European long-term (12 months) results after this procedure and data on the long-term durability of the repair. For that reason, new data are particularly relevant and interesting, as they have an impact on the further use and development of this technology.

Although single centres have published their experience of transapical mitral repair, there have been no publications comparing the results of conventional and transapical MV repair. Certainly, such a comparison would be very useful and relevant for assessing the safety and effectiveness of this new technique.

2. The aim of the research

The purpose of this study was to evaluate and compare the efficacy and early and long-term postoperative outcomes of conventional and transapical mitral valve repair.

3. The objectives of the research

1. An assessment of the effectiveness and safety of transapical MV repair (the incidence of adverse events in the early postoperative period) and comparison with the results of conventional MV repair.
2. An assessment of the long-term durability of the transapical MV repair and comparison with the results of conventional MV repair.
3. Determination of the association between the anatomical type of MV pathology and long-term durability of the MV repair.
4. Analysis of the causes leading to recurrent MR following transapical and conventional MV repair.

4. Scientific novelty

Currently, there is only one available publication (Colli et al., 2018) reporting long-term outcomes after transapical MV repair (12 months follow-up). At Vilnius University Hospital Santaros Klinikos, transapical MV repair was first performed in 2011, and the median follow-up for the patients included in this study was 39 months (IQR 20-51 months). This dissertation presents the long-term results of transapical MV repair, and demonstrates the long-term durability of the repair and its association with the initial anatomy of the MV. It also investigates the causes of recurrent MR after transapical and conventional MV repair.

Both transapical and conventional MV repair are used to treat degenerative MV disease. However, transapical MV repair is a completely new technique, while conventional MV repair has been already recognised as safe and effective way to treat degenerative MR. The dissertation compares the early and long-term outcomes of these two methods of treatment.

5. Practical significance of the research

The practical significance of this study lies in the possibility of using the results to optimise the selection of patients for transapical MV repair. This study demonstrates the long-term durability of transapical MV repair and its association with the initial MV anatomy. The best long-term durability of transapical MV repair was found to be achieved in patients with an isolated prolapse of the P2 segment.

It was also found that although the incidence of adverse events in the early postoperative period was lower after transapical MV repair, the long-term durability of conventional MV repair was better and did not relate to the initial anatomy of the MV.

Subjects and methods of the research

6. Research subjects

This retrospective non-randomised study was performed on a single centre's patient cohort at Vilnius University Hospital Santaros Klinikos during the period from 2014-2018. In order to conduct the study, permission No. 158200-16-829-345 was obtained from the Vilnius Regional Committee for the Ethics of Biomedical Research.

The study involved 202 patients who underwent surgical MV repair (both transapical and conventional) for severe MR of degenerative origin between December 2011 and December 2017 at the Centre of Cardiac and Thoracic Surgery of Vilnius University. Data were collected from in-patient and out-patient medical notes, including surgical and anaesthetic notes. Data collection was completed on 1st January 2018.

There were 266 patients who underwent MV repair for severe MR during the period of study. All patients had indications for surgical treatment according to ACC/AHA and ESC/EACTS guidelines. This study included only those patients (n=202) who underwent MV repair for degenerative MV disease. MV pathology in this group included single or bi-leaflet MV prolapse or flail, with or without chordal rupture. All patients (n=64) who had a restrictive mechanism of regurgitation (rheumatic disease, cardiomyopathy), ischemic mitral regurgitation, or MV infectious lesions, and patients with a central regurgitation jet were excluded from this study.

All patients with degenerative MV disease were discussed by the heart team. Patients who had a favourable MV anatomy and agreed to undergo transapical MV repair were selected for the NeoChord procedure (n = 94). The rest of the patients were selected for conventional MV repair (n = 108). Patients who underwent MV replacement were not involved in this study.

There were 3 patients who underwent transapical off-pump mitral repair following the failure of conventional MV repair with an annuloplasty ring. These three patients were not excluded from the study; however, they were described separately.

Thus, all patients were divided into two groups:

1. Conventional surgical MV repair (CS) group – 108 patients.
2. Transapical MV repair (TA) group – 91 patients.

Clinical examination of the patients, as well as laboratory and instrumental studies, was performed:

- prior to the procedure;
- at discharge;
- at follow-up visits at 1, 6 and 12 months after the procedure, and every 12 months after that.

Follow-up was not considered complete if patients missed last two or more visits.

All data collected after evaluation and analysis were presented as:

- Preoperative data and description of mitral pathology;
- Intraoperative results;
- Early postoperative outcomes (up to 30 days after the procedure);
- Late postoperative outcomes (1 month – until the end of the follow-up).

7. Statistical analysis

Statistical analysis was performed using the data collection and analysis software package SPSS 22.0 (IBM Corp., Armonk, NY, USA). The quantitative normality of continuous data was evaluated using the criteria of histograms, rectangular diagrams, and the Shapiro–Wilk's test ($p<0.05$). Quantitative data with a normal distribution are presented as a mean value ± standard deviation. The quantitative continuous data distributed outside the normal distribution were presented as the median and quartile intervals. The categorical data were expressed as a percentage. Freedom from mitral regurgitation >2+, freedom from reoperations and long-term survival in each group were estimated using the Kaplan–Meier method and compared using the Log Rank test. The censored data include patients who had their follow-up terminated. P-values <0.05 are considered statistically significant.

Results

8. Preoperative data: demographics and comorbidities

The study included 202 patients who underwent MV repair for degenerative MR between 1st December 2011 and 1st January 2018 at VUHSK. There were 108 patients in the CS group and 91 patients in the TA group. One patient from the TA group had undergone an aortic valve replacement 9 years ago. The rest of the patients had no history of previous cardiac surgery.

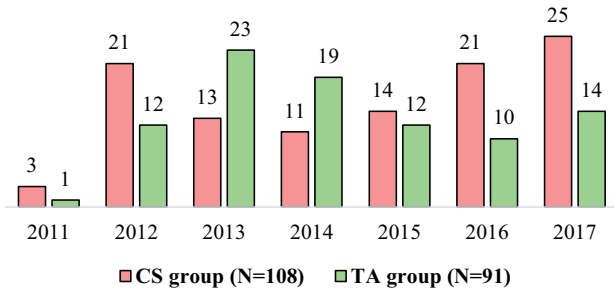
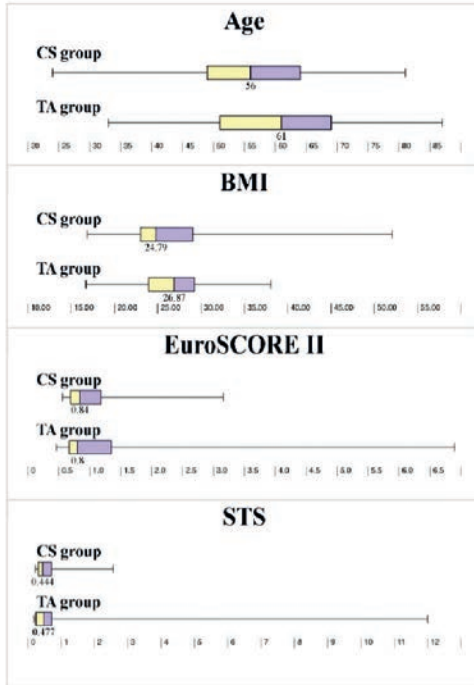


FIGURE 1. Number of procedures performed in each group during the study period



Preoperative characteristics of the patients are shown in Table 1. Patients in the TA group were slightly older (61 y.o. vs. 56 y.o.) and had a slightly higher body mass index (27 vs. 25). Male gender was dominant in both groups.

The preoperative risk was evaluated using STS and EuroSCORE II calculators of preoperative risk. The risk score in both the CS and TA groups was low: 0.44% and 0.48%, respectively, according to STS, and 0.84% and 0.8%, respectively, according to EuroSCORE II, with no significant difference between patient groups.

There were more patients with paroxysmal and chronic atrial fibrillation in the CS group: 21% and 19%, respectively, compared to the TA group: 2% and 3%, respectively ($p<0.001$).

The incidence of severe pulmonary hypertension (>55mmHg) was the same in both groups and was found in 15% of patients.

Preoperative renal function was similar in both groups. The median creatinine level was 80 μmol/l in the CS and 74 μmol/l in the TA group, with a different degree of renal insufficiency (GFR<85 ml/min) revealed in one-third of the patients in both groups.

TABLE 1. Demographics and comorbidities

		CS group	TA group	p value
Patients, n		108	91	
Male gender, n (%)		64 (59 %)	63 (69 %)	0.145
Age (years), median (IQR)		56 (49–64)	61 (51–69)	**0.008**
BMI (kg/m²), median (IQR)		25 (23–29)	27 (24–29)	0.079
BSA (m²), median (IQR)		1.9 (1.8–2.1)	2 (1.8–2.1)	0.315
STS score (%), median (IQR)		0.44 (0.32–0.71)	0.48 (0.24–0.72)	0.138
EuroSCORE II (%), median (IQR)		0.84 (0.69–1.18)	0.8 (0.67–1.35)	0.151
Functional classification	NYHA I, n (%)	2 (1.8%)	5 (5.5%)	0.165
	NYHA II, n (%)	21 (19.4%)	49 (53.8%)	**<0.001**
	NYHA III, n (%)	84 (77.8%)	36 (39.6%)	**<0.001**
	NYHA IV, n (%)	1 (0.9%)	1 (1.1%)	0.903
Paroxysmal AF, n (%)		23 (21%)	2 (2%)	**<0.001**
Permanent AF, n (%)		21 (19%)	3 (3%)	**<0.001**
Pulmonary hypertension (>55 mmHg), n (%)		16 (15%)	14 (15%)	0.911
Creatinine (μmol/l), median (IQR)		80 (68–92)	74 (69–88)	0.353
GFR (ml/min.), median (IQR)		98 (81–119)	98 (79–120)	0.804
GFR <85 ml/min., n (%)		30 (32%)	29 (32%)	0.995
Hypertension, n (%)		42 (39%)	45 (54%)	**0.024**
Coronary artery disease, n (%)		7 (6%)	8 (9%)	0.539
Diabetes, n (%)		5 (5%)	2 (2%)	0.354
Stroke, n (%)		2 (2%)	4 (4%)	0.296
COPD, n (%)		2 (2%)	3 (3%)	0.516
Permanent pacemaker, n (%)		1 (1%)	0	0.357
Previous cardiac surgery, n (%)		0	1 (1%)	0.275

The most common comorbidity in both groups was primary arterial hypertension, with a higher frequency in the TA group (39% vs. 54%). There was no statistically significant difference in the incidence of coronary artery disease, diabetes, chronic obstructive pulmonary disease and stroke. The incidence of these diseases was <10% in each of the groups.

9. Preoperative echocardiographic data

All patients had standard transthoracic and transoesophageal 2D and 3D echocardiography before surgery (Table 2).

The LV ejection fraction was similar in both groups, with a median of 55%. There was also no significant difference in LV systolic or diastolic measurements, as well as LA volume. The anteroposterior diameter of the MV annulus was bigger in the CS group: 39 mm vs. 36 mm (p=0.020). Also, there was a higher incidence of significant tricuspid regurgitation (≥2+) in the CS group: 13% vs. 4% (p=0.036).

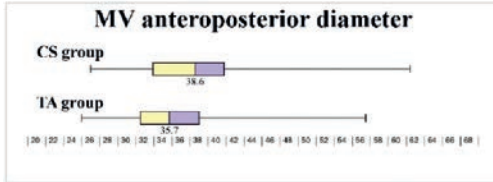


TABLE 2. Preoperative echocardiographic data

		CS group	TA group	p value
LVEF (%), median (IQR)		55 (55–60)	55 (55–55)	0.885
LVEDD (mm), median (IQR)		58 (56–63)	59 (54–63)	0.936
LVESD (mm), median (IQR)		36 (32–40)	35 (31–39)	0.537
LA volume (ml), median (IQR)		127 (102–168)	139 (110–166)	0.455
LA volume index (ml/m²), median (IQR)		68 (56–83)	68 (56–82)	0.904
MV annulus anteroposterior diameter (mm), median (IQR)		39 (34–42)	36 (33–39)	**0.020**
MV annulus mediolateral diameter (mm), median (IQR)		46 (42–52)	45 (42–49)	0.152
MR	3+, n (%)	81 (75%)	65 (71.4%)	0.570
	4+, n (%)	27 (25%)	26 (28.6%)	0.570
TR ≥2+, n (%)		14 (13%)	4 (4%)	0.036

10. Mitral valve pathology and anatomical stratification

The most common cause of MR in both groups was a chordal rupture in one of the MV segments. It was more common in the TA group compared to the CS group: 78% vs. 56% (p<0.001) (Table 3).

TABLE 3. Causes of mitral regurgitation

	CS group	TA group	p value
n	108	91	
Prolapse without chordal rupture	48 (44%)	20 (22%)	**<0.001**
Chordal rupture	60 (56%)	71 (78%)	**<0.001**

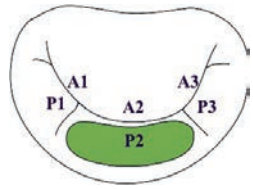
The majority of the patients had pathology of the posterior MV leaflet: 89% in the TA group and 54% in the CS group (p<0.001) (Table 4).

TABLE 4. Location of MV pathology

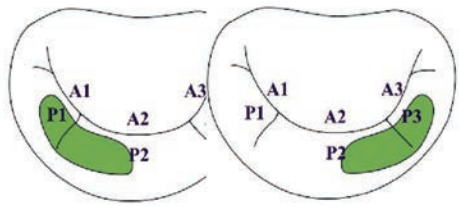
	CS group	TA group	p value
n	108	91	
AMVL	14 (13%)	5 (5%)	0.074
PMVL	58 (54%)	81 (89%)	**<0.001**
AMVL and PMVL	36 (33%)	5 (5%)	**<0.001**

According to the MV pathology location, all patients were stratified into four anatomical types (Figure 2):

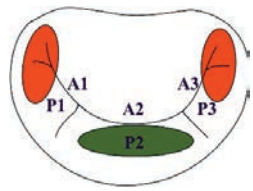
- Type A – isolated P2 prolapse. These patients are ideal for the transapical MV repair. There were 22 (24%) patients of this anatomical type in the TA group and 15 (14%) patients in the CS group.



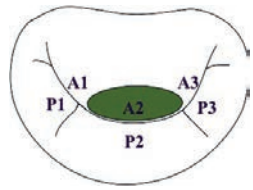
- Type B – P2 and adjacent segments disease. More than half (48 pts - 53%) of the patients in the TA group had this type of MV anatomy and 20 pts (19%) in the CS group.



- Type C – single or bi-leaflet prolapse with the pericommissural segments involved. The majority of patients in the CS group (70 pts - 65%), were stratified as type C. There were 16 pts (18%) of this type in the TA group.



- Type D – isolated A2 prolapse. It was the smallest anatomical group in this study: only 5 (5%) patients in the TA group and 3 (3%) patients in the CS group.



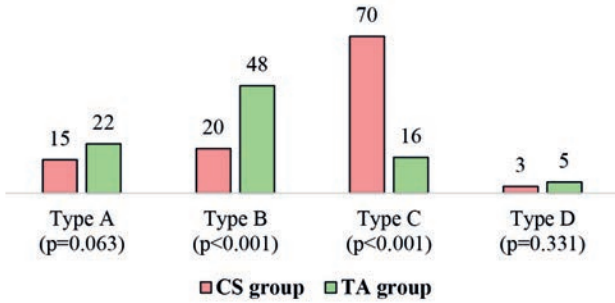


FIGURE 2. Anatomical stratification of the patients

All patients involved in this study were treated at the same centre; they were selected for one of the procedures and underwent surgery during the same timeframe. Therefore, patients with more favourable anatomy (type A and B) were mostly selected for transapical repair and patients with more complex anatomy (type C) – for conventional MV repair.

11. Intraoperative results

Duration of the procedure

The overall duration of the procedure in the TA group was much shorter: 120 min vs. 310 min (p<0.001). In the CS group, CPB time and aortic cross-clamp time were 176 min and 118 min, respectively (Table 5).

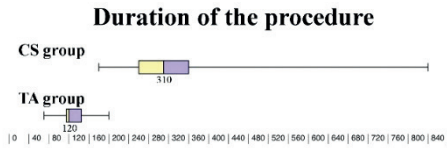


TABLE 5. Duration of the procedure

	CS group	TA group	p value
Duration of the procedure (min.), median (IQR)	310 (260–360)	120 (115–145)	<0.001
CPB time (min.), median (IQR)	176 (149–207)	-	-
Aortic cross-clamp time (min.), median (IQR)	118 (94–147)	-	-

Conventional MV repair and its components

Conventional MV repair was performed through median sternotomy in the majority of cases – 99 (91.7%) patients. In 8 (7.4%) cases it was done through lower partial sternotomy and 1 (0.9%) patient underwent a conventional repair through the right thoracotomy (Table 6).

One of the essential components of modern MV repair, annuloplasty ring, was implanted in 104 (96.3%) patients and the size of the rings ranged from 26 mm to 40 mm (Figure 3). In the rest of the patients, the annuloplasty ring has not been implanted due to a small MV annulus and unfavourable anatomical conditions.

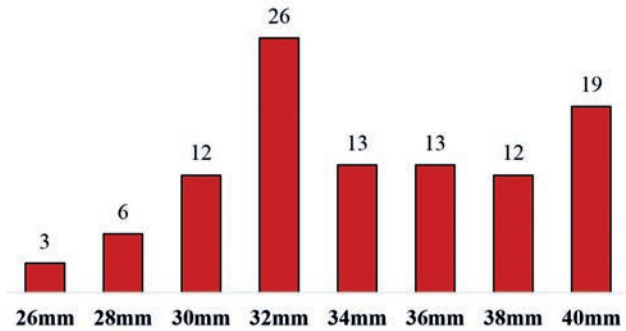


FIGURE 3. Conventional MV repair: range of anuloplasty ring sizes

Artificial ePTFE chords were implanted in 90 (83.3%) patients. Among the other MV repair components, there were triangular and quadrangular leaflet resections, closure of fissures, paracomissural stitches and central Alfieri stitch (Table 6).

TABLE 6. Intraoperative results: conventional MV repair and its components

		n (%)
Approach	Median sternotomy	90 (91.7%)
	Lower partial sternotomy	8 (7.4%)
	Right thoracotomy	1 (0.9%)
Components of the repair	Annuloplasty ring	104 (96.3%)
	ePTFE chords	90 (83.3%)
	Resection of the leaflet	13 (12.5%)
	Closure of fissures	35 (32.4%)
	Paracomissural stitch	18 (16.7%)
	Alfieri stitch (“edge-to-edge” repair)	6 (5.6%)
Repeated cardioplegia		5 (4.6%)
LA appendage occlusion		49 (45.4%)
TV repair	Bicuspidalisation	89 (82%)
	Ring annuloplasty	5 (4.6%)

Intraoperative TOE revealed a significant residual MR in five patients. For that reason, the aorta was cross-clamped again and MV repair was corrected. Systolic anterior motion caused a residual MR in one of the patients and Alfieri stitch was placed to fix it. In another 3 patients, SAM was managed medically.

Forty-nine (45.5%) patients in the CS group also had LA appendage occlusion performed as part of the procedure. TV repair was performed in 94 (87%) cases: ring annuloplasty in 5 (4.6%) patients and bicuspidalisation for the rest of the patients.

Transapical MV repair

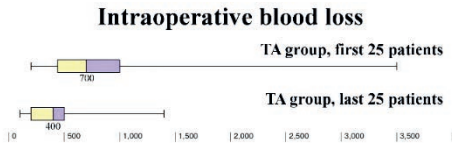
Transapical MV repair was performed through a small left thoracotomy at the level of the LV apex in all patients. Two standard purse-string sutures were placed at the entry site, which was determined by the echocardiographic guidance. After ventriculotomy, a NeoChord DS1000 device with an ePTFE suture was inserted into the LV and pushed towards the MV. All movements of the device as well as deployment of the artificial chordae to the edge of the prolapsing segment were performed under 2D and 3D TEE guidance.

The number of implanted ePTFE chordae ranged from 2 to 7 in the TA group (Figure 4). Two thirds of the patients received 3 or 4 chords. This number was assumed to be optimal for distribution of the load per artificial chord.

FIGURE 4. Number of implanted chords in TA group (N=87)

Transapical MV repair is performed without CPB; thus, it is associated with intraoperative blood loss. Median intraoperative blood loss in this group was 500ml (Table 7). Forty-five (52%) patients needed auto-transfusion of "Cell Caver" recycled blood and a median of 250 ml of washed erythrocytes were transfused. In 11 (12.6%) cases, intraoperative blood loss exceeded 1000 mL. This shows that severe intraoperative bleeding is one of the possible risks of the procedure.

Comparing the first 25 patients and the last 25 patients in the TA group, the median intraoperative blood loss decreased from 700 ml to 400 ml (p=0.001); how-



ever, the rate of blood transfusions remained the same - two patients in each of the groups.

TABLE 7. Intraoperative blood loss in TA group

Intraoperative blood loss (ml), median (IQR)	500 (300–700)
Intraoperative blood loss >1000 ml, n (%)	11 (12.6%)
Re-transfusion of washed erythrocytes (ml), median (IQR)	250 (180–400)
Re-transfusion of washed erythrocytes, n (%)	45 (52%)

Evaluation of intraoperative result: concept of a successful procedure

Intraoperative result of MV repair was evaluated on the basis of MVARC (Mitral Valve Academic Research Consortium) recommendations. According to them, MR reduction is considered optimal when post-procedure MR is reduced to trace or absent. MR reduction is considered acceptable when post-procedure MR is reduced by at least 1 class or grade from baseline and to no more than moderate (2+) in severity.

Mitral repair with an acceptable result in this study was considered to be a successful procedure.

All patients in the CS group underwent successful MV repair and achieved residual MR of less than 2+. In the case of failed MV repair, MV was replaced with a prosthetic valve; these patients were not included in this study.

Transapical MV repair was unsuccessful in 4 (4.4%) cases. Intraoperative conversion to median sternotomy followed by conventional MV repair was performed in two patients. Also, non-acceptable intraprocedural MR >2+ was achieved in two patients. One of the patients who underwent intraoperative conversion was stratified as anatomical type B, while the other three patients were type C.

The aim of this study was a comparison of early (up to 30 days) and late outcomes of successful conventional MV repair and successful transapical MV repair. For that reason, 108 patients from CS group and 87 patients from TA group (Diagram 1) were included for the further calculation of intraoperative and postoperative results.

Intraoperative residual MR

Intraoperative echocardiography revealed completely competent MV or trivial mitral regurgitation in 62.1% of patients after successful transapical repair and in 76.8% of patients in the CS group. Residual MR 1+ was achieved in one-third of

TABLE 8. Intraoperative residual mitral regurgitation

MR	CS group (n = 108)	TA group (n = 87)	p value
0+, n (%)	83 (76.8%)	54 (62.1%)	**0.025**
1+, n (%)	25 (23.2%)	28 (32.2%)	0.159
2+, n (%)	0	5 (5.7%)	
>2+, n (%)	0	2 (2.2%) *	
Intraoperative conversion, n (%)	–	2 (2.2%) *	
* – unsuccessful TA procedures; calculated for 91 pts			

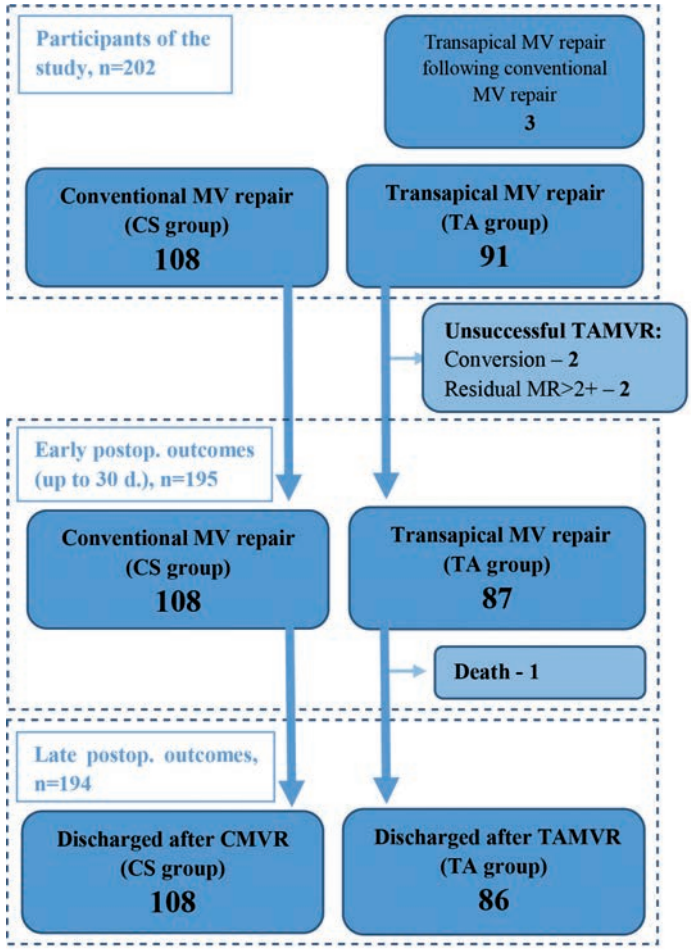


DIAGRAM 1. Patients included in the calculation of early and late postoperative outcomes

patients in the TA group and 23.2% of patients in the CS group. MR 2+ was revealed in five patients after transapical MV repair and an acceptable result was not achieved in two patients (Table 8).

There were two cases where transapical MV repair was converted to the conventional MV repair. In the first case, it happened due to an iatrogenic injury of the posterior MV leaflet caused by the "NeoChord" device. This complication happened only once, in the very first patient of the NeoChord program at VUHSK. In the second patient, despite successful transapical implantation of artificial chords, conversion was performed due to the dehiscence of the chords from the MV leaflet alongside with severe bleeding from the apex of the LV.

12. Early postoperative outcomes

Postoperative bleeding and transfusion of blood products

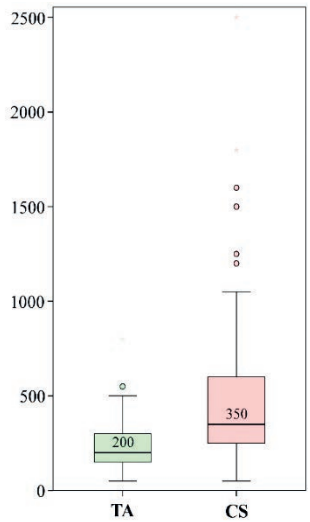


FIGURE 5. Postoperative drainage (ml)

Postoperative drainage was much lower in the TA group - the median was 200 ml vs. 350 ml in the CS group (p<0.001) (Figure 5). Also, surgical wound revision due to postoperative bleeding was less frequent in the TA group: 2.3% vs. 2.8% (Table 9).

One patient from the TA group (the patient was stratified as anatomical type D) underwent surgical re-exploration for severe bleeding on the second postoperative day. Large volume of blood and blood clots was found in the left pleural cavity; however, no obvious source of bleeding was found. The patient had massive transfusion of blood products due to hypovolaemia. After that, the patient developed asystole and direct cardiac compressions were carried out. During CPR, the front wall of the RV ruptured, and the procedure was converted to the median sternotomy. The front wall of the RV was repaired; however, the patient died of acute heart failure. This was the only death in the TA group and among all of the participants in the study.

The rate of blood product transfusions was much lower in the TA group – 5.8% vs. 43.5% (p<0.001). The transfusion of red blood cells was performed in only 4.6% of patients in this group, fresh frozen plasma was used in 3.4% of patients, and thrombocytes were transfused in only one (1.1%) patient (Figure 6).

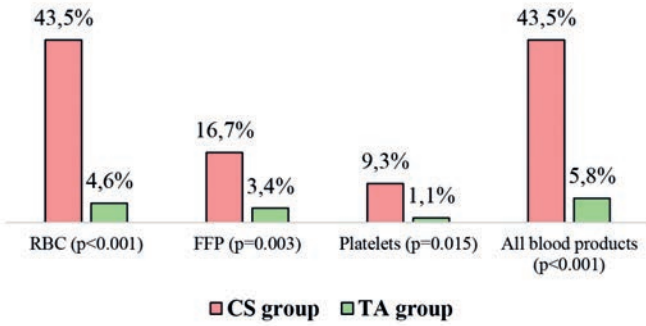


FIGURE 6. Transfusion of blood products

BARC (Bleeding Academic Research Consortium) classification was introduced in 2011 for the evaluation of postoperative bleeding following cardiac surgery. The majority of patients in the TA group (82 pts – 94.3%) were classified as Type 2 (prompting evaluation), 3 (3.4 %) patients as Type 3a (transfusion of blood products), 1 (1.1%) patient as Type 3b (bleeding requiring surgical intervention for control) and 1 (1.1%) patient as Type 5a (fatal bleeding). Transfusions of blood products were required more frequently in the CS group; therefore, more patients (47 pts – 44%) were classified as type 3a compared to the TA group.

Postoperative adverse events

The incidence of new atrial fibrillation and renal failure was much lower in the TA group (Table 9). Compared to the CS group, the rate of new AF was twice as low in the TA group: 37% *vs.* 17.4% (p=0.003). The postoperative elevation of creatinine level above 150% of the baseline was found in 3 (3.6%) patients of the TA group and in 17 (15.7%) patients of the CS group (p=0.006). No patients in the TA group required hemofiltration compared to three (2.8%) patients in the CS group.

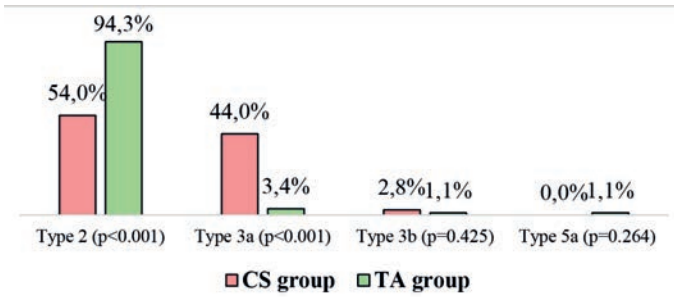


FIGURE 7. Postoperative bleeding: BARC classification

The rate of postoperative stroke in the CS group and the TA group was 1.9% and 1.1%, respectively. The patient who had a stroke post-transapical repair developed speech difficulties on the day after the operation, but he had completely recovered prior to discharge.

TABLE 9. Postoperative adverse events

Adverse event	CS group (n = 108)	TA group (n = 87)	p value
Surgical wound re-exploration for bleeding, n (%)	3 (2.8%)	2 (2.3%)	0.833
New AF, n (%)	40 (37%)	15 (17.4%)	**0.003**
Renal failure (creatinine elevation 150% and above from baseline), n (%)	17 (15.7%)	3 (3.6%)	**0.006**
Haemofiltration, n (%)	3 (2.8%)	0	0.117
Stroke, n (%)	2 (1.9%)	1 (1.1%)	0.692
Myocardial infarction, n (%)	0	0	
Wound infection, n (%)	1 (0.9%)	0	0.368
Mortality, n (%)	0	1 (1.1%)	0.368
PPM insertion, n (%)	13 (12%)	2 (2.3%)	**0.011**

None of the patients in this study had postoperative myocardial infarction. There was one patient who had superficial (above sternum) wound infection following conventional MV repair. There were no wound infections in the TA group.

Permanent pacemakers (PPM) were implanted in 13 (12%) patients following conventional repair and 2 (2.3%) patients in the TA group. Both patients from the TA group had a PPM implanted for preoperatively known sick sinus syndrome.

Postoperative course

Inotropic support postoperatively was required in almost all, 106 (98%) patients following conventional MV repair and in 22 (25.3%) patients in the TA group ($p<0.001$).

The duration of postoperative mechanical ventilation in the TA group was much shorter – 4 hours vs. 7 hours. Besides that, postoperative stay in ICU in this group was also shorter – 22 hours vs. 67 hours ($p<0.001$), as well as postoperative in-hospital stay – 8 days vs. 12 days ($p<0.001$) (Table 10, Figures 8-10).

FIGURE 8. FIGURE 9. FIGURE 10.

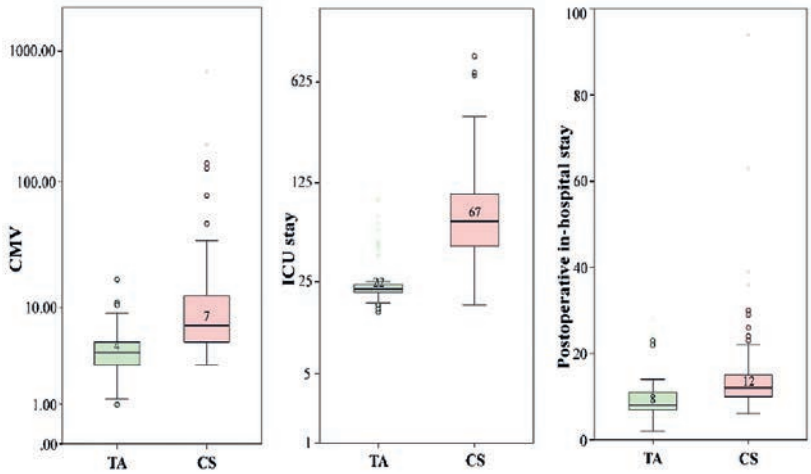


FIGURE 8. CMV duration (hours)

FIGURE 9. ICU stay (hours)

FIGURE 10. Postoperative in-hospital stay (days)

TABLE 10. Postoperative course

	CS group	TA group	p value
Inotropic support, n (%)	106 (98%)	22 (25.3%)	<0.001
IABP, n (%)	7 (6.5%)	0	0.015
ECMO, n (%)	1	0	0.368
CMV (h), median (IQR)	7 (5–13)	4 (3–5)	**<0.001**
Length of ICU stay (h), median (IQR)	67 (45–112)	22 (21–24)	**<0.001**
Post-procedure length of stay in hospital (days), median (IQR)	12 (10–15)	8 (7–11)	**<0.001**

13. Late postoperative outcomes

Postoperative follow-up

Follow-up visits for all patients were scheduled at 1, 6, 12 months and then every 12 months after surgery. All patients who were discharged from the hospital were involved in the postoperative follow-up: 108 pts in the CS group and 86 pts in the TA group. The median follow-up was similar in both groups: 34 months (IQR 19–64 months) in the CS group and 39 months (IQR 20–51 months) in the TA group. The follow-up was complete for 101 (94%) patients in the CS group and 82 (95%) in the TA group.

Long-term durability of the repair

Long-term durability of the repair in each group was assessed using the Kaplan–Meier method and compared using Log Rank test.

Long-term durability of the repair was considered to be good if MR did not exceed 2+. A comparison of the long-term durability of the MV repair between the two groups is presented in Figure 11. The long-term durability of the conventional MV repair was much better comparing to transapical MV repair. MR did not exceed 2+ in 99%, 97%, 97%, 95%, 92%, 90%, and 90% of the patients at 6, 12, 24, 36, 48, 60, and 72 months after surgery respectively. In the TA group, freedom from MR>2+ was 82%, 77%, 69%, respectively, and 68% for the remaining period of the follow-up. These data were obtained regardless of the anatomical stratification within the groups. The difference of long-term durability was statistically significant between the two groups (χ2 - 19.214, p=0.000).

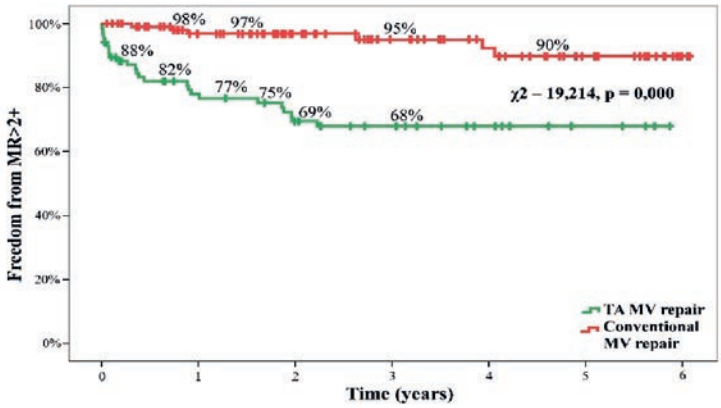


FIGURE 11. Long-term durability of the MV repair: TA and CS groups

There was a significant difference in long-term durability of the repair in both groups, therefore, long-term durability was compared between the CS group and each of the anatomical types in the TA group. Splitting the results of the CS group according to the anatomical types showed no major difference between the groups (χ2 - 0.106, p=0.991); for that reason, the results of the entire CS group were evaluated without grouping. The results are shown in Figures 12 and 13.

The best results were achieved in the group of anatomical type A– freedom from MR>2+ dropped down to 85% over first 6 months; later, this number remained stable throughout the observation period.

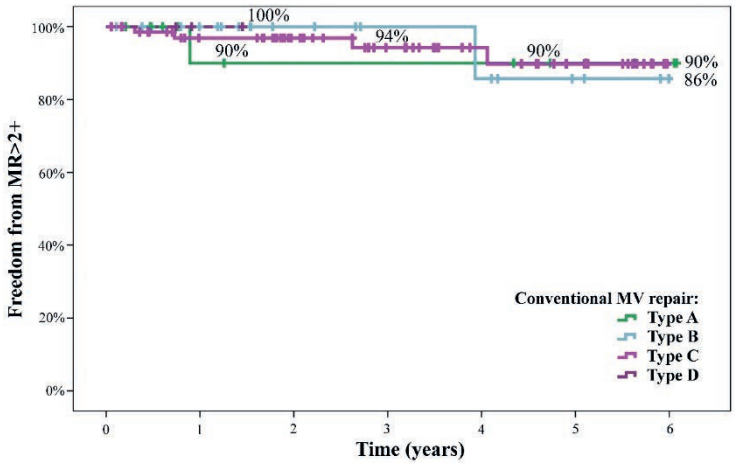


FIGURE 12. Long-term durability of the MV repair: CS group, anatomical types

The long-term durability was worse in the group of anatomical type B: freedom from MR>2+ was observed in 87% after 6 months, in 80% after a year, and 74% from 24 months until the end of the observation.

The results of anatomical group C, compared to groups A and B, are the worst. Severe recurrent MR was revealed in about half of the patients after six months, and freedom from severe MR was only 23% after 24 months.

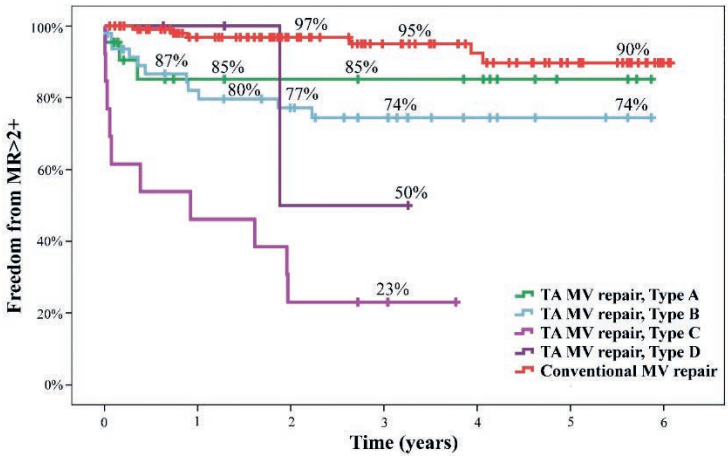


FIGURE 13. Long-term durability of the MV repair: CS and TA groups (anatomical types of the TA group shown separately)

There were only four patients in anatomical group D. Severe MR recurred in one of them. Due to the small number of patients, the long-term durability of the repair in this group was not analysed further.

The long-term durability of the repair in the CS group and different anatomical types of the TA group was compared using the Log Rank test. The results are shown in Table 11. A statistically significant difference was obtained between the CS group and anatomical types B, C and D.

TABLE 11. Comparison of the long-term durability between the groups

Compared groups	Log Rank test
CS vs. Type A (TA)	($\chi 2$ – 1.646, p = 0.200)
CS vs. Type B (TA)	($\chi 2$ – 10.736, **p = 0.001**)
CS vs. Type C (TA)	($\chi 2$ – 78.788, **p = 0.000**)
CS vs. Type D (TA)	($\chi 2$ – 4.961, **p = 0.026**)
Type A (TA) vs. Type B (TA)	($\chi 2$ – 0.483, p = 0.487)

The presented results show that the long-term durability of the repair in type C group is very poor; therefore, TA repair should not be performed in these patients. Group D was not analysed further due to the small number of patients. For the reasons mentioned above, long-term repair durability was further compared between the CS group and the two best anatomical types of the TA group – A and B. Long-term durability for these types is shown separately in Figure 14. The combined

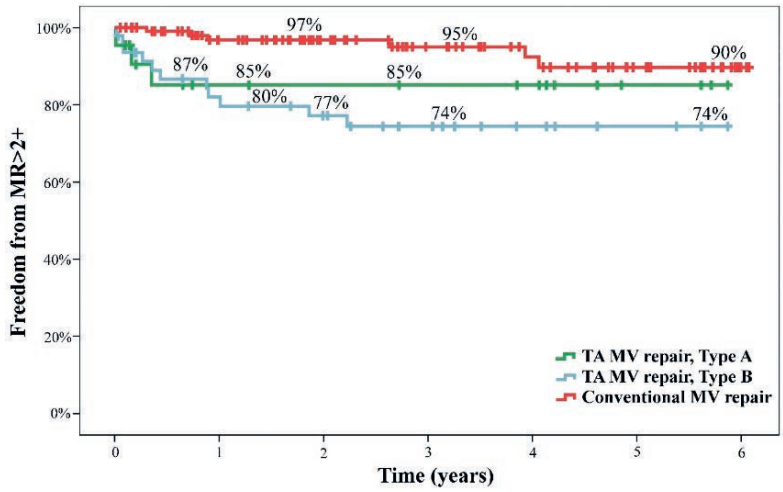


FIGURE 14. Long-term durability of the MV repair: CS and TA groups (Types A and B of the TA group shown separately).

results of groups A and B are shown in Figure 15. Freedom from severe MR in the combined A and B group remained 81% after 12 months and 77% after 28 months. It remains unchanged until the end of the observation.

The difference in long-term durability between the CS group and the TA group (combined results of the types A and B) was statistically significant ($\chi 2$ – 9.006, p = 0.003).

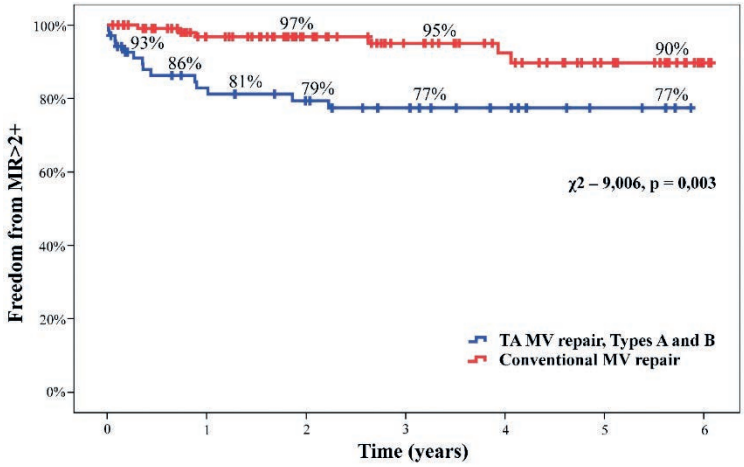


FIGURE 15. Long-term durability of the MV repair: CS and TA groups (Types A and B of the TA group are shown combined).

Rehospitalisation and its causes

One of the MVARC-recommended indicators of successful MV pathology treatment is rehospitalisation of the patient during the first 12 months after surgery. Suggested classification of the rehospitalisation causes includes three groups:

- Heart failure hospitalisation, cardiac-related or non-cardiac-related (for example, recurrence of MR);
- Other cardiovascular hospitalisation (for example, cardiac arrhythmias);
- Non-cardiovascular hospitalisation.

About 10% of the patients in each group were readmitted to hospital within the first 12 months after the procedure; however, the causes of rehospitalisation were different.

Seven (8.1%) patients after transapical MV repair were readmitted due to the recurrence of high-grade MR and 4 of them underwent surgical interventions during the same rehospitalisation. Only one (0.9%) patient was readmitted for that reason in the CS group and he underwent redo mitral surgery.

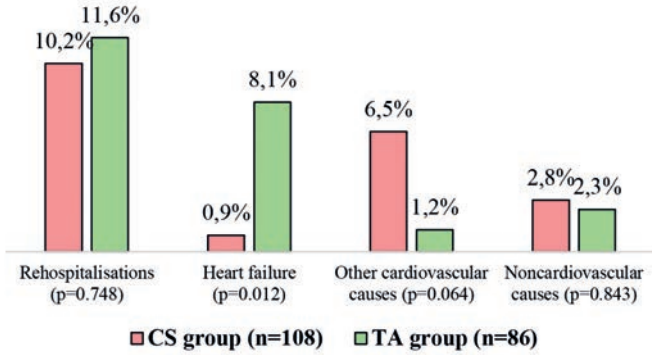


FIGURE 16. Causes of rehospitalisation

Atrial fibrillation and other rhythm disturbances were the main cause of rehospitalisation in the CS group, with 7 (6.5%) patients, while only one (1.2%) patient had this issue in the TA group.

The number of rehospitalisations for non-cardiovascular reasons was similar in both groups: 3 (2.8%) patients in the CS group and 2 (2.3%) in the TA group. Two patients were rehospitalised due to a wound infection several months after the conventional MV repair, while the third patient in this group was readmitted due to neurological deterioration (dizziness). One patient in the TA group underwent thyroid surgery and another patient was treated for cholangitis.

Recurrent MR and redo surgery: CS group

High-grade recurrent MR was revealed in 6 (5.5%) patients after conventional MV repair. Four of them underwent repeat surgical procedure: one underwent conventional MV repair and 3 patients were treated with transapical MV repair. Two other patients are currently under medical management (Diagram 2).

Recurrent MR and redo surgery: TA group

TA repair was unsuccessful in 4 patients. Also, there was one early postoperative death in this group. Therefore, 86 patients were discharged from the hospital out of the initial group of 91 patients. Twenty-two patients were stratified as anatomical type A, 47 as type B, 13 as type C and 4 as type D. Severe recurrent MR was revealed in 25 patients: 3 patients were categorised as anatomical type A, 11 as type B, 10 as type C and 1 as type D.

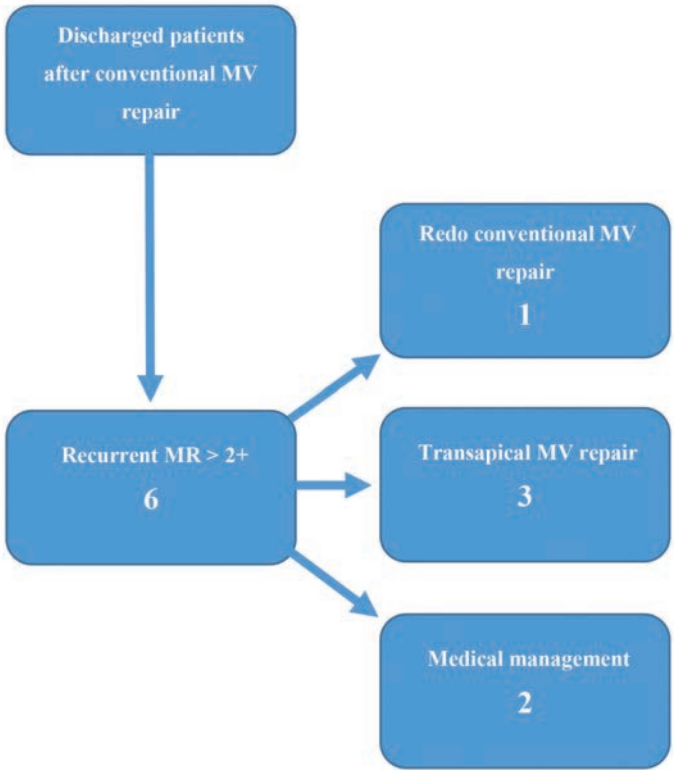


DIAGRAM 2. Management of the recurrent MR: CS group

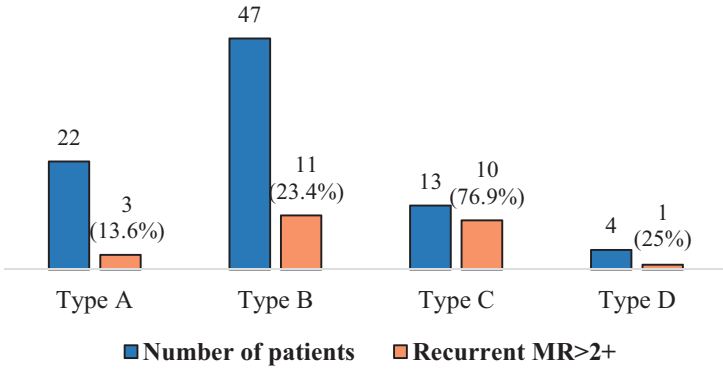


FIGURE 17. Recurrent MR: TA group, anatomical types

TABLE 12. Recurrent MR: comparison of the anatomical types of the TA group

Groups in comparison	p value
A vs. B	0.347
A vs. C	**<0.001**
A vs. D	0.562
B vs. C	**<0.001**
B vs. D	0.942
C vs. D	0.057

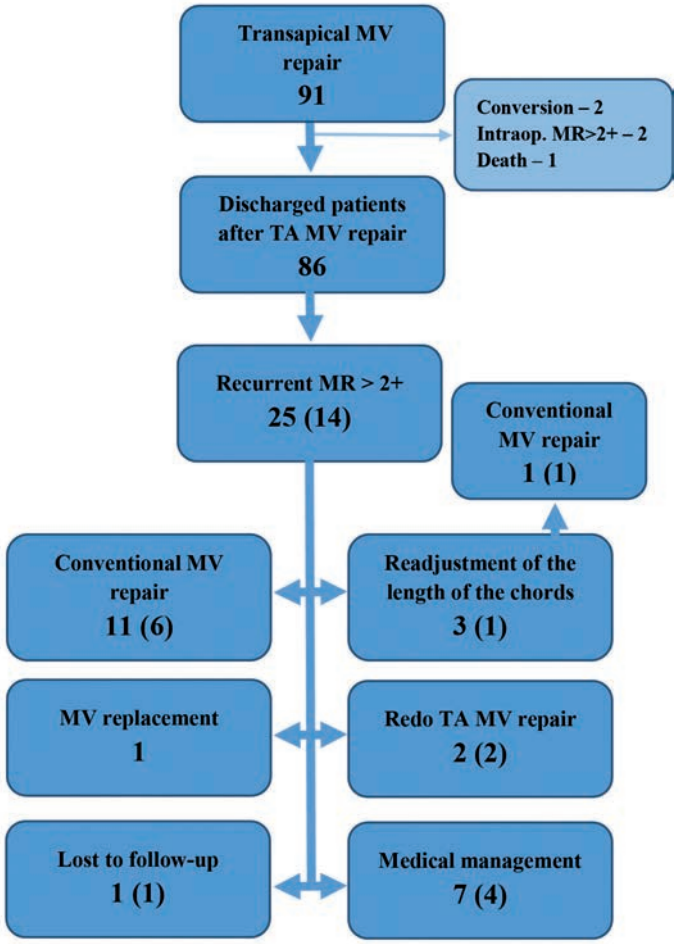


DIAGRAM 3. Management of the recurrent MR in the TA group (number of type A and B patients is shown in brackets).

Eleven patients in the TA group underwent conventional MV repair for severe recurrent MR. Also, one patient had MV replaced with a prosthetic valve. The length of the artificial chords was readjusted in 3 patients and redo transapical repair was performed in two patients. One of the patients who underwent length readjustment of the chords developed severe recurrent MR again and underwent conventional MV repair later. Seven patients were managed medically. One patient was lost to follow-up; therefore, the further course of his treatment is unknown.

The diagrams above show that not all patients with a recurrent MR were re-operated upon. Repeat surgery was performed on 4 out of 7 patients with recurrent MR in the CS group and in 17 out of 25 patients in the TA group. Nine out of 14 patients of type A and B in the TA group were re-operated upon. Figures 18 and 19 show the results of the CS and TA group (combined type A and B): the green curve shows freedom from severe MR, and red – freedom from reoperations. This difference was not statistically significant in either the CS ($\chi 2$ – 1.568, p=0.210) or in the TA group ($\chi 2$ – 1.120, p=0.290).

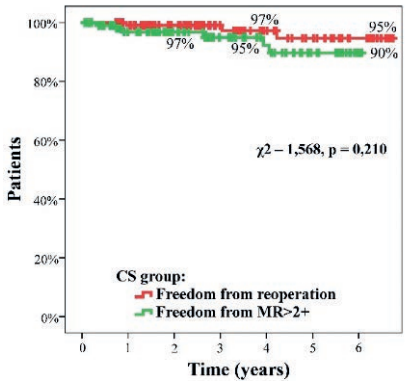


FIGURE 18. Long-term durability of the repair and reoperations: CS group

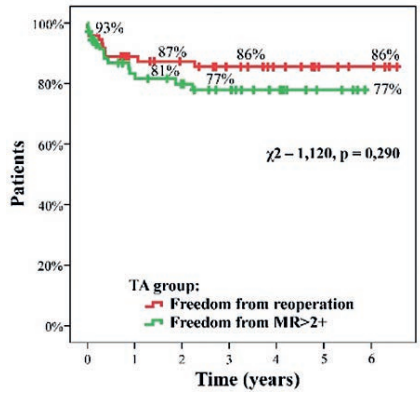


FIGURE 19. Long-term durability of the repair and reoperations: TA group (Type A and B)

Causes of the MR recurrence

MR recurrence in the CS group was caused by mitral prolapse due to a new rupture of the native chords. This diagnosis was confirmed mostly by echocardiography because there was only one patient who underwent a repeat surgery in this group.

The main causes of the MR recurrence in the TA group are listed in Table 13. Although sometimes recurrent MR was caused by a combination of factors, few main groups of causes can be identified:

- Relative elongation of the artificial chords. This most likely happens due to the reverse remodelling of the LV. There were 3 patients in the TA group who underwent readjustment of the length of the chords.
- Rupture of the native chords. Rupture of the AMVP chords was the most common in the TA group. The rupture could be spontaneous or induced by mechanical interactions with artificial chords.

TABLE 13. Recurrent MR causes in the TA group

Patient	Type	MR mechanism	Re-intervention
1	C	Relative elongation of the artificial chords	Conventional MV repair
2	C	Rupture of the native chords (P1)	Conventional MV repair
3	C	Relative elongation of the artificial chords	Conventional MV repair
4	B	Relative elongation of the artificial chords	None
5	C	Relative elongation of the artificial chords	Length readjustment
6	C	Leaflet restriction	None
7	C	Rupture of the artificial chords.	Conventional MV repair
8	B	Rupture of the native chords (AMVL) Dehiscence of the artificial chords	Conventional MV repair
9	B	Rupture of the artificial chords.	None
10	B	Rupture of the artificial chords.	Conventional MV repair
11	B	Relative elongation of the artificial chords	Length readjustment. Later – conv. MV repair
12	B	Rupture of the artificial chords.	Conventional MV repair
13	C	Relative elongation of the artificial chords	Length readjustment
14	C	Rupture of the artificial chords.	MV replacement
15	D	Relative elongation of the artificial chords	None
16	C	Rupture of the native chords (AMVL)	Conventional MV repair
17	B	Rupture of the native chords (AMVL)	None
18	B	Rupture of the artificial chords.	Conventional MV repair
19	A	Rupture of the native chords (AMVL)	Transapical MV repair
20	B	Rupture of the native chords (AMVL)	Lost to follow-up
21	B	Rupture of the native chords (AMVL)	Conventional MV repair
22	B	Rupture of the native chords (AMVL)	Transapical MV repair
23	A	Rupture of the artificial chords.	None
24	C	Rupture of the artificial chords.	None
25	A	Rupture of the native chords (AMVL)	Conventional MV repair

- Rupture of the artificial chords. Artificial chord ruptures in the TA group happened both in the middle part and at the level of the apex of the ventricle. This was most likely caused by mechanical damage of the chord during the implantation.
- Leaflet restriction caused a recurrent MR in one patient.
- Dehiscence of the artificial chords from the edge of the leaflet occurred in 2 patients. However, rupture of the native chords was also diagnosed in both patients and it remains unclear which of the MR causes was the primary one.

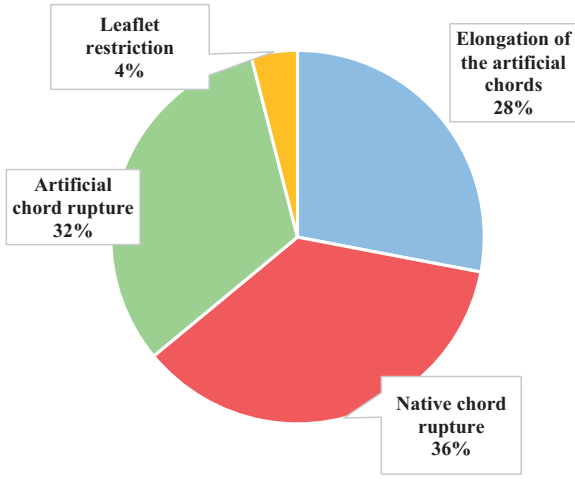


FIGURE 20. Causes of the MR recurrence in the TA group

Mortality and causes of death

No patients died in the CS group postoperatively.
There were 3 late deaths in the TA group:

- A 50-year-old patient died 32 months after TA repair. His initial mitral pathology was classified as anatomical type A. The patient had liver cirrhosis and died from hepatic failure. According to the latest echocardiography, his MR was <1+.
- A 64-year-old female died 34 months after the procedure. She was known to have type C mitral pathology. A recurrent MR was diagnosed 2.5 years after the transapical repair; for that reason, she underwent conventional MV repair, however, she died of sepsis and multiple organ failure 4 weeks later.
- A 72-year-old male died 20 months after the procedure. He was stratified as anatomical type C patient and had a successful transapical repair, how-

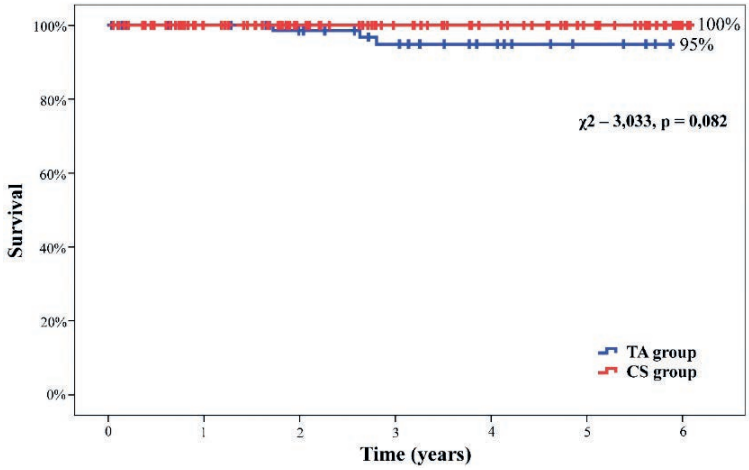


FIGURE 21. Survival: CS and TA groups

ever, a severe recurrent MR was revealed 1 month later. For that reason, he underwent MV replacement with a tissue valve 14 months later. After 4 months, he was readmitted to the hospital with an infective endocarditis of the prosthetic valve. Also, spontaneous intracerebral haemorrhage was also diagnosed. The patient was managed medically and died 1 month later.

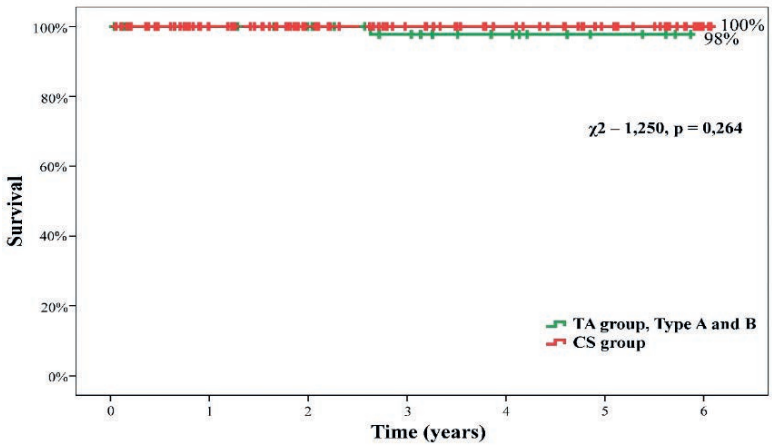


FIGURE 22. Survival: CS and TA (Type A and B) groups

The first death was classified as non-cardiac, and the other two were cardiac related. The survival of patients in the CS and TA groups is presented in Figure 21. The difference between survival in both groups was not statistically significant ($\chi 2$ – 3.033, $p = 0.082$).

The survival in the CS and TA group (combined types A and B only) after 6 years was 100% and 98%, respectively (Figure 22).

14. Transapical mv repair following previous cardiac surgery

The TA group included one patient who had a history of previous cardiac surgery – aortic valve replacement with a 21mm tissue valve 9 years ago. Eight years later, severe MR due to the rupture of P2 native chords was revealed. Mitral pathology was stratified as anatomical type A. The patient underwent successful transapical implantation of two artificial chords to the prolapsing P2 segment and 4 months later echocardiography showed MR 1+.

Severe MR recurred in 7 patients after conventional MV repair. Four of them underwent repeat surgical procedures: conventional repair was performed in one patient and 3 patients had transapical repair. The last three patients formed a small group which was separated from the main TA group, because these patients had previous MV reconstruction and had an annuloplasty ring implanted. A brief overview of the management of these patients is presented below:

1. A 35-year-old female underwent conventional MV repair for the myxomatous mitral degeneration five years ago: P2 and A2 segments were re-suspended with four artificial chordae and a 36mm annuloplasty ring was implanted. Despite the patient remaining asymptomatic a recurrence of severe mitral regurgitation (MR) was diagnosed at routine follow-up four years later. Echocardiography revealed A3 segment native chordae rupture. The patient underwent a successful transapical off-pump implantation of 3 neochordae into the A3 segment with a residual mild MR seen on intraoperative TEE. Although the patient had 1 unit of red blood cells transfused due to anaemia, her further postoperative course was rather uneventful and she was discharged on the 7th postoperative day. At the 10-month follow-up, transthoracic echocardiography showed no change in residual MR

2. A 35-year-old male underwent conventional MV repair for the bi-leaflet prolapse of myxomatous valve 3 years ago: segments A1-A3, P2 and P3 were re-suspended with six artificial chordae and 40mm annuloplasty ring was implanted. Two years later a severe MR due to new P2 (lateral) prolapse was revealed on follow-up TTE. The patient underwent successful transapical off-pump implantation of 2 neochordae into the prolapsing segment resulting residual trivial MR. The patient underwent re-exploration for bleeding postoperatively and had a fresh frozen plasma

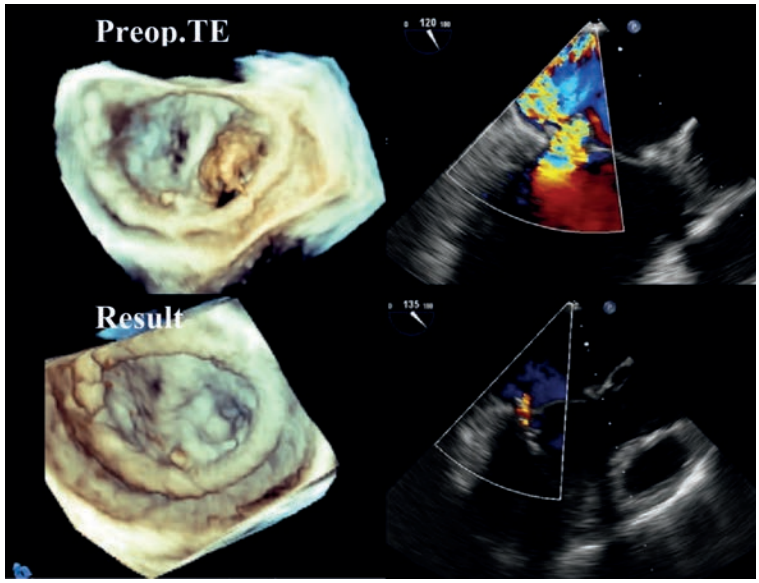


FIGURE 23. Transapical MV repair following failure of conventional MV repair (1st patient)

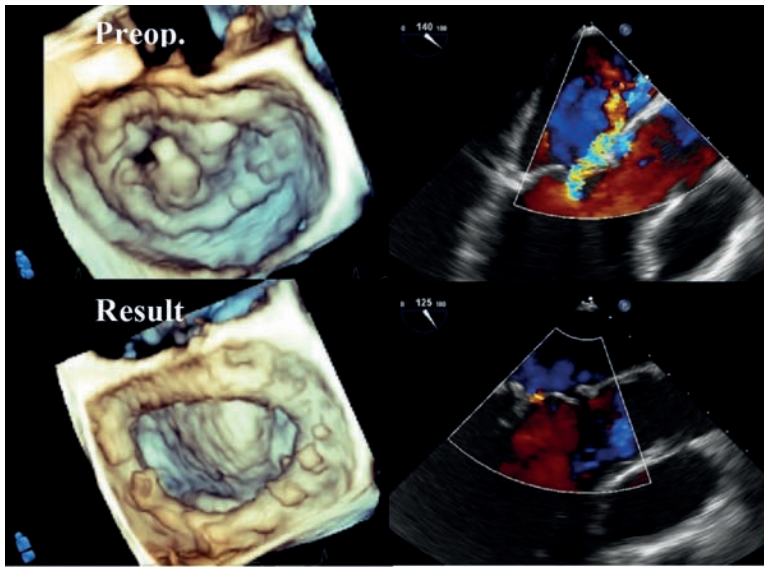


FIGURE 24. Transapical MV repair following failure of conventional MV repair (2nd patient)

transfusion. His further postoperative course was uneventful and he was discharged from the hospital on the 9th postoperative day. At the 1-month follow-up, transthoracic echocardiography showed trivial residual MR.

3. A 59-year-old male underwent conventional MV repair for the P2 prolapse caused by chordal rupture 3 years ago: the P2 segment was re-suspended with three artificial chordae and a 36 mm annuloplasty ring was implanted. Two years later the patient started to complain of shortness of breath and ecochardiography showed severe recurrent MR due to the prolapse of the middle of the P2 segment. A successful transapical repair was performed: two artificial chords were deployed into the prolapsing segment and a trivial MR was achieved. The patient underwent re-exploration for postoperative bleeding on day 2. The further course of treatment was uneventful and the patient was discharged on day 12. At the 4-month follow-up, transthoracic echocardiography showed mild MR.

Discussion

This study presents and compares the intraoperative, early and late postoperative results of conventional and transapical MV repair.

Conventional MV repair is a standard method to treat degenerative mitral regurgitation. Since the 1980s, the use of ePTFE artificial chords has become a standard tool in modern cardiac surgery and is one of the most commonly used components of the repair. MV repair, performed using artificial chords, is associated with good long-term durability and good haemodynamic parameters of the reconstructed valve.

In order to reduce the invasiveness of the procedure and number of CPB-related adverse events, a concept of transapical MV repair was developed. There are many publications presenting the late outcomes of the conventional MV repair, but the majority of publications about transapical repair present only intraoperative and early postoperative results. The longest long-term results were reported by Colli et al. (2018): the publication presents European experience of the transapical mitral repair with 12 months postoperative follow-up. This dissertation presents a much longer follow-up duration of the TA group – 39 months, which is important for proper assessment of the long-term durability of the repair. In addition, there are no publications comparing the outcomes of conventional and transapical MV repair. It is announced that a randomised study RECHORD has been launched aiming to compare the results of these two techniques, but no data have been published to date.

Intraoperative results of the transapical MV repair published by Colli et al. (2018) are similar to those presented in this study: the duration of the procedure

is about 120 minutes, three or four artificial chords were implanted in two thirds of the patients, about 2% of patients underwent a conversion to conventional MV repair; the procedure was successful in about 96% of patients. There were no intraoperative deaths in either the group of Colli et al. or in the TA group of this study. This shows that transapical MV repair is a short, effective and safe procedure. However, this study showed that intraoperative residual MR<1+ was achieved more often in the CS group: 76.8% vs. 62.1% (p=0.025). This most likely occurred due to the wider choice of components in the conventional repair, like annuloplasty rings, paracomissural stitches, closure of the leaflet fissures and others. In the majority of patients in the CS group, the tricuspid valve was repaired as well.

It is important to note that in the evaluation of MV repair effect, this study followed the recommendations of the MVARC, according to which the intraoperative result is considered acceptable if the MR is reduced by at least one grade and postoperative MR does not exceed 2+. However, in practice, such a result after conventional MV repair is generally considered unacceptable, and further valve reconstruction or valve replacement is performed.

One of the most relevant variables of the early postoperative period is postoperative bleeding and the incidence of blood product transfusions. The postoperative drainage in the TA group was significantly lower comparing to the CS group (200 ml vs. 350 ml (p<0.001)), and most of the patients in the TA group were classified as type 2 according to the BARC classification. Red blood cell transfusion was required in only 4.6% of the patients, and this figure is almost twice as low as that given in the article of Colli et al. (2018) - 8%. Red blood cell transfusion was much more common in the CS group, with 43.5% of patients, while the most commonly reported incidence is about 50% after conventional MV repair and about 40% after minimally invasive MV surgery.

Recent publications show that surgical re-exploration for postoperative bleeding following minimally invasive MV surgery is necessary in 3.5-7% cases and the rate is similar to that of conventional MV repair. The reported rate of re-explorations after transapical MV repair is lower – 1.4% of patients. In this study, re-exploration for bleeding was performed in 2.3% of patients in the TA group and slightly more often (2.8%) in the CS group. Low postoperative drainage, and a low rate of transfusions and re-explorations indicate that the risk of postoperative bleeding after transapical MV repair is low.

This study also confirms that one of the positive facts associated with the transapical repair is a lower rate of postoperative atrial fibrillation: 17.4% vs. 37% in the CS group. Colli et al. (2018) reported an incidence of this adverse event of 22.5% in his group of patients, while the rate reported by Samalavicius et al. (2017) was lower - 12%. Permanent pacemakers were also much less frequently implanted in

the TA group: 2.3% vs. 12%. Both pacemakers in the TA group were implanted for preoperatively known sick sinus syndrome.

The lower incidence of PPM implantation in the TA group could be explained by the fact that all manipulations are carried out further away from the locations at which the heart conductive system could be damaged. Similarly, Colli et al. reported only one patient who had a postoperative myocardial infarction and none of the patients in this study had this complication, because manipulations are carried out further away from major coronary arteries, so the risk of damaging them is low.

The incidence of postoperative renal failure was almost 4 times higher in the CS group, with 2.8% of the patients requiring haemofiltration postoperatively. Recent publications report rate of the latter adverse event following mitral surgery to be less than 3% (131,132). None of the patients in the TA group needed haemofiltration.

One of the most commonly reported CPB-related adverse events is stroke. Various authors report the rate of neurological complications ranging from 1% to 2.8%. In this study, this complication occurred in 1.9% of the patients in the CS group. One patient had a transitory speech disorder following transapical MV repair, but the patient completely recovered before he was discharged from the hospital.

Publications reporting the outcomes of transapical MV repair often emphasise that this is a minimally invasive procedure, which significantly reduces the duration of postoperative mechanical ventilation, length of stay at the intensive care unit and the length of postoperative in-hospital stay. Kavakli et al. (2016) reports these indicators to be 2.6 hours, 19.8 hours and 5 days, respectively, while Samalavicius et al. (2017) reported 4 hours, 22 hours and 8 days, respectively, and Colli et al. (2018) reported 3 hours and 8 days (duration of stay in the intensive care unit is not provided). This study showed similar results: 4 hours, 22 hours and 8 days, and it was much shorter compared to the CS group: 7 hours, 67 hours and 12 days.

In summary, most of the early postoperative results in the TA group are better and adverse events following transapical repair are rare, which shows safety of this treatment method. Patients recover from the operation faster and they leave the hospital earlier. This method is effective: acceptable intraoperative results were achieved in almost all patients. However, analysis of the late outcomes shows that long-term durability of the repair is better in the CS group, even when compared only with the results of the two best anatomical types of the TA group: freedom from severe MR was 90% after 5 years in the CS group, 85% for the type A patients and 74% for the type B patients in the TA group. The long-term durability was very poor in type C patients: it is only 23% after 2 years. Therefore, transapical MV repair should not be performed in type C patients.

Initial mitral valve anatomy was different in both groups. There were more type C patients in the CS group, while the majority of patients in the TA group were clas-

sified as types A and B. However, long-term durability was very similar in different anatomical types of the CS group, which shows that anatomical type is not relevant for the conventional repair. For this reason, the results were compared between the entire CS group and different anatomical types of the TA group. The statistical reliability of a direct comparison between the anatomical types in different patient groups would be much lower due to the significantly lower number of patients.

It should also be noted that cases of MR recurrence in the CS group are evenly distributed throughout the postoperative follow-up period. Meanwhile, the majority of MR recurrence events in the TA group are concentrated within the first 6 months after the procedure. Then, the incidence of repair failure becomes much lower and a "plateau" phase can be seen after 6 months in group A and after 24 months in group B when new events of failure no longer occur.

This study shows the safety and efficacy of transapical MV repair in the early postoperative period. Although this treatment method is associated with a lower incidence of transfusions and arrhythmias compared to conventional techniques, it also has a higher rate of failure of the repair, especially during the first 24 months.

Transapical MV repair is a completely new approach, which has been refined both technically, for example by changing the location of ventriculotomy in the TACT study, and theoretically, developing patient selection criteria and categorising patients into anatomical types. The main difference between conventional and transapical repair is that MV annuloplasty is not performed in the latter approach. Therefore, combining transapical procedures with endovascular or other minimally invasive annuloplasty could in theory improve the long-term durability of this repair. Currently, the thorough selection of patients based on characteristics of the mitral anatomy remains crucial for improving the long-term results of transapical mitral repair.

Findings

1. Transapical MV repair is an effective and safe method of treatment of degenerative mitral regurgitation characterised by the lower incidence of postoperative adverse events in the early postoperative period compared to conventional MV repair.
2. The long-term durability of transapical MV repair is statistically significantly poorer compared to the results of conventional MV repair (long-term durability of transapical MV repair (types A and B) was 77%, while durability of conventional MV repair – 90%).

3. The long-term durability of the conventional MV repair does not depend on the anatomical type of mitral pathology. The best long-term durability of transapical MV repair is achieved in patients with isolated P2 segment prolapse or pathology of the P2 segment and adjacent areas. Patients with MV pathology not limited to the P2 segment and adjacent areas should not undergo transapical MV repair due to poor long-term outcomes.
4. The recurrence of mitral regurgitation after transapical MV repair is usually caused by artificial and native chord rupture, as well as the relative elongation of artificial chords. The main cause of recurrent mitral regurgitation after conventional MV repair is the new rupture of native chords.

Practical recommendations

1. Transapical MV repair can only be performed in patients with a mitral valve pathology of anatomical type A or B.
2. Patients with MV pathology not limited to the P2 segment and adjacent areas should undergo conventional MV repair.
3. In order to improve the long-term durability of transapical MV repair, a thorough preoperative selection of patients based on echocardiographic data and anatomical characteristics of mitral valve is necessary.

List of publications

Samalavicius RS, Norkiene I, Drasutiene A, Lipnevicius A, Janusauskas V, Urbonas K, Zakarkaite D, Aidietis A, Rucinskas K. Anesthetic Management and Procedural Outcomes of Patients Undergoing Off-Pump Transapical Implantation of Artificial Chordae to Correct Mitral Regurgitation: Case Series of 76 Patients. Anesth Analg. 2018 Mar;126(3):776–784. doi: 10.1213/ANE.0000000000002767.

Colli A, Manzan E, Aidietis A, Rucinskas K, Bizzotto E, Besola L, Pradegan N, Pittarello D, Janusauskas V, Zakarkaite D, Drasutiene A, Lipnevicius A, Danner BC, Sievert H, Vaskelyte L, Schnelle N, Salizzoni S, Marro M, Rinaldi M, Kurnicka K, Wrobel K, Ceffarelli M, Savini C, Pacini D, Gerosa G. An early European experience with transapical off-pump mitral valve repair with NeoChord implantation. Eur J Cardiothorac Surg. 2018 Sep 1;54(3):460–466. doi: 10.1093/ejcts/ezy064.

SANTRAUKA

Įvadas

Tiriamoji problema, darbo aktualumas ir reikšmė

Degeneracinės kilmės mitralinio vožtuvo nesandarumas (MVN) yra labiausiai paplitusi širdies vožtuvų patologija išsivysčiusiose šalyse ir yra nustatomas 2–3 % populiacijos. Pagrindinis nesandarumo mechanizmas yra mitralinio vožtuvo (MV) burių ir povožtuvinio aparato morfologiniai pakitimai ir dėl to atsirandantis burių prolapsas. Negydomas MVN ilgainiui tampa širdies ritmo sutrikimų bei širdies nepakankamumo priežastimi, reikšmingai pablogėja ligonio gyvenimo kokybė.

Plačiausiai naudojamas ir efektyviausiu pripažintas degeneracinės kilmės MVN gydymo metodas šiuo metu yra MV atkuriamosios operacijos, kitaip dar vadinamos MV plastika. Šio gydymo tikslas – atlikti anatominę pažeisto MV rekonstrukciją ir sugrąžinti jo funkciją. Palyginti su MV protezavimu, MV plastika yra susijusi su geresniais pooperaciniais rezultatais, ilgesniu išgyvenamumu ir geresne kairiojo skilvelio funkcija, atkuria normalią gyvenimo kokybę bei trukmę, turi mažesnę infekcinio endokardito ir trombembolinių komplikacijų riziką.

Populiariausias MV plastikos metodas yra klasikinė MV plastika per vidurinę sternotomiją. Tokios operacijos visada atliekamos naudojant dirbtinę kraujo apytaką (DKA) ir kardioplegiją, t. y. operacija vyksta neplakančios širdies sąlygomis. Tokioms operacijoms yra būdingos komplikacijos, susijusios su DKA ir kardioplegija, t. y. insultas, sisteminis uždegiminis atsakas, pooperacinis širdies ir sisteminės kraujotakos nepakankamumas, širdies ritmo sutrikimai, inkstų ir kitų organų funkcijos nepakankamumas, infekcija, didesnis pooperacinis kraujavimas.

Nuo 1980-jų metų atliekant MV plastiką pradėtos naudoti ePTFE dirbtinės chordos. Jos yra implantuojamos į prolabuojančio burės segmento kraštą ir fiksuojamos papiliniuose raumenyse. Tokiu būdu šis segmentas yra nuleidžiamas iki neprolabuojančių segmentų aukščio ir grąžinamas vožtuvo sandarumas. Per kelis dešimtmečius dirbtinės chordos labai išpopuliarėjo ir dabar plačiai naudojamos atliekant MV plastiką.

Per pastaruosius 20 metų atsirado keletas mažiau invazinių MV plastikos metodų. Jie buvo sukurti siekiant sumažinti chirurginės procedūros traumą, tačiau išlaikant priimtiną saugumo ir korekcijos kokybės lygį. Nors šios operacijos neabejotinai turi tam tikrų privalumų, tačiau jas atliekant visgi išlieka rizika, susijusi su DKA naudojimu.

Tobulėjant technologijoms pasaulinėje praktikoje vis labiau jaučiama tendencija tobulinti mažiau invazines procedūras. Šį faktą nulemia tai, kad nuolat siekiama trumpinti hospitalizacijos ir nedarbingumo laikotarpį, be to, senstant populiacijai didėja ir MVN paplitimas tarp senyvo amžiaus žmonių. Tai skatina kurti naujus, mažiau invazinius metodus, kurie būtų saugesni ir sukeltų mažesnę pooperacinę traumą. Jie leistų darbingo amžiaus žmonėms greičiau pasveikti ir sugrįžti į darbą, be to, būtų tinkamesni senyviems pacientams, turintiems didesnę klasikinės chirurgijos riziką.

Vienas iš tokių naujoviškų alternatyvių gydymo metodų yra transapikalinė MV plastika naudojant „NeoChord DS1000™“ sistemą. Ši sistema yra unikalus chirurginis instrumentas, leidžiantis implantuoti dirbtines ePTFE chordas į prolabuojantį burės segmentą bei atkurti normalią vožtuvo funkciją. Ši procedūra yra atliekama per mažą pjūvį ir be DKA, todėl leidžia išvengti su DKA susijusių komplikacijų. Nuo 2011 m. tokios transapikalinės MV plastikos operacijos yra atliekamos Vilniaus universiteto ligoninėje Santaros klinikose (VULSK). Tuo pačiu laikotarpiu toliau buvo atliekamos klasikinės MV plastikos.

Transapikalinė MV plastika yra nauja procedūra, kuri nėra paplitusi pasaulyje ir atliekama tik pavieniuose širdies chirurgijos centruose, todėl duomenų apie šį gydymo metodą bei publikacijų, aprašančių pooperacinius rezultatus, yra labai mažai. Tik vienoje publikacijoje pateikiami vėlyvieji (12 mėn.) 213 ligonių rezultatai bei duomenys apie ilgalaikį plastikos efektyvumą. Todėl nauji ankstyvieji bei vėlyvieji rezultatai yra ypač aktualūs ir įdomūs, nes nuo to priklauso tolesnis šios technologijos naudojimas ir tobulinimas.

Nepavyko rasti nė vienos publikacijos, kurioje būtų palyginami klasikinės bei transapikalinės plastikos rezultatai, o tai yra labai svarbu siekiant įvertinti šio naujo gydymo metodo saugumą bei efektyvumą.

Tyrimo tikslas

Įvertinti ir palyginti klasikinės ir transapikalinės mitralinio vožtuvo plastikos efektyvumą, ankstyvuosius bei vėlyvuosius pooperacinius rezultatus.

Tyrimo uždaviniai

1. Įvertinti transapikalinės MV plastikos efektyvumą ir saugumą (komplikacijų skaičių ankstyvuoju pooperaciniu laikotarpiu) ir palyginti su klasikinės MV plastikos rezultatais.
2. Įvertinti transapikalinės MV plastikos ilgalaikį efektyvumą ir jį palyginti su klasikinės MV plastikos rezultatais.
3. Nustatyti ryšį tarp MV anatominio tipo ir ilgalaikio MV plastikos efektyvumo.
4. Išanalizuoti priežastis, lėmusias MVN atsinaujinimą po transapikalinės ir klasikinės MV plastikos.

Darbo naujumas

Šiuo metu yra tik viena publikacija, kurioje pateikiami 213 ligonių transapikalinės MV plastikos vėlyvieji (12 mėn.) pooperacinės stebėsenos rezultatai. Vilniaus universiteto ligoninėje Santaros klinikose transapikalinė MV plastika atliekama nuo 2011 metų ir į šį tyrimą įtrauktų ligonių pooperacinės stebėsenos mediana yra 39 mėn. (IKP 20–51 mėn.). Šioje disertacijoje pateikiami vėlyvieji transapikalinės MV plastikos rezultatai, analizuojamas ilgalaikis šios plastikos efektyvumas bei jo priklausomybė nuo MV anatominio tipo, taip pat aptariamos priežastys, sukėlusios MVN atsinaujinimą po transapikalinės ir klasikinės MV plastikos.

Nė vienoje iš publikacijų transapikalinės MV plastikos rezultatai nebuvo palyginti su klasikinės MV plastikos rezultatais. Abu šie gydymo metodai yra naudojami degeneracinei MV ligai gydyti, tačiau transapikalinė MV plastika yra visiškai naujas, o klasikinė chirurginė MV plastika – seniai ir plačiai taikomas metodas, kuris pripažintas saugiu ir efektyviu. Disertacijoje yra palyginami šių dviejų gydymo metodų ankstyvieji ir vėlyvieji rezultatai.

Praktinė darbo reikšmė

Šiame darbe buvo ištirtas ilgalaikis transapikalinės MV plastikos efektyvumas ir jo ryšys su pradine MV anatomija. Nustatyta, kad geriausias ilgalaikis transapikalinės MV plastikos efektyvumas pasiekiamas ligoniams, kuriems MVN sukėlė izoliuotas P2 segmento prolapsas.

Taip pat nustatyta, kad nors ankstyvuoju pooperaciniu laikotarpiu komplikacijų skaičius po transapikalinės MV plastikos buvo mažesnis, ilgalaikis klasikinės MV plastikos efektyvumas yra geresnis ir nepriklauso nuo MV anatomijos.

Tiriamieji ir tyrimo metodai

Tyrimas atliktas 2014–2018 m. Vilniaus universiteto ligoninėje Santaros klinikose (VULSK). Tyrimui atlikti gautas Vilniaus regioninio biomedicininių tyrimų etikos komiteto pritarimas Nr. 158200-16-829-345.

Tai retrospektyvusis kohortinis stebėsenos tyrimas. Į tyrimą buvo įtraukti 202 ligoniai, kuriems nuo 2011 m. gruodžio 1 d. iki 2018 m. sausio 1 d. VULSK buvo atlikta klasikinė chirurginė MV plastika arba transapikalinė MV plastika dėl degeneracinės kilmės MVN. Duomenys rinkti iš medicininės dokumentacijos.

Į šį tyrimą įtraukti ligoniai, kurie buvo operuoti dėl degeneracinės kilmės MVN: vienos arba abiejų burių prolapso (taip pat ir plevėsuojančios burės), su chordos plyšimu arba be jo. Visi ligoniai, kurių MVN buvo sukeltas burių restrikcijos (reumatas, kardiomiopatija), išeminės širdies ligos, arba MV infekcinio pažeidimo, taip pat ligoniai, kuriems buvo centrinė MVN srovė, į šį tyrimą neįtraukti.

Remiantis gautais echokardiografijos duomenimis buvo atrenkami ligoniai, tinkami klasikinei arba transapikalinei MV plastikai. Transapikalinė MV plastika buvo atlikta ligoniams (n = 94), kurių MV anatomija buvo palanki šiam gydymo metodui ir kurie sutiko šiai procedūrai. Kitiems ligoniams buvo atlikta klasikinė MV plastika (n = 108).

Transapikalinė MV plastika buvo atlikta 3 ligoniams, kuriems anksčiau buvo atlikta klasikinė MV plastika ir implantuotas anuloplastikos žiedas. Šie ligoniai buvo atskirti nuo pagrindinės ligonių grupės ir jų gydymo eiga buvo aprašyta atskirai.

Tokiu būdu buvo suformuotos dvi ligonių grupės:

1. Klasikinės chirurginės mitralinio vožtuvo plastikos (KC) grupė – 108 ligoniai.
2. Transapikalinės mitralinio vožtuvo plastikos (TA) grupė – 91 ligonis.

Tyrime buvo analizuojami duomenys, gauti atliekant klinikinį ištyrimą, instrumentinius bei laboratorinius tyrimus:

1. prieš procedūrą (pradinė būklė);
2. prieš išrašant iš ligoninės;
3. per stebėjimo apsilankymus praėjus 1 mėn., 6 mėn. ir 12 mėn. po operacijos, o vėliau – kas 12 mėn.

Statistinė analizė

Statistinė duomenų analizė atlikta naudojant duomenų kaupimo ir analizės programos paketą „SPSS 22.0“ (IBM Corp., Armonk, NY, USA). Kiekybinių tolydžiųjų duomenų normalumui patikrinti naudotos histogramos, stačiakampės diagramos, Šapiro ir Vilko (angl. *Shapiro–Wilk*) kriterijus ($p > 0,05$). Kiekybiniai pagal normalųjį skirstinį pasiskirstę duomenys išreikšti vidurkiu ± standartiniu nuokrypiu. Šių duomenų vidurkio palyginimui naudotas Stjudento t kriterijus nepriklausomoms imtims. Vidurkiams tarp daugiau negu dviejų grupių palyginti naudota vieno faktoriaus dispersinė analizė (ANOVA). Kiekybiniai tolydieji duomenys, pasiskirstę ne pagal normalųjį skirstinį, išreikšti mediana ir kvartilių intervalu. Šiems duomenims palyginti naudotas Mano, Vitnio ir Vilkoksono kriterijus (angl. *Mann–Whitney–Wilcoxon*). Kategoriniai duomenys išreikšti procentais. Kategoriniai kintamieji buvo palyginti χ^2 arba Fišerio (Fisher) tiksliuoju kriterijumi. Ligonių KC bei TA grupės vertintos Kaplano ir Mejerio (angl. *Kaplan–Meier*) metodu, o tarpusavyje ligonių grupės buvo palygintos taikant logranginį testą (angl. *Log Rank test*). Cenzūruoti duomenys – ligoniai, kurių stebėjimas baigtas. Dydžiai $p < 0,05$ buvo vertinami kaip statistiškai reikšmingi, o $0,05 < p \leq 0,1$ buvo laikomi tendencija.

Rezultatai

TA grupės ligoniai buvo kiek vyresni nei KC grupės – atitinkamai 61 m. ir 56 m. ($p = 0,008$). Taip pat TA grupės ligoniai turėjo šiek tiek didesnį kūno masės indeksą. Tiek KC, tiek TA grupės ligonių priešoperacinė rizika vertinama kaip maža: 0,44 % ir 0,48 % pagal STS, 0,84 % ir 0,8 % pagal EuroSCORE II, reikšmingo skirtumo tarp ligonių grupių nebuvo.

KC grupėje buvo daugiau ligonių, turinčių paroksizminį ir lėtinį prieširdžių virpėjimą – atitinkamai 21 % ir 19 %, o TA grupėje tokie ligoniai sudarė 2 % ir 3 % ($p < 0,001$). Dažniausia abiejų grupių ligonių gretutinė liga buvo pirminė arterinė hipertenzija, kiek dažnesnė ji buvo TA grupėje (39 % ir 54 %). Statistiškai reikšmingo skirtumo tarp sergamumo vainikinių arterijų liga, diabetu, lėtine obstrukcine plaučių liga bei insultu nenustatyta.

Visiems tyrimo dalyviams prieš operaciją buvo nustatytas ryškus MV nesandarumas: apie ¾ ligonių – MVN 3+ ir ¼ ligonių – MVN 4+ abiejose grupėse. KC grupėje reikšmingas (≥2+) triburio vožtuvo nesandarumas buvo dažnesnis: 13 % vs 4 % ($p=0,036$).

Pagal patologinio segmento lokalizaciją visi ligoniai buvo suskirstyti į keturis anatominius tipus:

A – izoliuotas P2 segmento prolapsas. TA grupėje tokių ligonių buvo 22 (24 %), o KC grupėje – 15 (14 %);

B – P2 segmento ir greta esančių sričių prolapsas. Šio tipo MV patologiją turėjo 48 (53 %) TA grupės ligoniai ir 20 (19 %) KC grupės ligonių;

C – vienos arba abiejų burių prolapsas, kai prolabuoja ir parakomisūriniai segmentai. Šie ligoniai sudarė daugumą KC grupėje – 70 (65 %). TA grupėje tokių ligonių buvo 16 (18 %);

D – izoliuotas A2 segmento prolapsas. TA grupėje tokių ligonių buvo 5 (5 %), o KC grupėje – 3 (3 %).

Dauguma klasikinių MV plastikos operacijų buvo atlikta per vidurinę sternotomiją – 91,7 %, anuloplastikos žiedas buvo implantuotas 96,3 % ligonių, o dirbtinės ePTFE chordos – 83,3 % ligonių.

Dviem trečdaliams TA grupės ligonių buvo implantuotos 3 arba 4 chordos.

TA grupėje buvo 4 (4,4 %) ligoniai, kuriems procedūra laikoma nesėkminga: dviem ligoniams buvo atlikta intraoperacinė konversija į klasikinę MV plastiką ir 2 ligoniams pasiektas MVN buvo didesnis nei 2+.

MV buvo visiškai sandarus arba turėjo minimalų nesandarumą 62,1 % ligonių, kuriems buvo sėkmingai atlikta transapikalinė MV plastika. KC grupėje tokių ligonių buvo 76,8 % ($p = 0,025$). Penkiems ligoniams po TA plastikos išliko MVN 2+.

Pooperacinis kraujavimas buvo daug mažesnis TA grupėje – 200 ml, KC grupėje – 350 ml. TA grupėje kraujo produktų transfuzijų dažnis buvo daug mažesnis – 5,8 % vs 43,5 % ($p < 0,001$). Naujas prieširdžių virpėjimas TA grupėje išsivystydavo rečiau: 37 % vs 17,4 %. Pooperacinis kreatinino koncentracijos padidėjimas >150 % buvo nustatytas tik 3,6 % TA grupės ligonių, o KC grupėje tokių ligonių buvo 15,7 %. Hemofiltracijos TA grupės ligoniams neprireikė.

Ligoniai po TA plastikos būdavo ekstubuojami gerokai anksčiau – po 4 val. vs 7 val., be to, intensyviosios terapijos skyriuje jie buvo gydomi trumpiau – 22 val. vs 67 val. Pooperacinės hospitalizacijos trukmė buvo taip pat mažesnė: 8 d. vs 12 d.

Pooperacinės stebėsenos trukmės mediana KC grupėje buvo 34 mėn., TA grupėje – 39 mėn.

Ilgalaikis klasikinės MV plastikos efektas buvo daug geresnis nei TA MV plastikos: praėjus 72 mėnesiams po operacijos MVN neviršijo 2+ 90 % ligonių. TA grupės ligonių šis rodiklis buvo 68 %.

Suskirsčius KC grupės rezultatus pagal anatominius tipus, didesnio skirtumo tarp grupių negauta ($\chi2$ – 0,106, $p = 0,991$), todėl toliau KC grupės rezultatai buvo vertinami kartu, neskirstant į grupes.

Palygintas plastikos efektyvumas tarp KC grupės ir TA grupės, pastarąją suskirsčius į anatominius A, B, C ir D tipus. Geriausi rezultatai buvo gauti A anatominėje grupėje – per pirmuosius 6 mėn. ligonių, kuriems išliko geras plastikos efektas, sumažėjo iki 85 %, o vėliau šis skaičius nesikeitė. Kiek prastesnis ilgalaikis efektyvumas buvo B grupėje: po 6 mėn. MVN ≤2+ išliko 87 % ligonių, po metų – 80 %, o nuo 24 mėn. iki stebėjimo pabaigos – 74 % ligonių. C grupės rezultatai buvo prasčiausi. Jau po pusės metų ryškus MVN atsinaujino maždaug pusei ligonių, o nuo 24 mėn. geras plastikos efektas išlieka tik 23 % ligonių.

Sudėjus A ir B anatominių grupių rezultatus, MVN ≤2+ išliko 81 % ligonių po 12 mėn. ir 77 % po 28 mėn. Toliau rezultatas nesikeičia iki stebėjimo pabaigos.

Po transapikalinės MV plastikos iš ligoninės išvyko 86 ligoniai, iš jų 25 (29 %) ligoniams MVN >2+ atsinaujino. Trys ligoniai buvo priskirti A anatominiam tipui, 11 – B tipui, 10 – C tipui ir 1 – D tipui.

Visais atvejais, kai atsinaujindavo MV nesandarumas KC grupėje, buvo diagnozuojamas naujas chordų plyšimas. Pagrindinės priežastys, sukėlusios MVN atsinaujinimą TA grupėje, buvo reliatyvus dirbtinių chordų pailgėjimas, natyvinių ir dirbtinių chordų plyšimas.

Išvados

1. Transapikalinė MV plastika yra efektyvus ir saugus degeneracinės kilmės MV nesandarumo gydymo metodas, pasižymintis mažesniu pooperacinių komplikacijų skaičiumi ankstyvuoju pooperaciniu laikotarpiu lyginant su klasikine MV plastika.
2. Ilgalaikis transapikalinės MV plastikos efektyvumas yra statistiškai patikimai prastesnis nei klasikinės chirurginės MV plastikos (A ir B anatominių tipų transapikalinės MV plastikos efektyvumas yra 77%, o klasikinės MV plastikos – 90%).
3. Klasikinės MV plastikos ilgalaikis efektyvumas nepriklauso nuo MV anatominio tipo. Geriausias ilgalaikis transapikalinės MV plastikos efektyvumas pasiekiamas ligoniams, turintiems izoliuotą P2 segmento prolapsą arba P2 segmento ir gretimų sričių patologiją, o ligoniams, kurių MV patologija neapsiriboja P2 segmento ir gretimų sričių prolapsu, transapikalinė MV plastika neturi būti atliekama dėl prastų vėlyvų rezultatų.
4. MVN atsinaujinimą po transapikalinės MV plastikos dažniausiai sukelia dirbtinių bei natyvinių chordų plyšimas, taip pat reliatyvus dirbtinių chordų pailgėjimas. Pagrindinė MVN atsinaujinimo po klasikinės MV plastikos priežastis – naujas natyvinių chordų plyšimas.

Praktinės rekomendacijos

1. Transapikalinė MV plastika gali būti atliekama tik tiems ligoniams, kurių mitralinio vožtuvo patologija yra A ar B anatominio tipo.
2. Ligoniams, kurių MV patologija neapsiriboja P2 segmento ir gretimų sričių prolapsu, turi būti atliekama klasikinė chirurginė MV plastika.
3. Siekiant pagerinti ilgalaikį transapikalinės MV plastikos efektyvumą, būtina griežta priešoperacinė ligonių atranka remiantis echokardiografijos duomenimis ir MV anatomijos ypatumais.

Disertacijos tema paskelbtų darbų sąrašas

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THE INTERRELATIONSHIP BETWEEN THE ALTERATION OF ARTERIAL MARKERS AND LEFT VENTRICULAR DIASTOLIC DYSFUNCTION IN METABOLIC SYNDROME SUBJECTS

ARTERIJŲ FUNKCIJOS IR STRUKTŪROS RODIKLIŲ POKYČIO IR KAIRIOJO SKILVELIO DIASTOLINĖS DISFUNKCIJOS SĄSAJŲ TYRIMAS METABOLINĮ SINDROMĄ TURINČIŲ ASMENŲ GRUPĖJE

Biomedical sciences, Medicine 06 B
Biomedicinos mokslai, Medicina 06B

Abbreviations

ABI – ankle-brachial index
AH – arterial hypertension
BMI – body mass index
BP – blood pressure
CAVI – cardio–ankle vascular index
CCA – common carotid artery
CI – confidence interval
CVDs – cardiovascular diseases
DM – diabetes mellitus
FPG – fasting plasma glucose
HDL-C – high-density lipoprotein cholesterol
HF – heart failure
HFpEF – heart failure with preserved left ventricular ejection fraction
hsCRP – high sensitivity C-reactive protein
IMT – intima-media thickness
IVST – interventricular septal end-diastolic thickness
LDL-C – low-density lipoprotein cholesterol
LitHiR – the Lithuanian High Cardiovascular Risk Primary Prevention Program
LPWT – left ventricular posterior wall end-diastolic thickness
LVDd – left ventricular end-diastolic dimension
LV – left ventricular
LVMI – left ventricular mass index
MetS – metabolic syndrome
OR – odds ratio
PP – pulse pressure
PWV – pulse wave velocity
QCS – quality carotid stiffness
RWT – relative wall thickness
SD – standard deviation
TC – total cholesterol
TG – triglycerides
WC – waist circumference
WHO – World Health Organization

1. Introduction

1.1 The Problem of the Study

Cardiovascular diseases (CVDs) are the main cause of mortality, morbidity and decreasing working capacity worldwide and accounts for 31% of all deaths [1]. According to the World Health Organization (WHO), around 17.5 million people died of CVDs in 2012. Myocardial infarction or vertebrobasilar circulatory disorders were the cause of death in 80% of these cases. Based on the data of the Lithuanian Department of Statistics, 56.2% of the residents of Lithuania died of vascular diseases in 2016. The majority of them died of ischemic heart disease (65.6%) and cerebrovascular disease (24.2%). Most of the deceased (i.e., 87%) who were diagnosed with vascular disorders were over 65 years old. Among them, 47.8% were males and 64.4% were females, which showed a higher mortality rate in women compared to men.

Risk factors of cardiovascular diseases are analyzed extensively in developed countries. According to the available scientific research, the following are some of the most important risk factors of CVDs: arterial hypertension (AH), glucose me-

tabolism disorders, especially, diabetes mellitus (DM), dyslipidemia, smoking and obesity. These risk factors are most often brought about by unhealthy diet, lack of physical activity and high levels of stress. INTER-HEART, a case-control study on the effect of potentially modifiable risk factors associated with myocardial infarction in 52 countries, revealed that standard risk factors accounted for approx. 90% of acute CVDs [2].

As soon as it was noticed that patients with several cardiometabolic risk factors had a higher probability of developing CVDs, a new term was suggested: "metabolic syndrome", also called "syndrome X" and "insulin resistance syndrome" [3, 4, 5].

Metabolic syndrome (MetS) is a cluster of risk factors (e.g. arterial hypertension, abdominal obesity, insulin resistance, atherogenic dyslipidemia) and is considered one of the major problems in our modern world. More economically developed countries deem it a priority to prevent and treat these pathological conditions in a timely manner, as cases of obesity, AH and MetS seem to keep increasing in number and frequency [6, 7]. Diagnosis of MetS has a high clinical significance as, on one hand, the syndrome can be slowed down by employing preventive means and treating patients in a timely manner, but on the other hand, the syndrome can cause or accelerate the development of type 2 diabetes and atherosclerosis, which are the top causes of death worldwide [8, 9]. With respect to the increase of the cases of people diagnosed with DM and MetS both in Europe and the United States, the prevalence of Mets is forecasted to grow as high as 40% on a global scale in 2020. The occurrence and progress of such highly prevalent diseases as CVDs and type 2 diabetes mellitus are closely connected to metabolic and hormonal disorders that constitute the basis of MetS [10, 11]. According to the results of epidemiological studies, MetS is highly prevalent and its risk group is constantly growing [12, 13].

The prevalence of MetS in Lithuania depends on the age, sex and type of respondents and, at the moment, the frequency of MetS cases tends to range from 10.3% to 36.6%. According to the data of the Lithuanian High Cardiovascular Risk (LitHiR) Primary Prevention Program, as many as 31.5% of the respondents in the group aged 40-65 years old were diagnosed with MetS [14].

Arterial stiffness is another important independent risk factor for CVDs and cardiovascular mortality, which is being researched more and more widely all over the world [15, 16, 17]. Arterial stiffness occurs as a consequence of aging and progressing atherosclerosis. It is related to certain functional and morphological changes in arterial walls which start with the endothelial dysfunction and may progress to intima-media thickening and, ultimately, formation of atherosclerotic plaques. Furthermore, current research suggests that increased arterial stiffness and atherosclerosis might be two interdependent but not identical pathways to increased cardiovascular morbidity and mortality [18]. Accumulating data suggests the link between

increased aortic stiffness with the occurrence and progressing of the left ventricular (LV) diastolic dysfunction [19, 20]. Higher arterial stiffness results in increased pulse wave velocity (PWV) and earlier (systolic rather than diastolic) arrival of reflected wave at the ascending aorta. Systolic blood pressure increases and the diastolic blood pressure drops in the aorta, the left ventricular afterload increases, myocardial hypertrophy starts developing, coronary perfusion worsens, and normal flow is replaced with pulsatile flow heading towards the small arteries in the peripheral vascular system. The small arteries degenerate causing microcirculation disorders and target organ (e.g. heart, kidney, brain, etc.) damage. Worsening coronary perfusion and LV myocardial hypertrophy result in abnormal ventricular relaxation and development of diastolic dysfunction. It has been proven that increased left ventricular stiffness is diagnosed more often in patients with increased arterial stiffness. Both of these factors are closely connected to the occurrence and progression of heart failure [21, 22].

About 40–50% of the patients with heart failure (HF) are also diagnosed with preserved left ventricular ejection fraction. Certain structural and functional changes in the left ventricle are inherent to heart failure with preserved LV ejection fraction (HFpEF). Abnormal LV diastolic function is one of the most important of such changes [23, 24] and is conditioned by various factors, e.g. aging, AH, DM, chronic kidney disease, metabolic syndrome and high arterial stiffness. LV diastolic function is an important factor causing HFpEF [25, 26]. Furthermore, it has been demonstrated that the increase of arterial stiffness in patients with HF is related to a worse health prognosis, thus, determination and decrease of arterial stiffness could become a goal in both prevention and treatment of heart failure [27].

Since LV diastolic dysfunction is considered to be an early preclinical marker of cardiovascular disorders which cause HF over time, the determination of the pathogenetic factors for LV diastolic dysfunction is of key importance for choosing an optimal medical treatment. Several studies confirm a significant connection between the LV diastolic dysfunction and the various indices of arterial stiffness (pulse wave velocity [PWV], cardio-ankle vascular index, augmentation index) in different CVDs risk groups (patients with AH, DM). W. P. Abhayaratna *et al* [28, 29] determined that high arterial stiffness in the older patients group was related to the LV diastolic dysfunction and that the strength of the correlation was dependent on the indices used for assessment of arterial stiffness. The PWV turned out to be a better marker of LV diastolic dysfunction, compared to the central or peripheral pulse pressure. Another population–based study on 1233 participants revealed that the LV diastolic dysfunction parameters have significant correlations with the carotid-femoral PWV and central pulse pressure measured by applanation tonometry [30]. Data of 5799 patients were analyzed during Framingham Heart Study. A significant

correlation was found between the carotid-femoral PWV, central pulse pressure and LV diastolic dysfunction parameters (e', E/e' ratio) [31]. Yet, data on the correlation between arterial stiffness and LV diastolic dysfunction in subjects with metabolic syndrome are insufficient: the studies are of a small size and only single indices of arterial stiffness are analyzed [32, 33].

Increasingly greater body of scientific evidence confirms the relevance of metabolic syndrome and its components for the development of heart failure [34]. The frequency of LV diastolic dysfunction occurrence was determined to be significantly higher in subjects with MetS [35, 36]. However, scientific literature lacks reliable data on the early clinical diagnosis of heart failure with preserved LV ejection fraction, and on its association with the progression of arterial stiffness, in subjects with metabolic syndrome.

1.2 Relevance and Significance

Scientific literature provides an impressive amount of evidence showing the link between arterial stiffness and the development and progression of CVDs. Over 50% of middle-aged Lithuanian residents are afflicted with arterial hypertension and related risk factors, such as MetS, that induce early arterial function impairment and over time result in the morphological changes of the arterial wall. In case of further progressing of this pathological condition, heart failure with preserved left ventricular ejection fraction (HFpEF) develops. Possibilities of treating HFpEF are low due to insufficient research of pathogenetic mechanisms; thus, early detection of left ventricular diastolic dysfunction is of utmost significance.

According to available research, LV diastolic dysfunction and heart failure with preserved ejection fraction are related to the increase in arterial stiffness [37]. However, it is not known whether an early prevention of the increase of arterial stiffness could in turn prevent the development of HF nor is it established how the slowing down of the increase in arterial stiffness affects the left ventricular remodeling. To answer these questions is important for practical cardiology as it would allow for choosing the optimal medications for treatment and forecasting the occurrence and progression of heart failure. In the search for an answer, examining the dynamics of arterial parameters (e.g., assessment of the arterial stiffness, endothelial function, intima-media thickness and plaque of the carotid arteries) and their correlation with the left ventricular diastolic dysfunction, HFpEF and other cardiac diseases is of crucial importance.

The results of research carried out during the past decade revealed that it was the central rather than peripheral arterial blood pressure that correlated better with the cardiovascular outcome in certain patient groups. However, the prognostic relevance of central blood pressure in patients with MetS was not widely researched.

Successful studies in this field would most probably change treatment plans: physicians would first choose antihypertensive drugs that effectively lower central blood pressure instead of the peripheral.

It should be noted that MetS is a potentially curable condition. Early diagnosis and adequate early intervention might dramatically reduce the prevalence of MetS, consequently decreasing the risk of developing CVDs and DM. The available research suggests that cardiovascular diseases associated with MetS comprise vascular and myocardial abnormalities that are initially manifested as impaired relaxation of the left ventricle. This myocardial dysfunction is characterized predominantly by diastolic dysfunction consisting of relaxation abnormalities that are prevalent and have prognostic importance in patients with MetS [38].

A number of cross-sectional studies have been carried out with the intent to analyze the relation between arterial stiffness and LV diastolic dysfunction. However, there is a lack of longitudinal studies, the results of which could demonstrate the predictive value of the increased arterial stiffness for the development of LV diastolic dysfunction, a potential precursor to heart failure in MetS subjects. Such studies could help to choose the optimal treatment geared towards the prevention of occurrence and progress of CVDs and heart failure.

The paper presents an analysis of a large cohort, comprised of 1208 subjects with MetS. Follow-up and repeated tests were carried out on 573 participants after three or more years of observation. The relationship between the arterial indices and their changes, and the dynamics of LV diastolic function were assessed.

Currently, little is known about an early development and early clinical diagnosis of heart failure with preserved ejection fraction (HFpEF) in subjects with MetS. Given the high prevalence of LV diastolic dysfunction in this population, it can be reasonably expected that a significant number of subjects with MetS already have unrecognized HFpEF. To uncover the early phase of HFpEF we tested a HF biomarker, BNP, and evaluated the exercise capacity of our cohort subjects by performing the cardiopulmonary stress test. Additionally, we examined an association of markers of arterial stiffness with early HFpEF.

1.3 Research Objective and Tasks

Research Objective

To evaluate the interrelationship between the alterations of arterial markers and left ventricular diastolic dysfunction in metabolic syndrome subjects.

Research Tasks

1. To evaluate the relationship between arterial markers and left ventricular diastolic dysfunction in the cross-sectional study.
2. To determine how the alteration of arterial stiffness affects the dynamics of left ventricular diastolic dysfunction during longitudinal observation.

3. To investigate the frequency of left ventricular diastolic dysfunction and to uncover the early phase of heart failure with preserved left ventricular ejection fraction in subjects with metabolic syndrome. Then, to examine an association of arterial markers with early heart failure with preserved left ventricular ejection fraction.

1.4 Statements to be Defended

1. Left ventricular diastolic dysfunction is significantly associated with elevated arterial stiffness in metabolic syndrome subjects.
2. The increase in arterial stiffness, measured as carotid to femoral pulse wave velocity, is significantly related to the deterioration of the left ventricular diastolic function.
3. A significant number of patients with metabolic syndrome already have left ventricular diastolic dysfunction and unrecognized early heart failure with preserved left ventricular ejection fraction. These subjects exhibit higher aortic augmentation index.

1.5 Novelty of the Study

One of the gratest methodological assets of our study was that, as compared to other studies, we analysed a relatively large group of MetS subjects. The paper presents the cross-sectional study of 1208 subjects with MetS. The study assessed the relationship between different indices of arterial wall function and structure (e.g. pulse wave velocity, aortic augmentation index, and central blood pressure indices, cardio-ankle vascular index, carotid artery stiffness and intima-media thickness) and LV diastolic dysfunction. The carotid-femoral PWV was found to be a significant independent prognostic marker of the LV diastolic dysfunction in metabolic syndrome subjects.

The longitudinal study was carried out by observing 573 participants. The effects of the change in arterial stiffness on the dynamics of the LV diastolic dysfunction were assessed (average monitoring duration: 3.8 ± 0.6 years). Our findings show that carotid-femoral PWV is more closely correlated with LV diastolic function parameters than other indices of arterial stiffness, namely AIx, ABI, CAVI or aortic

pulse pressure. The results of longitudinal observation allowed to better understand the relation between arterial stiffness and LV diastolic dysfunction. We found that the increase of carotid-femoral PWV and aortic augmentation index are strongly associated with negative dynamics of LV diastolic function. Aortic pulse pressure, heart ratio, BMI, weight, and calcium channel blockers might also have an important prognostic value. According to the published research available our study is the first of this type of the longitudinal observation study in MetS subjects.

The possibility of early detection of heart failure in subjects with MetS was analyzed for the first time. The study results revealed that patients diagnosed with early heart failure demonstrated increased arterial stiffness and abnormal oxygen metabolism. The assessment of pulmonary ventilation, oxygen metabolism during physical exertion and natriuretic peptide levels showed that more than half of the patients with MetS may develop heart failure with preserved left ventricular ejection fraction. Physical capacity test might be useful in early detection of heart failure. The results of our study are useful for timely detection of the heart failure with the preserved LV ejection fraction.

2. Methodology

2.1 Study Subjects

A study was carried among 1208 metabolic syndrome subjects. All of the study cohort participants were recruited between 2009 and 2015 years as paticipants of the Lithuanian High Cardiovascular Risk (LitHiR) primary prevention programme at the Vilnius University Hospital Santaros Clinics. This long-term programme has focused on women (aged 50–65 years) and men (aged 40–55 years) without overt cardiovascular disease [39, 40]. Subjects with systolic dysfunction (LV ejection fraction < 50%), significant valvular disease, cardiomyopathy, renal failure, atrial fibrillation, infection, malignancy were excluded. Prior to formal enrollment in the study, all interested subjects signed an informed consent form. The study was authorized by the Vilnius Regional Biomedical Research Ethics Commitee (No. 158200–13–641–205).

The cross sectional study included all the 1208 MetS subjects. The follow-up visit of 573 subjects was carried out after 3.8 ± 0.6 years. For additional assessment of cardiopulmonary stress test and BNP we prospectively enrolled 148 patients with MetS from 573 follow-up subjects. For comparison of study parameters between early and clinically obvious stages of HFpEF, 38 patients with MetS and confirmed HFpEF were enrolled retrospectively (Fig.1).

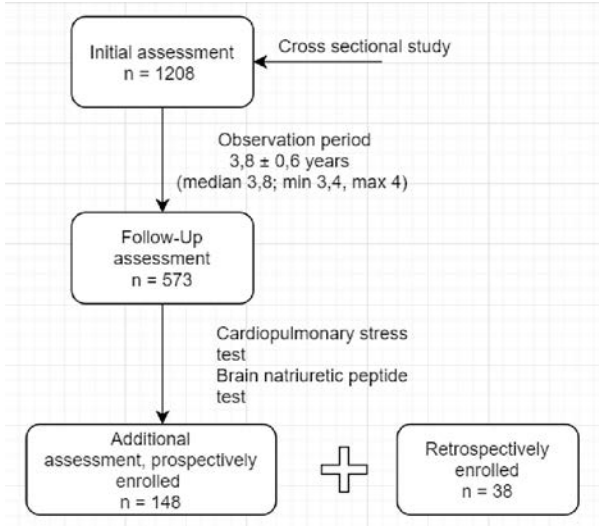


FIG. 1. Model of the study.

All participants at initial and follow-up assessments underwent a physical examination, risk profile (smoking) analysis, anthropometry (height, weight, waist circumference [WC] and body mass index [BMI], defined as weight in kilograms divided by height), blood pressure and pulse determination. Twelve lead electrocardiogram was registered. After a 12–hour fast serum total cholesterol (TC), high-density lipoprotein cholesterol (HDL–C), triglycerides (TG), low-density lipoprotein cholesterol (LDL–C), high sensitivity C–reactive protein (hsCRP) levels were assessed. Fasting plasma glucose (FPG) was sampled. The Abbott ARCHITECT immunoassay analyzer was used for the diagnosis of BNP concentration in plasma (units of measure – ng/l).

Each participant at initial and follow-up assessments also underwent echocardiography, and the detailed assessment of arterial function and wall properties. The latter consisted of evaluation of the ankle-brachial index (ABI) and cardio–ankle vascular index (CAVI), carotid wall assessment for the measurement of common carotid artery (CCA) intima-media thickness (IMT) and stiffness, aortic pulse wave velocity (PWV) for aortic stiffness evaluation, and the pressure wave reflection measurements. Vascular assessment was carried out in supine position after 20 minutes bed rest in a temperature–controlled room at 23°C. Study subjects abstained from drinking alcoholic beverages, coffee and vasoactive drugs for 12 hours before the examination.

Brain natriuretic peptide (BNP) test and cardiopulmonary stress test were performed for additional assessment in 148 subjects.

2.2 Definition of Metabolic Syndrome, Hypertension, Dyslipidaemia, and Smoking Status

MetS was diagnosed according to AHA/IDF updated National Cholesterol Education Program Adult Treatment Panel III definition [41]. Participants were classified as having MetS if they satisfied at least 3 of the following 5 criteria:

- Central (abdominal) obesity with a waist circumference > 102 cm in men, or > 88 cm in women;
- Triglycerides of ≥ 1.7 mmol/l;
- Fasting serum glucose ≥ 5.6 mmol/l;
- HDL-cholesterol level ≤ 1.03 mmol/l in men, and ≤ 1.29 mmol/l in women;
- Blood pressure of ≥ 130/85 mmHg.

Hypertensives were defined as subjects with a history of hypertension in whom the sustained elevation of blood pressure (> 140 mmHg systolic and/or > 90 mmHg diastolic BP) has been observed in at least three separate measurements obtained on different days (or antihypertensive treatment).

Dyslipidemia was defined as TC > 5mmol/L, or LDL–C > 3 mmol/L, or HDL–C < 1.0mmol/L in men or < 1.2 mmol/L in women, or TG > 1.7 mmol/L (or receiving of hypolipidaemic medications).

Smoking was classified as never, former, or current smoking.

2.3 Signs of Early Heart Failure With Preserved Left Ventricular Ejection Fraction

Objective parameters of oxygen uptake during peak exercise and biomarker of myocardial wall stress were used for the establishment of the initial phase of HF. Individuals were considered as having early HFpEF if during cardiopulmonary stress test the peak VO2 was ≤ 90% of predicted value or/and BNP was 35 ng/l or higher. Based on these two criteria prospective patients were divided into two subgroups: without (HFpEF–) and with signs of early HFpEF (HFpEF+).

2.4 Methods

2.4.1 Echocardiographic Examination

Two–dimensional echocardiography was performed using ultrasonic system equipped with a 1.0–5.0 MHz transducer (GE Vivid 4; GE Healthcare, New York, USA). The following measures were obtained: interventricular septal end-diastolic thickness (IVST), left ventricular posterior wall end-diastolic thickness (LPWT), and left ventricular end-diastolic dimension (LVDd).

The following formulas were used:

body surface area = 0,0061 × body height (cm) + 0.128 × body weight (kg) – 0.1529 (m^2),

left ventricular mass (LVM)= 0.8 × 1.04 × [(IVST + LPWT + LVDd)^3 – LVDd^3] + 0.6 (g),

and relative wall thickness (RWT) = (IVST + LPWT)/LVDd.

According to recommendations for chamber quantification LV hypertrophy was diagnosed when LV mass index was ≥ 95 g/m² in women and ≥ 115 g/m² in men and/or relative wall thickness ≥ 0.42 [42]. Based on measurments of left ventricular mass index (LVMI) and relative wall thickness (RWT) 3 patterns of LV remodeling were defined: concentric remodeling (normal LVMI and increased RWT), eccentric hypertrophy (increased LVMI and normal RWT) and concentric hypertrophy (increased LVMI and increased RWT).

Assessment of LV diastolic function included transmitral pulse wave Doppler with evaluation of peak velocities of early diastolic flow (E) and peak flow of atrial contraction (A), E/A ratio, E wave deceleration time (DT) as well as tissue Doppler imaging parameters with early (e') and late (a') diastolic mitral septal and lateral annular velocities, E/e' ratio. Isovolumic relaxation time (IVRT) was measured from the end of aortic flow to the beginning of mitral inflow with the simultaneous visualization of aortic and mitral flow.

The left atrial volume (LAV) was measured by the biplane area-length method and indexed to the body surface area.

Diagnosis of LV diastolic dysfunction

Left ventricular diastolic dysfunction was defined according to the 2016 ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure [43] (Table 1). Impaired relaxation was described as E/A < 1.0 and E/e' mean < 13. Parti-

TABLE 1. Left ventricular diastolic dysfunction.

Parameter	Impaired relaxation	Pseudonormal filling	Restrictive filling
E/A ratio	< 1.0	1.0 – 2.0	> 2.0
DecT, ms	> 220	140 – 220	< 140
IVRT, ms	> 110	60 – 100	< 60
e'septal, cm/s	< 8	< 8	< 8
e'lateral cm/s	< 10	< 10	< 10
E/e' mean ratio		≥ 13	≥ 13
Abbreviations: DecT – deceleration time, IVRT – isovolumetric relaxation time, e' – early diastolic tissue velocity of mitral annular.			

cipants were considered as having pseudonormal or restrictive diastolic dysfunction if the E/e' mean ratio was ≥ 13. In case of E/A > 1.0 and e'septal ≥ 8 cm/s and e'lateral ≥ 10 cm/s diastolic function was interpreted as normal.

According to the cardiac ultrasound findings study subjects were divided into two groups: with LV diastolic dysfunction (LV DD+, n = 915) and with normal LV diastolic function (LV DD–, n = 293).

2.4.2 Arterial Stiffness and Wave Reflection Measurements

Parameters of arterial stiffness and wave reflection was assessed by applanation tonometry (SphygmoCor, AtCor Medical, v.8.0, Sydney, Australia) system with high-fidelity micromanometer (Millar R, Millar Instruments, Houston, TX), which is placed on the skin surface at the projection of radial, carotid and femoral arteries in order to obtain pulse pressure wave curves [44]. Brachial blood pressure was recorded and the distance between the surface markings of the sternal notch and the femoral artery was measured. These data and simultaneously recorded electrocardiogram allows the system from pulse wave curves to compute the main parameters of arterial stiffness (carotid-femoral pulse wave velocity [cfPWV], also called aortic PWV), aortic augmentation index, and central blood pressure.

2.4.3 Carotid Artery Wall Assessment

Carotid intima-media thickness (IMT), cross-sectional carotid artery distensibility (both calculated in μm) and non-dimensional index Quality Carotid Stiffness (QCS) of common carotid artery (CCA) were measured using high-resolution echo-tracking technology (Art.Lab, Esaote Europe B.V., Maastricht, the Netherlands).

2.4.4 Assesment of the Ankle-brachial Index and Cardio-ankle Vascular Index

The ankle-brachial index (ABI) and cardio-ankle vascular index (CAVI) were calculated using the Fukuda Vascular Screening system VaSera VS-1000 (Fukuda Denshi Co. Ltd., Tokyo, Japan) [45]. CAVI < 8 and an ABI between 0.9 and 1.3 in both legs were considered as normal.

2.4.5 Cardiopulmonary Stress Test

The cycle ergometer was set to a ramp mode for individually fitted incremental tests, each increment varied from 15 to 30 W per minute. The exercise protocol was designed to last approximately 8-12 minutes. Throughout the test, blood pressure and constant eletrocardiogram monitoring was applied. Prior to the exercise test,

spirometry was performed for each subject using a manually calibrated spirometer (SensorMedics, USA). Expired gases during the test were measured and analyzed using a "breath-by-breath" respiratory mass spectrometry system (Vmax® Encore 229, SensorMedics, USA), that was automatically calibrated before each test. The anaerobic threshold and VAT were determined by the "V-slope" method (VCO2/VO2 ratio) – the first curve inflection [46]. Peak oxygen consumption (peak VO2) was considered to be achieved if VO2 reached a plateau in the presence of a growing power output (W) [47]. If the subject was exhausted before the attainment of peak VO2, the VO2 value was accepted as a reasonable maximum if the heart rate was > 85 % of the predicted maximum rate and a respiratory quotient (RQ) > 1.00 was recorded.

2.5 Statistical Analysis

Variables were presented as mean ± standard deviation (SD). Statistical analysis was performed using STATISTICA (StatSoft, version 10) and SPSS (version 17.0 *for Windows*). Comparison between subjects groups was made using t test or Wilcoxon test. Spearman's rank test was used to test the correlation between indices of arterial stiffness and the determinants of LV diastolic function and LV filling pressure (E/A ratio, E/e' ratio). Subsequently, significantly correlated variables in the univariate logistic regression analysis were further analyzed by the forward stepwise logistic regression analysis to assess independent association with LV diastolic dysfunction. Receiver-operating characteristic (ROC) curves were obtained to calculate the cut-off values of a statistical models to reach the best prediction of LV diastolic dysfunction. Optimal cut-off was defined as a threshold where the sum of sensitivity and specificity was maximal. To assess prediction of LV diastolic dysfunction conditional random forests analysis was performed. A key advantage of random forest variable importance measures, as compared to univariate screening methods, is that they cover the impact of each predictor variable individually as well as in multivariate interactions with other predictor variables. Random forests are able to better examine the contribution and behavior that each predictor has, even when one predictor's effect would usually be overshadowed by more significant competitors in simpler models. The statistical significance was set at $p < 0.05$ (two-sided tests), and for multiple testing we used a statistical significance of $p < 0.01$.

3. Results

3.1 The Relationship Between Arterial Markers and Left Ventricular Diastolic Dysfunction in the Cross-sectional Study

In the study cohort, there were 423 men (35%) and 785 women (65%), the average age 54.5 ± 6.17 years. Ninety five percent of study subjects were overweight (BMI > 25kg/m^2) and 60% had BMI > 30kg/m^2. Ninety eight percent of men and ninety nine percent of women with a BMI > 30 kg/m^2 had an inceased waist circumference > 102 cm in men and > 88 cm in women. High blood pressure was found in 92% of study subjects, increased fasting glucose in 58%, decreased HDL-C in 36.5%, and elevated TG in 58.5% of subjects. The carotid-femoral pulse wave velocity (cfPWV) was 8.78 ± 1.6 m/s, the mean stiffness of carotid artery was 3.82 ± 1.42, and the carotid artery mean IMT was 651.9 ± 103 μm. We found that 915 (75.7%) subjects had LV diastolic dysfunction (LV DD+) and 293 (24.3%) participants had normal LV diastolic function (LV DD–). The main characteristics of our cross-sectional cohort and its LV DD+ and LV DD– subgroups are presented in Tables 2 and 3.

TABLE 2. The clinical characteristics in groups of subjects with (LV DD+) and without (LV DD–) left ventricular diastolic dysfunction.

Parameters	All subjects (n=1208)	LV DD+ (n= 915)	LV DD– (n=293)	p value
Age, years	54.54 ± 6.17	54.7 ± 6.00	52.0 ± 6.28	< 0.0001
Sex, men, %	425 (35.1)	293 (32.0)	132 (45.2)	< 0.001
Hipertensives, %	1115 (93.3)	856 (93.7)	257 (88.3)	0.005
Diabetes mellitus, %	195 (16.1)	152 (15.7)	43 (14.7)	0.466
Never smoked, %	339 (28.1)	242 (26.6)	95 (32.9)	0.043
Dyslipidemia, %	1204 (99.7)	909 (99.7)	290 (99.7)	1.000
Weight, kg	88.84 ± 16.79	89.1 ± 16.69	87.9 ± 17.12	0.2941
BMI, kg/m2	31.72 ± 4.98	32.1 ± 5.06	30.5 ± 4.52	< 0.0001
Waist circumference, cm	106.04 ±10.74	106.8 ± 10.76	103.6 ± 10.40	< 0.0001
TC, mmol/L	6.98 ± 1.44	7.0 ± 1.45	7.0 ± 1.41	0.7586
LDL-C, mmol/L	4.61 ± 1.29	4.6 ± 1.29	4.6 ± 1.31	0.8125
HDL-C, mmol/L	1.33 ± 0.36	1.3 ± 0.35	1.3 ± 0.40	0.8255
TG, mmol/L	2.21 ± 1.31	2.2 ± 1.27	2.2 ± 1.42	0.8623
Fasting glucose, mmol/L	6.0 ± 1.22	6.0 ± 1.29	5.9 ± 0.99	0.0231
hsCRP, mg/dL	4.06 ± 28.83	4.5 ± 33.11	2.5 ± 3.08	0.0697

Abbreviations: BMI – body mass index; TC – total cholesterol; LDL-C – low density lipoprotein cholesterol; HDL-C – high density lipoprotein cholesterol; TG – triglycerides; hsCRP – high sensitivity C-reactive protein.

TABLE 3. The arterial and hemodynamic properties in subjects with (LV DD+) and without (LV DD–) left ventricular diastolic dysfunction.

Parameters	All subjects (n=1208) Mean ± SD	LV DD+ (n= 915) Mean ± SD	LV DD– (n=293) Mean ± SD	p value
HR, beats/min	65.77 ± 9.23	66.4 ± 9.44	63.8 ± 8.25	< 0.0001
cfPWV, m/s	8.78 ± 1.6	9.0 ± 1.65	8.2 ± 1.34	<0.0001
AIx/HR, %	24.36 ± 10.27	25.4 ± 9.96	21.1 ± 10.84	<0.0001
Arterial blood pressure, mmHg	107.36 ± 12.35	108.6 ± 12.85	103.4 ± 10.13	<0.0001
Aortic pulse pressure, mmHg	43.89 ± 10.68	44.5 ± 10.69	41.9 ± 10.80	0.0004
CAVI	7.99 ± 1.62	8.0 ± 1.67	7.8 ± 1.55	0.0721
CCA IMT, μm	651.92 ± 103.08	656.6 ±105.29	637.6 ± 98.49	0.0069
CCA stiffness	3.82 ± 1.42	3.9 ± 1.47	3.5 ± 1.32	<0.0001
Mean ABI	1.09 ± 0.09	1.1 ± 0.59	1.2 ± 0.55	0.5652

Abbreviations: HR – heart rate, cfPWV – carotid-femoral pulse wave velocity; AIx/HR – augmentation index, automatically normalized for a heart rate of 75 bpm; CAVI – cardio-ankle vascular index; CCA – common carotid artery; IMT – intima-media thickness; ABI – ankle-brachial index.

We found significant differences between the groups with LV diastolic dysfunction (LV DD+) and without LV diastolic dysfunction (LV DD–). Subjects with LV diastolic dysfunction were older (54.7 ± 6 vs. 52 ± 6.28 years, $p < 0.0001$), had higher BMI (32.1 ± 5.06 vs. 30.5 ± 4.52 kg/m^2, $p < 0.0001$) and WC (106.8 ± 10.76 vs. 103.6 ± 10.40 cm, $p < 0.0001$), arterial blood pressure (108.6 ± 12.85 vs. 103.4 ± 10.13 mmHg, $p < 0.0001$), heart rate (66.4 ± 9.44 vs. 63.8 ± 8.25 beats/min, $p < 0.0001$), and aortic pulse pressure (44.5 ± 10.69 vs. 41.9 ± 10.80 mmHg, $p = 0.0004$). The LV DD+ subjects, as compared to LV DD– group, had higher prevalence of hypertensives (93.7% of subjects in LV DD+ group compared to 88.3% in LV DD– group, $p = 0.005$), female subjects (68% in LV DD+ group compared to 54.8% in LV DD– group, $p < 0.001$), and smokers (73.4% of subjects in LV DD+ group compared to 67.1% in LV DD– group, $p = 0.043$).

Subjects with LV diastolic dysfunction displayed higher cfPWV (9.0 ± 1.65 vs. 8.2 ± 1.34m/s, $p < 0.0001$), AIx/HR (25.4 ± 10 vs. 21.1 ± 10.84%, $p < 0.0001$), and mean carotid artery IMT (656.6 ± 105.29 vs. 637.6 ± 98.49 μm, $p = 0.0069$) (Table 3). The LVMI (109.88 ± 24.56 vs. 101,96 ± 21.5 g/m^2, $p < 0.0001$) and RWT (0.43 ± 0.05 vs. 0.42 ± 0.05, $p = 0.0023$) were higher in the LV DD+ group (Table 4).

We found statistically significant correlations between arterial stiffness indices and LV diastolic function parameters: cfPWV and aortic augmentation index negatively correlated with E/A ratio ($r_{cfPWV} = -0.19$ [Figures 2], $r_{AIxHR75} = -0.15$, $p < 0.001$), and positively correlated with E/e' mean ratio ($r_{cfPWV} = 0.17$, $r_{AIxHR75} = 0.14$, $p < 0.001$).

TABLE 4. The echocardiographic parameters in subjects with (LV DD+) and without (LV DD–) left ventricular diastolic dysfunction.

Parameters	All subjects (n=1208) Mean ± SD	LV DD+ (n=915) Mean ± SD	LV DD– (n=293) Mean ± SD	p value
LV index, cm/m2	2.59 ± 0.27	2.59 ± 0.28	2.55 ± 0.26	0.142
LVMI, g/m2	107.96 ± 24.08	108.82 ± 24.04	97.11 ± 21.96	<0.001
RWT	0.43 ± 0.05	0.43 ± 0.05	0.41 ± 0.05	0.003
LA length, cm	5.25 ± 0.5	5.26 ± 0.51	5.19 ± 0.43	0.229
LA width, cm	4.73 ± 0.59	4.75 ± 0.58	4.48 ± 0.54	<0.001
LA volume index, ml/m2	32.64 ± 9.29	32.78 ± 9.38	30.84 ± 7.83	0.059
LA volume, ml	64.32 ± 20.25	64.66 ± 20.48	60.14 ± 16.55	0.044
Transmitral peak early diastolic flow (E-wave velocity), m/s	0.8 ± 0.17	0.8 ± 0.17	0.84 ± 0.15	0.014
Transmitral peak flow of atrial contraction (A-wave velocity), m/s	0.85 ± 0.17	0.87 ± 0.16	0.65 ± 0.12	<0.001
E/A ratio	0.97 ± 0.27	0.94 ± 0.25	1.32 ± 0.24	<0.001
Transmitral E-wave DT, millisec	210.81 ± 37,60	212.58 ± 38.48	199.4 ± 29.71	0.145
Early (e'sept) diastolic tissue velocity of septal mitral annular, cm/sec	7.15 ± 1.86	6.92 ± 1.68	9.97 ± 1.72	<0.001
Early (e'lat) diastolic tissue velocity of lateral mitral annular, cm/sec	9 ± 2.56	8.74 ± 2.39	12.18 ± 2.45	<0.001
E/e'sept ratio	11.91 ± 4.08	12.17 ± 4.09	8.65 ± 2.02	<0.001
E/e'lat ratio	9.63 ± 3.67	9.83 ± 3.7	7.18 ± 1.93	<0.001
E/e'mean ratio	10.47 ± 3.44	10.69 ± 3.46	7.72 ± 1.66	<0.001
Mean pulmonary capillary wedge pressure (mmHg) by Nagueh formula	13.84 ± 4.55	14.09 ± 4.59	10.81 ± 2.39	<0.001

Abbreviations: LV – left ventricular, LVMI – left ventricular mass index, RWT - relative wall thickness, LA - left atrium, DT - deceleration time.

Also the positive significant correlation was found between E/e'mean ratio and age ($r = 0.190$, $p < 0.001$), BMI ($r = 0.216$, $p < 0.001$), LVMI ($r = 0.324$, $p < 0.001$), arterial blood pressure ($r = 0.159$, $p < 0.001$), aortic pulse pressure ($r = 0.265$, $p < 0.001$) (Figure 3), and mean carotid artery IMT ($r = 0.163$, $p < 0.001$) (Table 5). The strongest correlation was found between the LVMI and E/e' mean ratio.

TABLE 5. The correlations between various variables and left ventricular diastolic function parameters.

Parameters	E/A ratio		E/e'mean ratio	
	r	p value	r	p value
Age, years	-0.178	<0.001	0.190	<0.001
BMI, kg/m2	-0.054	0.062	0.216	<0.001
cfPWV, m/s	-0.190	<0.001	0.167	<0.001
AIx/HR, %	-0.151	<0.001	0.136	<0.001
Arterial blood pressure, mmHg	-0.125	<0.001	0.159	<0.001
Aortic pulse pressure, mmHg	-0.053	0.068	0.265	<0.001
CCA IMT, µm	-0.014	0.632	0.163	<0.001
HR, beats/min	-0.198	<0.001	-0.062	0.031
CAVI	-0.065	0.024	0.003	0.922
LVMI, g/m2	-0.037	0.199	0.324	<0.001

Abbreviations: BMI – body mass index; cfPWV–- carotid-femoral pulse wave velocity; AIx/HR – augmentation index, automatically normalized for a heart rate of 75 bpm; CCA – common carotid artery; IMT – intima–media thickness; HR – heart rate; CAVI – cardio-ankle vascular index; LVMI – left ventricular mass index.

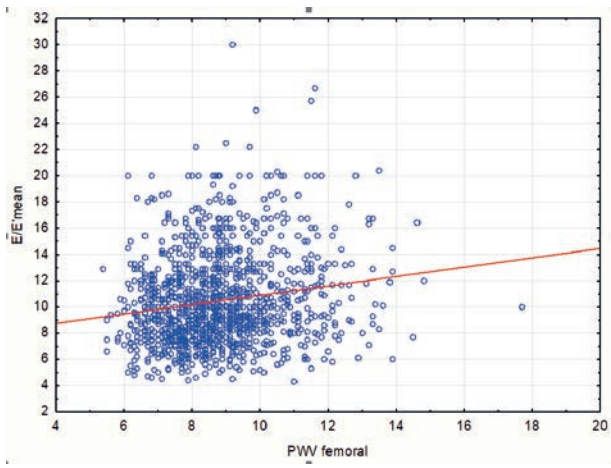


FIGURE 2. Scatter plot showing the assotiation between carotid-femoral pulse wave velocity and E/e'mean ratio in 1208 subjects.

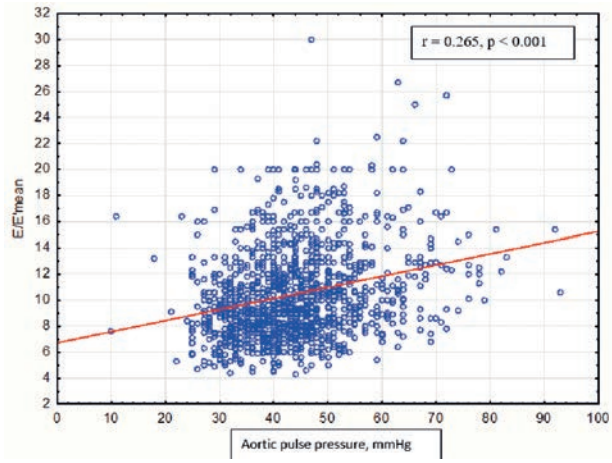


FIGURE 3. Scatter plot showing the assotiation between aortic pulse pressure and E/e'mean ratio in 1208 subjects.

Univariate logistic regression analysis (Table 6) revealed that the probability of LV diastolic dysfunction is increasing with age. Also, we found that the increase of carotid-femoral PWV, aortic augmentation index, heart rate, arterial blood pressure, aortic pulse pressure, carotid artery IMT, BMI, WC, LVMI, and hsCRP is associated with the presence of the LV diastolic dysfunction (all $p < 0.05$). Women also had a higher probability of the LV diastolic dysfunction than men, namely by 43%. We have found that the carotid-femoral PWV threshold value of 8.2 m/s helps to distinguish subjects with the increased risk for the development of LV diastolic dysfunction. An increase in carotid-femoral PWV higher then 8.2 m/s was associated with a significantly higher probability of LV diastolic dysfunction by 41% (AUC 0.642, sensitivity 0.715, specificity 0.596).

TABLE 6. Univariate logistic regression analysis for the association between LV diastolic dysfunction and various variables.

Characteristics	Cut-off	OR	95% CI	p value	AUC
Age, years	≥ 54	1.073	1.050 – 1.097	< 0.001	0.623
Sex	men	0.571	0.436 – 0.747	< 0.001	0.566
BMI, kg/m2	≥ 32.2	1.078	1.046 – 1.111	< 0.001	0.599
WC, cm	≥ 106	1.030	1.016 – 1.043	< 0.001	0.587
B–Ch, mmol/l	≥ 7.7	1.014	0.926 – 1.112	0.759	0.497
Fasting glucose, mmol/l	≥ 6.87	1.142	1.000 – 1.303	0.05	0.536
hsCRB, mg/dl	≥ 3.0	1.064	1.019 – 1.112	0.005	0.570
cfPWV, m/s	≥ 8.2	1.412	1.280 – 1.557	< 0.001	0.642
AIx/HR, %	≥ 19.6	1.041	1.027 – 1.054	< 0.001	0.617

Characteristics	Cut-off	OR	95% CI	p value	AUC
Mean arterial BP, mmHg	≥ 99.5	1.039	1.027 – 1.052	< 0.001	0.616
Aortic pulse pressure, mmHg	≥ 36.9	1.024	1.011 – 1.038	< 0.001	0.573
CAVI mean	≥ 8.4	1.078	0.993 – 1.171	0.072	0.558
IMT mean, μm	≥ 662	1.002	1.000 – 1.003	0.007	0.563
CCA stiffness mean	≥ 3.7	1.244	1.119 – 1.383	< 0.001	0.588
HR, beats/min	≥ 63.8	1.032	1.017 – 1.048	< 0.001	0.575
ABI mean	≥ 2.0	0.940	0.759 – 1.163	0.568	0.552
LVMI, g/m²	≥ 103	1.015	1.009 – 1.021	< 0.001	0.595
Smoking status	Current	0.741	0.557 – 0.986	0.040	0.531

Abbreviations: OR – odds ratio; CI – confidence interval; BMI – body mass index; TC – total cholesterol; hsCRP – high sensitivity C-reactive protein; HR – heart rate; cfPWV – carotid-femoral pulse wave velocity; AIx/HR – augmentation index, automatically normalized for a heart rate of 75 bpm; BP – blood pressure, CAVI – cardio-ankle vascular index; CCA – common carotid artery; IMT – intima–media thickness; ABI – ankle-brachial index; LVMI – left ventricular mass index; AUC – area under the curve.

In order to further clarify the significant predictors of LV diastolic dysfunction, a stepwise multiple logistic regression analysis with all significant variables was performed. Due to high correlation between BMI, WC, two stepwise logistic regression models were constructed including either BMI or WC (Table 7, Model 1 and Model 2). Age, gender and HR were included in all regression models as covariates. In Model 1, we included BMI, hsCRP, cfPWV, AIx, mean aortic BP, aortic PP, mean CCA IMT, mean ABI, LVMI, and smoking as independent variables. In Model 2, BMI was replaced with WC. Stepwise multiple logistic regression analyses demonstrated that cfPWV, age, sex (only in Model 1), BMI or WC, and LV MI remained significant predictors of the presence of LV diastolic dysfunction ($p < 0.05$).

TABLE 7. The predictors of left ventricular diastolic dysfunction in the cross-sectional data: multiple logistic regression analysis.

	Model 1			Model 2		
Characteristics	OR	95% CI	p	OR	95% CI	p
Age, years	1.068	1.031 – 1.107	0.003	1.053	1.023 – 1.083	<0.001
Sex, men	1.61	1.016 –2.551	0.043	No significance		
BMI, kg/m2	1.077	1.042 – 1.114	<0.001			
WC, cm				1.039	1.023 – 1.055	<0.001
hsCRB, mg/dl						
cfPWV, m/s	1.186	1.066 – 1.320	0.002	1.182	1.062 – 1.315	0.002
AIx/HR, %	1.031	1.014 – 1.049	<0.001	1.029	1.013 – 1.046	0.001

	Model 1			Model 2		
Arterial BP, mmHg						
Aortic pulse prssure, mmHg						
CCA IMT mean, μm						
HR, beats/min						
ABI mean						
LVMI, g/m²	1.031	1.014 – 1.048	<0.001	1.030	1.013 – 1.048	0.001
Smoking status, current						
AUC			0.706			0.707

Abbreviations: OR – odds ratio; CI – confidence interval; BMI – body mass index; WC – waist circumference; TC – total cholesterol; hsCRP – high sensitivity C-reactive protein; HR – heart rate; cfPWV – carotid-femoral pulse wave velocity; AIx/HR – augmentation index, automatically normalized for a heart rate of 75 bpm; BP – blood pressure, CAVI – cardio-ankle vascular index; CCA – common carotid artery; IMT – intima–media thickness; ABI – ankle-brachial index; LVMI – left ventricular mass index; AUC – area under the curve.

As the odds ratios in Table 7 show, carotid-femoral PWV was the strongest predictor of the LV diastolic dysfunction: increase of cfPWV by 1SD is associated with significantly higher probability of LV diastolic dysfunction, namely, by 18.6% and 18.2% in Models 1 and 2 respectively. The ROC curve analysis showed that the

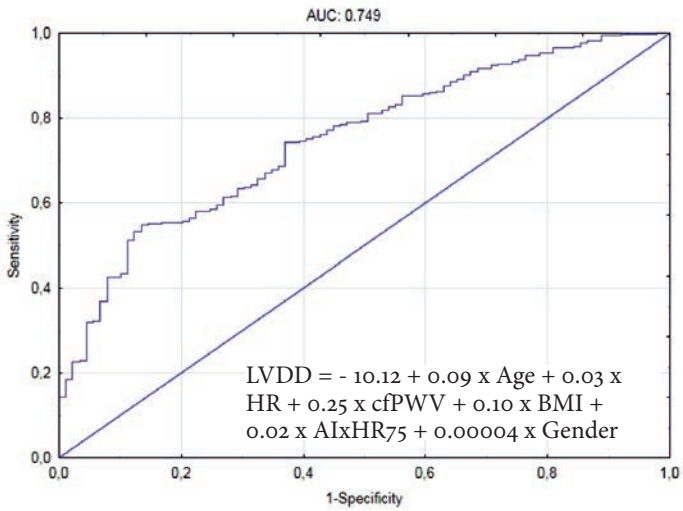


FIGURE 4. ROC curve of Model 1 for prediction of LV diastolic dysfunction (Sensitivity 0.566; Specificity 0.820)

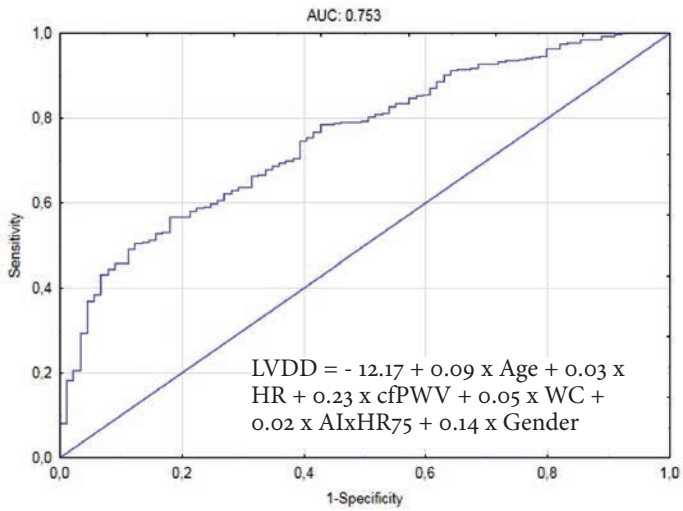


FIGURE 5. ROC curve of Model 2 for prediction of LV diastolic dysfunction. (Sensitivity 0.549; Specificity 0.865)

areas under the ROC (AUC) of the statistical models for predicting of LV diastolic dysfunction were 0.706 in Model 1 (Figure 4) and 0.707 in Model 2 (Figure 5), thus indicating slightly higher predictive value of WC over BMI in MetS subjects.

In conlusion, left ventricular diastolic dysfunction is significantly associated with elevated arterial stiffness in metabolic syndrome subjects. Non-invasively measured carotid-femoral pulse wave velocity, an index of aortic stiffness, is a significant and independent determinant of the LV diastolic dysfunction in patients with metabolic syndrome without overt cardiovascular disease. Age, BMI, aortic augmentation index, gender and LV mass index also are significant predictors of the presence of LV diastolic dysfunction.

3.2 The Influence of Arterial Stiffness Alteration on the Dynamics of Left Ventricular Diastolic Dysfunction during Longitudinal Observation

The longitudinal study of 3.8 ± 0.6 years was carried out in 573 subjects randomly selected from the general cohort of 1208 participants. The data of this study was used to analyze the effects of the alteration of arterial stiffness on the dynamics of the left ventricular diastolic dysfunction.

3.2.1 Longitudinal Observation: The Subjects Profile at Baseline and Follow-up Visit

In the longitudinal study cohort there were 36.8% (n = 211) men and 63.2% (n = 362) women, an average age of the study participants was 53.4 ± 5.8 years. High blood pressure was found in 92.7% (n = 531) of subjects. The obesity (BMI > 30 kg/m^2) was found in 62.8% (n = 360) of study subjects.

During the longitudinal observation, we have observed a significant reduction in waist circumference (from 105.77 ± 9.94 to 104.65 ± 10.99 cm, $p < 0.001$), total cholesterol (from 6.83 ± 1.45 to 6.57 ± 1.59 mmol/L, $p = 0.002$), LDL cholesterol (from 4.49 ± 1.25 to 4.27 ± 1.34 mmol/L, $p = 0.003$) and hsCRP (from 3.12 ± 4.13 to 2.58 ± 3.00 mg/L, $p = 0.009$) (Table 8).

TABLE 8. Baseline and follow-up indeces of obesity, lipid and fasting glucose profile, and inflammation.

Parameters	At baseline (n = 573) Mean ± SD	Follow-up (n = 573) Mean ± SD	Change Mean ± SD	p value
Weight, kg	89.38 ± 15.68	89.22 ± 16.03	0.25 ± 6.95	0.495
BMI, kg/m^2	31.53 ± 4.24	31.37 ± 4.28	– 0.16 ± 2.05	0.065
Waist circumference, cm	105.77 ± 9.94	104.65 ± 10.99	– 1.12 ± 6.74	< 0.001
TC, mmol/l	6.83 ± 1.45	6.57 ± 1.59	– 0.26 ± 1.67	0.002
LDL–C, mmol/l	4.49 ± 1.25	4.27 ± 1.34	– 0.22 ± 1.43	0.003
HDL–C mmol/l	1.31 ± 0.34	1.27 ± 0.33	– 0.03 ±0.03	< 0.001
TG, mmol/l	2.30 ± 1.91	2.39 ± 4.15	0.09 ± 3.75	0.623
Fasting glucose, mmol/l	5.99 ± 1.22	6.19 ± 1.42	0.19 ± 1.28	0.001
hsCRB, mg/l	3.12 ± 4.13	2.58 ± 3.00	– 0.54 ± 4.08	0.009

Abbreviations: BMI – body mass index; TC – total cholesterol; LDL-C – low density lipoprotein cholesterol; HDL-C – high density lipoprotein cholesterol; TG – triglycerides; hsCRP – high sensitivity C-reactive protein.

The treatment data were collected only at follow-up visit. In our follow-up cohort, 89.3% (n = 512) of subjects were on treatment. The most frequently used medications were ACE inhibitors, BB, CCB and statins (Table 9).

TABLE 9. The data of medications used.

Medication	n	%
ACE inhibitors	233	40,7
ARB	89	15,5
Diuretics	130	22,7
CCB	198	34,6
BB	206	36
α blockers	4	0,7
Other	32	5,6
Statins	412	71,9
Abbreviations: ACE – angiotensin converting enzyme inhibitors, CCB – calcium channel blockers, ARB – angiotensin receptor blockers, BB – beta blockers.		

3.2.2 Arterial Wall Functional and Structural Parameters at Baseline and Follow-up Visit

The baseline evaluation of arterial wall parameters revealed that the subjects with LV diastolic dysfunction (LV DD+) had higher heart rate (67.97 ± 10.81 vs. 64.70 ± 9.23 beats/min, p = 0.002), carotid-femoral PWV (8.71 ± 1.48 vs. 8.10 ± 1.20 m/s, p < 0.001), AIx/HR (23.90 ± 10.07 vs. 20.48 ± 10.48%, p < 0.001), mean arterial BP (106.63 ± 10.65 vs. 102.53 ± 10.17 mmHg, p < 0.001), aortic pulse

TABLE 10. The comparison of arterial wall parameters between subjects groups at baseline.

Parameters	LV DD+ (n = 418) mean ± SD	LV DD– (n = 155) mean ± SD	p value
HR, beats/min	67.97 ± 10.81	64.70 ± 9.23	0.002
cfPWV, m/s	8.71 ± 1.48	8.10 ± 1.20	< 0.001
AIx/HR, %	23.90 ± 10.07	20.48 ± 10.48	< 0.001
Mean arterial BP, mmHg	106.63 ± 10.65	102.53 ± 10.17	< 0.001
Aortic pulse pressure, mmHg	43.33 ± 9.80	40.57 ± 9.96	0.003
CAVI	7.73 ± 1.57	7.90 ± 1.69	0.258
CCA IMT , μm	663.26 ± 100.87	634.04 ± 95.44	0.002
CCA stiffness	3.92 ± 1.41	3.55 ± 1.31	0.006
ABI	1.07 ± 0.09	1.10 ± 0.09	0.002
Abbreviations: LV – left ventricular, DD – diastolic dysfunction, HR – heat rate, cfPWV – carotid-femoral pulse wave velocity; AIx/HR – augmentation index, automatically normalized for a heart rate; BP – blood pressure, CAVI – cardio-ankle vascular index; CCA – common carotid artery; IMT–- intima-media thickness; ABI – ankle-brachial index.			

pressure (43.33 ± 9.80 vs. 40.57 ± 9.96 mmHg, p = 0.003), carotid artery stiffness (3.92 ± 1.41 vs. 3.55 ± 1.31, p = 0.006), and carotid artery IMT (663.26 ± 100.87 vs. 634.04 ± 95.44 μm, p = 0.002) (Table 10).

After 3 years, we have observed a significant decrease in heart rate (– 3.61 ± 10.6 k/min, p < 0.001) and mean arterial BP (– 3.71 ± 15.22 mmHg, p < 0.001) and a significant increase in cfPWV (0.15 ± 1.59 m/s, p = 0.019), AIx/HR (1.44 ± 9.55 proc., p < 0.001), carotid artery stiffness (0.78 ± 1.46, p < 0.001) and carotid artery IMT (38.33 ± 90.86 μm, p < 0.001) (Table 11).

TABLE 11. The changes of haemodynamic and arterial wall parameters during the longitudinal observation.

Prameters	At baseline (n = 573) Mean± SD	Follow-up (n = 573) Mean ± SD	Change Mean ± SD	p value
HR, beats/min	67.00 ± 10.29	63.35 ± 8.67	– 3.61 ± 10.6	< 0.001
cfPWV, m/s	8.55 ± 1.44	8.71 ± 1.61	0.15 ± 1.59	0.019
AIx/HR, %	22.83 ± 10.37	24.27 ± 10.82	1.44 ± 9.55	< 0.001
Mean arterial BP, mmHg	105.63 ± 10.62	101.59 ± 14.72	– 3.71 ± 15.22	< 0.001
Aortic pulse pressure, mmHg	42.62 ± 9.94	43.31 ± 10.61	0.68 ± 11.15	0.146
CAVI	7.77 ± 1.61	7.78 ± 1.55	0.01 ± 1.9	0.916
CCA IMT , μm	652.77 ± 102.11	691.11 ± 103.04	38.33 ± 90.86	< 0.001
CCA stiffness	3.78 ± 1.40	4.56 ± 1.60	0.78 ± 1.46	< 0.001
ABI	1.08 ± 0.09	1.09 ± 0.10	0.01 ± 0.10	0.077

Abbreviations: cfPWV – carotid-femoral pulse wave velocity; AIx/HR – augmentation index normalized for a heart rate; BP – blood pressure, CCA – common carotid artery; IMT – intima–media thickness; HR – heart rate; CAVI – cardio-ankle vascular index; ABI – ankle-brachial index.

After 3 years, carotid-femoral PWV increased in 310 subjects, decreased in 241 subjects; aortic augmentation ndex increased in 295 and decreased in 218 subjects; and arterial BP increased in 221 and decreased in 318 subjects.

3.2.3 Echocardiographic Parameters and Their Changes during the Longitudinal Observation

At the baseline evaluation 72.9% (n = 418) of the longitudinal cohort subjects had LV diastolic dysfunction. The subjects with LV diastolic dysfunction (LV DD+) had higher LV mass index (106.77 ± 22.59 vs. 102.25 ± 21.99 g/m^2, p = 0.033) and relative wall thickness (0.43 ± 0.06 vs. 0.42 ± 0.06, p = 0.020) (Table 12).

TABLE 12. Comparison of echocardiographic parameters in subjects with (LV DD+) and without (LV DD–) left ventricular diastolic dysfunction at baseline.

Parameters	LV DD+ n = 418 Mean± SD	LV DD– n = 155 Mean± SD	p value
LV index, cm/m^2	2.55 ± 0.28	2.54 ± 0.24	0.481
LVMI, g/^{m}2	106.77 ± 22.59	102.25 ± 21.99	0.033
RWT	0.43 ± 0.06	0.42 ± 0.06	0.020
LA volume index, ml/m^2	33.13 ± 9.54	31.93 ± 7.56	0.120
LA volume, ml	65.89 ± 21.54	63.88 ± 18.02	0.264
E, cm/s	0.76 ± 0.16	0.91 ± 0.13	< 0.001
A, cm/s	0.87 ± 0.16	0.73 ± 0.13	< 0.001
E/A ratio,	0.89 ± 0.22	1.26 ± 0.18	< 0.001
e'_{sept}, cm/s	6.71 ± 2.87	8.65 ± 1.87	< 0.001
e'_{lat}, cm/s	8.39 ± 2.43	11.14 ± 2.34	< 0.001
E/e' mean ratio	10.85 ± 3.91	9.40 ± 2.01	< 0.001
E/e'_{lat} ratio	10.16 ± 6.55	8.52 ± 2.29	< 0.001
E/e'_{sept} ratio	12.32 ± 4.43	10.97 ± 2.86	< 0.001
PKpr, mmHg	14.50 ± 8.12	12.47 ± 2.83	< 0.001

Abbreviations: LV – left ventricular, LVMI – left ventricular mass index, RWT – relative wall thickness, LA – left atrium, E – transmitral peak early diastolic flow, A – transmitral peak flow of atrial contraction, sept – septal part of mitral annular, lat – lateral part of mitral annular, PKpr – mean pulmonary capillary wedge pressure (mmHg) by Nagueh formula.

After 3 years, we have observed the significant reduction of LV index from 2.55 ± 0.27 cm/m^2 to 2.51 ± 0.33 cm/m^2 (p = 0.008), LVMI from 105.56 ± 22.5 to 99.11 ± 23.67 (p < 0.001) and RWT from 0.43 ± 0.06 to 0.42 ± 0.06 (p = 0.002). Also, during the longitudinal observation, we have found the significant positive dynamics of LV diastolic function parameters: E/e'_{mean} decreased from 10.46 ± 3.55 to 9.43 ± 2.87 (p < 0.001), E/e'_{lat} decreased from 9.72 ± 5.77 to 8.54 ± 3.05 (p < 0.001), and E/e'_{sept} decreased from 11.95 ± 4.11 to 10.89 ± 3.19 (p < 0.001) (Table 13).

TABLE 13. Changes of echocardiographic parameters during the longitudinal observation.

Parameters	At baseline (n = 573) Mean± SD	Follow-up (n = 573) Mean ± SD	Change Mean ± SD	p value
LV index, cm/m²	2.55 ± 0.27	2.51 ± 0.33	– 0.03 ± 0.32	0.008
LVMI, g/m²	105.56 ± 22.5	99.11 ± 23.67	– 6.39 ± 26.28	< 0.001
RWT	0.43 ± 0.06	0.42 ± 0.06	– 0.01 ± 0.07	0.002
LA volume index, ml/m²	32.76 ± 9.02	33.36 ± 8.65	0.60 ±10.5	0.172
LA volume, ml	65.28 ± 20.6	66.42 ± 19.40	1.14 ± 21.49	0.206
E, cm/s	0.99 ± 0.27	0.93 ± 0.23	– 0.05 ± 0.27	< 0.001
A, cm/s	7.24 ± 2.77	7.14 ± 1.75	0.21 ± 2.74	0.402
E/A ratio,	9.13 ± 2.70	9.35 ± 2.56	– 0.10 ± 2.86	0.063
e'_{sept}, cm/s	10.46 ± 3.55	9.43 ± 2.87	– 1.03 ± 3.84	< 0.001
e'_{lat}, cm/s	9.72 ± 5.77	8.54 ± 3.05	– 1.18 ± 5.78	< 0.001
E/e' mean ratio	11.95 ± 4.11	10.89 ± 3.19	– 1.06 ± 4.57	< 0.001
E/e'_{lat} ratio	13.95 ± 7.15	12.49 ± 3.79	– 1.46 ± 7.17	< 0.001

Abbreviations: LV – left ventricular, LVMI – left ventricular mass index, RWT – relative wall thickness, LA – left atrium, E – transmitral peak early diastolic flow, A – transmitral peak flow of atrial contraction, sept – septal part of mitral annular, lat – lateral part of mitral annular, PKpr – mean pulmonary capillary wedge pressure (mmHg) by Nagueh formula.

The dynamics of LV diastolic function are presented in Table 14. During the longitudinal observation, in 243 (74.7%) subjects impaired LV relaxation remained unchanged, in 54 (16.6%) subjects LV diastolic dysfunction improved and in 28 (8.61%) – worsened.

Notably, in 77.2% (n = 72) subjects with advanced LV diastolic dysfunction (pseudonormal or restrictive LV filling) at baseline we observed the improvement of LV diastolic function and LV morphometric parameters, such as LVMI and RWT. In 16.6% (n = 54) subjects with impaired LV relaxation at baseline, we observed normal LV diastolic function at follow-up visit.

The baseline evaluation of LV morphometric parameters revealed normal LV geometry in 26.9% (n = 154) of longitudinal cohort subjects. LV remodeling was evaluated in 73.1% (n = 419) of subjects at baseline. The LV geometry at baseline and changes during the longitudinal observation presented in Figure 6. After 3 years, in 21.1% (n = 121) of subjects with LV remodeling at baseline, we found normal LV geometry. In 14% (n = 88) of subjects with normal LV geometry at baseline we found LV remodeling at follow-up visit.

TABLE 14. Dynamics of left ventricular diastolic function.

		Follow-up LV diastolic function			Overall
		Normal	Impaired relaxation	Pseudonormal filling	
At baseline LV diastolic function	Normal	73	69	13	155
	Impaired relaxation	54	243	28	325
	Pseudonormal LV filling	13	58	21	92
	Restictive LV filling	0	1	0	1
Overall		140	371	62	573

Abbreviations: LV – left ventricular.

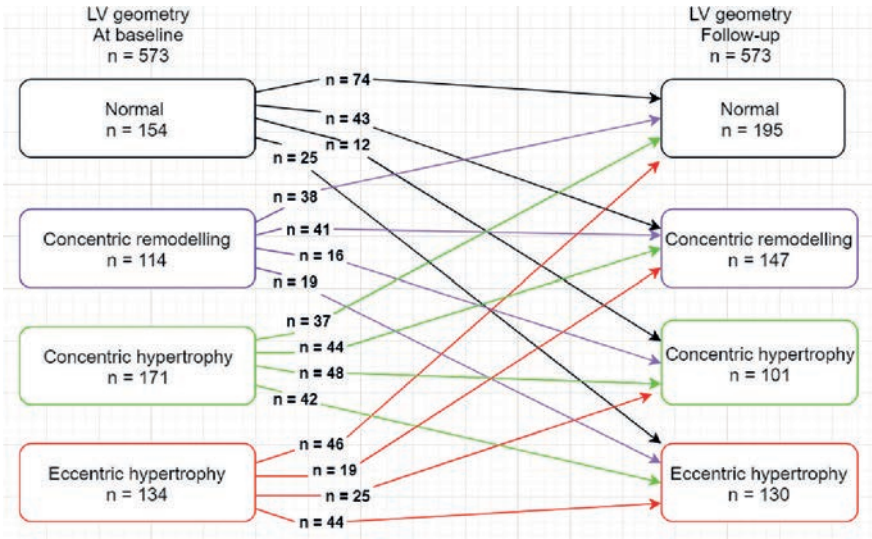


FIGURE 6. Changes in left ventricular geometry during the longitudinal observation.

3.2.4 Changes in Aortic Pulse Wave Velocity and Left Ventricular Diastolic Function Dynamics

Depending on the LV diastolic function dynamics, our further analysis focused on the comparison of the arterial hemodynamic markers, in the 3 subgroups (Figure 7):

- Group I: LV diastolic dysfunction improved (22.0, n = 126),
- Group II: LV diastolic function unchanged (58.8 %, n = 337),
- Group III: LV diastolic function worsened (19.2 %, n = 110).

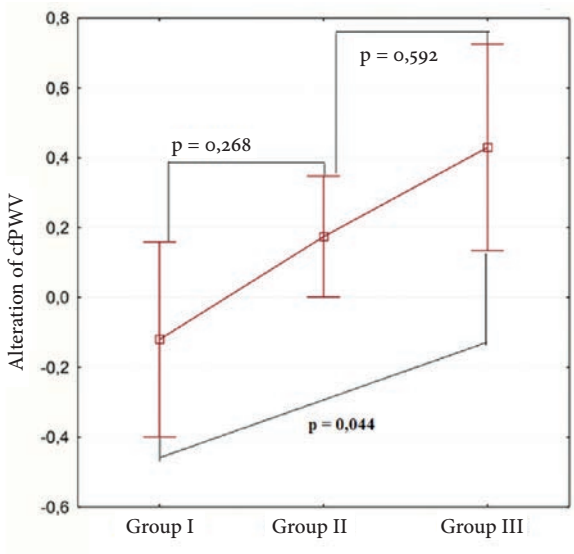


FIGURE 7. The dependence of the left ventri cular diastolic function on the alteration of carotid-femoral pulse wave velocity (cfPWV).

After 3 years, we have observed the following significant changes in the subjects of Group I (LV dastolic dysfunction improved): BMI decreased from 32.06 ± 4.34 to 31.60 ± 4.31 kg/m^2 (p = 0.013), and WC decreased from 106.85 ± 9.99 to 105.57 ± 10.34 cm (p = 0.040).

In Group III we have found a significant increase of weight from 88.21 ±15.58 to 89.35 ±16.85 kg (p = 0.041). Notably, BMI (31.02 ± 4.08 vs. 31.27 ± 4.45, p = 0,267) and WC (04.96 ± 8.88 vs. 103.88 ± 11.9, p = 0.133) did not change significantly.

After 3 years, in both Groups I and III we found the following change in the hemodynamic and arterial parameters (Table 15):

- A significantly decreased heart rate;
- A significantly decreased arterial blood pressure;
- A significantly increased carotid artery stiffness and IMT.

After 3 years, carotid-femoral PWV and aortic augmentation index significantly increased only in Group III. These results confirm, that the negative dynamics of LV diastolic function is associated with the progression of the arterial stiffening.

TABLE 15. Comparison of arterial and central haemodynamics indices Between Group I and Group III at baseline and follow-up.

Group I Improved LV DD (n = 126)				Group III Worsened LV DD (n = 110)		
At baseline (n = 573) Mean± SD	Follow-up (n = 573) Mean ± SD	p value	Parameters	At baseline (n = 573) Mean± SD	Follow-up (n = 573) Mean ± SD	p value
66.27 ±10.09	62.15 ±8.86	< 0.001	HR, beats/min	65.17 ±9.41	62.07 ±8.51	0.003
8.67 ±1.40	8.55 ±1.51	0.489	cfPWV, m/s	8.25 ±1.36	8.68 ±1.73	0.004
23.43 ±9.31	23.85 ±10.55	0.450	AIx/HR, %	23.16 ±9.94	25.16 ±10.08	0.035
107.60 ±10.89	100.48 ±16.55	< 0.001	Mean arterial BP, mmHg	104.07 ±10.94	99.73 ±13.17	0.010
43.14 ±9.41	43.18 ±10.77	0.978	Aortic pulse pressure, mmHg	43.30 ±10.08	43.55 ±10.73	1.000
7.76 ±1.61	7.95 ±1.37	0.314	CAVI	8.02 ±1.96	7.65 ±1.60	0.070
653.26 ±98.87	688.31 ±103.46	< 0.001	CCA IMT, µm	658.31 ±98.40	694.21 ±106.14	0.001
3.72 ±1.35	4.47 ±1.57	< 0.001	CCA stiffness	3.75 ±1.39	4.57 ±1.54	< 0.001
1.07 ±0.08	1.07 ±0.11	0.842	ABI	1.08 ±0.09	1.09 ±0.10	0.790

Abbreviations: LV – left ventricular, DD – diastolic dysfunction, SD – standart deviation, cfPWV – carotid-femoral pulse wave velocity; AIx/HR – augmentation index normalized for a heart rate; BP – blood pressure, CCA – common carotid artery; IMT – intima–media thickness; HR – heart rate; CAVI – cardio-ankle vascular index; ABI – ankle-brachial index.

The longitudinal observation has demonstrated the following significant changes in ehocardiographic parameters: in Group I a decrease in LVMI ($p < 0.001$) and RWT ($p = 0.001$). In Group III we found no significant changes of LVMI and RWT (Table 16).

TABLE 16. Comparison of echocardiographic parameters between Group I and Group III.

Group I Improved LV DD (n = 126)				Group III Worsened LV DD (n = 110)		
At baseline (n = 573) Mean ± SD	Follow-up (n = 573) Mean ± SD	p value	Parameters	At baseline (n = 573) Mean ± SD	Follow-up (n = 573) Mean ± SD	p value
5.08 ±0.45	5.02 ±0.51	0.193	LV diastolic diameter, cm	5.01 ±0.47	5,04 ±0,58	0,556
2.56 ±0.27	2.49 ±0.42	0.217	LV index, cm/m²	2.55 ±0.25	2,55 ±0,27	0,861
109.98 ±20.22	98.92 ±24.37	< 0.001	LVMI, g/m²	105.27 ±23.79	105,08 ±25,72	0,946
0.44 ±0.05	0.42 ±0.05	0.001	RWT	0.43 ±0.06	0,43 ±0,08	0,310
33.80±9.65	33.22 ±7.71	0.957	LA volume index, ml/m²	32.32 ±8.44	33,69 ±7,89	0,220
67.72 ±21.13	66.19 ±16.66	0.926	LA volume, ml	64.21 ±20.00	67,58 ±20,10	0,158
0.83 ±0.17	0.76 ±0.17	0.003	E, cm/s	0.87 ±0.16	0,76 ±0,15	< 0,001
0.85 ±0.16	0.78 ±0.14	< 0.001	A, cm/s	0.79 ±0.16	0,83 ±0,16	0,004
0.99 ±0.25	1.00 ±0.26	0.734	E/A ratio,	1.13 ±0.26	0,92 ±0,19	< 0,001
6.55 ±4.68	7.46 ±1.70	< 0.001	e'_{sept}, cm/s	8.10 ±2.05	6,61 ±1,63	< 0,001
7.73 ±2.53	9.77 ±2.44	< 0.001	e'_{lat}, cm/s	10.00 ±2.43	8,26 ±2,47	< 0,001
12.94 ±4.65	9.07 ±2.08	< 0.001	E/e' mean ratio	9.80 ±2.00	10,94 ±3,75	0,007
11.97 ±4.89	8.17 ±2.15	< 0.001	E/e'_{lat} ratio	9.07 ±2.32	10,12 ±3,93	0,023
14.53 ±5.11	10.64 ±2.81	< 0.001	LV index, cm/m²	11.25 ±3.06	12,20 ±4,03	0,099
16.75 ±6.06	12.03 ±2.67	< 0.001	PPKsp, mmHg	13.15 ±2.88	14,45 ±4,87	0,023

Abbreviations: SD – standart deviation, LV – left ventricular, DD – diastolic dysfunction, LVMI – left ventricular mass index, RWT – relative wall thickness, LA – left atrium, E – transmitral peak early diastolic flow, A – transmitral peak flow of atrial contraction, sept – septal part of mitral annular, lat – lateral part of mitral annular.

TABLE 17. Logistic regression analysis assessing the association between the change of carotid-femoral pulse wave velocity and left ventricular diastolic dysfunction.

	Coefficient of regression	OR	95% CI	p reikšmė
Δ cfPWV	0.222	1.248	1.045 - 1.492	0.015
Δ aortic BP	0.008	1.008	0.991 – 1.026	0.337

Abbreviations: CI – confidence interval, cfPWV – carotid-femoral pulse wave velocity, OR – odds ratio, BP – blood pressure, Δ – the change.

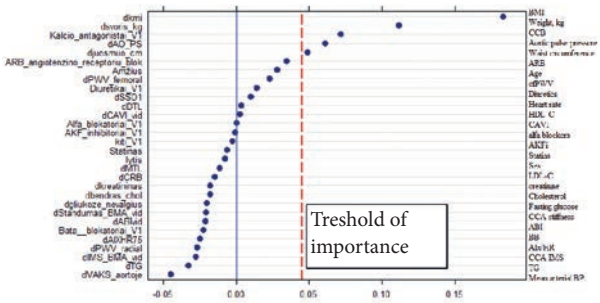
The logistic regression analysis was performed to determined which are the independent variables with a significant influence on the dynamics of LV diastolic function (Table 17). In our regression models, carotid-femoral PWV remained a significant predictor of LV diastolic dysfunction even after adjusting for arterial blood pressure changes.

For more accurate evaluation of the relationship between alterations of arterial stiffness indices and other analized parameters and the dynamics of LV diastolic function the conditional random forests analysis was performed.

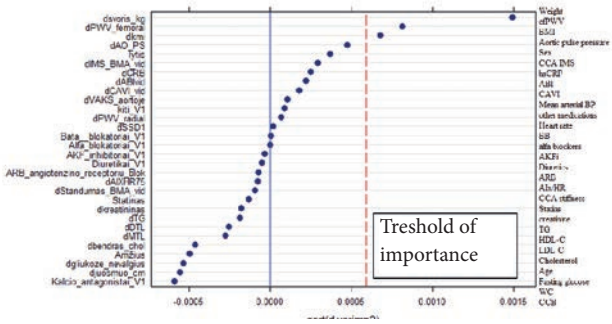
In this analysis, the effect of each variable is evaluated. Random forest can be very effective to find a set of predictors that best explains the variance in the response variable. The results of analysis are presented in importance diagrams. To ensure the stability of analysis 4 tests were performed: Test 1 and Test 2 with generated 1000 trees, Test 3 and Test 4 with 5000 trees.

During the analysis, we evaluate two prognostic models. One model was designed to evaluate which variables are most significant for worsening of LV diastolic function. The second model was constructed to evaluate the prognostic markers of E/e' alterations.

Random forests analysis demonstrated that carotid-femoral PWV, BMI, weight, and CAVI had prognostic significance for the worsening of LV diastolic function



Test 1
1000 trees



Test 2
1000 trees

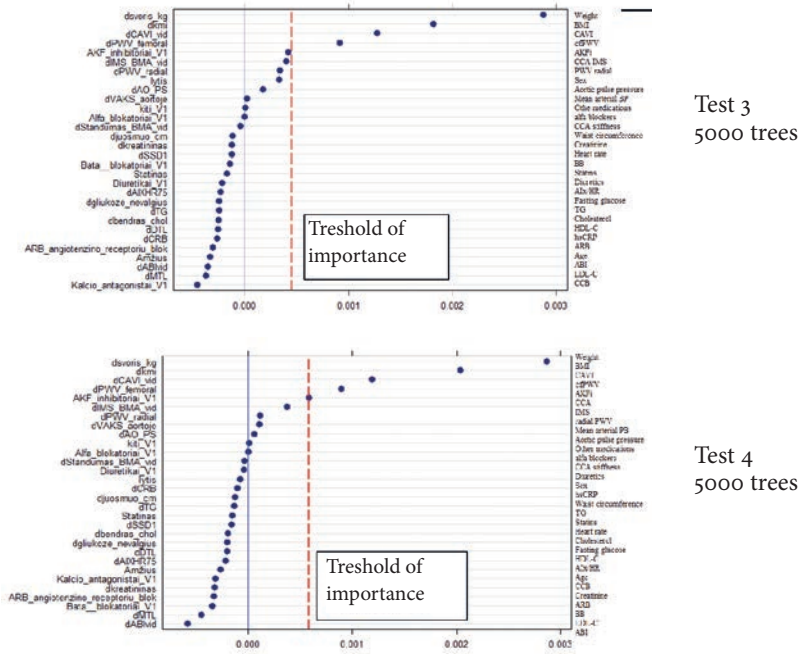
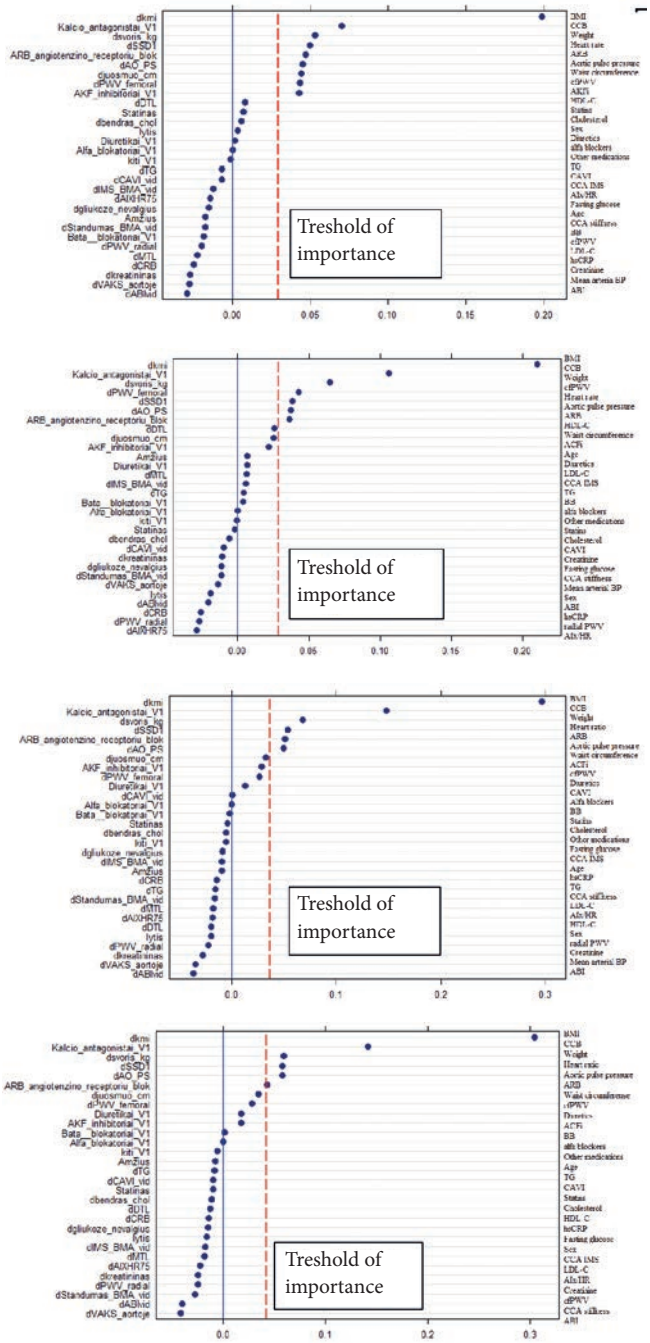


FIGURE 8. Importance diagrams of conditional random forests analysis for prognosis of LV diastolic function worsening.

Abbreviations and explanations: variables at the right of the threshold are the most important. cfPWV – carotid-femoral pulse wave velocity; AIx/HR – augmentation index normalized for a heart rate; BP – blood pressure, CCA – common carotid artery; IMT – intima–media thickness, CAVI – cardio-ankle vascular index; ABI – ankle-brachial index, BMI – body mass index; TC – total cholesterol; LDL-C – low density lipoprotein cholesterol; HDL-C – high density lipoprotein cholesterol; TG – triglycerides; hsCRP – high sensitivity C-reactive protein, ACFi – angiotensin converting enzyme inhibitors, CCB – calcium channel blockers, ARB – angiotensin receptor blockers, BB – beta blockers.

(Figure 8). The second random forests model demonstrated the significant association between the aortic pulse pressure, heart rate, BMI, weight, and calcium chanel blockers and the changes of E/e'_{mean} ratio (Figure 9).

In conclusion, the increase of carotid-femoral PWV and aortic augmentation index are significantly associated with negative dynamics of LV diastolic function. Aortic pulse pressure, heart rate, BMI, weight and calcium channel blockers might also have an important prognostic value.



Test 1
1000 trees

Test 2
1000 trees

Test 3
5000 trees

Test 4
5000 trees

FIGURE 9. Conditional random forests analysis importance diagrams assessing the association between the changes of E/e'_{mean} and evaluated parameters.

Abbreviations and explanations: variables at the right of the threshold are significantly associated with the changes of the E/e'_{mean}. cfPWV – carotid-femoral pulse wave velocity; AIx/HR – augmentation index normalized for a heart rate; BP – blood pressure, CCA – common carotid artery; IMT – intima–media thickness, CAVI – cardio-ankle vascular index; ABI – ankle-brachial index, BMI – body mass index; TC – total cholesterol; LDL-C – low density lipoprotein cholesterol; HDL-C – high density lipoprotein cholesterol; TG – triglycerides; hsCRP – high sensitivity C-reactive protein, ACFi – angiotensin converting enzyme inhibitors, CCB – calcium channel blockers, ARB – angiotensin receptor blockers, BB – beta blockers.

3.3 Detection of Incipient Heart Failure with Preserved Left Ventricular Ejection Fraction according to Evaluation by Means of Exercise Capacity and Biomarker Testing in Metabolic Syndrome Subjects

According to our cross-sectional study in 1208 subjects with metabolic syndrome, LV diastolic dysfunction is highly prevalent (75.7% [n = 915] of study subjects had LV diastolic dysfunction).

For the additional assessment of early HFpEF, 148 subjects of our follow-up cohort (n = 573) underwent a cardiopulmonary stress (spiroergometry) and an assessment of BNP. The average age of this „prospective" group was 56.4 ± 6.6 years, there were 46 (31.1%) men. For the sake of comparing our findings in this group with the clinical characteristics of the subjects with clinically HFpEF, we additionally enrolled 38 MetS subjects with confirmed HFpEF, an average age in this „retrospective" group was 61 ± 10.5 years, 16 (42.1%) men.

According to the BNP values and cardiopulmonary stress test results, subjects from prospective group were divided into two subgroups: with and without early HFpEF. Subjects were assigned to the early HFpEF subgroup (HFpEF+), if, in the cardiopulmonary stress test, they had the peak VO2 ≤ 90% of predicted value or/and BNP ≥ 35 ng/l. According to this classification, the rest of the subjects were considered as HFpEF free (HFpEF– subgroup).

Data on baseline characteristics, echocardiographic parameters, cardiometabolic risk factors, laboratory markers and exercise capacity are presented in Table 18. The most frequent components of metabolic profile were pathological waist circumference (96.0%), blood pressure (90.5%) and fasting serum glucose (70.3%) in the prospective subjects, while – high blood pressure (86.8%), low HDL-cholesterol level (84.2%) and increased fasting serum glucose (76.3%) dominated in retrospective group. In our study the majority of prospective and retrospective subjects presented with LV diastolic dysfunction, and more then half had LV hypertrophy.

As many as 96 subjects from the prospectively enrolled cohort (n = 148) demonstrated reduced exercise capacity and/or elevated BNP consistent with the early

TABLE 18. Metabolic risk factors, echocardiography, laboratory and exercise capacity data of study participants.

	Prospective sub-group without HFpEF– n = 52	Prospective sub-group with early HFpEF+ n = 96	Retrospective group with HF-pEF n = 38
Men	27 (51.9 %)	19 (19.8 %)	16 (42.1 %)
Age, years	55.4 ± 6.4	57.1 ± 6.6	61 ± 10.5
MetS components			
Waist circumference, men	108.8 ± 10.4	107.3 ± 7.3	107.5 ± 6.4
Waist circumference, women	**105.0 ± 11.2**	**98.7 ± 9.7***	103.0 ± 12.8
Triglycerides, mmol/l	2.6 ± 2.3	2.2 ± 2.0	2.4 ± 1.4
Fasting serum glucose, mmol/l	6.4 ± 1.3	6.1 ± 0.9	6.5 ± 1.5
HDL-cholesterol, mmol/l	**1.2 ± 0.3**	**1.3 ± 0.4***	**1.0 ± 0.3** ¢
Mean systolic BP, mmHg	152.2 ± 18.9	155.1 ± 21.8	**146.1 ± 15.5** ¢
Mean diastolic BP, mmHg	97.2 ± 11.7	95.6 ± 11.7	**90.2 ± 10.1** ¢
Symptoms, %			
Dyspnoea or fatigue	7 (13.5)	11 (11.5)	27 (71.1)
Chest pain	8 (15.4)	19 (19.8)	11 (28.9)
Arrhythmias	4 (7.7)	8 (8.3)	6 (15.8)
Echocardiographic parameters			
E/e'mean ratio	9.6 ± 2.6	**9.5 ± 3.0**	**11.4 ± 4.6** ¢
E/A ratio	0.9 ± 0.2	0.9 ± 0.2	1.4 ± 2.1
LA volume index, ml/m²	34.0 ± 9.0	**31.8 ± 9.1**	**46.7 ± 13.7** ¢
LVMI, man, g/m²	104.0 ± 27.8	101.8 ± 28.3	119.1 ± 25.5
LVMI, women, g/m²	97.5 ± 16.7	94.6 ± 22.2	104.5 ± 33.4
RWT	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
LVH, %	29 (55.8)	61 (63.5)	26 (68.4)
LV diastolic function, %			
Normal	2 (3.8)	3 (3.1)	1 (2.6)
Impaired LV relaxation	29 (55.8)	56 (58.3)	15 (39.5)
Pseudonormal LV filling	21 (40.4)	37 (38.5)	20 (52.6)
Restrictive LV filling	0 (0)	0 (0)	2 (5.3)
Cardiopulmonary stress test			
Peak VO2 %.	**103.1 ± 7.6**	**82.7 ± 14.0***	79.8 ± 22.1
VE/VCO2	28.3 ± 2.8	**29.1 ± 2.9**	**33.0 ± 4.4** ¢
BNP, ng/l	16.2 ± 6.6	31.7 ± 23.2*	191.2 ± 49.8 ¢

Abbreviations: HFpEF – heart failure with preserved ejection fraction; VE/VCO_2 - ventilation/carbon dioxide production slope; LVMI - left ventricular mass index; RWT - relative wall thickness; LAVI – left atrial volume index; LVH - left ventricular hypertrophy; BNP - brain natriuretic peptide.
early HFpEF was considered if peak VO2 was ≤ 90% of predicted value or/and BNP was 35 ng/l or higher;
* - statistically significant ($p < 0.05$) difference between prospective group without HFpEF– and with early HFpEF+;
¢ - statistically significant ($p < 0.05$) difference between prospective group with early HFpEF+ and retrospective group with HFpEF.

signs of HFpEF (Table 18). The BNP level was significantly higher in early HFpEF+ subgroup comparing with the HFpEF– subjects (31.7 ± 23.2 vs. 16.2 ± 6.6 ng/l, $p < 0.05$); in 44 participants (29.7%) of the prospective cohort BNP ≥ 35 ng/l was found. Importantly, retrospective patients with established HFpEF and prospective subgroup with early HFpEF+ demonstrated similarly decreased exercise tolerance, with no difference in oxygen uptake (82.7 ± 14.0 vs. 79.8 ± 22.1%, $p > 0.05$).

We have found that prospective subjects with early heart failure (HFpEF+ subgroup) were older (57.1 ± 6.6 to compared with 55.4 ± 6.4 years in HFpEF– subgroup), women dominated (80.2% to compared of 48.1% in HFpEF– subgroup). Notably, only 11.5% of HFpEF+ subgroup subjects had complaints, in contrast, 84.3% demonstrated decreased exercise tolerance during the cardiopulmonary stress test. Early HFpEF+ subjects were characterized by significantly lower waist circumference in women and higher HDL-cholesterol compared to HFpEF– subgroup subjects.

Remarkably, significantly higher values of BNP (191.2 ± 49.8 vs. 31.7 ± 23.2 ng/l, $p < 0.05$), E/e' ratio (11.4 ± 4.6 vs. 9.5 ± 3.0, $p < 0.05$), LA volume index (46.7 ± 13.7 vs. 31.8 ± 9.1 ml/m^2, $p < 0.05$), and VE/VCO_2 (33.0 ± 4.4 vs. 29.1 ± 2.9, $p < 0.05$) as well as significantly lower HDL-C was observed in retrospective group as compared to the HFpEF+ prospective subgroup.

The analysis of arterial indices in the prospective group revealed that the HFpEF+ subgroup had significantly higher aortic augmentation index as compared to

TABLE 19. Arterial indices of preclinical atherosclerosis in prospective group (n = 148).

	PROSPECTIVE GROUP		
	All prospective group n=148 Mean ± SD	Prospective group HFpEF– n=52 Mean ± SD	Prospective group HFpEF+ n=96 Mean ± SD
sBP, mmHg	152.2 ± 18.9	152.2 ± 18.9	155.1 ± 21.8
dBP, mmHg	97.2 ± 11.7	97.2 ± 11.7	95.6 ±11.7
cfPWV, m/s	9.0 ± 1.3	9.0 ± 1.3	9.0 ± 1.3
AIx/HR, %	29.8 ± 9.2	27.3 ± 7.4	31.2 ± 9.9*
CCA IMT, µm	670.1 ± 98.1	682.1 ± 88.7	663.4 ± 102.7
CCA stiffness	4.8 ± 1.5	4.7 ± 1.6	4.8 ± 1.5
ABI	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.1

Abbreviations: SD – standart deviation, cfPWV – carotid-femoral pulse wave velocity; sBP – systolic blood pressure, dBP – diastolic blood pressure, CCA IMT– common carotid artery intima–media thickness; ABI – ankle-brachial index.

* - statistically significant ($p < 0.05$) difference between prospective group without HFpEF– and with early HFpEF+.

the HFpEF– subgroup (31.2 ± 9.9 vs. 27.3 ± 7.4%, $p < 0.05$). There were no significant differences between carotid-femoral PWV, mean systolic and mean diastolic blood pressure in HFpEF+ subjects as compared with HFpEF– subjects. The data of the hemodynamic and arterial parameters are presented in Table 19.

An analysis on interdependence of the arterial indices and early heart failure markers revealed significant positve correlation between BNP and aortic augmentation index ($r = 0.148$, $p = 0.074$) and carotid artery stiffness ($r = 0.248$, $p = 0.003$). We also observed a significant negative correlation between peak VO2 and aortic augmentation index ($r = – 0.264$, $p = 0.001$) and carotid arterty stiffness ($r = – 0.270$, $p = 0.001$) (Table 20).

TABLE 20. Correlation between arterial indices and heart failure markers.

	BNP, ng/l		Peak VO2, %	
	r	p value	r	p value
Peak VO2, %	–0,354	< 0,001		
cfPWV, m/s	0,011	0,899	–0,071	0,393
AIx/HR, %	0,148	0,074	–0,264	0,001
CCA stiffness	0,248	0,003	–0,270	0,001
CCA IMT , μm	–0,014	0,632	0,030	0,718

Abbreviations: BNP - brain natriuretic peptide; cfPWV – femoral–carotid pulse wave velocity; AIx/HR – aortic augmentation index; CCA IMT– common carotid artery intima–media thickness.

* - statistically significant (p<0.05) difference between prospective group without HFpEF and with early HFpEF.

TABLE 21. Univariate logistic regression analysis assessing the association between arterial indices and early heart failure with preserved left ventricular ejection fraction.

	OR	95% CI	p value
BNP, ng/l	1,084	1,044 – 1,124	< 0,001
Peak VO2, %	0,076	0,032 – 0,179	< 0,001
cfPWV, m/s	1,003	0,775 – 1,299	0,982
AIx/HR, %	1,045	1,004 – 1,087	0,030
CCA stiffness	1,048	0,926 – 1,112	0,690
CCA IMT, μm	0,998	0,834 – 1,316	0,269

Abbreviations: CI – cofidence interval; OR – odds ratio; BNP - brain natriuretic peptide; cfPWV – femoral–carotid pulse wave velocity; AIx/HR – aortic augmentation index, automatically; CCA IMT– common carotid artery intima–media thickness.

In order to further investigate the dependence of the early HFpEF on arterial indices in the prospective cohort with MetS, we performed an univariate logistic regression analysis (Table 21). This analysis has demonstrated that the presence of an early HFpEF was significantly associated with aortic augmentation index ($p = 0.030$), BNP ($p < 0.001$), and peak VO2 ($p < 0.001$). However, in the stepwise multiple logistic regression analysis aortic augmentation index did not remain a significant predictor of early HFpEF (Table 22).

TABLE 22. Stepwise multiple logistic regression analysis assessing the association between significant variables and early heart failure with preserved left ventricular ejection fraction.

	OR	95% CI	p value
BNP, ng/l	1,068	1,024 - 1,114	0,002
Peak VO2, %	0,093	0,037 - 0,230	<0,001
AIx/HR, %	Not significant		

Abbreviations: CI – cofidence interval; OR – odds ratio; BNP - brain natriuretic peptide; AIx/HR – aortic augmentation index.

In conclusion, our results show that the considerable proportion of subjects with MetS have signs of the early stage of heart failure. In metabolic syndrome population initial stage of heart failure with preserved ejection fraction might be associated with increased arterial stiffness.

4. Discussion

Cardiovascular diseases remains one of the main causes of death worldwide. According to the World Health Organization (WHO), around 17.5 million people died of CVDs in 2012. Metabolic syndrome represents a cluster of cardiovascular risk factors, such as obesity, hypertension, diabetes, hyperlipidemia and has a growing prevalence worldwide. As a result, this syndrome contributes to development of cardiovascular events.

It has been demonstrated that increased arterial stiffness is associated with increased morbidity and both all-cause and cardiovascular mortality in hypertensive patients, in patients with impaired glucose tolerance and/or diabetes [48, 49, 50]. Besides, increased arterial stiffness is associated with multiple cardiovascular risk factors, such as hypertension, dyslipidemia, obesity, smoking, diabetes, and aging, which contribute to the development of atherosclerosis [6, 51]. These observations suggest that arterial stiffening might be mediating the adverse cardiovascular outcome in MetS subjects.

The research available suggests that cardiovascular diseases associated with MetS comprise vascular and myocardial abnormalities that are initially manifested as impaired relaxation of the left ventricle. This myocardial dysfunction is characterized predominantly by diastolic dysfunction consisting of relaxation abnormalities that are prevalent and have prognostic importance in subjects with MetS [8]. The correlation between the intensity of metabolic syndrome and both the presence and the grade of diastolic dysfunction is reported in several studies [52, 53, 54, 55].

Although the relationship between arterial stiffness and left ventricular diastolic function in patients with hypertension [56], diabetes mellitus [57], in general [58] or eldery population has been previously reported [59], data in subjects with high cardiovascular risk and MetS without overt cardiovascular disease are limited to small sample studies assessing some arterial wall properties [60, 61]. Therefore, in the present study, we evaluated the relationship of a variety of arterial stiffness parameters and left ventricular diastolic dysfunction development and longitudinal alterations in large cohort of MetS subjects. There are invasive and noninvasive methods of evaluating arterial stiffness, including vascular catheterization, ultrasound, magnetic resonant imaging and arterial tonometry [62, 44]. In our study we used an arterial tonometry method and measured the carotid-femoral PWV (the „gold standart" for aortic stiffness), aortic augmentation index, arterial blood pressure and aortic pulse pressure. Arterial tonometry is an accessible, nonivasive assessment of arterial stiffness that can be done at the bedside. For assessment of carotid artery stiffness and IMT we used a highly accurate echotracking system (Art. Lab, Esaote Europe B.V.) [63]. The research available of association between arterial markers and LV diastolic dysfunction have had ralatively small sampe sizes and showed the different strengths of the correlation of arterial indeces with LV diastolic dysfunction. In our study we have analysed much more numerous cohort of 1208 subjects with MetS. Currently, little is known about an early development and early clinical diagnosis of heart failure with preserved ejection fraction in subjects with metabolic syndrome. Given the high prevalence of diastolic dysfunction in this population, we hypothesized that a significant number of subjects with MetS already have unrecognized HFpEF. In our study we uncovered the early phase of HFpEF by means of exercise capacity evaluation and biomarker testing. Additionally, we evaluated an association of arterial indices with early HFpEF.

For assessment of the LV diastolic dysfunction we used Doppler echocardiography. Although cardiac catheterization and measuring of LV filling pressure during the procedure remain the primary methods for diagnosing LV diastolic dysfunction; however, the application of this invasive method is limited in daily clinical practice. It was determined that the values of LV filling pressure measured during cardiac ultrasound coincided with the results collected during cardiac catheteriza-

tion using the Swan-Ganz catheter [64]. The LV filling pressure can be assessed precisely by determining the ratio of the blood flow via the mitral valve and the speed of the mitral annular displacement (E/e' ratio) during cardiac ultrasound [242, 65]. LV filling pressure measured as E/e' ratio is an important diagnostic criterion in determining LV diastolic dysfunction [66]. In our study we evaluated the association between E/A ratio (an important echocardiography index for characterizing the severity and staging LV diastolic dysfunction) and E/e' ratio with arterial indices in MetS subjects.

4.1 Characteristics of the Study Participants

The cohort was comprised of 1208 subjects of both genders with MetS. Arterial hypertension (93.3%, n = 1115) and dyslipidemia (99.7%, n = 1204) were highly prevalent among them. Approximately 60% of the subjects (63% of men and 58% of women) were obese (BMI ≥ 30 kg/m^2) at the time of the evaluation. Impaired LV diastolic function was diagnosed in the majority of the participants (915 [75.7%] patients compared to 292 [24.2%] subjects with normal LV diastolic function). According to the scientific literature, the prevalence of LV diastolic dysfunction tends to increase with the presence of MetS and can reach as many as 35-65% compared to 11-27% without Mets in the general population of adults [67, 68]. Such a difference in results might be caused by the chosen diagnostic criteria. In our study the LV diastolic dysfunction was determined according to the most recent HF diagnosis and treatment recommendations released by the European Society of Cardiology in 2016 [282]. The studies described in scientific literature mostly used the criteria suggested by S. F. Nagueh *et al* in 2009 to determine the LV diastolic dysfunction.

The study subjects with impaired LV diastolic function were older than those without LV diastolic dysfunction (54.7 ± 6.0 vs. 52.0 ± 6.2 years , $p < 0.0001$). Age is one of the risk factors of LV diastolic dysfunction and early HF [69]. LV relaxation slows down with age in both males and females [70]. All organs are afflicted with normal aging which causes certain morphological and functional changes. The impairment of the LV diastolic function during normal aging is caused by structural (increase the size of cardiomyocytes, higher degree of apoptosis, decrease in the number of cardiomyocytes, impaired regulation of growth factors, accumulation of collagen in myocardium) and functional (impaired response to β-adrenergic stimulation, lower number and activity of Ca^{2+} ions transporting albumin) changes in the heart. Our study revealed a significant correlation between age and the ultrasound parameters of LV diastolic function, e.g. blood flow via the mitral valve (E/A ratio, $p < 0.001$) and LV filling pressure (E/e' ratio, $p < 0.001$). Age has remained significantly associated with LV diastolic dysfunction in stepwise logistic regression models ($p < 0.001$).

Our study revealed that the majority of the respondents with LV diastolic dysfunction were women (68%, n = 622, compared to 54.8% in subjects without LV diastolic dysfunction, $p < 0.0001$). However in stepwise logistic regression analysis sex in one of analysis models lost its significance. According to the available literature gender impact on LV diastolic dysfunction is controversial. F. Ceis *et al* determined that women sex is a significant risk factor in developing LV diastolic dysfunction and HFpEF. The prevalence of HFpEF among women tends to increase more with age compared to heart failure [71]. Based on available studies, sex influences both the arterial wall and LV stiffness. However S. A. Carrubbas *et al* analysed the 3936 subjects data did not reveal a significant sex effects on prevalence of LV diastolic dysfunction [72]. Probably gender can influence cardiac changes during physiological aging [73]. In our opinion, the gender impact on the development of LV diastolic dysfunction and HFpEF requires further detailed research that may be a continuation of this study.

Our study showed that 95% of subjects were overweight (BMI > 25 kg/m^2) and 60% had BMI > 30kg/m^2. Subjects with LV diastolic dysfunction displayed a higher BMI (32,1 ± 5,06 vs. 30,5 ± 4,52 kg/m^2, $p < 0,0001$) and waist circumference (106,8 ± 10,76 vs. 103,6 ± 10,40 cm, $p < 0,0001$). We found statistically significant correlations between BMI and LV filling pressure (E/e' ratio) ($r = 0,216$, $p < 0,001$). In stepwise logistic regression analysis BMI and waist circumference remained as predictors of LV diastolic dysfunction in MtS subjects. The studies discussed bellow showed that obesity is associated with LV diastolic dysfunction and heart failure. S. Kenchaiah et al in longitudinal study of 5881 subjects (average follow-up period was 14 years) determined a significant association between BMI and higher risk of heart failure [74]. The risk of heart failure increased in men by 5% and in women by 7% when BMI increase per 1 kg/m^2. M. R. Movahed *et al* determined a significant correlation between obesity (BMI ≥ 30 kg/m^2) and LA enlargement, LV hypertrophy LV mass and RWT [75]. In the case of BMI ≥ 30 kg/m^2 the risk of heart failure increased (the ORs were 2.5, $p < 0,0001$). The results of *Framinghame Heart Study* confirm the association of obesity and changes in KS geometry independently on age and arterial blood pressure [76]. It should be noted that insulin resistance, hyperinsulinemia and oxidative stress [77] can promote LV remodeling in obesity [78].

Our study has demonstrated that, as compared to subjects with normal LV diastolic function, subjects with LV diastolic dysfunction had higher fasting glucose (6.0 ± 1.29 vs. 5.9 ± 0.99 mmol/l, $p = 0,0231$). Earlier studies have shown that hyperglycaemia may cause arterial wall and myocardial damage and functional impairment. Hyperglycaemia increases the activity of inducible nitric oxide synthase and promotes inflammation, oxidative stress, AGE accumulation that can lead to atherosclerosis progression and myocardial damage [79]. M. K. Rutter *et al* found

a higher LV mass and RWT in subjects with impaired glucose tolerance [80]. Some studies indicate that hyperglycaemia may be a significant risk factor for the development of chronic heart failure [81]. In our study in univariate logistic regression analysis fasting glucose did not remained significant factor for LV diastolic dysfunction (p = 0,05).

4.2 The Relationship between Arterial Markers and Left Ventricular Diastolic Dysfunction in the Cross-sectional Study

This study represents the association of noninvasive indices of arterial wall parameters (carotid-femoral PWV, aortic augmentation index, mean arterial blood pressure, aortic pulse pressure, common carotid artery IMS and stiffness, cardio-ankle index) with ultrasound measurements of LV diastolic function in a large cohort (n = 1208) of MetS subjects without overt coronary artery disease. Notably, the majority of study participats were demonstrated to have subclinical LV diastolic dysfunction.

The relationship between arterial stiffness and LV diastolic function has been previously reported in subjects with hypertension, diabetes mellitus, in general or eldery populations [82, 83, 84], but similar data in MetS subjects are lacking. The main determinants of arterial wall pathological changes in MetS are endothelial dysfunction, increased collagen production, accumulation of collagen molecules and AGE's, activation of the renin-angiotensin-aldosterone system and inflammatory response [92, 93, 94, 95, 96]. It has been demonstrated that increased arterial stiffness is associated with increased morbidity and both all-cause and cardiovascular mortality [83]. Carotid-femoral PWV is the "gold standard" for measuring large artery stiffness. The additive value of PWV above and beyond traditional risk factors, including SCORE and the Framingham risk score, has been suggested by European Society of Cardiology [102]. As described earlier (pp. 9), the independent prognostic value of increased aortic stiffness for LV diastolic dysfunction and higher CV risk has been theoretically explained by the elevation of central systolic and pulse pressure and the diminishment of the diastolic component in the central blood pressure curves. Our study has demonstrated, that subjects with LV diastolic dysfunction displayed a higher cfPWV (8.8 ± 1.6 compared to 7.9 ± 1.34 m/s in group without LV diastolic dysfunction, $p < 0,0001$). The analysis of the relationship between arterial markers and LV diastolic function parameters showed statistically significant correlations between carotid-femoral PWV and E/A ratio ($r_{cfPWV} = -0.190$, $p<0.001$), and E/e' ratio ($r_{cfPWV} = 0.167$, $p<0.05$). Our results confirms those of the earlier studies conducted in several smaller and other than MetS cohorts. The study conducted by C. J. Roos *et al* revealed a significant negative correlation between aortic PWV

and E/A ratio (r = – 0,350, p < 0,001) in 148 patients with diabetes [85]. Abhayaratna *et al* [86] in 233 elderly adult, also Hack-Lyoung Kim at al. [87] in a healthy population, demonstrated a significant correlation between PWV and E/e'. A study of 70 hypertensive patients [88] showed a significant association between arterial stiffness measured as central pulse pressure and E/e', however, the significant correlation was found only in the univariate logistic regression analysis. In our study, there was a significant positive correlation between cfPWV and E/e' ratio in a larger cohort of 1208 participants and these findings suggests usefulness of cfPWV for early detection of increased LV filling pressure in MetS subjects.

In a multiple regression analysis, we have demonstrated that cfPWV, an index of aortic stiffness, is a significant and independent determinant of the LV diastolic dysfunction in MetS population (p = 0.002). Though, in a univariate logistic regression analysis, subjects with MetS and LV diastolic dysfunction also displayed higher aortic augmentation index, ABI, carotid artery IMT, LVMI, RWT, and left atrium width, after adjustment for other significant variables, these associations did not remain significant. Thus, the aortic PWV is an independent prognostic marker of LV diastolic dysfunction. Our results showed that increase of cfPWV by 1.3 m/s is associated with 18.6% higher probability of LV diastolic dysfunction (p = 0.002). The determined cfPWV threshold value 8.2 m/s allows to distinguish subjects with the higher risk of LV diastolic dysfunction (AUC 0.642, sensitivity 0.715, specificity 0.596).

In our study cohort subjects with LV diastolic dysfunction displayed a higher aortic augmentation index (25.4 ± 9.96 compared to 21.1 ± 10.84 % in LV DD–group, p < 0.0001), which was also reflected in the statistically significant correlations between AIx/HR and LV diastolic function parameters: E/A (r = – 0.151, p < 0.001) ir E/e' ratio (r = 0.136, p < 0.001). In the stepwise multiple logistic regression analysis aortic augmentation index remained significant predictor of LV diastolic dysfunction (p = 0.001). Previous studies have shown contradictory results in association between augmentation index and LV diastolic dysfunction. Similarly to our data, N. Cauwenberghs *et al* found that AIx was significantly related with increased LV filling pressure [89]; however, their study was carried out in general population, not MetS subjects. C. Luers *et al* [90] in 257 subjects group (mean age 66 years) demonstrated that increased AIx is related with increasing of LV filling pressure. In multiple regression analysis, however, after adjustment for age, gender and carotid-femoral PWV, association between AIx and increased LV filling pressure did not remain. In distinction, our study has shown that both aortic augmentation index and carotid-femoral PWV remained significant for prediction of LV diastolic dysfunction in middle aged subjects with MetS and therefore their combination should be taken into account for CV risk assessment.

In our study, we also investigated the relationship between arterial blood pressure, aortic pulse pressure and LV diastolic dysfunction. Some previous studies have shown that arterial blood pressure and aortic pulse pressure are clinically more significant in comparison to peripheral blood pressure. For instance, J. Hashimoto *et al* described the association between aortic pulse pressure and regression of LV remodeling [91]. Our findings complement these studies by showing that subjects with LV diastolic dysfunction displayed a higher arterial blood pressure (108.6 ± 12.85 vs. 103.4 ± 10.13 mmHg, $p < 0.0001$) and aortic pulse pressure (44.5 ± 10.69 vs. 41.9 ± 10.8 mmHg, $p = 0.0004$) compared to those with normal LV diastolic function. Furthermore, our results revealed a statistically significant positive correlation between arterial blood pressure, aortic pulse pressure and LV filling pressure, measured as E/e' ratio: $r_{aBP} = 0.159$, $r_{aPP} = 0.265$, $p < 0.001$. However, in the stepwise multivariate regression analysis, the relationship between these parameters of central BP and the presence of LV diastolic dysfunction did not remain significant, thus suggesting that the effect of increase central pulse pressure is not an independent predictor: it is derivative of aortic stiffness.

The relationship between carotid artery IMT, stiffness and LV diastolic dysfunction has been demonstrated in different populations other than MetS. In a cohort of the *Multi-Ethnic Study of Atherosclerosis* of 58 subjects free of cardiovascular diseases (mean average age 64 years), carotid artery IMT was associated with the incipient systolic and diastolic myocardial dysfunction [92]. Similar results were obtained by Garcia *et al* [93] in 48 healthy subjects in whom the relation between carotid artery IMT and LV diastolic dysfunction was evaluated. In contrast, Y. Mizuguch *et al* [94] have shown no significant association between carotid artery IMS and the echocardiographic LV systolic and diastolic parameters. In our cohort subjects with LV diastolic dysfunction displayed higher carotid artery IMT (656.6 ± 105.29 vs. 637.6 ± 98.49 μm, $p = 0.007$) and carotid artery stiffness (3.9 ± 1.47 vs. 3.5 ± 1.32, $p < 0.0001$) compared to participants with normal LV diastolic function. Once more, after adjustment for other significant variables by performing a stepwise logistic regression analyses, this relationship lost its statistical significance, foregrounding that, in contrast to aortic stiffness indices, the carotid IMT and the local stiffness in the carotid artery do not have an independent predictive value for LV diastolic dysfunction.

The cardio-ankle vascular index (CAVI) has been recently promoted as easy-to-obtain marker of aortic stiffness, which is automatically adjusted for the BP at the time of measurement. K. Sakane *et al* in total of 119 subjects (mean average age 62 ± 11 years) demonstrated significant assotiation between CAVI and E/A ratio, LA diameter, age, BMI and systolic blood pressure [95]. The study conducted by Y. Mizuguchi *et al* [96] in small 30 subjects group (mean average age 59 ± 5.7 years)

showed significant positive correlation between CAVI and the peak early diastolic transmitral flow velocity, E/A ratio, deceleration time of the early diastolic transmitral flow velocity, peak early diastolic mitral annular motion velocity. Our study showed no significant difference of CAVI values between LV DD+ and LV DD– groups subjects (8.0 ± 1.67 in LV DD+ subjects compared to 7.8 ± 1.55 in LV DD–, p = 0,0721). Likewise, in the univariate logitic regression analysis CAVI was not associated with the presence of LV diastolic dysfunction (p = 0,178). Thus, in our study, CAVI did not demonstrate superiority over other indices of arterial stiffness for predicting of LV diastolic dysfunction in MetS. This might be explained by the fact that CAVI is a derivative index that reflects a complex interaction of arterial properties beyond aortic PWV.

To sum up, our study has shown that left ventricular diastolic dysfunction is significantly associated with elevated arterial stiffness in metabolic syndrome subjects. Non–invasively measured carotid-femoral pulse wave velocity, an index of aortic stiffness, is a significant and independent determinant of the LV diastolic dysfunction. Based on our results, in subjects with MetS, the adequate measures for preventing the development of LV diastolic dysfunction are especially urgent when carotid-femoral PWV is higher than 8.2 m/s. Since LV diastolic dysfunction is highly prevalent in middle aged MetS subjects, implementing these measures is of key importance for preventing the future development of heart failure with preserved LV ejection fraction.

4.3 The Influence of Arterial Stiffness Alteration on the Dynamics of the Left Ventricular Diastolic Dysfunction during Longitudinal Observation

Left ventricular diastolic dysfunction is an early pre-clinical marker of cardiovascular diseases. Any kind of heart disease that leads to myocardial structural alteration may cause LV diastolic dysfunction [97]. Early subclinical LV diastolic dysfunction has leads to the elevation of LV filling pressures and has been shown to be associated with development of heart failure. To clarify the relationship between arterial stiffness and LV diastolic dysfunction we aimed to determine how the alteration of arterial stiffness affects the dynamics of LV diastolic dysfunction during longitudinal observation.

Our study has shown that over a 3 ± 0.6 years period, LV diastolic function remained unchanged in 58.8 % MetS subjects. In 22% of subjects LV diastolic dysfunction improved, and in 19% – has worsened. Some previous studies determined age–related changes in LV diastolic dysfunction dynamics. G. C. Kan *et al* in a cohort of 1402 subjects, over a 4 ± 0.3 years period, found unchanged LV diastolic dysfunction in 67.8% patients, and only in 8.8% subjects LV diastolic dysfunction

normalized and in 23.4% - worsened [98]. In their study the worsening of LV diastolic dysfunction was associated with age 65 years or older. Left ventricular diastolic dysfunction was also associated with incident heart failure during 6 years period after adjustment for age, hypertension, diabetes, and coronary artery disease. Our study showed no significant age difference between subjects with improved vs. worsened LV diastolic dysfunction at baseline (respectively 53.25 ± 5.28 vs. 53.16 ± 5.7, p = 0.857). During the longitudinal observation we found no significant difference in age changes between subjects with improved vs. worsened LV diastolic dysfunction (age changes 3.85 ± 0.54 vs. 3.9 ± 0.56 years, p > 0.05). Furthermore, in logistic regression analyses, age did not remained significant for dynamics of LV diastolic function (p = 0.605) neither for alteration of E/e' ratio (p = 0.653). This suggests that age–related changes might blurred and acceleratedby the presence of MetS and its various cluster. This hypothesis is supported by the acceleration of arterial stiffness in MetS subjects, recently demonstrated in large follow–up study [99].

In our study we evaluated how the alteration of arterial markers, LV geometry and LA echocardiographic parameters affect the improvement or the worsening of LV diastolic function. We have found that main factor associated with the worsening of LV diastolic function was the carotid-femoral PWV. In a group of improved LV diastolic dysfunction, cfPWV did not increase; there was, in fact a decrease in cfPWV, however, not statisticaly significant (p = 0.489). In a group of worsened LV diastolic dysfunction, cfPWV significantly increased (p = 0.004). Logistic regression analysis demonstrated that alteration of cfPWV remained a significant predictor of the deterioration of LV diastolic function even after adjustment for other potentially explanatory variables (p = 0,015). In order to more accuratly evaluate the prognostic markers of the LV diastolic dysfunction progression, we performed conditional random forests analysis. According to the available literature, random forests have become a popular and widely-used tool for non-parametric regression in many scientific areas. They show high predictive accuracy and are applicable even with highly correlated variables [100]. In our study conditional random forests analysis confirmed the high significance of the elevated arterial stiffness alteration for the development of LV diastolic dysfunction. It has been shown that cfPWV alteration stands out among other arterial stiffness indices as a significant predictor of the dynamics of the LV diastolic function.

Our study demonstrated significant reduction of LVMI (from 109.98 ± 20.22 to 98.92 ± 24.37, p < 0.001) and relative wall thickness (from 0.44 ± 0.05 to 0.42 ± 005, p = 0.001) in a group of improved LV diastolic dysfunction. According to the literature, development of an abnormal LV geometric pattern is associated with increased CVDs risk [101]. *Multi-Ethnic Study of Atherosclerosis* showed the significant progression in the increase of LV mass during the 9.4 years period [102]. In our study

progression of LV remodeling was found in 24.2% of cohort subjects, regression of LV remodeling – in 39.4% subjects. Findings from other studies demonstrated the association between AH with LV hypertrophy and LV diastolic dysfunction [103]. In a group of LV diastolic dysfunction, our study demonstrated the significant higher values of LVMI (106.77 ± 22.59 compared to 102.25 ± 21.99 g/m^2 in LV DD– group, p = 0.033) and RWT (0.43 ± 0.06 compared to 0.42 ± 0.06 in LV DD– group, p = 0,020) at baseline. We found significant correlation between LVMI and LV filling pressure, measured as E/e' ratio (r = 0.324, p < 0.001). In stepwise multiple logistic regession analysis, LVMI remained significant predictor of the LV diastolic dysfunction together with cfPWV, BMI, waist circumference, and age.

Previous studies revealed that left atrium volume index increases as an expression of LV diastolic dysfunction severity and is independently associated with age, LV hypertrophy, LV systolic dysfunction and increased LV filling pressure [104]. In our study, no sigificant difference was found in LV volume and LV volume index between LV DD+ and LV DD– groups at baseline. During the longitudinal observation, no significant differences were found in respect to the alteration of LA volume and volume index between subjects with improved LV diastolic dysfunction and LV diastolic function deterioration. It might be explained by the fact that, the majority of study patients (74.7%, n = 243) had an impaired LV relaxation with normal LV filling pressure at baseline.

It should be noted, that the conditional random forests analysis demonstrated a number of drugs used by our study subjects, such as calcium channel blockers, angiotensin converting enzyme inhibitors, and angiotensin receptor blockers might influence E/e' ratio. Univariate logistic regression analysis, however, revealed that alteration of E/e' ratio was associated only with calcium channel blockers: the use of calcium channel blockers during the follow–up period was related to LV diastolic dysfunction improvement (p = 0.012). No significant association was found between LV diastolic function dynamics and angiotensin converting enzyme inhibitors (p = 0.195) and angiotensin receptor blockers (p = 0.504). The possible limitation for an accurate evaluation of the treatment effect was that we collected data on medical treatment only at follow-up visit. Further longitudinal studies designed specifically to assess various medical treatment strategies are needed to determine the relationship between the dynamics of LV diastolic function and medications.

In summary, our findings show that the alterations of arterial indices, namely, carotid–femoral PWV and aortic pulse pressure are the independent predictors of LV diastolic dysfunction in MetS subjects. Our findings suggest that increased arterial stiffness is a pathogenetic factor of LV diastolic dysfunction. These results have important clinical implications. Since PWV is modifiable with medical therapy [105,106], it can be used as a surrogate endpoint for assessing the treatment efficiency

in subjects with MetS and LV diastolic dysfunction. Moreover, the identification of subjects with increased aortic stiffness should be considered in primary prevention of LV diastolic dysfunction, a condition associated with adverse prognosis in the community.

4.4 Detection of Early Heart Failure with Preserved Left Ventricular Ejection Fraction in Subjects with Metabolic Syndrome

To the best of our knowledge, this is the first study that addresses the early detection of HFpEF in MetS patients using a combination of BNP and peak VO_2. In our cross–sectional study of 1208 participants, highly prevalent of LV diastolic dysfunction was demostrated. We hypothesized that subjects, who are at high risk of heart failure development, a focused testing will reveal a very early phase of HFpEF. Indeed, up to two thirds of our prospectively enrolled MetS subjects (n = 148) without established overt cardiovascular disease, had an impaired functional capacity, elevated neurohormonal activation and ultrasound evidence of elevated LV filling pressure. Heart failure diagnosis in such patients is largely in accordance with HFpEF definition by the recent guidelines [107]; the components of the diagnosis include increased BNP, left ventricular mass, diastolic dysfunction and objectively documented impaired exercise capacity. A certain peculiarity is the use of exercise test in order to elicit objective decrease of exercise tolerance, which frequently is not adequately appreciated by the patients themselves.

It has been shown that the usage of self-rated health (SRH), assessed through a single question [108, 109], is of limited value in patient with left ventricular diastolic dysfunction for a subjetive self–evaluation tends to underestimate the heart failure symptoms. A Swedish study has shown that the majority of participants with asymptomatic left ventricular diastolic dysfunction rated their health status as good or very good, despite the high prevalence of co-morbidities [110]. Therefore, there is a need for comprehensive diagnostic methods that would help to highlight the symptoms. Cardiopulmonary stress test is an established tool for the evaluation of the disease severity and prognosis in heart failure patients. Our study suggests its value for the early detection of reduced exercise tolerance in subjects enrolled in the prevention program. Though only 11.5% of patients with the signs of early HFpEF had documented complaints of dyspnoea, the cardiopulmonary stress test revealed reduced exercise tolerance in 83.3% of these patients.

The latest ESC heart failure guidelines require the elevated BNP level for establishment of HFpEF diagnosis. In our study 96 (64.8%) subjects from 148 prospectively enrolled participants with MetS, have shown modest but statistically significant elevation of BNP: 31.7 ± 23.2 in the subgroup with early HF compared to 16.2 ± 6.6 ng/l in the subgroup without early HF ($p < 0.05$). BNP ≥ 35 ng/l was

found in 28.3% subjects with early HFpEF. It can be expected that subjects with hidden HFpEF could be identified with even greater accuracy if not the inverse impact of MetS on BNP – as it was previously demonstrated, BNP values are significantly lowered in persons affected by MetS [111]. Several hypotheses attempt to explain the inverse relationship between BNP levels and obesity. Renal theory states that BNP levels are depressed because of higher glomerular filtration rates which lead to more efficient molecular clearing [112]. Adipose tissue theory states that the reason for lowered BNP values in MetS is natriuretic peptide clearance receptor-C (NPR-C) [113]. On the contrary, the *Framingham Heart Study* [114] and the *Dallas Heart Study* [115] questioned this theory showing that NT–proBNP values in obesity are also reduced even though it is not cleared by NPR-C. The Dallas Heart Study authors found that both BNP and NT-proBNP values were more closely associated with lean mass than with BMI. Due to this finding they theorized that not the adipose tissue itself is the main factor causing lowered BNP values but a substance produced in the lean mass which could suppress either BNP synthesis either its release from cardiomyocytes.

Two MetS criteria, insulin resistance and lipid abnormalities, are considered as the main components that influence progression of the disease, whereas obesity and high blood pressure are linked to the outcomes in HF patients [116]. Recently presented new paradigm for HFpEF emphasizes the importance of obesity as one of the main comorbidities which induces a systemic inflammatory state that sequentially leads to diastolic LV dysfunction [117].

Left ventricular diastolic dysfunction as a leading mechanism in the formation of HFpEF was detected in most of the study patients. Only 3.4% of prospective and 2.6% of retrospective individuals had normal diastolic function. The most common grade of diastolic dysfunction in both prospective subgroups was impaired relaxation, whereas retrospective group mostly presented with pseudo-normal filling.

Arterial markers of preclinical atherosclerosis are used for early prediction of cardiovascular risk and the impact of MetS on arterial elastic properties is discussed in many current scientific papers [118, 119]. In our study we found a markedly higher AIxHR75 in early HFpEF patients compared to those without (31.2 ± 9.9% vs. 27.3 ± 7,4%, $p < 0,05$). The analysis of the relationship between arterial markers and the markers of early HFpEF has shown a statistically significant correlation between aortic augmentation index and peak VO2 ($r = -0.264$, $p = 0.001$). However, in the multiple logistic regression analysis, the association between AIx/HR and the presence of HFpEF lost its significance. This controversial finding might be explained by the relatively small number of participants, and the negative association between BNP and obesity. Thus, increasing the sample size and using an heart failure biomarker which is not affected by obesity is desirable in further studies.

5. Conclusions

1. In metabolic syndrome subjects without overt cardiovascular disease, arterial stiffness, asessed by measuring the "gold standard" index of carotid-femoral pulse wave velocity, is significantly and independently associated with the LV diastolic dysfunction.
2. In subjects with metabolic syndrome, the progression in arterial stiffening, assessed as an increase in carotid-femoral pulse wave velocity, significantly predicts the worsening of the left ventricular diastolic function. Our findings suggest that increased arterial stiffness is a pathogenetic factor of left ventricular diastolic dysfunction.
3. A considerable portion of subjects with metabolic syndrome exhibit signs of an early heart failure with preserved left ventricular ejection fraction. In metabolic syndrome population, initial stage of heart failure with preserved ejection fraction might be associated with the increased arterial stiffness.

6. Recommendations for clinical practice

1. Based on our results, in subjects with metabolic syndrome, the adequate measures for preventing the development of LV diastolic dysfunction are especially urgent when carotid–femoral PWV is higher than 8.2 m/s. Since LV diastolic dysfunction is highly prevalent in the middle aged MetS subjects, implementing these measures is of key importance for preventing the future development of heart failure with preserved LV ejection fraction.
2. Carotid–femoral pulse wave velocity can be used as a surrogate endpoint for assessing the treatment efficiency in subjects with metabolic syndrome and LV diastolic dysfunction.
3. In addition to the assessment of clinical complaints, cardiopulmonary stress test can be used for the early detection of the reduced exercise tolerance and for identifying of an early heart failure with the preserved LV ejection fraction in subjects with metabolic syndrome.

7. Publication

Articles:

1. Solovjova S, Ryliškytė L, Čelutkienė J, Badarienė J, Navickas R, Puronaitė R, Bieliauskaitė G, Skiauterytė E, Lisaitė G, Laucevičius A. Aortic stiffness is an independent determinant of left ventricular diastolic dysfunction in metabolic syndrome patients. Blood Pressure 2016;25(1):11-20.

2. Laucevičius A, Ryliškytė L, Balsytė J, Badarienė J, Puronaitė R, Navickas R, Solovjova S. Association of cardio-ankle vascular index with cardiovascular risk factors and cardiovascular events in metabolic syndrome patients. Medicina 2015, 51:152-158.

3. Čelutkienė J, Jakštaitė AM, Badarienė J, Solovjova S, Slivovskaja I, Navickas R, Kazėnaitė E, Rinkūnienė E, Čypienė A, Misiūra J, Ryliškytė L, Laucevičius A, Coats AJS. Detection of Early Heart Failure with Preserved Ejection Fraction (HFpEF) in Metabolic Syndrome Patients Detected as Part of a National Screening Programme in Middle Aged Subjects. Priimtas spausdinimui į "The International Cardiovascular Forum Journal".

Theses, and Poster Presentations:

1. Ryliškytė L, Čelutkienė J, Puronaitė R, Badarienė J, Solovjova S, Navickas R, Petravičiūtė M, Kundrotaitė S, Skujaitė A, Laucevičius A. Aortic Stiffness is an Independent Determinant of Left Ventricular Diastolic Dysfunction in Metabolic Syndrome Patients. The 61st Annual Conference of the Israel Heart Society, April 30 – May 1, 2014, Tel Aviv. Abstract book:65. (elektroninis pranešimas)

2. Navickas R, Ryliškytė L, Puronaitė R, Solovjova S, Badarienė J, Laucevičius A. Aortic pulse wave velocity is an independent cardiovascular event predictor in hight cardiometabolic risk group. Artery 14, the MECC, 9–11 October 2014, Maastricht, the Netherlands. Artery Research 2014;8(4):160.

3. Solovjova S, Puronaitė R, Stuopelytė M, Ryliškytė L. The coherence between arterial stiffness and left ventricular diastolic function in patients with metabolic syndrome: longitudinal study. Fourth international conference dedicated to the 100th Anniversary of the Restoration of Lithuania's Independence „Lithuania evolutionary medicine: health and diseases in changing environment". 5–10 June 2018, Vilnius Lithuania.

4. Solovjova S, Puronaitė R, Jakštaitė A, Ryliškytė L, Čelutkienė J, Laucevičius A. The Association between Metabolis syndrome components, arterial markers of early atherosclerosis and left ventricular diastolic dysfunction. 12-14 October 2017, Pisa, Italy.

5. Čelutkienė J, Solovjova S, Ryliškytė L, Puronaitė R, Badarienė J, Navickas R, Laucevičius A. Aortic stiffness is a significant determinant of left ventricular diastolic dysfunction in metabolic syndrome patients. ESC Congress 2015, 29 August – 02 September 2015, London, United Kingdom.

SANTRAUKA

Įvadas

Širdies ir kraujagyslių ligos (ŠKL) – tai pagrindinė pasaulio gyventojų mirtingumo ir sergamumo bei mažėjančio darbingumo priežastis, lemianti 31 proc. visų mirčių. Pagal Pasaulinės sveikatos organizacijos (PSO) duomenis, 2012 metais nuo ŠKL mirė apie 17,5 milionų žmonių, 80 proc. mirčių sąlygojo miokardo infarktas ir galvos smegenų kraujotakos sutrikimai. Lietuvos Respublikos Statistikos departamento duomenimis, 2016 metais nuo kraujotakos sistemos ligų mirė 56,2 proc. gyventojų. Didžiausią dalį sudarė asmenys, mirę nuo išeminės širdies ligos (65,6 proc.) ir cerebrovaskulinių ligų (24,2 proc.). Didžioji dalis (87 proc.) mirusiųjų nuo kraujotakos sistemos ligų buvo 65 metų ir vyresnio amžiaus asmenys.

Širdies ir kraujagyslių ligų rizikos veiksniai (RV) plačiai tyrinėjami ekonomiškai išsivysčiusiose šalyse. Metabolinis sindromas (MetS) – rizikos veiksnių (arterinė hipertenzija, pilvinio tipo nutukimas, atsparumas insulinui ir aterogeninė dislipidemija) derinys – viena iš opiausių problemų šiuolaikiniame pasaulyje. Dėl didėjančio nutukimo, AH ir MetS dažnio, šių patologinių būklių profilaktika ir laiku suteiktas gydymas tampa prioritetu ekonomiškai stipriose šalyse. MetS nustatymas turi didelę klinikinę reikšmę, kadangi, viena vertus, tai sindromas, kurio pasireiškimą taikant prevencines priemones ir laiku suteiktą gydymą galima sulėtinti, kita vertus, jis sąlygoja 2 tipo CD ir aterosklerozinių ligų, pirmaujančių pasaulyje tarp gyventojų mirties priežasčių, atsiradimą ir progresavimą.

Kitas stiprus ir nepriklausomas ŠKL sergamumo ir mirtingumo rizikos veiksnys – arterijų standumas – vis plačiau tyrinėjamas pasaulyje. Svarbiausi ikiklinikiniai arterijų sienelės funkciniai ir morfologiniai pakitimai apima endotelio disfunkciją, arterijų standumo padidėjimą, intimos-medijos sluoksnio storėjimą, tačiau arterijų standumui šiuo metu skiriamas didžiausias dėmesys. Esant MetS, padidinto arterijų standumo reikšmė ypač pabrėžiama. Kaupiasi duomenys, rodantys, kad padidėjęs aortos standumas daro įtaką kairiojo skilvelio (KS) diastolinės disfunkcijos atsiradimui bei progresavimui. Padidėjus arterijų sienelės standumui, didėja pulsinės bangos plitimo greitis (PBG) ir ankstyvas (sistolėje, o ne diastolėje) atspindėtos pulsinės bangos grįžimas į kylančią aortą. Aortoje didėja sistolinis, mažėja diastolinis kraujospūdis, didėja kairiojo skilvelio pokrūvis, vystosi miokardo hipertrofija, blogėja koronarinė perfuzija, pulsuojanti, ne vientisa tėkmė pasislenka toliau periferijos, smulkiųjų arterijų link. Smulkios arterijos degeneruoja, sutrinka mikrocirkuliacija, vystosi organų–taikinių pažeidimas (inkstų, smegenų ir kt.). Blogėjanti koronarinė perfuzija ir kairiojo skilvelio miokardo hipertrofija lemia KS atsipalaidavimo sutrikimą bei diastolinės disfunkcijos atsiradimą. Atliktų tyrimų duomenys rodo, kad pacientams, padidėjus arterijų standumui, dažniau nustatomas sutrikęs KS atsipalaidavimas, ir abu šie veiksniai kartu su arterijų senėjimu (angl. *vascular aging*) yra siejami su širdies nepakankamumo atsiradimu ir progresavimu.

Tarp sergančiųjų širdies nepakankamumu apie 40–50 proc. pacientų nustatoma išsaugota kairiojo skilvelio išstūmio frakcija. Išsaugotos kairiojo skilvelio išstūmio frakcijos širdies nepakankamumui (IIFŠN) būdingi tam tikri kairiojo skilvelio struktūriniai ir funkciniai pokyčiai, kurių vienas svarbiausių – sutrikusi KS diastolinė funkcija. Įvairūs veiksniai, tokie kaip

senėjimas, arterinė hipertenzija, cukrinis diabetas, lėtinė inkstų liga, metabolinis sindromas ir padidėjęs arterijų sienelės standumas sąlygoja kairiojo skilvelio diastolinės funkcijos sutrikimą ir gali būti svarbūs vystantis IIFŠN. Arterijų standumo didėjimas pacientams su ŠN siejamas su blogesne prognoze, tad arterijų standumo nustatymas ir jo sumažinimas galėtų būti taikiniu ir ŠN prevencijoje, ir gydyme.

Kadangi kairiojo skilvelio diastolinė disfunkcija literatūroje įvardinama ankstyvuoju ikiklinikiniu širdies ir kraujagyslių sistemos sutrikimų žymeniu, kuriai progresuojant išsivysto ŠN (o tai savo ruožtu sąlygoja blogėjančią pacientų gyvenimo kokybę mažėjant fiziniam pajėgumui bei tolerancijai krūviui), jos patogenetinių veiksnių nustatymas yra svarbus parenkant tinkamą medikamentinį gydymą. Tačiau vis dar trūksta duomenų apie arterijų standumo ir kairiojo skilvelio diastolinės funkcijos sąsajas tarp asmenų su nustatytu metaboliniu sindromu: atlikti tyrimai yra mažos imties, analizuojami tik atskiri arterijų standumo rodikliai.

Kaupiasi duomenys, patvirtinantys metabolinio sindromo ir jo komponentų svarbą išsivystant širdies nepakankamumui. Esant MetS, nustatytas reikšmingai didesnis kairiojo skilvelio diastolinės disfunkcijos dažnis. Tačiau literatūroje vis dar trūksta duomenų apie IIFŠN vystymosi eigą ir ankstyvąją klinikinę diagnostiką asmenims, turintiems metabolinį sindromą. Iki šiol nebuvo tirtos galimybės taikyti tikslingą ištyrimą (kardiopulmoninį krūvio mėginį, natriuretinio peptido koncentracijos vertinimą), siekiant nustatyti tikrąjį ankstyvojo širdies nepakankamumo dažnį asmenų, turinčių MetS, grupėje.

Nors pasaulyje atlikta nemažai skerspjūvio tyrimų, analizuojančių ryšį tarp arterijų standumo ir KS diastolinės disfunkcijos, vis dar trūksta išilginio stebėjimo tyrimų, išaiškinančių ryšį tarp arterijų standumo didėjimo ir KS diastolinės disfunkcijos progresavimo bei širdies nepakankamumo išsivystymo. Tokių tyrimų atlikimas padėtų geriau parinkti optimalų gydymą sumažinant ŠKL ir širdies nepakankamumo atsiradimą ir progresavimą.

Darbo tikslas

Darbo tikslas – nustatyti sąsajas tarp arterijų funkcijos ir struktūros rodiklių pokyčių ir kairiojo skilvelio diastolinės disfunkcijos asmenų, turinčių metabolinį sindromą, kohortoje.

Darbo uždaviniai

1. Įvertinti sąsajas tarp arterinių rodiklių ir kairiojo skilvelio diastolinės disfunkcijos skerspjūvio tyrime.
2. Išilginio stebėjimo tyrime nustatyti, kaip arterijų standumo pokytis daro įtaką kairiojo skilvelio diastolinės disfunkcijos dinamikai.
3. Ištirti ankstyvojo širdies nepakankamumo paplitimą tarp asmenų, turinčių metabolinį sindromą, vertinant krūvio deguonies suvartojimą ir natriuretinio peptido koncentraciją. Įvertinti sąsajas tarp arterinių rodiklių ir išsaugotos kairiojo skilvelio išstūmio frakcijos širdies nepakankamumo.

Ginamieji teiginiai

1. Asmenims, turintiems metabolinį sindromą, kairiojo skilvelio diastolinė disfunkcija reikšmingai siejasi su padidintu arterijų standumu.

2. Arterijų standumo, išmatuoto vertinant miego-šlaunies arterijų pulsinės bangos greitį, didėjimas reikšmingai siejasi su kairiojo skilvelio diastolinės funkcijos blogėjimu.
3. Esant metaboliniam sindromui, dalis asmenų turi neatpažintą ankstyvąjį širdies nepakankamumą, objektyviai nustatomą vertinant fizinio pajėgumo ir neurohormoninės aktyvacijos žymenis.

Tyrimo naujumas ir aktualumas

Šiame darbe atlikta didelės 1208 asmenų, turinčių MetS, kohortos duomenų skerspjūvio analizė, kurios dėka įvertintos skirtingų arterijų sienelės struktūros ir funkcijos rodiklių (pulsinės bangos greičio, aortos augmentacijos indekso, centrinio AKS, centrinio pulsinio spaudimo, širdies-kulkšnies indekso, bendrosios miego arterijos standumo ir intimos-medijos storio) sąsajos su KS diastoline disfunkcija. Nustatyta, kad miego-šlaunies arterijų pulsinės bangos greitis yra reikšmingas nepriklausomas veiksnys prognozuojant KS diastolinę disfunkciją asmenims, turintiems metabolinį sindromą. Kaip minėta, MetS turinčiųjų kohortoje, tokios didelės apimties tyrimų šioje srityje iki šiol labai trūksta.

Atliktas 573 asmenų išilginio stebėjimo tyrimas, kuriame įvertinta arterijų standumo pokyčio įtaką KS diastolinės disfunkcijos dinamikai (stebėjimo trukmės vidurkis 3,8 ± 0,6 metai). Nustatyta, kad arterijų standumo didėjimas yra nepriklausomas KS diastolinės funkcijos blogėjimo prognostinis veiksnys. Taip pat atskleista, kad KS diastolinės disfunkcijos dinamika reikšmingai siejasi su kūno masės indekso ir širdies susitraukimų dažnio pokyčiais. Remiantis šiuo metu prieinamų mokslinių tyrimų duomenimis, mūsų darbas yra pirmas tokio pobūdžio stebėjimo tyrimas asmenų, turinčių MetS, grupėje.

Mūsų darbe tai pat pirmą kartą objektyviai įvertinti ankstyvojo širdies nepakankamumo požymiai asmenų, turinčių MetS, grupėje. Vertinant pikinį deguonies suvartojimą per fizinį krūvį ir natriuretinio peptido (BNP) koncentraciją, nustatyta, kad daugiau nei pusė mūsų tirtų asmenų turėjo širdies nepakankamumo su išsaugota KS išstūmio frakcija požymių.

Šiame darbe naujai atskleistos ir patvirtintos sąsajos gali ženkliai pasitarnauti mokslo tiriamajame ir klinikiniame darbe, ypač užtikrinant savalaikį širdies nepakankamumo nustatymą ir perspėjant jo vystymąsį.

Metodika

Biomedicininiam tyrimui atlikti gautas Vilniaus regioninio biomedicininių tyrimų etikos komiteto leidimas Nr. 158200-13-641-205. Visi tyrime dalyvaujantys asmenys buvo informuoti apie tyrimo eigą ir pasirašė informuoto asmens sutikimo formą.

Tiriamųjų grupę sudarė abiejų lyčių asmenys, 2009 - 2015 metais tirti Vilniaus universiteto ligoninėje Santaros klinikose pagal Asmenų, priskirtų ŠKL didelės rizikos grupei, atrankos ir prevencijos priemonių finansavimo programą (angl. *The Lithuanian high cardiovascular risk primary prevention program, LitHiR*). Į tyrimą buvo įtraukti 50 – 65 metų moterys ir 40 – 55 metų vyrai, kuriems buvo nustatytas metabolinis sindromas.

Metabolinis sindromas nustatytas remiantis 2005 metų NCEP ATP III modifikuotais kriterijais (kai nustatyti bet kurie trys iš žemiau išvardintų penkių kriterijų):

- Liemens apimtis: vyrams ≥ 102 cm, moterims ≥ 88 cm;
- Arterinis kraujo spaudimas: ≥ 130/85 mmHg, arba gydymas vaistais, esant AH anamnezėje;
- Gliukozės koncentracija kraujyje (nevalgius): ≥ 5,6 mmol/l, arba gydymas vaistais esant hiperglikemijai;
- DTL – Ch koncentracija serume: vyrams < 1,03 mmol/l; moterims < 1,30 mmol/l;
- TG koncentracija serume : ≥1,7 mmol/l, arba gydymas vaistais.

Pirmame etape atlikta 1208 tiriamųjų duomenų skerspjūvio analizė. Antrame etape, po 3,8 ± 0,6 metų, 573 tiriamieji buvo ištirti pakartotinai. Tiek pradinio, tiek pakartotino vertinimo metu atliktas pilnas asmenų ištyrimas pagal LitHiR programos protokolą. Pakartotino ištyrimo metu, siekiant įvertinti ankstyvojo ŠN dažnį, 148 tiriamiesiems papildomai nustatyta B tipo natriuretinio peptido (BNP) koncentracija kraujyje ir atliktas kardiopulmoninis krūvio mėginys (spiroergometrija).

Atlikti antropometriniai matavimai – matuotas tiriamųjų svoris, ūgis, juosmens apimtis, apskaičiuotas kūno masės indeksas (KMI), matuotas arterinis kraujo spaudimas (AKS), atlikta didelio jautrumo C-reaktyvaus baltymo (djCRB), BNP, lipidograma, gliukozė nevalgius. Atliktas arterijų sienelės parametrų vertinimas: pulsinės bangos greičio (PBG), vidutinio arterinio kraujo spaudimo, pulsinio spaudimo, augmentacijos indekso aortoje matavimas aplanacinės tonometrijos metodu, miego arterijų intimos ir medijos sluoksnio storio ir miego arterijų standumo nustatymas ultragarsiniu metodu, kulkšnies-žasto ir širdies-kulkšnies indeksų matavimas. Atlikus echokardiografiją vertinta KS diastolinė funkcija, KS geometrija. Atliekant spiroergometriją vertintas fizinis pajėgumas, degonies suvartojimas per laiko vienetą.

Rezultatai ir jų aptarimas

Šiame darbe pirmą kartą Lietuvoje įvertinom KS diastolinės disfunkcijos paplitimą didelės kardiovaskulinės rizikos asmenų grupėje, esant nustatytam MetS. Tyrimo kohortą sudarė 1208 abiejų lyčių asmenys. Atlikus echokardiografiją didžiajai tirtų asmenų daliai (75,7 proc., n = 915) nustatėm sutrikusią KS diastolinę funkciją.

Atlikę tyrimą ir palyginę tiriamųjų grupes nustatėme, kad tiriamieji, esant sutrikusiai KS diastolinei funkcijai, buvo vyresnio amžiaus, vyravo moterys, turėjo didesnę gliukozės koncentraciją nevalgius. Taip pat šioje grupėje nustatėme didesni AH sergančiųjų procentą (93,7 proc. vs. 88,3 proc., p = 0,005).

Tiriamųjų, esant sutrikusiai KS diastolinei funkcijai, grupėje nustatyti reikšmingai didesni KMI (32,1 ± 5,06 vs. 30,5 ± 4,52 kg/m^2, p < 0,0001) ir liemens apimtis (106,8 ± 10,76 vs. 103,6 ± 10,40 cm, p < 0,0001). Vertinant analizuojamų kintamųjų tarpusavio priklausomybes ir skaičiuojant koreliacijos koeficientus nustatėme, kad KMI reikšmingai susijęs su E/e'_{vid} santykiu (r = 0,216, p < 0,001). Pažingsninės daugialypės regresinės analizės modeliuose, prognozuojant KS diastolinę disfunkciją, KMI ir liemens apimtis išliko reikšmingais rizikos veiksniais (p < 0,001).

Siekiant įvertinti sąsajas tarp KS diastolinės funkcijos ir arterijų sienelės rodiklių atlikome 1208 tiriamųjų duomenų skerspjūvio analizę. Mūsų žiniomis tai pirmas didelės kohortos,

esant MetS, tyrimas, per kurį vertinom neinvaziniais metodais išmatuotus pagrindinius arterijų sienelės struktūros ir funkcijos rodiklius – miego-šlaunies arterijų PBG, augmentacijos indeksą, centrinį kraujospūdį, centrinį pulsinį spaudimą, bendrosios miego arterijos standumą ir intimos-medijos storį, širdies-kulkšnies indeksą.

Mūsų darbe tiriamųjų, esant sutrikusiai KS diastolinei funkcijai, grupėje miego-šlaunies arterijų PBG buvo reikšmingai didesnis (9,0 ± 1,65 vs. 8,2 ± 1,34 m/s, $p < 0,0001$). Nustatėme reikšmingas koreliacijas tarp miego – šlaunies arterijų PBG ir KS diastolinės finkcijos parametrų: atvirkštinis ryšys su E/A ($r = – 0,190$, $p < 0,001$) ir teigiamas ryšys su E/e'_{vid} ($r = 0,167$, $p < 0,001$) santykiais. Mūsų darbe atskleidėme, jod miego-šlaunies arterijų PBG yra reikšmingas veiksnys prognozuojant KS diastolinę disfunkciją asmenų, esant nustatytam MetS, grupėje. Atliekant žingsninės daugialypės logistinės regresijos analizę kartu su arterijų sienelės rodikliais įvertinome klasikinius ŠKL rizikos veiksnius (KMI ir liemens apimtį, centrinį vidutinį AKS, rūkymą). Koregavus analizės rezultatus pagal amžių, lytį ir ŠSD, miego-šlaunies arterijų PBG išliko reikšmingu nepriklausomu KS diastolinės disfunkcijos išsivystymo rizikos veiksniu ($p = 0,002$). Nustatėme, kad padidėjus miego-šlaunies arterijų PBG per 1,3 m/s, tikimybė pasireikšti KS diastolinei disfunkcijai padidėja 18,6 proc..

Mūsų tiriamųjų grupėje, esant KS diastolinei disfunkcijai, nustatytas didesnis augmentacijos indeksas aortoje (25,4 ± 9,96 vs. 21,1 ± 10,84 proc., $p < 0,0001$). Augmentacijos indeksas reikšmingai koreliavo su KS diastolinės funkcijos parametrais: E/A ($r = – 0,151$, $p < 0,001$) ir E/e'_{vid} ($r = 0,136$, $p < 0,001$). Daugialypės regresinės analizės modeliuose AIx, automatiškai koreguotas pagal ŠSD, šalia mšPBG, išliko reikšmingai susijęs su KS diastoline disfunkcija.

Mūsų darbe tiriamųjų grupėje, esant KS diastolinei disfunkcijai, nustatėme reikšmingai didesni centrinis vidutinis AKS (108,6 ± 12,85 vs. 103,4 ± 10,13 mmHg, $p < 0,0001$) ir centrinis pulsinis spaudimas (44,5 ± 10,69 vs. 41,9 ± 10,8 mmHg, $p = 0,0004$). Taip pat atskelidėme, kad centrinis AKS ir centrinis pulsinis spaudimas reikšmingai susiję su KS prisipildymo spaudimu, vertintu kaip E/e'_{vid} santykis ($r_{cAKS} = 0,159$, $p < 0,001$; $r_{cPP} = 0,265$, $p < 0,001$). Tačiau, tęsiant analizę, pažingsninės daugialypės regresijos modeliuose centrinio AKS ir centrinio pulsinio spaudimo sąsajos su KS diastoline disfunkcija neišliko reikšmingos.

Mūsų darbe įvertinome centrinės hemodinamikos ir arterijų sienelės rodiklių pokytį ir jo įtaką KS diastolinės funkcijos dinamikai. Šiai užduočiai atlikti po 3,8 ± 0,6 metų laikotarpio pakartotinai ištyrėme 573 asmenys iš bendros 1208 tiriamųjų kohortos. Nustatėme, kad pagrindinis veiksnys, susijęs su KS diastolinės funkcijos blogėjimu, yra miego-šlaunies arterijų PBG. Šio rodiklio pokytis buvo skirtingas tarp tiriamųjų grupių: KS diastolinės disfunkcijos gerėjimo grupėje stebėtas, nors ir statistiškai nereikšmingas, mšPBG sumažėjimas. Tuo tarpu KS diastolinės disfunkcijos blogėjimo grupėje mšPBG reikšmingai didėjo ($p = 0,004$). Įdomu tai, kad ne tik visoje pakartotinai tirtų asmenų kohortoje, bet ir KS diastolinės funkcijos blogėjimo grupėje nustatytas reikšmingas ŠSD (nuo 65,17 ± 9,41 iki 62,07 ± 8,51 k/min, $p = 0,003$) ir centrinio vidutinio AKS (nuo 104,07 ± 10,94 iki 99,73 ± 13,17 mmHg, $p = 0,010$) sumažėjimas. Tačiau atlikę logistinės regresijos analizę, jos modeliuose tik miego-šlaunies arterijų PBG išliko reikšmingu, prognozuojant KS diastolinės funkcijos blogėjimą ($p = 0,015$).
Mūsų stebėjimo rezultatai patvirtina skerspjūvio analizės išvadas, kad mšPBG yra KS diastoli-

nės disfunkcijos atsiradimą lemiantis patogenetinis veiksnys. Manome, kad atsakėme į svarbų klausimą – ar aortos standumo didėjimas lemia KS diastolinės funkcijos sutrikimą nepriklausomai nuo amžiaus, AKS ir kitų tradicinių ŠKL rizikos veiksnių. Galime teigti, kad mšPBG (aortos standumo rodiklis) yra KS diastolinės disfunkcijos atsiradimą lemiantis patogenetinis veiksnys. Todėl terapinės priemonės, kurios galėtų sulėtinti arba sustabdyti amžinius ir (arba) patologinius arterijų sienelės pakitimus ir standumo didėjimą, turėtų būti vertingos KS diastolinės disfunkcijos iš ŠN prevencijai ir regresijai.

Mūsų žiniomis, šiame darbe pirmą kartą pabandyta nustatyti galimybę aptikti ankstyvąjį ŠN, remiantis deguonies apykaita ir plaučių ventiliacija per fizinį krūvį bei BNP koncentracija kraujyje, tiriamųjų, esant MetS, grupėje. Atlikus tyrimą, didžiajai daliai (64,8 proc.) tiriamųjų šalia didelio KS diastolinės disfunkcijos dažnio (73,6 proc.) nustatėme sumažintą fizinio krūvio toleranciją ir (arba) padidintą natriuretinio peptido koncentraciją. Šiems asmenims mes diagnozavom ankstyvąjį ŠN.

Palyginus tiriamųjų duomenis, ankstyvojo širdies nepakankamumo pogrupyje nustatėme patikimai didesnį augmentacijos indeksą (31,2 ± 9,9 vs. 27,3 ± 7,4 proc., $p < 0,05$). Augmentacijos indeksas buvo reikšmingai susijęs tik su $VO2_{piko}$ ($r = -0,264$, $p = 0,001$). Atlikę vienfaktorinę logistinę regresiją nustatėme, kad ankstyvojo ŠN buvimas reikšmingai susijęs su BNP, $VO2_{piko}$ ir augmentacijos indeksu aortoje. Tačiau įtraukus šiuos reikšmingus kintamuosius į pažingsninės logistinės regresijos analizę, augmentacijos indeksas prarado reikšmingumą prognozuojant ankstyvąjį ŠN.

Išvados

1. Skerspjūvio tyrime nustatyta, kad trims ketvirtadaliams tiriamųjų, turinčių metabolinį sindromą, randama sutrikusi kairiojo skilvelio diastolinė funkcija.
2. Asmenims, turintiems metabolinį sindromą, arterijų standumas, vertintas matuojant miego-šlaunies pulsinės bangos greitį, yra nepriklausomas kairiojo skilvelio diastolinės disfunkcijos prognostinis veiksnys, kurio reikšmingumas išlieka net ir atsižvelgus į tradicinius kardiometabolinės rizikos veiksnius.
3. Miego-šlaunies pulsinės bangos greičio didėjimas, esant metaboliniam sindromui, yra reikšmingas kairiojo skilvelio diastolinės funkcijos blogėjimo veiksnys. Tai atskleidžia jo svarbą kairiojo skilvelio diastolinės disfunkcijos patogenezėje.
4. Pritaikius objektyvų fizinio pajėgumo ir neurohormoninės aktyvacijos vertinimą, daugiau nei pusei tirtų asmenų, turinčių metabolinį sindromą, nustatytas ankstyvasis išsaugotos kairiojo skilvelio išstūmio frakcijos širdies nepakankamumas. Ankstyvojo širdies nepakankamumo asmenų grupėje nustatytas statistiškai reikšmingas arterijų standumo, vertinto matuojant augmentacijos indeksą aortoje, padidėjimas.

Praktinės rekomendacijos

1. Efektyviam asmenų, turinčių metabolinį sindromą, kardiovaskulinės rizikos įvertinimui ir gydymo taktikos parinkimui tikslinga nustatyti arterijų standumo ir centri-

nės hemodinamikos rodiklius, ypač pulsinės bangos greitį aortoje ir centrinį pulsinį spaudimą.

2. Remiantis šio darbo rezultatais nustatyta slenkstinė 8,2 m/s miego-šlaunies pulsinės bangos greičio reikšmė, padedanti atskirti asmenis, esant didesnei kairiojo skilvelio diastolinės disfunkcijos galimybei. Nustačius miego-šlaunies arterijų pulsinės bangos greitį > 8,2 m/s asmenims, turintiems metabolinį sindromą, rekomenduojame taikyti ankstyvesnę ir efektyvesnę kardiovaskulinių ligų prevenciją. Tai padėtų sumažinti kairiojo skilvelio diastolinės disfunkcijos išsivystymo riziką.
3. Asmenims, turintiems metabolinį sindromą, šalia klinikinių simptomų vertinimo ir echokardiografijos, siekiant laiku aptikti besivystantį išsaugotos kairiojo skilvelio išstūmio frakcijos širdies nepakankamumą, būtų tikslinga nustatyti natriuretinių peptidų koncentraciją kraujyje ir atlikti kardiopulmoninį krūvio mėginį.

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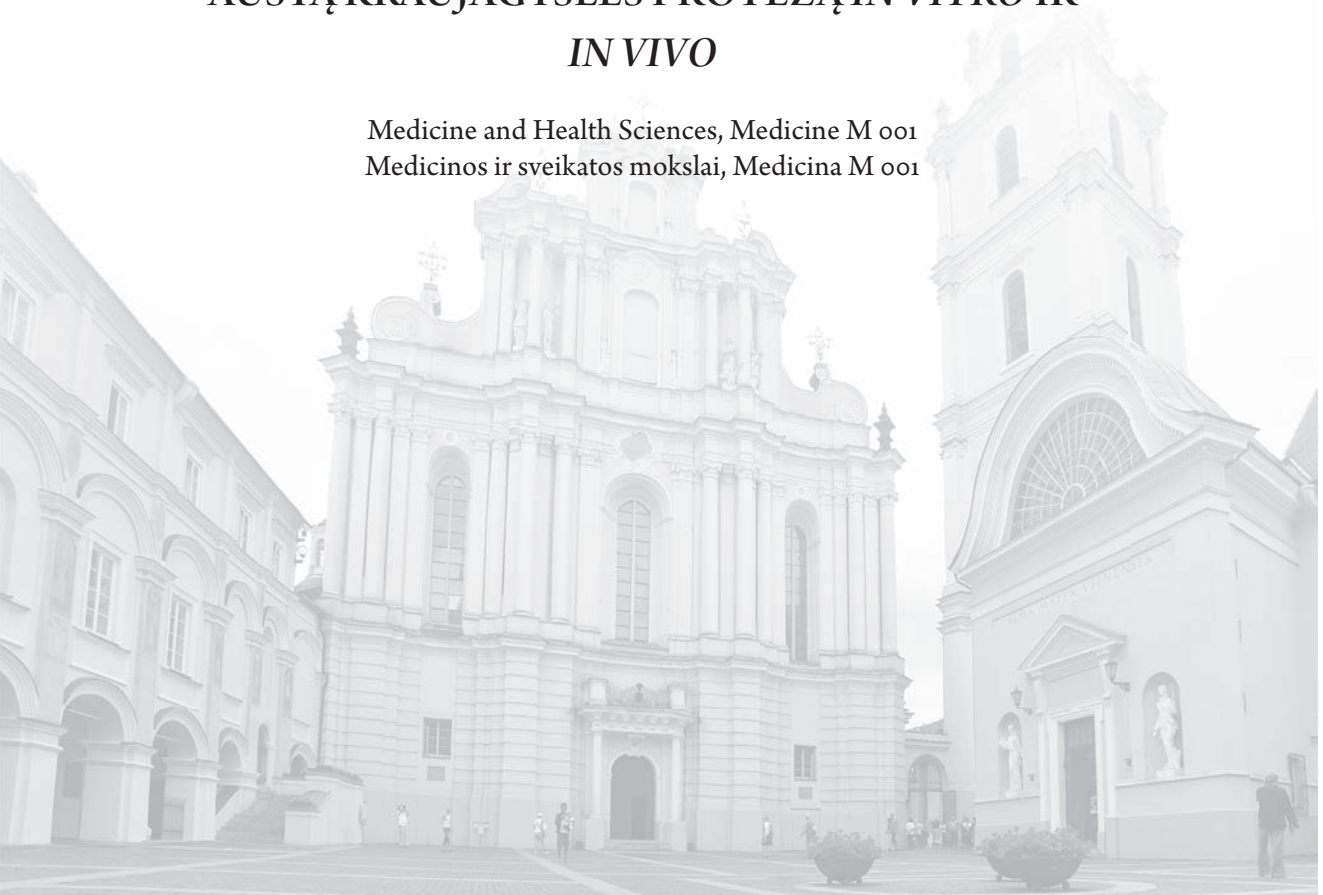
Elvyra Stanevičiūtė

THE COMPARISON OF THE EFFICACY OF THE ANTISEPTIC SOLUTIONS IN TREATMENT OF *STAPHYLOCOCCUS AUREUS* INFECTED WOVEN VASCULAR GRAFT *IN VITRO* AND *IN VIVO*

ANTISEPTIKŲ VEIKSMINGUMO PALYGINIMAS VEIKIANT *STAPHYLOCOCCUS AUREUS* INFEKUOTĄ AUSTĄ KRAUJAGYSLĖS PROTEZĄ *IN VITRO* IR *IN VIVO*

Medicine and Health Sciences, Medicine M 001
Medicinos ir sveikatos mokslai, Medicina M 001



The list of abbreviations

CFU – colony forming units
IQR – interquartile range
MF – Faculty of Medicine
MLM – modified Lubbock's medium
NPWT – negative pressure wound therapy
OD – optical density
PBS – phosphate buffer solution
PMN – polymorphonuclears
PVGI – prosthetic vascular graft infection
SD – standard deviation
SEM – scanning electron microscopy
TSA – tryptic soy agar
TSB – tryptic soy broth
VU – Vilnius University

1. Introduction

1.1 Clinical relevance of the study

Prosthetic vascular graft infection (PVGI) is a rare but threatening complication in vascular surgery. It is estimated that PVGI occurs to 0.5-9.5% of the patients undergoing vascular graft implantation [1-8]. Regardless of the relatively low incidence rate, if left untreated, this complication often results in life-threatening hemorrhage or sepsis [9]. Despite all the measures of treatment, the outcomes of PVGI include high numbers of limb amputations (5-70% of all cases) and death (10-75% of all cases) [4, 9-14].

PVGI may develop perioperatively (due to breaches in asepsis) and postoperatively (mostly due to the infection of the surgical site or systemic bacteremia). The most common infectious agents include *Staphylococci, Escherichia, Enterococci and Salmonella* species. *Staphylococcus aureus* is responsible for the majority of PVGIs in the early postoperative period. Its tendency to form bacterial biofilms on the surface of the graft aggravates the course of treatment [2, 15-18].

There are no widely accepted guidelines or algorithms for the treatment of PVGI. An aggressive surgical approach, including removal of the infected graft, debridement of the infected perigraft tissues, systemic antibiotic therapy and forming an extra-anatomic bypass was considered a "golden standard" for a long time [4]. This approach is not suitable in some cases, for example, when a patient is critically ill and would not survive a major surgical intervention or the graft is connected to the aorta or other vitally important arteries. Therefore, in some cases PVGI is chosen to be treated conservatively. Successful case reports or reviews on small series of patients, who received conservative treatment, are published in scientific literature [19, 20]. If a conservative treatment approach is chosen, usually several treatment methods are applied together to achieve better results: long-term antibiotic therapy, negative pressure wound therapy (NPWT), local wound washings and drainage ap-

plying constant or repetitive irrigations [1, 3, 9, 20]. The wounds can be washed with sterile saline [21], antibiotic or antiseptic solutions. Although antibiotics are a must in the treatment of PVGI, only some of them are capable of penetrating biofilms, formed by the bacteria on the graft. The formation of a bacterial biofilm on one of the most commonly used prosthetic vascular grafts – Dacron – usually leads to a disastrous scenario. Since Dacron is made of polyester microfilaments, which create an uneven graft surface and increase the area of adhesion for bacteria to cling and cluster, the accessibility of antimicrobials and the host's immune cells greatly decrease [16, 22].

Two main problems are faced when PVGI is treated with antibiotics. First, due to the formation of bacterial biofilms the penetration of antibiotics is reduced. Second, bacteria develop resistance to antibiotics, so alternative antimicrobials – antiseptic solutions – are becoming more appealing, because the bacteria's resistance to them remains minimal [23]. Even though antiseptics are usually used for prophylaxis [24], introducing them into the treatment of complicated infections wound allow limiting the use of ineffective antibiotic therapy.

1.2 Aim of the study

To evaluate the efficacy of routinely used antiseptic solutions in the treatment of *Staphylococcus aureus* infected woven vascular graft *in vitro* and *in vivo.*

1.3 Study objectives

1. To compare a biofilm-disrupting activity of 0.1% octenidine dihydrochloride, 10% povidone-iodine and 0.02% chlorhexidine digluconate on S. aureus biofilms in vitro.
2. To compare the antimicrobial efficacy of 0.1% octenidine dihydrochloride, 10% povidone-iodine and 0.02% chlorhexidine digluconate on S. aureus biofilms in a simulated wound environment in vitro.
3. To design an experimental PVGI model in vitro and to compare the antimicrobial efficacy of 0.1% octenidine dihydrochloride, 10% povidone-iodine and 0.02% chlorhexidine digluconate on S. aureus biofilms, situated on the patches of vascular graft.
4. To design an experimental PVGI model in vivo for Wistar rats and to compare the efficacy of 0.1% octenidine dihydrochloride, 10% povidone-iodine and 0.02% chlorhexidine digluconate, treating S. aureus infected surgical wounds with patches of woven vascular graft.
5. To determine the duration of the treatment needed to eradicate S. aureus from the wounds with infected vascular grafts and to perform cytological and histological analysis of the perigraft tissues.

1.4 Statements to be defended

1. Antiseptic solutions are able to affect *S. aureus* biofilms *in vitro*.
2. Irrigations with antiseptic solutions are effective in treating *S. aureus* infected surgical wounds with patches of woven vascular graft in rats.

1.5 Novelty of the study

In the era of quickly developing bacterial resistance to antibiotics, the medical community is in constant search of alternative ways to treat various infections. A conservative method to treat PVGI – local wound irrigation with antiseptic solutions – is used from time to time, but is not a well established treatment method. After thoroughly reseaching this topic, it was discovered that there is a lack of representative, wound-simulating *in vitro* models, which would assist studying an interaction of biofilm-producing *S. aureus* and routinely used antiseptic solutions on woven vascular grafts. No publications were found on *in vivo* studies where antiseptic solutions would be used for treatment of PVGI, but not for prophylaxis.

Therefore, a novel *in vitro* model, simulating the wound environment and PVGI, was designed to study antimicrobial efficacy of antiseptics on *S. aureus* biofilms. In order to test if wound irrigation with antiseptic solutions is an effective treatment method, a new experimental model of PVGI was developed for Wistar rats.

2. Materials and methods

The *in vitro* experiments, studying the efficacy of routinely used antiseptic solutions on *S. aureus* biofilms were performed at Vilnius University (VU) Faculty of Medicine (MF) Institute of Biomedical Science Department of Physiology, Biochemistry, Microbiology and Laboratory Medicine Division of Microbiology and Lithuanian Center of Non-formal Youth Education laboratory from May 2017 to October 2018. Three strong biofilm-producing *S. aureus* strains (215N, A7189 and ATCC 25923) were selected for the experiments. The biofilm-disrupting activity and antimicrobial efficacy of 0.1% octenidine dihydrochloride (Octenisept®, Schulke&Mayr GmbH, Germany), 10% povidone-iodine (Betadine®, EGIS Pharmaceuticals LTD, Hungary) and 0.02% chlorhexidine digluconate (Fresenius Kabi AG, Germany) on *S. aureus* biofilms were tested in three different *in vitro* settings. Phosphate buffer solution (PBS) was used for control groups.

The *in vivo* experiments, evaluating the efficacy of the antiseptics for treatment of infected surgical wounds with vascular grafts in rats were carried out in State Research Institute Centre for Innovative Medicine Department of Immunology, VU

Life Sciences Center and VU MF Institute of Biomedical Science Department of Physiology, Biochemistry, Microbiology and Laboratory Medicine Division of Microbiology from February 2012 to July 2018. All researchers attended courses of Laboratory Animal Science prior to the experiments. The permissions to perform the studies were granted by State Food and Veterinary Service on January 4, 2012 and April 25, 2016 (permit no. 0222 and G2-43).

The experiments were performed on Wistar rats of both genders. All rats underwent a surgery when a patch of woven vascular graft was implanted under the skin. The wounds were inoculated with a biofilm-producing *S. aureus* 215N strain. A conservative method to treat PVGI – wound irrigation with antiseptic solutions (0.1% octenidine dihydrochloride, 10% povidone-iodine and 0.02% chlorhexidine digluconate) was tested. Sterile saline solution was used for the control group. Microbiological tests of the wound washouts were performed repeatedly to evaluate the efficacy of the treatment.

2.1 Materials and methods for *in vitro* experiments

2.1.1 Qualitative and quantitative analysis of *S. aureus* biofilm production

In order to select strong biofilm-producers for the further experiments, ten different *S. aureus* strains were tested. The strains were selected randomly from the storage of VU MF Institute of Biomedical Science Department of Physiology, Biochemistry, Microbiology and Laboratory Medicine Division of Microbiology. Qualitative and quantitative evaluation of biofilm formation were performed according to *Rewatkar, Wadher* [25], *Darwish and Asfour* [26]. The cultures were cultivated on tryptic-soy agar (TSA, Liofilchem®, Italy) for 24 h at 35°C±2°C. After cultivation, one colony of each strain was transferred to Congo red agar and incubated for 24 h at 35°C±2°C. *S. aureus* strains, which produced black colonies on Congo red agar, were considered biofilm-producing strains [20, 190].

Quantitative selection of *S. aureus* isolates was performed using a modified crystal violet absorbtion assay [27]. 100 µL of each strain's suspension were transferred to a 96-well plate (Thermo Scientific™, Nunc ™) (Figure 1 A) and cultivated for 24 h at 35°C±2°C. After the incubation the medium was aspirated from the wells and they were washed three times with PBS to eliminate planktonic bacteria. Biofilms, which were stuck to the bottom of the wells, were fixed with 96% ethanol solution and colored with aqueous crystal violet dye. The well plate was washed again and left to dry. Finally, each well was filled with 300 µL of 30% acetic acid to extract crystal violet from the biofilms. After 30 minutes, 200 µL of each extract was transferred to a clean 96-well plate. The optical density (OD) of the solutions was determined using a spectrophotometer (Dynex MRX) at 595 nm. The wells with tryptic soy broth

(TSB) without bacteria served as a negative control. Each strain was tested 12 times. The mean value of OD was used to evaluate the mass of a produced biofilm.

According to the qualitative and quantitative biofilm-formation assays, three strains were found to be strong biofilm-producers: 215N, A7189 and ATCC 25923. They were used in the further experiments.

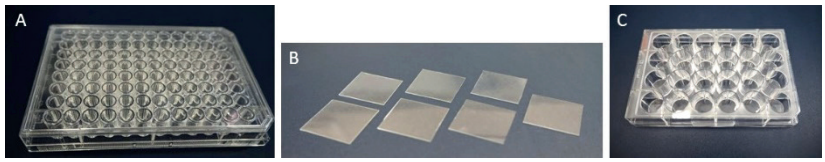


FIGURE 1. A – 96-well microplate; B – glass coverslips; C – 24-well plate.

2.1.2 Microscopic evaluation of the integrity of *S. aureus* biofilms

OBJECTIVE 1: to compare a biofilm-disrupting activity of 0.1% octenidine dihydrochloride, 10% povidone-iodine and 0.02% chlorhexidine digluconate on *S. aureus* biofilms *in vitro*

215N, A7189 and ATCC 25923 *S. aureus* strains were used for the experiment. The biofilms were grown in TSB on borosilicate glass coverslips (Figure 1 B) in Petri dishes for 48 h and then treated with 0.1% octenidine dihydrochloride, 10% povidone-iodine and 0.02% chlorhexidine digluconate solutions for 10 minutes. PBS was used for the control groups. The integrity of the biofilm was visualized by scanning the glass coverslips with a Scan Scope XT Slide Scanner (Leica Aperio Technologies, Vista, CA, USA). The actual area of the coverslips, covered with the biofilms, was analysed and quantified using Halo Area Quantification v1.0 algorithm and expressed in percent. The area of the coverslips, covered with the biofilms in control groups was compared to the area of the coverslips, covered in biofilms after the application of the antiseptics.

2.1.3 Antimicrobial efficacy of different antiseptic solutions on *S. aureus* biofilms

OBJECTIVE 2: to compare the antimicrobial efficacy of 0.1% octenidine dihydrochloride, 10% povidone-iodine and 0.02% chlorhexidine digluconate on *S. aureus* biofilms in a simulated wound environment *in vitro*

The methodology of the experiment was based on Lubbock's chronic wound pathogenic biofilm model (further – Lubbock's model), described by *Sun et al.* [28].

The essence of the model is to mimick a wound environment *in vitro*, using a medium, which consists of blood components and nutrients. We modified the model and used medium, which consisted of 50% of sheep blood plasma, 5% of cold lysed horse erythrocytes, 45% of brain-heart infusion and 1% of type B gelatin (further – modified Lubbock's medium, MLM).

The biofilms of 215N, A7189 and ATCC 25923 *S. aureus* strains were grown in a MLM in the 24-well plates (Thermo Scientific™, Nunc ™) (Figure 1 C). The biofilms were treated with 0.1% octenidine dihydrochloride, 10% povidone-iodine and 0.02% chlorhexidine digluconate solution for 10 minutes. PBS was used for control groups. After the time of the exposition, the colony forming units (CFU) of *S. aureus*, remaining in the biofilms, were evaluated microbiologically on Mannitol-salt agar (Liofilchem®, Italy) plates.

2.1.4 Antimicrobial efficacy of different antiseptic solutions on *S. aureus* biofilms on the vascular grafts

OBJECTIVE 3: to design an experimental PVGI model *in vitro* and to compare the antimicrobial efficacy of 0.1% octenidine dihydrochloride, 10% povidone-iodine and 0.02% chlorhexidine digluconate on *S. aureus* biofilms, situated on the patches of vascular graft

Lubbock's model [28] was modified and adapted to design an innovative experimental PVGI model *in vitro*. The biofilms of *S. aureus* 215N, A7189, ATCC 25923 strains were grown in MLM on the patches of a non-impregnated woven Dacron graft (Twillweave®, Vascutek Terumo) (Figure 2 A). The patches of the vascular graft, covered in biofilms (Figure 2 B) were treated with 0.1% octenidine dihydrochloride, 10% povidone-iodine, 0.02% chlorhexidine digluconate solution for 10 minutes. PBS was used for control groups. After the time of the exposition, the amount of *S. aureus* remaining in the biofilms on the vascular grafts, was evaluated microbiologically on Mannitol-salt agar plates.

For *in vitro* experiments, depicted in sections 2.1.2, 2.1.3 and 2.1.4 the standardised inocula of *S. aureus* strains were used (Table 1). Identification of *S. aureus*

TABLE 1. *S. aureus* inocula used for *in vitro* experiments

S. aureus strain	Inoculum for 2.1.2 experiment, CFU*/mL	Inoculum for 2.1.3 experiment, CFU/mL	Inoculum for 2.1.4 experiment, CFU/mL
215N	1.49×10^6	8.18×10^7	1.49×10^6
A7189	7.63×10^7	7.63×10^7	7.63×10^7
ATCC 25923	6.91×10^7	6.91×10^7	6.91×10^7

*CFU – colony forming units

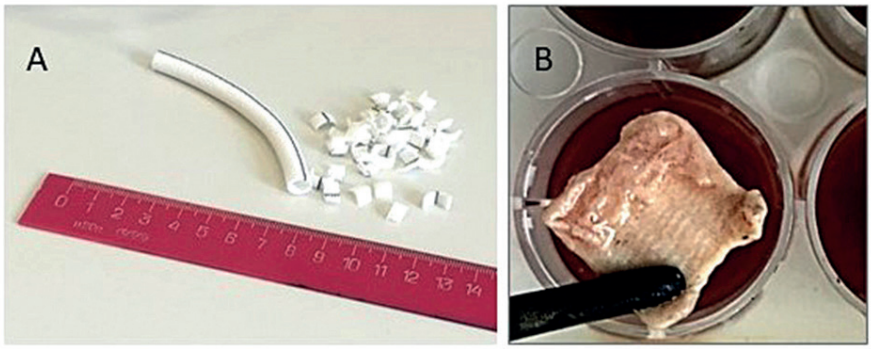


FIGURE 2. A – a non-impregnated woven Dacron graft and its patches; B – a patch of Dacron graft, covered by *S. aureus* biofilm.

was based on latex agglutination, plasma coaguliation tests and light microscopy. *S. aureus* ATCC 29213 was used as a control strain.

2.1.5 Scanning electron microscopy of the vascular grafts

A scanning electron microscope (SEM, HITACHI TM-1000, Japan, accelerating voltage 15 kV) was used to visualize the structure of a non-impregnated Dacron vascular graft. Three patches of clean and three patches of biofilm-covered grafts were scanned. Prior to scanning, patches of the vascular graft with S. aureus biofilms were fixed in 5% glutaraldehyde solution for 12 hours. All patches of the graft were gold-coated before scanning. Every patch was photographed 10 times to get a representative image of the graft with and without the biofilm.

2.2 Materials and methods for *in vivo* experiments

A new experimental rat model was designed to study the efficacy of antiseptics in the treatment of PVGI. A biofilm-producing *S. aureus* 215N strain, previously used for *in vitro* experiments, was chosen to induce the infection. The treatment of the wounds was performed by applying daily irrigations with either 0.1% octenidine dihydrochloride, 10% povidone-iodine, 0.02% chlorhexidine digluconate solution or sterile saline (for control group).

Wistar rats of both genders, 3-6 months of age and 239-450 g of body mass were used for the study. Prior to the main experiment, a pilot study was performed to estimate a proper *S. aureus* dose to cause a suppuration of a surgical wound, which would persist for at least two weeks.

The main study was performed in two stages. The first series of *in vivo* experiments was performed to test a newly designed rat model for the treatment of PVGI caused by *S. aureus* and to evaluate the efficacy of three different antiseptic solutions. The second series of *in vivo* experiments was performed with the most effective antiseptic solution to evaluate the repeatability of the newly designed model, to achieve *S. aureus* eradication from the wound washouts and to evaluate both cytological and histological wound findings.

2.2.1 The first series of *in vivo* experiments

OBJECTIVE 4: to design an experimental PVGI model *in vivo* for Wistar rats and to compare the efficacy of 0.1% octenidine dihydrochloride, 10% povidone-iodine and 0.02% chlorhexidine digluconate, treating *S. aureus* infected surgical wounds with patches of woven vascular graft

The rats (n=48) were placed into separate cages one week prior to the experiment. Balanced standardised food, tap water and same controlled conditions were ensured. The anaesthesia was induced using 10% ketamine 40 mg/kg ("Bio-ketan", Vetoquinol Biowet Sp. Zoo, Poland) and 2% xylazin solution 5 mg/kg ("Xylazin" 2%, "Bela-Pharm GmbH & Co. KG", Germany), injected intraperitoneally. A patch

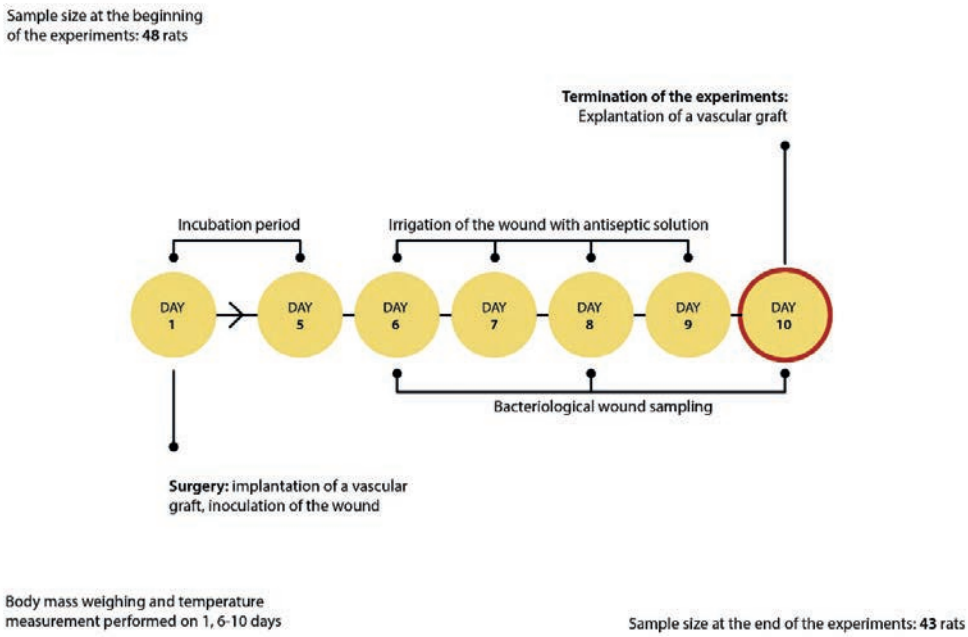


FIGURE 3. The first series of *in vivo* experiments.

of a sterile non-impregnated woven Dacron graft was implanted under the skin for each rat on the right side of the spine and the wound was inoculated with 1 mL of *S. aureus* suspension, equivalent to 5 McFarland standard. After five days of incubation, wound washouts were taken for the microbiological evaluation. The rats were randomly grouped into four groups of 12 and daily wound irrigations were started with different antiseptic solutions (0.1% octenidine dihydrochloride, 10% povidone-iodine and 0.02% chlorhexidine digluconate solution or sterile saline (control group). The treatment was carried out for four days. To evaluate the dynamics, wound washouts were taken again on day eight and 10 of the experiment. After the termination of the experiment, the grafts were removed from the wounds and tested microbiologically. The detailed course of the first series of *in vivo* experiments is depicted in Figure 3.

2.2.2 The second series of *in vivo* experiments

OBJECTIVE 5: to determine the duration of the treatment needed to eradicate *S. aureus* from the wounds with infected vascular grafts and to perform cytological and histological analysis of the perigraft tissues

Using the same methodology (described in section 2.2.1) a group of 10 rats underwent a vascular graft implantation and wound inoculation with *S. aureus*. The suppurative wounds were treated with daily irrigations of 0.1% octenidine dihydrochloride for seven days. The cytological examinations of the wound washouts were performed along with the microbiological tests. For enumeration of *S. aureus* in the wound washouts, samples were plated on Mannitol-salt agar plates.

The second series of *in vivo* experiments were terminated on day 13. For microbiological evaluation the patches of vascular graft were removed from the wounds. Blood samples were taken via cardiopuncture. The cutaneous fistulas and perigraft tissues were resected for microbiological and histological analysis. The detailed course of the second series of *in vivo* experiments is depicted in Figure 4.

For both the first and the second series of *in vivo* experiments identification of *S. aureus* colonies was based on latex agglutination and plasma coaguliation tests and light microscopy. *S. aureus* ATCC 29213 was used as a control strain.

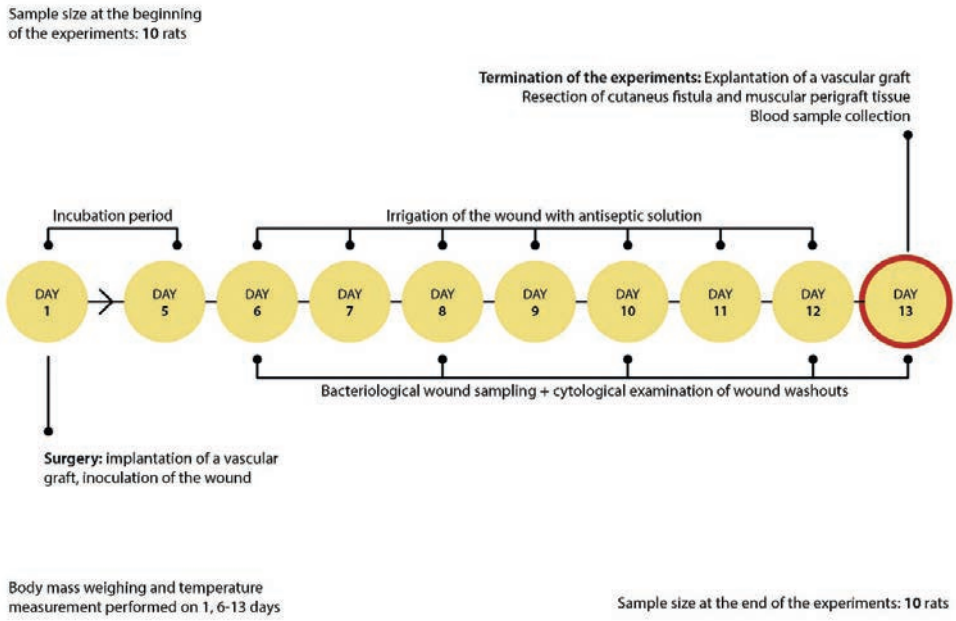


FIGURE 4. The second series of *in vivo* experiments.

2.3 Statistical analysis

The data were analyzed using parametric and non-parametric tests. Parametric tests were used when data were assumed to be normally-distributed (paired sample T test and independent samples T test). Non-parametric tests were used when data were assumed to be not normally distributed (Mann Whitney U, Kruskal Wallis H and Wilcoxon T tests). Correlation analysis was performed using Spearman's Rho test. The data were processed using SPSS 24.0 and Microsoft Excel 2016 programs. Statistical significance was assigned when p values were less than 0.05.

3. Results

3.1 The results of *in vitro* experiments

3.1.1 Qualitative and quantitative evaluation of biofilm-producing *S. aureus* strains

Nine out of 10 *S. aureus* strains produced black colonies on Congo red agar and thus were considered biofilm-producing strains. Red colonies of *S. aureus* were produced by I-1242 strain, therefore it was considered a non-biofilm producer (Figure 5).

Quantitative evaluation of *S. aureus* biofilm production was assessed by a spectrophotometric crystal violet absorbtion assay (Table 2) [27]. Three *S. aureus* strains, which showed signs of strong biofilm production (215N, A7189 and ATCC 25923) were selected for further *in vitro* experiments.

FIGURE 5. Differentiation of biofilm-producing *S. aureus* strains on Congo red agar.

TABLE 2. Quantitative evaluation of the biofilm production by different *S. aureus* strains

S. aureus strain	Mean OD_{595} nm (SD)*	Biofilm production
215N	**0.73**±0.04	**Strong**
A1152	0.47±0.14	Weak
A4192	0.49±0.08	Weak
A6132	0.49±0.13	Weak
A7189	**0.69**±0.12	**Strong**
I-1242	0.15±0.04	None
I-1717	0.51±0.09	Moderate
I-1867	0.57±0.05	Moderate
I-1975	0.53±0.09	Moderate
ATCC 25923	**0.86**±0.04	**Strong**
Control well	0.04±0.02	-

*OD – optical density; SD – standard deviation

3.1.2 Evaluation of the biofilm-disrupting activity of different antiseptic solutions on *S. aureus* biofilms

S. aureus biofilms successfully formed on the glass coverslips in all experimental groups. The area of the coverslips, covered by a biofilm, was homogenous in the control groups of all three *S. aureus* strains (215N, A7189 and ATCC 25923) (Kruskal Wallis H test, p=0.33) (Table 3).

TABLE 3. The area of the glass coverslips, covered by a biofilm for different *S. aureus* strains

S. aureus strain	The area of the glass coverslips, covered by *S. aureus* biofilm in the control groups, %				p value
	Minimum	Maximum	Median	IQR*	
215N	71.6	76.8	72.85	3.57	0.33
A7189	74.2	86.18	82.18	7.3	
ATCC 25923	67.66	85.65	70.32	12.43	

*IQR – interquartile range

A significant biofilm-disrupting activity for all three *S. aureus* strains was observed only after application of chlorhexidine digluconate (Kruskal Wallis H test, 215N strain p=0.02, A7189 strain p<0.001, ATCC 25923 strain p=0.02 (Figure 6).

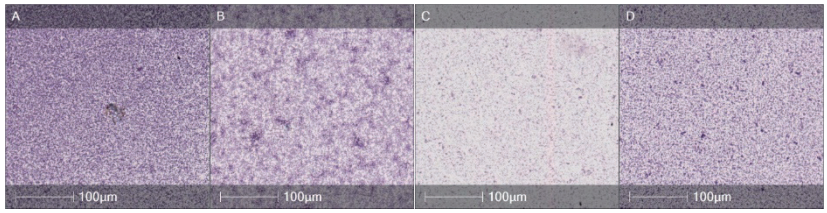


FIGURE 6. Scanned images of the glass coverslips covered by the biofilms of *S. aureus* 215N strain after application of the antiseptics (Scan Scope XT scanner, one representative image for a group): A – control group; B – octenidine dihydrochloride group; C – chlorhexidine digluconate group; D – povidone-iodine group.

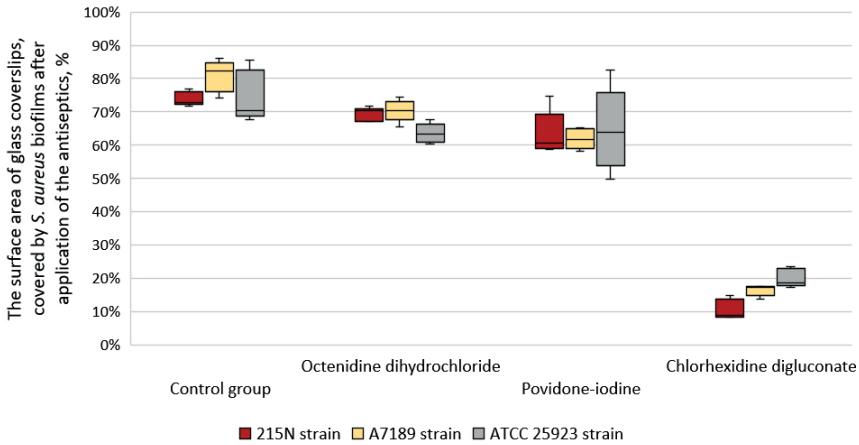


FIGURE 7. The biofilm-disrupting activity of different antiseptics on *S. aureus* biofilms, formed on the glass coverslips. The area of the biofilms on the coverslips did not change significantly after the application of octenidine dihydrochloride (p=0.2) and povidone-iodine (p=0.4), but decreased significantly after the application of chlorhexidine digluconate (p=0.02, p=0.02 and p<0.001, respectively for strains 215N, A7189 and ATCC 25913).

3.1.3 Evaluation of the antimicrobial efficacy of different antiseptic solutions on *S. aureus* biofilms

In a MLM all *S. aureus* strains (215N, A7189 and ATCC 25923) produced biofilms which were similar in texture and mass (p=0.7). The CFUs of *S. aureus* in the biofilms were enumerated for the control groups of all the strains (Table 4) and then compared to the number of the CFUs, left in the biofilms after the application of the antiseptics.

TABLE 4. The number of *S. aureus* CFUs in the biofilms, produced in a modified Lubbock's medium

S. aureus strain	*S. aureus* in the biofilm, CFU/mL			
	Minimum	Maximum	Median	IQR*
215N	$3.5x10^{7}$	$7.2x10^{9}$	$4.57x10^{8}$	$2.56x10^{9}$
A7189	$1.00x10^{6}$	$2.00x10^{12}$	$6.00x10^{11}$	$1.29x10^{12}$
ATCC 25923	$4.00x10^{10}$	$7.00x10^{14}$	$2.00x10^{14}$	$4.99x10^{14}$

*IQR – interquartile range

The application of the antiseptic solutions on the biofilms, produced by *S. aureus* 215N strain, resulted in:

- a decrease of *S. aureus* CFUs in the biofilms by three orders of magnitude after application of 0.02% chlorhexidine digluconate solution (showing median (M) and interquartile range (IQR) (from $M=4.57x10^{8}$, $IQR=2.56x10^{9}$ to $M=2.77x10^{5}$, $IQR=4.86x10^{5}$ CFU/mL, p=0.002);
- no *S. aureus* was detected after the application of 0.1% octenidine dihydrochloride and 10% povidone-iodine (p=0.002).

The application of the antiseptic solutions on the biofilms, produced by *S. aureus* A7189 strain, resulted in:

- a decrease of *S. aureus* CFUs in the biofilms by five orders of magnitude (from $M=6.00x10^{11}$, $IQR=1.29x10^{12}$ to $M=1.00x10^{6}$, $IQR=7.00x10^{5}$ CFU/mL, p=0.02) after the application of 0.02% chlorhexidine digluconate solution and nine orders of magnitude (from $M=6.00x10^{11}$, $IQR=1.29x10^{12}$ to $M=1.05x10^{2}$, $IQR=2.09x10^{2}$ CFU/mL, p=0.002) after the application of 10% povidone-iodine;
- no *S. aureus* was detected after the application of 0.1% octenidine dihydrochloride (p=0.002).

The application of the antiseptic solutions on the biofilms, produced by *S. aureus* ATCC 25923 strain, resulted in:

- no significant change of the number of *S. aureus* CFUs in the biofilms after the application of 0.02% chlorhexidine digluconate (decreased from M=2.00x10^{14}, IQR=4.99x10^{14} to M=4.50x10^{13}, IQR 2.92x10^{14} CFU/mL, p=0.82);
- no *S. aureus* was detected after the application of 0.1% octenidine dihydrochloride (p=0.002); after the application of 10% povidone-iodine no *S. aureus* was detected in five out of six biofilms, 7 CFU/mL were detected in one out of six biofilms (p=0.002).

The results of this experiment are represented in Figure 8.

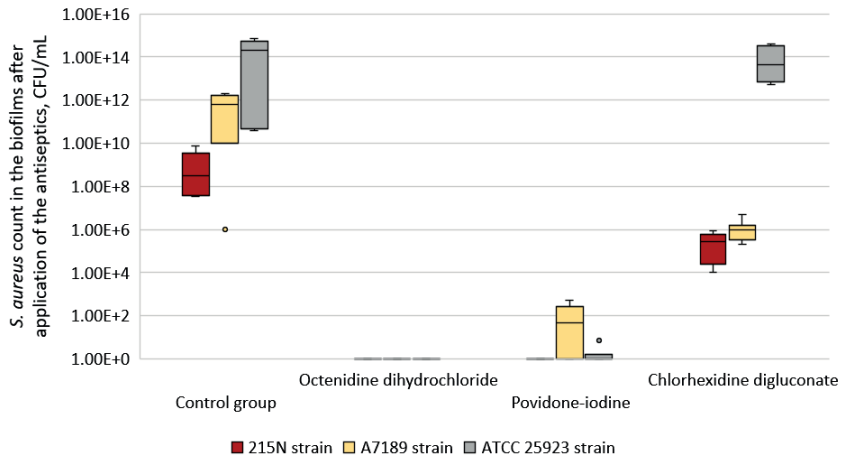


FIGURE 8. The antimicrobial efficacy of different antiseptic solutions on *S. aureus* biofilms, grown in a MLM. After the application of octenidine dihydrochloride the bacteria of all three strains of *S. aureus* were eradicated (p=0.002). After the application of povidone-iodine, no bacteria of 215N strain were found (p=0.002); the number of bacteria of A7189 and ATCC 25923 strains significantly decreased (p=0.002). After the application of chlorhexidine digluconate the number of bacteria of 215N and A7189 strains significantly decreased (p=0.002 and p=0.02, respectively), but did not change for ATCC 25923 strain (p=0.82).

3.1.4 PVGI on scanning electron microscopy

Due to the components of the medium, which resemble a wound *in vivo*, a modified Lubbock's model was used to design an innovative model of infected vascular graft *in vitro*. After the time of incubation, the patches of a non-impregnated

woven vascular prosthesis (Figure 9 A) were successfully colonized by *S. aureus* biofilms (Figure 9 B, C and D) and treated with antiseptic solutions.

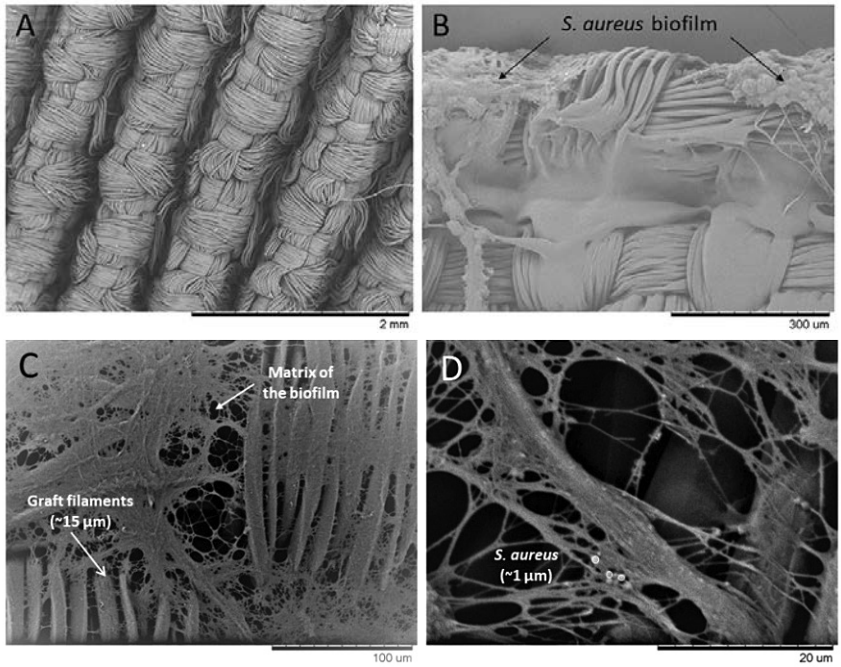


FIGURE 9. Images of a non-impregnated woven vascular graft (Dacron) by SEM. A – image of a vascular graft at 50x; B – image of a vascular graft with *S. aureus* biofilm at 150x; C – matrix of the biofilm at 800x; D – cocci at 5000x.

3.1.5 Evaluation of the antimicrobial efficacy of different antiseptic solutions on *S. aureus* biofilms in PVGI model *in vitro*

The CFUs of *S. aureus* in the biofilms on the vascular grafts were enumerated for the control groups of all the strains (Table 5) and then compared to the number of CFUs left in the biofilms after the application of the antiseptics.

TABLE 5. *S. aureus* CFUs in the biofilms, produced in a MLM on the vascular grafts

S. aureus strain	*S. aureus* in the biofilms on the vascular grafts, CFU/mL, x10[14]			
	Minimum	Maximum	Median	IQR*
215N	1.20	8.00	3.035	4.1
A7189	1.00	9.00	4.5	6.5
ATCC 25923	3.00	8.00	7.5	1.0

*IQR – interquartile range

After the application of antiseptic solutions on the grafts with *S. aureus* biofilms, the results were as follows:

- in comparison with the control group, all three antiseptic solutions demonstrated a similar antimicrobial efficacy in a newly designed PVGI model in vitro for all three S. aureus strains (p=0.002);
- *S. aureus* counts in the biofilms on the vascular grafts decreased by seven orders of magnitude after application of all three antiseptic solutions;
- the efficacy of 0.1% octenidine dihydrochloride, 10% povidone-iodine and 0.02% chlorhexidine digluconate was similar for *S. aureus* strains A7189 and ATCC 25923 (p>0.05);
- 0.1% octenidine dihydrochloride was more effective than 10% povidone-iodine (p=0.02) and 0.02% chlorhexidine dihydrochloride (p=0.04) for *S. aureus* 215N strain. The efficacy of povidone-iodine and chlorhexidine digluconate was similar (p=0.94);
- octenidine dihydrochloride was more effective for *S. aureus* 215N strain than for ATCC 25923 strain (Mann Whitney U test, p=0.02). The results of the experiment are represented in Figure 10.

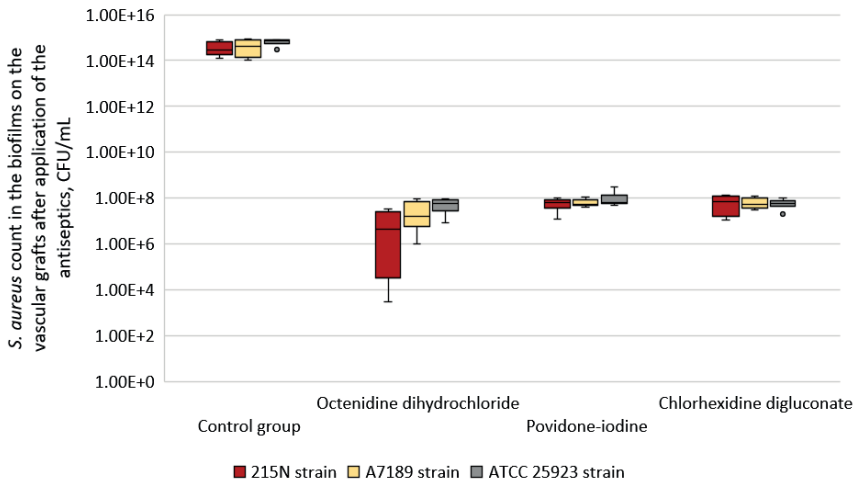


FIGURE 10. The antimicrobial effect of different antiseptic solutions on *S. aureus* biofilms, grown in a MLM on the vascular grafts. In comparison with control group, all antiseptic solutions demonstrated similar antimicrobial effect in a newly designed PVGI model *in vitro* (p=0.002).

3.2 The results of *in vivo* experiments

3.2.1 The final sample size

One rat, which was randomly assigned to the control group, did not recover from anaesthesia (possible anaesthetic overdose). Two rats, which were randomly assigned to povidone-iodine group and one rat, which was assigned to chlorhexidine's group did not reach adequate level of suppuration (based on the results of microbiological tests on day six of the experiment before starting the treatment) and were excluded from the study. One rat, which was assigned to chlorhexidine's group, died on day eight of the experiment. The loss of appetite and body mass (from 355 g to 188 g), reduced physical activity and pus around the eye area distinguished this rat from the other rodents.

The final sample size in the first series of experiments was 43 rats:

- 12 rats in octenidine dihydrochloride's group;
- 10 rats in povidone-iodine's group;
- 10 rats in chlorhexadine digluconate's group;
- 11 rats in control group.

In the second series of *in vivo* experiments the final sample size was 10 rats.

3.2.2 Macroscopic findings in the first series of *in vivo* experiments

During the time of the first series of experiments the fluctuations of mean body temperature for rats remained in the normal range (35.8-37.5°C) and did not differ significantly between the study groups. The mean body mass for the rats on day one of the experiment was 317.6±48.1 g. The experimental groups were homogenous according to the body mass (dispersion analysis, p=0.97). The changes in rats' body mass during the course of the study are represented in Table 6.

TABLE 6. The changes in rats' body mass, related to the antiseptic used for the treatment of PVGI

Antiseptic solution	Octenidine dihydrochloride	Povidone-iodine	Chlorhexidine digluconate	Control group
Mean body mass at day 1, g	318.5±40.1	323.1±58.2	312.5±48.6	316.2±52.0
Mean body mass at day 10, g	299.3±44.1	306.2±60.6	276.4±54.3	313.9±50.0
Mean change of body mass, g	-19.25±59.6	-16.9±83.9	-36.1±72.9	-2.3±72.1
Effect's size, Cohen's d	-0.32	-0.2	-0.49	-0.03
Mean change of body mass, %	-5.9	-5.6	-11.5	-0.1
p value	0.05	0.004	0.04	0.57

On day six of the experiments all rodents presented with a hardened swelling at the incision site (Figure 11). There were no suture leaks or eritema. Some rats had developed crusts at the incision site and cutaneous fistulas with purulent discharge. Once wound irrigations with antiseptic solutions were started, all rats developed crusts at the site of the abscess and cutaneous fistulas.

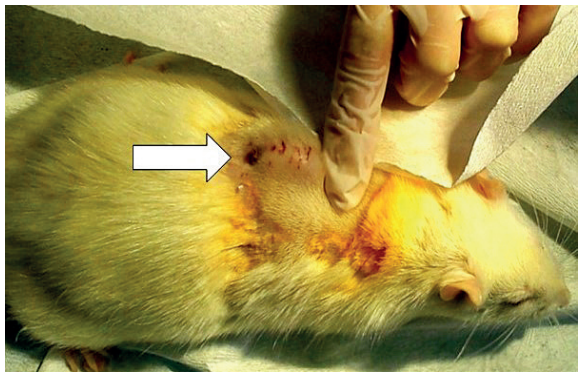


FIGURE 11. A hardened swelling and cutaneous fistula with purulent discharge at the site of a vascular graft implantation on day six of the experiment (before starting the treatment).

3.2.3 Microbiological findings of the first series of *in vivo* experiments

The efficacy of the antiseptics in treatment of *S. aureus* infected wounds with implanted patches of vascular grafts for rats was evaluated by comparing the changes of *S. aureus* CFUs in the wound washouts before the beginning of treatment and after the course of treatment. In four days of treatment, the number of *S. aureus* CFU/mL in the wound washouts significantly decreased in all treatment groups, but insignificantly increased in control group (Table 7, Figure 12). The best results were achieved by treatment with octenidine dihydrochloride – the count of bacteria in the wound washouts decreased by 99.98% ($p<0.001$).

TABLE 7. The results of microbiological tests from rats' wound washouts

Antiseptic solution	CFU/mL before the treatment, M (IQR)*	CFU/mL after the treatment, M (IQR)*	Change in CFU, %	p value
Control group	1.13×10^7 (1.63×10^7)	9.70×10^6 (1.61×10^7)	↑19.72	0.77
Octenidine dihydrochloride	1.77×10^7 (1.17×10^7)	1.14×10^3 (2.91×10^3)	↓99.98	<0.001
Povidone-iodine	1.59×10^7 (1.41×10^7)	9.39×10^4 (2.97×10^5)	↓90.73	0.002
Chlorhexidine digluconate	1.22×10^7 (2.19×10^7)	2.16×10^6 (3.17×10^6)	↓65.97	0.004

* M – median; IQR – interquartile range

In comparison with the control group, only the treatment with octenidine dihydrochloride significantly reduced *S. aureus* CFU counts in the wound washouts (p=0.02). The efficacy of povidone-iodine came close to the limit of statistical significance (p=0.06). The efficacy of chlorhexidine digluconate did not differ from sterile saline in the control group (p=0.13).

Total eradication of *S. aureus* from the wound washouts was achieved for two rats, treated with octenidine dihydrochloride and one rat, treated with povidone-iodine. High counts of *S. aureus* (variating from 1.08×10^2 to 8.05×10^7 CFU/mL) were detected on the patches of vascular grafts removed from the wounds. No correlation was found between *S. aureus* cell counts found on the grafts and in the wound washouts for the same rat.

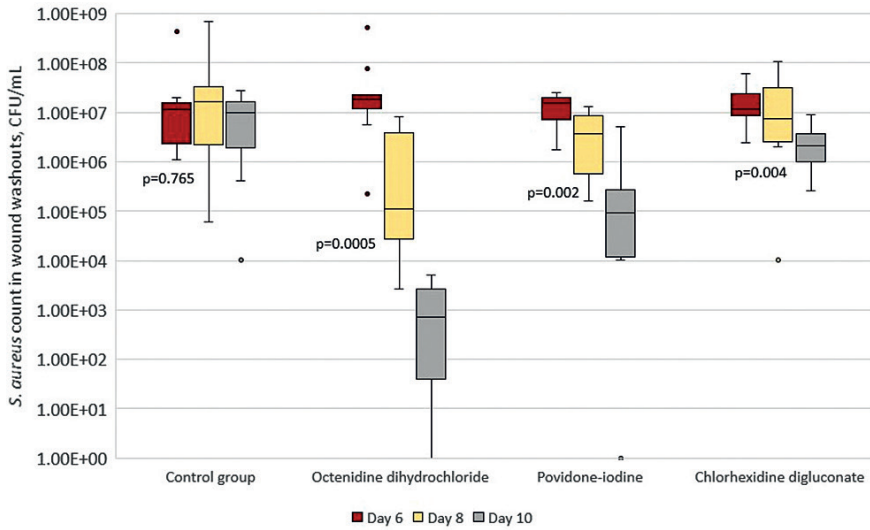


FIGURE 12. The changes in *S. aureus* counts in the wound washouts during the time of treatment. In four-day treatment course the count of *S. aureus* CFUs decreased for rats, treated with octenidine (n=12, p<0.001), povidone-iodine (n=10, p=0.002) and chlorhexidine (n=10, p=0.004). A nonsignificant increase of *S. aureus* CFUs was registered in the control group (n=11, p=0.77).

3.2.4 The second series of *in vivo* experiments

An additional series of experiments was run with a group of 10 rats. All of them were treated with octenidine dihydrochloride. All rodents survived the surgery. No significant fluctuations in body temperature were registered.

On day six of the experiment a hardened swelling was observed at the site of the incision for all rodents. There was no eritema around the suture line. For most of the rats there was a crust at the incision site. Two rats presented with cutaneous fistulas. From day 10 of the experiment all rodents presented with crusts of varying sizes and purulent fistulas (Figure 13). At day 12 of the experiment a massive excretion of purulent discharge was noticed for all rodents through the fistulas.

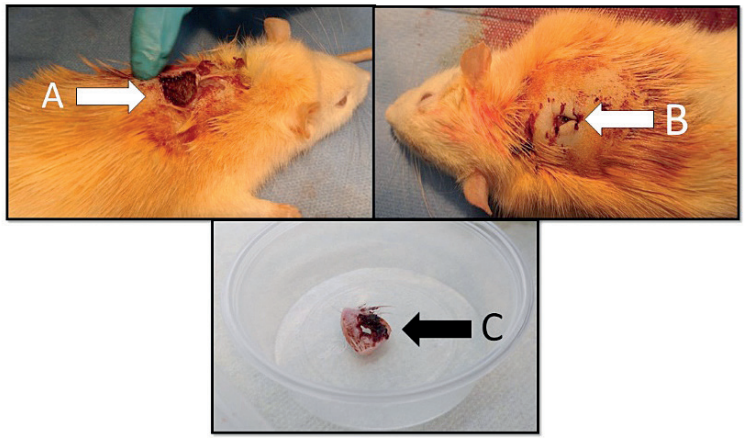


FIGURE 13. Macroscopic findings for a rat, treated with octenidine dihydrochloride (day 13 of the experiment): A – a crust at the incision site; B – a cutaneous fistula at the incision site; C – a cutaneous fistula, resected for histological and microbiological evaluation.

A significant reduction of the body mass by 9.27% was registered during the course of the experiment (paired samples T test, $p<0.001$). The mean body mass at day one of the experiment was 267.3±19.6 g, and at day 13 – 242.2±18.3 g.

Before starting wound irrigations with octenidine dihydrochloride the median of *S. aureus* CFU/mL in the wound washouts was $1.5x10^6$ (IQR=$6.17x10^6$ CFU/mL). After completing a seven-day treatment course, *S. aureus* was eradicated from the wound washouts for nine out of 10 rats. For one rat 15 CFU/mL were detected in the wound washouts (Figure 14). The results of the treatment showed high statistical significance (Wilcoxon signed-rank test, $p=0.002$).

At the end of the experiment (day 13) the patches of vascular graft were explanted for seven rats. For three other rats the patches of vascular graft had been expelled from the wounds through the cutaneous fistulas before the termination of the experiment, and thus were not microbiologically evaluated. For one rat, no *S. aureus* was found on the graft. For six other rats the counts of *S. aureus* on the graft varied from 20 CFU/mL to $2x10^7$ CFU/mL (M=$8.0x10^1$, IQR=$8.33x10^6$ CFU/mL).

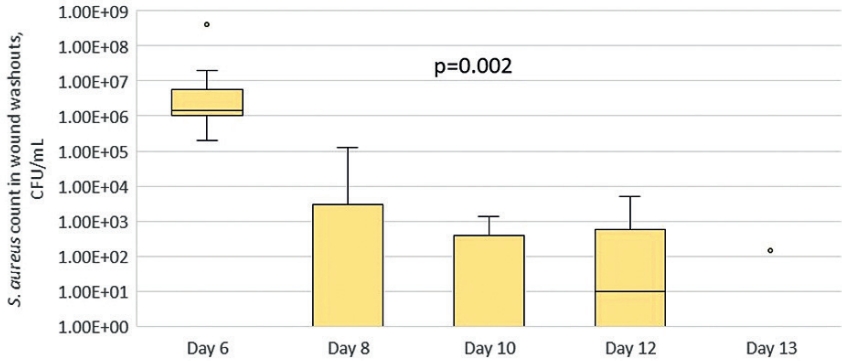


FIGURE 14. The results of the second series of *in vivo* experiments. A seven-day treatment course resulted in a significant decrease of *S. aureus* CFUs in the wound washouts (n=10, p=0.002). *S. aureus* was eradicated for nine out of 10 rats. For one rat 15 CFU/mL of *S. aureus* were detected in the wound washouts on day 13.

A digital analysis of Gram-stained cytological smears (Figure 15) revealed that higher number of inflammatory cells in the wound washouts on day 13 significantly correlated with shorter *S. aureus* eradication time from the wound washouts (number of days, taken for *S. aureus* CFU/mL in the wound washouts to decrease to zero) (Spearman's correlation, r=-0.74, p=0.01, Figure 16).

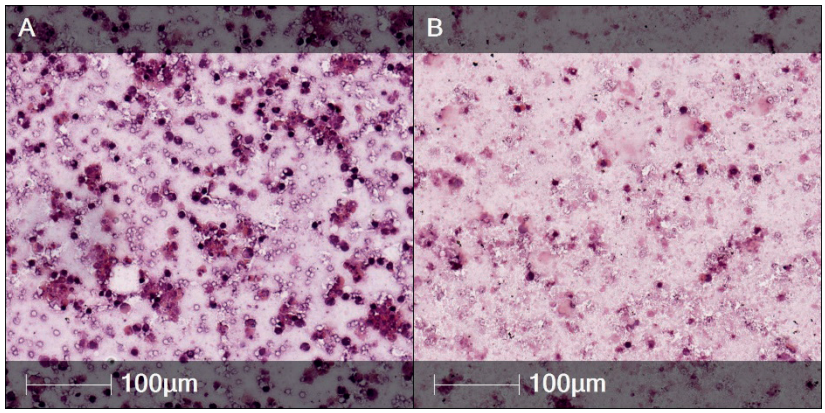


FIGURE 15. Gram-stained cytological smears of the wound washouts. A – dense infiltration of inflammatory cells; B – moderate density of inflammatory cells.

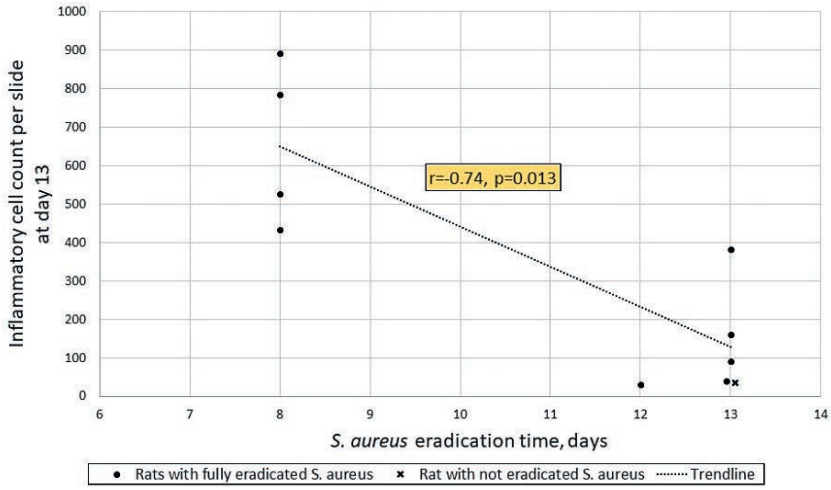


FIGURE 16. Correlation between high inflammatory cell count in the wound smears and faster *S. aureus* eradication time from the wound washouts. Spearman's correlation, r=-0.74, p=0.01.

No dissemination of *S. aureus* into the bloodstream was detected. *S. aureus* was detected in nine out of 10 samples of the cutaneous fistulas (M=1.2x10^3, IQR=2.85x10^4 CFU/mL/g).

The area of necrosis in the samples of cutaneous fistulas varied from 1.87% to 49.07% (M=17.33%, IQR=17.14%) without significant relation to the rats' level of suppuration or response to treatment. Two samples of fistulas presented with no inflammation, two – with minor polymorphonuclear (PMN) infiltration, four – with mild PMN infiltration and two – with dense PMN infiltration. The grade of

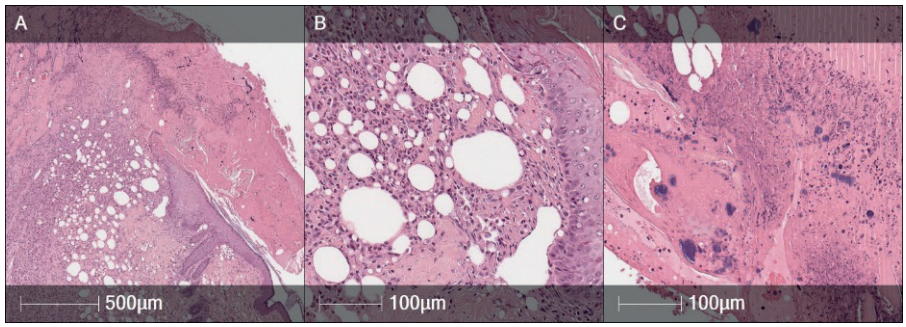


FIGURE 17. Images of hematoxylin and eosin-stained cutaneous fistulas. A – epidermis with a crust; B – PMN infiltration in the derma; C – clusters of bacteria in the crust.

inflammation did not correlate with the area of necrosis in the fistula tissues (Spearman's correlation, r=-0.07, p=0.84). Histological images of the cutaneous fistulas are shown in Figure 17.

The area of tissue necrosis in soft tissue specimens varied from 0 to 22.48% (M=1.5%, IQR=11.59%). According to the type and severity of the inflammation four rats had developed xanthogranulomatous inflammation, three rats had mild, one had minor and two had no PMN infiltration (Table 8, Figure 18).

TABLE 8. Histological evaluation of the soft tissue samples for rats treated with octenidine dihydrochloride

Rat number	The area of necrosis in soft tissue specimen, %	Type of inflammation in soft tissue specimen
1	1.90	Minor PMN
2	0	Xanthogranulomatous
3	11.58	Xanthogranulomatous
4	0	Xanthogranulomatous
5	1.17	None
6	20.77	Minor PMN
7	0	Xanthogranulomatous
8	0	None
9	22.48	Mild PMN
10	10.04	Minor PMN

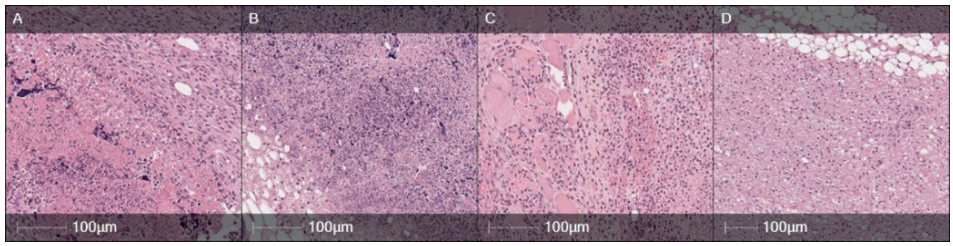


FIGURE 18. Images of hematoxylin and eosin-stained soft tissue specimens. A – soft tissue necrosis (left), PMN infiltration, proliferation of fibroblasts (right); B – soft tissue necrosis; C – PMN infiltration in the fibers of skeletal muscle (left), red blood cells and PMN infiltration (right); D – infiltration of macrophages (xanthogranulomatous inflammation).

Discussion

Despite all efforts to treat PVGI, it still remains a disastrous complication in vascular surgery. This research was designed to investigate one of PVGI's conservative treatment options – wound irrigation with antiseptic solutions, as its role in the treatment of PVGI is not yet defined. The efficacy of three commonly used antiseptic solutions was tested in an innovative *in vitro* setting, mimicking PVGI and in an *in vivo* setting for rats with implanted patches of vascular graft. The biofilm-producing strains of *S. aureus* were chosen to induce the infection, as bacterial biofilms are recognized as one of the main reasons of ineffective antimicrobial treatment and infection recurrence. In clinical cases, bacterial biofilms are frequently found to be situated on implants, such as vascular grafts [24, 25]. The formation of a biofilm on a vascular prosthesis is one of the main hassles in the treatment of PVGI, because bacteria in a biofilm are more resistant to the host's immune system and antimicrobials.

In this research Lubbock's chronic wound pathogenic biofilm model [28] was successfully modified and used to design a PVGI model *in vitro.* Although all of the tested antiseptics were able to suppress the multiplying of bacteria on the surface of the vascular graft, their effectiveness was much lower than in the experiments, performed in the same media on *S. aureus* biofilms without vascular prostheses. The results of this study confirm the hypothesis that the presence of the vascular graft in a wound and in an *in vitro* setting as well increases the surface area for bacterial adhesion and worsens the susceptibility of the antiseptic.

Further research on antiseptics' ability to fight biofilm-producing *S. aureus* on a vascular graft was continued in an *in vivo* setting on Wistar rats. We succeded in producing a flourishing infection of a surgical wound, which was confirmed by clinical findings (formation of subcutaneous abscesses and cutaneous fistulas with purulent discharge), microbiological and cytological tests (*S. aureus* and inflammatory cells in the wound washouts) and histological analysis of cutaneous and perigraft tissues (infiltration of inflammatory cells – PMN leukocytes as signs of acute inflammation and/or foamy macrophages as signs of xanthogranulomatous inflammation, clusters of bacteria in the crusts and tissue necrosis).

Despite an outstanding effect of octenidine dihydrochloride which managed to eradicate 99-100% of *S. aureus* CFUs from the wound washouts in a seven-day treatment course, eradication of the infection from the graft itself was only detected for one rat out of 10. Using the same methodology and octenidine dihydrochloride as an irrigation solution, we have managed to sterilize one more vascular graft for a rat in the previous studies [29].

The duration of our study was limited due to rats' tendency to heal on their own. Nevertheless, high counts of inflammatory cells for rats in the control group

remained in the wound washouts until the end of the experiment, so we are certain that rats did not start to recover during the course of the experiment.

It is important to note that experimental models *in vitro* and animal models as well cannot clearly represent a clinical situation of PVGI, so drawing conclusions about antiseptics' efficacy in treatment of human PVGI based solely on our findings would be inaccurate. In order to establish this as an effective treatment method of PVGI clinical studies are necessary.

Conclusions

1. A significant biofilm-disrupting activity on *S. aureus* biofilms *in vitro* was observed exclusively after the application of 0.02% chlorhexidine digluconate solution.
2. In a simulated wound environment, containing blood components, the strongest antimicrobial efficacy on *S. aureus* biofilms was observed by 0.1% octenidine dihydrochloride solution. The efficacy of 10% povidone-iodine was almost as great as octenidine's, but the effect of chlorhexidine digluconate was poor.
3. Lubbock's chronic wound pathogenic biofilm model was modified and adapted to design a novel PVGI model *in vitro* with woven Dacron graft and biofilm-producing strains of *S. aureus*. The formation of the biofilms on the vascular grafts was confirmed by SEM. All three tested antiseptics presented with similar antimicrobial efficacy in a newly developed model of PVGI.
4. A new *in vivo* model of PVGI was designed for Wistar rats, using a biofilm-producing *S. aureus* 215N strain and a woven Dacron graft. 0.1% octenidine dihydrochloride solution, applied by daily irrigation, proved to be the most effective antiseptic solution, treating suppurative surgical wounds with *S. aureus* infected patches of Dacron vascular grafts in rats.
5. A seven-day treatment course with 0.1% octenidine dihydrochloride was sufficient to eradicate *S. aureus* from the wound washouts for nine out of 10 rats and one vascular graft. Cytological and histological analysis of the perigraft tissues confirmed active inflammation in the perigraft tissues.

Practical recommendations

1. For researchers studying the effectiveness of local antiseptics and antibiotics on bacterial biofilms we recommend using our newly-designed experimental models *in vitro* and *in vivo*.

2. For physicians treating *S. aureus* infected wounds with a fair amount of organic matter (leucocytes, detrite) we recommend applying wound irrigations with 0.1% octenidine dihydrochloride or 10% povidone-iodine solutions.
3. For vascular surgeons treating a PVGI, caused by *S. aureus,* we recommend applying wound irrigations with 0.1% octenidine dihydrochloride solution. Once the wound is sterilised (based on microbiological tests from the wound washouts), infected vascular graft may be replaced with another vascular substitute.

Future perspectives

Based on the results and repeatability of our experiments, our newly-designed *in vitro* and *in vivo* models for studying antiseptics' efficacy on the treatment of PVGI proved to be successful. Since curing PVGI remains a challenge, we believe that it would be useful to continue our research further.

Studying the efficacy of antiseptic solutions on *S. aureus* biofilms revealed strong biofilm-disrupting activity of chlorhexidine digluconate and outstanding antimicrobial efficacy of octenidine dihydrochloride and povidone-iodine. Given the possibility that antiseptics could act synergically, it would be useful to try using a combination of these antiseptics on biofilm-covered vascular grafts *in vitro*.

Since a single application of antiseptics suppressed the multiplying of bacteria, but was not sufficient to eradicate *S. aureus* biofilms from the surface of the vascular grafts, we believe that it would be useful to carry out a new series of *in vitro* experiments, mimicking PVGI, and apply antiseptics in a repetitive mannier to achieve bacterial eradication from the surface of the vascular grafts.

During *in vivo* experiments, outstanding results were achieved using octenidine dihydrochloride solution for irrigation of infected surgical wounds with patches of vascular graft. A seven-day treatment course resulted in nine out of 10 sterile wound cultures from the wound washouts. Despite this, only one vascular graft was found sterile. Therefore, we believe that repeating the experiment and increasing the sample size wound be beneficial. First, it would reveal if sterilising one vascular graft was a single case success, or the number of sterilised grafts increases with the sample size. Second, after obtaining negative cultures from the wound washouts it would be useful to resect all perigraft tissues and to perform their microbiological analysis. After making sure there were no residing bacteria, it would be possible to implant a new sterile patch of a vascular graft (bare, silver-coated or rifampicin-impregnated) *in situ* and observe, if it stays uninfected. Once wound irrigation with octenidine dihydrochloride is acknowledged to be able to sterilise the wound with a vascular graft and it is safe to implant a new vascular substitute *in situ*, we suggest starting a clinical study of PVGI and using octenidine dihydrochloride for wound irrigations.

In order to evaluate octenidine dihydrochloride's cytotoxic effect for graft surrounding tissues, it would be useful to form a control group of rats which wound be treated with sterile saline. After the treatment course it would be possible to compare histological analysis of the tissues treated with octenidine and sterile saline and distinguish the tissue damage caused by the infection from the cytotoxic effect of an antiseptic. It would allow us to evaluate the antiseptic and define its role in the treatment of PVGI.

SANTRAUKA

Įvadas

Kraujagyslės protezo infekcija (KPI) yra gana reta, tačiau viena iš sunkiausiai gydomų komplikacijų kraujagyslių chirurgijoje. Ją patiria nuo 0,5 proc. iki 9,5 proc. visų ligonių, kuriems implantuojami sintetiniai kraujagyslių pakaitalai [1-8]. Negydant ši infekcija dažnai komplikuojasi gyvybei pavojingu kraujavimu ar sepsiu [9]. Tačiau net ir gydant neišvengiama pačių blogiausių išeičių – galūnių amputavimo (5-70 proc. visų atvejų) bei mirties (10-75 proc.) [4, 9-14].

Dažniausiai KPI sukelia fakultatyviniai anaerobai: stafilokokai, žarnyno lazdelės, enterokokai, salmonelės ir kt. Stafilokokai yra ypač dažni – jie sudaro nuo 24 iki 50 proc. visų KPI tiek ankstyvuoju, tiek vėlyvuoju pooperaciniu laikotarpiu [11, 15-18]. Kasdienėje kraujagyslių chirurgijos praktikoje ypač aktualus yra *Staphylococcus aureus* sukeltos KPI gydymas, nes ši bakterija ne tik dažniausiai sukelia KPI ankstyvuoju pooperaciniu laikotarpiu, bet ir yra linkusi formuoti bioplėveles, kurios dar labiau apsunkina gydymo eigą [2, 15-18].

Gydymo būdo, efektyvaus visais KPI atvejais, nėra. Įprastai rekomenduojama agresyvi chirurginė taktika: pašalinti infekcijos apimtus audinius kartu su pačiu kraujagyslės protezu, suformuoti ekstraanatominį šuntą, apeinant infekuotą vietą, skirti sisteminę antibiotikų terapiją didelėmis dozėmis [4]. Tačiau ši gydymo taktika ne visada įmanoma, ypač kai infekcijos apimtas protezas yra įsiūtas į aortą ar kitas gyvybiškai svarbias arterijas, kai ligonis išsekęs nuo gretutinių ligų ir neatlaikytų didelės apimties operacijos arba kai infekcijos sukėlėjas yra atsparus daugeliui antimikrobinių medžiagų. Dėl šių priežasčių tam tikrais atvejais bandoma taikyti konservatyvius gydymo metodus, leidžiančius išsaugoti protezą: ilgalaikę sisteminę antibiotikų terapiją, neigiamo slėgio žaizdų terapiją ir vietinį žaizdos drenažą nuolatiniais arba cikliškais plovimais [1, 3, 9, 20]. Žaizdos gali būti plaunamos steriliu 0,9 proc. natrio chlorido tirpalu [21], antibiotikų arba antiseptikų tirpalais.

Nors antibiotikai yra neatsiejama KPI gydymo dalis, taikant sisteminę antibiotikų terapiją susiduriama su keliomis problemomis: pirma, dauguma antibiotikų sunkiai prasiskverbia pro bakterijų suformuotas bioplėveles, antra – vis dažniau pasitaiko antibiotikams atsparių bakterijų padermių. Šiandien pranašesnės tampa kitos antimikrobinės medžiagos – antiseptikai, nes bakterijų atsparumas jiems išlieka minimalus [23].

Darbo tikslas

Įvertinti įprastai klinikinėje praktikoje naudojamų antiseptinių tirpalų efektyvumą, veikiant *Staphylococcus aureus* užkrėstą austą kraujagyslės protezą *in vitro* ir *in vivo*.

Darbo uždaviniai

1. Palyginti skirtingų *S. aureus* padermių bioplėvelių struktūrą ardantį 0,1 proc. oktenidino dihidrochlorido, 10 proc. povidono-jodo ir 0,02 proc. chlorheksidino digliukonato poveikį *in vitro.*
2. Palyginti 0,1 proc. oktenidino dihidrochlorido, 10 proc. povidono-jodo ir 0,02 proc. chlorheksidino digliukonato antimikrobinį veiksmingumą skirtingų *S. aureus* padermių suformuotoms bioplėvelėms *in vitro* sąlygomis imituojant žaizdos aplinką.
3. Sukurti eksperimentinį infekuoto austo kraujagyslės protezo modelį *in vitro* ir palyginti 0,1 proc. oktenidino dihidrochlorido, 10 proc. povidono-jodo ir 0,02 proc. chlorheksidino digliukonato tirpalų antimikrobinį veiksmingumą skirtingų *S. aureus* padermių bioplėvelėms ant kraujagyslės protezo lopinėlių.
4. Sukurti eksperimentinį infekuoto austo kraujagyslės protezo modelį *in vivo* Wistar klono žiurkėms ir palyginti 0,1 proc. oktenidino dihidrochlorido, 10 proc. povidono-jodo ir 0,02 proc. chlorheksidino digliukonato tirpalų veiksmingumą, gydant *S. aureus* užkrėstas žiurkių žaizdas su kraujagyslės protezo lopinėliais.
5. Įvertinti žiurkių gydymo trukmę, reikalingą pašalinti *S. aureus* iš kraujagyslės protezo aplinkos, atlikti citologinę bei histologinę žaizdos audinių analizę.

Ginamieji teiginiai

1. Antiseptiniai tirpalai *in vitro* sąlygomis geba paveikti *S. aureus* bioplėveles.
2. Žaizdų plovimas antiseptikais yra efektyvus metodas, gydant *S. aureus* sukeltą žaizdos infekciją su kraujagyslės protezu.

Tyrimo naujumas

Sparčiai besivystant bakterijų atsparumui antimikrobinėms medžiagoms, ieškoma alternatyvių būdų, kaip kovoti su infekcijomis. Konservatyvus KPI gydymo metodas – vietinis žaizdos plovimas antiseptiniais tirpalais – vis labiau diegiamas į praktiką, tačiau visuotinai neįsitvirtinęs kovos su KPI būdas. Išnagrinėjus šia tema paskelbtas publikacijas pastebėta, kad trūksta reprezentatyvių, žaizdai artimų *in vitro* modelių, kurie padėtų įvertinti antiseptikų poveikį bioplėveles formuojančiam *S. aureus* ant kraujagyslės protezų. Taip pat nerasta paskelbtų tyrimų, kur antiseptiniai tirpalai *in vivo* sąlygomis būtų panaudojami KPI gydymui, o ne profilaktikai.

S. aureus bioplėveles ardančio bei antimikrobinio antiseptikų poveikio vertinimui sukurtas naujas *in vitro* modelis, artimas žaizdai ir KPI. Tam, kad būtų įvertintas dar plačiai klinikinėje praktikoje netaikomas konservatyvus KPI gydymo metodas – žaizdų plovimas antiseptiniais tirpalais – sukurtas naujas eksperimentinis modelis, panaudojant Wistar klono žiurkes.

Darbo metodika

In vitro eksperimentai, tyrinėjantys antiseptikų efektyvumą veikiant *S. aureus* bioplėveles, atlikti VU MF Biomedicinos mokslų instituto Fiziologijos, biochemijos, mikrobiologijos ir laboratorinės medicinos katedros Mikrobiologijos skyriuje ir Lietuvos mokinių neformaliojo švietimo centro Jaunojo tyrėjo laboratorijoje 2017 m. gegužės – 2018 m. spalio mėn. Eksperimentams atrinktos trys gausiai bioplėveles formuojančios *S. aureus* padermės: 215N, A7189 ir ATCC 25923. Bioplėvelėms susiformuoti ir antiseptikų veiksmingumui testuoti sukurtos skirtingos aplinkos sąlygos: mitybinė terpė, žaizdos aplinką imituojanti terpė su kraujo komponentais ir žaizdos aplinką imituojanti terpė su kraujo komponentais bei austo kraujagyslės protezo lopinėliais. Bioplėvelės veiktos 0,1 proc. oktenidino dihidrochlorido, 10 proc. povidono-jodo ir 0,02 proc. chlorheksidino digliukonato tirpalais. Kontrolinei grupei naudotas fosfatinio buferio tirpalas (FBT). Eksperimentų metu vertintas antiseptikų poveikis bioplėvelių struktūrai ir bakterijų gyvybingumui.

In vivo eksperimentai, tyrinėjantys antiseptikų veiksmingumą gydant infekuotas chirurgines žiurkių žaizdas su kraujagyslės protezais, vykdyti Valstybinio mokslinių tyrimų instituto Inovatyvios medicinos centro Imunologijos departamente, VU Gyvybės mokslų centre ir VU MF Fiziologijos, biochemijos, mikrobiologijos ir laboratorinės medicinos katedros Mikrobiologijos skyriuje 2012 m. vasario mėn. – 2018 m. liepos mėn. Prieš pradedant eksperimentą, visi dalyvavę tyrėjai išklausė vieno semestro trukmės Laboratorinių gyvūnų mokslo kursus ir gavo leidimus dirbti su laboratoriniais gyvūnais. 2012 m. sausio 4 d. ir 2016 m. balandžio 25 d. gauti Valstybinės maisto ir veterinarijos tarnybos leidimai (Nr. 0222 ir G2-43) tyrimams atlikti.

Eksperimentui naudotos Wistar klono abiejų lyčių žiurkės. Visoms žiurkėms po oda implantuoti austo poliesterinio kraujagyslės protezo lopinėliai. Žaizdos infekuotos bioplėvelę formuojančia *S. aureus* paderme. Tyrimo metu įvertintas konservatyvaus KPI gydymo būdo – žaizdų plovimo antiseptiniais tirpalais veiksmingumas. Tyrimui pasirinkti 0,1 proc. oktenidino dihidrochlorido, 10 proc. povidono-jodo ir 0,02 proc. chlorheksidino digliukonato tirpalai. Kontrolinei grupei naudotas sterilus 0,9 proc. natrio chlorido tirpalas.

Identifikuojant *S. aureus* kolonijas remtasi latekso agliutinacijos, plazmakoaguliazės testais, šviesine mikroskopija. Kontroline *S. aureus* paderme pasirinkta ATCC 29213.

Statistinė duomenų analizė

Duomenys išanalizuoti naudojant parametrinius ir neparametrinius statistinius testus. Parametriniai testai naudoti tais atvejais, kai buvo galima taikyti normaliojo duomenų pasiskirstymo prielaidą – taikytas dviejų suporuotų imčių t-testas bei dviejų atskirų imčių t-testas. Kitais atvejais naudoti neparametriniai testai: Wilcoxon t-testas analizuojant dvi suporuotas imtis, Mann – Whitney U testas analizuojant dvi nesuporuotas imtis ir Kruskal – Wallis H testas analizuojant daugiau negu dvi imtis tarpusavyje. Duomenų koreliacija įvertinta Spearman's Rho testu. Skaičiavimai atlikti naudojantis SPSS 24.0 bei Microsoft Excel 2016 programomis. Pasirinkta statistinio reikšmingumo riba – $p < 0,05$.

Rezultatai

In vitro eksperimentų rezultatai

Remiantis kokybine ir kiekybine (spektrofotometrine) skirtingų *S. aureus* padermių bioplėvelių formavimo analize, gausiai bioplėveles formuojančios padermės (215N, A7189 ir ATCC 25923) buvo atrinktos tolimesniems *in vitro* tyrimams.

Ant dengiamųjų stikliukų mitybinėje terpėje susiformavus trijų skirtingų padermių *S. aureus* bioplėvelėms ir paveikus jas antiseptiniais tirpalais, reikšmingas bioplėvelę ardantis poveikis stebėtas tik po veikimo chlorheksidinu (Kruskal – Wallis H testas, 215N padermei $p = 0,02$, A7189 padermei $p < 0,001$, ATCC 25923 padermei $p = 0,02$).

Trijų skirtingų *S. aureus* padermių bioplėvelėms susiformavus modifikuotoje Lubbock'o terpėje ir paveikus jas antiseptikais nustatyta, kad po bioplėvelių ekspozicijos oktenidino dihidrochloridu, 215N, A7189 ir ATCC 25923 padermių *S. aureus* bakterijų nerasta ($p = 0,002$). Po ekspozicijos povidono-jodu 215N padermės bakterijų nerasta ($p = 0,002$), A7189 ir ATCC 25923 padermių bakterijų žymiai sumažėjo ($p = 0,002$). Po ekspozicijos chlorheksidino digliukonatu 215N ir A7189 padermių bakterijų žymiai sumažėjo (atitinkamai $p = 0,002$ ir $p = 0,02$), o ATCC 25923 padermei bakterijų skaičius nepakito ($p = 0,82$).

Modifikavus ir adaptavus Lubbock'o modelį sukurtas inovatyvus infekuoto austo kraujagyslės protezo modelis *in vitro*. Bioplėveles ant kraujagyslės protezų lopinėlių paveikus antiseptiniais tirpalais, lyginant su kontroline grupe, visoms *S. aureus* padermėms visi trys testuoti antiseptikai pademonstravo efektyvų antimikrobinį veikimą – visų naudotų antiseptikų atveju *S. aureus* bakterijų ant kraujagyslės protezo sumažėjo septyniomis eilėmis ($p = 0,002$).

In vivo eksperimentų rezultatai

Dėl anestetikų perdozavimo, nepasiekus adekvataus supūliavimo lygio ar nugaišus iki eksperimento pabaigos į galutinę analizę dalis žiurkių neįtrauktos. Galutinis imties dydis pirmojo etapo eksperimentuose – 43 žiurkės: 12 žiurkių oktenidino dihidrochlorido, 10 – povidono-jodo, 10 – chlorheksidino digliukonato ir 11 – kontrolinėje grupėje. Antrojo etapo eksperimentuose, kur testuotas vien oktenidino dihidrochlorido veikimas, galutinis imties dydis – 10 žiurkių.

Antiseptikų veiksmingumas, gydant *S. aureus* infekuotas žiurkių žaizdas su kraujagyslės protezo lopinėliais vertintas lyginant *S. aureus* kolonijas formuojančių vienetų (KFV) skaičiaus pokyčius žaizdų nuoplovose prieš pradedant gydymą ir baigus gydymo kursą. Per keturias gydymo antiseptikais dienas KFV/ml žaizdų nuoplovose reikšmingai sumažėjo visose antiseptikais gydytų žiurkių grupėse, tačiau kontrolinėje grupėje – nereikšmingai padidėjo. Geriausių rezultatų pasiekta gydant oktenidino dihidrochloridu – bakterijų skaičius žaizdų nuoplovose gydymo kurso metu sumažėjo 99,98 proc.

Lyginant su kontroline grupe, tik gydymas oktenidino dihidrochloridu statistiškai reikšmingai sumažino *S. aureus* KFV skaičių žaizdų nuoplovose ($p = 0,02$). Povidono-jodo veiksmingumas buvo arti statistinio reikšmingumo ribos ($p = 0,06$), o gydymo chlorheksidinu rezultatai nesiskyrė nuo kontrolinės grupės ($p = 0,13$).

Visiškas *S. aureus* išnaikinimas iš žaizdų nuoplovų buvo mikrobiologiškai patvirtintas dviem žiurkėms, gydytoms oktenidinu, ir vienai žiurkei, gydytai povidono-jodu. Dideli *S. aureus* KFV kiekiai (svyruojantys nuo 1,08 x 10^2 iki 8,05 x 10^7 KFV/ml) buvo aptikti po gydymo kurso pašalintuose kraujagyslės protezų lopinėliuose. Nerasta ryšio tarp *S. aureus* kiekio, rasto ant kraujagyslės protezų lopinėlių, ir tos pačios žiurkės *S. aureus* kiekio žaizdų nuoplovose.

Atlikus papildomą analogiškų eksperimentų seriją, gydymui naudojant vien oktenidino dihidrochloridą, po septynių gydymo dienų devynioms iš dešimties žiurkių *S. aureus* bakterijos buvo išnaikintos iš žaizdų nuoplovų. Vienai žiurkei po gydymo kurso rasta 15 KFV/ml Gydymo rezultatai – aukšto statistinio patikimumo (Wilcoxon t-testas, p = 0,002).

Pabaigus eksperimentą (13-ąją dieną), septynioms žiurkėms iš žaizdų pašalinti kraujagyslės protezo lopinėliai. Kitoms trims žiurkėms kraujagyslės protezo lopinėliai išpūliavo ir iškrito pro fistules dar nepasibaigus eksperimentui, todėl nebuvo galimybės juos mikrobiologiškai ištirti.

Vienos žiurkės kraujagyslės protezo lopinėlyje nebuvo rasta nei vienos *S. aureus* bakterijos. Kituose šešiuose protezo lopinėliuose KFV skaičius svyravo nuo 20 KFV/ml iki 2 x 10^7 KFV/ml (nurodoma mediana, M ir tarpkvartilinis plotis, TKP) (M = 8,0 x 10^1, TKP = 8,33 x 10^6 KFV/ml).

Skaitmeninė Gramo būdu dažytų neskiestų žaizdų nuoplovų citologinė analizė atskleidė, kad didesnis uždegiminių ląstelių skaičius 13-ąją eksperimento dieną reikšmingai koreliavo su trumpesniu *S. aureus* išnaikinimo laiku (dienų skaičiumi, užtrukusiu pašalinti *S. aureus* iš žaizdų nuoplovų iki 0) (Spearman'o koreliacija, r = -0,74, p = 0,01).

S. aureus diseminacijos kraujyje nerasta nei vienai žiurkei.

Bakterijų rasta devyniose iš dešimties odos-poodžio fistulių. *S. aureus* KFV skaičiaus mediana fistulėse buvo 1,2 x 10^3 (TKP = 2,85 x 10^4) KFV/ml/g.

Histologiškai nekrozės plotai odos-poodžio fistulių mėginiuose varijavo nuo 1,87 proc. iki 49,07 proc. (M = 17,33 proc., TKP = 17,14 proc.) be reikšmingo ryšio su žiurkės supūliavimo laipsniu ar atsaku į gydymą.

Histologiškai įvertinus fistulių bioptatus, dviejuose uždegimo nenustatyta (0 laipsnis), dviejuose rasta pavienė PMN infiltracija (I laipsnis), keturiuose – vidutinio tankio PMN infiltracija (II laipsnis), dviejuose – didelio tankio PMN infiltracija (III laipsnis). Uždegimo laipsnis nekoreliavo su nekrozės plotu fistulių audiniuose (Spearman'o koreliacija, r = -0,07, p = 0,84).

Nekrozės plotas minkštųjų audinių mėginiuose varijavo nuo 0 iki 22,48 proc. (M = 1,5 proc., TKP = 11,59 proc.). Pagal uždegimo tipą ir laipsnį, keturioms žiurkėms nustatytas ksantogranuliomatozinis uždegimas, trims – vidutinė, vienai – reta, dviem – jokios PMN infiltracijos.

Išvados

1. *In vitro* sąlygomis reikšmingas *S. aureus* bioplėvelių struktūrą ardantis poveikis nustatytas veikiant 0,02 proc. chlorheksidino digliukonato tirpalu.

2. *In vitro* sąlygomis imituojant pūlingą žaizdą su *S. aureus* bioplėvele stipriausias antimikrobinis efektas nustatytas veikiant 0,1 proc. oktenidino dihidrochlorido tirpalu. Labai stipriu antimikrobiniu aktyvumu pasižymėjo 10 proc. povidono-jodas, tuo tarpu 0,02 proc. chlorheksidino digliukonato efektyvumas buvo menkas.
3. Eksperimentinis infekuoto austo kraujagyslės protezo modelis *in vitro* sukurtas modifikavus ir adaptavus literatūroje aprašytą Lubbock'o lėtinės žaizdos patogeninių bioplėvelių modelį, panaudojant austo dakrono lopinėlius ir bioplėveles formuojančias *S. aureus* padermes. Bioplėvelių susiformavimas ant kraujagyslės protezo lopinėlių patvirtintas skenuojančiu elektroniniu mikroskopu. KPI modelyje *in vitro* visi naudoti antiseptikai pasižymėjo panašiu antimikrobiniu efektu.
4. Panaudojant bioplėvelę formuojančią 215N *S. aureus* padermę sukeltas chirurginių žaizdų su austo kraujagyslės protezo lopinėliais supūliavimas Wistar klono žiurkėms. Kasdieniais plovimais gydant pūlingas žiurkių žaizdas su austo kraujagyslės protezo lopinėliais efektyviausiu pripažintas 0,1 proc. oktenidino dihidrochlorido tirpalas.
5. Plaunant žaizdas 0,1 proc. oktenidino dihidrochloridu, septynių dienų gydymo kursas buvo pakankamas sterilizuoti devynių iš 10 žiurkių protezo aplinkos audinius ir vieną kraujagyslės protezą. Citologiniai žaizdų nuoplovų tepinėliai ir audinių preparatų histologinė analizė patvirtino aktyvų uždegiminį procesą protezo aplinkos audiniuose.

Praktinės rekomendacijos

1. Eksperimentatoriams: tiriant vietiškai veikiančių medžiagų (antiseptikų, antibiotikų) efektyvumą gydant infekcijas rekomenduojame pasinaudoti mūsų sukurtais eksperimen- tiniais *in vitro* ir *in vivo* modeliais.
2. Gydytojams: *S. aureus* infekuotas žaizdas, kuriose gausu baltyminių produktų (leukocitų, detrito), rekomenduojama plauti su 0,1 proc. oktenidino dihidrochlorido arba 10 proc. povidono-jodo tirpalais.
3. Kraujagyslių chirurgams: gydant *S. aureus* sukeltą žaizdos infekciją su implantuotu kraujagyslės protezu rekomenduojame žaizdą plauti su 0,1 proc. oktenidino dihidrochlorido tirpalu, o žaizdą sanavus (remtis mikrobiologinių pasėlių rezultatais iš žaizdų nuoplovų) – infekuotą protezą šalinti ir pakeisti nauju.

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1. Poster presentation at MAC 2016 in Munich, Germany (December 1-3, 2016) "Animal experiment: conservative treatment of prosthetic vascular graft infection caused by *Staphylococcus aureus*".

2. Poster presentation at "Evolutionary medicine: health and diseases in changing environment" Vilnius, Lithuania (June 5-8, 2018) "*In vitro* evaluation of antiseptics against *Staphylococcus aureus* biofilms formed in different surroundings".

3. Poster presentation at ESVS 2018 Valencia, Spain (September 25-28, 2018) "*In vitro* evaluation of several antiseptic solutions against *Staphylococcus aureus* biofilms: Antiseptics are able to fight *Staphylococcus aureus* biofilms on vascular grafts".

4. Oral presentation at a conference of Lietuvos žaizdų gydymo asociacija „Holistinis požiūris į opų ir žaizdų gydymą" (April 26, 2019) „Antiseptikų poveikio *Staphylococcus aureus* suformuotai bioplėvelei palyginimas".

Acknowledgements

The success of experimental studies relies on the work of a big team. I would like to express my sincere appreciation for everyone who contributed to this research by means of ideas, professional, organizational and technical support. My special gratitude goes to:

Prof. Habil. Dr. Vytautas Jonas Sirvydis and Prof. Habil. Dr. Vytautas Jonas Triponis, who shared their valuable experience in the field of experimental research, encouraged to renew this type of studies in VU MF and helped develop a minor student project into a doctoral dissertation. I thank you sincerely for your professional help, constructive critisism, infinite time for valuable discussions, support, intelligence and goodwill every step of the way;

Mindaugas Danilevičius – for generating the original idea of the study and contributing to designing the research;

the first team of researchers: **Andrej Podkopajev, Vilma Kuzminskaitė and Otilija Kutanovaitė** – for unstoppable determination to work in an unfamiliar field and an extraordinary team spirit;

the second team of researchers: **Inga Urtė Builytė, Linas Davainis, Alvita Ginčaitė and Gabrielė Valiūnaitė** – for willingness to continue *in vivo* experiments, contributing to increasing the sample size of the final study and winning Ist place in VU MF Students Scientific Society Conference Plenary Session; the third team of researchers: **Martynas Ridziauskas and Lukas Kevličius** – for thoroughness, commitment, gumption and endless patience working in a microbiology lab and winning Ist place in VU MF Students Scientific Society Conference Section of Infectious diseases and Microbiology;

Povilas Kavaliauskas and Rūta Prakapaitė – for innovative ideas and design of the methodology for *in vitro* experiments and qualified technical assistance in a microbiology lab;

VU MF Institute of Biomedical Science Department of Physiology, Biochemistry, Microbiology and Laboratory Medicine Division of Microbiology: **Assist. Dr. Agnė Kirkliauskienė** – for the insight teaming up a group of great researchers and professional, quick and constructive help in every stage of the research, **Assoc. Prof. Dr. Tomas Kačergius** – for valuable professional advices and providing with specific lab devices, **specialists Halina Gluchovienė and Olga Purakevič** – for their skilled technical assistance;

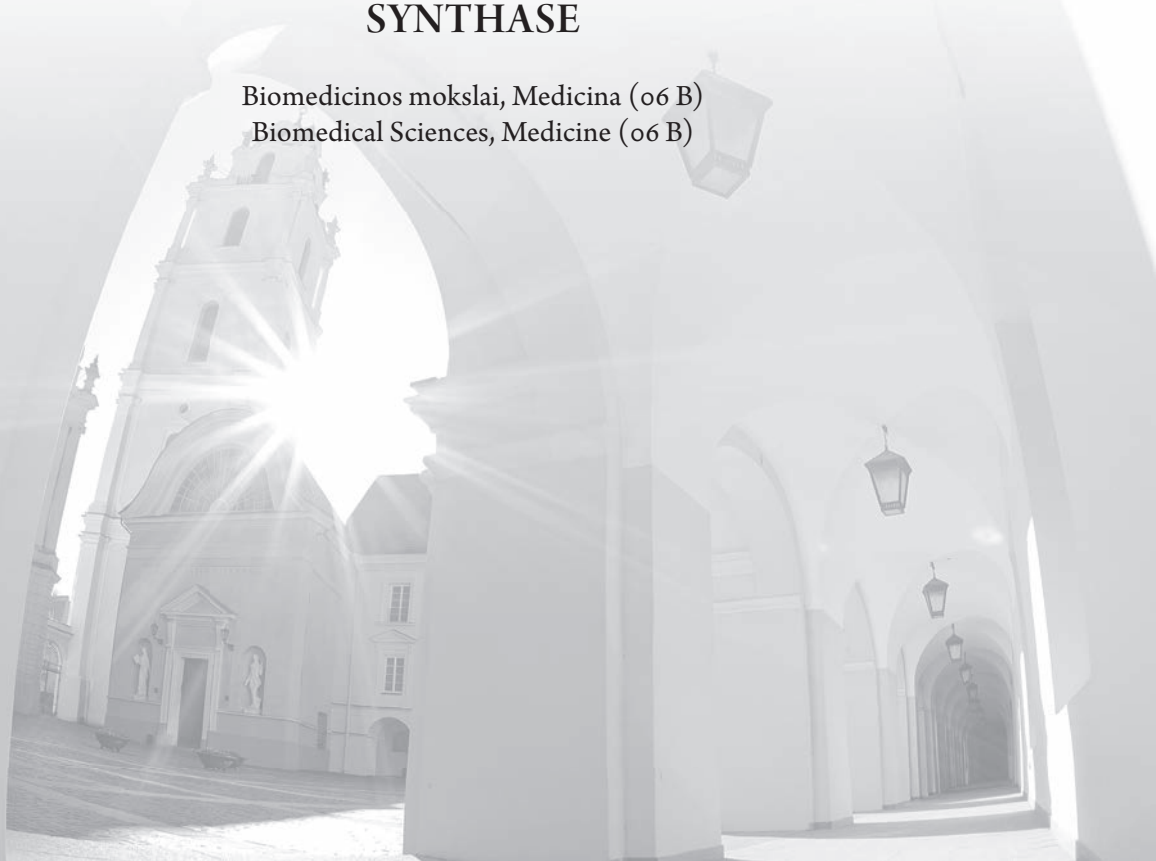
reviewers **Prof. Dr. Lilija Kalėdienė, Dr. Virginija Bukelskienė and Dr. Vilius Janušauskas** – for valuable comments and recommendations.

Birutė Zablockienė

GRIPO VIRUSINĖS PNEUMONIJOS PREVENCIJA NAUDOJANT ZANAMIVIRĄ IR PRIEŠPRASMINĮ OLIGONUKLEOTIDĄ PRIEŠ INDUKUOJAMĄ AZOTO OKSIDO SINTAZĘ

PREVENTION OF INFLUENZA VIRAL PNEUMONIA USING ZANAMIVIR AND ANTISENSE OLIGONUCLEOTIDE AGAINST INDUCIBLE NITRIC OXIDE SYNTHASE

Biomedicinos mokslai, Medicina (06 B)
Biomedical Sciences, Medicine (06 B)



Santrumpos

ANOVA – vienfaktorinė dispersijos analizė
AT-PGR – atvirkštinės transkripcijos - polimerazės grandininė reakcija
BAL – bronchoalveolinis lavažas
CPE – citopatogeninis efektas
DNR – deoksiribonukleorūgštis
FT – fiziologinis tirpalas
IFN-γ – interferonas gamma
iNOS – indukuojama azoto oksido sintazė
iRNR – informacinė ribonukleorūgštis
JAV – Jungtinės Amerikos Valstijos
$LKID_{50}$ – 50 proc. ląstelių kultūros infekcinė dozė
LSD testas – mažiausiai reikšmingo skirtumo (angl. *least significant difference*) testas
MDCK – Madin–Darby šuns inkstų ląstelės
NI – neuraminidazės inhibitoriai
NO – azoto oksidas
NO_2^- – nitritai
O.T. – optinis tankis
PO – priešprasminis oligonukleotidas
PSO – Pasaulio sveikatos organizacija
RB – rūgštinis baltymas
RNR – ribonukleino rūgštis
SPSS – socialinių mokslų statistinis paketas
SP – standartinė paklaida
TNF-α – tumoro nekrozės faktorius alfa

1. Įvadas

Gripo infekcija yra visame pasaulyje aktuali sveikatos problema dėl galimų komplikacijų ir mirties atvejų. Per kasmetines epidemijas pasaulyje 3–5 milijonai žmonių suserga sunkia gripo forma, iš jų 290 000–650 000 miršta (PSO, 2018).

Dauguma mirčių gripo metu būna susiję su viruso (Taubenberger, Morens, 2008; Kuiken, Taubenberger, 2008) ar bakterijų sukeltu plaučių pažeidimu (Morens ir kt., 2008; Klugman ir kt., 2009; Ruuskanen ir kt., 2011). Gripo viruso sukelto sunkaus plaučių uždegimo patogenezėje svarbus ne tik tiesioginis viruso citopatinis poveikis, bet ir pasigaminę uždegimo mediatoriai (de Jong ir kt., 2006; Kash ir kt., 2006).

Dėl gripo viruso sukelto imuninio atsako išskiriami uždegimo citokinai ir chemokinai („citokinų audra“), gausėja makrofagų ir neutrofilų plaučiuose (Van Reeth, 2000; Jayasekera ir kt., 2006; La Gruta ir kt., 2007; Kumar ir kt., 2014; Short ir kt., 2014). Citokinai, tokie kaip interferonas gama (IFN-γ) arba tumoro nekrozės faktorius alfa (TNF-α), skatina indukuojamos azoto oksido sintazės (iNOS) gamybą, dėl to gaminamas didelis azoto oksido (NO) kiekis (Short ir kt., 2014; Akaike ir kt., 1996; Suliman ir kt., 2001; Bosca ir kt., 2005). Yra žinoma, kad, sergant gripu, didesnė laisvųjų radikalų, pvz., NO ir reaktyviojo azoto radikalų gamyba sukelia plaučių pažeidimą (La Gruta ir kt., 2007; Short ir kt., 2014; Zaki ir kt., 2005).

Šiuo metu gripui gydyti skiriami neuraminidazės inhibitoriai (NI) slopina viruso replikaciją ir plitimą, taip padeda išvengti sunkių ligos formų ir mirties, ypač

anksti pradėjus gydymą. Tačiau net ir ankstyvas gydymas neapsaugo nuo komplikacijų didelės rizikos ligonių ar užsikrėtusių paukščių gripo viruso potipiu A(H5N1) (Hayden, 2009; 2013). Daugėjant NI atsparių gripo virusų potipių, reikalingi nauji vaistai gripo prevencijai ir jam gydyti (Moscona, 2008; Barik, 2012; Han ir kt., 2016; Hussain ir kt., 2017).

Gyvūnų tyrimuose, kuriuose buvo slopinama iNOS funkcija ir NO gamyba, buvo mažesnis gripo sukeltas plaučių pažeidimas (Jayasekera ir kt., 2006; Akaike ir kt., 1996; 2003). NI, slopindami viruso plitimą, galėtų netiesiogiai mažinti ir iNOS raišką bei NO gamybą. NI slopino NO gamybą gripo virusu užkrėstuose ir IFN-γ aktyvintuose RAW 264,7 makrofaguose *in vitro* (Kačergius ir kt., 2006). Tačiau nėra žinoma, ar NI geba slopinti NO gamybą ir taip mažinti plaučių pažeidimą gripo infekcijos metu *in vivo*.

Priešprasminis iNOS oligonukleotidas, slopindamas iNOS geno raišką ir NO gamybą, galėtų būti nauja gripo gydymo galimybe. Šiame moksliniame darbe siekėme įvertinti, ar gripo A viruso sukeltą plaučių uždegimą pelėms slopina priešprasminis oligonukleotidas prieš iNOS iRNR bei jo derinys su zanamiviru.

2. Mokslinio darbo tikslas

Įvertinti, ar zanamiviras ir(ar) priešprasminis oligonukleotidas prieš iNOS slopina gripo sukeltą plaučių uždegimą.

3. Mokslinio darbo uždaviniai

1. Įvertinti gripo viruso poveikį pelių plaučių gripo viruso RNR, citokinų IFN-γ ir TNF-α, iNOS iRNR santykiniam lygiui, nitritų kiekiui BAL skystyje, pelių svorio pokyčiui ir plaučių histologiniam pažeidimui.
2. Įvertinti gydymo zanamiviru poveikį pelių plaučių gripo viruso RNR, citokinų IFN-γ ir TNF-α, iNOS iRNR santykiniam lygiui, nitritų kiekiui BAL skystyje, pelių svorio pokyčiui bei plaučių histologiniam pažeidimui.
3. Įvertinti gydymo priešprasminiu oligonukleotidu prieš iNOS poveikį pelių plaučių gripo viruso RNR, citokinų IFN-γ ir TNF-α, iNOS iRNR santykiniam lygiui, nitritų kiekiui BAL skystyje, pelių svorio pokyčiui ir plaučių histologiniam pažeidimui.
4. Įvertinti gydymo zanamiviro ir priešprasminio oligonukleotido prieš iNOS derinio poveikį pelių plaučių gripo viruso RNR, citokinų IFN-γ ir TNF-α, iNOS iRNR santykiniam lygiui, nitritų kiekiui BAL skystyje, pelių svorio pokyčiui ir plaučių histologiniam pažeidimui.

4. Mokslinio darbo naujumas

Šiame moksliniame darbe pirmą kartą buvo vertintas zanamiviro gebėjimas sumažinti plaučių pažeidimą slopinant NO gamybą gripo infekcijos metu *in vivo*. Taip pat pirmą kartą tirtas pelių, užkrėstų gripo virusu, gydymo priešprasminiu oligonukleotidu prieš iNOS iRNR ar jo derinio su zanamiviru poveikis.

5. Mokslinio darbo praktinė reikšmė

Šiuo metu gripui gydyti dažniausiai vartojami neuraminidazės inhibitoriai, tačiau jie gali būti nepakankamai efektyvūs sunkiai sergantiems, didelės rizikos ligoniams ar esant paukščių gripo viruso potipio A(H5N1) infekcijai. Daugėja gripo viruso potipių, atsparių priešvirusiniams vaistams. Todėl reikalingi nauji vaistai nuo gripo, veikiantys naujus taikinius ir kitais mechanizmais. Priešprasminis oligonukleotidas prieš iNOS, slopindamas gripo viruso sukeltą plaučių pažeidimą, derinyje su priešvirusiniais vaistais galėtų būti nauja gydymo galimybė.

6. Ginamieji disertacijos teiginiai

1. Užkrėtus BALB/c peles A/PR/8/34 gripo virusu, sukeliamas plaučių audinio pažeidimas, kuris yra susijęs su pelių svorio mažėjimu, didesne citokinų bei iNOS iRNR raiška ir nitritų koncentracija plaučiuose.
2. Zanamiviras slopina plaučių pažeidimą mažindamas gripo viruso replikaciją ir plitimą, dėl to mažiau gaminama NO gripu užkrėstų pelių plaučiuose.
3. Priešprasminis oligonukleotidas prieš iNOS iRNR slopina gripu užkrėstų pelių plaučių uždegimą mažindamas NO gamybą.
4. Zanamiviro ir priešprasminio oligonukleotido prieš iNOS derinys mažina gripu užkrėstų pelių plaučių uždegimą slopindamas gripo viruso replikaciją ir NO gamybą.

7. Medžiagos ir metodai

7.1 Gyvūnai

Moteriškos lyties, 6–12 savaičių amžiaus, 18–20 g svorio BALB/c pelių buvo įsigyta iš Charles River laboratorijos (Vilmingtonas, Masačusetso v., JAV). Pelės buvo laikomos atskiroje patalpoje mikroizoliaciniuose narvuose. Visos procedūros buvo atliekamos biosaugumo kabinete, turinčiame antrą biosaugumo lygį. Prieš kiekvieną procedūrą – užkrėtimą virusu, gydymą vaistais ir eutanaziją – pelėms buvo atliekama anestezija suleidžiant į pilvaplėvės ertmę ketamino (50 mg/kg) ir ksilazino

(50 mg/kg) mišinio. Visi eksperimento protokolai buvo patvirtinti Rytų Virdžinijos aukštosios medicinos mokyklos Gyvūnų priežiūros ir naudojimo komitete, Norfolk'as, Virdžinijos v., JAV (etikos leidimo Nr. 07-003).

7.2 Gripo virusas ir jo infektyvumo titro nustatymas

Pelėms adaptuotas gripo A/Puerto Rico/8/34 [A/PR/8/34] (H1N1) virusas buvo gautas iš Floridos universiteto Medicinos koledžo [Gainesville'as, Floridos v., JAV] (dr. Bradley S. Bender dovana). Virusas buvo padaugintas 10 dienų amžiaus vištos kiaušinių embrionų alantojaus ertmėse pagal literatūroje aprašytą metodiką (Lennette, 1995). Kad būtų nustatytas gripo A/PR/8/34 viruso infektyvumo titras, vientisi MDCK ląstelių (Amerikietiško tipo ląstelių kultūrų kolekcijos Nr. CCL-34, Manasas, Virdžinijos v., JAV) monosluoksniai buvo inokuliuoti su 10 kartų serijiniu būdu praskiestais viruso mėginiais. 50 proc. ląstelių kultūros infekcinė dozė ($LKID_{50}$) įvertinta pagal viruso citopatogeninio efekto (CPE) išplitimo dydį monosluoksniuose praėjus 96 val., o viruso infektyvumo titras buvo apskaičiuotas Kärber metodu (Lennette, 1995). Nustatyta gripo infektyvumo titro vertė buvo $10^{8,4}$ $LKID_{50}$/ml. Po to įvairiais serijiniu būdu praskiestais viruso mėginiais intranazaliai užkrėstos pelės, skiriant vienkartinę 20 µl viruso suspensijos dozę (po 10 µl į šnervę). Pelės stebėtos 5 dienas, kad būtų nustatytas viruso infektyvumo titras, kuris sukelia ryškius ligos simptomus, tačiau nėra letalus. Pagal pelių svorio sumažėjimą ir išgyvenamumą buvo nustatytas $10^{7,6}$ $LKID_{50}$/ml viruso infektyvumo titras, kuris ir buvo naudotas eksperimente.

7.3 Tiriamosios vaistinės medžiagos

Zanamiviras (5-(acetilamino)-4-((aminoiminometil)-amino)-2,6-anhidro-3,4,5-trideoksi -D-glicero-D-galakto-ne-2-enoinė rūgštis) buvo gautas iš „GlaxoSmithKline" (Tyrimų trikampio parkas, Šiaurės Karolinos v., JAV) kaip Relenza™, susidedantis iš 5 mg zanamiviro ir 20 mg laktozės miltelių mišinio lizdinėje plokštelėje. Priešprasminis oligonukleotidas (PO) prieš iNOS buvo sintezuotas „Sigma-Aldrich" bendrovėje (St. Louis'as, Misūrio v., JAV) kaip nemodifikuotas oligodeoksiribonukleotidas su fosfodiesterinėmis tarpnukleotidinėms jungtimis, sudarytas iš 21 nukleotidų sekos: 5′-CAAGCCATG TCTGAGACTTTG-3′, atitinkančios pelės iNOS iRNR transliacijos iniciacijos vietą nuo 1 iki 21 bazės, kaip aprašyta Ding'o ir kt. (Ding ir kt., 1998). Laktozė buvo sintezuota „Sigma-Aldrich bendrovėje" (St. Louis'as, Misūrio v., JAV). Vaistinės medžiagos buvo ištirpintos steriliame fiziologiniame tirpale, pH 7,4, ir 20 µl tirpalo intranazaliai (po 10 µl į kiekvieną šnervę) buvo skiriamas pelėms. Zanamiviro (2mg/kg) ir PO (5 mg/kg) dozės buvo parinktos atsižvelgiant į literatūros duomenis (Ryan ir kt., 1994; Tarbet ir kt., 2014; Li ir kt.,

2014; Ding ir kt., 1998; Templi ir kt., 2000). Laktozės (placebo) dozė buvo 8 mg/kg. Vaistinės medžiagos pelėms buvo skiriama vieną kartą per dieną, kad būtų išvengta žalojančio kartotinės intervencijos poveikio.

7.4 Eksperimento planas

Po anestezijos pelės buvo užkrėstos intranazaliai 20 µl gripo A/PR/8/34 viruso suspensija su viruso $10^{7,6}$ $LKID_{50}$/ml infektyvumo titru. Praėjus 3 valandoms po užkrėtimo, pelės buvo pradėtos gydyti ir gydytos 3 arba 5 dienas zanamiviru (2 mg/kg, 3 d.: n = 5, 5 d.: n = 5), PO (5 mg/kg, 3 d.: n = 6, 5d.: n = 6) arba abiejų vaistų deriniu tomis pačiomis dozėmis (3 d.: n = 6, 5 d.: n = 6). Tiriamieji vaistai buvo skiriami kasdien su 20 µl fiziologinio tirpalo intranazaliai vieną kartą per dieną. Neigiamos kontrolinės grupės pelės gripu užkrėstos nebuvo, joms kasdien intranazaliai buvo skiriama arba 20 µl fiziologinio tirpalo (5 d.: n = 3), arba vaistinė medžiaga, ištirpinta 20 µl fiziologinio tirpalo: laktozė (3 d.: n = 3, 5 d.: n = 3), zanamiviras (3 d.: n = 3, 5 d.: n = 3), PO prieš iNOS (3 d.: n = 3, 5 d.: n = 3) arba zanamiviro ir PO prieš iNOS derinys (3 d.: n = 4, 5 d.: n = 4) tomis pačiomis dozėmis. Teigiamos kontrolinės grupės pelės buvo užkrėstos gripo virusu, paskui joms kasdien intranazaliai buvo skiriama 20 µl fiziologinio tirpalo (5 d.: n = 3), laktozė ištirpinta 20 µl fiziologinio tirpalo (3 d.: n = 4, 5 d.: n = 4) arba neskirta nieko (3 d.: n = 3, 5 d.: n = 3). Pelės kasdien buvo sveriamos, kad būtų įvertintas jų sergamumas. Po 3 arba 5 dienų visoms pelėms buvo atlikta eutanazija sukeliant terminalinį nukraujavimą. Paskui pelių plaučiai buvo išpjauti ir atliktas bronchoalveolinis lavažas (BAL). BAL buvo atliktas du kartus su 1 ml fiziologinio tirpalo pro į trachėją įkištą vamzdelį. Surinktas BAL skystis buvo centrifuguotas 735 × g 5 minutes, gautas supernatantas buvo užšaldytas –80 °C iki nitritų koncentracijos nustatymo. Tuoj po BAL procedūros pelių kairieji plaučiai buvo įdėti į 4 % paraformaldehido tirpalą (Thermo Fisher Scientific, Waltham'as, Masačusetso valstija, JAV) ir laikomi 4 °C iki histologinių tyrimų. Dešinieji plaučiai buvo įmerkti į TRIzol™ reagentą (Thermo Fisher Scientific, Valtham'as, Masačusetso valstija, JAV) ir homogenizuoti ant sauso ledo naudojant audinių smulkintuvą (Dremel, Racin'as, Viskonsino valstija, JAV). Plaučių audinio homogenatai centrifuguoti 12 000 × g 5 minutes 4 °C temperatūroje, ir supernatantai buvo užšaldyti –80 °C. Vėliau jie buvo naudojami gripo viruso RNR bei iNOS, citokinų IFN-γ, TNF-α ir beta (β)-aktino iRNR nustatyti taikant dauginę tikrojo laiko kiekybinę atvirkštinės transkripcijos - polimerazės grandininę reakciją (AT-PGR).

7.5 RNR išskyrimas ir nustatymas dauginiu tikrojo laiko kiekybiniu AT-PGR tyrimu

Visa RNR iš plaučių homogenatų buvo išskirta naudojant Trizol® reagentą pagal instrukcijas, suteiktas „Thermo Fisher Scientific“ (Valtham'as, Masačusetso v., JAV). Gripo viruso RNR (koduojanti gripo A viruso polimerazės rūgštinį baltymą (RB)), iNOS, IFN-γ, TNF-α ir beta (β)-aktino iRNR lygiai buvo nustatyti dauginiu tikrojo laiko kiekybiniu AT-PGR tyrimu, naudojant SensiFAST® ™ Probe No-ROX vienos pakopos rinkinį (Bioline Reagents Ltd, Londonas, Didžioji Britanija). Buvo naudojami šie pradmenys ir zondai (pagaminti Biolegio B.V., Nijmegen'as, Olandija): gripo A viruso RB tiesioginės krypties pradmuo 5′-TCTCAGCGGTCCAAATTC CTGC -3′, gripo A viruso RB atvirkštinės krypties pradmuo 5′-GGTTTAACAACATTGGGTTCCTTC CAT-3′, gripo A viruso RB zondas 5′-HEX-TGA GGACCCAAGTCATGAAGGAGAGGGA-BHQ1-3′; iNOS tiesioginės krypties pradmuo 5′-CTTGTGCTGTTCTCAGCCCAACAATA-3′, iNOS atvirkštinės krypties pradmuo 5′-TGGAACATT CTGTGCTGTCCCAG-3′, iNOS zondas 5′-FAM-TGGCTCCCCGCAGCTCCTC-BHQ1-3′; IFN-γ tiesioginės krypties pradmuo 5′-GCCAAGTTT GAGGTCAACAACCC-3′, atvirkštinės krypties pradmuo 5′-TGGAACATTCTGTGCTGTCCCAG-3′, zondas 5′-FAM-TCATCCGAGTGGTCCACCAGC TGT-BHQ1-3′; TNF-α tiesioginės krypties pradmuo 5′-CCAGACCCTCACACTCAGATCATC-3′, atvirkštinės krypties pradmuo 5′-CTCCTCCACTTG GTGGTTTGCTAC-3′, zondas 5′-HEX-TCGAGTGAC AAGCCTGTAGCCCACGT-BHQ1-3′; β-aktino tiesioginės krypties pradmuo 5′-GCACMATGA AGATCAAGATCATTGCTCC-3′, β-aktino atvirkštinės krypties pradmuo 5′-TCRTACTCC TGCTTGCTGATCCAC-3′ ir β-aktino zondas 5′-ROX-TCCTGGCCTCRCTGTCCACCTTCC-BHQ2-3′. Visos vienos pakopos reakcijos buvo atliktos 15 µl reakcijos tūriuose su 1 µl RNR iš kiekvieno mėginio, 200 nM koncentracijos pradmenimis ir 100 nM koncentracijos zondais. Dauginės vienos pakopos AT-PGR buvo atliktos tikrojo laiko termocikleriu Rotor-Gene Q 5plex su 1.7 versijos kompiuterių programa (Qiagen GmbH, Hilden'as, Vokietija) tokiomis sąlygomis: pirmoji komplementarios DNR grandinė buvo sintezuota 48 °C temperatūroje 10 min. (1 ciklas) ir paskui denatūruota 95 °C temperatūroje 2 min. (1 ciklas); o toliau buvo 50 ciklų DNR denatūracijos 95 °C temperatūroje 10 s ir prijungimo / pratęsimo 60 °C temperatūroje 1 min. β-aktino iRNR raiška buvo panaudota kaip vidinė kontrolė. $2^{-\Delta\Delta CT}$ algoritmas buvo naudojamas santykinio PGR amplifikacijos produkto kiekiui, atspindinčiam santykinę tikslinės RNR ir iRNR raišką (Livak ir kt., 2001), apskaičiuoti.

7.6 Nitritų nustatymas

Nitritų (NO_2^-) lygis, kaip ląstelių gaminamo NO žymuo, buvo nustatytas BAL skystyje panaudojant modifikuotą Griess reagentą, įsigytą iš „Sigma-Aldrich" bendrovės (St. Louis'as, Misūrio v., JAV), pagal pateiktas gamintojo instrukcijas. Optinis tankis (O.T.) išmatuotas parinkus 540 nm bangos ilgį, PowerWawe X 340 mikroplokštelių skaitytuvu-spektrofotometru (Bio-Tek Instruments Inc., Winooski's, Vermonto v., JAV). Nitritų koncentracija buvo apskaičiuota su KC4 statistinės programos 2.5 versija (Bio-Tek Instruments Inc., Winooski's, Vermonto v., JAV) lyginant nitritų O.T. mėginiuose su standartinio praskiedimo natrio nitritų tirpalų O.T. Žemutinė NO_2^- nustatymo riba buvo 0,12 μmol/ml.

7.7 Histologinis tyrimas

Pelių plaučiai buvo įlieti į parafiną, supjaustyti 5 μm pjūviais ir nudažyti hematoksilinu ir eozinu naudojant standartinę techniką. Užkoduotus plaučių preparatus šviesaus lauko mikroskopija įvertino gydytojas patologas aklu metodu. Įvertinti šie plaučių histopatologijos parametrai: perivaskulinė limfocitų, peribronchinė limfocitų, židininė ir difuzinė leukocitų infiltracija; alveolių makrofagai; alveolių / bronchų epitelio pažeidimas; hiperemija; kapiliarų trombozė; bronchiolitas; pleuritas; alveolių hemoragija; atelektazė; fibrozė; edema ir hialininės membranos. Visi šie parametrai įvertinti subjektyviai skalėje nuo 0 (nėra patologinių pakitimų) iki 3 (maksimalūs pakitimai). Kiekvienos pelės histologinę skalę sudarė minėtų parametrų įvertinimo balais suma.

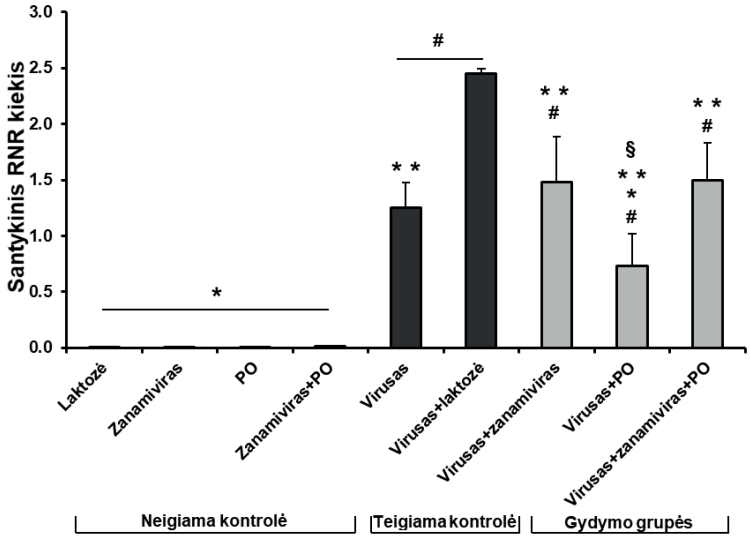
7.8 Statistinė analizė

Statistinis duomenų įvertinimas buvo atliktas Socialinių mokslų statistinio paketo (SPSS) kompiuterių programa (21.0 versija, IBM Corp., Armonk'as, Niujorko v., JAV). Eksperimentinių grupių vidurkiams palyginti naudota vienfaktorinė dispersijos analizė (ANOVA) su *post hoc* mažiausiai reikšmingo skirtumo (LSD) testu. Duomenys pateikti kaip vidurkiai ± standartinė paklaida (SP). Kintamųjų koreliacijai įvertinti naudota Spirmano koreliacijos analizė. Mažesnė už 0,05 p vertė buvo vertinama kaip statistiškai reikšminga.

8. Rezultatai

8.1 Santykinė gripo A viruso RNR raiška pelių plaučiuose

Trečią dieną po užkrėtimo AT-PGR metodu gripo viruso RB RNR nebuvo nustatyta neigiamos kontrolinės grupės pelių plaučiuose, bet RB RNR raiška buvo padidėjusi visų gripu užkrėstų pelių grupių plaučiuose (1 paveikslas).



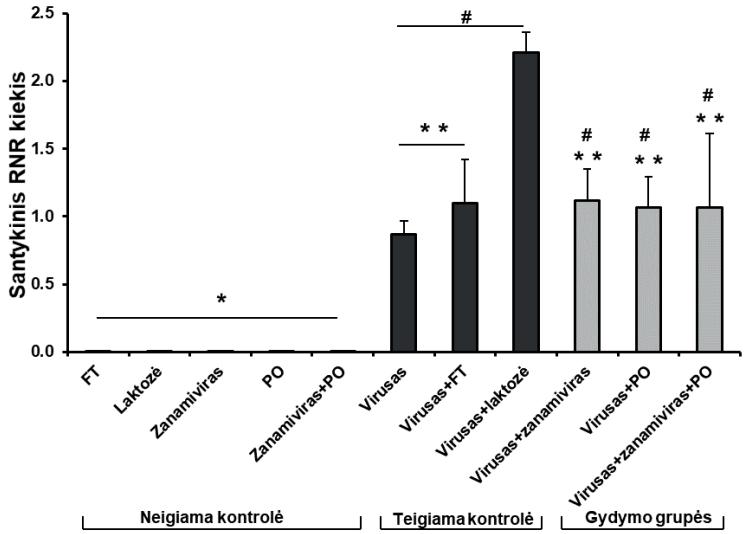
1 PAVEIKSLAS. Kontrolinių ir gydymo grupių pelių santykinė gripo viruso RP RNR raiška trečią dieną po užkrėtimo

Duomenys pateikti kaip vidurkiai ± SP. Statistiškai reikšminga ($p < 0{,}05$) #vs. neigiama kontrolinė grupė, n = 11, *vs. teigiama kontrolinė grupė, n = 5, **vs. gripu užkrėstos ir laktoze gydytos pelės, n = 3 ir §vs. gripu užkrėstos ir zanamiviru gydytos pelės, n = 4.

Gripu užkrėstos, PO gydytos, n = 4; gripu užkrėstos, zanamiviro ir PO deriniu gydytos, n = 4.

Teigiamos kontrolinės grupės virusu užkrėstų ir gydytų laktoze pelių plaučiuose santykinė gripo viruso RB RNR raiška buvo reikšmingai didesnė nei tik virusu užkrėstos pelių grupės ($p = 0{,}013$). Visų gripu užkrėstų gydymo grupių rasta reikšmingai mažesnė viruso RB RNR santykinė raiška, palyginti su gripu užkrėstomis ir laktoze gydytomis pelėmis. Virusu užkrėstų ir PO prieš iNOS gydytų pelių plaučiuose RB RNR santykinė raiška buvo mažesnė nei užkrėstų ir zanamiviru gydytų pelių plaučiuose ($p = 0{,}039$).

Penktą eksperimento dieną viruso RB RNR nebuvo nustatyta neigiamos kontrolinės grupės pelių plaučiuose, bet RB RNR raiška buvo padidėjusi visų grupių gripu užkrėstų pelių plaučiuose (2 paveikslas).



2 PAVEIKSLAS. Kontrolinių ir gydymo grupių pelių santykinė gripo viruso RP RNR raiška penktą dieną po užkrėtimo

Duomenys pateikti kaip vidurkiai ± SP. Statistiškai reikšminga ($p < 0,05$) #vs. neigiama kontrolinė grupė, n = 15, *vs. teigiama kontrolinė grupė, n = 7, **vs. gripu užkrėstos ir laktoze gydytos pelės, n = 2.

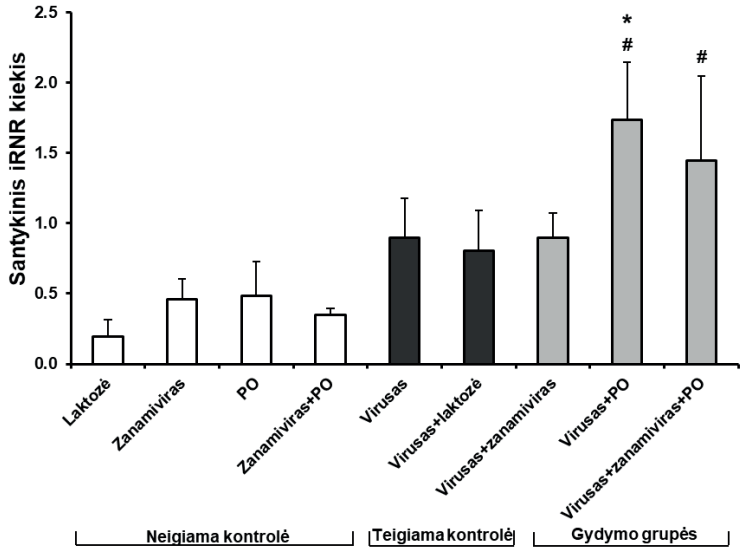
Gripu užkrėstos ir zanamiviru gydytos pelės, n = 4; gripu užkrėstos, PO gydytos, n = 4; gripu užkrėstos, zanamiviro ir PO deriniu gydytos, n = 4.

Teigiamos kontrolinės grupės virusu užkrėstų ir gydytų laktoze pelių plaučiuose santykinė gripo viruso RB RNR raiška buvo reikšmingai didesnė nei tik virusu užkrėstos pelių grupės (p = 0,012) ar virusu užkrėstų ir fiziologiniu tirpalu (FT) gydytų pelių grupės (p = 0,021). Visų trijų gripu užkrėstų gydymo grupių pelių rasta reikšmingai mažesnė viruso RB RNR santykinė raiška, palyginti su gripu užkrėstomis ir laktoze gydytomis pelėmis (p = 0,017, palyginti su zanamiviro, p = 0,013, palyginti su PO, p = 0,013, palyginti su vaistų derinio gydymo grupe).

8.2 Santykinė IFN-γ iRNA raiška pelių plaučiuose

Santykinis IFN-γ iRNA kiekis buvo 55,1 proc. didesnis teigiamos (užkrėstos pelės) negu neigiamos (neužkrėstos pelės) kontrolinės grupės trečią eksperimento dieną, tačiau tai nebuvo statistiškai reikšminga (3 paveikslas). Užkrėstų ir zanamiviru gydytų pelių plaučiuose santykinis IFN-γ iRNR kiekis buvo panašus kaip ir kontrolinių grupių. Užkrėstų ir PO gydytų pelių grupės santykinis IFN-γ iRNR kiekis buvo reikšmingai didesnis nei abiejų kontrolinių grupių (p = 0,001 vs. neigiama

kontrolė ir p = 0,034 vs. teigiama kontrolė), o vaistų derinio gydymo grupėje – vs. neigiama kontrolinė grupė (p = 0,005).

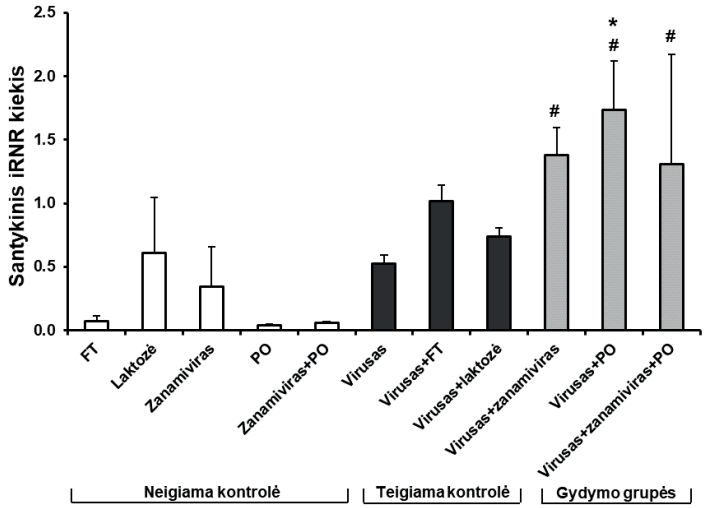


3 PAVEIKSLAS. Kontrolinių ir gydymo grupių pelių santykinė IFN-γ iRNR raiška trečią dieną po užkrėtimo

Duomenys pateikti kaip vidurkiai ± SP. Statistiškai reikšminga (p < 0,05) #vs. neigiama kontrolinė grupė, n = 11, *vs. teigiama kontrolinė grupė, n = 5.

Gripu užkrėstos ir zanamiviru gydytos pelės, n = 4; gripu užkrėstos, PO gydytos, n = 4; gripu užkrėstos, zanamiviro ir PO deriniu gydytos, n = 4.

Penktą eksperimento dieną, panašiai kaip ir trečią, santykinis IFN-γ iRNR kiekis buvo 72,0 proc. didesnis teigiamos nei neigamos kontrolinės grupės, tačiau tai nebuvo statistiškai reikšminga (4 paveikslas). Visų gydymo grupių IFN-γ iRNR santykinis kiekis buvo reikšmingai didesnis negu neigiamos kontrolinės grupės (p = 0,006, palyginti su užkrėstomis ir zanamiviru gydytomis, p = 0,001, palyginti su užkrėstomis ir PO gydytomis, ir p = 0,01 palyginti su užkrėstomis ir vaistų deriniu gydytomis pelėmis). Didžiausias santykinis IFN-γ iRNR kiekis buvo nustatytas užkrėstų ir PO gydytų pelių plaučiuose, jis buvo 20,7 proc. didesnis nei zanamiviro gydymo grupėje ir reikšmingai didesnis nei teigiamos kontrolinės grupės pelių plaučiuose (p = 0,04).



4 PAVEIKSLAS. Kontrolinių ir gydymo grupių pelių santykinė IFN-γ iRNR raiška penktą dieną po užkrėtimo

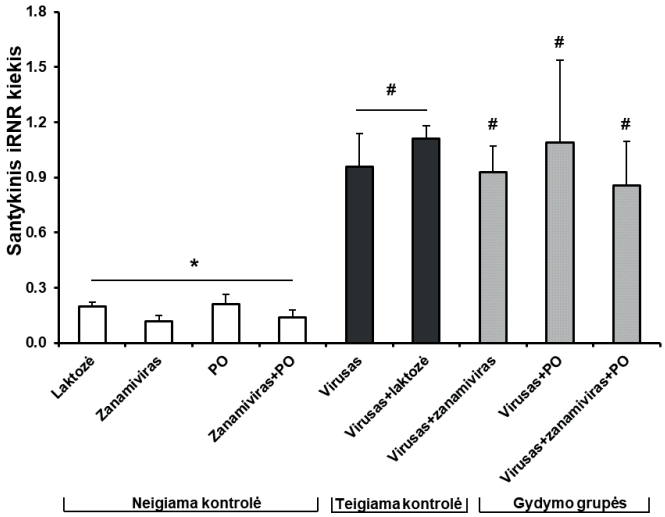
Duomenys pateikti kaip vidurkiai ± SP. Statistiškai reikšminga (p < 0,05) #vs. neigiama kontrolinė grupė, n = 15, *vs. teigiama kontrolinė grupė, n = 7.

Gripu užkrėstos ir zanamiviru gydytos pelės, n = 4; gripu užkrėstos, PO gydytos, n = 4; gripu užkrėstos, zanamiviro ir PO deriniu gydytos, n = 4.

8.3 Santykinė TNF-α iRNR raiška pelių plaučiuose

Santykinis TNF-α iRNR kiekis pelių plaučiuose trečią eksperimento dieną vaizduojamas 5 paveiksle. TNF-α iRNR santykinė raiška buvo 84,2 proc. didesnė teigiamos negu neigiamos kontrolinės grupės (p <0,001). Visų trijų užkrėstų pelių gydymo grupių santykinis TNF-α iRNR kiekis buvo panašus kaip ir teigiamos kontrolinės grupės, ir reikšmingai didesnis nei neigiamos kontrolinės grupės (p = 0,003, palyginti su zanamiviro, p < 0,001, palyginti su PO, p = 0,006, palyginti su vaistų derinio gydymo grupe).

Penktą eksperimento dieną santykinis TNF-α iRNR kiekis buvo 76,9 proc. didesnis teigiamos, palyginti su neigiamos kontrolinės grupės (p = 0,001). TNF-α iRNR santykinė raiška visų trijų gydymo grupių buvo didesnė nei neigiamos kontrolinės grupės (p = 0,004 užkrėstų ir zanamiviru gydytų, p < 0,000 užkrėstų ir PO gydytų ir p = 0,019 užkrėstų ir vaistų deriniu gydytų) pelių plaučiuose.

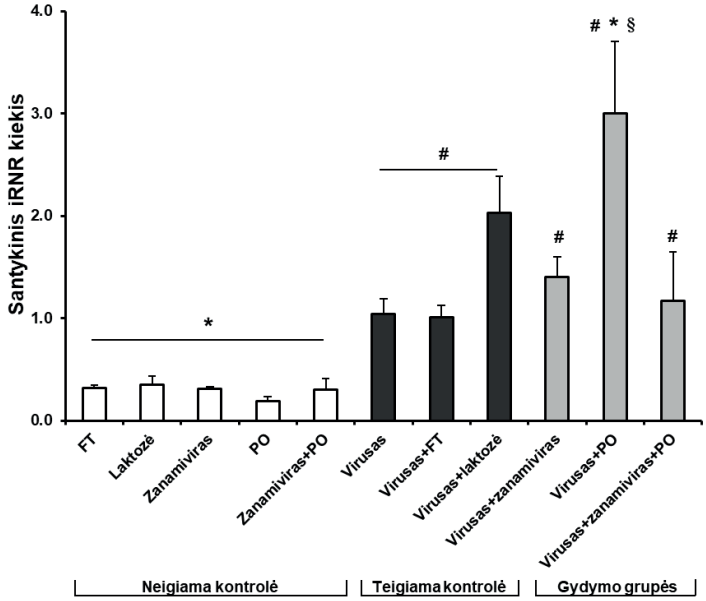


5 PAVEIKSLAS. Kontrolinių ir gydymo grupių pelių santykinė TNF-α iRNR raiška trečią dieną po užkrėtimo

Duomenys pateikti kaip vidurkiai ± SP. Statistiškai reikšminga ($p < 0{,}05$) #vs. neigiama kontrolinė grupė, n = 11, *vs. teigiama kontrolinė grupė, n = 5.

Gripu užkrėstos ir zanamiviru gydytos pelės, n = 4; gripu užkrėstos, PO gydytos, n = 4; gripu užkrėstos, zanamiviro ir PO deriniu gydytos, n = 4.

Didžiausias santykinis TNF-α iRNR kiekis buvo nustatytas užkrėstų ir PO gydytų pelių grupės plaučiuose: jis buvo 53,0 proc. didesnis nei užkrėstų ir zanamiviru gydytų pelių grupės ($p = 0{,}001$) bei didesnis nei teigiamos kontrolinės grupės pelių plaučiuose ($p < 0{,}001$). Užkrėstų ir vaistų deriniu gydytų pelių plaučiuose santykinis TNF-α iRNR kiekis buvo reikšmingai mažesnis ($p < 0{,}001$) nei užkrėstų ir PO gydytų pelių, ir 17,0 proc. mažesnis nei užkrėstų ir zanamiviru gydytų pelių plaučiuose (6 paveikslas).



6 PAVEIKSLAS. Kontrolinių ir gydymo grupių pelių santykinė TNF-α iRNR raiška penktą dieną po užkrėtimo

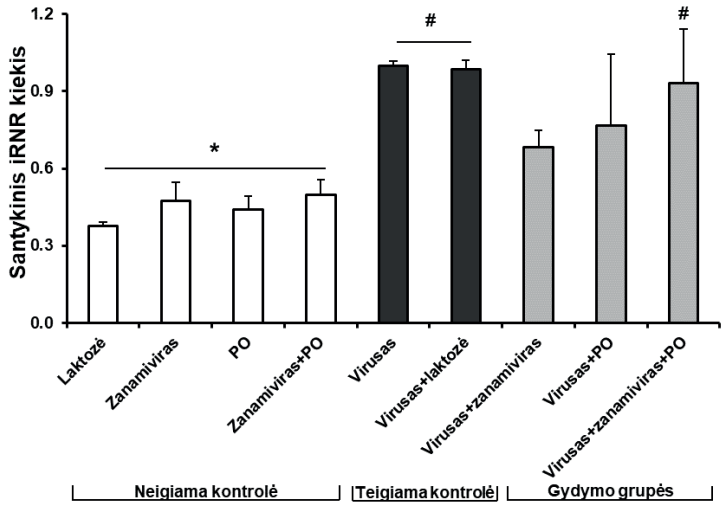
Duomenys pateikti kaip vidurkiai ± SP. Statistiškai reikšminga ($p < 0{,}05$) #vs. neigiama kontrolinė grupė, n = 15, *vs. teigiama kontrolinė grupė, n = 7, §vs. gripu užkrėstos ir zanamiviru gydytos pelės, n = 4.

Gripu užkrėstos, PO gydytos, n = 4; gripu užkrėstos, zanamiviro ir PO deriniu gydytos, n = 4.

8.4 Santykinė iNOS iRNR raiška pelių plaučiuose

Trečią eksperimento dieną iNOS iRNR santykinė raiška buvo reikšmingai didesnė teigiamos kontrolinės grupės (gripu užkrėstų) pelių plaučiuose negu neigiamos kontrolinės grupės (neužkrėstų pelių) (54,0 proc. padidėjimas, $p = 0{,}001$) (7 paveikslas). Tarp trijų gydymo grupių mažiausia santykinė iNOS iRNR raiška buvo užkrėstų ir zanamiviru gydytų pelių plaučiuose: 31,3 proc. mažesnė negu teigiamos kontrolinės grupės pelių ir panaši kaip neigiamos kontrolinės grupės pelių.

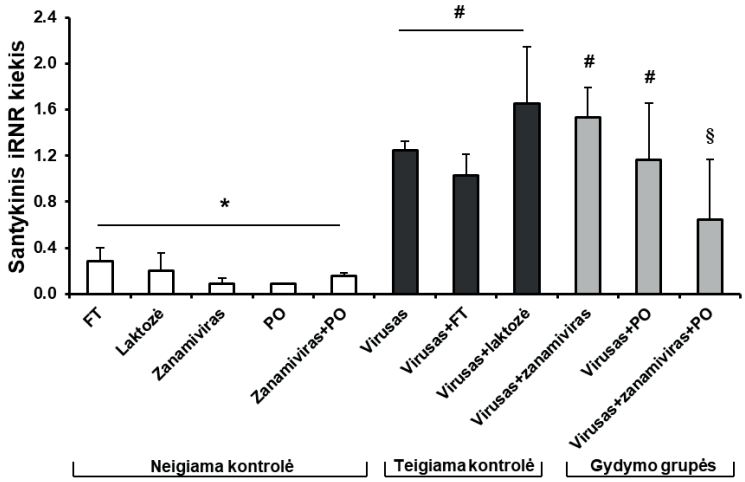
proc. Užkrėstų ir PO gydytų pelių plaučiuose santykinis iNOS iRNR kiekis buvo 22,2 proc. mažesnis negu teigiamos kontrolinės grupės pelių ir taip pat panašus kaip neigiamos kontrolinės grupės pelių. Vaistų derinio gydymo grupės iNOS iRNR santykinė raiška buvo reikšmingai didesnė nei neigiamos kontrolinės grupės ($p = 0{,}005$).



7 PAVEIKSLAS. Kontrolinių ir gydymo grupių pelių santykinė iNOS iRNR raiška trečią dieną po užkrėtimo

Duomenys pateikti kaip vidurkiai ± SP. Statistiškai reikšminga ($p < 0{,}05$) #vs. neigiama kontrolinė grupė, n = 11, *vs. teigiama kontrolinė grupė, n = 5.

Gripu užkrėstos ir zanamiviru gydytos pelės, n = 4; gripu užkrėstos, PO gydytos, n = 4; gripu užkrėstos, zanamiviro ir PO deriniu gydytos, n = 4.



8 PAVEIKSLAS. Kontrolinių ir gydymo grupių pelių santykinė iNOS iRNR raiška penktą dieną po užkrėtimo

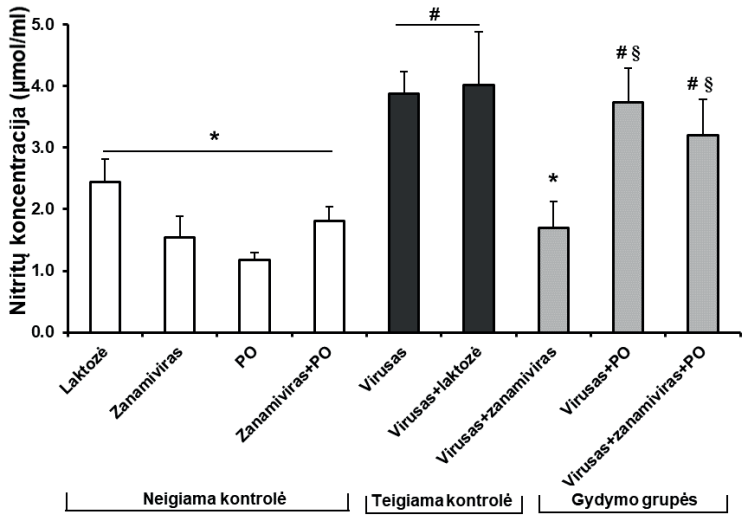
Duomenys pateikti kaip vidurkiai ± SP. Statistiškai reikšminga ($p < 0{,}05$) #vs. neigiama kontrolinė grupė, n = 15, *vs. teigiama kontrolinė grupė, n = 7, §vs. gripu užkrėstos ir zanamiviru gydytos pelės, n = 4.

Gripu užkrėstos, PO gydytos, n = 4; gripu užkrėstos, zanamiviro ir PO deriniu gydytos, n = 4.

Penktą eksperimento dieną taip pat reikšmingai didesnis santykinis iNOS iRNR kiekis buvo nustatytas teigiamos nei neigiamos kontrolinės grupės pelių plaučiuose (87,8 proc. padidėjimas, $p < 0{,}001$) (**8 paveikslas**). Užkrėstų ir zanamiviru arba PO gydytų pelių plaučiuose rasta reikšmingai didesnė iNOS iRNR raiška negu neigiamos kontrolinės grupės pelių plaučiuose ($p < 0{,}001$ ir $p = 0{,}002$ atitinkamai). Nors santykinis iNOS iRNR kiekis užkrėstų ir PO gydytų pelių plaučiuose buvo 8,5 proc. mažesnis negu teigiamos kontrolinės grupės, šis skirtumas nebuvo reikšmingas. Užkrėstų ir vaistų deriniu gydytų pelių plaučiuose santykinis iNOS iRNR kiekis nesiskyrė nuo neigiamos kontrolinės grupės. Tarp gydymo grupių didžiausias santykinis iNOS iRNR kiekis buvo nustatytas užkrėstų ir zanamiviru gydytų pelių plaučiuose. iNOS iRNR santykinis kiekis buvo 23,7 proc. mažesnis užkrėstų ir PO gydytų ir 57,2 proc. ($p = 0{,}027$) mažesnis užkrėstų ir vaistų deriniu gydytų pelių plaučiuose.

8.5 Nitritų koncentracija BAL skystyje

Trečią eksperimento dieną didesnė NO gamyba ($p < 0{,}001$) buvo nustatyta teigiamos kontrolės (gripu užkrėstų) pelių BAL skystyje, palyginti su neigiamos kontrolės (neužkrėstomis pelėmis) (9 paveikslas). Nitritų koncentracija buvo reikš-

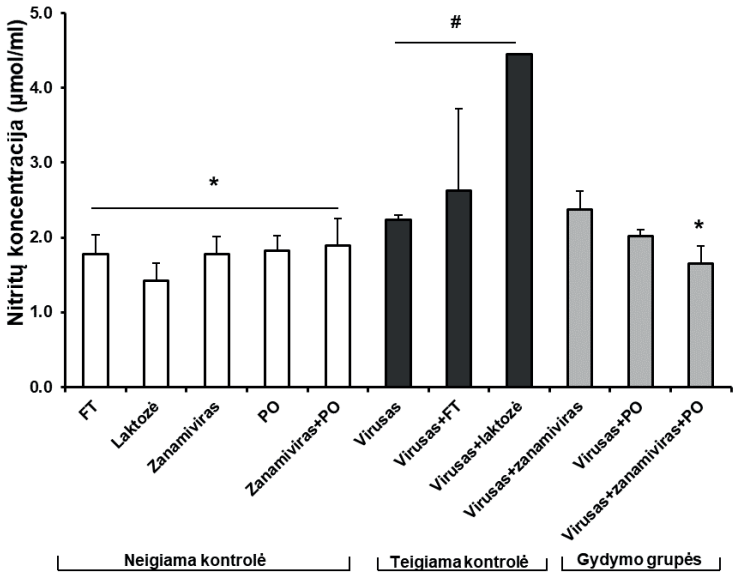


9 PAVEIKSLAS. Nitritų (NO_2^-) koncentracija pelių bronchoalveoliniame skystyje trečią dieną po užkrėtimo

Duomenys pateikti kaip vidurkiai ± SP. Statistiškai reikšminga ($p < 0{,}05$) #vs. neigiama kontrolinė grupė, n = 13, *vs. teigiama kontrolinė grupė, n = 6, §vs. gripu užkrėstos ir zanamiviru gydytos pelės, n = 4. Gripu užkrėstos, PO gydytos, n = 4; gripu užkrėstos, zanamiviro ir PO deriniu gydytos, n = 4.

mingai didesnė užkrėstų ir PO gydytų (p = 0,001) bei vaistų deriniu gydytų (p = 0,007) pelių grupių, palyginti su neigiama kontroline grupe. Užkrėstų ir zanamiviru gydytų pelių grupės nitritų koncentracija buvo reikšmingai mažesnė nei teigiamos kontrolinės grupės (57,5 proc. sumažėjimas, p = 0,001) ir nei užkrėstų ir PO gydytų (54,1 proc. sumažėjimas, p = 0,005) bei vaistų deriniu gydytų (46,9 proc. sumažėjimas, p = 0,026) pelių BAL skystyje.

Nitritų koncentracija pelių BAL skystyje penktą dieną po užkrėtimo vaizduojama 10 paveiksle. Panašiai kaip trečią eksperimento dieną, sustiprėjusi NO gamyba buvo rasta teigiamos kontrolinės grupės, palyginti su neigiama kontroline grupe (p = 0,003). Gripu užkrėstų ir vaistų deriniu gydytų pelių BAL skystyje buvo nustatyta reikšmingai mažesnė nitritų koncentracija, palyginti su teigiamos kontrolinės grupės pelių (39,3 proc. sumažėjimas, p = 0,005).



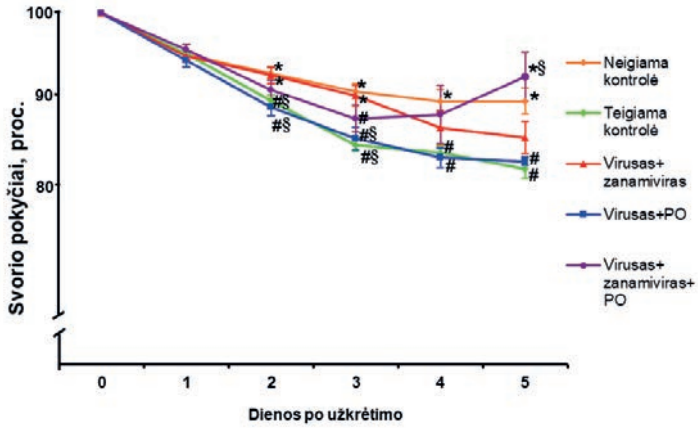
10 PAVEIKSLAS. Nitritų (NO_2^-) koncentracija pelių bronchoalveoliniame skystyje penktą dieną po užkrėtimo

Duomenys pateikti kaip vidurkiai ± SP. Statistiškai reikšminga (p < 0,05) #vs. neigiama kontrolinė grupė, n = 11, *vs. teigiama kontrolinė grupė, n = 5.

Gripu užkrėstos ir zanamiviru gydytos pelės, n = 4; gripu užkrėstos, PO gydytos, n = 3; gripu užkrėstos, zanamiviro ir PO deriniu gydytos, n = 5.

8.6 Pelių svorio pokyčiai

Pelių sv orio pokyčiai penkių dienų eksperimento laikotarpiu pateikiami 11 paveiksle. Teigiamos kontrolinės grupės (užkrėstų) pelių svoris buvo reikšmingai mažesnis negu neigiamos kontrolinės (neužkrėstų) grupės pelių nuo antros eksperimento dienos. Užkrėstos ir zanamiviru gydytos pelės neteko mažiau svorio (9,4 proc.) negu teigiamos kontrolinės grupės pelės (15,0 proc., $p = 0{,}002$) ir negu užkrėstos ir PO gydytos pelės (14,3 proc., $p = 0{,}009$) trečią eksperimento dieną; tačiau penktą dieną reikšmingų svorio netekimo skirtumų tarp šių grupių nenustatyta. Užkrėstos ir PO gydytos pelės neteko svorio reikšmingai daugiau nei neigiamos kontrolinės grupės pelės ir trečią dieną (svorio sumažėjimas 14,3 proc. ir 8,9 proc. atitinkamai, $p < 0{,}001$), ir penktą dieną (svorio sumažėjimas 16,9 proc. ir 10,0 proc. atitinkamai, $p = 0{,}02$). Užkrėstos ir vaistų deriniu gydytos pelės neteko 12,0 proc. svorio trečią dieną, tai buvo daugiau nei neigiamos kontrolinės grupės pelių, kurios neteko tik 8,9 proc. ($p = 0{,}037$).



11 PAVEIKSLAS. Kontrolinių ir gydymo grupių pelių svorio pokyčiai penkių dienų laikotarpiu po užkrėtimo

Svoris pavaizduotas procentais nuo pradinio svorio 0 dieną. Duomenys pateikti kaip vidurkiai ± SP. Statistiškai reikšminga ($p < 0{,}05$) #vs. neigiama kontrolinė grupė, n = 29 trečią dieną, n = 16 penktą dieną; *vs. teigiama kontrolinė grupė, n = 16 trečią dieną, n = 8 penktą dieną; §vs. gripu užkrėstos ir zanamiviru gydytos pelės, n = 10 trečią dieną, n = 5 penktą dieną.

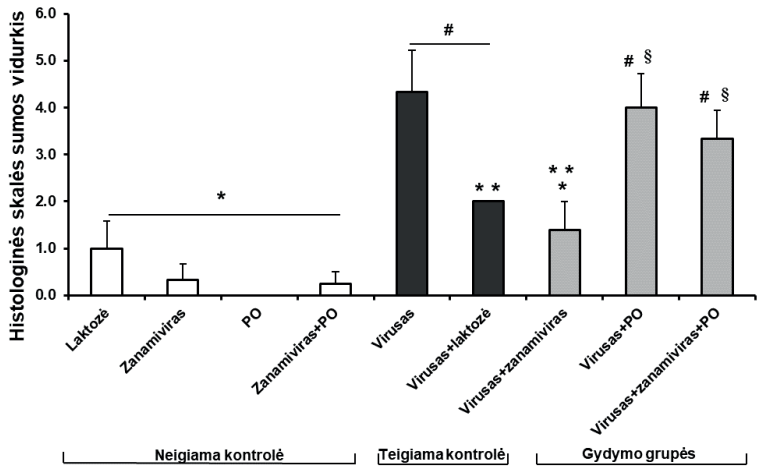
Gripu užkrėstos, PO gydytos, n = 12 trečią dieną, n = 4 penktą dieną; gripu užkrėstos, zanamiviro ir PO deriniu gydytos, n = 12 trečią dieną, n = 5 penktą dieną.

Įdomu, kad užkrėstos pelės, gydytos vaistų deriniu, netgi pradėjo priaugti svorio, ir penktą dieną jų svoris buvo reikšmingai didesnis nei teigiamos kontrolinės grupės pelių ($p = 0{,}001$) ir užkrėstų zanamiviru gydytų pelių ($p = 0{,}036$).

8.7 Pelių plaučių histologiniai pokyčiai

Pelių kontrolinių ir gydymo grupių plaučių pažeidimo histologinės skalės sumos vidurkiai trečią dieną po užkrėtimo vaizduojami **12 paveiksle**. Teigiamos kontrolinės grupės histologinės skalės vidurkis buvo reikšmingai didesnis negu neigiamos kontrolinės grupės pelių ($p < 0{,}001$). Užkrėstų ir zanamiviru gydytų pelių skalės vidurkis buvo mažesnis nei teigiamos kontrolinės grupės ($p = 0{,}018$). Užkrėstų ir PO ar vaistų deriniu gydytų pelių plaučių pažeidimo histologinės skalės sumos vidurkiai buvo panašūs kaip teigiamos kontrolinės grupės ir reikšmingai didesni nei užkrėstų ir zanamiviru gydytų pelių.

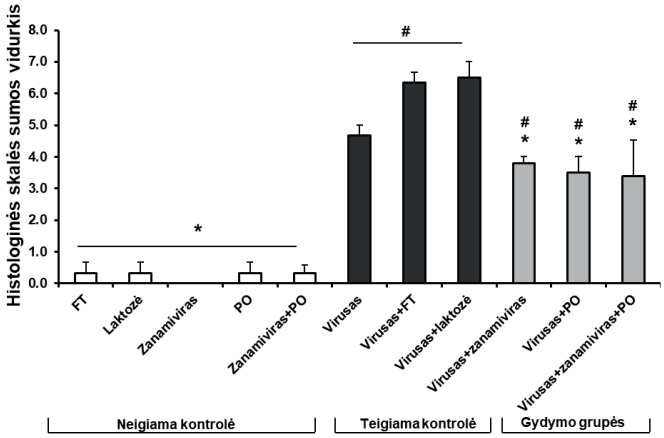
Penktą eksperimento dieną histologinės skalės vidurkis taip pat buvo reikšmingai didesnis teigiamos kontrolinės grupės negu neigiamos kontrolinės grupės ($p < 0{,}001$) (13 paveikslas). Visų užkrėstų pelių gydymo grupių histologinės skalės vidurkiai buvo reikšmingai mažesni nei teigiamos kontrolinės grupės pelių (užkrėstų zanamiviru gydytų pelių, $p = 0{,}006$; užkrėstų ir PO gydytų, $p = 0{,}003$ ir užkrėstų vaistų deriniu gydytų, $p = 0{,}001$).



12 PAVEIKSLAS. Kontrolinių ir gydymo grupių pelių plaučių pažeidimo histologinės skalės sumos vidurkiai trečią dieną po užkrėtimo

Duomenys pateikti kaip vidurkiai ± SP. Statistiškai reikšminga ($p < 0{,}05$) #vs. neigiama kontrolinė grupė, n = 13, *vs. teigiama kontrolinė grupė, n = 6, **vs. gripu užkrėstos ir negydytos pelės, n = 3 ir §vs. gripu užkrėstos ir zanamiviru gydytos pelės, n = 5.

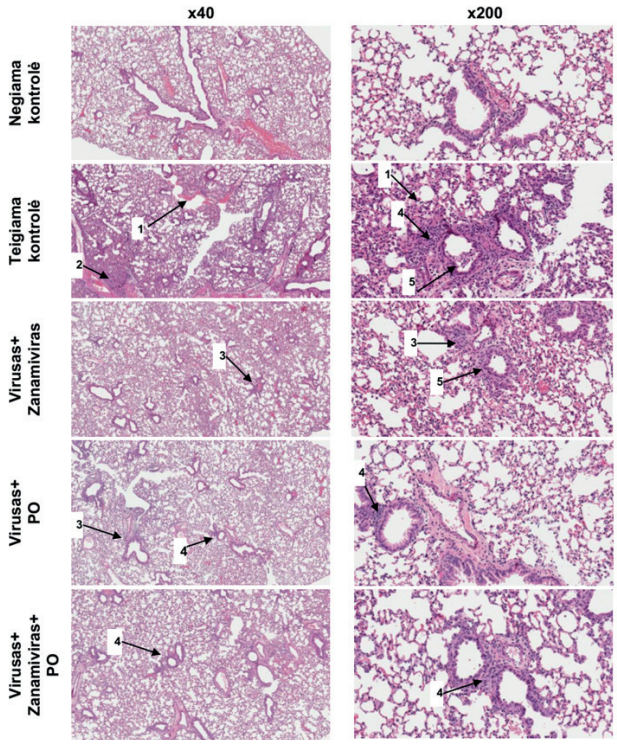
Gripu užkrėstos, PO gydytos, n = 6; gripu užkrėstos, zanamiviro ir PO deriniu gydytos, n = 6.



13 PAVEIKSLAS. Kontrolinių ir gydymo grupių pelių plaučių pažeidimo histologinės skalės sumos vidurkiai penktą dieną po užkrėtimo

Duomenys pateikti kaip vidurkiai ± SP. Statistiškai reikšminga ($p < 0{,}05$) #vs. neigiama kontrolinė grupė, n = 16, *vs. teigiama kontrolinė grupė, n = 8, ir $^{\$}$vs. gripu užkrėstos ir zanamiviru gydytos pelės, n = 5.

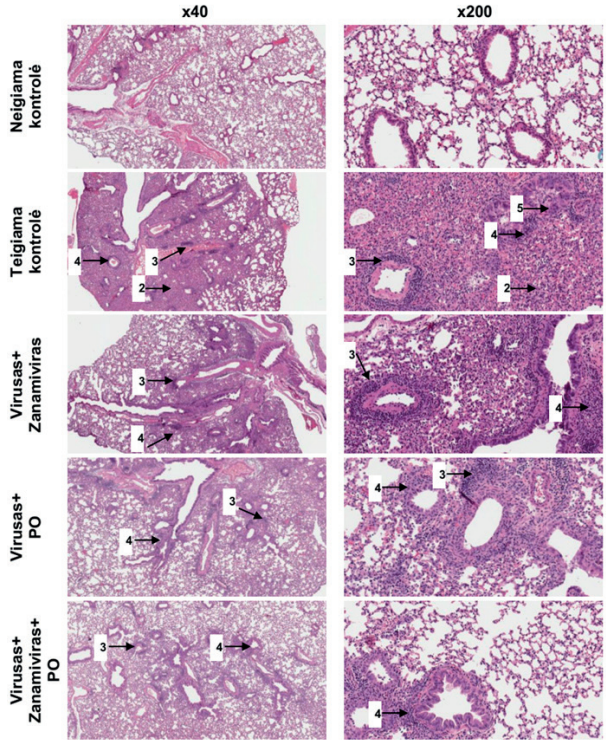
Gripu užkrėstos, PO gydytos, n = 4; gripu užkrėstos, zanamiviro ir PO deriniu gydytos, n = 5.



14 PAVEIKSLAS. Histologiniai BALB/c pelių plaučių preparatai, nudažyti hematoksilinu ir eozinu trečią dieną po užkrėtimo

Rodyklėmis pažymėta: (1) alveolių hemoragija, (2) židininė leukocitų infiltracija, (3) perivaskulinė limfocitų infiltracija, (4) peribronchinė limfocitų infiltracija, (5) bronchiolitas.

Trečios eksperimento dienos pelių plaučių histologinių preparatų nuotraukos pateikiamos 14 paveiksle, o penktos eksperimento dienos – 15 paveiksle.



15 PAVEIKSLAS. Histologiniai BALB/c pelių plaučių preparatai, nudažyti hematoksilinu ir eozinu penktą dieną po užkrėtimo

Rodyklėmis pažymėta: (1) alveolių hemoragija (nebuvo penktos dienos preparatų nuotraukose), (2) židininė leukocitų infiltracija, (3) perivaskulinė limfocitų infiltracija, (4) peribronchinė limfocitų infiltracija, (5) bronchiolitas.

8.8 Plaučių pažeidimo ir svorio pokyčio, nitritų koncentracijos, gripo viruso RNR, citokinų bei iNOS iRNR pelių plaučiuose koreliacija

Trečios eksperimento dienos pelių plaučių pažeidimo histologinės skalės sumos vidurkio ir svorio pokyčio, nitritų koncentracijos, gripo viruso RNR, citokinų bei iNOS iRNR pelių plaučiuose koreliacijos koeficientai pateikiami 1 lentelėje, o penktos dienos – 2 lentelėje.

1 LENTELĖ. Plaučių pažeidimo histologinės skalės sumos vidurkio ir svorio pokyčio, nitritų koncentracijos, gripo viruso RNR, IFN-γ ir TNF-α bei iNOS iRNR pelių plaučiuose koreliacija trečią eksperimento dieną

Kintamasis	Histologinės skalės sumos vidurkis	
	r	*p* vertė
Svoris 3 dieną	-0,428*	0,009
Nitritai	0,546*	0,001
Gripo viruso RB RNR	0,595*	0,001
iNOS iRNR	0,428*	0,023
IFN-γ iRNR	0,476*	0,010
TNF-α iRNR	0,746*	<0,001

Naudota Spirmeno koreliacijos analizė: r – koreliacijos koeficientas. *P* vertė mažesnė nei 0,05 buvo vertinta kaip statistiškai reikšminga (*).

2 LENTELĖ. Plaučių pažeidimo histologinės skalės sumos vidurkio ir svorio pokyčio, nitritų koncentracijos, gripo viruso RNR, IFN-γ ir TNF-α bei iNOS iRNR pelių plaučiuose koreliacija penktą eksperimento dieną

Kintamasis	Histologinės skalės sumos vidurkis	
	r	*p* vertė
Svoris 5 dieną	-0,495*	0,002
Nitritai	0,355	0,05
Gripo viruso RB RNR	0,822*	<0,001
iNOS iRNR	0,594*	<0,001
IFN-γ iRNR	0,571*	<0,001
TNF-α iRNR	0,690*	<0,001

Naudota Spirmeno koreliacijos analizė: r – koreliacijos koeficientas. *P* vertė mažesnė nei 0,05 buvo vertinta kaip statistiškai reikšminga (*).

9. Išvados

1. Užkrėtus peles A/PR/8/34 gripo virusu, reikšmingai padidėjo TNF-α, iNOS iRNR raiška plaučiuose, nitritų kiekis BAL skystyje, sumažėjo pelių svoris bei plaučių pažaida buvo didesnė nei neužkrėstų pelių.
2. Gydant zanamiviru gripo virusu užkrėstas peles ankstyvas gripo viruso replikacijos ir NO gamybos plaučiuose slopinimas lėmė mažesnį plaučių pažeidimą ir pelių svorio netekimą.
3. Gydant gripu užkrėstas peles priešprasminiu oligonukleotidu prieš iNOS, viruso replikacijos slopinimas buvo ankstyvas, bet apsauginis plaučių pažaidos poveikis vėlyvas ir nesusijęs su NO gamybos plaučiuose slopinimu.

Padidėjusi citokinų raiška plaučiuose galimai buvo susijusi su reikšmingu pelių svorio mažėjimu.

4. Gydant gripu užkrėstas peles zanamiviro ir priešprasminio oligonukleotido prieš iNOS deriniu, viruso replikacijos slopinimas buvo ankstyvas, tačiau plaučių pažaidos apsauginis poveikis vėlyvas, susijęs su NO gamybos plaučiuose slopinimu. Mažesnė citokinų raiška galimai buvo susijusi su mažesniu pelių svorio netekimu.

10. Praktinės rekomendacijos

Tyrime nustatėme keletą naujienų. Pirma, gydant zanamiviru gripu užkrėstas peles, stebėjome ankstyvą NO gamybos slopinimą plaučiuose. Antra, gydant gripu užkrėstas peles priešprasminiu oligonukleotidu prieš iNOS iRNR, plaučių pažeidimas buvo mažesnis, o jo derinys su zanamiviru lėmė mažesnį plaučių pažeidimą, NO gamybą ir pelių svorio netekimą, todėl tikimasi, kad šis gydymo derinys galėtų praversti ateityje gripui gydyti. Tačiau lieka keletas neaiškių klausimų dėl vaistinių medžiagų veikimo mechanizmų, todėl ateityje būtų tikslinga atlikti išsamesnius tyrimus.

1. Kadangi vėlyvojo (penktos dienos) zanamiviro poveikio slopinti NO gamybą nebuvo nustatyta, reikėtų tolesnių tyrimų, kuriuose būtų naudojamos skirtingos zanamiviro dozės ir jų skyrimo schemos.
2. Priešprasminis oligonukleotidas prieš iNOS mūsų eksperimente turėjo ir priešvirusinį poveikį. Šio poveikio mechanizmas nėra aiškus, todėl reikėtų tęsti tyrimus.
3. Mūsų tyrime priešprasminis oligonukleotidas prieš iNOS reikšmingai NO sintezės neslopino, tačiau tikslingi ilgesnės trukmės tyrimai naudojant skirtingas šios vaistinės medžiagos dozes. Taip pat būtų tikslinga tirti ne tik iNOS iRNR raišką, bet ir iNOS baltymo koncentraciją pelių plaučiuose.
4. Nėra aiškus mechanizmas, kodėl priešprasminio oligonukleotido prieš iNOS gydymo grupėje buvo mažesnė uždegimo ląstelių infiltracija plaučiuose po užkrėtimo gripo virusu, todėl būtų naudingi tolesni patikslinamieji tyrimai.

Publikacijų ir pranešimų disertacijos tema sąrašas

Publikacijos recenzuojamuose žurnaluose

1. **Zablockienė B**, Ambrozaitis A, Kačergius T. Gripo gydymo galimybės ir ateities perspektyvos. *Vaikų pulmonologija ir alergologija,* 2017 (11); 1: 6208–6227.

2. **Zablockienė B**, Ambrozaitis A, Kačergius T, Žurauskas E, Bratchikov M, Gravenstein S. Gripo viruso sukelta azoto oksido gamyba pelių plaučiuose ir jos slopinimo metodas. *Vaikų pulmonologija ir alergologija,* 2017 (20); 2: 6306–6313.

3. **Zablockienė B**, Kacergius T, Ambrozaitis A, Zurauskas E, Bratchikov M, Jurgauskienė L, Zablockis R, Gravenstein S. Zanamivir Diminishes Lung Damage in Influenza A Virus-infected Mice by Inhibiting Nitric Oxide Production. *In Vivo* 2018; 32: 473–478.

Pranešimai vietiniuose ir tarptautiniuose mokslo reinginiuose

Doktorantė mokslinio darbo tyrimų rezultatus pristatė:

1. Dviejuose vietiniuose moksliniuose renginiuose:

1) **Zablockienė B**. Žodinis pranešimas „Gripo gydymas: naujos galimybės" konferencijoje „Apsinuodijimų ir infekcinių susirgimų sąlyčio taškai". 2017 m. vasario 9 d., Vilnius, Lietuva.

2) **Zablockienė B**. Žodinis pranešimas „Gripo virusai ir jų sukeliamos visuomenės sveikatos problemos" konferencijoje „Individualus požiūris į visuomenės sveikatą šaltuoju metų sezonu". 2017 m. spalio 7 d., Kaunas, Lietuva.

2. Keturiuose tarptautiniuose moksliniuose renginiuose:

1) Ambrozaitis A, **Zablockiene B**, Kacergius T, Zurauskas E, Gravenstein S. Prevention of influenza-virus induced pulmonary histopathology in mice using antivirals and antisense oligonucleotides to indicible nitric oxide synthase. Santrauka ir stendinis pranešimas konferencijoje „11th Annual Conference of the Baltic Network Against Life-Threatening Viral Infections". 2014 m. balandžio 24–27 d., Vilnius, Lietuva.

2) **Zablockiene B**, Kacergius T, Ambrozaitis A, Zurauskas E, Bratchikov M, Gravenstein S. Treatment with zanamivir and antisense oligonucleotides to inducible nitric oxide synthase decreases infuenza A virus-induced lunk inflamation. Santrauka ir stendinis pranešimas konferencijoje „Nordic Society of Clinical Microbiology and Infectious Diseases 2016". 2016 m. rugsėjo 15–18 d., Rovaniemi, Suomija.

3) **Zablockienė B**. Žodinis pranešimas „New perspectives in the therapy of influenza", konferencijoje „Baltic Young Infectologists Conference 2017", 2017 m. kovo 24–25 d., Ryga, Latvija.

4) **Zablockienė B**, Kacergius T, Ambrozaitis A, Zurauskas E, Bratchikov M, Gravenstein S. Zanamivir Reduces Lung Pathology by Inhibiting Nitric Oxide Production in Influenza A Virus-Infected Mice. Stendinis pranešimas konferencijoje „Life Science Baltics 2018", 2018 m. rugsėjo 26–27 d., Vilnius, Lietuva.

SUMMARY

Introduction

Influenza remains a serious public health problem that causes severe illness and death in high risk populations. The various studies associate severe disease with the development of influenza viral pneumonia (Taubenberger, Morens, 2008; Kuiken, Taubenberger, 2008) with or without secondary bacterial pneumonia (Morens et al., 2008; Klugman et al., 2009; Ruuskanen et al., 2011). Some of the pulmonary abnormalities of fatal influenza viral pneumonia might be induced by the release of host inflammatory mediators, rather than by a direct viral cytopathic effect (de Jong et al., 2006; Kash et al., 2006).

Influenza infection induces a cascade of host immune responses that involves production of proinflammatory cytokines and recruitment of inflammatory cells in infected lungs (Van Reeth, 2000; Jayasekera et al., 2006; La Gruta et al., 2007; Kumar et al., 2014; Short et al., 2014). Cytokines such as interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) stimulate the production of inducible nitric oxide synthase (iNOS) leading to high-output synthesis of nitric oxide (NO), predominantly in macrophages (Short et al., 2014; Akaike et al., 1996; Suliman et al., 2001; Bosca et al., 2005). In turn, the NO and excessive amounts of reactive nitrogen intermediates can lead to significant lung immunopathology (La Gruta et al., 2007; Short et al., 2014; Zaki et al., 2005). Reducing or preventing infection or NO production could prevent such immunopathology.

Animal experiments that affect iNOS function and NO production in host cells show therapeutic promise for the control of lung inflammation and damage from influenza (Jayasekera et al., 2006; Akaike et al., 1996; 2003). Suppression of virus spread within the respiratory tract by NIs could indirectly cause a reduction in iNOS expression and NO synthesis. NIs reduced NO generation in influenza virus-infected and IFN-γ-activated RAW 264.7 macrophage cell line *in vitro* (Kacergius et al., 2006). However, it is unknown whether NIs have the capacity to suppress net NO production during influenza virus infection and, in this way, reduce lung tissue damage under *in vivo* conditions.

A relatively new approach to inhibit iNOS activity involves antisense-mediated gene knock-down. In the present study the hypothesis that influenza A virus (IAV) induced lung inflammation in mice can be reduced using antisense oligodeoxynucleotide targeting iNOS mRNA or its combination with NI zanamivir was investigated.

Aim of the study

To evaluate if zanamivir and/or antisense oligonucleotide against inducible nitric oxide synthase prevents influenza viral pneumonia.

Objectives of the study

1. To evaluate the effect of A/PR/8/34 influenza virus infection of BALB/c mice on IAV RNA, IFN-γ, TNF-α and iNOS mRNA relative levels in lungs, nitrite concentration in bronchoalveolar lavage (BAL) fluid, weight changes and lung histological damage.

2. To evaluate the zanamivir's treatment effects on IAV RNA, IFN-γ, TNF-α and iNOS mRNA relative levels in lungs, nitrite concentration in BAL fluid, weight changes and lung histological damage with A/PR/8/34 influenza virus infection.
3. To assess the antisense oligonucleotide against inducible nitric oxide synthase treatment effects on IAV RNA, IFN-γ, TNF-α and iNOS mRNR relative levels in lungs, nitrite concentration in BAL fluid, weight changes and lung histological damage during A/PR/8/34 influenza virus infection.
4. To evaluate the combination of Zanamivir and antisense oligonucleotide against inducible nitric oxide synthase treatment effects on IAV RNA, IFN-γ, TNF-α and iNOS mRNA relative levels in lungs, nitrite concentration in BAL fluid, weight changes and lung histological damage during A/PR/8/34 influenza virus infection.

Scientific novelty of the study

Several scientific novelties are presented in the dissertation. First, the capacity of zanamivir to suppress net NO production during influenza virus infection and, in this way, to reduce lung tissue damage under *in vivo* conditions, was analysed for the first time. Second, the effect of antisense oligodeoxynucleotide to iNOS mRNA or its combination with zanamivir treatment of influenza infected mice has not been previously evaluated.

Practical value of the study

The neuraminidase inhibitors are currently the only option in most clinical settings, however they may be insufficient for seriously ill, high-risk patients or in influenza A(H5N1) disease. An increasing number of drug resistant viruses have been discovered recently. Thus there is a continuing need for new influenza therapies using novel targets and creative strategies. As treatment with antisense oligodeoxynucleotide to iNOS mRNA can reduce lung inflammation caused by influenza virus infection in mice, this more targeted approach, in combination with antiviral strategies, could be a potential new option in the treatment of influenza virus infection.

Defended statements of the dissertation

1. Infection of BALB/c mice with A/PR/8/34 influenza virus induces lung histological damage, which is associated with weight loss, higher expression of cytokines, iNOS mRNA and nitrite concentration in lungs.
2. Zanamivir reduces lung damage by suppressing IAV replication, with a result of less NO production in influenza infected mice.
3. Antisense oligodeoxynucleotide to iNOS mRNA reduces lung inflammation caused by influenza virus infection in mice by inhibiting NO production.
4. Zanamivir and antisense oligodeoxynucleotide to iNOS mRNA combination treatment diminishes lung inflammation by suppressing IAV replication and inhibiting NO production in influenza infected mice.

Materials and methods

Animals. Female BALB/c mice (6–12 weeks of age) weighing 18–20 g were obtained from the Charles River Laboratories, Inc., Wilmington, MA, USA. The mice were housed within microisolator cages in an isolation room, and all experimental procedures were performed in a biosafety cabinet using biosafety level 2 containment. The animals were anesthetized by intraperitoneal injection a mixture of ketamine (50 mg/kg) and xylazine (50 mg/kg) before every procedure. All protocols involving experiments with mice were approved by the Institutional Animal Care and Use Committee, Eastern Virginia Medical School, Norfolk, VA, USA (permit no. 07-003).

Influenza virus infectivity. The mouse-adapted influenza A/Puerto Rico/8/34 (A/PR/8/34) (H1N1) virus was propagated in the allantoic cavities of 10-day-old embryonated chicken eggs as previously described (Lennette, 1995). For the determination of influenza A/PR/8/34 virus infectivity titer, confluent monolayers of Madin-Darby canine kidney cells (American Type Culture Collection No. CCL-34, Manassas, VA, USA) were inoculated with the virus stock of serial 10-fold dilutions. The 50% tissue culture infectious dose ($TCID_{50}$) was evaluated according to the extent of viral cytopathic effect in monolayers after 96 h. The virus infectivity titer was calculated using Kärber method, and it was determined as $10^{8.4}$ $TCID_{50}$/mL (Lennette, 1995). Afterwards, different serial dilutions of the virus stock were tested on mice by challenging them with a single 20 μL dose of the virus suspension (10 μL/nostril) for five days in order to determine the virus infectivity titer that produces a significant infection without causing death. According to the body weight loss and survival rate of mice, the virus infectivity titer of $10^{7.6}$ $TCID_{50}$/mL was determined and selected for further experiments.

Compounds. Zanamivir (5-(acetylamino)-4-((aminoiminomethyl)-amino)-2,6-anhydro-3,4,5-trideoxy-D-glycero-D-galacto-non-2-enonic acid) was obtained from GlaxoSmithKline, Research Triangle Park, NC, USA as drug Relenza™ consisting of a powder mixture of 5 mg of zanamivir and 20 mg of lactose per blister. The antisense oligonucleotide to iNOS was synthesized as an unmodified oligodeoxyribonucleotide (ODN) with phosphodiester internucleotide linkages at Sigma-Aldrich, St. Louis, MO, USA. The antisense ODN sequence comprised of 21 nucleotides: 5′-CAAGCCATGTCTGAGACTTTG-3′, corresponding to bases 1 through 21 of the translation initiation site of mouse iNOS mRNA, as reported by Ding et al. (Ding et al., 1998). Lactose was obtained from Sigma-Aldrich, St. Louis, MO, USA. The compounds were prepared in sterile phosphate-buffered saline (PBS), pH 7.4, for intranasal administration of 20 μL (10 μL/nostril) to the mice. The dosage of zanamivir (2 mg/kg) and antisense ODN (5 mg/kg) for the mice was based on therapeutic dose ranges of these compounds provided in the literature (Ryan et al., 1994; Tarbet et al., 2014; Li et al., 2014; Ding et al., 1998; Templi et al., 2000). In this regard, the doses of zanamivir and antisense ODN for the local delivery to target tissues are in the ranges of 0.3–12.5 mg/kg and 5–20 mg/kg, respectively. The dose of lactose (placebo) was 8 mg/kg. The compounds were administered in a single-dose regimen to minimize the animal distress caused by the treatment procedures.

Experimental design. The anesthetized mice were inoculated intranasally with 20 μL of influenza A/PR/8/34 virus suspension containing infectivity titer of $10^{7.6}$ $TCID_{50}$/mL. At

3 h post-infection, mice were treated daily with 2 mg/kg dose of zanamivir, 5 mg/kg dose of antisense ODN or a combination of zanamivir (2 mg/kg) plus antisense ODN (5 mg/kg) for 3 or 5 days. These doses of zanamivir and antisense ODN were administered to the anesthetized mice in 20 μL of PBS using a single-dose regimen. The negative controls were uninfected mice treated with either 20 μL of PBS, lactose, zanamivir, antisense ODN or combination of zanamivir and antisense ODN in the same dosages. The positive control groups consisted of infuenza infected only, infected and 20 μL of PBS or lactose treated mice in the same dosages. Mice were monitored daily for morbidity, as measured by weight loss. After 3 or 5 days, mice were euthanized by axillary bleeding (terminal exsanguination), and then the lungs of mice were excised and subjected to bronchoalveolar lavage (BAL). For the BAL procedure, the trachea was cannulated and the lavage of lungs was performed twice with 1 mL of PBS. The collected BAL fluids were centrifuged at 735 × *g* for 5 min, and the supernatants were frozen at –80 °C until determination of nitrite levels. Immediately after the BAL procedure, the left lung was placed in 4% paraformaldehyde (Thermo Fisher Scientific, Waltham, MA, USA) at 4 °C until processing for histological examination. The right lung was immersed in the TRIzol™ reagent (Thermo Fisher Scientific, Waltham, MA, USA) and homogenized on dry ice by using a Tissue Tearor (Dremel, Racine, WI, USA). The lung tissue homogenates were centrifuged at 12 000 × *g* for 5 min at 4 °C, and the supernatants were frozen at –80 °C. Afterwards, they were used for isolation of RNA followed by determination of the levels of viral RNA, iNOS and cytokine (*i.e.*, IFN-γ, TNF-α) mRNA applying a multiplex real-time quantitative reverse transcription (qRT)-PCR.

Nitrite determination. The levels of nitrite (NO_2^-), an indicator of NO synthesis in biological systems, were determined in BAL fluids using a modified Griess reagent (Sigma-Aldrich, St. Louis, MO, USA) as directed by the manufacturer. A microplate-reader spectrophotometer PowerWave X 340 (BioTek Instruments, Inc., Winooski, VT, USA) was employed to measure the optical density (OD) of BAL fluid samples at a wavelength of 540 nm. Nitrite concentrations were calculated using the KC4 software, version 2.5 (BioTek Instruments, Inc., Winooski, VT, USA), by comparison OD of the samples containing formed NO_2^- to a standard curve of sodium nitrite ($NaNO_2$). The lower limit of NO_2^- detection was 0.12 μmol/mL, and the upper limit of NO_2^- detection was 15.63 μmol/mL.

Histological examination. The harvested lung tissues were embedded in paraffin, cut into 5 μm sections and stained with hematoxylin and eosin (H&E). Coded lung samples were examined using light microscopy by a pathologist in the blinded approach. The following indicative parameters of pulmonary histopathology were evaluated: perivascular lymphocytic, peribronchial lymphocytic, focal and diffuse leucocytic infiltrations; praesense of alveolar macrophages; damage of alveolar/bronchial epithelium; hyperemia; capillary thromboses; bronchiolitis; pleuritis; alveolar hemorrhage, collapse, fibrosis, edema and hyaline membranes. Each of these parameters was graded subjectively by scoring on a scale from 0 (absence of pathological changes) to 3 (maximum extent of pathological changes), and the sum of scores comprised the histological score for each mouse.

RNA isolation and multiplex real-time quantitative reverse transcription (qRT)-PCR. Total RNA was isolated from lung homogenates using TRIzol™ reagent according to the instructions provided by Thermo Fisher Scientific, Waltham, MA, USA. The levels of viral RNA (encoding influenza A virus (IAV) polymerase acidic (PA) protein), iNOS, IFN-γ, TNF-α and beta (β)-actin mRNA were determined by the multiplex real-time qRT-PCR using SensiFAST™ Probe No-ROX One-Step Kit (Bioline Reagents Ltd, London, UK). Primers and hydrolysis probes were synthesized by Biolegio B.V., Nijmegen, Netherlands. Primer and probe sequences used in the multiplex real-time quantitative reverse transcription (qRT)-PCR were: IAV PA forward primer 5′-TCTCAGCGGTCCAAATTCCTGC-3′, reverse primer 5′-GGTTTAACAACATTGGGTTCCTTCCAT-3′, probe TGAGGACC-CAAGTCATGAAGGAGAGGGA -BHQ1-3′, amplicon size 146 bp; iNOS sequences were: forward primer 5′-CTTGTGCTGTTC TCAGCCCAACAATA-3′, reverse primer 5′-TGGA-ACATTCTGTGCTGTCCCAG-3′, probe 5′-FAM-TGGCTCCCCGCAGCTCCTC-BHQ1-3′, amplicon size 95 bp; IFN-γ forward primer 5′-GCCAAGTTTGAGGTCAACAACCC-3′, reverse primer 5′-TGGAACATTCTGTGCTGTCCCAG-3′, probe 5′-FAM-TCATCCGA-GTGGTCCACCAGCTGT -BHQ1-3′, amplicone size 101 bp; TNF-α forward primer 5′-CCAGACCCTCACACTCAGATCATC-3′, reverse primer 5′-CTCCTCCACTTG GTGGTTTGCTAC-3′, probe 5′-HEX-TCGAGTGAC AAGCCTGTAGCCCACGT-BHQ1-3′, amplicon size 85 bp; β-actin forward primer 5′-GCACMATGAAGATCAA-GATCATTGCTCC-3′, reverse primer 5′-TCRTACTCC TGCTTGCTGATCCAC-3′, probe 5′-ROX-TCCTGGCCTCRCTGTCCACCTTCC-BHQ2-3′, amplicon size 118 bp. All multiplex one-step reactions were carried out in a total volume of 15 μL using 1 μL of the isolated RNA sample and primers at a concentration of 200 nM each, and probes at a concentration of 100 nM each. The multiplex one-step qRT-PCR assays were performed employing a real-time thermocycler Rotor-Gene Q 5plex model with software version 1.7 (Qiagen GmbH, Hilden, Germany) under the following conditions: first strand cDNA was synthesized at 48 °C for 10 min and then denatured at 95 °C for 2 min; followed by 50 cycles of denaturation at 95 °C for 10 s and annealing/extension at 60 °C for 1 min. The expression of β-actin mRNA was used as an internal standard for normalization of the viral PA RNA and target mRNA levels between different samples. The $2^{-\Delta\Delta C_T}$ algorithm was applied for calculation of the relative quantities of PCR amplification product reflecting the relative levels of viral PA RNA and target mRNA (Livak et al., 2001).

Statistical analysis. Data were analyzed using the Statistical Package for Social Science (SPSS) software version 21.0 for Windows (IBM Corp., Armonk, NY, USA). Differences between the experimental groups were evaluated applying one-way analysis of variance (ANOVA) followed by a *post hoc* least significant difference (LSD) test for multiple comparisons. Data are presented as means ± standard error of the mean (SEM). Spearman's correlation analysis was used for evaluation of correlation between the variables. A p value less than 0.05 was considered to indicate a statistically significant difference.

Results

Relative influenza A viral RNA levels in mouse lungs. IAV PA RNA was not detected in negative control mice whilst increased viral loads were present in the lungs of all groups of IAV-infected mice as quantified by multiplex one-step RT-qPCR assay on day 3 *p.i.* Between the two infected (positive) control groups, the infected group treated with lactose had significantly (p=0.013) higher IAV PA RNA level compared with the group that was infected without any treatment. All non-control treatment groups showed significantly less relative IAV PA RNA level vs. IAV infected lactose treated mouse group (p=0.016, p<0.001, p=0.017 respectively). IAV infected and zanamivir or zanamivir + ODN treated mouse viral loads were similar, however IAV infected and ODN treated mice relative IAV PA RNA levels were significantly smaller vs. IAV + zanamivir treatment group (p=0.039).

On day 5, viral PA RNA was not detected in negative mouse control group and increased viral loads were present in the lungs of all groups of IAV-infected mice. Of the positive control group, the infected group treated with lactose had significantly higher IAV PA RNA level compared with infected-only (p=0.012) or infected and PBS treated (p=0.021) mouse groups. All treatment groups showed similar IAV PA RNA levels and were significantly less vs. the IAV infected and lactose treated mouse group (p=0.017, p=0.013, p=0.013 respectively).

Relative IFN-γ mRNA levels in mouse lungs. The relative IFN-γ mRNA level was 55.1% higher in positive (infected) versus negative (uninfected) control groups on day 3, however this difference did not reach significance. In infected and zanamivir treated mouse lungs IFN-γ mRNA level did not differ significantly from control groups. However, virus + ODN treatment group had the significantly more IFN-γ mRNA expression vs. both control groups (p=0.001 vs. negative control and p=0.034 vs. positive control), and virus + Zanamivir + ODN treatment group - vs. negative control group (p=0.005).

Like on the 3rd day, the relative IFN-γ mRNA level was 72.0% higher in positive versus negative control groups on day 5, but this difference did not reach the significance. In all treatment groups significantly higher IFN-γ mRNA expressions vs. negative control were found (p=0.006 in infected and zanamivir treated, p=0.001 in infected and ODN treated and p=0.01 in infected and zanamivir + ODN treated mouse lungs). The highest relative level of IFN-γ mRNA was found in Virus + ODN treated mouse lungs, it was 20.7% higher than in Virus + Zanamivir treatment group and significantly higher vs. positive control group (p=0.04) mouse lungs.

Relative TNF-α mRNA level in mouse lungs. The expression of TNF-α mRNA was 84.2% greater in positive than in negative control groups (p<0.001). Mouse lung TNF-α mRNA expression was similar in all IAV infected treatment groups and the infected control group (p=NS), but significantly higher than in the negative non-infected control groups (p=0.003, p<0.001, p=0.006 for infected treatment of zanamivir, ODN and zanamivir + ODN combination, respectively).

On the 5th day of the experiment, the relative TNF-α mRNA level was 76.9% higher in positive than in negative control groups (p=0.001). TNF-α mRNA expression was higher in all IAV infected treatment groups vs. negative control, (p=0.004 in infected and zanamivir

treated, $p<0.000$ in infected and ODN treated, and $p=0.019$ in infected and zanamivir + ODN treated mouse lungs). However, the highest relative level of TNF-α mRNA was found in Virus + ODN treatment group mouse lungs: it was 53.0% higher than in Virus + Zanamivir treatment group ($p=0.001$) and higher vs. positive control group ($p<0.001$) mouse lungs. The level of TNF-α mRNA in combination treatment group was significantly smaller ($p<0.001$) than in IAV+ODN and had a tendency to lessen (17.0% decrease) compared to IAV+Zanamivir treatment groups.

Relative iNOS mRNA level in mouse lungs. IAV infection caused significant up-regulation of the iNOS gene by day 3, resulting in 54.0% higher mRNA levels in mice from the positive control group vs. the negative control group ($p=0.001$). The greatest effect in reducing iNOS mRNA level between the treatment groups was observed in the IAV+Zanamivir treatment group (31.3% smaller vs. positive control group) and was similar to that found in the negative control group. In the IAV+ODN treatment group, iNOS mRNA level was 22.2% lower as compared to the positive control group and also was similar to the negative control group. The iNOS mRNA expression in IAV infected and zanamivir + ODN was similar to the positive control group and significantly higher than that found in the negative control group mouse lungs ($p=0.005$).

Significantly higher relative levels of iNOS mRNA were detected in the positive vs. the negative control group on the 5th day of the experiment (87.8%, $p<0.001$). IAV-infected and zanamivir or ODN treated mice showed significantly higher iNOS mRNA expression vs. negative control group ($p<0.001$ and $p=0.002$ respectively). Although the relative levels of iNOS RNA were less in the Virus+ODN mouse group on the 5th day (8.5% decrease) compared to the positive control group, it did not reach significance. The infected and zanamivir + ODN treated mouse groups there were similar to the negative control group. Between the treatment groups, the highest relative iNOS mRNA level was found in the zanamivir treatment group. The level was 23.7% less in the Virus+ODN treatment group, and 57.2% ($p=0.027$) in the Virus+Zanamivir+ODN treatment group vs. the Virus+Zanamivir treatment group.

Nitrite concentration in BAL fluid. The enhanced NO synthesis in the lungs was observed in influenza infected (positive control) mice vs. uninfected (negative control) on day 3 ($p<0.001$). NO concentration was significantly higher in influenza infected and ODN ($p=0.001$) or Zanamivir+ODN ($p=0.007$) treatment groups compared to negative control group. In the group of influenza infected and zanamivir treated mice the concentration of NO was significantly smaller vs. positive control group (57.5%, $p=0.001$), and vs. treatment groups with ODN (54.1%, $p=0.005$) or Zanamivir+ODN (46.9%, $p=0.026$).

Similarly to day 3, on day 5 the enhanced NO synthesis was observed in influenza infected (positive control) mice vs. uninfected (negative control) ($p=0.003$). In influenza infected and Zanamivir+ODN treatment group mouse lungs had significantly lower nitrite concentrations as compared to those in the positive control group (39.3%, $p=0.005$).

Body weight changes. The positive mouse control group (influenza infected) lost significantly more weight vs. negative mouse control group (uninfected) starting from day 2. The infected and zanamivir treated mouse weight loss (9.4%) was smaller than for the positive

control group (15.0%, p=0.002) and the influenza+ODN treatment group (14.3%, p=0.009) on day 3; there was no significant difference by day 5 (14.2%, p=0.22). The infected and ODN treated mice lost weight similarly to the positive mouse control group, and differed significantly from the negative mouse control group on days 3 (14.3% and 8.9%, p<0.001) and 5 (16.9% and 10%, p=0.02). The infected and zanamivir + ODN combination treated mouse group on day 3 lost 12.0% of their weight, more than the negative mouse control group, which lost only 8.9%, (p=0.037). Interestingly, mice in the combination treatment group started to gain weight, and by day 5 their weight loss was only 7.2%, significantly more than the positive mouse control group (7.7%, p=0.001) and the Zanamivir treatment group mice (14.2%, p=0.036).

Mouse lung histopathology. The histological score was significantly greater in the positive control vs. the negative control mouse group lungs (p<0.001). There histological score was essentially unaffected in the negative control group, however inside the positive control group, the IAV-infected and the untreated mouse group had histological lung damage scores larger than that of the IAV-infected and lactose treated mice (p=0.007). The score in IAV-infected and zanamivir treated mice was significantly smaller (p=0.018), but there were no differences in the IAV-infected and ODN or zanamivir plus ODN treated mice vs. the positive control mouse group.

On the 5th day, the histological score was significantly higher in the positive control vs. the negative control mouse group lungs (p<0.001). There were no differences within either the negative (non-infected) or the positive (infected) control groups. All treatment groups' (IAV-infected and zanamivir treated, p=0.006; IAV-infected and ODN treated, p=0.003, and IAV-infected and zanamivir plus ODN treated, p=0.001) mean cumulative histological scores were significantly smaller than that of the positive control mouse group.

The correlation between lung damage and analysed parameters. A strongly positive correlation was found between lung damage score and TNF-α mRNA relative levels in mouse lungs. In addition, the mean cumulative histological score was found to be moderately positively correlated with relative nitrite concentration in BAL fluid, IAV PA RNA, iNOS mRNA and IFN-γ mRNA levels in mouse lungs and negatively correlated with mouse weight on day 3.

Strongly positive correlations were found between the lung damage score and the relative IAV PA RNA and TNF-α mRNA levels in mouse lungs. The mean cumulative histological score was found to be moderately positively correlated with iNOS mRNA and IFN-γ mRNA relative levels in mouse lungs and moderately negatively correlated with mouse weight on day 5.

Conclusions

1. Infection of mice with A/Puerto Rico/8/34 influenza virus induced lung inflammation, which was associated with significantly greater TNF-α mRNA, iNOS mRNA expression in lungs, higher NO concentration in BAL fluid, and more mouse weight loss.

2. Treatment of influenza infected mice with zanamivir caused the early suppression of viral replication and NO synthesis, and it was associated with less lung damage and weight loss.
3. The suppression of viral replication was early, but the protective lung damage effect was late and not associated with inhibition of NO synthesis in lungs in the treatment of influenza infected mice with antisense oligodeoxynucleotide to iNOS group. The increased expression of TNF-α mRNA was associated with significant mouse weight loss.
4. Treatment of influenza infected mice with the combination of zanamivir and antisense oligodeoxynucleotide to iNOS resulted in early suppression of viral replication, but the protective lung damage effect was late and associated with inhibition of NO synthesis in lungs. The lower expression TNF-α mRNA could explain reduced weight loss.

Practical recomendations

The study demonstrated several new findings. First, that treatment with zanamivir posesses the early capacity to suppress NO synthesis in mouse lungs after influenza infection. Second, that treatment with antisense oligodeoxynucleotide to iNOS mRNA can reduce lung inflammation caused by influenza virus infection in mice, and its combination with zanamivir has clinical benefit; thus, this treatment approach could be a potential new option in the treatment of influenza virus infection. However, there are several unresolved questions to fully understand the mechanisms of action of medications and additional studies should may be worthful.

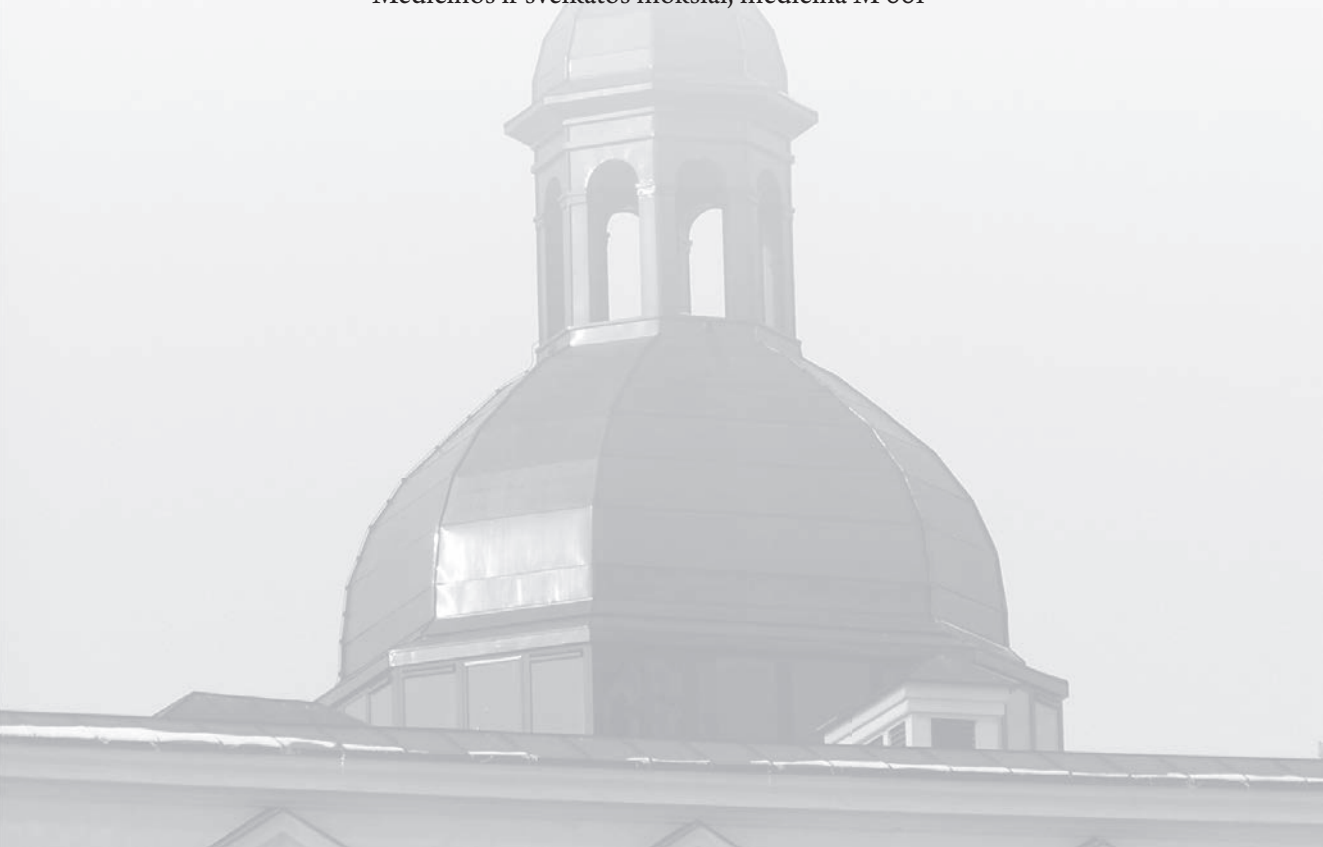
1. As the later effect (on day 5) of zanamivir's capacity to suppress NO synthesis was not observed, further experiments with different doses and schemes of administration of zanamivir are needed.
2. The observed antiviral mechanism of antisense ODN to iNOS in our experiment is not fully clear and should be evaluated in further studies.
3. We found no significant effect of antisense ODN to iNOS in suppressing NO synthesis, but different doses of medication and treatment and testing for additional days should be studied. Detection of not only iNOS mRNA level, but the iNOS protein concentration in mouse lungs should also help our understanding.
4. The exact mechanisms of the antisense ODN to iNOS-dependent potential to reduce inflammatory cells accumulation in mouse lungs post-influenza infection are unknown and should be evaluated in further studies.

Agnė Jagelavičienė

RELATION BETWEEN VACCINATION AND ALLERGIC DISEASES IN PRESCHOOL CHILDREN IN EVERYDAY PRIMARY CARE PRACTICE

IKIMOKYKLINIO AMŽIAUS VAIKŲ SKIEPIJIMO IR ALERGINIŲ LIGŲ SĄSAJOS KASDIENĖJE AMBULATORINĖJE PRAKTIKOJE

Medicine and Health Sciences, Medicine M 001
Medicinos ir sveikatos mokslai, medicina M 001



Abbreviations

AB	Antibiotics
AD	Atopic dermatitis
AR	Allergic rhinitis
BA	Bronchial asthma
PCP	Primary care practitioner
BCG	Tuberculosis vaccine: *Bacillus Calmette–Guérin*
CCDA	Center of Communicable Diseases and AIDS
DTaP-IPV-Hib, DTaP	Diphteria, tetanus, acellular pertussis – inactivated poliomielytis – *Haemophilus influenzae b* vaccine
DTaP1	First dose of the DTaP-IPV-Hib vaccine
HB	Hepatitis B vaccine
MMR	Measles, mumps, rubeola vaccine
NIPC	
PCP	National Immunoprophylaxis Calendar
Primary care practitioner	
SPT	Skin prick test

Introduction

The rise in the prevalence of allergic diseases worldwide during the recent decades is a concerning issue. The influence of genetic factors on the development of allergic diseases (bronchial asthma, allergic rhinitis, atopic dermatitis) is unquestionable. The growth of allergic sensitization and allergic diseases in society has been actively analyzed in the context of environmental factors (1). The first vaccines fall in the period of the "window of opportunity" for the prevention of allergic diseases – it lasts from conception to the sixth month of life (2–4). As a result, immunization, as a planned target effect on a particular response of the immune system during infancy and as an additional risk factor for the development of allergic diseases, peaks the interest of scientists and the public.

The methodologies of studies that analyze the link between vaccination and allergic diseases are different and difficult to compare; the conclusions are controversial as well (5–10). During the analysis of the link between vaccination and allergic diseases, a hypothesis has been made that vaccination may disturb the balance of cytokines and direct the response of the immune system toward T_H2 and, at the same time, stimulate the excretion of immunoglobulin E (IgE), including the progress of reactions associated to it, to usually harmless environmental stimuli (allergens) (11, 12). This hypothesis was refuted, and evidence was provided that vaccination affects a specific part of the immune system and leads only to the production of cytokines related to the specific vaccine that has no significant effect on the development of allergies or atopy (13).

Although during the recent years the focus on vaccination of allergic children has increased across the global scholarly literature, this problem still lacks sufficient attention in Lithuania. Physicians and parents still have doubts as to whether allergic diseases that have manifested shortly after vaccination or coincided in its time are related to the vaccination or not. The frequency of the registered adverse events after vaccination in Lithuania is not higher than in other countries, but there still is some fear that allergic children have a higher risk of side effects after vaccination, and this may lead to untimely and incomplete vaccination (14). There are no contraindications for the vaccination of allergic children, but the absence of clear guidelines at the national level allows the uncertainty and beliefs – not substantiated by scientific evidence – to flourish and permits the spread of fake news.

Relevance and Scientific Novelty

According to the position paper of the European Academy of *Allergists* and Clinical Immunologists (2017), there still is a lack of data from prospective and retrospective studies on the potential impact of vaccines on the development of allergies. There is a shortage of validated studies or tests allowing to predict and define the clinical expression of the adverse reactions to vaccines (5, 15, 16). There is also a lack of research on how immunization time is related to the development of allergic diseases. The changing composition of the vaccines and their production processes that are being improved prompt researchers to re-examine their safety, efficacy, and links with various diseases, including allergic ones (17).

The relation between vaccination and allergic diseases in Lithuania was analyzed only in one prospective study of a small sample and one retrospective study of statistical data (18, 19). There were no studies in Lithuania that analyzed whether the frequency and severity of post-vaccination reactions are the same among children with allergic diseases and children without allergic diseases. There are not many such studies in the world. However, they are necessary and relevant in order to assess and define the safety of the immunization of allergic children.

Our work is a "real-life" study that reveals the links between vaccination and allergic diseases diagnosed by primary health care phycisians. Real-life studies reflect everyday issues and allow scholars to look into the problems of clinical practice and search for solutions. We have established that atopic dermatitis, diagnosed by a general practitioner or pediatrician during infancy, is associated with the disorders of the routine immunization plan. This study is the first analysis of the links between vaccination and allergies in Lithuania that uses a digital statistical database of a primary health care institution. On the global scale, digital databases are identified as a potential tool for the recording and analysis of the adverse events of vaccination.

The symptoms that manifest shortly after vaccination, similar to those associated with allergic diseases, can be directly linked to the vaccination; therefore, the precise definition, registration, and testing of these events are of paramount importance (20).

Primary prevention of infectious diseases plays a crucial role in children's health. Allergic children deserve access to the same publicly recommended immunizations as non-allergic patients. Our work has added insights into the ongoing discusion about the risks of allergy developent due to vaccination. A comprehensive analysis of the relationship between vaccination and allergic diseases in outpatient practice conducted in our study has revealed how different and complementary to each other outcomes for the same population can be.

Aim of the Study

To thoroughly analyze the links between vaccination and allergic diseases diagnosed under real working conditions in a primary healthcare facility during the preschool age, and to assess the safety of vaccinating children diagnosed with atopic dermatitis.

Objectives

1. Evaluate the overall frequency of allergic diseases diagnosed at a primary health care institution among vaccinated, partially vaccinated, and non-vaccinated children according to a routine immunization schedule.
2. Analyze the links between atopic dermatitis, allergic rhinitis, or bronchial asthma, diagnosed by primary care practitioners and specialists, and the disorders of a routine immunization schedule.
3. Analyze the links between the start of vaccination during the first 6 months of life and the allergic diseases diagnosed under 6 years of age.
4. Compare the frequency of adverse reactions after vaccination according to the National immuno-prophylaxis calendar among children diagnosed with atopic dermatitis and children with no diagnosed allergic diseases.
5. Determine whether vaccination is a possible independent factor significantly related to the development of allergic diseases.
6. Determine what other factors, acting in parallel with vaccination, are associated with the development of allergic diseases.

Propositions to be Defended

1. Allergic diseases are not more frequently diagnosed in fully vaccinated children in comparison to non-vaccinated or partially vaccinated children.
2. The vaccination of children diagnosed with atopic dermatitis is safe.
3. Routine immunization is not independently associated with the frequency of diagnosing allergic diseases in everyday primary healthcare practice.

1. Materials and methods

The study was conducted from 2015 to 2017 in the Central Polyclinic of Vilnius – partner of the Clinic of Children's Diseases at the Institute of Clinical Medicine of the Faculty of Medicine of Vilnius University.

An authorization to conduct the study was received from the Vilnius Regional Biomedical Research Ethics Committee (09-01-2015 No. 158200-15-757-284) with a supplement to the authorization (04-07-2017 No. 158200-757-PP1-22).

1.1 Phase I: Analysis of the Primary Health Care Institution's Statistical Database

Statistical data about 1294 children born in 2009, retrieved from the polyclinic's database "MEDIS," were analyzed. The following data about the research subjects were collected: month of birth, gender, diagnoses of allergic diseases determined by primary care practitioners and physicians-specialists before the children had reached six years of age; periods of their determination (up to 1 year, from 1 to 2 years, from 2 to 6 years, and overall – from birth to 6 years); the vaccination plan (inoculated vaccines, the time of inoculation, and doses), number of visits to PSP and specialists (pediatric pulmonologist, allergist, otorhinolaryngologist). Visits to physicians were picked according to codes of health care specialist services. Diagnoses were picked according to the codes of ICD-AM-10. Only data that have been included in statistical forms of medical histories were retrieved from the electronical database.

Allergic Diseases

Based on the diagnostic codes specified in the protocols for "Diagnosis and Treatment of Children's Atopic Dermatitis," "Diagnostics and Treatment of Children's Allergic Rhinitis," "Diagnostics and Treatment of Children's Bronchial Asthma" of Lithuania (21–23), diagnoses of alergic diseases were selected from the CP database according to the International Classification of Diseases (ICD-10), diagnosed by physicians under actual working conditions of the primary health care institution. The following disease codes have been selected: atopic dermatitis (L20.8, L20.9), asthma (J45.9, J45.0, J45.1, J45.8), and allergic rhinitis (J30.1, J30.2, J30.3, J30.4). Diagnoses of atopic dermatitis have been analyzed in both of such cases: diagnosed by PCP or verified by an allergist; asthma – only if verified by a pediatric pulmonologist; allergic rhinitis – verified by an allergist or otorhinolaryngologist. The diseases with the asthma code (J45.9) in patients under one year of age in our study were named the wheezing diseases. All specialists had consulted children at

the same polyclinic after referral from PCP. In order for the study to meet the actual working conditions of the primary health care institution as best as possible, we did not recheck the diagnoses determined by physicians.

1.1.1 Differences in the Scope of Vaccination of Allergic and Non-Allergic Children up to 2 Years and up to 6 Years of Age

In order to assess the differences in the vaccination of allergic and non-allergic children, we checked whether children diagnosed with allergic diseases (atopic dermatitis, allergic rhinitis, and bronchial asthma) are in general different from children who did not have these diseases based on the number of doses of vaccines inoculated before 2 years of age and before 6 years of age. We subsequently analyzed whether vaccination with individual vaccines according to the routine immunization schedule is related to the development of allergic diseases (AD, AR, BA) from birth to 6 years of age.

Children born in 2009 were vaccinated according to the Lithuanian Children's Prophylactic Vaccination Calendar, which was in force at that time based on the National Immunoprophylaxis Program for 2009–2013 (24). According to it, all children in CP were vaccinated with vaccines supplied in a centralized manner: BCG (BCG, SSI, Denmark) – within the first 24 hours, HB (Engerix B, GSK) – 2–3 days, 1 month, and 6-months-old, DTaP-IPV-Hib (Infanrix-IPV + Hib, GSK) – 2, 4, 6, and 18 months, MMR (Priorix, GSK) – 15–16.5 months.

According to the general scope of vaccination, patients were classified into groups. (1) Fully vaccinated – children who completed the vaccination schedule before the age of 2 years: one dose of the tuberculosis (BCG) vaccine, three doses of the hepatitis B vaccine (HB1, HB2, HB3), four doses of the DTaP-IPV-Hib vaccine, as well as first dose of the measles, mumps, and rubella vaccine (MMR1). (2) Partially vaccinated: before 2 years of age – children vaccinated according to the schedule but not inoculated with all vaccines specified in the calendar before 2 years of age. (3) Non-vaccinated children: not vaccinated with any of the state-reimbursed vaccines according to the immunization calendar before 2 years of age.

Respectively, children were classified based on receiving vaccination under 6 years of age.

1.1.2 Links of Diagnosed Allergic Diseases with the Time of Each Vaccine Inoculation and the Number of Doses Inoculated

Number of Doses of Vaccine Received before 7 Months of Age

In order to evaluate the links between the disorders of the routine immunization schedule under 7 months of age and allergic diseases, the population was

analyzed for each vaccine separately. There were 36 non-vaccinated children with BCG. Depending on the received HB vaccine doses, the children were divided into groups of those completely vaccinated with three doses of HB, partially vaccinated, and non-vaccinated with HB under 7 months of age. According to the inoculated doses of the DTaP-IPV-Hib vaccine, children were devided as follows: completely vaccinated – received all 3 doses of the vaccine; partially vaccinated – received one or two doses over the same period; non-vaccinated – did not receive any dose of the DTaP-IPV-Hib vaccine before 7 months of age.

Starting Time of Vaccination

According to the vaccination with the BCG vaccine, the population of the research subjects was divided into groups as follows: vaccinated on time (at the maternity hospital) or with a 1 month delay. According to the vaccination with the HB vaccine: the group of children who had received the first dose on time (within the first day) and those vaccinated 1 month later. According to the vaccination with the DTaP-IPV-Hib vaccine: vaccinated with the first dose on time (at 2 months of age), with a 1 month delay, i.e., at 3 months of age, and with the vaccination delayed until 4–6 months of age.

1.2 Phase II: Analysis of Medical Histories. Case-Control Study

The medical histories of 289 of children born in 2009 who were diagnosed by specialists with allergic diseases (atopic dermatitis, allergic rhinitis, allergic asthma) were selected from the database (relying on the ICD-AM-10 classification). For the control group, 269 children who had no recorded allergic diseases were selected. A total of 558 medical histories were selected for a retrospective case-control study. The selection of the research sample subjects was based on the availability and completeness of data and the presence of a clear diagnosis of an allergic disease verified by a specialist.

1.2.1 Analysis of Reactions after Vaccination

In order to determine whether vaccination is equally safe for allergic and non-allergic children, we compared the frequency of recorded reactions after vaccination in children with atopic dermatitis diagnosed by an allergist (n=250) and in children who had no diagnosis of any allergic disease (n=269).

Only children with atopic dermatitis with or without diagnosis of asthma or allergic rhinitis were included into analysis. We decided not to include children who have been diagnosed only with allergic rhinitis or asthma, because the period of experienced post-vaccination reactions occurs earlier than the manifestation of these diseases, as opposed to the atopic dermatitis prevalent during the first year of life, when the vaccination is most intense.

The following definition of an adverse reaction after vaccination was used: it is a temporary or permanent health condition disorder that causes changes in physical signs, symptoms and/or laboratory parameters, that commence after immuno-prophylaxis and is believed to be caused by it.

1.2.2 Vaccination as a Risk Factor of Allergic Diseases

We intended to examine whether, in addition to already known factors (allergic anamnesis of the family, harmful environmental effects, factors affecting the development of the fetus in the uterus and during the first months of life, etc.), immunization could be associated with the risk of allergic diseases diagnosed in a primary health care facility. We included into our analysis the method of delivery (natural or SC), birth weight, breastfeeding and its duration, number of siblings, prescription of antibiotics (prescription time, number of prescribed antibiotic courses in different age periods), the mother's age, the presence of other allergic diseases.

Research subjects were classified into groups according to the allergic diseases: AD, BA, and AR. People who were never diagnosed with any allergic disease were deemed non-allergic.

2. Statistical analysis

Statistical data analysis was performed using the computer software SPSS (21.0 version) statistical data analysis package (IBM, USA) and Microsoft Excel 2013.

The distribution of the data of quantitative variables was tested by using the Shapiro-Wilk test. Differences in the averages of the independent variables distributed normally were measured by using the t test, or the Mann-Witney U test, if the assumption of normality was not met. Averages ± standard deviation were calculated for quantitative variables distributed according normal distribution; in other cases, the median with a quartile difference was calculated.

The spread of the categorical variable frequency was compared using the Chi-square or Fisher's exact tests. The odds ratio and the 95% confidence interval were calculated for the analysis of binary variables.

The possible influence of factors, its size, and significance for the diagnosis of allergic diseases were initially analyzed in models of univariate binary logistic regression. Models of multivariate binary logistic regression were developed in order to identify independent prognostic factors as well as the magnitude and significance of their impact on the diagnosis of allergic diseases. The odds ratio (OR) was calculated with 95% of the confidence interval (CI).

A p-value of <0.05 was considered as statistically significant.

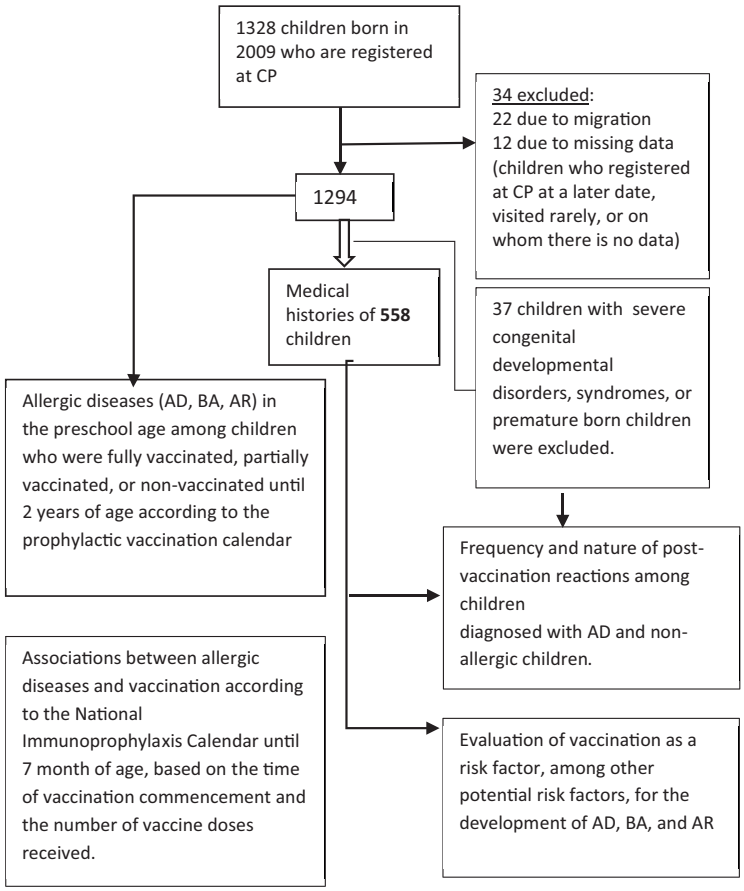


FIGURE 1. General scheme of the study.

3. Results

3.1 Phase I: Analysis of the Primary Health Care Institution statistical Database

According to a specially formulated request by the researchers, data of 1328 children who were born in 2009 and registered at the polyclinic were retracted by CP Technology and Statistics department specialists. Data of 1294 patients who met the inclusion criteria were analyzed. The cohort of the research subjects consisted of 650 girls and 644 boys.

At least one allergic disease – atopic dermatitis, asthma or allergic rhinitis – was diagnosed at least once in 417 (32%) children before 6 years of age. All three allergic diseases affected 30 (2.3%) children, two were diagnosed in 84 (6.5%) children, and 303 (23.5%) children were diagnosed with one of these diseases.

The predominant final diagnosis in the CP database was atopic dermatitis, confirmed by an allergist in 21.0% (n=272) of children under 6 years of age. The second most frequent final diagnosis was bronchial asthma – 14.2%. Allergic rhinitis has been diagnosed in 9.6% of children. These diagnoses were collected only from the records of specialists (pediatric pulmonologists, allergists, and otorhinolaryngologists). Atopic dermatitis in the pre-school age was diagnosed by a PCP at least once in 357 (27.6%) children, in which 73.1% (n = 272) of cases were confirmed by an allergist.

3.1.1 Dynamics of Diagnosed Allergic Diseases

PCPs have diagnosed atopic dermatitis in 205 (15.5%) of children in infancy. An allergist confirmed this diagnosis in 126 (61.5% of PCP cases) of children younger than 1 year of age. In the second year of life, a PCP had diagnosed atopic dermatitis in 123 children, and an allergist had confirmed 116 new AD cases during that year (Table 1). Table 1 shows the dynamics in the diagnoses of bronchial asthma and allergic rhinitis diagnosed by specialists at different age periods as well.

TABLE 1. The frequency of diagnoses of allergic diseases in different age periods.

Events of atopic diseases	No. of cases	%
Atopic dermatitis until 6 y by a PCP	357	27.6
Until 1 year	205	57.4
1–2 years	123	34.5
2–6 years	29	8.1
AD diagnosed by an allergist until 6 y	272	21.0
Until 1 year	126	46.4
1–2 years	116	42.6
2–6 years	30	11.0
Asthma until 6 years	184	14.2
Wheezing illness until 1 y of age	11	6.0
1–2 years	60	32.6
2–6 years	113	61.4
Allergic rhinitis	124*	9.6
Allergic rhinitis until 1 year	0	-
1–2 years	14 (8)*	11.3
2–6 years	110	88.7

*Starting from the 2nd year of life, 8 from 14 children with allergic rhinitis were proved to have a disease, and 6 were neglected. Therefore, the general number of allergic rhinitis from the database during the first six years of life is 124, although only 118 of children had it finally confirmed.

3.1.2 Scope of Vaccination: Completely Vaccinated, Non-Vaccinated, and Partially Vaccinated Children

We evaluated the overall vaccination coverage according to the NIPC in the study population up to 2 years of age and up to 6 years of age. Until 2 years of age, there were 17.6% of children who were partially vaccinated or non-vaccinated (Figure 2), but the proportion of such children up to 6 years of age decreased to 6.2% (Figure 3).

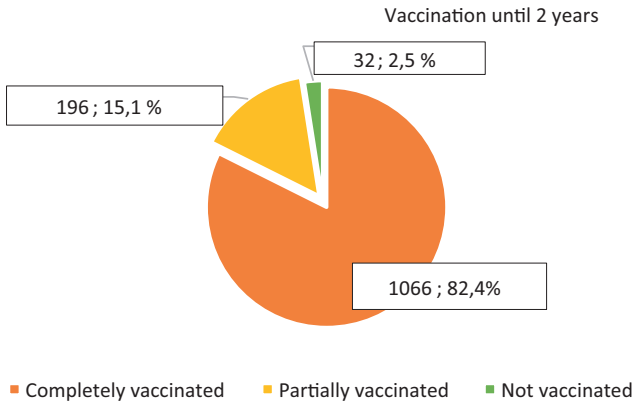


FIGURE 2. Vaccination until 2 years of age.

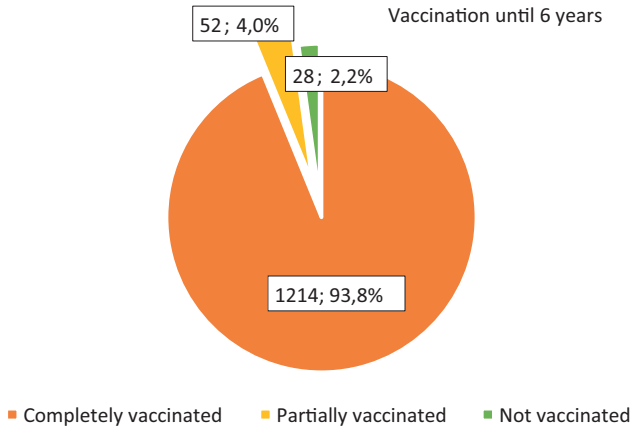


FIGURE 3. Vaccination until 6 years of age.

The relative majority of specialist-confirmed diagnoses of all analyzed allergic diseases over the period of 6 years has been among the non-vaccinated children until 2 years of age. However, comparing all three groups of children (completely vac-

cinated, non-vaccinated, and partially vaccinated according to NIPC), the overall frequency of diagnosed allergic diseases did not differ significantly ($p > 0.05$ among each group) (Figure 4).

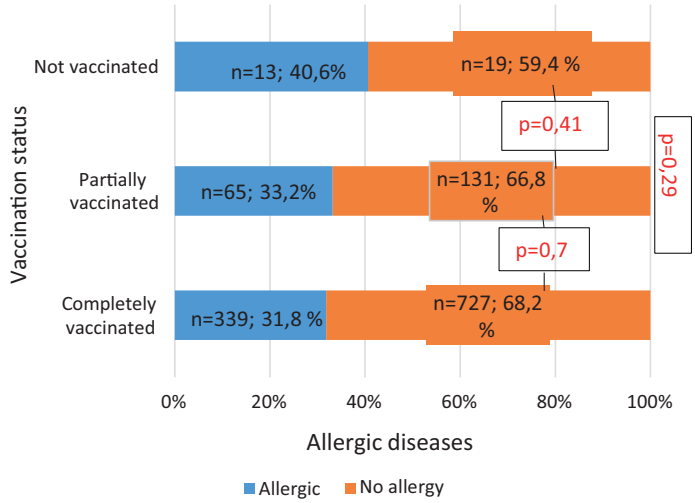


FIGURE 4. The frequency of allergic diseases in the final statistical data of the digital databases of clinics among completely vaccinated, partially vaccinated, and non-vaccinated children until 2 years of age.

3.1.3 Association between the Diagnoses of Allergic Diseases and Vaccination Coverage according to the NIPC until 2 Years of Age

The total number of allergic diseases among completely vaccinated, non-vaccinated, and partially vaccinated children have no significant differences, but we identified certain differences when analyzing each allergic disease individually. Of the 196 children who were partially vaccinated before the age of 2, 21.9% ($n = 43$) were diagnosed with AD by a PCP during their first year of life. When compared to the group of completely vaccinated children, AD before 1 year of age was diagnosed in 14.4% ($p = 0.008$). And up until 6 years of age, respectively, an AD diagnosis was determined in 36.2% of partially vaccinated and 25.8% of completely vaccinated children ($p = 0.003$). PCPs and allergists have diagnosed AD in non-vaccinated children more frequently at the age of 2–6 years, but this group did not differ statistically significantly from partially vaccinated or completely vaccinated children. The frequency of other diseases diagnosed among the fully vaccinated, non-vaccinated, and partially vaccinated children had no statistically significant differences (Table 2).

TABLE 2. The proportions of separate allergic diseases between chidlren who were non-vaccinated, partially vaccinated, and completely vaccinated until 2 years of age.

Diagnosis	Non-vaccinated	Partially vaccinated	Completely vaccinated	p value
No. of chidlren	32	196	1066	
	YES(%)/No	YES(%)/No	YES(%)/No	
Atopic dermatitis by PCP				
Until 6 years	11 (34.4%) /21	71 (36.2%) /125*	275(25.8%) /791*	0.008
Until 1 year	8 (25.0)/24	43 (21.9) /153**	154 (14.4) /912**	0.016
1–2 years	8 (25.0)/24	35(17.9)/161	167 (15.7)/899	0.29
2–6 years	5 (15.6)/ 27***	19 (9.7)/176	76(7.1)/991 ***	0.09
Atopic dermatitis by allergist				
Until 6 years	10(31.3)/22	46 (23.5) /150	216(20.3) /850	0.21
Until 1 year	3 (9.4)/29	22(11.22)/ 174	101 (9.5)/965	0.75
1–2 years	7 (21.9)/25	24(12.2)/ 172	133(12.5)/933	0.28
2–6 years	4 (12.5)/28	12 (6.1)/184	59 (5.5)/1007	0.25
Bronchial asthma				
Until 6 years	1 (3.1)/31	31(15.8)/165	152(14.3)/914	0.16
Wheezing illness until 1 year	0/32	3 (1.5)/192	8 (0.8)/1057	-
BA at 1–2 years	1(3.1)/30	10 (5.1)/183	50 (4.7)/1016	0.031
BA at 2–6 years	3 (9.4)/29	23(11.7)/172	132(12.4)/934	0.28
Allergic rhinitis				
Until 6 years	3 (9.4)/29	19 (9.7)/177	102 (9.6)/964	0.98
1–2 years	1(3.1)/30	2 (1.0)/192	11 (1.0)/1055	0.012
2–6 years	3 (9.4)/29	18 (9.2)/177	96 (9.0)/970	0.99

AD until 6 years was diagnosed by a PCP statisticaly signficantly more often in partially vacinated children than in completely vaccinated children until 2 years of age (*p=0.003). An AD diagnosis until 1 year of age by a PCP was more often made in the partially vaccinated group (**p=0.008). In the age group of 2–6 years, AD was diagnosed by a PCP more often in non-vaccinated than in completely vaccinated children (***p=0.07).

The links between allergic diseases and each vaccine separately were analyzed further. The scope of vaccination of children under 2 years of age not with all vaccines was 90%, but children at six years of age had been given at least 95% of the NIPC's coverage. Based on the data belonging to the Lithuanian Department of Statistics, vaccination coverage with individual vaccines according to the NIPC in 2009–2010 at CP did not differ from the general Lithuanian data.

3.1.4 Association between Vaccination according to the National Immunoprophylaxis Calendar under 7 Months of Age and Diagnosed Allergic Diseases under 6 Years of Age

The number of children based on vaccination time and the number of doses given with every vaccine that should be inoculated until the 7th month are shown in Table 3.

TABLE 3. Vaccination starting time and number of vaccine doses received.

Vaccine	No. of children	%
BCG		
BCG vaccination time	1294	
On time	1157	89.4
During the first month	92	7.1
Later (2–15 months)	9	0.7
Non-vaccinated	36	2.8
HB		
Doses of HB received until the 7th month	1238	
Completely vaccinated (3 doses)	1014	78.4
Partially vaccinated (1–2 doses)	224	17.3
Non-vaccinated:	56	4.3
Not until the 7th month, but vaccinated later	20	35.7
Never vaccinated	36	64.3
Time of the first HB dose	1258	
On time	1201	95.5
During the first month of life	40	3.2
From the second month	17	1.3
DTaP-IPV-Hib		
Doses of DTaP-IPV-Hib received until the 7th month		
Completely vaccinated (3 doses)	1016	78.5
Partially vaccinated	214	16.5
1 dose	52	24.3
2 doses	162	75.7
Non-vaccinated	64	5.0
Until the 7th month	25	39.0
Never vaccinated	39	61.0
Time of the 1st DTaP-IPV-Hib dose:	1230	95.0
On time	851	69.2
Slightly delayed	324	26.3
Delayed	55	4.5

3.15 Links of Diagnosed Allergic Diseases with the Time of Each Vaccine Inoculation and Number of Doses Inoculated

We found that children who were inoculated with the BCG vaccine one month later were diagnosed with atopic dermatitis by an allergist 2.83 times more often at the age of 2–6 years than those vaccinated on time (p=0.03). BCG inoculation time was not significantly linked to the incidence of asthma and allergic rhinitis (Table 4).

Children whose vaccination with the first dose of the hepatitis B vaccine was delayed by a month were diagnosed with a case of asthma 2.3 times more frequently (p = 0.02) (Table 5). The increase in the chance of allergic rhinitis with respect to the time of vaccination in this age group was statistically insignificant.

We established that atopic dermatitis was diagnosed by PCPs 2.2 times more often in the group of children who had not been vaccinated with the HB vaccine than children who had received all 3 doses of the vaccine (p=0.02). The group of children under one year of age stood out: OR 2.57 (95% CI: 1.24-5.34), p=0.01. This tendency was also seen in the case of AD diagnosed by an allergist in children of 1 to 2 years of age: the diagnosis of atopic dermatitis was relatively more frequent among non-vaccinated children (p=0.03) (Table 6).

There was no difference in the relative frequency of atopic dermatitis between children in the analyzed age groups vaccinated with DTaP1 on time and those vaccinated one month later. However, in general, asthma in the pre-school age was diagnosed more frequently in the group of children vaccinated with a one month delay: OR 0.64 (95% CI: 0.43-0.95), p = 0.03. The frequency of allergic disease diagnosing did not change when comparing the group with an even further delayed start of DTaP vaccination to the children vaccinated on time (Table 7).

In the group of partially vaccinated children, the frequency of AD diagnosed by a PCP (OR 1.53 (95% CI: 1.05-2.23); p = 0.03), and the wheezing disease (J45.9) frequency (OR 4.02 (95% CI: 1.22-13.29); p = 0.01) in the group of children younger than one year of age, was statistically significantly higher in comparison to completely vaccinated children. Atopic dermatitis, diagnosed by an allergist, was relatively more prevalent in the group of partially vaccinated children under 1 year of age (OR 1.24 (95% CI: 0.77-1.99), but no significant difference from completely vaccinated children was found (p = 0.38) (Table 8).

TABLE 4. Distribution of the diagnosed atopic diseases in different age periods according to BCG vaccination timing and fact.

	BCG on time	BCG 1 month later	OR (95% CI)	p value	Non-vaccinated BCG	OR (95% CI)	p value
Number of children Overall: 1285	1157 (90.0%)	92 (7.2%)			36 (2.8%)		
Diagnosis of allergic disease	n (%)	n (%)	1 month later vs. on time		n (%)	Non-vaccinated vs. vaccinated on time	
AD 0–6 years PCP overall	316 (27.3)	28 (30.4)	1.16 (0.73-1.45)	*0.52*	12 (33.3)	1.33 (0.66-2.69)	0.43
Until 1 year	177 (15.3)	19 (20.7)	1.44 (0.85-2.45)	*0.17*	9 (25.0)	1.85 (0.85-3.99)	0.11
1–2 years	114 (9.9)	6 (6.5)	0.64 (0.27-1.49)	*0.30*	2 (5.6)	0.54 (0.13-2.27)	0.39
2–6 years	25 (2.2)	3 (3.3)	1.53 (0.45-5.15)	*0.49*	1 (2.8)	1.29 (0.17-9.82)	0.80
AD 0–6 years allergist	239 (20.7)	21 (22.8)	1.14 (0.68-1.89)	*0.62*	11 (4.4)	1.69 (0.82-3.48)	0.15
Until 1 year	112 (9.7)	11 (12.0)	1.27 (0.66-2.45)	*0.48*	3 (8.3)	0.85 (0.26-2.81)	0.79
1–2 years	104 (9.0)	5 (5.4)	0.58 (0.23-1.47)	*0.25*	6 (16.7)	2.03 (0.82-4.98)	0.12
2–6 years	23 (2.0)	5 (5.4)	2.83 (1.05-7.64)	***0.03***	2 (5.6)	2.90 (0.66-12.8)	0.14
Asthma 0–6 years overall	167 (14.4)	13 (14.1)	1.02 (0.61-1.72)	*0.94*	2 (5.6)	2.60 (0.67-10.01)	*0.13*
Wheezing illness until 1 y.	11 (1.0)	0 (0)	-	*0.35*	0 (0)	-	*0.56*
1–2 years	57 (4.9)	5 (5.4)	0.91 (0.37-2.21)	*0.83*	0 (0)	-	*0.17*
2–6 years	101 (8.7)	8 (8.7)	1.00 (0.50-1.20)	*0.99*	2 (5.6)	1.57 (0.40-6.12)	*0.50*
AR 0–6 years overall	107 (9.2)	14 (15.2)	0.61 (0.36-1.02)	*0.06*	3 (8.4)	1.11 (0.37-3.33)	*0.85*
1–2 years	12 (1.0)	2 (2.2)	0.48 (0.11-2.10)	*0.32*	0 (0)	-	*0.54*
2–6 years	94 (8.1)	12 (13.0)	0.62 (0.36-1.09)	*0.10*	3 (8.3)	0.97 (0.32-2.93)	*0.96*

TABLE 5. Association between the allergic diseases and the time of the first HB dose.

	HB1 vaccinated 1 month later	HB1 vaccinated on time	OR (95% PI)	p value
Number of children	N=40 (3.2%)	N=1201 (96.8%)		
Allergic disease	n (%)	n (%)		
AD 0–6 years PCP overall	14 (35.0)	324 (27.0)	1.46 (0.75-2.83)	0.26
Until 1 year	10 (25.0)	184 (15.3)	1.84 (0.89-3.83)	0.10
1–2 years	3 (7.5)	117 (9.7)	0.75 (0.23-2.47)	0.64
2–6 years	1 (2.5)	23 (2.8)	1.31 (0.17-9.97)	0.79
AD 0–6 years allergist	8 (20.0)	249 (20.7)	0.96 (0.44-2.10)	0.91
Until 1 year	6 (15.0)	117 (9.7)	1.63 (0.67-3.98)	0.27
1–2 years	1 (2.5)	108 (9.0)	0.26 (0.03-1.91)	0.15
2–6 years	1 (2.5)	24 (2.0)	1.26 (0.17-9.53)	0.82
Asthma 0-6 m. overall				
Wheezing illness	11 (27.5)	170 (14.2)	2.30 (1.13-4.69)	***0.02***
until 1 y.	0 (0)	11 (9.1)	-	*0.54*
1–2 years	4 (10.0)	57 (4.7)	2.23 (0.77-6.48)	*0.13*
2–6 years	7 (17.5)	104 (8.7)	2.24 (0.97-5.18)	*0.05*
AR 0–6 years overall	8 (20.0)	111 (9.2)	2.45 (1.10-5.46)	***0.02****
1–2 years	2 (5.0)	11 (0.9)	5.69 (1.22-26.56)	***0.01****
2–6 years	6 (15.0)	99 (8.2)	1.96 (0.81-4.79)	*0.13*
AR 2–6 years overall	6 (15.0)	106 (8.8)	1.82 (0.75-4.44)	*0.18*

* Allergic rhinitis is not a usual diagnosis in children under 2 years; therefore, we suggest to rely on the frequency of AR at the age of 2–6 years rather than the period from birth to 6 years.

TABLE 6. Distribution of the allergic diseases between the children who have received all three doses of HB and those who had not completed vaccination until the 7th month.

	Non-vaccinated HB	All three doses of HB until 7 months	OR (CI 95%)	p value
Number of children	N=36 (3.4%)	N=1014 (96.6%)		
Allergic disease	n (%)	n (%)		
AD 0–6 years by PCP overall	16 (44.4)	267 (26.3)	2.24 (1.14-4.38)	***0.02***
Until 1 year	11(30.6)	148 (14.6)	2.57 (1.24-5.34)	***0.01***
1–2 years	3 (8.3)	99 (9.8)	0.84 (0.25-2.79)	*0.78*
2–6 years	2 (5.6)	20 (2.0)	2.92 (0.66-13.01)	*0.14*
AD 0-6 years by allergist	12(33.3)	205 (20.2)	1.97 (0.97-4.01)	*0.06*
Until 1 year	3 (8.3)	93 (9.2)	0.90 (0.27-2.99)	*0.86*
1–2 years	7 (19.4)	91 (9.0)	2.45 (1.04-5.75)	***0.03***
2–6 years	2 (5.6)	20 (2.0)	2.92 (0.66-13.01)	*0.14*
Asthma 0–6 years overall	1 (2.8)	148 (14.6)	0.17 (0.02-1.23)	*0.05*
Wheezing illness until 1 y.	0 (0)	7 (0.7)	-	*0.62*
1–2 years	0 (0)	51 (5.0)	-	*0.17*
2–6 years	1 (2.8)	90 (9.0)	0.29 (0.04-2.17)	*0.20*
AR 0–6 years overall	3 (8.3)	97 (9.6)	0.86 (0.26-2.85)	*0.80*
1–2 years	0 (0)	12 (1.2)	-	*0.51*
2–6 years	3 (8.3)	84 (8.3)	1.01 (0.30-3.35)	*0.99*

TABLE 7. Allergic diseases at different age periods according to the time of the first DTaP-IPV-Hib dose inoculation.

	DTaP the 1st dose on time	DTaP the 1st dose 1 month later	OR (95% PI) 1 month later vs. on time	DTaP-IPV-Hib delayed	OR (95% PI) Delayed vs. on time
Number of children N=1230	N=851(69.2%)	N=324 (26.3%)		N=55 (4.5%)	
Allergic disease	n (%)	n (%)		n (%)	
AD 0–6 years by PCP overall	235 (27.6)	81 (25.0)	0.87 (0.65-1.17)	19 (34.5)	1.38 (0.78-2.46)
Until 1 year	134(15.7)	47 (14.5)	0.91 (0.63-1.30)	10 (18.2)	1.19 (0.58-2.42)
1–2 years	79 (9.3)	29 (9.0)	0.96 (0.61-1.50)	9 (16.4)	1.91 (0.90-4.05)
2–6 years	22 (2.6)	5 (1.5)	0.59 (0.22-1.57)	0 (0)	-
AD 0–6 years by allergist	181(21.3)	60 (18.5)	0.84 (0.61-1.16)	12 (21.8)	1.03 (0.53-2.00)
Until 1 year	87 (10.2)	27 (8.3)	0.80 (0.51-1.25)	4 (7.3)	0.69 (0.24-1.95)
1–2 years	73 (8.6)	26 (8.0)	0.93 (0.58-1.48)	8 (14.5)	1.81 (0.83-3.99)
2–6 years	21 (2.5)	7 (2.2)	0.87 (0.37-2.07)	0 (0)	-
Asthma 0–6 years overall	132 (15.5)	34 (10.5)	**0.64 (0.43-0.95)***	12 (21.8)	1.52 (0.78-2.96)
Wheezing illness until 1 y.	10 (1.2)	1 (0.3)	0.26 (0.03-2.04)	1 (1.8)	1.56 (0.20-12.38)
1–2 years	40 (4.7)	10 (3.1)	0.65 (0.32-1.31)	4 (7.3)	1.59 (0.55-4.62)
2–6 years	81 (8.3)	22 (6.8)	0.69 (0.42-1.13)	7 (12.7)	1.39 (0.61-3.16)
AR 0–6 years overall	85 (10.0)	25 (7.7)	0.75 (0.47-1.20)	6 (10.9)	1.10 (0.46-2.65)
1–2 years	11 (1.3)	3 (0.9)	0.71 (0.19-2.57)	0 (0)	-
2–6 years	74 (8.7)	22 (6.8)	0.76 (0.47-1.25)	6 (11.0)	1.29 (0.53-3.10)

If the 1st dose of DtaP-IPV-Hib was delayed by 1 month, the risk for an asthma diagnosis decreased in comparison to timely vaccinated children (* p=0.03).

TABLE 8. Distribution of the diagnosed allergic diseases between the children who have received all three doses of DTaP-IPV-Hib with those who did not complete their vaccinations until the 7th month or were not vaccinated at all.

	Completely vaccinated with 3 doses of DTaP until 7 month	Partially vaccinated until the 7th month	OR (95% CI)	p value	Non-vaccinated with DTaP until the 7th month	OR (95% CI)	p value
Number of children: N=1294	N=1016 (82.6%)	N=214(17.4%)			N=64 (5.9%)		
Allergic disease	n (%)	n (%)	Partially vs. completely		n (%)	Non-vaccinated vs. completely	
AD 0–6 years by PCP overall	268 (26.4)	67 (31.3)	1.27 (0.92-1.75)	*0.14*	22 (34.4)	1.46 (1.86-2.49)	*0.16*
Until 1 year	147 (14.5)	44 (20.6)	1.53 (1.05-2.23)	***0.03***	14 (21.9)	1.66 (0.89-3.07)	*0.11*
1–2 years	99 (9.7)	18 (8.4)	0.85 (0.50-1.44)	*0.55*	6 (9.4)	0.96 (0.40-2.28)	*0.92*
2–6 years	22 (1.7)	5 (2.3)	1.08 (0.40-2.89)	*0.88*	2 (3.1)	1.46 (0.34-6.34)	*0.61*
AD 0–6 years by allergist	208 (20.5)	45 (21)	1.03 (0.72-1.49)	*0.85*	19 (29.7)	1.64 (0.94-2.86)	*0.08*
Until 1 year	94 (9.3)	24 (11.2)	1.24 (0.77-1.99)	*0.38*	8 (12.5)	1.40 (0.65-3.03)	*0.39*
1–2 years	91 (9.0)	16 (7.5)	0.82 (0.47-1.43)	*0.49*	9 (14.0)	1.66 (0.80-3.48)	*0.17*
2–6 years	23 (2.3)	5 (2.3)	1.03 (0.39-2.75)	*0.95*	2 (3.1)	1.39 (0.32-6.04)	*0.66*
Asthma 0–6 years overall	145 (14.3)	33 (15.4)	1.10 (0.73-1.65)	*0.66*	6 (9.4)	0.62 (0.26-1.47)	*0.27*
Wheezing illness until 1 y.	6 (0.6)	5 (2.3)	4.02 (1.22-13.29)	***0.01***	0 (0)	-	*0.54*
1–2 years	48 (4.7)	11 (5.1)	1.09 (0.56-2.14)	*0.80*	3 (4.7)	0.99 (0.30-3.28)	*0.99*
2–6 years	92 (9.1)	18 (8.4)	0.92 (0.54-1.56)	*0.76*	3 (4.7)	0.49 (0.15-1.60)	*0.23*
AR 0-6 years overall	95 (9.4)	21 (9.8)	1.05 (0.64-1.73)	0.83	8 (12.5)	1.38 (0.64-2.99)	*0.41*
1–2 years	13 (1.3)	0 (0)	-	*0.09*	1 (1.6)	1.24 (0.16-9.67)	*0.83*
2–6 years	81 (8.0)	21 (9.8)	1.26 (0.76-2.08)	*0.37*	7 (10.9)	1.42 (0.63-3.21)	*0.40*

3.2 Phase II: Analysis of Medical Histories. Case-Control Study

The group of 250 children diagnosed with AD with or without other allergic diseases included 116 girls and 134 boys. The group of children who had not been diagnosed with allergic diseases as confirmed by the allergist included 142 girls and 127 boys. There was no statistically significant difference between the distribution of genders. Some of the children in the group diagnosed with AD were also diagnosed with other allergic diseases: AD and BA were diagnosed in 41 (16.4%) child, AD with AR were diagnosed in 28 (11.2%) children, AD with AR and BA in 26 (10.4%). More than a half of children with the diagnosis of AD, i.e., 134 (52.6%) were diagnosed with atopic dermatitis during the first year of life.

3.2.1 Frequency and Nature of Adverse Reactions to Vaccination, Recorded in Medical Histories, in Children under 24 Months of Age

After reviewing all medical histories of the research subjects and control group (n=519), reactions after vaccination identified by physicians were found in 28 children under 2 years of age. Post-vaccination reactions to one vaccine were documented in 26 children, and in 2 children – to two inoculations of the same vaccine (one to DTaP2 and DTaP3, another to DTaP1 and DTaP3). Thus, the total number of adverse reactions to vaccines, documented in medical histories, was 30. Post-vaccination reactions were recorded in 10 (35.7%) girls and 18 (64.3%) boys (p = 0.141). Adverse reactions to vaccines were reported in 16 (6.4%) children with AD and 8 (3.0%) children without any allergic diseases. Children diagnosed with AD

TABLE 9. Comparison of the adverse reactions rate after vaccination, between children with atopic dermatitis and non-allergic children.

Vaccine	Number of reactions/ number of children vaccinated	In children with AD	In non-allergic children	OR (CI 95%)
BCG	3/496	1/241	2/255	0.53 (0.05-5.82)
HB1	0/495	0/240	0/255	-
HB2	1/491	1/237	0/254	p=0.30
HB3	1/481	1/235	0/246	p=0.31
DTaP1	6/491	2/240	4/251	0.52 (0.09-2.88)
DTaP2	7/485	5/237	2/248	2.62 (0.50-13.61)
DTaP3	5/485	4/237	1/248	4.12 (0.46-37.71)
DTaP4	4/471	2/231	2/240	1.04 (0.15-7.44)
MMR1	3/476	2/234	1/242	2.07 (0.17-22.97)
Iš viso:	30/4371	18/2132	12/2239	1.58 (0.76-3.28)

experienced 18 adverse reactions to vaccination, while non-allergic children – 14 ($p = 0.063$). There was no statistically significant difference between groups in the frequency of adverse reactions after vaccination with every vaccine (Table 9).

Egg Allergy and Vaccination

Sensitization to food or environmental allergens was confirmed for 139 children in the group diagnosed with AD, of those 43 (30.9%) were sensitized to eggs (sensitization confirmed by positive SPT). Two of the 43 egg-sensitized children were not vaccinated with the MMR vaccine, although contraindications were not indicated neither in their medical histories nor in the database system. Of the 43 children, 39 were completely vaccinated and 4 were vaccinated partially. Of the 4 partially vaccinated children one was vaccinated only with the BCG vaccine, two children were not vaccinated with the DTaP4 vaccine, and another child was not vaccinated with the MMR1 and DTaP4 vaccines.

3.2.2 Association between Early Life Factors, Including Vaccination, and Allergic Diseases

We have selected medical histories of 289 children who have been diagnosed with allergic diseases. We excluded 49 children from the group, who (1) were diagnosed with AD by an allergist before 2 months of age, i.e., prior to the vaccination with DTaP-IPV-Hib (24 children); (2) were diagnosed with AD by a PCP before 2 months of age, which was subsequently confirmed by an allergist (22 children); (3) commenced vaccination at the time of AD diagnosis (2 children); (4) were diagnosed with AD after 2 months of age, but prior to the commencement of vaccination (1 child).

Of the 240 children selected, 203 were diagnosed with AD, 62 were diagnosed with AR, and 76 were diagnosed with BA. Of 203 children diagnosed with atopic dermatitis, 47 (23.2%) were diagnosed with asthma and 43 (21.2%) with allergic rhinitis. Atopic dermatitis was diagnosed by an allergist in 88 children in the first year of life, in 74 children in the second year of life, and in 41 child after their second birthday. The control group consisted of 269 children with no diagnoses of allergic diseases.

Association between Early Risk Factors and Allergic Diseases

For this part of the study, we have selected the factors that undoubtedly occurred before the onset of allergic diseases: the method of delivery (birth through natural means as a protective factor), gender (male), birth pathology, presence of

older siblings, time of vaccination with DTaP1, breastfeeding, weight at birth and weight at discharge (difference by one hundred grams); we later added the prescription of antibiotics during the first year of life.

Analyzing one factor after another in univariate binary logistic regression models, we found that the risk of allergic disease diagnosis was slightly increased before 7 months of age by every dose of inoculated vaccines (OR 1.337; 95% CI: 1.005-1.778, p=0.046). A DTaP1 delay by a month later than specified in the NIPC tended to the 0.786 lower risk of diagnosis of allergic disease, but was statistically insignificant (p = 0.217). In the univariate binary logistic regression model, allergic diseases were diagnosed more frequently (OR 5.050; 95% CI: 1.672-15.25, p = 0.004) if congenital infection was diagnosed at birth, but in the models of multivariate analysis, this factor was not statistically significant. The antibiotic prescription fact during infancy – OR 1.609 (95% CI: 1.123-2.306, p=0.010), same as the number of prescribed courses of antibiotics during the infancy – OR 1.480 (95% CI: 1.200-1.824, p=0.0001), were associated with a higher overall risk of having a diagnosis of allergic disease at a preschool age. But in the multivariate binary logistic regresion model, only the number of antibiotic prescriptions was associated with a higher risk of allergic disease: OR 1.463 (95% PI: 1.15-1.903, p=0.002) (Table 10).

TABLE 10. Early-life factors associated to the diagnoses of the allergic diseases.

N=509	Univariate analysis		Multivariate analysis	
Factor	OR (95% CI)	*p*	OR (95% CI)	p
Vaccination			I model: N=442	
DTaP1 vaccination	1.649 (0.746-3.647)	0.216		
DTaP1 on time vs. not on time	1.174 (0.582-2.368)	0.654		
DTaP1 timing: 1 month later	0.768 (0.505-1.168)	0.217		
BCG vaccination	1.407 (0.599-3.317)	0.432		
HB1 vaccination	1.358 (0.598-3.083)	0.464		
DTaP1 timing from 2 month	1.001 (0.958-1.047)	0.948		
DTaP doses received until the 7th month	1.167 (0.957-1.423)	0.127		
DTaP1 on time vs. non-vaccinated	1.668 (0.753-3.694)	0.207		
DTaP3 on time vs. delayed	1.020 (0.652-1.597)	0.930		
HB3 on time	1.034 (0.649-1.616)	0.918		
Demographic data			II model: N= 372	
Gender: boy	1.295 (0.913-1.835)	0.147		
Month of birth (1)	1.036 (0.982-1.092)	0.195		

N=509	Univariate analysis		Multivariate analysis	
Factor	OR (95% CI)	*p*	OR (95% CI)	p
Birth circumstances				
Birth at hospital	0.891 (0.220-3.600)	0.871		
Delivery (SC)	1.011 (0.647-1.681)	0.961		
Birth weight (100 g)	1.023 (0.987-1.060)	0.212		
Weight at the discharge (100 g)	1.038 (0.997-1.080)	0.073	1.045(0.997-1.094)	0.067
Birth pathologies	1.606 (0.959-2.690)	0.072		
Congenital infection	5.050 (1.672-15.25)	0.004		
Mothers age (a year)	0.995 (0.962-1.029)	0.763		
Early life factors			III model: N= 347	
Number of siblings	1.006 (0.780-1.297)	0.964		
Breastfeeding	0.392 (0.119-1.289)	0.123		
AB prescription in infancy	1.609 (1.123-2.306)	0.010		
Frequency of AB prescription in infancy	1.480 (1.200-1.824)	0.0001	1.463 (1.15-1.903)	0.002

Risk Factors for Atopic Dermatitis

In the univariate logistic regresion models, we found that birth pathologies were associated with a 1.724 times higher risk of AD diagnosis. Congenital infection increased the risk of AD diagnosis by 5.325 times. The fact of antibiotics prescription and the frequency of prescriptions were also associated with a higher risk of AD diagnosis. We found that breastfeeding was a protective factor for AD diagnosis: OR 0.332 (95% CI: 1.101-1.093).

Each additionaly prescribed course of antibiotics during the first year of life independently increased the risk of diagnosis of AD by 1.448 times (p = 0.003), but the fact of the prescription of antibiotics itself was not associated with an increase in AD risk in multiple binary logistic regression models. Congenital infection with the effect of other factors was not a statistically significant risk factor for atopic dermatitis (Table 11).

TABLE 11. Association between early life factors and atopic dermatitis.

N= 472	Univariate analysis (for AD without BA and AR comparing to non-allergic children).		Multivariate analysis	
Factor	OR (95% CI)	*p*	OR (95% CI)	*p*
Vaccination			I model: N=413	
BCG vaccination	1.338(0.550-3.254)	0.520		
DTaP1 vaccination	1.546 (0.680-3.516)	0.299		

N= 472	Univariate analysis (for AD without BA and AR comparing to non-allergic children).		Multivariate analysis	
DTaP1 on time/ delayed	1.242 (0.588-2.625)	0.570		
DTaP1 time from 2 months (monthly)	1.007 (0.963-1.054)	0.755	-	
DTP1 time: 1 month later	0.841 (0.545-1.298)	0.434		
HB1 vaccination	1.273 (0.546-2.970)	0.577		
HB1 monthly	0.711 (0.235-2.158)	0.547	-	
HB3 on time	0.901 (0.381- 2.130)	0.813	-	
DtaP3 on time / delayed	0.818 (0.334-2.008)	0.662	-	
Demographic data			II model: N=346	
Gender: boy	1.295 (0.913-1.835)	0.147		
Month of birth (1)	1.036 (0.982-1.092)	0.195	-	
Birth circumstances				
Birth at hospital	1.006 (0.223-4.547)	0.993		
Delivery (SC)	1.145 (0.724-1.810)	0.564		
Birth weight (100 g)	1.024 (0.987-1.063)	0.209		
Weight at the discharge (100 g)	1.037 (0.995-1.081)	0.087	II 1.048 (0.998-1.099) III 1.045 (0.994-1.098)	0.058 0.082
Birth pathologies	1.724 (1.015-2.930)	0.044		
Congenital infection	5.325 (1.737-16.22)	**0.003**		
Early life factors			III model: N=324	
Number of siblings	1.037 (0.796-1.352)	0.786		
Breastfeeding	0.332 (0.101-1.093)	0.070		
AB prescription in infancy	1.561 (1.072-2.275)	**0.020**		
Frequency of AB prescription in infancy	1.460 (1.183-1.824)	**0.001**	1.448 (1.124-1.866)	0.003
Allergy				

Allergological anamnesis is not included in the models of multiple logistic regression due to the small sample of research subjects who had such data.

Risk Factors for Bronchial Asthma

In the models of the multivariate binary logistic regression, we established that antibiotic prescription in infancy twice (p = 0.009) and each additional antibiotic prescription increased the risk of asthma diagnosis by 1.59 times (p = 0.002) in the

preschool age. We observed a trend that every month that the inoculation of DTaP1 was postponed was related to the reduction of asthma risk, but it was statistically insignificant. Postponing the first dose of DTaP from the second to the third month of life, i.e., by one month, was significantly linked with a reduction in the risk of asthma diagnosis: OR 0.385 (95% CI: 0.186-0.795), p = 0.01. The male gender was associated with a 1.5 time higher risk of asthma diagnosis, but this factor did not reach the level of statistical significance (Table 12).

DTaP1 delay for 1 month in the multavariate regresion model with all analyzed factors included did not remain significant for asthma risk reduction. The only factor remaining in the complete model, which was significantly linked to the increase of asthma risk by 1.5 times, was the frequency of antibiotics prescription during infancy (p = 0.033) (Table 12).

TABLE 12. Association between early life factors and bronchial asthma.

N = 345	Univariate analysis		Multivariate analysis	
Factor	OR (95% CI)	*p*	OR (95% CI)	*p*
Vaccination			I model: N=248	
BCG vaccination	4.118 (0.533-31.827)	0.175		
BCG on time vs. 1 month later	0.821 (0.312-2.164)	0.691		
DTaP1 on time/ delayed	1.163 (0.419-3.226)	0.772		
DTaP1 time from 2 months (monthly)	0.808 (0.590-1.107)	0.184		
DTP1 time: 1 month later	0.385 (0.186-0.795)	**0.010**	I 0.397 (0.192-0.823) II 0.479 (0.234-1.057)	0.013 0.069
HB1 vaccination	4.429 (0.576-34.082)	0.153		
HB1 monthly	0.945 (0.782-1.142)	0.557		
HB3 on time	1.168 (0.594-2.296)	0.659		
DTaP3 time monthly	0.976 (0.926-1.029)	0.374		
DTaP until the 7th month according to the vaccination plan vs. plan disturbed	1.397 (0.756-2.579)	0.286		
Number of vaccine doses received until 7 months	1.499 (0.865-2.569)	0.149		
Vaccinated vs. partially and non-vaccinaed	1.699 (0.864-3.344)	0.125		
Demographic data			II model: N=234	
Gender: boy	1.584 (0.947-2.650)	0.080		
Month of birth (1)	1.037 (0.959-1.121)	0.366		

N = 345	Univariate analysis		Multivariate analysis	
Factor	OR (95% CI)	*p*	OR (95% CI)	*p*
Birth circumstances				
Delivery (SC)	1.256 (0.675-2.337)	0.471		
Birth weight (100 g)	1.013 (0.962-1.067)	0.616		
Weight at the discharge (100 g)	1.032 (0.973-1.095)	0.290		
Birth pathologies	1.708 (0.839-3.476)	0.140		
Congenital infection	4.762 (1.242-18.25)	**0.023**	-	
Discharge from the hospital day	1.053 (0.939-1.179)	0.378		
Mother's age (year)	0.984 (0.937-1.033)	0.521		
Early life factors			III model: N=226	
Number of siblings	0.738 (0.476-1.146)	0.716		
AB prescription in infancy	2.000 (1.193-3.353)	**0.009**		
Frequency of AB prescription in infancy	1.591 (1.188-2.130)	**0.002**	1.484 (1.032-2.135)	0.033

Risk factors for the Diagnosing of Allergic Rhinitis

Although bronchial asthma and allergic rhinitis are related conditions (up to 30% of children with allergic rhinitis had asthma as well), a one-month delay in DTaP1 had no effect on the frequency of allergic rhinitis diagnoses. Antibiotic prescription during the first year of life increased the risk of AR by 1.6 times (in the models of multivariate binary logistic regression) (p = 0.02) (Table 13).

TABLE 13. Association between early life factors and allergic rhinitis.

N=331	Univariate analysis		Multivariate analysis	
Factors	OR (95% CI)	*p*	OR (95% CI)	*p*
Vaccination			I model: N=282	
BCG vaccination	3.349 (0.432-25.96)	0.247		
BCG on time vs. 1 month later	0.655 (0.247-1.737)	0.395		
DTaP1 vaccination	2.151 (0.486-9.525)	0.313		
DTaP1 on time/delayed	1.147 (0.375-3.504)	0.810		
DTaP1 time from 2 months (monthly)	0.896 (0.721-1.114)	0.323		
DTP1 time: 1 month later	0.640 (0.318-1.287)	0.210		
HB1 vaccination	3.602 (0.467-27.80)	0.219		
HB1 monthly	0.885 (0.331-2.364)	0.807		
HB3 on time	1.224 (0.579-2.589)	0.596		

N=331	Univariate analysis		Multivariate analysis	
Factors	OR (95% CI)	*p*	OR (95% CI)	*p*
Demographic data			II model: N=240	
Gender: boy	1.229 (0.707-2.136)	0.465		
Month of birth (1)	1.009 (0.927-1.098)	0.844	-	
Birth circumstances	0.921 (0.101-8.384)	0.942		
Birth at hospital	0.940 (0.458-1.933)	0.867		
Delivery (SC)	1.059 (0.999-1.123)	0.055	(II) 1.100 (1.002-1.183) (III) 1.094 (1.012-1.182)	0.011 0.024
Birth weight (100 g)	1.065 (1.000-1.136)	0.051		
Birth pathologies	1.226 (0.531-2.830)	0.633		
Congenital infection	5.556 (1.444-21.38)	0.013		
Discharge day	1.007 (0.879-1.153)	0.924		
Mothers age (a year)	0.991 (0.939- 1.045)	0.737		
Early life factors			III model: N=216	
Number of siblings	0.878 (0.572- 1.345)	0.549		
Breastfeeding	0.444 (0.079-2.487)	0.356		
AB prescription in infancy	2.000 (1.143-3.498)	**0.015**	(III) 1.610 (1.078-2.406)	0.02
AB prescription times during infancy	1.593 (1.178-2.154)	**0.003**		

4. Discusion

A comparative analysis of studies analyzing vaccination links with allergic diseases is difficult due to the different combinations of vaccine components examined (25–28). Even the research subjects included in the sample of the same study may differ according to the components of the inoculated vaccines (e.g., some research subjects are vaccinated with whole cell pertussis vaccine, while others with an acellular vaccine), the national prophylactic vaccination calendars of children are also supplemented with new vaccines and some research subjects are vaccinated according to one plan, while others are vaccinated according to another (28, 29). In our study, all children were vaccinated according to the NIPC of Children of the Republic of Lithuania, which was in effect in 2009–2010, the number of planned vaccines and the composition of specific vaccines did not differ for any child. Study results may also vary due to regional-socio-economic and genotypic peculiarities of the study populations (30).

4.1. Discussion of the Phase I results

Vaccination Coverage and Frequency of Allergic Diseases

In the first phase of the study, we determined that the frequency of diagnoses of allergic diseases before the age of 6 years is not related to the coverage of vaccination before the age of 2 years and before the age of 6 years. This means that children vaccinated with NIPC vaccines are not more allergic than non-vaccinated or partially vaccinated children. No associations with national immunization rates were found in the International Study of Asthma and Allergies in Childhood (31), as well as in a more recent large-scale prospective study (32).

Disorders in the Routine Immunisation Schedule

By analyzing the data in the polyclinic's electronical database and further examining each allergic disease separately, we found that AD was diagnosed by PCPs in non-vaccinated or partially vaccinated children more frequently than in children up to 2 and up to 6 years of age who were fully vaccinated according to the routine immunization schedule. In the group of partially vaccinated children of up to 2 years, 21.9% of children under 1 year of age were diagnosed with AD by a PCP when compared to 14.4% in the group of completely vaccinated children ($p = 0.008$). This trend remained all the way up to 6 years of age. To sum up these results, we can say that vaccinated children suffer from allergic diseases as often as non-vaccinated ones, but atopic dermatitis, diagnosed by primary care practitioners, may be associated with disorders of the routine immunization schedule.

We assumed that the diagnosing of allergic diseases in the primary health care may be related to the disorders of the NIPC. Therefore, we analyzed in detail the links between vaccination with individual vaccines and atopic dermatitis, diagnosed both by PCPs and allergists (as the first atopic disease diagnosed at the earliest), as well as with the diagnoses of BA and AR diagnosed by the specialists.

There was no statistically significant difference in the frequency of PCP diagnoses of allergic diseases among children vaccinated with BCG and children who were not vaccinated with it. A MIRIAM study (in Germany) with a sample size similar to ours ($n = 1673$) did not identify links of BCG vaccination with any allergic diseases in studied children from 5 to 7 years of age (33). The results of studies conducted in Germany, Spain, and the United Kingdom have demonstrated the protective effect of BCG on the development of asthma in pre-school children (30, 34, 35). Systematic reviews have not identified the protective effects of BCG against the development of asthma (36, 37).

We found that the atopic dermatitis was diagnosed by PCP in the group of children who were not vaccinated with the HB vaccine statistically significantly more

often when compared to children under the age of 7 months who received all 3 doses of the vaccine (95% CI: 1.14-4.38), p = 0.02. This trend was also seen in children who were diagnosed with AD by an allergist at the age of 1 to 2 years: the diagnosis of atopic dermatitis was 2.5 times more frequent in the group of non-vaccinated children (p = 0.03). Such results allow to presume that the HB vaccine is not related to the development of allergic diseases. There is very little research on the link between the hepatitis B vaccine and atopic diseases. Usually this vaccine is analyzed in combination with others (29, 38). To date, no significant correlation between vaccination with the HB vaccine and allergic diseases has been established.

It is likely that vaccination for children, who were suspected of having atopic dermatitis during the first months of their life, could have been delayed more often during the first six months of their life. The increase of atopic dermatitis by 1.5 times (p = 0.03) and asthma by 4 times (p = 0.01) in the first year of life in the group of children who were not fully vaccinated with the DTaP-IPV-Hib vaccine before 7 months of age when compared to the fully vaccinated, which was established in our study, may have been caused by a refusal to vaccinate due to transient disorders of the skin and respiratory system. In a study of children hospitalized for wheezing, E. Ozkaya found that wheezing episodes during the first three years of life increased the likelihood that children would not be fully vaccinated with BCG, HB, Hib, and MMR even up to 10.6 times (39).

In order to evaluate whether vaccination disorders might have been associated with wheezing in infancy due to recurent infections (which may "hide" under the diagnosis of asthma until the first year of age) or with a PCP-diagnosed atopic dermatitis (in order to avoid exacerbations), we compared the frequency of allergic diseases between children vaccinated and not vaccinated with DTaP-IPV-Hib before 7 months of age. There was no difference in the ratio of PCP- and allergist-diagnosed AD in vaccinated and non-vaccinated children. This indicates that prophylactic vaccination of infants with vaccines containing the acellular pertussis component is not associated with a more frequent diagnosing of allergic diseases. We can assume that interferences of the routine vaccination plan, same as in the study conducted by E. Ozkaya and co-authors, were related to transient health conditions.

Links of Diagnosed Allergic Diseases with Time of Each Vaccine Inoculation

The first six months of life are a certain "window of opportunity" where the impact of allergic disease-inducing or protective factors is crucial. AD is usually diagnosed during the first year of life, which coincides with the intense vaccination of infants. We did not study the reasons for postponing or delaying vaccination but

analyzed the links between the postponing of vaccination with individual vaccines and allergic diseases.

We found that BCG vaccination delay by a month was associated with atopic dermatitis as diagnosed by an allergist 2.8 times more often at the age of 2 to 6 years (p = 0.03). However, there was no statistically significant difference in the frequency of PCP-diagnosed AD during the same age period, between children vaccinated on time and those vaccinated 1 month later. It should be noted that at all age periods analyzed, PCPs had diagnosed atopic dermatitis in a higher number of children than allergists, except for the period of 2 to 6 years, so it could mean that the allergists later verified the diseases suspected by PCPs. According to the results of the research of a cross-section by D. Dilli and co-authors, the time of vaccination with BCG and HB vaccines during the first two months of life was not associated with the development of atopy, but it was noticed that the risk of wheezing was lower in the group of children who were previously vaccinated with BCG (right after birth) when compared to children vaccinated with this vaccine at 2 months of age (40).

In our study, the children whose vaccinations with the first HB dose were delayed by 1 month were diagnosed with asthma over the period of 2–6 years of age 2.8 times more often (95% CI: 1.38-5.78, p=0.003). Same as in the case of the delayed BCG vaccination, we believe that there may have been reasons (such as congenital pathology, etc.) that could lead to the delay of vaccination and have an effect on the development of allergic diseases. We also found that allergic rhinitis was diagnosed 2.45 times more often in children who were vaccinated with HB1 a month later, but we would be critical of this result. The diagnosis of allergic rhinitis at an early age (1–2 years) is questionable, and a significant difference in proportions may be due to the very small sample of of the group of children that were vaccinated later (n = 8).

The frequency of asthma diagnosis from birth to 6 years of age was generally lower if children were vaccinated with DTaP-IPV-Hib one month later: OR 0.64 (95% CI: 0.43-0.95, p=0.03). However, there was no statistically significant difference in the frequency of asthma diagnoses for children who had been vaccinated with DTaP-IPV-Hib even later when compared to children who were vaccinated on time. The negative correlation between the reception of the first dose of DTP-IPV-Hib at infancy and asthma was first of all described by K. L. McDonald. The postponing of vaccination with the first dose of DTP reduced the risk of diagnosing asthma before 7 years of age. This trend was even more clear if all three first doses of the vaccine were postponed (OR 0.39; CI 95%: 0.18-0.86) (41). However, the results of the study caused doubt for other scientists, as the asthma description chosen by the researchers was based on the need of parents to contact a medical facility when a child with asthma experienced the symptoms (42). The results of the meta-analysis study did not indicate any correlation between the time of vaccination and asthma (43).

The results of our study resemble the results of the most recent study with a representative sample (n = 4433) cohort conducted in Australia. It was established that vaccination with DTaP (combined with IPV, Hib and HB) when delayed by one month (109 (2.5%) children of the general sample) is associated with a decrease in eczema (atopic dermatitis) (OR 0.55; 95% CI: 0.33-0.91) and reduced the need for eczema medication (OR 0.41, 95% CI: (c. p. 0.22-0.76) at one year of age (44).

According to the Global Infant Immunization Program, the expected start of vaccination with DTaP-IPV-Hib in Lithuania is at 2 months of age, which is a common practice in Europe and in many countries around the world. We could speculate that a slight delay in vaccination, by no more than one month, could be a protective factor for an allergic march – in children with a risk of atopy, for example. However, the lack of strong evidence leaves the question open of whether the delayed vaccination with DTaP-IPV-Hib would be beneficial to individual patients and how it would affect universal immune protection (45). It must be kept in mind that the postponement of vaccination times and the delay of the vaccine-induced protection increases the risk of contracting the diseases that are managed with vaccines, and the benefits of early vaccination outweigh any possible or only suspected risk of progression of allergic diseases (46, 47).

4.2 Discussion of the Phase II Results

Adverse Reactions to the Vaccines

Changes in health conditions that manifest shortly after vaccination are very rarely associated with allergic reactions. Whatever they are, they have a major impact on parents' decisions on the further vaccination of their children (39). One of our research subjects had experienced a post-vaccination reaction after a dose of DTaP2 – sluggishness and drowsiness. After this reaction, the parents stopped further vaccination of the child.

The frequency of adverse reactions to vaccines reflect the safety of vaccines. The fact that after the review of 519 medical histories during the study we found 30 documented post-vaccination reactions experienced by 28 children over 1.5 years, when one BCG, three HB, four DTaP-IPV-Hib, and one MMR vaccine doses are inoculated, showed that the reporting of reactions after immunization is not sufficient, or that most reactions are mild, do not pose a threat to health, and quickly pass on their own. According to the data of CCDA, 36 to 80 post-vaccine reactions are registered every year in Lithuania, both for children and adults. Based on the frequency of officially registered post-vaccination reactions, our country does not differ from other countries (48).

Recent data suggest that children who are allergic to an egg white can be vaccinated safely with the MMR vaccine at primary health care centers (49–51). In addi-

tion, the latest results from the *Europrevall* study showed that an egg white allergy is not as common as previously thought, and more than half of all children who were allergic to egg whites are starting to tolerate it even before their first birthday (52).

Based on the data of our study, children who were allergic to the egg white and who were vaccinated did not experience any adverse effects after the vaccination. Four children who were allergic to egg whites were vaccinated only partially, although no contraindications were specified in medical histories. Based on the results of the study conducted in Ireland, during the vaccination of children with a possibly increased risk, for whom an allergy was suspected, only 1.3% experienced adverse reactions to the MMR vaccine; all of those were mild and were not caused by the immune mechanisms of the allergy (49).

The difference between the reactions after vaccination recorded in the medical histories of the polyclinic and the CCDA data shows that the current recording of such reactions is rather complicated and inadequate. The recording of adverse reactions after vaccination could be facilitated by the digital data storage and automatic forwarding systems as well as mobile applications.

Association between Early Life Factors (Including Vaccination) and Allergic Diseases

For the second phase of the study, the medical histories of children whose diagnoses of allergic diseases raised no doubts were selected. During the first year of life, none of the selected children were diagnosed with allergic rhinitis or bronchial asthma. Furthermore, children who were diagnosed with atopic dermatitis even before the vaccination with the DTaP-IPV-Hib vaccine were excluded from the group of research subjects. Therefore, vaccination as a factor in all cases was prior to the diagnosing of an allergic disease and could be analyzed as a protective or risk factor.

It is difficult to analyze the links between vaccination during the first year of life and allergic diseases over the time in the same sample of children in different age periods. This could be the reason why there are not many studies of such kind (35, 41, 53, 54). Usually children of different ages are examined at one specific point in time or by questionnairing parents, but no continuous or periodic observations of the same children are performed (26, 55–58).

After a comprehensive multiple binary logistic regression analysis, the postponement of first dose of DTaP-IPV-Hib by one month was not statistically significantly associated with a reduction in asthma risk, and postponement of HB1 and BCG were not a significant risk factor for the diagnosis of allergic diseases. A recent study has established links between a one-month postponement of immunization with the DTaP vaccine and the development of eczema (44). Hence, there is a particular focus on the early effects of various factors related to the development of the

immune system. However, when assessing the vaccination in the context of other factors, the evidence that delaying of vaccination with one vaccine or another would be significant to the prevention of the development of allergic diseases is insufficient.

We analyzed the fact of antibiotic prescription, which we were able to verify by conducting an analysis of medical histories. We found that during the first year of life, each course of antibiotics prescribed increased the risk of the analyzed allergic diseases. A frequent prescription of antibiotics is possibly associated with a higher morbidity with respiratory infections, but that was not a subject of our study. Retrospectively, it was difficult to assess whether certain diseases or antibiotics themselves were associated with further developments of allergies. It was shown that infection may be associated with the progression of allergic diseases (59).

The first year of life is a crucial time for beginning antibiotic consumption. We did not find any association between the diagnosis of asthma or atopic dermatitis before 6 years of age and the antibiotic administration fact. However, these diseases were diagnosed more often in children who were prescribed antibiotics in their first year of life more often: BA – 1.48 times ($p = 0.033$), AD – 1.45 times ($p = 0.008$). A continuous study of parents and children by AVON found that children who were more often prescribed antibiotics at 0–2 years of age were diagnosed with asthma 1.75 times more often at 7.5 years of age. (60). According to a prospective birth cohort study PASTURE, exposure to antibiotics in the first year of life (aOR 2.73; 95% CI 1.66-4.49) and the number of doses received were strongly positively associated with AD up to 4 years. A tendency of a possitive association between antibiotic intake in infancy and asthma between 3 and 6 years was also observed (aOR 1.65; 95% CI 0.95-2.86) (61).

In analyzing the pathology immediately after birth, we found that a congenital infection was associated with an increase in the frequency of all allergic diseases examined in the models of the univariate binary logistic regression, i.e. it could be one of the potential risk factors for allergic diseases. However, in the multivariate logistic regression models, congenital infections did not appear as an independent significant risk factor for the diagnosing of allergic diseases. It has been established that the impact of early infection on the microbiota, which is important for the development of the immune balance right after birth, can result in a development of atopic diseases as the individual grows (62, 63). T. Sobko and co-authors showed that sepsis in the newborn is associated with a higher risk of asthma and eczema, and that an early prescription of antibiotics may increase this risk even more (64).

The results of our study revealed what a comprehensive subject is the link between vaccination and allergic diseases, and how one or the other conclusion cannot be interpreted unambiguously. It is expedient to continue prospective research on this subject by including more factors that act at the same time, such as the morbid-

ity of infectious diseases during infancy, the confirmation of antibiotics consumption, the duration of consumption, etc. The adaptation of digital databases of health care institutions for such research would expand the prospects of not only regional but international research as well.

Conclusions

1. The frequency of allergic diseases in general, diagnosed among children vaccinated according to the prophylactic vaccination calendar and among non-vaccinated children, did not differ.
2. Atopic dermatitis diagnosed by primary care physicians during infancy and wheezing diseases diagnosed by pulmonologists are associated with disorders of the routine immunization plan for the DTaP-IPV-Hib vaccine. In children who have not been vaccinated with the HB vaccine before 7 months of age, atopic dermatitis was diagnosed more frequently by primary care physicians during infancy, and by allergists – during the second year of life.
3. For children vaccinated with BCG a month later, an allergist has diagnosed atopic dermatitis at 2 to 6 years statistically significantly more frequently. For children vaccinated with HB1 a month later, asthma was diagnosed at 2 to 6 years of age statistically significantly more frequently. The inoculation of the first dose of DTaP-IPV-Hib delayed by one month was associated with a lower frequency of asthma diagnosis in the pre-school age (compared to the children who were vaccinated on time).
4. There was no difference in the frequency of adverse reactions to vaccines among children diagnosed with atopic dermatitis and children with no allergic diseases diagnosed.
5. The first dose of the DTaP-IPV-Hib vaccination, when delayed by one month, is not an independent protective factor for bronchial asthma. BCG delay by a month is not an independent risk factor for atopic dermatitis, and a HB1 delay is not an independent risk factor for asthma.
6. The number of antibiotic courses prescribed during infancy is a significant independent risk factor for atopic dermatitis and asthma, and the fact of antibiotics prescription during infancy is a significant independent risk factor for allergic rhinitis.

Recomendations

1. Based on the findings of our study and international recommendations – allergic diseases, allergic reactions, or sensitization are not related to the components of vaccines; therefore, children with allergic diseases can be safely vaccinated. It is advisable to supplement the National recommendations on the absolute and relative, both permanent and temporary contraindications for the immunisation of children with allergies.
2. Based on the results of our real-life study, children vaccinated with the DTaP-IPV-Hib vaccine with the delay of a month later than specified in the NIPC were diagnosed with asthma more rarely, but the delay of vaccination is not an independent protective factor for asthma. It would be advisable to expand and to conduct the study prospectively.
3. We recommend that children with atopic dermatitis be vaccinated according to the national immuno-prophylaxis calendar – their vaccination is safe, as the frequency of adverse reactions after vaccination does not differ from that of children without allergic diseases.
4. The adaptation of digital statistical databases and systems used in health care institutions to record adverse reactions to vaccines and their automatic forwarding to CCDA and other responsible institutions would greatly facilitate the analysis of these events. It is advisable to improve data collection and to ensure the possibility for collaboration with researchers who use digital health databases abroad.
5. It is necessary to follow the protocols for diagnosis and treatment of diseases or algorithms established and approved by health care institutions in order to improve the quality of the prescription of antibacterial preparations in Lithuanian health care institutions. It is fundamental to prescribe antibiotics to children responsibly, especially during infancy.

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Jekaterina Byčkova

THE FUNCTIONAL RESULTS AND PROGNOSTIC FACTORS OF PEDIATRIC COCHLEAR IMPLANTATION

VAIKŲ KOCHLEARINĖS IMPLANTACIJOS FUNKCINIAI REZULTATAI IR PROGNOSTINIAI VEIKSNIAI

Biomedical Sciences, Medicine 06 B
Biomedicinos mokslai, medicina 06 B

Introduction

Hearing loss (HL) is the most frequent congenital disability in developed countries. Out of every 1000 children, 1 to 3 are born with sensorineural hearing loss, and another 1 to 2 out of 1000 children develop hearing loss later. According to the World Health Organization, in 2018, approximately 466 million people all over the world were diagnosed with HL, 34 million of them being children. (*Deafness and Hearing Loss*, http://www.who.int/en/news-room/fact-sheets/detail/deafness-and-hearing-loss). According to the Compulsory Health Insurance Fund data, 2156 Lithuanian children were diagnosed with deafness or hearing loss in 2017 (4.25 of 1000 children) (*Lietuvos gyventojų sveikata ir sveikatos priežiūros įstaigų veikla 2016 m.*).

One third of congenital sensorineural hearing loss cases are profound or severe. Such a hearing loss has long-term consequences in both the child's and the family's life. Hearing loss has a distinct negative effect on the development of the spoken language, and that consequently affects everyday communication and limits learning and literacy; therefore, a child's achievements and abilities of employment suffer, causing psychosocial problems. (Schroeder et al. 2006; Marschark & Spencer 2010). A lack of auditory information in early childhood interrupts with the development of the auditory system of deaf children, as in the absence of acoustic stimulation, the auditory cortex reorganizes to receive information from other senses (vision, for example) – cross-modal reorganization occurs (Sharma & Glick 2016a). When the critical period of brain development ends, the auditory cortex can no longer process auditory information, even though primary centers frequently react to the stimulation of the cochlear nerve after cochlear implantation (CI) (Nishimura et al. 1999) It is essential that the period from the onset of bilateral deafness until medical intervention – hearing rehabilitation with hearing aids (HA) or cochlear implants (CI) – is as short as possible (Nicholas & Geers 2007). Universal hearing screening dramatically improved the diagnosis of congenital hearing loss (CHL) and the speech and language results of children with hearing impairment (Shani J. Dettman, Pinder, Briggs, Dowell & Leigh 2007).

A cochlear implant is an electronic medical device used to restore hearing for deaf people. A CI processor transforms environmental sounds into an electric signal, and the electrode array inserted into the inner ear cochlea transmits this signal to the cochlear nerve. The impulse then travels to the cerebral cortex, and the implanted person is able to hear because of this. In recent decades, revolutionary solutions in HL diagnostics, implant technology, sound processing, surgical techniques, programming and special education gave the possibility for deaf children to not only understand spoken language but to speak as well. This progress proved cochlear implantation is a safe and effective method for restoring hearing (F. Forli 2011).

CI results are widely studied. CI gives the opportunity to hear, positively affects speech and language development, improves educational achievements, employment possibilities and quality of life. The effect on society manifests by the decreased expenses for the education of the deaf and increased work productivity (Bond et al. 2009). The results of cochlear implantation are assessed in steps: first – hearing and speech perception, later – speech and language development, and then – integration to the general education, quality of life and others. Based on the scientific literature, speech perception and speech intelligibility levels of half of children who are CI users can be the same as of their hearing peers (A. E. Geers, Moog, Biedenstein, Brenner, & Hayes 2009). In a 2016 review, Monteiro claims that the speech perception of 81% of children aged 12–48 months after implantation was within normal limits, and the speech and language development of 57% of children matched the results of their peers (C. G. Monteiro, Cordeiro, Silva & Queiroga 2016).

Individual results still differ a lot. A huge variability in speech perception and language development results is observed (Ann E. Geers, Strube, Tobey, Pisoni & Moog 2011a; Tobey et al. 2013). It was proved that age at implantation is the most important prognostic factor of cochlear implantation – the earlier the implantation is performed, the better the results are. Other important factors are: internal and biological – the etiology of deafness, a child's intelligence; external and technical – implant characteristics, programming; social – the possibilities of speech and language therapy, parents' engagement in the learning process, and others (Driver & Jiang 2017). The establishment of prognostic factors helps to predict results for an individual patient and build real expectations for the family, plan implantation and rehabilitation process after the CI to achieve the maximal benefit (Peterson, Pisoni & Miyamoto 2010).

The first cochlear implantation in Lithuania was performed in 1998 in the Lithuanian University of Health Sciences by Professor S. Harris. Today, there are about 370 CI users in Lithuania.

Only early postoperative results of CI were evaluated in Lithuania (Byčkova, Gradauskienė, Lesinskas, Mikštienė & Utkus 2012). Long-term results, such as speech perception, speech and language development, integration into general education have not yet been studied in the Lithuanian population. Such factors as the etiology of deafness, the radiological anatomy of the inner ear and social factors that might affect the effectiveness of CI were not studied as well. The genomics of Lithuanian congenital hearing loss were recently studied by Violeta Mikstiene, in 2017. However, the etiology of pediatric deafness in general has not been studied yet. Half to two thirds of congenital HL cases are hereditary. The remaining cases are nonheritable – environmental or of unknown etiology. Mutations of the *GJB2* gene are found in 30% of cases of congenital deafness (Chan & Chang 2013). A

congenital CMV (cCMV) infection is the second most frequent cause of CHL and accounts for 10–30% of CHL cases. Usually, a cCMV infection is asymptomatic; therefore, specific tests are not performed at birth. However, HL can develop after several months or even years. The detection of the prevalence of cCMV infection is still a relevant problem all over the world (Rawlinson et al. 2017) Different populations have characteristic geographical, ethnic, social, medical, and genetic factors that generate a unique etiologic profile of HL. This is the first study to determine the etiological profile of deaf Lithuanian children who are CI users, and this study will enable the evaluation of the prevalence of genetic factors, cCMV infections and other risk factors as well as the establishment of recommendations for the creation of CI program guidelines.

It is known that best results are demonstrated in countries with a CI program, including early HL diagnostics, preoperative preparation, surgical implantation procedures and full postimplantation service: technical, medical, psychological, social, and financial support (Moeller, Carr, Seaver, Stredler-Brown & Holzinger 2013). Despite the fact that pediatric CI has been performed in Lithuania for already two decades, such a program is still lacking. This study attempts to prepare recommendations for developing a Lithuanian CI program. This program will help optimize the selection of candidates and postoperative rehabilitation, and improve the social integration of deaf children with CI.

In conclusion, it can be claimed that the evaluation of CI results and the establishment of prognostic factors is a relevant clinical and scientific problem. This study was the first in Lithuania to assess the etiology of hearing loss, postoperative results, and prognostic factors amongst pediatric CI users. The results of this doctoral dissertation might widen the clinical, social and educational means for optimizing the results of pediatric CI in Lithuania and improve indices of public health.

Aims of the study

To assess the functional results of cochlear implantation in children and determine their prognostic factors.

Objectives of the study

1. To identify the etiology of hearing loss amongst pediatric cochlear implant users by performing genetic testing, detecting CMV DNA in dried blood spots, analyzing perinatal risk factors and, therefore, establishing the etiological profile of the study sample.
2. To evaluate the anatomical malformations of the inner ear of pediatric cochlear implant users by analyzing images of the temporal bone made by computed tomography.

3. To evaluate the speech perception results of deaf children after cochlear implantation.
4. To evaluate the results of speech and language development after pediatric cochlear implantation.
5. To determine prognostic factors for the outcomes of pediatric cochlear implantation.

1. Materials and methods

This multicenter, interdisciplinary, cross-sectional study was performed during 2013–2018 in Vilnius University, Faculty of Medicine, at the Clinic of the Ear, Nose, Throat and Eye Diseases, as well as in the Children's Hospital, affiliate of Vilnius University Hospital Santaros Klinikos (VUHSC), at the Children's Otorhinolaryngology and Ophthalmology Department. The local Vilnius Regional Biomedical Research Ethics Committee approved the protocol of this study (No. 158200-15-786-298)). An individual informed consent form was read and signed by parents or caregivers of each study participant prior to the inclusion to the study. The ethical principles of the Declaration of Helsinki for medical research involving human subjects were fulfilled.

The scheme of the study was planned at the beginning of the research, and data were collected based on the design of the cross-sectional study – the preoperative, surgical, and postoperative data of each participant were collected, etiologic factors tested, and postoperative hearing and speech results assessed all at the same time:

- In order to evaluate the *preoperative, surgical*, and *postoperative* factors that might influence CI results, the parents of participants were interviewed, and medical documentation reviewed: demographic, medical, audiological, surgical, and family data, as well as data regarding rehabilitation and education, were collected;
- To determine the *etiology* of hearing loss, anamnestic risk factors of hearing loss were assessed, genetic testing performed, and CMV DNA detected in dried blood spots;
- To determine any *inner ear malformations* that might influence CI results, a thorough analysis of preoperative temporal bone CT images was performed;
- Postoperative *hearing results* were evaluated using scales, the sound field warble tone and speech audiometry;
- Postoperative *speech and language results* were assessed during evaluations performed by teachers of the deaf.

After analyzing the collected demographic, medical, audiologic, surgical, and family data, as well as data about hearing rehabilitation, educational settings and postoperative results, the prognostic factors of pediatric CI were defined (Fig. 1).

The objectives of the scientific study were achieved in collaboration between an otorhinolaryngologist-audiologist (author of the study), a nurse audiometrist, a geneticist, radiologists, an otorhinolaryngologist, teachers of the deaf, speech and language therapists, a statistician, a laboratory medicine doctor and a medical student.

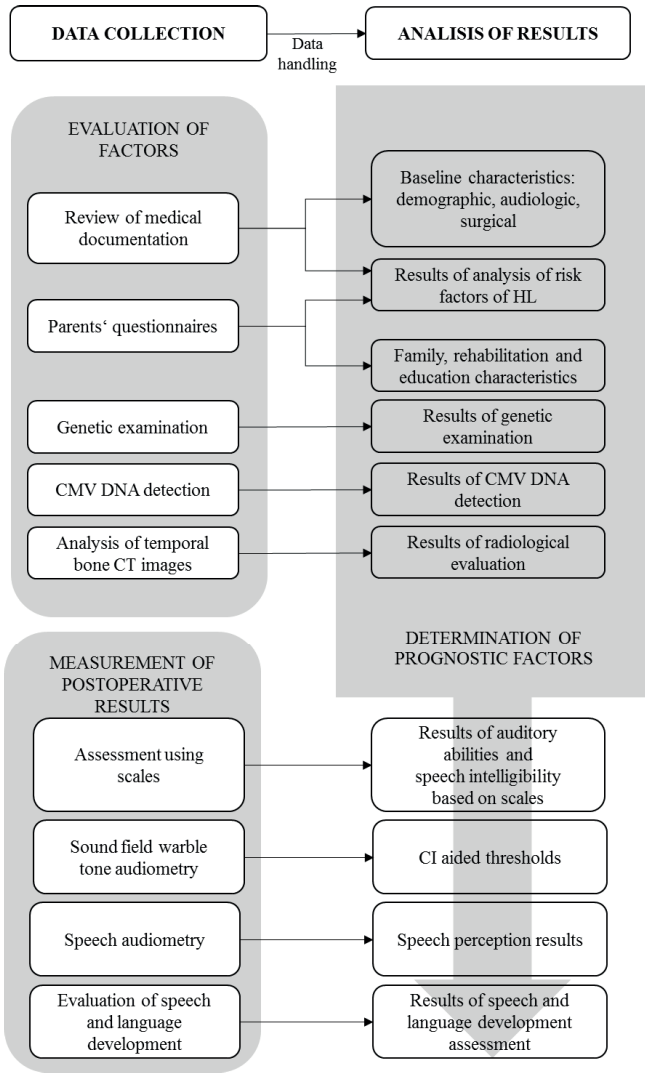


FIGURE 1. Scheme of the study.

1.1. Sampling

The sample of the study included children who underwent unilateral or bilateral cochlear implantations in Vilnius University Hospital Santaros Klinikos, in the Clinic of Ear, Nose, Throat and Eye Diseases. Participants were chosen from the list of CI surgeries performed in 2005–2017 in Vilnius University Hospital Santaros Klinikos, in the Department of the Ear, Nose and Throat Diseases. Patients who met the inclusion criteria were informed about the study and suggested to participate during a scheduled visit to an otorhinolaryngologist-audiologist in the Children's Hospital, affiliate of Vilnius University Hospital Santaros Klinikos. Families of children who did not come to a visit were invited to participate by contacting the parents or caregivers via a phone call, using contact data found in medical documentation. One hundred thirty children with CI were found, 122 of them met the inclusion criteria:

1. Deaf children, who underwent one or two cochlear implantation surgeries;
2. Participants aged 1–18 years;
3. Cochlear implantation surgery performed in Vilnius University Hospital Santaros Klinikos at the Clinics of the Ear, Nose, Throat and Eye Diseases;
4. Unilateral or the first one of the bilateral CI surgeries performed not later than prior to 6 months;
5. Parents or caregivers of children agreed for their child to participate in the study and signed the informed consent form.

Cochlear implantation was performed for all participants in Vilnius University Hospital Santaros Klinikos during the period from July 5, 2005 to July 10, 2017. Implantation surgery was performed according to a general procedure, when, based on the description of the CI reimbursement procedure, the necessity of surgery and an additional processor was ascertained by the medical consultation of three otorhinolaryngologists of the Santaros clinics. All surgeries were performed by one experienced otosurgeon. Processors were turned on and regulated, and their technical support maintained according to the manufacturers' guidelines by the representatives of the CI manufacturers MED-EL, Cochlear and Advanced Bionics in Lithuania.

Data of all **122** participants were used to assess general, family, hearing rehabilitation and educational characteristics and to determine the etiologic profile. In addition to this, all children participated when performing sound-field audiometries and evaluations of auditory abilities and speech intelligibility according to scales. A sound-field speech audiometry was only performed for children older than 5 years who were implanted at least 2 years ago – they composed a group of **95** participants. Fourteen more participants (children with severe additional disabilities, and children with an onset of HL after their 3rd birthdays) were excluded when assessing

speech and language development and determining prognostic factors for speech perception and speech and language development (N=81) (Fig. 2).

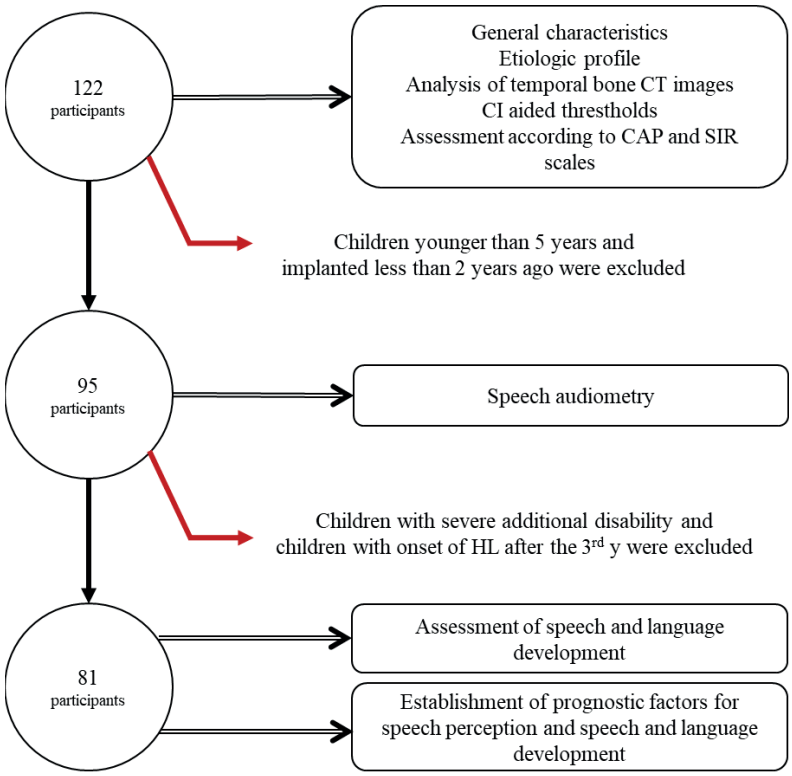


FIGURE 2. Flow-chart of the study.

1.2. Data Collection from Questionnaires and Medical Documentation

Questionnaires were filled in during the visit to an otorhinolaryngologist-audiologist; medical documentation reviewed from the in-patient and out-patient medical records, the electronic medical records of Vilnius University Hospital Santaros Klinikos and medical records from other healthcare institutions. According to the questionnaire prepared for the research, (a) medical, (b) hearing, (c) surgical, (d) family, (e) hearing rehabilitation and education data were collected.

(a) **Medical documentation**. Prenatal, perinatal and postnatal risk factors for HL were recorded according to the list proposed by the Joint Committee of Infant Hearing from the medical documentation:

- Prematurity <32 weeks of gestation;

- Birth weight less than 1500 g;
- Severe perinatal hypoxia;
- Congenital TORCH infections: toxoplasmosis, syphilis, rubella, CMV, herpes;
- Newborn sepsis;
- Hyperbilirubinemia requiring blood transfusion;
- Ototoxic medication;
- Meningitis.

Data about any additional diseases that might have influence on postoperative results (visual impairment, cerebral palsy, epilepsy, developmental delay, autism, syndromes and other) were searched for as well.

(b) Audiological data were used to assess residual hearing before the implantation. Following hearing tests results and anamnesis, data were collected from the medical records:

- Age at diagnosis;
- Otoacoustic emission results;
- Brainstem Electric Response Audiometry (BERA) thresholds;
- Auditory Steady State Audiometry (ASSR) thresholds;
- Pure tone audiometry thresholds;
- HA aided thresholds;
- Duration of the hearing rehabilitation using HA;
- Onset and progression of HL.

Hearing thresholds prior to the CI were determined based on BERA results in case of the congenital HL, and based on age-appropriate last preoperative audiograms in case of acquired HL. Mean hearing thresholds were calculated in a better hearing ear as well as in an implanted ear. Lower than 90dB hearing thresholds in the implanted ear (or in one of implanted ears) or in the contralateral ear were considered as residual hearing.

(c) Medical documents were reviewed for these **surgical and implant-associated** data:

- Date of the first/second implantation surgery;
- Side of the implantation/unilateral/bilateral;
- Age at first (second) implantation;
- CI device manufacture/processor/electrode array;
- Insertion of the electrode array;
- Complications (intraoperative, early and late postoperative);
- Programming of the speech processor;
- Speech coding strategy;

- Issues with the use of the CI processor (in case medical documentation included and/or parents declared about the inconsistent use of the processor or fitting problems during the first postoperative year).

(d) Family data were collected according to the Nottingham Children's Implant Profile (NChIP) using questionnaire:

- Size and structure of family;
- Parents' education level (different educational levels were grouped to three categories: higher education – at least 14 years of education (higher education); secondary education – 10 – 13 years of education (post-secondary, special secondary, secondary vocational, and secondary education); incomplete secondary education – less than 9 years of education (vocational school, lower-secondary and primary education)
- Parents' understanding of the CI process (based on the Pre-/Post-Implant Family Assessment Profile for PCI candidates (Hickson & Black 2012) Hickson, & Black, 2012: parents understand the CI process when they have reasonable expectations consistent with hearing loss, demonstrate a good understanding of the CI process and post-operative rehabilitation requirements, demonstrate good understanding of the level of commitment required and the impact this will have on the child's well-being; parents do not fully understand the CI process when they have some unreasonable post-operative expectations, demonstrate a sound understanding of CI processes and post-operative rehabilitation requirements, require additional information to aid understanding; parents do not understand the CI process when they demonstrate little understanding of the CI processes and post-operative rehabilitation requirements, have unreasonable high expectations and a reluctance to consider a professional's views, and are strongly reluctant to engage in the process);
- Frequency of follow-up (based on the recommendations for a follow-up of children after CI proposed by the Children's Hospital, affiliate of Vilnius University Hospital Santaros Klinikos, visits were considered sufficient if at least 5 visits were recorded over a 2-year period; insufficient – if 3–4 visits were recorded over a 2-year period; family did not attend consultations – 2 or less visits recorded during the first two years after the implantation);

(e) The following data about **hearing rehabilitation and education** were collected:

- Parents' engagement in the child's learning process (based on the NChIP profile: active participation – parents were interested in the child's outcomes and constantly communicate and interact with the child; passive participation – parents were interested with the result, but do not communicate with

the child; no participation – parents were not interested with the result and do not communicate with the child);

- Communication mode (spoken language – when parents use only spoken language to communicate with the child, no additional visual cues are used; total communication – when both, spoken and sign languages are used, child is lip-reading; sign language – when a child does not understand any words, signs only are used for communicating);
- The availability of speech and language therapy (based on the NChIP profile: available – there is a constant possibility to consult an experienced teacher of the deaf; moderately available – there is a possibility to consult an unexperienced teacher of the deaf; unavailable – there is no possibility to consult a teacher of the deaf);
- Intensity of the speech and language therapy (based on the frequency of visits to the teacher of the deaf per week during the two first years after the CI);
- Educational placement settings, program at school.

1.3. Evaluation of the Etiology of Hearing Loss

In order to establish all causes of hearing loss, risk factors were determined, genetic testing performed and CMV DNA extracted from dried blood spots.

1.3.1. Analysis of Risk Factors of Hearing Loss

An analysis of prenatal, perinatal and postnatal risk factors of hearing loss was performed using data retrieved from the questionnaires and medical records according to the aforementioned list of perinatal, prenatal and postnatal risk factors.

1.3.2. Genetic Examination

Genetic counselling and testing were performed by geneticist in Vilnius University Hospital Santaros Klinikos, at the Center for Medical Genetics (VUHSC CMG). Participants were divided into subgroups and examined based on the HL type. In case of isolated (non-syndromic) HL, *GJB2* gene sequencing was performed according to the procedure established by the VUHSC CMG. In the absence of two pathogenic states, in case of signs of mitochondrial inheritance in genealogy or for patients with a complex perinatal anamnesis who were treated with aminoglycosides, the pathogenic state of the mitochondrial genome *MT-RNR1* 1555A>G was tested. Genes associated with a specific syndrome were tested for patients with a syndromic HL when a monogenic disease was suspected. *GJB2* gene sequencing was also performed for some patients with the syndromic HL to differentiate the

cause of HL. When a chromosomal syndrome was suspected, karyotyping test or comparative genomic hybridization were performed. If the cause of HL remained unknown, a sequencing of 126 genes was performed in case of an isolated HL and positive genealogy (Fig. 3). Genomic DNA used for testing was extracted from the peripheral blood leukocytes using the standard phenol chloroform method.

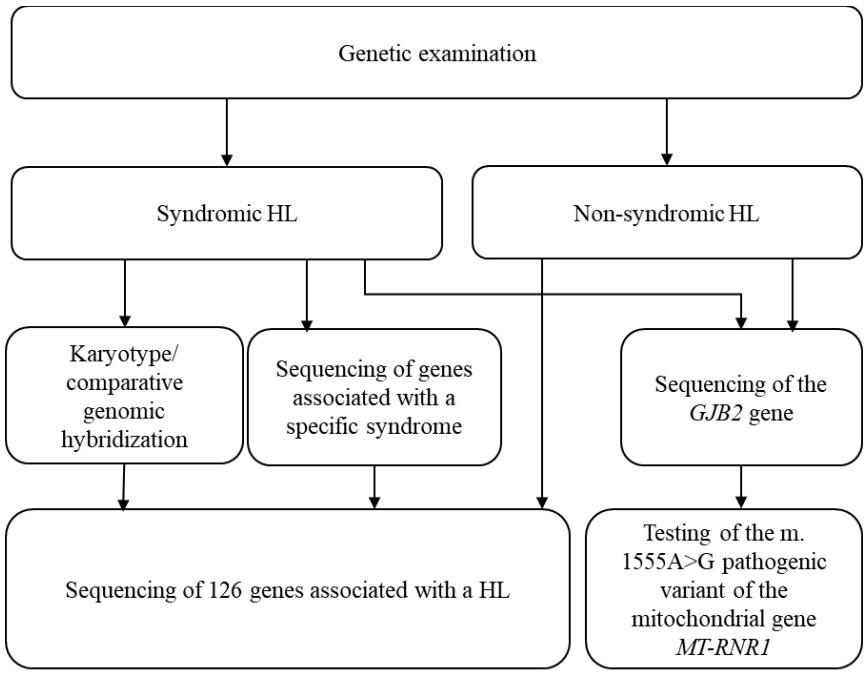


FIGURE 3. Scheme of the genetic examination.

1.3.3. Detection of the CMV DNA in Dried Blood Spot

In order to discover a possibly congenital cytomegalovirus (CMV) infection amongst the sample of the study, CMV DNA was detected in dried blood spots on the Guthrie cards. The examination was performed from September 2017 to April 2018 in Vilnius University Hospital Santaros Klinikos, at the Center of Laboratory Medicine (VUHSC CLM). Guthrie cards are filter-paper cards used for a universal neonatal screening of inherited metabolic disorders. The capillary blood of a newborn was collected onto a blood spot card on the 2nd – 5th day of life in a hospital. After screening for the metabolic disorder, cards with the remaining biologic content are kept in a VUHSC CLM archive. We used half of a dried blood spot for a every single DNA extraction (the diameter of a full spot is 10 mm), each sample was

proceeded in triplicate. Scissors used for cutting the DBSs were cleaned with 70% ethanol between cards to avoid contamination. In each DNA extraction, blank DBSs were included for contamination control. CMV DNA was amplified using primers targeting the 105 bp region of the major immediate early (*MIE*) gene (artus CMV QS-RGQ, Qiagen). Real time PCR (Polymerase Chain Reaction) was performed on DNA triplicates. Results were assessed qualitatively. DBS sample considered positive when two or more DNA triplicates were CMV DNA positive (Fig. 4).

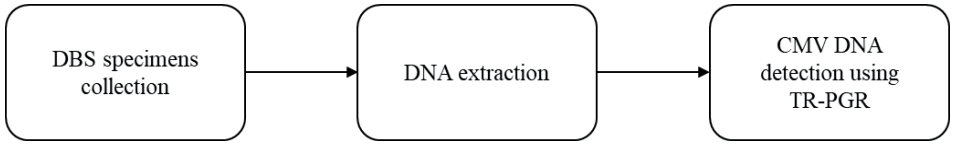


FIGURE 4. Scheme of the CMV DNA detection in dried blood spot.

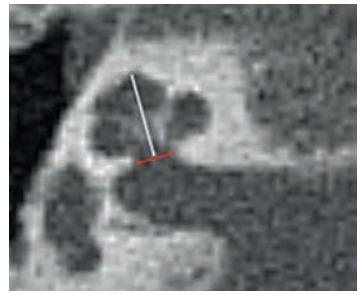
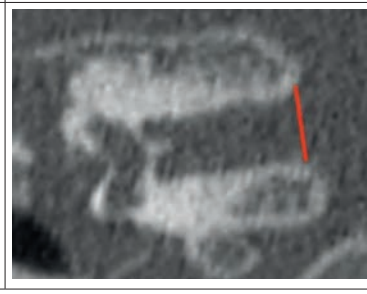
1.4. Evaluation of the Inner Ear Anatomy on Temporal Bone CT

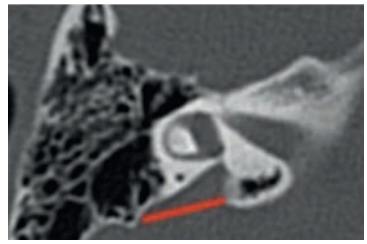
A retrospective analysis of preoperative temporal bone CT images, archived in the electronic medical system of VUHSC, was performed. High definition temporal bone CT images with <1-mm (0,7 mm on average) slice thickness were analyzed by an experienced radiologist in the VUHSC, at the Center of Radiology and Nuclear Medicine, using *Picture Archiving and Communications System* (PACS). *MultiPlanar Reconstructions* (MPR) of inner ear were performed as well. Evaluation was performed in two ways: major inner ear malformations (IEM) were assessed visually, small structures that might have effect on the CI results were measured additionally (Table 1). The architecture of the cochlea was considered abnormal in case of cystic cochlear changes and a decreased number of cochlear turns. The vestibule was considered abnormal in case it was dilated – when its transverse diameter was larger and the vestibule more rounded. Semicircular ducts were considered abnormal in case of defected integrity. Also, the radiologist measured all inner ear structures in detail in order to discover any delicate IEM that might have influence on postoperative results. All measurements were made in precisely described MPR planes – height of the cochlea, diameter of the bony cochlear nerve canal (BCNC), diameter of the internal acoustic meatus, diameter of the vestibular aqueduct. The descriptions by other authors about the technique of measurements were used to compare the result (Table 2). The cochlea was considered hypoplastic when its height was less than 3.3 mm. The BCNC was considered stenotic in case its diameter was equal or smaller than 1.4 mm. The internal acoustic meatus was considered stenotic when its diameter was smaller than 2 mm. The vestibular aqueduct was considered dilated when its width was more than 1.9 mm (D'Arco et al. 2017).

TABLE 1. An analysis of temporal bone CT images.

VISUAL EVALUATION	Architecture of the cochlea	Abnormal in case of decrease in number of turns, cystic cochlea
	Vestibule	Dilated in case transverse dimension is bigger, and vestibule rounder
	Semicircular canals	Abnormal in case of defected integrity
MEASUREMENTS	Height of the cochlea	Hypoplastic cochlea – height of the cochlear is smaller than 3.3 mm
	Diameter of the bony cochlear nerve canal	BCNC stenosis – diameter was equal or smaller than 1.4 mm
	Width of the internal acoustic meatus	Stenotic – when the width is smaller than 2 mm
	Width of the vestibular aqueduct	Dilated – when width is more than 1.9 mm

TABLE 2. Planes of radiologic measurements.

No	STRUCTURE	DESCRIPTION OF PLANES	IMAGES OF PLANES
1	Height of cochlea	The MultiPlanar Reconstruction depicts an X-shaped modiolus, cochlear nerve canal, cochlear canal. Measured is the distance from the center of the cochlear nerve canal to the apex of cochlea (Teissier, Van Den Abbeele, Sebag & Elmaleh-Berges 2010)age range from 3 days to 16 years between February 1999 and July 2004. A control group (n = 88.	
2	Diameter of the cochlear nerve canal	Measured is the distance between the osseous parts of the canal aperture (Teissier, Van Den Abbeele, Sebag & Elmaleh-Berges 2010)age range from 3 days to 16 years between February 1999 and July 2004. A control group (n = 88.	
3	Width of the internal acoustic meatus	The internal acoustic meatus is presented in the MultiPlanar Reconstruction. Perpendicularly measured is the distance between the osseous canal walls at the level of the *porus acusticus internus* (Shim, Snin, Chung, Lee, 2006).	

4	Width of the vestibular aqueduct	The MultiPlanar Reconstruction depicts the biggest width of the vestibular aqueduct. It is measured perpendicularly to the bony walls of the aqueduct (dilated vestibular aqueduct) (Vijayasekaran et al., 2007).	

1.5. Evaluation of Postoperative CI Results

A cross-sectional study was arranged to evaluate the postoperative results. All results, except for speech and language development, were assessed at the Children's Otorhinolaryngology and Ophthalmology Department of the Children's Hospital, affiliate of VUHSC. According to the hierarchic speech perception and speech and language development evaluation methodology, the assessment method was chosen based on the child's age. Hearing thresholds with CI in sound-field were tested for all participants as well as evaluated according to the CAP and SIR scales. Sound-field speech audiometry with CI, as well as an assessment of speech and language development, was performed only in the case the child was at least 5 years of age and was implanted at least 2 years ago.

1.5.1. Evaluation of Aided Thresholds and Speech Perception

CI-aided thresholds and open-set speech perception in quiet surroundings were evaluated during hearing assessments. Audiometric tests were performed with a diagnostic audiometer *Interacoustics AC 40* (Denmark), corresponding these standards: EN 60645-1/ANSI S3.6, 1 type, EN 60645-2/ANSI S3.6, A or A-E type, EN 60645 4/ANSI S3.6, EN 60601-1, I class, B type, EN 60601-1-2, calibrated according to ISO 389-1, ISO 389-3, ISO 389-7, IEC645-2. All hearing tests were performed in an audiometric booth, in which environmental noise did not exceed the permissible limits according to ISO 8253-2. All tests were performed by an audiometrist with long experience in pediatric audiology. The results were analyzed by the author.

The assessments of CI-aided thresholds were performed using warble tones following the standard procedure when the lowest sound intensity in 250, 500, 1000, 2000, and 4000 Hz frequencies is established for each ear separately. Loudspeakers were placed at a 45° azimuth in a 1 m distance from the child. When testing binaurally, in the case of bilateral CI, the loudspeaker was placed 1 m in front of the child at a 0° azimuth. Based on the child's age and psychomotor development, CI-aided sound field thresholds were assessed using visual reinforcement or play audiometry methods according to British Society of Audiology recommendations.

Open-set speech audiometry was performed in an audiometric booth according to standard procedure to assess speech perception levels using one or two CIs. Every child was presented with the list of 25 disyllabic phonetically balanced words at 65 dB SPL in quit with the loudspeaker positioned at a 0° azimuth in a 1 m distance from the child. The child was instructed to repeat words that he or she had heard. The speech perception score was calculated based on the number of correct words in percentages. Later, the results were classified according to speech perception levels (Table 3).

TABLE 3. Speech perception levels based on the results of speech audiometry.

Speech perception score (%)	Speech perception level
100–90	Excellent
89–75	Good
74–60	Average
59–50	Weak
<50	Very weak

Auditory abilities and the speech intelligibility of each child were assessed by an otorhinolaryngologist-audiologist during a visit using scales at least 6 months after surgery. Auditory abilities were evaluated using the *Categories of Auditory Performance* (CAP) scale proposed by Archbold and colleagues in 1995. CAP is a hierarchic scale reflecting the development of auditory abilities according to eight categories – from 0 to 7 (Table 4).

TABLE 4. Categories for auditory performance scale.

Category	Description
0	No awareness of environmental sounds
1	Awareness of environmental sounds
2	Responds to speech sounds
3	Recognizes environmental sounds
4	Discriminates at least two speech sounds
5	Understands common phrases without lipreading
6	Understands conversation without lipreading with a familiar talker
7	Can use the telephone with a familiar talker

Speech intelligibility was evaluated according to the *Speech Intelligibility Rating* (SIR) scale (Allen et al. 1998). SIR is a hierarchic scale, representing the develop-

ment of speech intelligibility according to five categories – from 1 to 5 (Table 5).

TABLE 5. Speech intelligibility rating scale

Category	Description
5	Connected speech is intelligible to all listeners. The child is understood easily in everyday contexts.
4	Connected speech is intelligible to a listener who has little experience of a deaf person's speech. The listener does not need to concentrate unduly.
3	Connected speech is intelligible to a listener who concentrates and lip-reads within a known context.
2	Connected speech is unintelligible. Intelligible speech is developing in single words when context and lip-reading cues are available.
1	Pre-recognizable words in spoken language. The child's primary mode of everyday communication may be manual.

1.5.2. Evaluation of Speech and Language Development

In this study, the methodology for the evaluation of speech and language development, the *Book for Speech and Language Therapists* was used. It is designed for speech and language therapists working in educational psychological services and for teachers of the deaf working in Lithuanian Educational Center for the Deaf and Hard of Hearing (prepared by Gaulienė et al. in 2008). Speech and language development of children CI users was evaluated by speech and language therapists from educational psychological services according to the child's residence or by teachers of the deaf from the Lithuanian Educational Center for the Deaf and Hard of Hearing, who were taught the technique in specialized courses and are allowed to use this method. Six speech and language skills were assessed: speech intelligibility, vocabulary, grammar skills, pronunciation, phonological awareness and sound analysis. Speech and language therapists evaluated speech and language development by filling standard protocols of speech and language development. Final assessments were made by the group of independent experts – teachers of deaf with long experience in teaching and assessing deaf children with CIs. The experts had no interests associated with this study, they were only motivated to improve the integration of deaf Lithuanian children CI users. These experts analyzed the filled protocols taking into consideration the children's chronologic and hearing ages and described each speech and language area based on one out of four developmental levels: very good, good, satisfactory, and unsatisfactory. This was determined based on the nature of the mistakes the child made according to the qualitative analysis of results. The general speech and language development level was established after summarizing all 6 speech and language areas. In case of disagreement, a lower level was chosen. An

agreement was reached when at least two out of three experts agreed. The speech and language development level was *very good* if a child's speech was developed, a child fulfilled all tasks covering different areas of speech and language development without making any mistakes. Speech and language development was considered *good* when a child's speech was developed but the child pronounced one or several sounds incorrectly when performing given tasks, made one or several mistakes in phonemic perception, word building, word changing or combination in a sentence, mixed the meaning of infrequently heard words. Speech and language development was considered *sufficient* if a child's speech was developed sufficiently, the child made frequent, repeated mistakes in pronunciation, phonemic perception, vocabulary, grammatical speech structure, and speech perception. Speech and language development was considered *insufficient* when a child's speech was not developed: the child would not understand the majority of tasks and could not perform them. The *Book for Speech and Language Therapists* was chosen as it covers a wide age range; it is well mastered by speech and language therapists working in educational psychological services and is comprehensive as well. All evaluations were performed with the permission from the Lithuanian Bioethics Committee, when a collaboration agreement was signed between the National Center for Special Needs Education and Psychology, the Lithuanian Educational Center for the Deaf and Hard of Hearing and VUHSK, and only after the parents of participants signed the informed consent form.

1.6. Establishment of Prognostic Factors for Pediatric CI

The prognostic factors of pediatric CI were established using methods of statistical analysis meant to find dependence between different demographic, audiologic, surgical, etiologic, radiological, family, rehabilitation and educational variables and results of speech perception and speech and language development. Univariate and multivariate regression analyses were used to establish and evaluate factors that might be prognostic as significantly associated with the results of CI.

1.7. Statistical Analysis

Descriptive statistics (mean, standard deviation, minimal and maximal values, median, and mode) were used to systematize the results. The distribution normality of qualitative indices was verified by carrying out the Kolmogorov-Smirnov test and evaluating the histogram.

Possible associations between variables were determined using the Pearson correlation coefficient for nominal variables, and the Spearman correlation coefficient – for categorical variables. The nonparametric Mann-Whitney-Wilcoxon

test (two independent samples) or the Kruskal-Wallis test (more than two independent samples) were used to verify the hypotheses concerning intergroup differences among variables. The results were considered significant when p value is <0.05.

The logistic regression analysis was used to define the influence of individual factors which were used in the case of the binary categorical dependent variable. The logistic regression model was considered appropriate if the χ^2 and Wald criterion p-value was less than 0.05, at least 50 percent of values were correctly classified and the chosen determination coefficient R^2 was equal or greater than 0.2. The Odds Ratio indicated the likelihood of *Y* to reach 1 varies.

Data handling and analysis were performed with MS Excel, IBM Statistical Package of the Social Sciences (SPSS) version 21.0 software (SPSS Inc., Chicago, IL) and the MedCalc 18.11.3 software.

2. Results

2.1. General Characteristics of the Participants

The study population consisted of 122 children (70 male, 57% and 52 female, 43%) who underwent one or two cochlear implantation surgeries in Vilnius University Hospital Santaros Klinikos (VUHSC), at the Center of the Ear, Nose, Throat and Eye Diseases. The study population constituted 43.1% of the general population of deaf Lithuanian children who had underwent CI surgery during the years 1999 to 2017 (N=283) (Mataitytė-Diržienė et al. 2018).

TABLE 6. The demographic characteristics of the participants.

	Age at first implantation, months	Age at second implantation, months	Duration of the implant use, years	Age at the time of the study, years
N	122.0	57.0	122.0	122.0
Mean	32.5	32.3	4.9	7.6
Median	21.0	23.0	5.0	8.0
Mode	11.9	12.0	5.0	8.0
Standard deviation	26.9	22.1	2.6	3.3
Minimum	10.3	10.0	0.6	2.0
Maximum	162.7	112.0	12.0	17.0

Sixty-five (53.3%) children received unilateral CIs (the right ear was implanted in 47 cases, the left – in 18 cases). Fifty-seven (46.7%) – bilateral CIs (sequential surgeries were performed in 34 (59.6%) cases, simultaneous CI – in 23 (40.4%) cases).

Three participants underwent reimplantation due to the malfunction of an implant (2 of them – after a head trauma). An electrode array was inserted fully in all 179 ears implanted.

The mean age of children at the time of inclusion to the study was 7.6 ± 3.3 years (Table 6, Fig. 5). The majority of the participants – 86 (70.5%) – were preschoolers and primary school-age children.

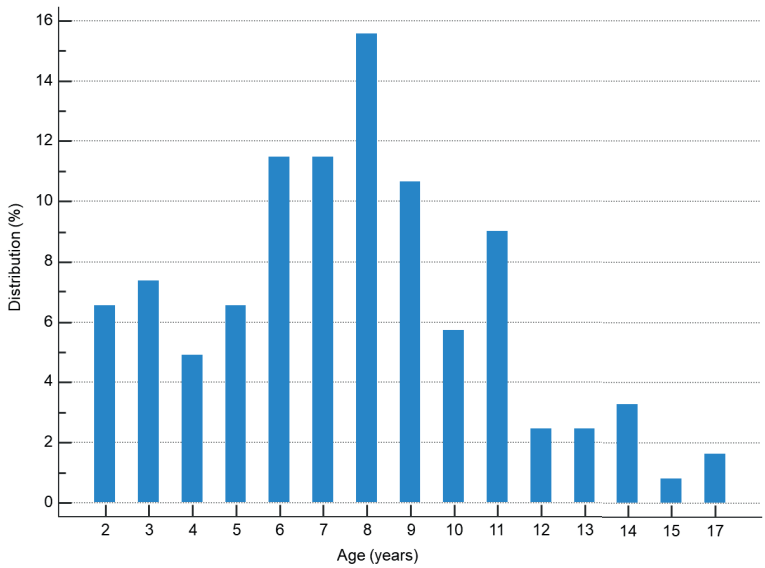


FIGURE 5. Age distribution amongst participants at the time on inclusion to the study (years).

Mean age during the first CI surgery was 32.5 ± 26.9 months. The youngest implanted child was 10 months of age, the oldest – 162 months old (Table 6, Fig. 6). The mean age of children born after 2014, when universal newborn hearing screening was introduced in Lithuania, was 14.56 ± 4.91 months during the first CI surgery and differed significantly compared to the age at first implantation of children born before 2014 (36.47 ± 28.1 months), p<0,001. On average, participants used their CI for 4.9 ± 2.6 years (Table 6, Fig. 7).

The majority of the participants (66 children) lived in five biggest Lithuanian cities; 11 children lived in cities with a population of 20 000 to 99 000 inhabitants; 4 – in cities with 10 000 to 19 900 inhabitants; 3 – in cities with less than 10 000 inhabitants in municipality centers; 13 – in other cities with less than 10 000 inhabitants; the last 25 participants resided in the rural areas (Fig. 8).

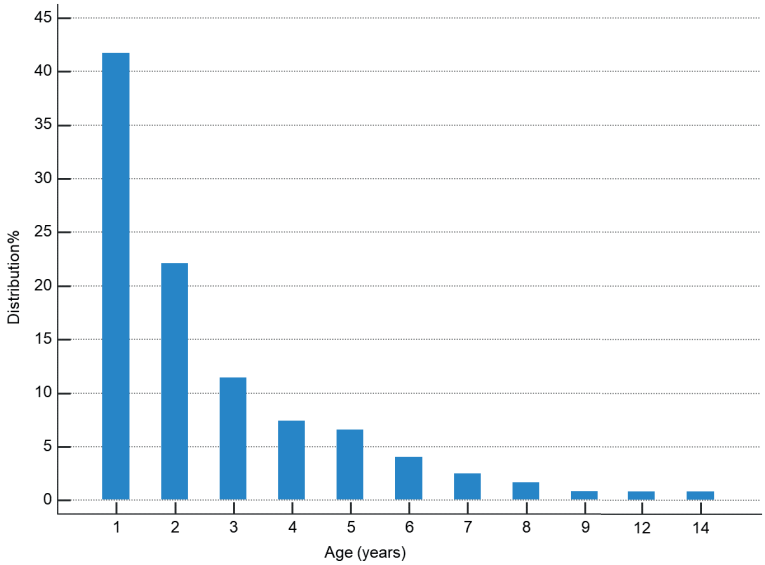


FIGURE 6. Distribution of age at first cochlear implantation amongst participants (years).

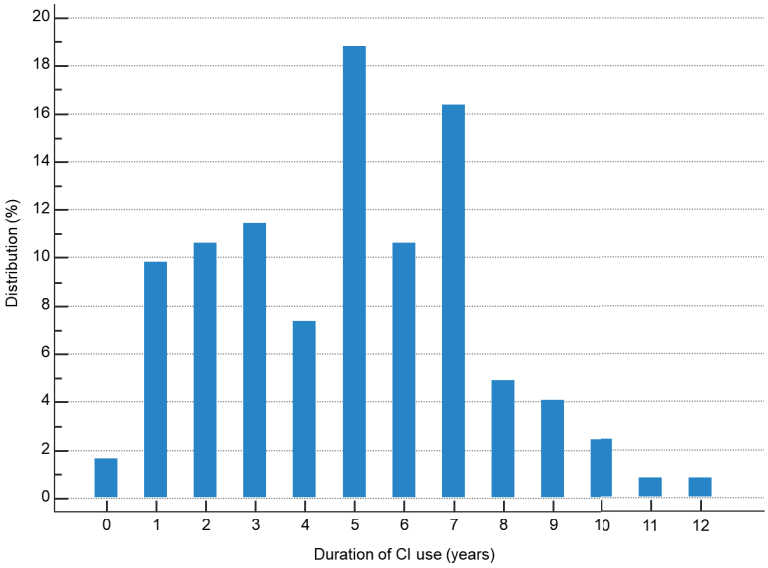


FIGURE 7. Distribution of the duration of the implant use amongst participants (years).

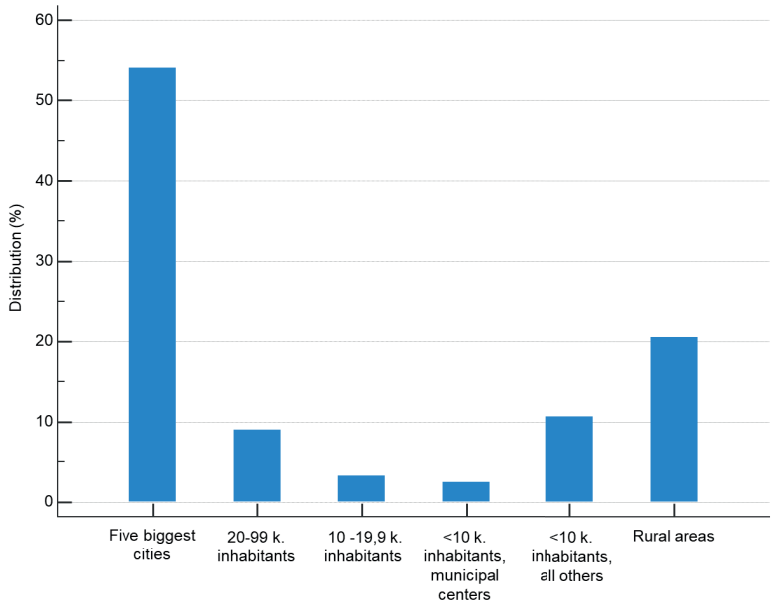


FIGURE 8. Distribution of a residential location amongst participants.

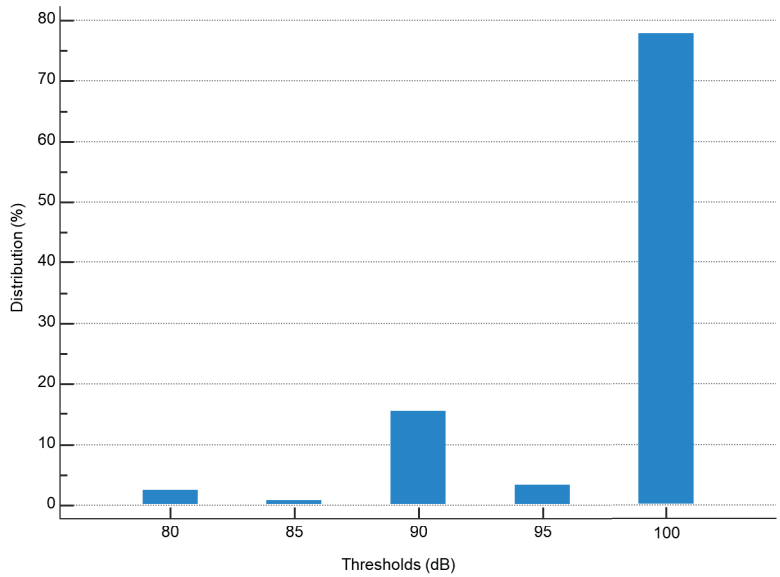


FIGURE 9. The distribution of hearing thresholds in the implanted ear before the surgery amongst participants (dB).

Analyzed was the distribution of participants based on the manufacturer of the cochlear implant: 103 (84%) children used MED-EL, 18 (14.8%) children – Cochlear, and 1 (0.8%) child – Advanced Bionics implants.

Forty-four (36%) participants were diagnosed during the newborn hearing screening, 23 (52.3%) of them were born in 2014 or later, when newborn hearing screening was implemented in Lithuania. Of all children, 118 (96.7%) were diagnosed with congenital hearing loss (HL) or their hearing loss progressed before 3 years of age; in 4 (3.3%) cases, the onset of hearing loss occurred after 3 years of age. Progressive HL was diagnosed in 21 (17.2%) cases. Congenital or prelingual HL was diagnosed at a mean age of 19.4 ± 16.5 months. When comparing the age of diagnosis between children born in 2014 and later, as well as children born earlier than 2014, a significant difference was observed (7.7 ± 3.4 months and 24.6 ± 22.5 months, respectively (p <0,001)).

Eighty-four (68.9%) of children were diagnosed with HL in the Children's Hospital, affiliate of VUHSC, and 38 (31.1%) – in the Lithuanian University of Health Sciences. Hearing thresholds before the CI were determined based on BERA results in case of congenital HL or based on the last preoperative audiogram and were 95,5 ± 7,8 dB in the better hearing ear and 97.7 ± 4.8 dB in the implanted ear, or one of the implanted ears in case of a bilateral CI (Fig. 9).

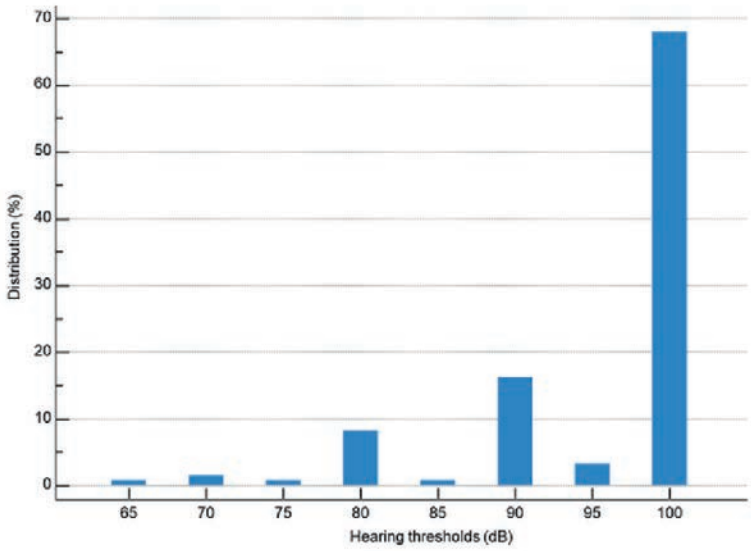


FIGURE 10. The distribution of hearing thresholds in the better hearing ear before the surgery participants (dB).

Average hearing thresholds were equal to or higher than 100 dB in the better hearing ear in the majority of cases (83 cases, 68%) (Fig. 10). Twenty (16.4%) children had residual hearing in the implanted or one of the implanted ears. Twenty (30.8%) participants with unilateral CI had residual hearing in the contralateral ear.

Seventy-nine (64.8%) children used two hearing aids at least 3 months prior to CI surgery. After the implantation, out of 65 unilateral CI users, 20 (30.8%) children permanently used hearing aids in the contralateral ear and the mean of aided thresholds using only HA was 51 ± 13 dB.

After analyzing the use of the CI processor, it was noticed that 63 (96.9%) unilateral CI users wore the device constantly, 2 children with an additional disability wore the device inconsistently, one bilaterally implanted recipient wore only one speech processor. During the first year after the surgery, 18 (14.8%) children reported issues with the processor (for example: inconsistent use of the device, programming issues of the device). Seven (38.9%) of these 18 children had severe additional disabilities.

2.2. Family Characteristics of Participants

Of all children, 13.1% lived in single-parent families, 0.8% were raised by caregivers, and the remaining 85.2% lived in a nuclear family. Of all children, 42.6% of children had no siblings, 44.3% lived in two-children families, and 13.1% had two or more siblings.

TABLE 7. The family characteristics of the participants.

Variable	Number of participants (percent)
Family composition	
Nuclear family	104 (85.2)
Single-parent	16 (13.1)
Caregivers	1 (0.8)
Number of children in family	
One	52 (42.6)
Two	54 (44.3)
Three or more children	16 (13.1)
Education of mother	
Higher education level	63 (52.5)
Secondary education level	53 (44.2)
Incomplete secondary education level	4 (3.3)
Education of father	
Higher education level	49 (40.2)
Secondary education level	57 (46.7)
Incomplete secondary education level	14 (11.5)

Variable	Number of participants (percent)
Parents' understanding of the CI process	
Sufficient	53 (43.4)
Insufficient	45 (36.9)
Do not understand	24 (19.7)
Visits to the CI center (N=108)	
Sufficient	46 (42.6)
Insufficient	41 (38.0)
Did not visit	21 (19.4)

The mothers of 52.5% of children had acquired higher education, 44.2% – secondary education, and 3.3% – incomplete secondary education. The fathers of 40.2% of participants had acquired higher education, 46.7% – secondary education, and 11.5% – incomplete secondary education.

When analyzing the understanding of the CI process, 43.4% of families were assessed to understand the process, 36.9% – to partially understand it, and 19.7% of families did not understand the CI process at all. After the implantation surgery, the majority of the participants' families (41%) visited the CI centers sufficiently, 36.9% – insufficiently, and 22.1% did not visit the CI centers at all (Table 7).

2.3. Rehabilitation and Education Characteristics of Participants

An analysis of hearing rehabilitation and educational placement settings of CI users revealed that 49.8% of families were actively engaged in their children's learning process, 33.6% participated passively, and 17.2% of families did not participate in their children's learning. The majority of the parents (71.3%) used spoken language to communicate with their children, 23.8% of families used both spoken and sign languages, and 4.9% communicated only in sign language. Speech and language therapy was accessible in 53.3% of cases, moderately accessible in 27.9% of cases, and not accessible in 18.9% of cases. Of all children, 12.3% attended speech and language therapy 5 times per week, 9.8% – 3 times per week, 36.9% – twice per week, and 28.7% – once per week. Lastly, 12.3% of children did not attend speech and language therapy for the first two years after the surgery.

Of all children, 48.4% attended general kindergartens, 36.1% – specialized kindergartens for deaf and hard of hearing, 5.7% of preschoolers did not go to any kindergarten, and 9.8% were too young at the time of the study to attend kindergarten. Out of 74 participants, 67.6% attended regular schools, and 28.4% – specialized schools for deaf and hard of hearing; 4.1% had been home-schooled. Seventy% of children who attended regular schools followed the mainstream educational program, 26% followed an adapted program, and 4% – an individual program. In general, 59% of study participants attended general, and 28.7% – specialized kindergar-

tens or schools; 2.5% had been home-schooled, while 9.8% were too young to attend any educational institution (Table 8, Fig. 11).

TABLE 8. The characteristics of hearing rehabilitation and education.

Variable	Number of participants (percent)
Parents' engagement in the learning process	
Active	60 (49.8)
Passive	41 (33.6)
Do not participate	21 (17.2)
Communication mode	
Spoken language	87 (71.3)
Total communication	29 (23.8)
Sign language	6 (4.9)
Accessibility of speech and language therapy	
Good	65 (53.3)
Moderate	34 (27.9)
Poor	23 (18.9)
Intensity of speech and language therapy	
5 times per week	15 (12.3)
3 times per week	12 (9.8)
2 times per week	45 (36.9)
Once per week	35 (28.7)
Did not attend	15 (12.3)
Preschool	
General	59 (48.4)
Special	44 (36.1)
Do not attend	7 (5.7)
Too young to attend	12 (9.8)
School N=74	
General school	50 (67.6)
Specialized school	21 (28.4)
Home-schooling	3 (4.1)
Educational institution attended at the time of the study	
General	72 (59.0)
Specialized	35 (28.8)
Home-schooling	3 (2.5)
Too young to attend	12 (9.8)

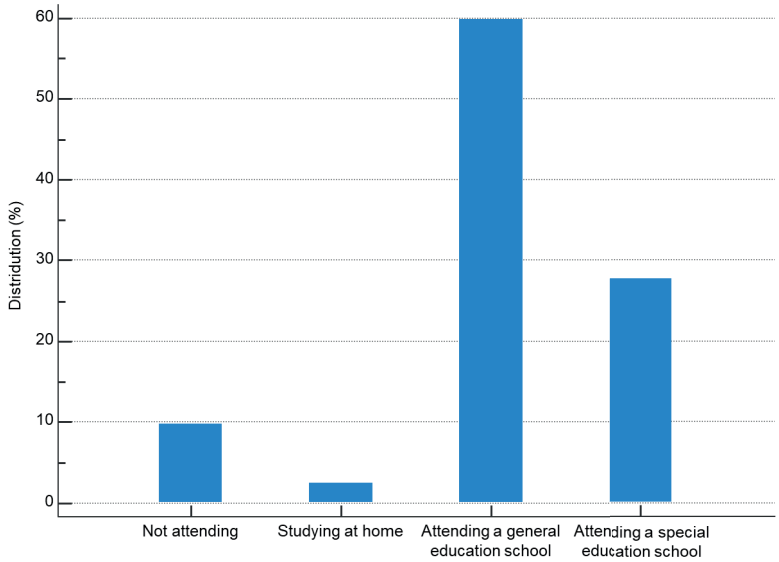


FIGURE 11. The distribution of educational institutions attended at the time of the study.

2.4. Etiologic Profile of HL amongst Lithuanian Children CI Users

2.4.1. Results of Genetic Testing

Of 122 children, 104 (85.2%) were consulted and examined by the geneticist (101 children had genetic testing, 3 children were only consulted), and the remaining 18 (14.8%) children were not consulted by the geneticist.

Out of 101 children consulted by the geneticist, 91 (90.1%) were attributed to the isolated HL group, 10 (9.9%) – to the syndromic HL group. In the isolated HL group, 13 (14.8%) children had perinatal risk factors for a HL (prematurity, hypoxia, sepsis), and 2 (2.2%) children were clinically diagnosed and confirmed by laboratory tests to have a congenital citomegalovirus (cCMV) infection. *GJB2* sequencing was performed in all 91 cases of isolated HL. Pathogenic homozygous or compound heterozygous variants of the *GJB2* gene were determined in 58 (63.7%) individuals in the isolated HL group, one (1.1%) child was diagnosed with only one heterozygous variant of the *GJB2* gene (which is not enough to confirm the molecular diagnosis), and 32 (35.2%) children were not found to have any pathogenic variants of the *GJB2* gene (Fig. 12). 35delG was the most frequent pathogenic variant of the *GJB2* gene found – the frequency of its allele composed 68.9% of all pathogenic alleles. The second most frequent pathogenic variant of the *GJB2* gene was c.313_326del14, its frequency composed 29.3% of all pathogenic *GJB2* alleles. Other pathogenic vari-

ants of the *GJB2* gene were identified on a much rarer basis – the frequency of the alleles was less than 2%.

In the group of isolated HL, 7 participants with a positive genealogy and negative *GJB2* gene mutation underwent the next generation sequencing analysis of 126 genes. Alterations in other genes causing non-syndromic HL were identified in 5 participants (Table 9). The *MT-RNR1* gene of the mitochondrial genome was tested in 3 children treated with aminoglycosides in the anamnesis, 1555A>G pathogenic variants were not identified. In the group of syndromic HL, 8 syndromes were confirmed, and genetic testing in 2 children still remains unfinished; therefore, the exact syndrome is not yet identified (Table 10).

In conclusion, after the genetic examination of 101 children, 63 (62.4%) cases of non-syndromic HL and 10 (9.9%) cases of the syndromic HL were identified. Generally, 73 (59.8%) of children out of the study population (122 participants) were diagnosed with genetic causes of the congenital HL.

TABLE 9. The distribution of pathogenic variants causing non-syndromic deafness in the group of isolated HL (N=63).

Gene	Number of participants (%)
GJB2, two pathogenic variants	58 (92.1)
MYO15A, two pathogenic variants	3 (4.8)
TMPRSS3, two pathogenic variants	2 (3.2)

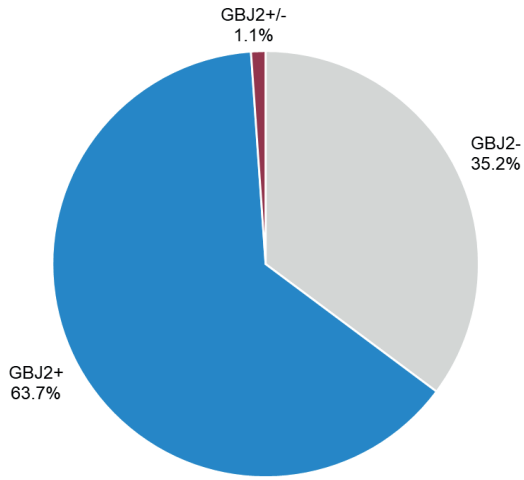


FIGURE 12. *GJB2* gene mutations testing results in the group of isolated HL.

TABLE 10. Number of hereditary syndromes in the study sample.

Syndrome	Number of cases
Pendred syndrome	2
Usher syndrome	2
Roger syndrome	1
Jacobsen syndrome	1
CHARGE syndrome	1
Coffin-Lowry syndrome	1
Unidentified syndrome	2

2.4.2. Results of the CMV DNR Testing

Dried blood spots were able to be received in 117 cases out of 122 (95.9%), and CMV DNA PCR tests performed. A retrospective DBS-based real-time PCR analysis showed 14 patients being positive for CMV DNA at birth. All 5 children who were diagnosed with a cCMV infection in newborn period were confirmed with a positive CMV DNA from a dried blood spot. Nine new cases of a cCMV infection were detected. Seven of these children had no confirmed etiologic factor of a HL prior to this research – *GJB2* gene mutations, a perinatal pathology, infection

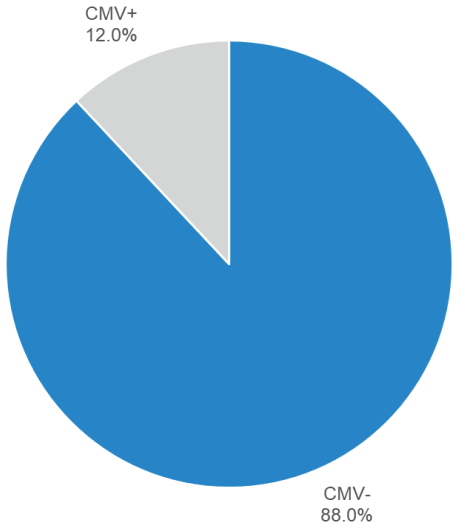


FIGURE 13. CMV DNA testing results amongst study participants.

or postnatal infection were all unconfirmed. One child suffered a severe perinatal pathology; however, a cCMV infection was detected only during our research. Two children were confirmed with a positive CMV DNA and a pathologic *GJB2* gene mutation. In total, 14 new cases of a cCMV infection were detected; a symptomatic form was diagnosed in 6 (42.9%) cases, asymptomatic – in 8 (57.1%) cases (Fig. 13). Three children with a symptomatic form of the disease underwent the treatment specific in infancy.

When analyzing annual data presented by the Center for Communicable Diseases and AIDS, from the beginning of 2003 to the end of 2016, cCMV was registered only 3 times, whereas we found 13 cases of a cCMV infection in the study population in the same time period (*Lietuvos gyventojų sveikata ir sveikatos priežiūros įstaigų veikla 2017 m.*, 2018).

2.4.3. Results of the Analysis of Risk Factors of HL

An analysis of the distribution of *perinatal* risk factors of HL amongst participants revealed that 11 (9%) children were premature, born before 32 weeks of gestation, 11 (9%) children weighed less than 1500g, 16 (13.1%) suffered severe perinatal hypoxia, 2 (1.6%) were diagnosed with hyperbilirubinemia requiring blood transfusion, 16 (13.1%) were diagnosed with sepsis, 16 (13.1%) were prescribed with ototoxic medication in infancy, and 1 (0.8%) was diagnosed with meningitis in infancy. All these children had more than one perinatal risk factor that could have been the cause of the HL, and all were treated for more than 5 days in the Newborn Intensive Care Unit.

TABLE 11. Prenatal, perinatal and postnatal risk factors of the HL amongst study participants.

Risk factor	Number of participants
Prematurity <32 weeks of gestation	11
Newborn weight <1500g	11
Severe perinatal hypoxia	16
Congenital TORCH infections: Toxoplasmosis, Rubella, CMV, Herpes, Syphilis	5 cases of CMV infection diagnosed in infancy
Newborn sepsis	16
Hyperbilirubinemia requiring blood transfusion	2
Ototoxic medication in infancy	16
Ototoxic medication in early childhood	2
Newborn meningitis	1
Meningitis in infancy or early childhood	3

Five children were diagnosed with a symptomatic cCMV infection in infancy. No other *prenatal* risk factors, except for the cCMV infection, were discovered.

An analysis of *postnatal* risk factors showed 3 participants suffered purulent meningitis in infancy or early childhood; 1 child was diagnosed with severe pulmonary hypertension and sepsis. Two of these children were prescribed with ototoxic medication (Table 11).

2.4.4. Results of the Etiological Profile of HL

In order to thoroughly evaluate the etiologic profile of deaf children who are CI users, the causes of HL were estimated after genetic molecular testing, CMV DNA detection and analysis of prenatal, perinatal and postnatal risk factors. Out of 33 participants who did not have any pathologic mutations of the *GJB2* gene, 12 children were found to have severe perinatal risk factors in their medical histories (prematurity, hypoxia, newborn sepsis), 1 child suffered post-meningitis deafness, 6 children were diagnosed with a cCMV infection after the CMV DNA analysis, 2 cases of clinically diagnosed cCMV infections were confirmed after the CMV DNA analysis, and 5 children were diagnosed with other than GJB2 gene pathogenic mutations. Therefore, only 7 cases out of 33 *GJB2* negatives were left with the unknown cause of their HL.

By concluding results of the genetic testing, CMV DNA analysis, and data of the analysis of perinatal, prenatal and postnatal, risk factors, the etiology of deafness

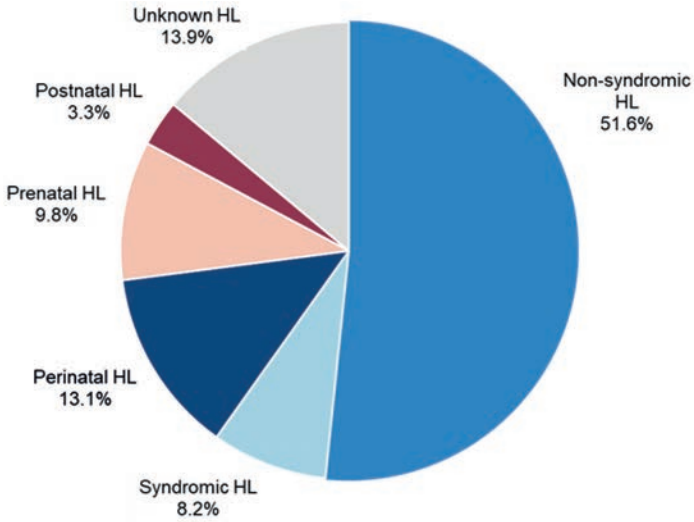


FIGURE 14. The etiologic profile of hearing loss amongst participants.

of all participants was attributed to one of the 6 following categories: non-syndromic HL, syndromic HL, prenatal HL (caused by a cCMV infection), perinatal HL, postnatal HL, and HL of unknown origin.

Most frequently diagnosed was the non-syndromic HL. It was diagnosed in 63 (51.6%) of cases. The second most frequent cause were the perinatal risk factors – they were found in 16 (13.1%) children. The third most common cause was a cCMV infection occurring in 12 (9.8%) cases. The fourth place was taken by the syndromic HL – 10 (8.2%) cases, and the fifth – by the postnatal risk factors, which caused HL in 4 (3.3%) children, 3 (2.5%) of whom had meningitis. In the final etiological classification, children diagnosed with several etiological factors (i.e., *GJB2* gene mutations and prenatal risk factors) were attributed to one of the factor that was most probable cause of a child's HL. HL of unknown origin was observed in 17 (13.9%) cases (Fig. 14). It is possible that the number of patients in this group will decrease as the etiologic examination continues.

2.5. Results of an Analysis of Temporal Bone CT Images

We had CT images of 109 children; 13 images were excluded due to the insufficient quality. Measurements were performed on 103 cases (205 ears). Cochlear ossification was found in 3 patients. Complex anatomical radiological changes of

TABLE 12. Inner ear malformations amongst participants.

Anatomical changes detected by visual inspection		Number of cases (bilateral)	Prevalence (percent)
Abnormal architecture of cochlea	Incomplete cochlear partition IP type I	2 (2) cases	3.9
	Incomplete cochlear partition IP type II	2 (2) cases	
Malformation of vestibule / semicircular canals		2(1) / 16(10) cases	18.9
Anatomical changes detected after the measurements		Number of cases (bilateral)	
Hypoplastic cochlea		16 (8) cases	15.5
Bony cochlear nerve canal stenosis		14 (8) cases	13.6
Internal auditory canal stenosis		0	0
Enlarged vestibular aqueduct		2 (1) cases	1.9
In total		34 patients (21 bilateral changes)*	33%

*Some of the participants were found to have several inner ear malformations; therefore, a general number of patients with at least one malformation is presented.

the inner ear (such as an incomplete cochlear partition) were classified separately for the analysis of CT images. Minor unclassified malformations were described as cases per study population, as one child might have several minor anomalies. In general, inner ear malformations were found in 34 cases (21 of them were bilateral, 16 – unilateral) (Table 12).

Calculated were the average dimensions of inner ear structures of the study population (Table 13). We also calculated the average dimensions of observed anatomical changes: the average height of the hypoplastic cochleae found in 24 ears (16 children) was 3.11 ± 0.13 mm. The average width of the bony cochlear nerve canal in case of the bony cochlear nerve canal stenosis found in 22 ears (14 patients) was 1.08 ± 0.53 mm. We found no cases of the internal auditory canal stenosis.

TABLE 13. The average dimensions of the inner ear structures of participants.

Inner ear structure	Average dimensions (±SD)
Height of the cochlea	3.73 mm (± 0.32mm)
Diameter of the bony cochlear nerve canal	1.76 mm (± 0.35mm)
Width of the internal auditory canal	4.22 mm (± 0.85mm)
Width of the vestibular aqueduct	0.84 mm (± 0.7mm)

TABLE 14. The distribution of inner ear malformations amongst different etiologic groups.

Malformation	Non syndromic HL	Syndromic HL	Prenatal HL	Perinatal HL	Postnatal HL	HL of unknown origin
IP type I	–	2	–	–	–	–
IP type II	1	1	–	–	–	–
Malformation of vestibule/semicircular canals	-/4	3/5	–/–	1/2	1/3	-/4
Hypoplastic cochlea	8	2	–	2	–	4
Bony cochlear nerve canal stenosis	4	3	2	1	1	3
Enlarged vestibular aqueduct	1	3	–	1	–	1
Cochlear ossification	–	–	–	–	3	–

After excluding 3 cases of cochlear ossification, in the sample of 103 participants, an analysis of temporal bone CT images revealed a 33% general prevalence of inner ear malformations.

Assessed was the distribution of inner ear malformations in different etiological groups. Two cases of the IP were associated with the Pendred syndrome, one – with an unidentified syndrome, and another one – with pathogenic *GJB2* gene mutations (Table 14).

2.6. Postoperative Hearing Results

Mean aided thresholds with one or two CI in the sound field were 36.3 ± 7.8 dB. Assessing children with one and two implants separately, the average CI aided thresholds were 38.1 ± 8.2 dB and 34.3 ± 6.8 dB, respectively, and the difference was statistically significant (p=0,033).

2.6.1. Results of Auditory Abilities and Speech Intelligibility

Auditory abilities, according to the CAP scale, were assessed on average 4.9 ± 2. 6 years after the CI – it was found that 41.8% of children reached the scale's ceiling and can easily talk to a familiar person on the phone. The results of the auditory abilities are presented in Table 15 and Fig. 15.

TABLE 15. An evaluation of listening skills according to the CAP scale (N=122).

Category	Description	Number of participants (%)
0	No awareness of environmental sounds	0
1	Awareness of environmental sounds	0
2	Responds to speech sounds	3 (2.5)
3	Recognizes environmental sounds	9 (7.4)
4	Discriminates at least two speech sounds	24 (19.7)
5	Understands common phrases without lipreading	23 (18.9)
6	Understands conversation without lipreading with a familiar talker	12 (9.8)
7	Can *use the telephone with* a familiar talker	51 (41.8)

Speech intelligibility, according to the SIR scale, was assessed on average 4.9 ± 2.6 years after the CI; it was found that 41% of 122 children reached the highest category in the scale – they developed such a speech that is easily understood by all listeners during casual activities (Table 16, Fig.16).

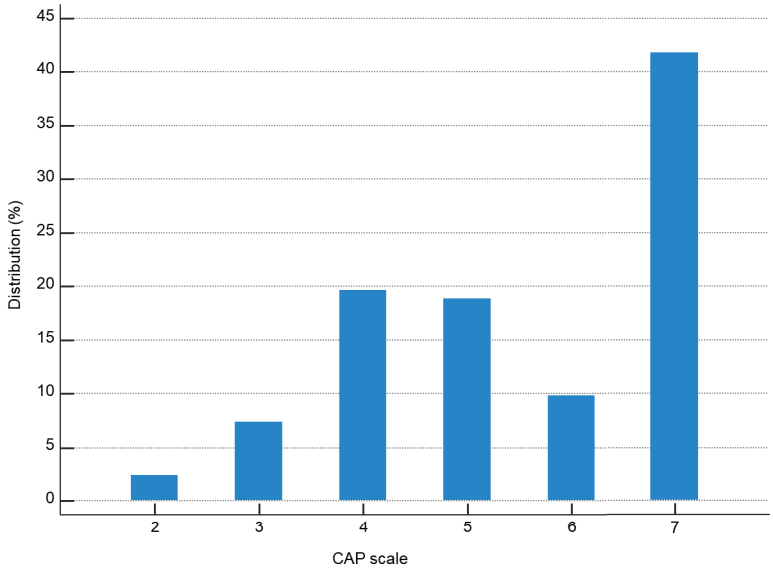


FIGURE 15. The distribution of the auditory abilities score according to the CAP scale.

TABLE 16. Speech intelligibility categories according to the SIR scale (N=122).

Category	Description	Number of participants (%)
5	Connected speech is intelligible to all listeners. The child is understood easily in everyday contexts	50 (41)
4	Connected speech is intelligible to a listener who has little experience of a deaf person's speech. The listener does not need to concentrate unduly.	17 (13.9)
3	Connected speech is intelligible to a listener who concentrates and lip-reads within a known context.	17 (13.9)
2	Connected speech is unintelligible. Intelligible speech is developing in single words when context and lip-reading cues are available.	28 (23)
1	Pre-recognizable words in spoken language. The child's primary mode of everyday communication may be manual.	10 (8.2)

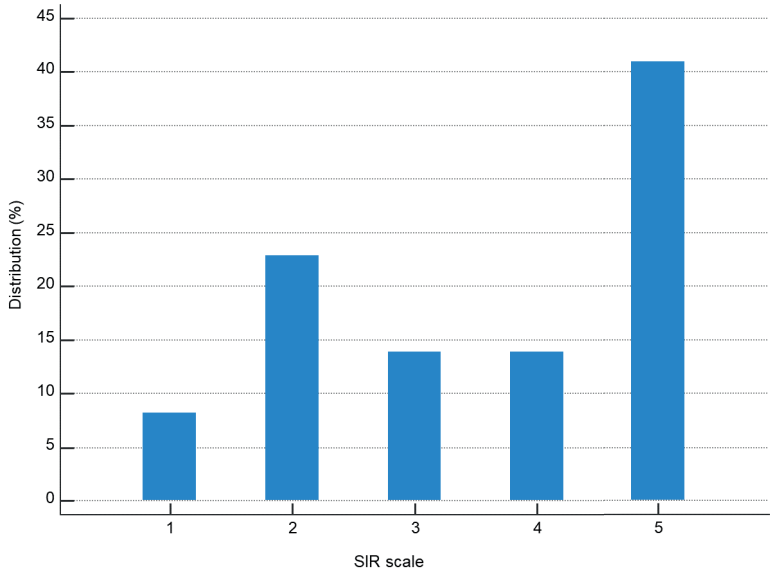


FIGURE 16. The distribution of the speech intelligibility category based on the SIR scale.

2.6.2. Results of Speech Audiometry

The speech audiometry results of 95 children were estimated to evaluate post-operative speech perception. The average score of the speech audiometry of these children was 63.9 ± 29.3%. The distribution of the participants, based on the speech perception level, is presented in Table 17.

TABLE 17. Speech recognition levels (N=95).

Speech perception level	Speech perception score in %	Number of participants (%)
None	Patient could not be tested with an open-set speech audiometry	11 (11.6)
Very weak	<50	14 (14.7)
Weak	59–50	5 (5.3)
Moderate	74–60	18 (18.9)
Good	89–75	30 (31.6)
Excellent	100–90	17 (17.9)

After excluding children with severe additional disabilities and patients with an onset of HL after 3 years of age, this group decreased to 81 children, whose speech perception levels were distributed as follows: excellent speech perception was reached by 16 (19.8%) children, good – 29 (35.8%), moderate – 16 (19.8%), weak – 5 (6.2%), and very weak – 11 (13.6%) children; 4 (4.9%) children could not be tested using open-set speech audiometry (Fig. 17). The average speech perception level in this group was 69.6 ± 24.2%. The average age at first implantation of these children was 33,1 ±22,2 months, age at the time of the study - 8,7 ±2,7 years and duration of the implant use 5,9 ±2,2 years.

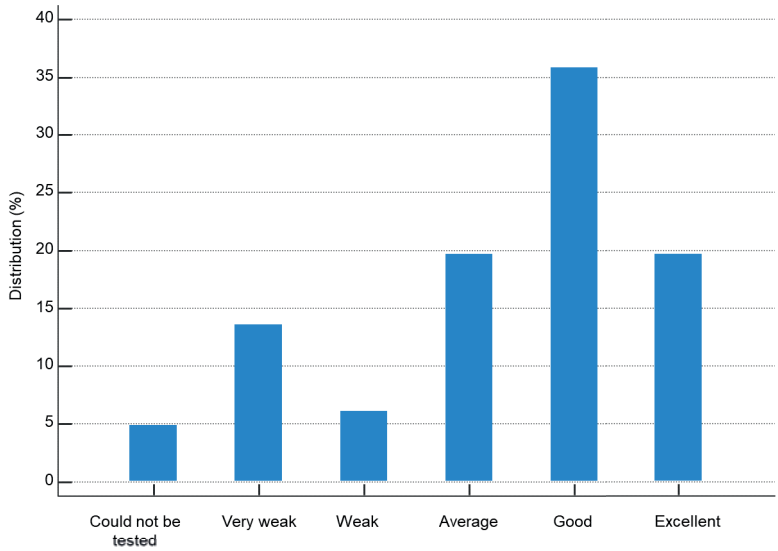


FIGURE 17. The distribution of patients based on the speech perception level (N=81).

2.6.3. Results of the Speech and Language Assessment

The results of the speech and language development assessment of 81 children were analyzed in order to evaluate postoperative speech and language achievement. Of them, 20 (24.7%) participants reached a very good speech and language development level, 22 (27.2%) – a good level, 21 (25.9%) – a satisfactory one, and 18 (22.2%) – an unsatisfactory level (Fig. 18).

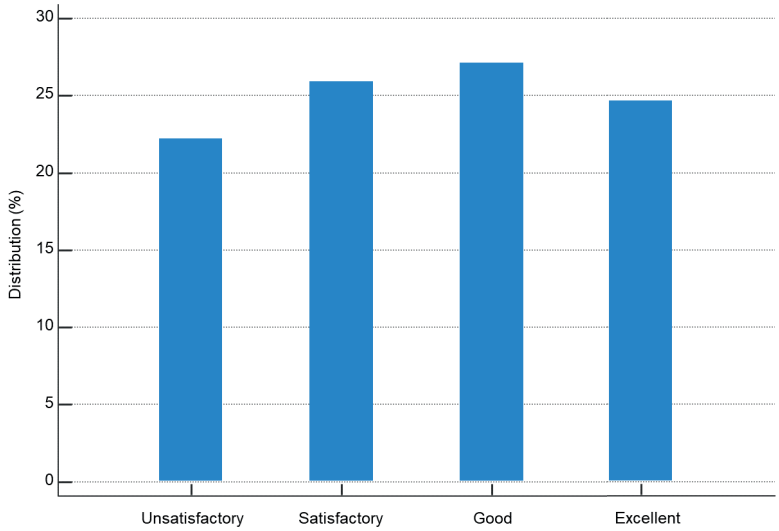


FIGURE 18. The distribution of participants based on speech and language development levels (N=81).

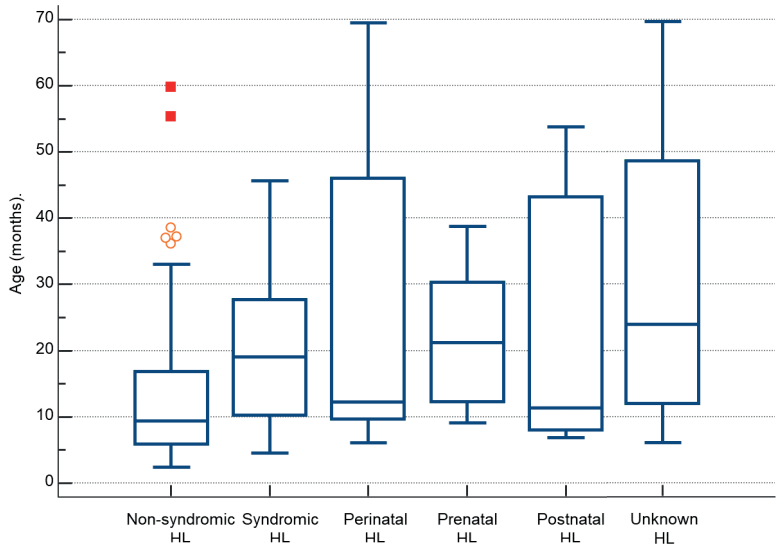


FIGURE 19. The distribution of age at diagnosis amongst different etiologic groups.

2.6.4. Relations between Etiological Factors and Age at Diagnosis and Operation

An analysis of the age at diagnosis and first implantation surgery amongst 6 different etiologic groups of participants (after excluding children with the postlingual HL) revealed that age at diagnosis and surgery differed significantly between different etiological groups (p=0,003 and p=0,023, respectively). Children with the non-syndromic HL were diagnosed and implanted earlier compared to children with HL of other etiologies (syndromic, perinatal, prenatal, postnatal, and unknown) (Figs. 19, 20).

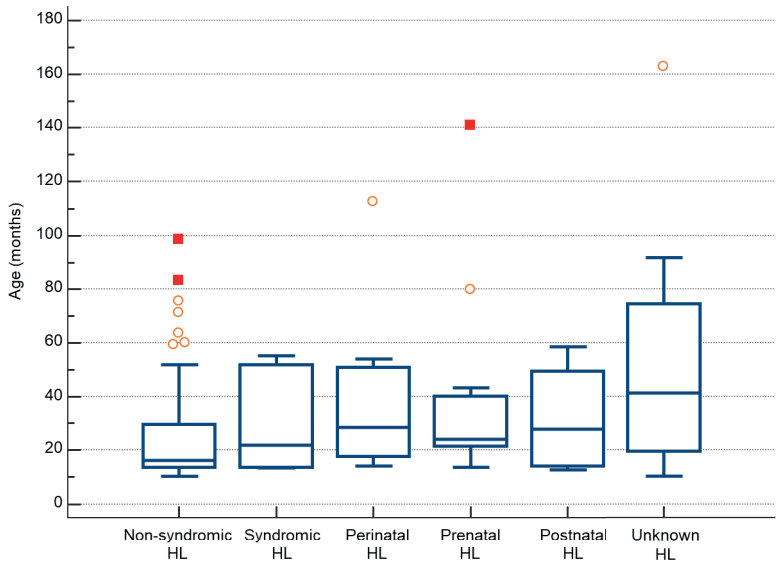


FIGURE 20. The distribution of age at implantation amongst different etiologic groups.

2.6.5. Relations between Etiological Factors and Speech Perception

In order to assess whether speech perception, evaluated using speech audiometry, differed comparing children with non-syndromic HL and children with HL of other etiologies, two etiological groups were picked – non-syndromic HL and HL of other etiologies (syndromic, prenatal, perinatal, postnatal, and unknown). The influence of the age at implantation on the speech perception results was reduced by including into the analysis only children implanted before 3.5 years of age (period of maximal neuroplasticity). Excluded were children with postlingual hearing loss. The "Non-syndromic HL" group included 38 participants, and the group "HL of

other etiologies" included 30 children. It was found that the results of the speech recognition of children with "non-syndromic HL" were significantly better compared to children with the "HL of other etiologies" (p=0,013) (Fig. 21). However, this difference became insignificant when children with severe additional disabilities were excluded from the analysis (p=0,21).

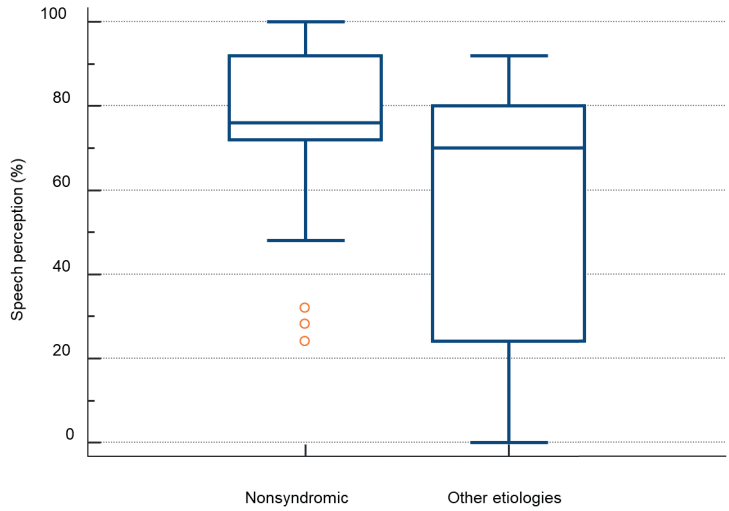


FIGURE 21. The distribution of speech perception results in "nonsyndromic hearing loss" and "hearing loss of other etiologies" groups.

2.6.6. Postoperative Results of Children with Additional Disability

Fourteen (11.5%) out of 122 children had severe additional disability, such as cerebral palsy, autism, epilepsy and others. All of them were diagnosed with developmental delay (Table 18). Twelve (85.7%) had more than two additional diagnoses. Severe additional disabilities were as follows: a CMV infection in 2 cases; congenital syndromes – in 5 cases; profound prematurity – in 2 cases; severe hypoxia at birth – in 1 case; severe postnatal infection – in 1 case; an unknown cause of severe addi-

TABLE 18. The distribution of the severe additional disability amongst study population (N=14).

Severe additional disability	Number of cases (percent)
Autism	4 (28.6)
Cerebral palsy	4 (28.6)
Hereditary syndrome	5 (35.7)
Severe isolated developmental disorder	1 (7.4)

TABLE 19. The results of auditory abilities and speech intelligibility amongst children with additional disabilities.

Case	Age at implantation, months	Duration of CI use, years	Additional disability	Other diseases	Cause of additional disability/hearing loss	CAP	SIR
N1	27	7.4	Autism	–	Prematurity	4	2
N2	15	6.6	Jacobsen syndrome	Vision impairment	Syndrome	4	1
N3	17	7.5	Autism	–	Unknown/*GJB2*	5	2
N4	25	7.1	CP	Epilepsy	cCMV	2	1
N5	16	6.9	Autism	–	Unknown/*GJB2*	5	2
N6	35	5.1	CP	Epilepsy	Unknown	3	2
N7	13	5.9	Unknown syndrome	–	Syndrome	3	1
N8	19	4.8	CP	Epilepsy	Hypoxia	4	2
N9	22	4.5	Roger syndrome	Diabetes mellitus, anaemia, vision impairment	Syndrome	5	1
N10	58	3.5	Severe postnatal infection	Pulmonary hypertension	Severe postnatal infection	4	2
N11	41	3.3	CHARGE syndrome	Multiple developmental defects	Syndrome	5	2
N12	22	1.1	CP	Epilepsy	cCMV	2	1
N13	29	2.1	CP	Epilepsy, blindness	Prematurity	3	2
N14	22	1.1	Coffin-Lowry syndrome	Hipothyreosis	Syndrome	4	1

Notes: CP – cerebral palsy.

tional disability – in 3 cases (2 of them were diagnosed with a GJB2 gene mutation that had caused HL).

In addition to this, 20 (16.4%) more children were diagnosed with minor additional disabilities that had no big influence on the child's psychomotor development, for example: Pendred syndrome, balance disorder, renal polycystosis, heart defect and others. In general, there were 34 (27.9%) of children CI users diagnosed with additional disabilities.

The general characteristics of children with severe additional disabilities revealed that these children were implanted at the mean age of 24.8 ± 12.8 months, used their CI on average 4.71 ± 2.3 years, and their mean age at the time of the study was 6.86 ± 2.2 years. Twelve (85.7%) of participants with severe additional disability use their CI permanently.

An analysis of postoperative auditory abilities of children with severe additional disabilities revealed that 4 (28.6%) children reached the 5th category on the CAP scale, 5 (37.7%) – the fourth scale; 3 (21.4%) – the third scale; and 2 (14.3%) children managed only the second category. Speech intelligibility scores according to the SIR scale were as follows: 8 (57.1%) children managed the second category; remaining 6 (42,7%) had the lowest – first – category (Table 19). A comparison of the group of children with severe additional disabilities with the group of children without a severe disability based on CAP and SIR scales revealed significant differences between the two groups (p=0,007 and p<0,001, respectively).

2.7. Determination of Prognostic Factors of CI

2.7.1. Comparison of Groups with Different Speech Perception Results

In order to determine which factors determine worse speech perception, two groups were formed: children with a speech perception score of ≥ 60% were included into the "good speech perception group," and the remaining – with the speech perception score less than 60% – into the "poor speech perception" group. Therefore, the "good speech perception" group included participants with excellent, good, and moderate speech perception levels; the "poor speech perception" group included participants with poor, very poor speech perception levels and children who could not be tested with an open-set speech audiometry. Sixty children formed the "good speech perception" group, the and 21 – the "poor speech perception" group.

Thirty prognostic factors (demographic, audiologic, surgical, implant-associated, etiological, radiological, family, rehabilitation and educational) were analyzed in this study in order to find the relation to postoperative results of speech perception.

Comparing *demographic* data, groups did not differ neither based on the gender (p=0,837) nor on the age at the time of the study (p=0,057). However, groups

differed significantly based on the residential location – the majority of children in the "good speech perception" group lived in the main cities (60%), whereas 47.6% of children from the "poor speech perception" group resided in small cities and the rural areas (p=0,034).

An analysis of the influence that *audiologic* factors have on speech perception results showed that in the group of "poor speech perception," congenital HL was diagnosed significantly later (p<0,001). The two groups did not differ neither in comparing preoperative hearing thresholds (p=0,767) nor in comparing residual preoperative hearing in the implanted ear (p=0,467), nor comparing use of HA in the contralateral ear after surgery (p=0,261) or in comparing the number of children in each group diagnosed with progressive HL (p=0,675). Postoperative mean CI-aided thresholds were significantly worse in the group of "poor speech perception" (p<0,001) (Fig. 22)

In evaluating *surgical, implant-associated and processor-associated* factors, it was revealed that age at implantation was significantly older in the group of "poor speech perception" (p<0,001). The two groups did not differ neither based on the duration of CI use (p=0,723) nor based on the number of bilateral cochlear implantations (p=0,19). However, children with the worse speech perception had significantly more issues with CI use (p<0,001).

After assessing the influence of the *etiologic factors*, it was found that the groups did not differ in comparing the number of *GJB2* gene mutations (p=0,754) or comparing all six etiologic groups of HL (p=0,552).

An analysis of the *radiologic factors* revealed that the patients with the worse speech perception tended to have a significantly narrower BCNC (p=0,021). The groups did not differ according to the cochlear height (p=0,114) and the diameter of the internal acoustic meatus (p=0,093) (Fig. 23).

An evaluation of the influence of *family factors* demonstrated that these groups did not differ comparing family structure (p=0,061). The education of the fathers and mothers was poorer in the group of children with the worse speech perception (p<0,001 and p<0,001, respectively), their parents tended to misunderstand the CI process more often (p<0,001), and their families visited the CI center less frequently after surgery (p<0,001).

A comparison of the *rehabilitation and educational factors* revealed that the parents of children with a worse speech perception participated in the child's learning process less (p<0,001). Children with "poor speech perception" attended special kindergartens and schools more often as well (p<0,001 and p<0,001, respectively); in addition to this, they used total communication more often (p<0,001). The groups differed significantly based on the accessibility of speech and language therapy: children with poor speech perception were less exposed to speech and language therapy (p<0,001), and it was significantly less intensive (p=0,029) (Table 20).

TABLE 20. A comparative analysis of the "good speech perception" and "poor speech perception" groups.

Variable	Good speech perception group (N=60) N (%) or M (±SD)	Poor speech perception group (N=21) N (%) or M (±SD)	P value
Demographic factors			
Gender Male Female	 30 (50) 30 (50)	 14 (66.7) 7 (33.3)	0,837
Residential location: Five biggest cities Cities with 20 000–99 000 inhabitants Cities with 10 000–19 900 inhabitants Cities with <10 000 inhabitants and rural areas	 36 (60.0) 6 (10.0) 1 (1.7) 17 (21.0)	 7 (33.3) 1 (4.8) 3 (14.3) 10 (47.6)	0,034
Age at the time of the study, years	8.32 (±2.56)	9,67 (±2.9)	0,057
Audiological factors			
Age at diagnosis, months	16.89 (±13.1)	32,10 (±17.3)	**<0,001**
Mean preoperative hearing thresholds, dB	94.25 (±9.2)	95,24 (±7.5)	0,767
Residual preoperative hearing in the implanted ear: Present Absent	 13 (21.7) 47 (78.3)	 3 (14.3) 18 (85.7)	0,467
Use of the residual hearing in the contralateral ear with the HA after CI: Yes No	 14 (46.7) 16 (53.3)	 4 (28.6) 10 (71.4)	0,261
Progression of HL: Yes No	 11 (18.3) 49 (81.7)	 3 (14.3) 18 (85.7)	0,675
CI aided thresholds, dB	33.23 (±5.0)	40.5 (±9.4)	**<0,001**
Surgical, implant and processor-associated factors			
Age at implantation, months	28.19 (±21.2)	47.16 (±19.4)	**<0,001**
Duration of the CI use, years	5.95 (±2.1)	5.62 (±2,4)	0,723
Unilateral CI Bilateral CI	30 (50) 30 (50)	14 (66.7) 7 (33.3)	0,190
Issues with the usage: Present Absent	 1 (1.7) 59 (98.3)	 10 (47.6) 11 (52.4)	**<0,001**

Variable	Good speech perception group (N=60) N (%) or M (±SD)	Poor speech perception group (N=21) N (%) or M (±SD)	P value
Etiologic factors			
GJB2 gene mutation (N=68): Positive Negative	 33 (63.5) 19 (36.5)	 10 (62.5) 6 (37.5)	0,754
Non-syndromic	34 (56.7)	11 (52.4)	0,552
Syndromic	4 (6.7)	1 (4.8)	
Prenatal cCMV	6 (10.0)	2 (9.5)	
Perinatal	8 (13.3)	3 (14.3)	
Postnatal	1 (1.7)	0 (0)	
Unknown	7 (11.7)	4 (19.0)	
Radiological factors (N=64)			
Diameter of the bony cochlear nerve canal, mm	1.8 (±0.2)	1.5 (±0.6)	**0,021**
Cochlear height, mm	3.6 (±0.3)	3.5 (±0.3)	0,114
Width of the internal acoustic meatus, mm	5.0 (±0.9)	4.9 (±1.9)	0,093
Family factors			
Family composition: Nuclear family Single-parent Caregivers	 52 (86.7) 7 (11.7) 1 (1.7)	 15 (71.4) 6 (28.6) 0 (0)	0,061
Family size: One child Two children 3 or more children	 23 (38.3) 29 (48.3) 8 (13.3)	 10 (47.6) 7 (33.3) 4 (19.0)	0,738
Father's education level: Higher education Secondary education Incomplete secondary education	 32 (55.2) 23 (39.7) 3 (5.2)	 1 (4.8) 15 (71.4) 5 (23.8)	**<0,001**
Mother's education level: Higher education Secondary education Incomplete secondary education	 39 (66.1) 20 (33.9) 0 (0)	 5 (23.8) 14 (66.7) 2 (9.5)	**<0,001**
Parents' understanding about the CI process: Sufficient Insufficient Did not understand	 37 (61.7) 23 (38.3) 0 (0)	 1 (4.8) 8 (38.1) 12 (57.1)	**<0,001**

Variable	Good speech perception group (N=60) N (%) or M (±SD)	Poor speech perception group (N=21) N (%) or M (±SD)	P value
Family visits to the CI center:			**<0,001**
Sufficient	34 (56.7)	1 (4.8)	
Insufficient	24 (40.0)	4 (19.0)	
Did not visit	2 (3.3)	16 (76.2)	
Rehabilitation and educational factors			
Parents' engagement in the learning process:			**<0,001**
Active	42 (70.0)	1 (4.8)	
Passive	18 (30.0)	8 (38.1)	
Did not participate	0 (0)	12 (57.1)	
Communication mode:			**<0,001**
Spoken language	59 (98.3)	4 (19.0)	
Total communication	1 (1.7)	14 (66.7)	
Sign language	0 (0)	3 (14.3)	
Accessibility of speech and language therapy:			**<0,001**
Good	46 (76.7)	4 (19.0)	
Moderate	12 (20)	10 (47.6)	
Bad	2 (3.3)	7 (33.3)	
Intensity of speech and language therapy:			**0,029**
Did not attend	0 (0)	7 (33.3)	
Once per week	12 (20)	3 (14.3)	
2 times per week	32 (53.3)	6 (28.6)	
3 times per week	7 (11.7)	3 (14.3)	
5 times per week	9 (15)	2 (9.5)	
Educational placement settings:			**<0,001**
General education	56 (93.3)	5 (23.8)	
Special education	4 (6.7)	16 (76.2)	
Home-schooling	0	0 (0)	
Does not attend yet	0	0 (0)	
Kindergarten:			**0,001**
General	41 (68.3)	6 (28.6)	
Special education	19 (31.7)	13 (61.9)	
Did not attend	0 (0)	2 (9.5)	
Does not attend yet	0 (0)	0 (0)	
School/program (N=57):			**<0,001**
General school/mainstream program	31 (79.5)	1 (5.6)	
General school/adapted program	7 (17.9)	5 (27.8)	
Special education school	1 (2.6)	12 (66.7)	
Home-schooling	0 (0)	0 (0)	

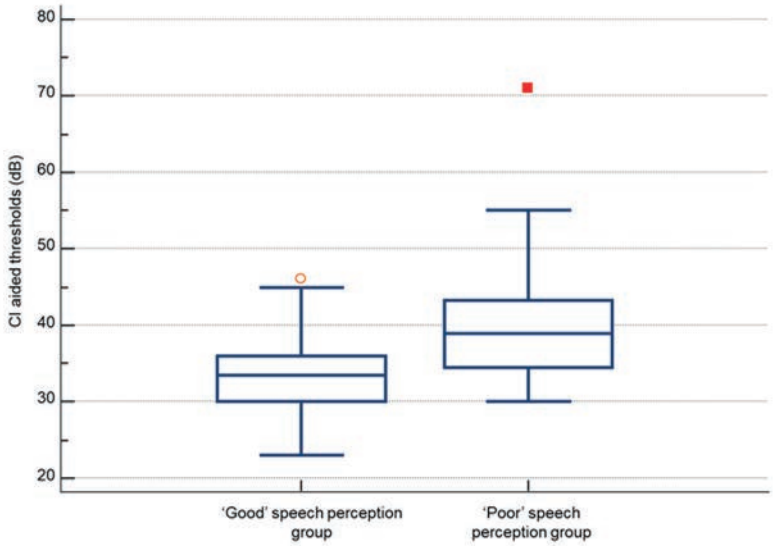


FIGURE 22. CI-aided thresholds with CI in different speech perception groups.

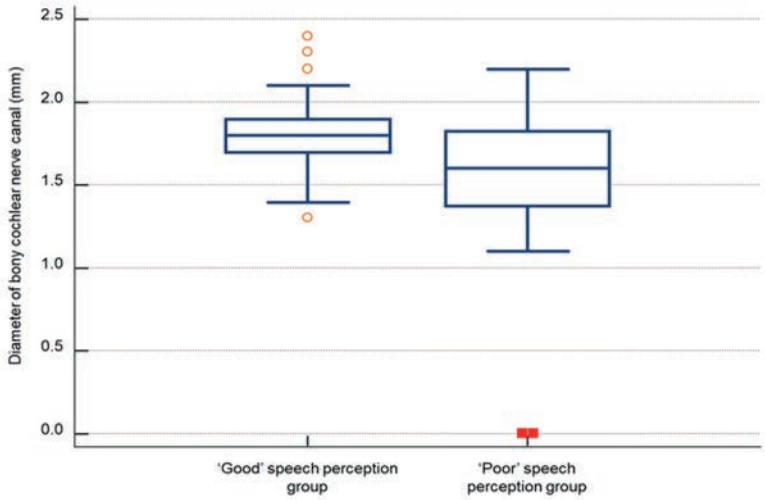


FIGURE 23. The diameter of the bony cochlear nerve canal in different speech perception groups.

A comparison of the "good" and "poor" speech perception groups based on other CI results revealed that children with poor speech perception showed significantly worse auditory abilities (p<0,001) and speech intelligibility (p<0,001) as estimated using CAP and SIR scales. In addition to this, children with poor speech perception showed significantly worse language development levels (p<0,001) (Table 21).

TABLE 21. Auditory abilities, speech intelligibility and and speech and language development amongst two grups of children with different speech perception levels.

Variable	Good speech perception N (%)	Poor speech perception N (%)	P value
CAP category			
1	0 (0)	0 (0)	**<0,001**
2	0 (0)	1 (4.8)	
3	0 (0)	3 (14.3)	
4	1 (1.7)	6 (28.6)	
5	4 (6.7)	10 (47.6)	
6	8 (13.3)	1 (4.8)	
7	47 (78.3)	0 (0)	
SIR category			
1	0 (0)	2 (9.5)	**<0,001**
2	2 (1.7)	6 (28.6)	
3	2 (3.3)	10 (47.6)	
4	11(18.3)	3 (14.3)	
5	46 (76.7)	0 (0)	
Language development levels			
Very good	20 (33.3)	0(0)	**<0,001**
Good	22 (36.7)	0(0)	
Sufficient	16 (26.7)	5 (23.8)	
Insufficient	2 (3.3)	16 (76.2)	

2.7.2. Determination of Prognostic Factors for Speech Perception Using Logistic Regression

The detection of prognostic factors for the speech perception of children who are CI users was performed using logistic regression analysis. The univariate analysis included all demographic, surgical/implant-associated, audiological, radiological, family, rehabilitation and educational variables that differed significantly between the "good" and "poor" speech perception groups.

A univariate logistic regression demonstrated that speech perception is influenced by the residential location (OR: 1,506, CI: 1,044–2,173, p=0,029). Age at diagnosis (OR: 1,064, CI 1,027–1,101, p=0,001), age at implantation (OR: 1,038;

CI: 1,014–1,064; p=0,002) and CI-aided thresholds (OR: 1,203; CI: 1,078–1,342; p=0,001) are all factors that have an effect on speech perception diagnosed using speech audiometry. Children who have issues with CI use have a 53-times bigger risk of insufficient speech perception (OR: 53,636; CI: 6,223–462, 33; p<0,001).

The diameter of the bony cochlear nerve canal is also associated with the speech perception results – as the diameter decreases by 1 mm, the risk for a child to have poor speech perception increases 12 times (OR: 11,928; CI: 1,292–110,129; p=0,029).

In the group of family factors, variables that have influence when predicting speech perception results are: the father's education (OR: 6,944; CI: 2,403–20,066; p<0,001), the mother's education (OR: 6,416; CI: 2,170–18,968; p=0,001), the parents' understanding of the CI process (OR: 42,745; CI 5,765–316,933; p<0,001) and the frequency of family visits to the CI center (OR: 24,444; CI: 6,234–95,855; p<0,001). The parents' engagement in a child's learning (OR: 44,230; CI: 6,069–322,372; p<0,001), the accessibility of speech and language therapy (OR: 7,076; CI: 2,837–17,652; p<0,001), intensity of speech and language therapy (OR: 1,639; CI: 1,030–2,609; p=0,037) and type of the preschool institution (OR: 5,067; CI: 1,812–14,171; p=0,002) were educational variables that were associated with speech perception results (Table 22).

TABLE 22. A univariate logistic regression analysis for detecting factors associated with postoperative speech perception.

Variable	Odds ratio		P
	Value	95% confidence interval	
Residential location	1,506	1,044–2,173	0,029
Age at diagnosis	1,064	1,027––1,101	0,001
Postoperative hearing thresholds with CI	1,203	1,078–1,342	0,001
Age at implantation	1,038	1,014–1,064	0,002
Issues with the use of CI	53,636	6,223–462,33	<0,001
Diameter of the bony cochlear nerve canal, mm	11,928	1,292–110,129	0,029
Father's education	6,944	2,403–20,066	<0,001
Mother's education	6,416	2,170–18,968	0,001
Parents' understanding of the CI process	42,745	5,765–316,933	<0,001
Family visits to the CI center	24,444	6,234–95,855	<0,001
Parents' engagement in the learning process	44,230	6,069–322,372	<0,001

Variable	Odds ratio		P
	Value	95% confidence interval	
Accessibility of speech and language therapy	7,076	2,837–17,652	<0,001
Intensity of speech and language therapy	1,639	1,030–2,609	0,037
Pre-school educational institution	5,067	1,812–14,171	0,002

After assuring a significant relation between postoperative speech perception and different chosen variables in a univariate regression analysis, the following variables were chosen in the multivariate regression analysis: age at implantation, aided thresholds, the diameter of the bony cochlear nerve canal and the mother's education. Age at diagnosis was not included in the multiple regression analysis despite its statistical significance, because it is associated with the age at implantation and depends on it. The father's education, the parents' understanding of the CI process, family visits to the CI centers and the parents' engagement in the learning process were not included in the multivariate analysis as well – they were related to and dependent on the mother's education.

The multivariate regression analysis revealed that age at implantation (OR: 0,927: CI: 0,877– 0,980; p=0,008), postoperative aided thresholds with CI (OR: 0,721; CI 0,570–0,911; p =0,006) and the diameter of the bony cochlear nerve canal (OR: 24,215; CI: 1,227–477,77; p=0,036) are all independent prognostic factors for speech perception after pediatric CI.

2.7.3. Comparison of Groups of Children with Different Speech and Language Development Levels

In order to find the factors that determine better or worse speech and language development results, two groups were formed: children who demonstrated very good and good speech and language development levels were attributed to the "good speech and language development" group, and children who demonstrated satisfactory or unsatisfactory language development levels were attributed to the "insufficient speech and language development" group. The "good speech and language development" group included 42 children; the "insufficient speech and language development" group included 39 children.

Thirty prognostic factors (demographic, audiological, surgical, implant-associated, etiological, radiological, family, rehabilitation and educational) were analyzed in this study in order to find their relation to the postoperative results of speech and language development levels.

An analysis of the *demographic* factors showed no significant difference between the groups neither based on the gender (p=0,212) nor on the age at the time of the study (p=0,394). Two groups differed significantly based on the residential location (p=0,013) – children included into the "good speech and language development" group more frequently lived in big cities (69%), while children from the "insufficient speech and language development" group – in small cities and rural areas (41%).

After analyzing influence of the *audiological* factors, it was found that age at the time of diagnosis is significantly lower in a "good speech and language development" group compared to the "insufficient speech and language development" group (p=0,011). The two groups did not differ based on the mean preoperative hearing thresholds (p=0,465), preoperative residual hearing in the implanted ear (p=0,344), or based on the number of progressive HL cases (p=0,309). However, children with estimated "insufficient speech and language development" tended to not use residual postoperative hearing in the contralateral ear by using HA (p=0,039). CI-aided thresholds were significantly higher in the "insufficient speech and language development" group (p=0,001).

An evaluation of *surgical, implant-associated and processor-associated* factors revealed that age at implantation was significantly older in the "insufficient language development" group (p=0,005). Groups did not differ comparing duration of the implant use (p=0,612) or the number of bilateral CIs performed (p=0,421). However, children with estimated "insufficient speech and language development" had significantly more issues with CI use (p<0,001).

No significant difference was observed between groups comparing the effect of the *etiologic* factors: neither comparing the number of GJB2 positive cases (p=0,947), nor other causes of HL (p=0,723).

An analysis of the *radiologic* factors showed no difference between two groups based on the diameter of the bony cochlear nerve canal (p=0,448), cochlear height (p=0,324) and the diameter of the internal acoustic meatus (p=0,145).

After analyzing *family-related* factors, it was observed that groups did not differ based on the family structure (p=0,185) and size of the family (p=0,793). In the "insufficient speech and language development" group, the father's and mother's education were significantly worse (p<0,001 in both cases), the parents' understanding of the CI process was poorer (p<0,001), and their families tended to significantly less frequently visit the CI center after the implantation surgery (p<0,001).

A comparison of groups based on *educational and hearing rehabilitation-related* factors revealed a significantly poorer parents' engagement in the child's learning process (p<0,001) in the "insufficient speech and language development" group. These children also attended specialized educational kindergartens more often

(p=0,002), used total language to communicate more often (p<0,001), found it more difficult to reach speech and language therapy services (p<0,001), the latter also being less intensive (p=0,029) (Table 23).

TABLE 23. A comparative analysis of "poor" and "good" speech and language development groups.

Variable	Good speech and language development group N=42 N (%) or Mean	Insufficient speech and language development group N=39 N (%) or Mean	P value
Demographic factors			
Gender Male Female	 20 (47.6) 22 (52.4)	 24 (61.5) 15 (38.5)	0,212
Residential location: Five biggest cities Cities with 20 000–99.000 inhabitants Cities with 10 000–19 900 inhabitants Cities with <10 000 inhabitants and the rural areas	 29 (69) 1 (2.4) 1 (2.4) 11 (26.2)	14 (35.9) 6 (15.4) 3 (7.7) 16 (41)	0,013
Age at the time of the study, years	8.36 (±2.3)	9.01 (±3.0)	0,394
Audiological factors			
Age at diagnosis, months	16.67 (±13.8)	25.32 (±16.6)	**0,011**
Preoperative mean hearing thresholds, dB	94.05 (±8.9)	95.0 (±8.7)	0,465
Residual preoperative hearing in the implanted ear: Present Absent	 10 (23.8) 32 (76.2)	 6 (15.2) 33 (84.6)	0,344
Use of residual hearing in the contralateral ear with the HA after CI: Yes No	 12 (57.1) 9 (42.9)	 6 (26.1) 17 (73.9)	0,039
Progression of hearing loss Present Absent	 9 (21.4) 33 (78.6)	 5 (12.8) 34 (87.2)	0,309
Postoperative hearing thresholds with CI, dB	32.60 (±5)	37.85 (±8)	**0,001**
Surgical, implant and processor-related factors			
Age at implantation, months	28,38 (±22.7)	38,20 (±20.7)	**0,005**
Duration of CI use, years	5,95 (±1.8)	5,77 (±2.5)	0,612

Variable	Good speech and language development group N=42 N (%) or Mean	Insufficient speech and language development group N=39 N (%) or Mean	P value
Unilateral CI Bilateral CI	21 (50) 21 (50)	23 (59) 16 (41)	0,421
Issues with the use of CI Present Absent	 0 (0) 42 (0)	 11 (28.2) 28 (71.8)	**<0,001**
Etiologic factors			
GJB2 (N=68) Positive Negative	 22 (62.9) 13 (37.1)	 22 (63.6) 12 (36.4)	0,947
Non-syndromic	23 (54.8)	22 (56.4)	0,723
Syndromic	4 (9.5)	1 (2.6)	
Prenatal cCMV	6 (14.3)	5 (12.8)	
Perinatal	4 (9.5)	4 (10.3)	
Postnatal	0 (0)	1 (2.6)	
Unknown	5 (11.9)	6 (15.4)	
Radiological factors N=64			
Diameter of the bony cochlear nerve canal, mm	1.77 (±0.2)	1,63 (±0.5)	0,448
Cochlear height, mm	3.62 (±0.3)	3,53 (±0.3)	0,324
Width of the internal acoustic meatus, mm	5.07 (±0.9)	4,87 (±1.5)	0,145
Family factors			
Family composition: Nuclear family Single-parent Caregivers	 38 (90.5) 4 (9.5) 0 (0)	 29 (74.4) 9 (23.1) 1 (2.6)	0,185
Family size: One child Two children 3 or more children	 17 (40.5) 20 (47.6) 5 (11.9)	 16 (41) 16 (41) 7 (17.9)	0,793
Father's education level: Higher education Secondary education Incomplete secondary education	 28 (70) 12 (30) 0 (0)	 5 (12.8) 26 (66.7) 8 (20.5)	**<0,001**
Mother's education level: Higher education Secondary education Incomplete secondary education	 31 (75.6) 10 (24.4) 0 (0)	 13 (33.3) 24 (61.5) 2 (5.1)	**<0,001**

Variable	Good speech and language development group N=42 N (%) or Mean	Insufficient speech and language development group N=39 N (%) or Mean	P value
Parents' understanding of the CI process:			**<0,001**
Sufficient	31 (73.8)	7 (17.9)	
Insufficient	11 (26.2)	20 (51.3)	
Did not understand	0 (0)	12 (30.8)	
Family visits to the CI center:			**<0,001**
Sufficient	30 (71.4)	5 (12.8)	
Insufficient	11 (26.2)	17 (43.6)	
Did not visit	1 (2.4)	17 (43.6)	
Rehabilitation and educational factors			
Parents engagement in the learning process:			**<0,001**
Active	34 (81)	9 (23.1)	
Passive	8 (19)	18 (46.2)	
Do not participate	0 (0)	12 (30.8)	
Communication mode:			**<0,001**
Spoken language	42 (100)	21 (53.8)	
Total communication	0 (0)	15 (38.5)	
Sign language	0 (0)	3 (7.7)	
Accessibility of speech and language therapy:			**<0,001**
Good	37 (88.1)	13 (33.3)	
Average	4 (9.5)	18 (46.2)	
Poor	1 (2.4)	8 (20.5)	
Intensity of speech and language therapy:			**0,029**
Did not attend	0 (0)	7 (17.9)	
Once per week	4 (9.5)	11 (28.2)	
2 times per week	28 (66.7)	10 (25.6)	
3 times per week	4 (9.5)	6 (15.4)	
5 times per week	6 (14.3)	5 (12.8)	
Educational placement settings:			**<0,001**
General education	41 (97.6)	20 (51.3)	
Special education	1 (2.4)	19 (48.7)	
Home-schooling	0(0)	0(0)	
Does not attend yet	0(0)	0(0)	
Kindergarten:			**0,002**
General education	31 (73.8)	16 (41)	
Special education	11 (26.2)	21 (53.8)	
Too young to attend	0(0)	2 (5.1)	
School/program N=57			
General/mainstream program	27 (96.4)	4 (13.8)	**<0,001**
General/adapted program	1 (3.6)	11 (37.9)	
Special education school	0	14 (48.3)	
Home schooling	0	0	

Comparing groups based on speech perception, listening and speech recognition skills (measured using CAP and SIR scales), it was noticed that children with better speech and language developments demonstrated better speech perception (p<0,001) and higher scores of CAP and SIR scales (p<0,001) (Fig. 24)

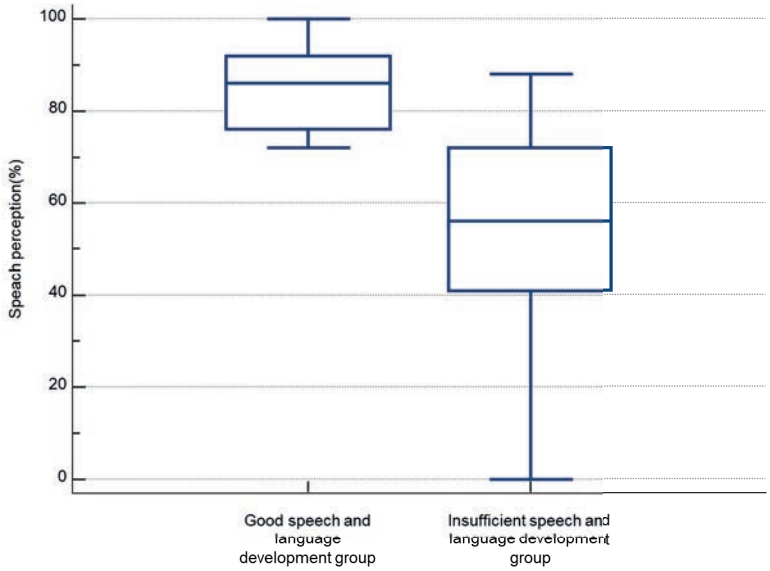


FIGURE 24. Speech perception amongst different speech and language development groups.

2.7.4. Determination of Prognostic Factors for Speech and Language Development Using Logistic Regression

The abovementioned variables, which differed significantly between two language development groups, were tested using the logistic regression analysis in order to determine associations between them and postoperative language development results

A univariate logistic regression analysis demonstrated that residential location (OR: 1,450; CI: 1,042-2,017; p=0,028), age at the time of diagnosis (OR: 1,039; CI: 1,007-1,072; p=0,017) and CI aided thresholds (OR: 1,166; CI: 1,057-1,286; p=0,002) are the factors that influence postoperative language development results. They are also affected by the father's education (OR: 13,679; CI: 4,508- 41,508; p<0,001), mother's education (OR: 6,013, CI: 2,319-15,596, p<0,001), parents' understanding about the CI process (OR: 10,306; CI: 3,867-27,466; p<0,001) and the frequency of visits to the CI center (OR: 9,742; CI: 3,822- 24,834; p<0,001). Language development is also influenced by the parents' engagement in the learning process (OR:

8,310; CI: 3,037-22,738; p<0,001), accessibility of the speech and language therapy (OR: 8,310; CI: 3,037 - 22,738 0,370; p<0,001) and preschool institution (OR: 3,175; CI: 1,558-9,522; p=0,006) (Table 24).

TABLE 24. Univariate regression analysis to determine variables associated with postoperative language development results.

Variable	Odds ratio		P
	Value	95% CI	
	Univariate		
Residential location	1,450	1,042 - 2,017	0,028
Age at diagnosis	1,039	1,007 - 1,072	0,017
Postoperative hearing thresholds with CI	1,166	1,057 - 1,286	0,002
Age at implantation	1,022	1,000 - 1,044	0,053
Father's education	13,679	4,508 - 41,508	<0,001
Mother's education	6,013	2,319 - 15,596	<0,001
Parents' understanding about the CI process	10,306	3,867 - 27,466	<0,001
Family visits at the CI center	9,742	3,822 - 24,834	<0,001
Parents' engagement in the learning process	10,544	3,917 - 28,381	<0,001
Accessibility of the speech and language therapy	8,310	3,037 - 22,738	<0,001
Intensity of the speech and language therapy	1,353	0,958 - 1,909	0,086
Preschool institution	3,175	1,558 - 9,522	0,006

After assuring a significant relation between postoperative language development and the different chosen variables in a univariate regression analysis, the following variables were chosen for the multivariate regression analysis: age at the time of diagnosis, postoperative hearing thresholds with CI, parents' engagement in the learning process, and accessibility of speech and language therapy. Education and understanding about the CI process of the parents, family visits to the CI center were not included in the multiple regression analysis despite their statistical significance, as they were associated with the parents' engagement in the learning process and depend on it.

A multivariate regression analysis revealed that only parents' engagement in the learning process (OR: 6,255; CI: 1,846- 21,191; p=0,003) and accessibility of speech and language therapy (OR: 3,295; CI: 1,112- 9,763; p=0,031) are two independent prognostic factors in language development after pediatric CI.

Conclusions

1. A unique etiologic profile of Lithuanian children who are CI users was established. The most common etiologic factor amongst children who are CI users was non-syndromic HL (63 cases, 51.6%). The second most common cause were perinatal factors (16 cases, 13.1%). Prenatal factors – a congenital CMV infection – were in the third place (12 cases, 9.8%). Syndromic HL was in the fourth place (10 cases, 8.2%) children; postnatal factors – in the fifth place (4 cases, 3.3%). The cause of HL remained unknown in 17 (13.9%) cases.
2. The prevalence of inner ear malformations in the group of pediatric CI users was established after performing an analysis of temporal bone CT images; the prevalence of inner ear malformations was at 33%.
3. Speech perception results after CI were evaluated. On average, 5.9 years after the CI surgery, excellent and good speech perception levels were demonstrated by 19.8 and 35.8% of children, respectively; average levels – by 19.8%, weak and very weak – by 6.2 and 13.6%, respectively; speech perception without any visual cues of 4.9% of children was equal to 0. The mean speech perception level of the study sample was 69.6 ± 24.2%.
4. Speech and language results after the CI were evaluated: out of 81 children examined, on average, 5.9 years after the CI surgery, 24.7% of participants reached a very good speech and language development level; 27.2% – a good level; 25.9% – a sufficient level; 22.2% – an insufficient level of speech and language perception.
5. A univariate logistic regression analysis revealed that results of speech perception and language development of pediatric CI users are mostly associated with family, education and rehabilitation – related factors.

A multivariate regression analysis proved that age at implantation, postoperative CI aided thresholds and the diameter of the BCNC are three independent prognostic factors of speech perception after pediatric CI. Results of speech and language development depend on parents' engagement in child's learning process and accessibility of speech and language therapy.

Recommendations

1. The preoperative preparation for CI should include the evaluation of etiologic, medical, anatomical, audiologic, surgical, implant–associated, family, education and rehabilitation–related factors that might influence results after CI.

2. The preoperative parental counselling should accentuate family's role in the CI process, and the influence of family–related factors on the postoperative results.
3. The evaluation of the etiology of congenital hearing loss should begin with the genetic counselling, and a retrospective diagnosis of a cCMV infection should be established by the detection of CMV DNA from a dried blood spot.
4. The preoperative evaluation of the temporal bone should include a detailed measurement of delicate inner ear structures, such as the cochlear height and the diameter of the bony cochlear nerve canal, on CT scan images.
5. Pediatric CI users should regularly visit specialists after the implantation to measure postoperative results. When insufficient CI results are identified, it is recommended to determine factors that might have influenced it, inform the family, and discuss with the CI team changes should be made in the intervention manner.
6. The CI program should be established to coordinate collaboration between different institutions and ensure the long term monitoring of pediatric CI users.

SANTRAUKA

Įvadas

Klausos sutrikimas (KS) yra dažniausia įgimta patologija išsivysčiusiose šalyse. Iš 1000 naujagimių 1–3 gimsta turėdami neurosensorinį klausos sutrikimą, dar 1–2 vaikų klausa sutrinka vėliau.

Trečdaliu atvejų įgimtas neurosensorinis klausos sutrikimas yra sunkus bei ypač sunkus (gilus). Toks KS turi ilgalaikių padarinių vaiko ir jo šeimos gyvenimui. Labiausiai kurtumas veikia sakytinės kalbos raidą, tai riboja kasdienį bendravimą, menkina mokymosi galimybes ir raštingumą, dėl to ne tik nukenčia vaiko mokymosi pasiekimai ir galimybė ateityje įsidarbinti, bet ir kyla psichosocialinių problemų. Svarbu, kad laikotarpis nuo abipusio kurtumo pradžios iki medicininės intervencijos – klausos reabilitacijos klausos aparatais arba kochleariniais implantais – būtų kuo trumpesnis.

Kochlearinis implantas (KI) – tai elektroninis medicininis prietaisas, kuriuo kurtiesiems grąžinama klausa. KI procesorius transformuoja aplinkos garsus į elektroninį signalą, o implanto elektrodas, įstatytas į vidinėje ausyje esančią sraigę, perduoda šį signalą klausos nervui. Toliau impulsas keliauja į smegenų žievę ir asmuo ima girdėti. Tyrimais įrodyta, kad kochlearinė implantacija – saugus ir efektyvus būdas kurtumui gydyti.

Kochlearinės implantacijos rezultatai vertinami etapais: pirmiausia – girdėjimas ir kalbos suvokimas, tuomet – kalbos įgūdžiai, vėliau – integracija į bendrojo lavinimo įstaigą, gyvenimo kokybė ir kiti. Mokslinės literatūros duomenimis, pusės vaikų, besinaudojančių KI, kalbos suvokimo ir kalbinių įgūdžių lygis gali siekti normaliai girdinčių bendraamžių.

Vis dėlto pastebima didelė individualių pasiekimų įvairovė kalbos suvokimo ir kalbos išsivystymo srityse. Įrodyta, kad vaiko amžius KI operacijos metu yra svarbiausias veiksnys, lemiantis pooperacinius rezultatus, – rezultatai geresni tuomet, kai operacija atliekama kuo jaunesniam vaikui. Ne mažiau svarbūs veiksniai yra vidiniai biologiniai – kurtumo etiologija ir vaiko intelektas; išoriniai techniniai – implanto savybės, implanto programavimas; socialiniai – specialiojo ugdymo galimybės, tėvų įsitraukimas padedant vaikui mokytis ir kiti. Įvardijus įtaką darančius veiksnius, lengviau prognozuoti konkretaus paciento rezultatus, o šeimai tai leidžia išsikelti realius lūkesčius bei planuoti gydymą ir reabilitaciją po kochlearinės implantacijos taip, kad būtų pasiekta maksimali nauda

Lietuvoje pirmąją kochlearinę implantaciją atliko švedų profesorius S. Harris 1998 m. Kauno medicinos universiteto klinikose. Dabar Lietuvoje yra daugiau kaip 370 KI naudotojų

Iki šiol Lietuvoje vaikų KI ilgalaikiai rezultatai – kalbos suvokimas, kalbos raida, integracija į bendrojo lavinimo įstaigas – nebuvo tiriami. Iki šio tyrimo nebuvo analizuojami ir tokie galimi KI efektyvumo veiksniai, kaip kurtumo etiologija, ausies radiologinė anatomija, socialiniai aspektai. Žinoma, kad nuo pusės iki dviejų trečdalių įgimto klausos sutrikimo (ĮKS) atvejų lemia paveldėjimas. Likusią dalį sudaro nepaveldėtas, t. y. aplinkos veiksnių sukeltas arba nežinomos kilmės, klausos sutrikimas. Įgimta citomegalo viruso (ĮCMV) infekcija yra antra pagal dažnį ĮKS priežastis ir lemia apie 10–30 proc. ĮKS atvejų. Dažniausiai ĮCMV infekcija yra besimptomė, todėl specifiniai tyrimai naujagimiams neatliekami, tačiau KS gali išsivystyti po kelių mėnesių ar metų. Todėl tebėra aktualu nustatyti ĮCMV infekcijos paplitimą įvairiose populiacijose.

Žinoma, kad geriausi KI rezultatai yra pasiekiami tose šalyse, kuriose yra sukurta KI sistema, apimanti ankstyvą KS diagnostiką, priešoperacinį pasiruošimą, chirurginę implantacijos procedūrą bei visapusišką medicininę, pedagoginę, techninę, psichologinę, socialinę bei finansinę pagalbą po implantacijos. Nors Lietuvoje vaikų KI atliekama beveik du dešimtmečius, tačiau iki šiol tokios sistemos mūsų šalyje nėra. Šiuo tyrimu siekta parengti rekomendacijas Lietuvos KI sistemai kurti. Remiantis KI sistema bus galima optimizuoti kandidatų atranką bei pooperacinę reabilitaciją, savo ruožtu pagerinti kurčiųjų vaikų socialinę integraciją.

Apibendrinant galima teigti, kad vaikų KI rezultatų vertinimas ir prognostinių veiksnių nustatymas išlieka svarbi klinikinė ir mokslinė problema. Šiame tyrime pirmą kartą Lietuvoje buvo vertinama KI naudojančių vaikų bendrosios, šeimos ir lavinimo charakteristikos, kurtumo etiologija, pooperaciniai rezultatai bei juos lemiantys veiksniai. Manoma, kad disertacinio darbo rezultatai padės išplėsti klinikines, socialines, pedagogines priemones, leidžiančias optimizuoti vaikų KI rezultatus.

Darbo tikslas

Nustatyti vaikų kochlearinės implantacijos funkcinius rezultatus ir jų prognostinius veiksnius.

Darbo uždaviniai

1. Nustatyti kochlearinius implantus naudojančių vaikų kurtumo priežastį, atliekant genetinius, CMV DNR sauso kraujo lašo tyrimus, klausos sutrikimo rizikos veiksnių analizę, bei įvertinti gautą etologinį profilį.
2. Įvertinti kochlearinius implantus naudojančių vaikų vidinės ausies anatominius pokyčius, atliekant smilkinkaulių kompiuterinės tomografijos vaizdų analizę.
3. Nustatyti kurčių vaikų kalbos suvokimo rezultatus po kochlearinės implantacijos, atliekant kalbinę audiometriją.
4. Nustatyti kurčių vaikų kalbos raidos rezultatus po kochlearinės implantacijos, atliekant kalbos raidos vertinimą.
5. Nustatyti vaikų kochlearinės implantacijos rezultatų prognostinius veiksnius.

Darbo metodika

Mokslinis daugiacentris tarpdisciplininis skerspjūvio tyrimas atliktas 2013–2018 metais VU MF Ausų, nosies, gerklės ir akių ligų klinikoje bei VUL SK filialo Vaikų ligoninės Vaikų ausų, nosies, gerklės ir akių ligų skyriuje. Tiriamieji – kurtieji vaikai, kuriems atlikta KI. 122 vaikai atitiko numatytus įtraukimo į tyrimą kriterijus: kurtieji vaikai, kuriems buvo atlikta viena arba dvi KI operacijos; tiriamieji 1–18 metų amžiaus; KI operacija(-os) atlikta(-os) VUL SK Ausų, nosies, gerklės ir akių klinikoje; vienpusė arba pirmoji iš dviejų KI atlikta ne vėliau nei prieš 6 mėn.; vaikų tėvai ar globėjai sutiko, kad jų vaikas dalyvautų tyrime, ir pasirašė informuoto asmens sutikimo formą.

Pagal numatytą skerspjūvio tyrimo dizainą – priešoperaciniai, operacijos ir pooperaciniai tiriamojo duomenys rinkti bei etologiniai kurtumo veiksniai tirti ir pooperaciniai klausos ir kalbos rezultatai vertinti lygiagrečiai tuo pačiu metu:

- siekiant nustatyti tiriamojo *priešoperacinius, operacijos ir pooperacinius veiksnius*, galinčius veikti KI rezultatus, apklausti tiriamųjų tėvai bei peržiūrėta medicininė dokumentacija – surinkti tiriamojo demografiniai, medicininiai, audiologiniai, operacijos, šeimos, lavinimo ir ugdymo duomenys;
- siekiant nustatyti *kurtumo priežastį*, vertinti kurtumo rizikos veiksniai, atliktas genetinis ištyrimas bei CMV DNR tyrimas sauso kraujo lašo ėminyje;
- siekiant nustatyti *anatominius vidinės ausies pokyčius*, galinčius veikti KI rezultatus, atlikta išsami prieš operaciją atliktos smilkinkaulių KT vaizdų analizė;
- siekiant nustatyti pooperacinius *klausos rezultatus*, atliktas vertinimas pagal skales, toninė audiometrija ir kalbinė audiometrija laisvame garso lauke naudojant KI;
- siekiant nustatyti pooperacinius *kalbos rezultatus*, buvo atliktas kalbos raidos vertinimas.

Vėliau, įvertinus turimus demografinius, medicininius, audiologinius, operacijos, šeimos, lavinimo ir ugdymo duomenis bei pooperacinius rezultatus, nustatyti vaikų KI rezultatų *prognostiniai veiksniai*.

Tyrimų rezultatams sisteminti naudota aprašomoji statistika, vidurkis, standartinis nuokrypis, minimali ir maksimali reikšmės, mediana, moda. Hipotezėms apie kintamųjų tarpgrupinius skirtumus tikrinti taikyti neparametriniai Mano, Vitnio ir Vilkoksono (dviem nepriklausomoms imtims) arba Kruskalo ir Voliso (daugiau negu dviem nepriklausomoms imtims) kriterijai. Rezultatai laikyti statistiškai reikšmingais, kai $p<0,05$. Ryšiams tarp kintamųjų nustatyti buvo skaičiuojami Pirsono ir Spirmeno koreliacijos koeficientai. Norint apibrėžti atskirų rodiklių priklausomybę ir prognozuoti analizuojamų rodiklių reikšmes, taip pat buvo naudotas logistinės regresijos metodas. Statistinė duomenų analizė atlikta naudojant MS Excel, IBM SPSS 21.0 ir MedCalc 18.11.3 programas.

Rezultatai

Tiriamųjų grupę sudarė 122 vaikai, kuriems buvo atlikta viena arba dvi KI operacijos VUL SK Ausų, nosies, gerklės ir akių ligų centre. Tiriamųjų imtis sudarė 43,1 proc. bendros Lietuvos kurčiųjų vaikų, kuriems KI operacija buvo atlikta nuo 1999 m. iki 2017 m. pabaigos, populiacijos. 65 (53,3 proc.) vaikams buvo atlikta vienpusė KI), 57 (46,7 proc.) vaikams – abipusė KI, iš jų 34 (59,6 proc.) vaikai operuoti nevienmomentiškai, 23 (40,4 proc.) – vienmomentiškai. Vidutinis tiriamųjų amžius tyrimo metu buvo 7,6 ± 3,3 metų. Didžiausią imties dalį 86 (70,5 proc.) sudarė priešmokyklinio amžiaus vaikai ir pradinukai. Vidutinis tiriamųjų amžius pirmos operacijos metu buvo 32,5 ± 26,9 mėn. Vaikų, gimusių 2014 m. ir vėliau, kai Lietuvoje pradėta visuotinė naujagimių klausos patikra, amžius pirmos KI metu buvo 14,56 ± 4,91 mėn. ir statistiškai reikšmingai skyrėsi nuo vaikų, gimusių iki 2014 m., amžiaus pirmos operacijos metu – 36,47 ± 28,1 mėn. ($p<0,001$). Dauguma tiriamųjų vaikų, naudojančių KI, gyveno penkiuose Lietuvos didmiesčiuose. Klausos slenksčių vidurkiai prieš KI vidutiniškai sudarė 95,5 ± 7,8 dB geriau girdinčioje ausyje, 97,7 ± 4,8 dB operuotoje arba vienoje iš operuotų ausų abipusės operacijos atveju. 16,4 proc. vaikų turėjo likutinę klausą operuotoje arba vienoje iš operuotų ausų. Po operacijos iš 65 vienpusių KI naudotojų 30,8 proc. vaikų nuolat naudojo KA kontralateralinėje ausyje. Pirmais pooperaciniais metais 14,8 proc. vaikų pasireiškė procesoriaus naudojimo problemų. Tiriamųjų vaikų šeimos po operacijos KI centre dažniausiai lankėsi pakankamai – 42,6 proc., 38 proc. šeimų lankėsi nepakankamai ir 19,7 proc. nesilankė KI centre, vertinant apsilankymų skaičių per pirmus 2 metus po KI. Vertinant vaikų, KI naudotojų, klausos ir kalbos lavinimo ir ugdymo aspektus nustatyta, kad 49,8 proc. vaikų šeimos aktyviai dalyvavo lavinant vaiką, 33,6 proc. šeimų dalyvavimas buvo pasyvus ir 17,2 proc. šeimų nedalyvavo vaiko lavinime. Dauguma tėvų bendraudami su vaiku vartojo tik sakytinę kalbą (71,3 proc.), 23,8 proc. šeimų vartojo ir sakytinę kalbą, ir gestus, 4,9 proc. šeimų su vaiku bendravo gestų kalba. Surdopedagoginė pagalba buvo gerai prieinama 53,3 proc. vaikų, vidutiniškai – 27,9 proc. ir blogai – 18,9 proc. vaikų. Tyrimo metu 59 proc. vaikų lankė bendrojo lavinimo ir 28,7 proc. – specialiojo lavinimo darželį ar mokyklą, 2,5 proc. mokėsi namuose ir 9,8 proc. vaikų dar nelankė ugdymo įstaigos dėl amžiaus.

GJB2 koduojančios sekos tyrimas atliktas visiems izoliuotą KS turintiems tiriamiesiems – 91 vaikui. Patogeniniai homozigotiniai arba sudėtiniai heterozigotiniai *GJB2* geno variantai nustatyti 58 (63,7 proc.) izoliuoto KS atvejais. 5 pacientams nustatyti kitų genų patogeniniai variantai, lemiantys nesindrominį KS. Atlikus 101 vaiko genetinį ištyrimą, nustatyti 63 (62,4 proc.) nesindrominio KS ir 10 (9,9 proc.) sindrominio KS atvejų.

117 vaikų buvo gauti sauso kraujo lašo ėminiai ir atlikti CMV PGR tyrimai, 14 (12 proc.) ėminių nustatyta CMV DNR.

Ištirtas vaikų, naudojančių KI, etologinis profilis. Dažniausias etologinis veiksnys – paveldimas nesindrominis klausos sutrikimas – nustatytas 51,6 proc., perinataliniai veiksniai – 13,1 proc., prenataliniai veiksniai, t. y. įgimta CMV infekcija, – 9,8 proc., sindromai – 8,2 proc., postnataliniai veiksniai – 3,3 proc. vaikų. Kurtumo priežastis liko neaiški 13,9 proc. vaikų.

Analizuojant smilkinkaulių KT vaizdus, 4 vaikams rastos sraigės nebaigtinio pasidalijimo anomalijos, 18 vaikų – prieangio ir pusratinių kanalų anomalijų, 16 vaikų – sraigės hipoplazija, 14 vaikų – kaulinio sraigės nervo kanalo stenozė ir 2 vaikams – prieangio vandentiekio išsiplėtimas. Bendras įgimtų vidinės ausies anomalijų dažnis buvo 33 proc.

Vertinant KI efektyvumą, laisvame garso lauke nustatytų klausos slenksčių vidurkis su vienu arba dviem KI buvo 36,3 ±7,8 dB. Vertinant klausymosi įgūdžius pagal KAK skalę, praėjus vidutiniškai 4,9 ±2,6 metų po KI operacijos, nustatyta, kad 41,8 proc. vaikų pasiekė skalės „lubas“ ir gali kalbėtis telefonu su pažįstamu pašnekovu. Vertinant kurčiųjų vaikų, KI naudotojų, kalbos suprantamumą pagal KSS skalę, praėjus vidutiniškai 4,9 ±2,6 metų po KI, paaiškėjo, kad iš 122 vaikų 41 proc. pasiekė aukščiausią skalės kategoriją – jiems išsivystė kalba, kuri yra suprantama visiems klausytojams kasdienės veiklos metu.

Vertinant kalbos suvokimą vaikų be sunkios negalios, vyresnių nei 5 metų amžiaus ir operuotų mažiausiai prieš 2 metus, nustatyta, kad puikų kalbos suvokimo lygį pasiekė –19,8 proc. tiriamųjų, gerą – 35,8 proc., vidutinį – 19,8 proc., silpną – 6,2 proc., labai silpną – 13,6 proc. vaikų; 4,9 proc. vaikų negalėjo būti tiriami atvirojo tipo kalbinės audiometrijos metodu. Šios grupės kalbos suvokimo vidurkis buvo 69,6±24,2 proc.

Vertinant kalbėjimo ir kalbos pasiekimus vaikų be sunkios negalios, vyresnių nei 5 metų amžiaus ir operuotų mažiausiai prieš 2 metus, nustatyta, kad 24,7 proc. tiriamųjų pasiekė labai gerą kalbos raidos lygį, 27,2 proc. – gerą, 25,9 proc. – patenkinamą ir 22,2 proc. – nepatenkinamą.

Nesindrominį KS turintiems vaikams diagnozė buvo nustatyta bei pirma KI operacija atlikta anksčiau nei kitų etologijų (sindrominės, perinatalinės, prenatalinės, postnatalinės ir nežinomos) grupių vaikams (atitinkamai p=0,003 ir p=0,023). Iš 122 vaikų 14-ai (11,5 proc.) diagnozuota sunki gretutinė negalia: vaikų cerebrinis paralyžius, autizmas, epilepsija ir kt.

Siekiant išsiaiškinti, kokie veiksniai lemia blogesnį kalbos suvokimą, nustatytą kalbinės audiometrijos metodu, buvo suformuotos dvi grupės: gero ir silpno kalbos suvokimo. Tyrime nagrinėta 30 (demografinių, audiologinių, operacijos ir implanto, etologinių, radiologinių, šeimos, lavinimo bei ugdymo) veiksnių, siekiant išsiaiškinti sąsajas su pooperaciniais kalbos suvokimo rezultatais. Visi kintamieji, kurie patikimai skyrėsi gero ir silpno kalbos suvokimo grupėse, buvo įtraukti į vienaveiksnės regresijos analizę. Regresinė analizė atskleidė, kad vaikų

po KI prastesni *kalbos suvokimo* rezultatai yra susiję su gyvenamąja vieta kaime, vyresniu amžiumi nustatant diagnozę, vyresniu amžiumi operacijos metu, didesniais klausos slenksčiais po KI, siauresniu kauliniu sraigės nervo kanalu, KI naudojimo problemomis, žemesniu tėvų išsilavinimu, prastesniu tėvų supratimu apie KI procesą, retu šeimos lankymusi KI centre, mažesniu tėvų dalyvavimu lavinant vaiką, specialiojo lavinimo darželio lankymu ir nepakankamu surdopedagoginės pagalbos prieinamumu ir intensyvumu. Daugiaveiksnės žingsninės regresijos metodu nustatyta, kad amžius operacijos metu, pooperaciniai klausos slenksčiai naudojant KI ir kaulinio sraigės nervo kanalo spindis yra nepriklausomi kalbos suvokimo prognostiniai veiksniai po KI operacijos.

Siekiant išsiaiškinti, kokie veiksniai lemia geresnius arba blogesnius kalbos raidos rezultatus, buvo suformuotos dvi grupės: geros ir nepakankamos kalbos raidos. Visi demografiniai, operacijos ir implanto, audiologiniai, radiologiniai, šeimos bei lavinimo ir ugdymo kintamieji, kurie patikimai skyrėsi geros ir nepakankamos kalbos raidos grupėse, įtraukti į vienaveiksnės regresijos analizę. Regresinė analizė parodė, kad vaikų po kochlearinės implantacijos nepakankami *kalbos raidos* rezultatai yra susiję su gyvenamąja vieta kaime ar mažame miestelyje, vyresniu amžiumi nustatant diagnozę, didesniais klausos slenksčiais po KI, žemesniu tėvų išsilavinimu, nepakankamu tėvų supratimu apie KI procesą, retesniu šeimos lankymusi KI centre, mažesniu tėvų dalyvavimu lavinant vaiką, specialiosios ikimokyklinės ugdymo įstaigos lankymu ir nepakankamu surdopedagoginės pagalbos prieinamumu. Daugiaveiksnės žingsninės regresijos metodu nustatyta, kad kalbos raidos rezultatai priklauso nuo tėvų įsitraukimo į mokymo procesą ir surdopedagoginės pagalbos prieinamumo.

Išvados

1. Nustatytas unikalus Lietuvos vaikų, naudojančių KI, etiologinis profilis. Dažniausias etiologinis veiksnys – paveldimas nesindrominis klausos sutrikimas – nustatytas 51,6 proc., perinataliniai veiksniai – 13,1 proc., prenataliniai veiksniai, t. y. įgimta CMV infekcija, – 9,8 proc., sindromai –8,2 proc., postnataliniai veiksniai – 3,3 proc. vaikų. Kurtumo priežastis liko neaiški 13,9 proc. vaikų.
2. Kurčiųjų vaikų, naudojančių kochlearinius implantus, vidinės ausies anomalijų dažnis, nustatytas analizuojant smilkinkaulių KT vaizdus, siekia 33 proc.
3. Praėjus vidutiniškai 5,9 metų po kochlearinės implantacijos, kurčiųjų vaikų kalbos suvokimo vidurkis buvo 69,6 proc. Puikų ir gerą kalbos suvokimo lygį pasiekė atitinkamai 19,8 proc. ir 35,8 proc. vaikų, vidutinį – 19,8 proc., silpną ir labai silpną – atitinkamai 6,2 proc. ir 13,6 proc. vaikų, naudojančių KI. Po kochlearinės implantacijos 4,9 proc. vaikų atvirojo tipo kalbos suvokimas be vizualinių užuominų buvo lygus nuliui.
4. Iš kurčiųjų vaikų, kuriems vidutiniškai prieš 5,9 metų atlikta kochlearinė implantacija, labai gerą kalbos raidos lygį pasiekė 24,7 proc., gerą kalbos raidos lygį – 27,2 proc., o patenkinamą ir nepatenkinamą – atitinkamai 25,9 proc. ir 22,2 proc. tiriamųjų.
5. Vienaveiksnė regresinė analizė parodė, kad KI naudojančių vaikų ir kalbos suvokimo, ir kalbos raidos rezultatai daugiausia yra susiję su vaiko šeimos bei ugdymo

ir lavinimo veiksniais. Daugiaveiksnės žingsninės regresijos metodu nustatyta, kad amžius operacijos metu, pooperaciniai klausos slenksčiai naudojant KI ir kaulinio sraigės nervo kanalo spindis yra nepriklausomi kalbos suvokimo prognostiniai veiksniai po KI operacijos, o kalbos raidos rezultatai priklauso nuo tėvų dalyvavimo lavinant vaiką ir surdopedagoginės pagalbos prieinamumo.

Rekomendacijos

Remdamiesi atlikto darbo rezultatais ir kitų autorių duomenimis, suformulavome šias praktines rekomendacijas Lietuvos kochlearinės implantacijos sistemai kurti:

1. Ruošiant vaiką kochlearinei implantacijai, priešoperaciniame etape rekomenduojame vertinti etiologinius, medicininius, anatominius, audiologinius, su operacija ir implantu, su šeima bei lavinimu ir ugdymu susijusius veiksnius, galinčius turėti įtakos implantacijos rezultatams.
2. Prieš operaciją reikėtų informuoti tėvus apie šeimos vaidmenį šiame procese ir pooperacinių rezultatų priklausomybę nuo šeimos veiksnių.
3. Vertinant kurtumo priežastis, etiologinę diagnostiką rekomenduojame pradėti nuo genetiko konsultacijos, o įgimtos citomegalo viruso (CMV) infekcijos retrospektyviajai diagnostikai atlikti rekomenduojame sauso kraujo lašo CMV DNR tyrimą.
4. Vertinant vidinės ausies anatomiją, į priešoperacinį smilkinkaulių kompiuterinės tomografijos vaizdų vertinimo protokolą rekomenduojame įtraukti detalų vidinės ausies struktūrų – sraigės aukščio ir kaulinio sraigės nervo kanalo spindžio matavimą.
5. Vertinant kochlearinės implantacijos rezultatus, reikėtų užtikrinti reguliarų pooperacinį lankymąsi KI centre. Identifikavus nepakankamus rezultatus, siūlome įvertinti veiksnius, kurie gali turėti tam įtakos, informuoti šeimą, kartu su KI komandos specialistais spręsti dėl intervencijos būdo pakeitimo.
6. Tikslinga sukurti ir įdiegti tarpdisciplininę kochlearinės implantacijos sistemą, kuri koordinuotų skirtingų institucijų bendradarbiavimą ir užtikrintų kochlearinius implantus naudojančių vaikų ilgalaikę stebėseną.

Publications and presentations

Publications

Violeta Mikstiene, Jurgita Songailiene, Jekaterina Byckova, Giedre Rutkauskiene, Edita Jasinskiene, Rasa Verkauskiene, Eugenijus Lesinskas, Algirdas Utkus. Thiamine Responsive Megaloblastic Anemia Syndrome: A Novel Homozygous SLC19A2 Gene Mutation Identified. *American Journal of Medical Genetics*, 2015 Jul; 167(7):1605-9.

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suggests its possible origin in ancestors of Lithuanian population. *BMC Genetics*, 2016 Feb 19; 17:45.

Jekaterina Byčkova, Justė Simonavičienė, Vaiva Mickevičienė, Eugenijus Lesinskas. Evaluation of quality of life after paediatric cochlear implantation. *Acta Medica Lituanica*, 2018. Vol. 25. No. 3. P. 125–136

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Mikstiene V, Byckova J, Jakaitiene A, Lesinskas E, Utkus A, "The contribution of GJB2 gene mutations to development of early onset hearing loss in affected group of patients in Lithuanian population." Lithuanian–Polish ENT Congress, 2013, Druskininkai, Lithuania.

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Mikstiene V, Songailiene J, Byckova J, Rutkauskiene G, Jasinskiene E, Verkauskiene R, Lesinskas E, Utkus A, "Rogers's syndrome (thiamine responsive megaloblastic anemia syndrome): the success of multidisciplinary approach)." The 6th Baltic Otorhinolaryngology Congress, 2014, Kaunas, Lithuania.

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Aistė Kadziauskienė

MORPHOLOGIC CHANGES OF LAMINA CRIBROSA IN GLAUCOMATOUS EYES AFTER TRABECULECTOMY

AKYTOSIOS PLOKŠTELĖS MORFOLOGINIAI POKYČIAI PO TRABEKULEKTOMIJOS GLAUKOMA SERGANČIŲJŲ AKYSE

Medical and Health Sciences, Medicine M 001
Medicinos ir sveikatos mokslai, Medicina M 001

Abbreviations

AL - Axial length
BCVA - Best corrected visual acuity
BM - Bruch's membrane
BMO - Bruch's membrane opening
CCT - Central corneal thickness
CI - Confidence interval
GSI - Global shape index
IOP - Intraocular pressure
LC - Lamina cribrosa
LCD - Lamina cribrosa depth
MD - Mean deviation
N-T - Nasal-temporal
OCT - Optical coherence tomography
ONH - Optic nerve head
PACG - Primary angle-closure glaucoma
PEXG - Pseudoexfoliative glaucoma
POAG - Primary open-angle glaucoma
PSD - Pattern standard deviation
RNFL - Retinal nerve fiber layer
S-I - Superior-inferior
VF - Visual field
VIF - Variance inflation factor

Introduction

Glaucoma is a heterogeneous group of disorders characterized by a cupping of the optic nerve head (ONH) and corresponding visual field defects.[1] It is the leading cause of irreversible blindness worldwide.[2,3] The global prevalence of glaucoma for people aged 40–80 years is 3.54% (95%CI, 2.09–5.82) and it constantly increases due to the growing and aging population.[4,5] In 2040, the prevalence of glaucoma worldwide for this age is expected to be 111.8 million.[4]

To date, elevated intraocular pressure (IOP) is proven to be the principal manageable risk factor for the development and progression of glaucoma.[6-10] However, the exact mechanism of IOP contributing to the glaucomatous injury remains incompletely understood. The lamina cribrosa (LC) is implicated to be the principal site of this damage.[11-16] IOP is thought to affect the structures of the optic nerve head (ONH) directly as the determinant of the translaminar pressure gradient and indirectly via the induced forces of the sclera.[17-19] Therefore, the connective tissues of the ONH, including the LC, scleral canal, and peripapillary sclera, constantly bear the biomechanical load, which in certain circumstances results in stress and strain to the tissue.[20] The biomechanical paradigm of glaucoma postulates that elevated IOP causes LC compression, stretch, and shear, which lead to lamina deformations, strains on glial cells, and subsequent damage of the retinal ganglion cell axons.[21,22] LC morphology is related to the onset and progression of glaucoma.[23-26] Thus, all morphologic parameters describing the biomechanics of LC in relation to IOP are of great importance to provide insights into the fundamental mechanisms of glaucoma pathogenesis and treatment.

For many years, the assessment of the IOP effect on LC was limited to *ex vivo* histomorphometrical studies, experimental animal studies, or theoretical models.[11,12,27-35] Advanced imaging technologies, such as enhanced depth imaging spectral domain optical coherence tomography (OCT), swept source OCT, and adaptive optics, allowed in vivo visualization and quantitative evaluation of the deep ONH structures.[36-41] Since then, the structural and biomechanical characteristics of LC in glaucomatous eyes have been widely investigated. A posterior displacement, thinning, and local defects of the LC have been suggested to occur more often in glaucoma-affected eyes and to be related to the progression of the disease.[24,26,42-47] A number of studies have reported the anterior or posterior displacement of LC after the IOP change in glaucoma, which depends upon the geometrical and material properties of the ONH connective tissues.[19,48-56]

So far, the main morphologic parameter in the analysis of the LC and IOP relationship has remained the depth of the LC, which has limitations because of its reference to the plane of Bruch's membrane opening (BMO) and subsequent reliance on the shifting thickness of the choroid.[57-60] In this context, the LC parameters describing the LC curvature and shape could play an important role as potential morphologic biomarkers independent of the reference plane. However, data about the IOP effect on the curvature and shape of the LC are limited. Only one study investigated the LC posterior bowing after glaucoma surgery and suggested that an LC curvature index might have value as a parameter relevant to ONH biomechanics.[61] The examined curvature index did not correspond to the actual LC curvature, and the authors referred to the change of the LC configuration as a change in curvature. At present, no research has addressed the global shape of the LC after IOP reduction, and this relationship remains to be determined.

The aim of this prospective observational study was to analyze the long-term changes of the LC shape, curvature, and depth after trabeculectomy. A recently introduced morphologic measure, the global shape index (GSI), was chosen to characterize the geometrical shape of the anterior LC surface as a whole independently from the BMO plane.[62] Actual LC curvatures were assessed along vertical and horizontal meridians. To our knowledge, this is the first study to assess the response of the overall shape of the LC and its actual curvature to the reduction of IOP in glaucomatous eyes.

Purpose and objectives of the research

Purpose of the Research

To evaluate the morphologic changes of the LC in glaucoma-affected eyes after the IOP reduction following trabeculectomy as well as to assess their relationship to biometric and clinical ocular parameters.

Objectives of the Research

1. To evaluate the long-term changes of the mean and sectoral LC depth, horizontal and vertical curvatures, and global shape after trabeculectomy in glaucomatous eyes.
2. To analyze the relationships of the postoperative LC changes with the possibly associated factors, including IOP, age, biometric ocular parameters, and baseline LC morphology.
3. To assess the associations of the morphologic LC changes after trabeculectomy with the clinical parameters of glaucoma progression.
4. To compare the post-trabeculectomy morphologic LC changes with respect to the type and severity of glaucoma.

Scope and methodology of the research

Subjects and Study Design

A prospective observational study was performed from 2014 to 2017 at Vilnius University Hospital Santaros Klinikos. The approval of the Regional Research Ethics Committee was obtained, and all investigations followed the tenets of the Declaration of Helsinki. Consecutive patients who met the eligibility criteria and signed a written informed consent were enrolled in the study.

The inclusion criteria were as follows: (1) diagnosis of open-angle or primary angle-closure glaucoma; (2) indicated trabeculectomy; (3) best-corrected visual acuity of 0.1 or greater in Snellen decimal scale; (4) refractive error from -6.0 D to +6.0 D of sphere and ±3.0 D of cylinder. Exclusion criteria were being under 18 years of age, any prior intraocular surgery (except phacoemulsification with intraocular lens implantation), other ophthalmological or neurologic diseases affecting the visual field (VF), and poor image quality of the LC.

Initially, 130 glaucomatous eyes of 124 patients planned for trabeculectomy were enrolled; of these, 12 patients (12 eyes) were excluded because of poor LC visibility (6 eyes), failure to attend more than 2 follow-up visits (3 eyes), failed trabeculectomies (2 eyes), and postoperative complications (1 eye). Finally, we analyzed the data of 112 patients (118 eyes) according to the study protocol (Fig. 1). Ten of these patients missed 1 follow-up visit, and 1 patient missed 2 follow-up visits.

Patients were grouped with respect to the severity of glaucomatous injury into an early-to-moderate glaucoma group (early stage and moderate stage glaucoma) and an advanced glaucoma group (advanced stage glaucoma) as well as with respect to the glaucoma diagnosis – a primary open-angle glaucoma (POAG) group, a primary angle-closure glaucoma (PACG) group, and a pseudoexfoliative glaucoma (PEXG) group.

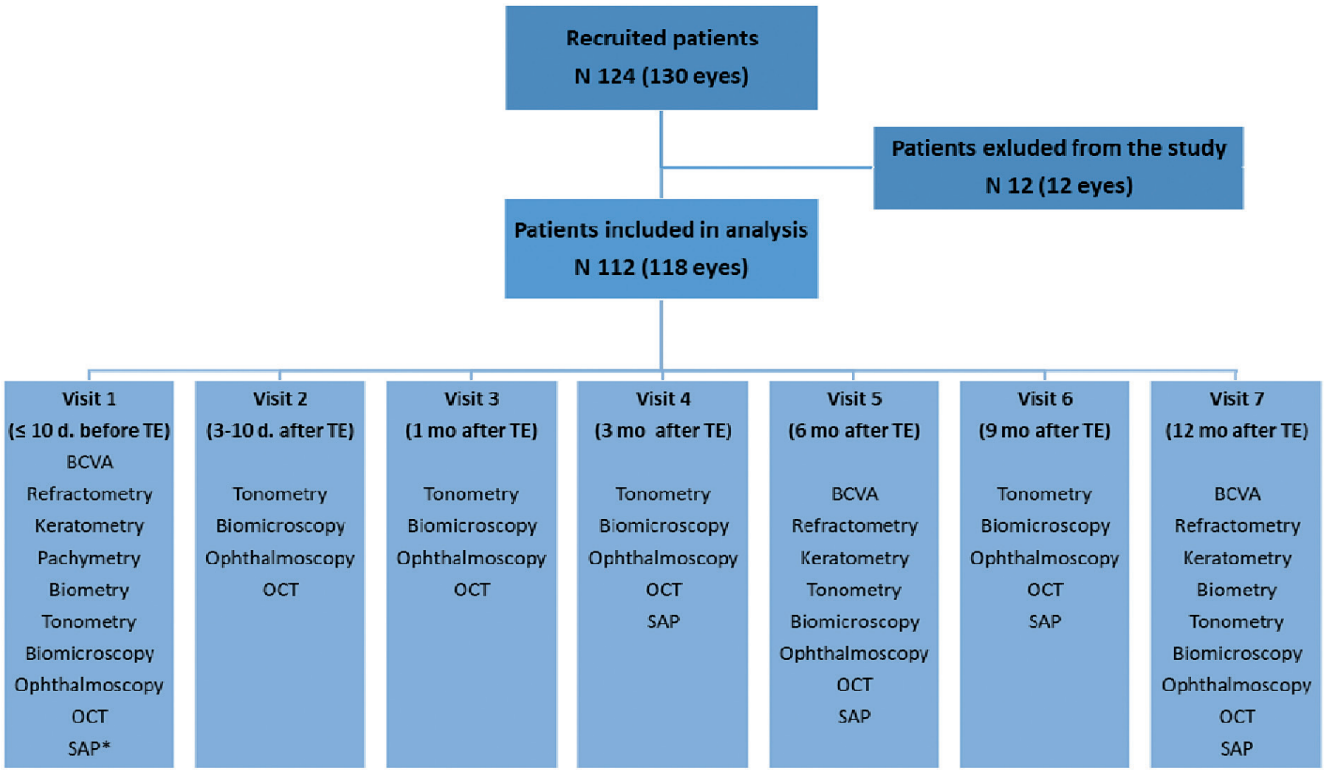

FIG. 1. Study design

BCVA = best corrected visual acuity, OCT = optical coherence tomography, SAP = standard automated perimetry, TE = trabeculectomy.
* SAP within 1 month before TE.

Examinations and Measurements

Each participant underwent the baseline examination, including an assessment of best-corrected visual acuity using the Snellen chart, an autorefractometry (Topcon KR-1 auto kerato-refractometer Topcon Medical Systems, Tokyo, Japan), an applanation tonometry (Goldmann tonometer, Haag-Streit AG, Koeniz, Switzerland), slit lamp biomicroscopy, a dilated stereoscopic examination of the fundus, a partial optical coherence interferometry (IOL Master, Carl Zeiss Meditec, Dublin, USA), and spectral-domain OCT (Heidelberg Spectralis, Heidelberg Engineering, Dossenheim, Germany). Two baseline VF tests of achromatic automated perimetry using the 30-2 Swedish Interactive Threshold Algorithm Standard strategy (Humphrey visual field analyzer, Carl Zeiss Meditec, Dublin, USA) were performed within 1 month before the trabeculectomy.

Glaucoma diagnosis was based on the presence of glaucomatous optic neuropathy (neuroretinal rim thinning, notching, or retinal nerve fiber layer [RNFL] defects) with or without associated glaucomatous VF defects.[63] The stages of glaucoma were defined on the basis of the Hodapp-Parrish-Anderson criteria of standard automated perimetry.[64]

All patients underwent a limbal-based trabeculectomy, with or without adjunctive 5-fluorouracile, following the same surgical protocol by 1 of 4 surgeons. Subsequently, needling with 5-fluorouracile was performed if a failure of the filtrating bleb occurred. Only patients with reduced postsurgical IOP continued the study. In all patients a target pressure was defined based on their baseline IOP, their risk factors, and the progression of glaucoma prior to inclusion. Medical treatment was administered during the postoperative period if the target's IOP was exceeded (19.49% of all studied eyes).

We evaluated the morphologic parameters of the LC and IOP within 10 days preoperatively and postoperatively, and at 1, 3, 6, 9, and 12 months postoperatively (Fig. 1). The mean of 2 IOP measurements was calculated. If 2 measurements differed by more than 2 mmHg, we took a third reading and averaged the 2 closest values. VF tests were repeated at three-month intervals postoperatively. The VF test was considered reliable if false-positive and false-negative errors were less than 33% and fixation losses were less than 20%. Nine patients lacked VF data at the 12-month visit, eighteen patients – at 3-month and 6-month visits, and twenty patients – at the 3-month visit.

Spectral Domain Optical Coherence Tomography

We performed enhanced depth imaging spectral domain OCT to visualize the LC.[36,39] The ONH was scanned using the Spectralis OCT system by centering

a 15° × 10° rectangle scan on the ONH. Each OCT volume consisted of 49 serial horizontal B scans (4.5-mm long lines, 40 images averaged) spaced at approximately 63-μm intervals.

The RNFL thickness was measured automatically from the circumferential OCT scan of 3.4 mm diameter centered on the ONH (single circle B scan of 12°, 100 images averaged) using Spectralis software (Eye Explorer, Heidelberg Engineering, Germany). The same type of the scan was repeated using the enhanced depth imaging for the visualization of the peripapillary choroid.

We imaged the retina performing a single horizontal linear scan (100 images averaged) and posterior pole scan (61 raster lines spaced 120 μm apart, 20 images averaged), both centered on the fovea. The subfoveal macular thickness was measured automatically from the single linear scan. The macular map analysis protocol was selected to display numeric averages of the measurements for the central macular subfield (the innermost 1-mm-diameter circle, defined by the Early Treatment Diabetic Retinopathy Study).

At least 2 OCT scans of each type were taken, and the one with the best quality was chosen. Images with a quality score of 15 or less were excluded. The baseline OCT scan was set as a reference, and all subsequent scans done were adherent to it (Spectralis Active Eye Tracking system). Any potential magnification errors were avoided by entering the corneal curvature and refraction of the eye before the OCT scanning.

Image Delineation, Three-Dimensional Reconstruction, and the Morphologic Measurements of Lamina Cribrosa

We enhanced raw OCT images using adaptive compensation (Reflectivity software, version 3.4, Ophthalmic Engineering & Innovation Laboratory, National University of Singapore, Singapore). Such post-processing has been shown to remove shadows, enhance tissue contrast, and improve the visibility of the LC.[65-67] Afterward, 2 ophthalmologists delineated and reconstructed the ONH volumes using semi-automated software Morphology 1.0 (version 1.0, Ophthalmic Engineering & Innovation Laboratory, National University of Singapore, Singapore). The anterior border of the LC was defined and manually marked on each B-scan as the upper margin of hyper-reflective tissue below the ONH tissues, extending laterally up to the LC insertions to the sclera (Fig. 2). The regions with an undistinguishable LC border were not delineated. The Morphology 1.0 software determined the border of the LC area as the contour of all delineated points. On average, there were 259 (median 258, range 148–433) marked points per ONH scan included in the analysis. We also defined and delineated the BMO as the end points of hyper-reflective Bruch's membrane layer on either side of the ONH in each B-scan.

Subsequently, the Morphology 1.0 software 3D reconstructed the LC anterior surface and BMO plane and automatically calculated the BMA area and LC morphologic parameters according to the earlier established protocols as follows.[62,68] The average LC visibility (LC coverage of BMO area evaluated by the software and expressed in %) of 130 enrolled eyes was 89.8% (median 94.2, range 29.8–97.8). Finally, 118 ONH with LC visibility of greater than 70% (mean 92.4, median 95.1, range 71.3-97.8) were analyzed.

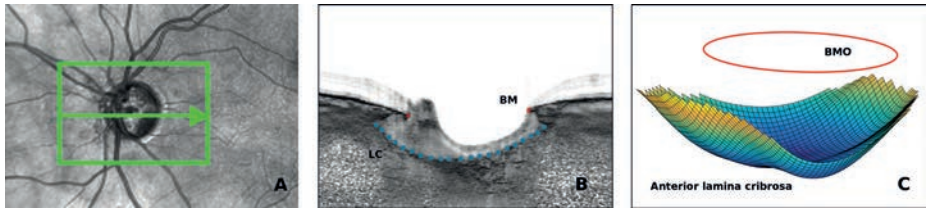

FIG. 2. The imaging, delineation, and reconstruction of the lamina cribrosa (LC).

We scanned the optic nerve head (ONH) by serial horizontal B scans spaced at approximately 63-mm intervals using enhanced depth imaging OCT. (**A**) Infrared image of the ONH demonstrates the place of the horizontal section where the B scan was located. (**B**) A cross-sectional view of the ONH after the adaptive compensation (Reflectivity software) and delineation of the anterior LC and the end points of Bruch's membrane (BM) using Morphology 1.0 software. (**C**) A three-dimensional view of the reconstructed LC and plane of the Bruch's membrane opening (BMO).

Lamina Cribrosa Depth (LCD). The software measured the LCD as the distance between the BMO plane and the anterior surface of the LC, which was reconstructed from the delineated LC points.[68] The mean LCD was calculated as the mean depth of all points on the LC surface. The lamina was automatically divided with respect to the center of the BMO ellipse into six sectors, comprising four 45° sectors (superotemporal, superonasal, inferotemporal, and inferonasal) and two 90° sectors (temporal and nasal). The sectoral LCD were assessed.

Lamina Cribrosa Curvature. The LC curvature was measured automatically in two principal meridians: superior-inferior (S-I) and nasal-temporal (N-T). Morphology 1.0 first intersects the anterior LC surface with 180 radial cross-sections that pass through the center of the BMO ellipse perpendicularly to it. An LC curve for each cross-section is generated.[62] Then, a circular arc is fitted to the LC curves along S-I and N-T directions. This approach enables estimation of LC curvatures along different radial orientations in a global sense without compromising robustness.[62] We expressed the values in mm,[1] with negative values describing a posteriorly curved LC and positive values indicating an anteriorly curved LC.

Lamina Cribrosa Global Shape Index. The LC GSI assessment was based on global curvature measurements along radial LC directions and defined as:

$$\text{LC GSI} = (2/\pi)\tan^{-1}((\kappa_1 + \kappa_2)/(\kappa_1 - \kappa_2)); \quad (\kappa_1 \geq \kappa_2).$$

where κ_1 and κ_2 are maximum and minimum principal arc curvatures of LC, respectively.[62] The value of the LC GSI varies between -1 and 1 and corresponds to a transition from a spherical cup (posteriorly curved LC; GSI = -1) through a symmetric saddle-shaped LC (GSI = 0) to a spherical cap (anteriorly curved LC; GSI = 1). Fig. 3 demonstrates the scale of LC GSI with the corresponding LC shapes. This index has previously been validated as an LC global shape parameter by Thakku et al. [62]

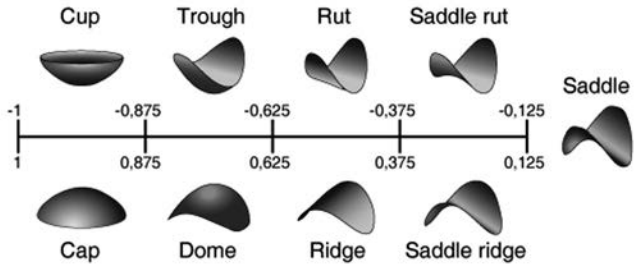

FIG. 3. A global shape index scale of the lamina cribrosa representing 9 intervals with corresponding shapes. Modified from Thakku et al.[62]

Measurements of the Peripapillary Choroid

The peripapillary choroidal thickness was measured manually from the circumferential enhanced depth imaging OCT scan 1.7 mm superiorly, temporally, inferiorly, and nasally to the center of the ONH with a caliper of Spectralis software. The choroidal thickness was defined as the distance from the hyper-reflective outer border of the retinal pigment epithelium to the hypo-reflective subchoroidal space or choroidal-scleral interface. The mean peripapillary choroidal thickness from the measurements at four listed locations was calculated.

Statistical Analysis

We analyzed the data using R statistical software. The study sample was calculated using the G*Power statistical package.[69] Continuous

variables were described by mean and standard deviation (SD). We used the Friedman rank sum test and the Nemenyi post-hoc test to compare the preoperative and postoperative measurements. The quantitative parameters of independent samples were compared using the Kruskal-Wallis test and the Two-sample Wilcoxon test, while the qualitative parameters – using the Chi-Square test. The significance test for the Pearson correlation coefficient was used to evaluate the correlation in between the changes of morphologic LC parameters at each time point. We per-

formed a linear mixed model analysis for longitudinal data to evaluate the associations between the changes of LC morphology with possible explanatory variables. In addition to age and sex, variables with P < 0.1 from the univariate models were included in the multivariate model. We used generalized estimating equation models to evaluate the relationship between the clinical parameters of glaucoma progression with the postoperative LC changes. A P value of < 0.05 was considered statistically significant.

The reproducibility of the LC measurements was determined on the basis of the LC GSI and LCD evaluation in 20 ONH scans and showed good intergrader (intraclass correlation coefficient (ICC) 0.948, 95% confidence interval (CI): 0.875-0.979; ICC 0.991, 95%CI: 0.977-0.996, respectively) and intragrader (ICC 0.986, 95%CI: 0.966-0.994; ICC 0.999, 95%CI: 0.998-1.0, respectively) agreements.

Results

In total, we analyzed the data of 118 eyes (112 patients). The demographic and clinical baseline characteristics of the patients are presented in Table 1. The mean (± SD) age of the patients was 67.6 ± 8.8 years and 52% were female. More than half of the patients had an advanced stage of glaucoma – 79 (66.95%), with PEXG being the most common diagnosis in the study sample – 89 (75.42%).

TABLE 1. Patient demographic and clinical characteristics.

Baseline characteristic	Value
Age, years	67.6 ± 8.8
Male/female	54/58
BCVA*	0.67 ± 0.25
Refractive error, D	-0.4 ± 2.0
Central corneal thickness, µm	520 ± 33
Axial eye length, mm	23.63 ± 0.92
Visual field MD, dB	-14.69 ± 8.91
Global RNFL thickness, µm	53.73 ± 14.01
Glaucoma type, % (eyes)	
Pseudoexfoliative	75.42 (89)
Primary open-angle	17.8 (21)
Primary angle-closure	6.78 (8)
Glaucoma severity, % (eyes)	
Mild-to-moderate	33.05 (39)
Mild	16.95 (20)
Moderate	16.1 (19)
Advanced	66.95 (79)

BCVA = best corrected visual acuity; D = diopters; MD = mean deviation; RNFL = retinal nerve fiber layer. Values expressed as mean ± SD unless otherwise indicated. * - decimal notation.

The mean IOP was reduced at all follow-up visits (P < 0.001) (Fig. 4, Table 2). At the first follow-up, the IOP reduction was the greatest when compared with later visits (P < 0.001); it stabilized after the first postoperative month (P > 0.05 in between the subsequent visits).

TABLE 2. Intraocular pressure before and after glaucoma surgery.

	Baseline	Postoperative					
		3-10 days	1 month	3 months	6 months	9 months	12 months
IOP, mmHg	27.6 ± 6.7	9.2 ±3.9	12.8 ±4.2	12.6 ±3.4	12.6 ±3.2	13.2 ±3.4	13.5 ±3.7
IOP reduction,* mmHg	-	18.3 ±7.7	15.0 ±7.4	15.1 ±7.3	15.1 ±7.5	14.5 ±7.3	14.3 ±7.7
IOP reduction,* %	-	65.0 ±16.9	51.9 ±17.3	52.2 ±16.1	52.0 ±16.4	49.8 ±17.5	48.5 ±18.8

IOP = intraocular pressure. The values are expressed as mean ± SD. * IOP reduction from baseline, P < 0.001 at all follow-up visits (pairwise comparisons of the Nemenyi multiple comparison test).

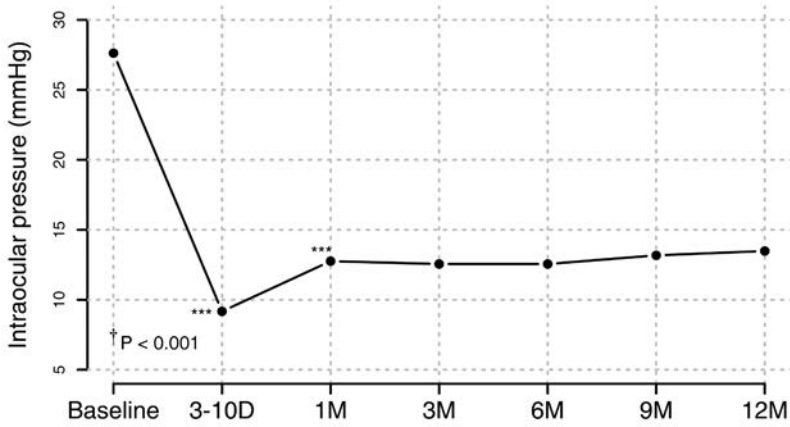

FIG. 4. A line graph showing the dynamics of intraocular pressure during the follow-up.

The mean IOP reduced at all follow-up visits compared to baseline (P < 0.001). † P value of the Friedman rank sum test. * P values for comparison with the previous follow-up visit obtained using the Nemenyi multiple comparison test, *** P ≤ 0.001.

Changes in Morphologic Parameters of Lamina Cribrosa

Lamina Cribrosa Depth

The mean and sectoral LCD decreased (shallowed) at all follow-up visits after the trabeculectomy ($P < 0.001$) (Fig. 5, Table 3). After the mean LCD decreased until the sixth month, there were no significant changes in between the later visits ($P > 0.05$). The trend of temporal sequence of LCD seemed to be consistent for all LCD parameters (Fig. 5).

Before the trabeculectomy, the superonasal sector of the LC was the deepest and the temporal sector was the shallowest when compared with the others ($P < 0.001$). During the early postoperative period, the LCD decreased more significantly in both superior sectors when compared with the inferior, nasal, and temporal ones ($P \leq 0.02$). After a year, the superonasal LCD showed the greatest decrease compared with other sectors ($P \leq 0.001$), except the superotemporal sector ($P = 0.21$); the LCD change was the least in the temporal sector when compared with others ($P \leq 0.006$).

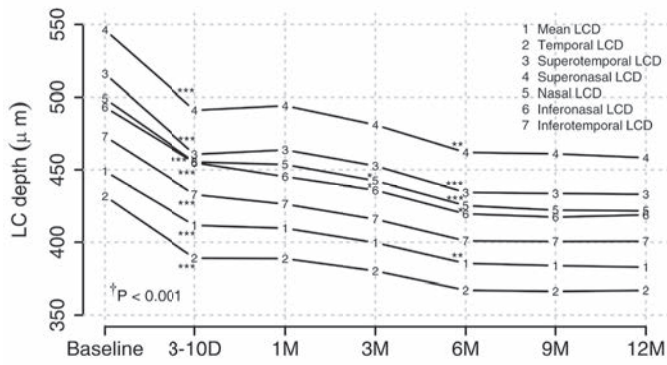

FIG. 5. A line graph demonstrating the time course of mean and sectoral lamina cribrosa (LC) depth after trabeculectomy.

All LC depth (LCD) parameters decreased from baseline at all postoperative follow-ups ($P < 0.001$). The mean and sectoral LCD, except the temporal and inferotemporal, were deeper at 1 week, 1 month, and 3 months when compared with 6, 9, and 12 months postoperatively ($P \leq 0.02$). There were no changes in between the visits after 6 months. † P values of the Friedman rank-sum test for all LCD parameters. * P values for comparison with the previous follow-up visit obtained using the Nemenyi multiple comparison test, * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

The postoperative LCD decreased from the baseline (shallowing of the LC) in the majority of the cases. However, 28 patients (35 visits) showed an increase in their LCD from the baseline (deepening of the LC) in at least one visit during the

TABLE 3. Shape, curvature, and depth parameters of the lamina cribrosa during the post-trabeculectomy year.

Parameter	Baseline	Postoperative						P* value
		3-10 days	1 month	3 months	6 months	9 months	12 months	
N-T curvature, mm^{-1}	-617 ±191	-547 ±196	-566 ±186	-547 ±167	-527 ±162	-531 ±167	-526 ±163	<0.001
S-I curvature, mm^{-1}	-334 ±655	-273 ±699	-238 ±512	-199 ±455	-191 ±471	-183 ±417	-156 ±419	0.006
Global shape index	-0.622 ±0.18	-0.583 ± 0.19	-0.613 ± 0.19	-0.611 ±0.19	-0.617 ±0.18	-0.617 ±0.18	-0.605 ±0.19	0.03
Mean LCD, µm	449 ±129	412 ±112	410 ±108	400 ±104	385 ±100	384 ±100	383 ±101	<0.001
Sectoral LCD, µm								
Temporal	432 ±146	389 ±123	389 ±119	380 ±113	367 ±108	366 ±109	367 ±109	<0.001
Superotemporal	516 ±171	461 ±171	464 ±138	453 ±132	434 ±126	434 ±128	433 ±127	<0.001
Superonasal	546 ±173	491 ±146	494 ±140	481 ±134	462 ±129	461 ±131	458 ±128	<0.001
Nasal	499 ±153	455 ±131	454 ±124	442 ±119	425 ±113	422 ±114	422 ±115	<0.001
Inferonasal	493 ±153	455 ±137	445 ±127	436 ±125	420 ±119	417 ±119	419 ±122	<0.001
Inferotemporal	473 ±153	433 ±134	426 ±127	416 ±123	401 ±117	400 ±117	401 ±119	<0.001

LCD = lamina cribrosa depth, N-T = nasal-temporal, S-I = superior-inferior. The values are expressed as mean ± standard deviation. The values are expressed as mean ± SD. * P values were obtained with the Friedman rank-sum test.

follow-up period. Most of those with an increase in the LCD were observed during the first postoperative month and reduced significantly over time (*P* for trend < 0.001) (Table 4). In the group with a decreased postoperative LCD, the patients tended to be younger (66.4 ± 8.1 y, *P* = 0.002) and have thinner baseline RNFL (51.9 ± 12.6 µm, *P* = 0.04) compared with those eyes with an increased postoperative LCD (71.2 ± 9.3 y, 59.7 ± 16.7 µm, respectively). There were no significant differences in terms of their gender, baseline IOP, IOP reduction, axial length, VF MD, or baseline LC parameters between the eyes with decreased and increased postoperative LCD (*P* > 0.05). IOP reduction was related to a greater LCD change in both groups. However, the effect was greater in the eyes in which the LC became shallower (β = 1.67; 95% CI, 1.1 – 2.24; P < 0.001) than in those eyes in which the LCD became deeper (β = 0.84; 95% CI, 0.31 – 1.36; P = 0.002) after the surgery.

In other words, eyes with shallower LCD compared with baseline responded to IOP reduction with greater movement anteriorly than eyes with deeper LCD (P = 0.002).

TABLE 4. The proportions of eyes according to the change of lamina cribrosa depth during follow-up.

Number of eyes N (%)	Postoperatively					
	3-10 days	1 month	3 months	6 months	9 months	12 months
LCD decrease	107 (92.2)	103 (89.6)	112 (94.9)	110 (95.7)	116 (99.1)	114 (98.3)
LCD increase*	9 (7.8)	12 (10.4)	6 (5.1)	5 (4.3)	1 (0.9)	2 (1.7)

LCD = lamina cribrosa depth. * The number of eyes with LCD increase reduced over time (P for trend < 0.001, Cochran-Armitage test for trend).

Lamina Cribrosa Curvature

There was a flattening of both N-T and S-I curvatures of the LC after the trabeculectomy (P < 0.001, P = 0.006, respectively) (Fig. 6, Table 3). The N-T curvature flattened from baseline at all follow-up visits (P < 0.001). It was more curved at the

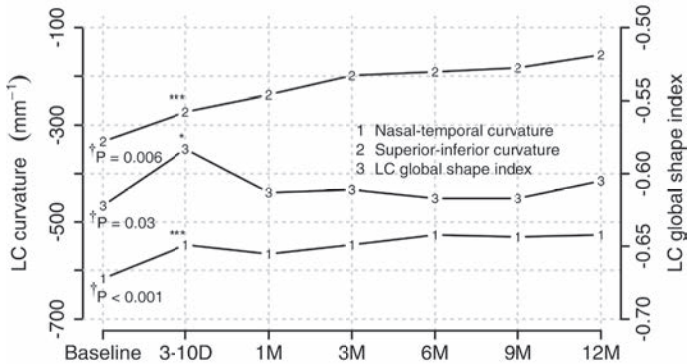

FIG. 6. A line graph showing the time course of the lamina cribrosa (LC) shape index and curvatures after trabeculectomy.

The N-T curvature flattened from the baseline at all follow-up visits (P < 0.001); a flattening of the S-I curvature was significant only at the first and last postoperative visits (P_1 = 0.001, P_2 = 0.003, respectively). The GSI increased from the baseline to the first follow-up (P = 0.02) and then decreased from the first follow-up to 3, 6, and 9 months (P_1 = 0.04, P_2 = 0.049, P_3 = 0.02, respectively). † P values of the Friedman rank-sum test. * P values for comparison with the previous follow-up obtained using the Nemenyi multiple comparison test, * P ≤ 0.05; ** P ≤ 0.01; *** P ≤ 0.001.

first month when compared with the curvature at 6, 9, and 12 months after surgery ($P \leq 0.02$) and at the 3rd month when compared to 12 months ($P = 0.03$). There were no changes in between the visits after the 6th month ($P > 0.05$). The flattening of S-I curvature was significant at 1 week and 12 months postoperatively ($P = 0.001$, $P = 0.003$, respectively); no changes were found in between other follow-ups.

Lamina Cribrosa Shape

The mean GSI changed from the baseline only in the early postoperative period ($P = 0.02$) when the IOP reduction was the greatest (Figure 6, Table 3). Its positive shift represented a change toward the saddle-shaped LC (less concave N-T meridian and closer to flat or anteriorly curved S-I axis). There were no statistically significant differences in the frequencies of LC GSI intervals between the visits ($P > 0.05$).

Changes in Choroidal and Retinal Thickness

The peripapillary choroidal thickness and retinal thickness at the macular central subfield decreased at all follow-up visits after the trabeculectomy ($P_1 < 0.001$, $P_2 \leq 0.007$, respectively), meanwhile the foveal retinal thickness did not change significantly (Table 5). A significant thinning of the mean RNFL compared to the baseline was observed from the 3rd to 12th months of the follow-up ($P < 0.001$) (Table 5).

TABLE 5. The dynamics of choroidal and retinal thickness during the follow-ups.

		Thickness of the parameter (μm)					
	Baseline	Postoperative					
Parameter		3–10 days	1 month	3 months	6 months	9 months	12 months
RNFL	53.73 ± 14.01	54.27 ±14.28	52.44 ±14.05	51.40 ±13.57†††	50.96 ±13.59†††	50.11 ± 13.28†††	49.68 ±12.86†††
Macular central subfield	266 ±18	270 ±18†††	267 ±18††	268 ±18†††	269 ±19†††	267 ±18†††	267 ±19††
Fovea	226 ±16	228 ±19	225 ±16	226 ±16	226 ±17	225 ±16	225 ±17
Peripapillary choroid	120 ±51	153 ±61†††	138 ±57†††	135 ±55†††	131 ±56†††	131 ±56†††	130 ±56†††

RNFL = retinal nerve fiber layer. The values are expressed as mean ± SD. ††† $P < 0.001$; †† $P < 0.01$, P values for comparison with baseline (Nemenyi multiple comparison test).

Factors Associated with Lamina Cribrosa Changes

Lamina Cribrosa Depth

After trabeculectomy, a greater decrease of the mean LCD was associated with a younger age, greater IOP reduction, more anteriorly-posteriorly and nasally-temporally curved preoperative LC, and greater baseline GSI (Table 6). The association of the decrease of the mean LCD with a lower baseline IOP was nearly significant (β = -1.89 μm/mmHg, 95% CI, -3.84 to 0.05, P = 0.059). To avoid multicollinearity, the effect of the axial length shortening on the LCD changes was analyzed in a separate multivariate model from the IOP reduction. The axial length shortening remained associated with the LC shallowing β = 106.62 μm/mm; 95% CI, 37.52 – 175.72; P = 0.003) (VIF < 2).

The sectoral associations were also tested. The greater decrease in the inferotemporal, superotemporal, and superonasal LCD were related to the thinner corresponding regional baseline RNFL (inferotemporal: β = -0.43 μm /μm; 95% CI,

TABLE 6. A regression analysis of the factors associated with the postoperative changes of the mean lamina cribrosa depth.

Variable	Change of mean LC depth			
	Univariate		Multivariate	
	***β* (95% *CI*)**	***P***	***β* (95% *CI*)**	***P***
Age, year	-2.76 (-3.88 to -1.65)	**<0.001**	-2.27 (-3.05 to -1.50)	**<0.001**
Male sex	-6.17 (-26.32 to 13.98)	0.550	-9.81 (-22.1 to 2.48)	0.121
AL preoperative, mm	3.40 (-8.01 to 14.80)	0.561		
IOP preoperative, mmHg	2.11 (0.67 to -3.55)	**0.005**	-1.89 (-3.84 to 0.05)	0.059
IOP reduction,* mmHg	2.55 (1.33 to 3.77)	**<0.001**	2.78 (1.07 to 4.49)	**0.002**
CCT preoperative, μm	-0.15 (-0.45 to 0.16)	0.350		
VF MD preoperative, dB	-0.82 (-1.94 to 0.30)	0.156		
VF MD decrease,* dB	-1.62 (-6.38 to 3.14)	0.506		
RNFL preoperative, μm	-0.81 (-1.54 to -0.09)	**0.030**	-0.0003 (-0.48 to 0.48)	0.999
RNFL thinning,* μm	1.20 (-1.21 to 3.61)	0.330		
BMO preoperative, μm^2	-27.37 (-52.76 to -1.98)	**0.037**	6.10 (-11.23 to 23.43)	0.492
GSI preoperative, μm	-55.27 (-109.57 to -0.97)	**0.049**	52.29 (12.72 to 91.86)	0.011
N-T curvature preoperative, mm^{-1}	-0.15 (-0.20 to -0.11)	**<0.001**	-0.08 (-0.11 to -0.04)	**<0.001**
S-I curvature preoperative, mm^{-1}	-0.06 (-0.07 to -0.04)	**<0.001**	-0.05 (-0.06 to -0.03)	**<0.001**

AL = axial length; BMO = Bruch's membrane opening; CI = confidence interval; CCT = central corneal thickness; GSI = global shape index; IOP = intraocular pressure; LC = lamina cribrosa; MD = mean deviation; N-T = nasal-temporal; RNFL = retinal nerve fiber layer; S-I = superior-inferior; VF = visual field. * Change at the 12th month of follow-up.

-0.82 – 0.03; P = 0.038; superotemporal: β = -0.67 μm /μm; 95% CI, -1.32 – -0.01; P = 0.049; superonasal: β = -0.84 μm/μm; 95% CI, -1.65 – -0.03; P = 0.045). However, these relationships became insignificant after adjusting for age and gender.

There was no association of the LCD change neither with retinal nor choroidal thickness (at baseline or posto perative change) after adjusting for age and gender (P > 0.05).

Lamina Cribrosa Curvature

A multivariate regression analysis showed an association between the flattening of the N-T curvature with younger age, lower baseline IOP, greater IOP reduction, and RNFL thinning over a year (Table 7). The postoperative flattening of S-I curvature was associated with more negative baseline GSI, deeper baseline LC, and flatter baseline N-T curvature (Table 7). To avoid multicollinearity, the effect of axial length shortening on the changes of the N-T curvature was analyzed in a separate model from the IOP reduction. The axial length shortening stayed significantly associated with the LC flattening (β = -272.08 mm^{-1}/mm; 95% CI, -401.95 – -142.21; P < 0.001; VIF < 2).

Postoperatively, there was a positive relation of the N-T flattening with the greater thickening of the peripapillary choroid (β = 0.9 mm^{-1}/μm; 95% CI, 0.54 – 1.27; P < 0.001) and central macular subfield (β = 1.51 mm^{-1}/μm; 95% CI, 0.68 – 2.34; P = 0.003) after adjusting for age and gender. No association of the S-I flattening with the retinal and choroidal thickness (at the baseline or change) was found (P>0.05).

Lamina Cribrosa Shape

The postoperative GSI change toward a positive value (the direction of cap) was associated with the more superiorly-inferiorly curved preoperative LC, RNFL thinning over a year, and IOP reduction (after adjusting for age, gender, and baseline RNFL thickness) (Table 8).

Postoperatively, there was a positive relation of the GSI increase with a greater thickening of the peripapillary choroid (β = 0.001 /μm; 95% CI, 0.006 – 0.01; P < 0.001) and central macular subfield (β = 0.003 /μm; 95% CI, 0.003 – 0.001; P = 0.005) after the adjustment for age and gender. No association with the baseline choroidal or retinal parameters was found.

TABLE 7. A regression analysis of factors associated with the postoperative changes of the lamina cribrosa curvature.

	Change of N-T curvature				Change of S-I curvature			
Variable	Univariate		Multivariate		Univariate		Multivariate	
	***β* (95% *CI*)**	***P***	***β* (95% *CI*))**	***P***	***β* (95% *CI*)**	***P***	***β* (95% *CI*)**	***P***
Age, year	3.50 (1.91 to 5.09)	**<0.001**	3.23 (1.75 to 4.71)	**<0.001**	6.06 (-4.53 to 16.64)	0.265	-1.04 (-9.94 to 7.86)	0.819
Male sex	-3.51 (-31.69 to 24.67)	0.808	1.24 (-22.12 to 24.60)	0.917	1.29 (-173.08 to 175.66)	0.988	44.15 (-99.55 to 187.8)	0.548
AL pre-operative, mm	-3.47 (-19.41 to 12.47)	0.671			-43.82 (-142.17 to 54.52)	0.384		
IOP pre-operative, mmHg	-3.01 (-5.02 to -1.00)	**0.004**	4.26(0.49 to 8.03)	**0.029**	-13.99 (-26.63 to -1.35)	**0.032**	2.85 (-19.13 to 24.84)	0.800
IOP re-duction,* mmHg	-3.59 (-5.30 to -1.89)	**<0.001**	-5.92 (-9.14 to -2.67)	**<0.001**	-14.69 (-25.69 to -3.68)	**0.010**	-11.69 (-31.21 to 7.83)	0.243
CCT pre-operative, µm	0.14 (-0.29 to 0.56)	0.534			0.15 (-2.51 to 2.80)	0.915		
VF MD preopera-tive, dB	1.16 (-0.40 to 2.73)	0.148			3.94 (-5.80 to 13.68)	0.429		
VF MD decrease,* dB	5.49 (-1.27 to 12.25)	0.115			10.86 (-29.57 to 51.28)	0.600		
RNFL preopera-tive, µm	1.09 (0.07 to 2.10)	**0.038**	0.85 (-0.14 to 1.84)	0.905	3.67 (-2.68 to 10.03)	0.260		
RNFL thinning,* µm	-3.93 (-7.23 to -0.63)	**0.021**	-4.22 (-7.59 to -0.85)	**0.016**	6.86 (-13.99 to 27.71)	0.520		
BMO pre-operative, μm^2	36.49 (0.98 to 72.01)	**0.047**	22.21 (-8.82 to 53.23)	0.164	221.36 (1.51 to 441.21)	**0.051**	194.86 (-4.94 to 394.67)	0.059
LCD pre-operative, µm	-0.13 (-0.24 to -0.02)	**0.017**	-0.01 (-0.11 to 0.08)	0.774	-1.85 (-2.44 to -1.27)	**<0.001**	-2.25 (-3.10 to -1.40)	**<0.001**
GSI pre-operative	24.57 (-52.51 to 101.64)	0.533			814.46 (362.19 to 1266.72)	**<0.001**	489.19 (80.62 to 897.76)	**0.021**
N-T cur-vature preopera-tive, mm^{-1}	-	-			0.68 (0.24 to 1.12)	**0.003**	-0.77 (-1.36 to -0.17)	**0.013**

S-I curvature pre-operative, mm^{-1}	0.02 (-0.01 to 0.04)	0.135			-	-		
AL = axial length; BMO = Bruch's membrane opening; CCT = central corneal thickness; CI = confidence interval; GSI = global shape index; IOP = intraocular pressure LC = lamina cribrosa; LCD = lamina cribrosa depth; MD = mean deviation; N-T = nasal-temporal; RNFL = retinal nerve fiber layer; S-I = superior-inferior; VF = visual field. * Change at the 12th month of follow-up.								

Interrelations of Morphologic Lamina Cribrosa Changes

The magnitude of the changes in GSI correlated with flattening of the S-I curvature (ρ_1 = 0.517, ρ_2 = 0.724, P < 0.001) and shallowing of the mean LCD (ρ_1 = 0.363, ρ_2 = 0.231, P < 0.05) at the first and last visits, respectively. The correlation of change in GSI with the changes in the N-I curvature was significant only in the early postoperative period (ρ = 0.351, P < 0.001). There was a positive correlation between the flattening of both LC curvatures and the shallowing of LC during the entire follow-up period (ρ_1 = 0.647, ρ_2 = 0.515, P < 0.001, N-T and S-I curvatures after 12 months, respectively). The flattening of the LC at both principal meridians correlated between each other (ρ_1 = 0.258, P_1 = 0.005; ρ_2 = 0.232, P_2 = 0.012 the first and the last follow-ups, respectively).

Associations of Lamina Cribrosa Changes with Glaucoma Progression

A GEE model analysis revealed that a greater RNFL thinning during a year after trabeculectomy was related to a greater postoperative LC shallowing (β = 0.016 μm/μm, 95% CI, 0.01-0.02, P < 0.001), N-T flattening (β = 0.005 μm/mm-1, 95% CI, 0.001-0.009, P = 0.007) (Fig 7), and GSI change (β = 0.002 μm/e^{-3}, 95% CI, 0.0002-000.4, P = 0.048) (adjustment for age, gender, and IOP). The association of the RNFL thinning with the LC shallowing was stronger in the early-to-moderate glaucoma group (β = 0.031 μm/μm, 95% CI 0.02-0.04, P < 0.001) than in the advanced glaucoma group (β = 0.011 μm/μm, 95% CI, 0.004-0.02, P = 0.003) (P = 0.014).

The relationships between the postoperative changes of VF parameters and the changes of LC morphologic parameters were significant only in the subgroups. After trabeculectomy the worsening of the VF pattern standard deviation (PSD) was related to the LC shallowing in the early-to-moderate glaucoma group (β = 0.004 μm/dB, 95% CI, 0.0004 – 0.007, P = 0.028), while the worsening of the VF MD showed a mild association with the positive change of the GSI (β = 0.003 dB/e^{-3}, 95% CI 0.0002-0.005, P = 0.035).

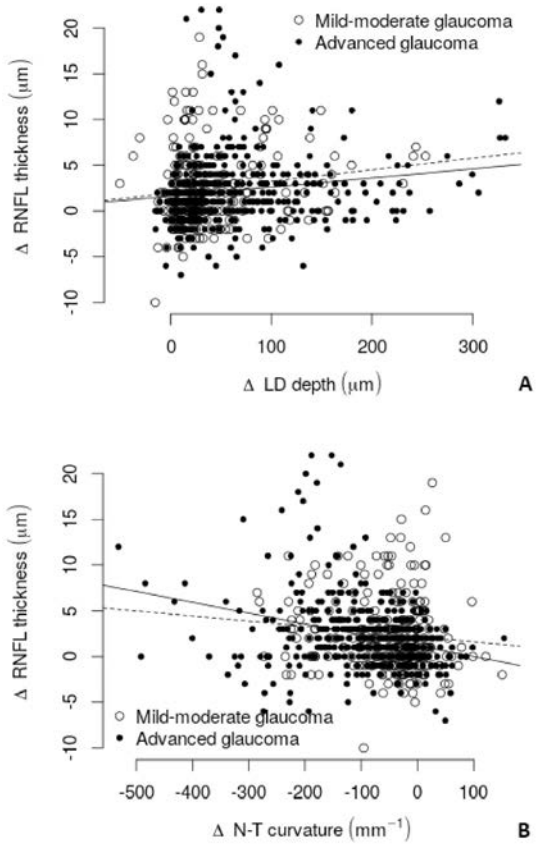

FIG. 7. A scatter plot showing the relationship between the thinning of the retinal nerve fiber layer (RNFL) with (A) a decrease in lamina cribrosa (LC) depth and (B) a flattening of nasal-temporal (N-T) curvature during a year after trabeculectomy.

TABLE 8. A regression analysis of factors associated with the postoperative changes of the lamina cribrosa global shape index.

	Change of LC global shape index			
Variable	Univariate		Multivariate	
	***β* (95% *CI*)**	***P***	***β* (95% *CI*)**	***P***
Age, 10 year	-0.0007 (-0.02 to 0.02)	0.952	-0.008 (-0.03 to 0.01)	0.491
Male sex	0.020 (-0.019 to 0.058)	0.324	0.028 (-0.006 to 0.062)	0.111
AL preoperative, mm	0.015 (-0.007 to 0.037)	0.175		
IOP preoperative, mmHg	-0.002 (-0.005 to 0.001)	0.186		
IOP reduction,* mmHg	-0.002 (-0.005 to 0.0001)	**0.061**	-0.003 (-0.006 to -0.001)	**0.007**

	Change of LC global shape index			
Variable	Univariate		Multivariate	
	***β* (95% *CI*)**	***P***	***β* (95% *CI*)**	***P***
CCT preoperative, µm	0.0001 (-0.0005 to 0.0007)	0.643		
VF MD preoperative, dB	0.001 (-0.001 to 0.003)	0.306		
VF MD decrease,* dB	0.003 (-0.007 to 0.012)	0.609		
RNFL thickness preoperative, µm	0.0007 (-0.0008 to 0.002)	0.368	-0.0006 (-0.002 to 0.001)	0.408
RNFL thinning,*µm	0.005 (0.0001 to 0.009)	**0.047**	0.007 (0.003 to 0.012)	**0.003**
BMO area preoperative, μm^2	0.026 (-0.023 to 0.076)	0.302		
LCD preoperative, 100 µm	-0.02 (-0.03 to -0.004)	**0.013**	0.003 (-0.02 to 0.02)	0.719
N-T curvature preoperative, 100 mm^{-1}	0.006 (-0.004 to 0.02)	0.249		
S-I curvature preoperative, 100 mm^{-1}	0.007 (0.004 to 0.01)	**<0.001**	0.006 (0.003 to 0.01)	**0.001**

AL = axial length; BMO = Bruch's membrane opening; CCT = central corneal thickness; CI = confidence interval; IOP = intraocular pressure; LC = lamina cribrosa; LCD = lamina cribrosa depth; MD = mean deviation; N-T = nasal-temporal; RNFL = retinal nerve fiber layer; S-I = superior-inferior; VF = visual field. * Change at the 12th month of follow-up. In addition to age, sex, and baseline RNFL thickness, variables with P < 0.1 from the univariate model were included in the multivariate model. * Change at the 12th month of follow-up.

Comparison of Lamina Cribrosa Changes with respect to the Severity and Type of Glaucoma

There were no statistical differences in the age, gender, visual acuity, refractive error, baseline IOP, and IOP reduction between the groups with respect to the glaucoma diagnosis and the severity of the disease (P > 0.05). In the group of advanced-stage glaucoma, the central cornea thickness (515 ± 34 µm) was less, the VF MD (19.72 ± 6.38 dB) was worse and the RNFL (47.03 ± 9.17 µm) was thinner when compared to the group of early-to-moderate glaucoma (529 ± 28 µm, 5.49 ± 4.86 dB and 66.63 ± 12.67 µm, respectively) (P < 0.001). The ocular axial length was shorter in the group of PACG (22.73 ± 0.52 mm) than in the group of POAG (23.56 ± 0.92) and PEXG (23.73 ± 0.91) (P = 0.012).

No differences in the LCD and GSI were found between the groups of early-to-moderate and advanced glaucoma before the trabeculectomy (P > 0.05). Meanwhile, the N-S curvature at baseline was greater in the eyes with the advanced disease (-650 ± 201 mm^{-1}) compared to those with early-to-moderate disease (-554 ± 154 mm^{-1}) (P = 0.015).

After the trabeculectomy there was a greater LC shallowing (3-10d, 1, 6, and 9 months postoperatively, P ≤ 0.045) and N-T flattening (6, 9 and 12 months post-

operatively, $P \leq 0.043$) in the advanced glaucoma group compared to the early-to-moderate glaucoma group (68.2 ± 66.7 μm vs 48.4 ± 52.4μm, $P = 0.027$; -99.9 ± 96.4 mm^{-1} v s-60.8 ± 80.7 mm^{-1}, $P = 0.043$; the change of LCD and N-T curvature after 6 months, respectively). The postoperative morphologic LC parameters did not differ significantly between the groups ($P > 0.05$).

There were no differences in the baseline LC parameters or in the postoperative changes of the LCD, curvature, and GSI between the groups of POAG, PACG, and PEXG ($P > 0.05$).

Discussion

In the present study, we evaluated the quantitative shape, curvature, and positional changes of the LC in glaucoma-affected eyes during the first year after trabeculectomy. A long-term flattening of the LC curvature, as well as shallowing of mean and sectoral LCD, were observed. However, the overall LC shape, characterized by GSI, changed significantly only in the early postoperative period. To the best of our knowledge, this is the first study to describe the IOP reduction-induced changes of the actual lamina curvature and its global shape.

Thus far, the clinically measurable quantitative LC parameters related to the ONH biomechanics have been limited. Most of the studies addressing the LC morphology evaluated the position rather than the shape of the lamina. The LC was reported to be deeper in glaucomatous eyes compared with healthy ones[24,25,70-72] and deeper LC were related to greater glaucomatous damage.[24-26] However, the predominant measurement of the LC morphology was LCD, which was defined with reference to the BMO plane and therefore carried potential bias. The reason is that the BMO position is influenced by the choroidal thickness, which has been shown to vary with age, diurnal phase, and systemic health as well as after IOP reduction.[57,58,60,73] Moreover, although the change in the LCD after the IOP reduction reflected the positional reversal of the lamina, it did not necessarily described the change in the LC curvature or shape, because the migration of the LC insertion might be possible due to active ONH remodeling.[22,74] Examining other LC parameters characterizing its morphology independently from shifting anatomic landmarks remained an important scientific goal.

Recently, increased attention has been given to the curvature of the LC, because it describes the LC topography and is not affected by the choroidal thickness. Kim and colleagues have reported that the LC posterior bowing, expressed as a LC curvature index, was increased in primary open-angle glaucoma when compared with healthy control subjects and in hypertension glaucoma when compared with normal tension glaucoma.[75,76] Later, the LC curvature index was shown to have a better dis-

criminating capability between POAG and healthy eyes than the LCD.[77] One study has addressed the effect of IOP reduction on the LC curvature.[61] Lee et al. evaluated eyes with POAG after trabeculectomy and found a decrease of the LC curvature index in the horizontal ONH sections after surgical IOP reduction.[61] The latter findings are in agreement with our study demonstrating the flattening of both principal LC curvatures after trabeculectomy; however, the results cannot be compared directly because in the previous study, the LC curvature index was defined as an inflection of the curve (μm) representing a section of the LC and did not correspond exactly to the actual LC curvature.[61] Furthermore, the measurements were limited to seven horizontal ONH cross-sections. In our study, a circular arc was fitted to each of the LC curves of radial cross-sections at principal meridians and allowed an estimation of the LC curvature (mm^{-1}) along radial orientations in a global sense.[62]

The LC GSI was introduced several years ago as a new morphologic parameter for characterizing the geometrical shape of the anterior lamina. LC GSI does not depend on the BMO reference plane and gives a quantitative, easily comparable value. In a healthy Indian population, the majority of the LC were reported to have the shape of a rut or saddle-rut and deeper LC insertions in the superior and inferior LC regions.[62] The GSI was shown to be smaller (more negative) in POAG eyes than in healthy or ocular hypertensive subjects.[68] In agreement, we also found the LC to be more curved in the nasal-temporal meridian compared with the superior-inferior one and to be deeper in the superior and inferior LC sectors of glaucomatous eyes. However, our mean GSI values were smaller than previously described, indicating a shape shift toward the form of a cup. These differences are most likely attributable to a higher, long-standing IOP and more advanced glaucoma of our subjects, since the risk factors of glaucoma, such as the vertical cup-to-disc ratio and minimum rim width, have been shown to be associated with more negative LC GSI.[62]

In our study, the increase in LC GSI after trabeculectomy was observed only in the early postoperative period at the time of the greatest IOP reduction. It is likely that the later postoperative changes of LC curvatures in both axis occurred concomitantly, since the GSI did not change significantly. Thus, our results suggests that parameters such as LC curvature and LCD are clinically more relevant than GSI in describing the longitudinal morphologic LC changes after trabeculectomy. However, it should be noted that the GSI parameter, but not the one of LCD, has been earlier reported to change during an IOP elevation in healthy and glaucomatous eyes.[68] Furthermore, eyes with POAG tended to have a more negative GSI following acute IOP increase compared to normal subject.[68]

The positional responses of the LC to IOP changes have been evaluated in experimental[11,35,78-80] and clinical[49-55,81-84] studies reporting anterior-posterior displacements. Many of them have observed a decrease in the mean LCD after IOP reduc-

tion.[50-53,55,84] However, several studies have previously reported that both posterior and anterior movements of the LC can occur after IOP lowering, which is in agreement with our data.[49,54,81-83] The different methodologies of LCD measurements and variable IOP reduction prevent any direct comparison of the magnitude of LC change between the studies. Many research groups evaluated the LCD only in a selected number of the ONH scans at the deepest, central, or paracentral locations of LC surface and reported bigger LCD values than ours.[51,52,83-85] In our study, an improved LC visibility after adaptive compensation and custom-written software allowed us to cover approximately 90% of the lamina surface and to assess the mean LCD including peripheral LC. The high coverage of LC also enabled us to measure the sectoral LCD. We found the LCD to decrease more in the superior and inferior sectors when compared with the temporal segment. The smaller density of the supporting connective tissue and glial cells in the superior and inferior quadrants have been reported and could explain the sectoral differences in LC mobility.[86]

The mean postsurgical LCD in our study is similar to the baseline data of Tun et al., who evaluated LCD after IOP elevation in glaucomatous eyes using the same methodology.[68] The LCD change that we found is also comparable to the results of the study reporting the LC shallowing after trabeculectomy or deep sclerectomy in POAG eyes with at least ¾ of ONH area measured.[50] We observed the mean LCD to decrease until the sixth postoperative month, and, thereafter, the LC position stabilized. These results are consistent with other authors reporting a similar tendency over a 2-year follow-up period.[52,82] Because IOP did not differ significantly between the visits after the first postoperative month, the subsequent changes of LCD and LC curvatures are likely to be a result of connective tissue remodeling and collagen arrangement under new postoperative hydrostatic pressure circumstances.[22,87]

We have observed the inward and outward movements of the LC after trabeculectomy. In the majority of our patients, the LC became shallower. However, there was a small group of the eyes that displayed a deepening of the LC. Most of the cases with deeper LC compared with the baseline occurred within the first postoperative month and reduced in number over time. Such a bidirectional biomechanical response of the LC to IOP change is a consequence of 2 major forces: the translaminar pressure gradient and IOP induced circumferential forces of the peripapillary sclera affecting the LC.[17] Because of the decrease in translaminar and transscleral pressure gradients, we would assume the shallowing of the LC and inward movement of the peripapillary sclera after the IOP reduction. However, because the surrounding sclera and LC respond as a single unit, the altered scleral forces can subsequently influence the stresses and strains on the LC and, thus, induce its outward movement. The biomechanical behavior of LC and peripapillary sclera is not fully understood because of the complex interaction between the IOP, material properties,

and the geometry of LC and sclera.[34] This biomechanical variability of the LC is in agreement with model predictions by Sigal et al., where the increase in LCD after IOP lowering could be explained by a reduction in scleral canal size, leading to the posterior movement of the LC.[88] Previous clinical studies have corroborated our findings that the LC moves anteriorly or posteriorly after a decrease in IOP.[54,82,83,89] Quigley et al. showed that the anterior LC position became shallower or deeper or stayed unchanged with IOP reduction.[54] The displacement of LCD was greater at lower IOP but was not associated with the magnitude of IOP lowering. Girard et al. evaluated the 3-dimensional ONH displacements and strain relief in vivo and found that the LC displaced posteriorly, anteriorly, or not at all; however, no factors related to different LC behavior were identified.[89] In our study, we demonstrated that eyes with shallower LCD compared with baseline responded to IOP reduction with greater movement anteriorly than eyes with deeper LCD.

We also evaluated the relationships of the morphologic LC changes and their possible predictors. A younger age was a significant factor for the greater flattening and shallowing of the postoperative LC. The age-related differences in the extracellular matrix of the sclera and LC potentially leading to the changes in their material properties have been reported and could at least partly explain this association.[90] Studies in animal and human eyes have demonstrated that the sclera becomes stiffer with age and therefore is subject to higher stresses but lower strains at different levels of IOP.[91-94] Albon et al. showed age-related changes in the collagenous and non-collagenous components of the LC and observed that the mechanical compliance of the human LC decreased with age.[95,96] However, because the biomechanics of the LC and sclera are highly interdependent, it is difficult to isolate the effects on the biomechanical properties of the LC alone. The reduction of IOP was also associated with a decrease in LCD, curvature, and GSI after the surgery. Our findings are in agreement with the studies reporting younger age and larger IOP reduction to be related to greater changes in the LCD and LC curve after trabeculectomy[51,53,61,83,84,97,98] However, it should be mentioned that in some studies, the IOP association was not confirmed, potentially because of a smaller sample size and less IOP reduction.[54,55,97] Quigley et al. showed that the LCD change was more related to the level of IOP than to the magnitude of IOP change.[54]

We found RNFL thinning to be associated with greater changes in the LCD, curvature, and shape during one year after trabeculectomy. Similar results concerning the postoperative LCD changes and RNFL thinning in POAG patients have been recently reportered.[50,81] Regardless of the performed procedure (a trabeculectomy or a non-penetrating deep sclerectomy), the magnitude of the decrease in the LCD was associated with significant focal RNFL thinning.[50] Furthermore, after the postoperative follow-up of 12 to 29 months, the authors concluded that a thinner RNFL thick-

ness after glaucoma surgery was associated with a greater rate of the LCD reduction, not with position of the LC at the final visit.[81] Lee et al., however, found no relationship between the postoperative changes of the LCD and rate of RNFL thinning. The authors concluded that eyes with sustained LCD reduction over a long period had a slow rate of progressive RNFL thinning after trabeculectomy.[82]

Intuitively, the shallowing (anterior movement) of the LC after trabeculectomy is related to a relief of compressed axons of the optic nerve when compared to the preoperative condition. Strains of the ONH have been in vivo shown to be relieved after trabeculectomy.[89] However, the data about the positive association between the postoperative RNFL thinning with the magnitude and rate of LCD change suggest that a rapid and significant anterior LC re-displacement after glaucoma surgery can itself be potentially related to the damage of axons, which pass through the distorted LC pores. To the best of our knowledge, no other study has reported the association between the changes in the LC curvature and global shape with the glaucoma progression after the surgical IOP reduction.

In our study, the morphologic LC changes after trabeculectomy were greater in the group of advanced glaucoma compared to the group of early-to-moderate disease. Since the demographic parameters and reduction of IOP did not differ significantly between the groups, we hypothesize that several factors, including progression-associated LC thinning[85,99,100] and the potential changes in the material properties of LC in advanced-stage disease, might play a role in the different biomechanical response.

The semi-automated measurements of the global LC shape and the actual geometrical curvature, as well as the prospective design of the study with a long follow-up period, are the obvious strengths of our study. However, we should note some potential limitations. First, we performed only a horizontal scanning of the ONH, and the superior and inferior lamina were poorly visualized in a part of the peripheral scans. The mean LC visibility of the 130 enrolled eyes was 89.8%, which may be improved if one uses a radial scan approach. To minimize the limitation, only scans with LC visibility better than 70% (mean 92.4%) were analyzed. Second, the LC curvatures were measured only in two meridians, whereas the superior-temporal and inferior-temporal regions, which are prone to glaucomatous injury, were not evaluated. However, it has been previously reported that the sections containing the principal arc curvatures of LC are aligned to the vertical and horizontal directions.[62] Furthermore, a comparison of the GSI values, computed from the entire reconstructed LC with the GSI values based on the N-T and S-I curvatures, showed very good agreement.[62] These results suggest that an evaluation of the vertical and horizontal meridians alone would be sufficient to calculate the GSI representing the LC global anterior shape. Third, the GSI was evaluated globally by fitting the arc curvature to

the entire cross-section; thus, it did not reflect the local changes of LC shape. However, no widely accepted globally and locally sensitive parameter for LC geometry is available. Fourth, the mean and sectoral postoperative LCD measurements could be affected by the changing choroidal thickness, as was described previously.[60] Fifth, since the majority of the glaucoma surgery patients had the pseudoexfoliative syndrome, the study sample mostly reflected the morphologic changes of LC in the pseudoexfoliative glaucoma. This could have potentially influenced the results of the LC biomechanical response if considering a possible pseudoexfoliation-specific elastinopathy of the LC and the findings that the lamina is thinner and less stiff in eyes with the pseudoexfoliation syndrome.[101,102] Further studies are needed to overcome these limitations and to investigate the long-term structural changes of the post-trabeculectomy LC morphology and its association with glaucomatous injury.

Conclusions

1. In most eyes, trabeculectomy resulted in a long-term flattening and shallowing of the lamina cribrosa, which tended to stabilize after 6 months. Notably, in some eyes, the lamina cribrosa deepened from baseline, revealing bidirectional behavior after the surgery. Changes in the global shape of the lamina cribrosa appeared to be temporal.
2. Reduction of intraocular pressure played an important role in the early phase of postoperative lamina cribrosa change. However, in the later phase, the remodeling of the lamina cribrosa may have played a crucial role, with intraocular pressure being stable.
3. The greater morphologic changes of the lamina cribrosa after trabeculectomy were significantly related to the structural and functional progression of glaucoma, with the relationship being influenced by the severity of the disease.
4. The biomechanical response of the lamina cribrosa to trabeculectomy was associated with other intraocular- pressure-dependent structural changes – an axial length shortening as well as choroidal and macular thickening. The changes of the depth, curvature, and global shape of the lamina cribrosa positively correlated in-between and with the magnitude of the corresponding morphologic parameter at baseline. A younger age had a positive effect on the flattening and shallowing of the lamina cribrosa.
5. Eyes with advanced glaucoma tended to have a more curved lamina cribrosa at baseline and showed a greater reversal of the lamina cribrosa curvature and depth following trabeculectomy compared to the earlier stages of glaucoma.

Practical recommendations

1. An enhanced depth imaging spectral domain optical coherence tomography enables visualization of the deep ocular structures, including the lamina cribrosa and choroid. This imaging benefits to objective analysis and documentation of the morphologic characteristics of the mentioned ocular structures.
2. Morphology 1.0 software is useful for a quantitative evaluation of the anterior surface of the lamina cribrosa. A complex morphologic assessment of the lamina is valuable for glaucoma research since it provides comprehensive knowledge about the geometry and biomechanics of the lamina cribrosa, which is relevant to insights into the mechanisms of glaucoma pathogenesis.
3. Trabeculectomy should aim for a safe target intraocular pressure of the patient, avoiding inappropriately low postoperative levels. Inadequately high surgical reduction of intraocular pressure could lead to a considerable and abrupt shallowing and flattening of the lamina cribrosa, which, in the long-term, is related to the progression of glaucoma.

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Approbation of the results

Publications

1. Kadziauskienė A, Jašinskienė E, Ašoklis R, Lesinskas E, Rekašius T, Chua J, Cheng CY, Mari JM, Girard MJA, Schmetterer L. Long-Term shape, curvature, and depth changes of the lamina cribrosa after trabeculectomy. *Ophthalmology*. 2018 Nov;125(11):1729–1740.

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Oral Presentations and Posters

1. Kadziauskienė A., Jašinskienė E., Ašoklis R., Lesinskas E., Schmetterer L. "The Displacement of Lamina Cribrosa after Glaucoma Surgery." Poster. International conference "Evolutionary Medicine: Health and Diseases in a Changing Environment," June 5–8, 2018, Vilnius, Lithuania.

2. Kadziauskienė A., Strelkauskaitė E., Ašoklis R., Lesinskas E., Girard M.J.A., Schmetterer L. "The Shape and Positional Changes of Lamina Cribrosa after Trabeculectomy in Pseudoexfoliative and Primary Open Angle Glaucoma." Poster. International conference "European Association for Vision and Eye Research Congress 2017 (EVER)." September 27–30, 2017, Nice, France.

3. Drukteinienė E., Kadziauskienė A., Strelkauskaitė E., Ašoklis R., Lesinskas E., Schmetterer L. "Macular Thickness after Intraocular Pressure Reduction following Trabeculectomy." Poster. International conference "European Association for Vision and Eye Research Congress 2017 (EVER)." September 27–30, 2017, Nice, France.

4. Kadziauskienė A. "Akytoji plokštelė ir glaukoma." Oral presentation. Conference "Metinė Lietuvos akių ligų gydytojų draugijos konferencija." September 22–23, 2017, Kaunas, Lithuania.

5. Kadziauskienė A., Strelkauskaitė E., Ašoklis R., Schmetterer L. "Struktūriniai akytosios plokštelės pokyčiai po trabekulektomijos." Oral presentation. Conference "Glaukomų perspektyva: vakar, šiandien, rytoj." May 19, 2017, Kaunas, Lithuania.

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Main publications

1. Kadziauskienė A, Jašinskienė E, Ašoklis R, Lesinskas E, Rekašius T, Chua J, Cheng CY, Mari JM, Girard MJA, Schmetterer L. Long-Term shape, curvature, and depth changes of the lamina cribrosa after trabeculectomy. *Ophthalmology*. 2018 Nov;125(11):1729–1740.
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Aistė Paškonienė

IMPORTANCE OF CLINICAL TESTS AND RADIOLOGICAL FINDINGS ON THE PERIPHERAL VESTIBULAR FUNCTION FOR THE DIAGNOSIS OF MENIERE'S DISEASE

PERIFERINĖS VESTIBULINĖS SISTEMOS INSTRUMENTINIŲ IR RADIOLOGINIŲ TYRIMŲ REIKŠMĖ DIAGNOZUOJANT MENJERO LIGĄ

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List of abbreviations

MD – Meniere's disease
MRI – magnetic resonance imaging
Gd – gadolinium-based contrast
EH – endolymphatic hydrops
IT-Gd – intratympanic injection of Gd
IV-Gd – intravenous injection of Gd
PTA – pure tone audiometry
VNG – videonystagmometry
UW – unilateral weakness
DP – directional preponderance
BPPV – benign paroxysmal positional vertigo
RFI – risk fall index
CP – computer posturography
vHIT – video head impuls test
HIT – head impuls test
HST – head shaking tes

Introduction

Meniere's disease (MD) is defined by a classic triad of symptoms: spontaneous episodes of vertigo, tinnitus or aural fullness, and fluctuating hearing loss [Gürkov et al., 2016]. The exact pathogenesis of MD is still not clear, but histopathological studies demonstrate an indisputable link with the endolymphatic hydrops (EH) [Foster et al., 2013].

Reported prevalence rates of MD vary widely, with estimates as low as 3.5 and as high as 513 per 100,000 population [Alexander et al., 2010]. Attacks of the disease are observed at almost all ages: since early childhood to a venerable age. Diagnostic criteria for the MD were first proposed by the American Academy of Ophthalmology and Otolaryngology more than 45 years ago in 1972, and later revised by the American Academy of Otolaryngology – Head and Neck Surgery in 1985 and 1995. In 2015, the Barany Society – the International Society for Neuro-Otology published a different version of diagnostic criteria, which distinguished only two types of MD, 'probable' or 'definite', based on patient's subjective symptoms including vertigo, hearing loss and tinnitus. However, it has been received controversially by medical society since they contained several shortcomings and ignored the role of the endolymphatic hydrops.

Pure tone audiometry (PTA) is the only objective, however, not specific diagnostic tool used based on the Barany's criteria. Therefore, there is a need for a specific objective instrument to diagnose the MD. Recently, the rapid development of technology has made it possible to visualize the delicate anatomical structures of the inner ear *in-vivo*. New studies demonstrate that high-resolution magnetic resonance imaging (MRI) with a gadolinium-based contrast (Gd) enables clinicians to

detect EH of the inner ear [Cho YS et al., 2018]. Such a diagnostic method in clinical practice is rather new, and there is still no generally accepted methodology and diagnostic criteria. In 2008, Nakashima and colleagues proposed a grading system of the EH to standardize the evaluation of the EH using MR imaging [Nakashima et al., 2009]. EH was determined by the ratio of the area of endolymphatic space to the vestibular fluid space and classified as none, mild and significant. Liu and colleagues (2011) compared this ratio between healthy volunteers and Meniere's patients and concluded that EH was visible in all cases of MD, but the ratio remained normal within healthy population [Liu et al., 2012]. In 2013, Young et al. studied MR images of 26 patients after the intratympanic injection of Gd – 81% of patients with a definite MD had EH in the affected cochleae, 69% had visualized EH of the vestibular space, and only one had normal endolymphatic space [Young et al., 2013]. Authors concluded that such an imaging technique is superior in diagnosing cochlear and vestibular hydrops; and that EH presented on MR images may be a reliable tool to diagnose MD. Nevertheless, study populations are usually quite small, proposed techniques differ, and results are inconsistent.

1.1. Aim of the study

We aimed to assess the significance of radiologic examination, peripheral vestibular system and balance testing when diagnosing Meniere's disease.

1.2. Objectives of the study

1. To assess the results of a complex (clinical and instrumental neuro-otology) evaluation of the peripheral vestibular system in different types of Meniere's disease.
2. To evaluate changes in the endolymphatic system in 3T delayed contrast enhancement magnetic resonance images of the temporal bone in case of different types of Meniere's disease.
3. To determine the prognostic value of instrumental and radiological examination of the peripheral vestibular system when diagnosing Meniere's disease.
4. To propose a grading system of 3T delayed contrast enhancement magnetic resonance images of the temporal bone in case of Meniere's disease.
5. To determine tests that are of higher value when diagnosing Meniere's disease, thus should be performed when making the diagnosis.

1.3. Relevance and scientific novelty of the research

During the research, values of the caloric test on video-nystagmography (VNG) and prognostic value of the test were determined in case of a *definite* and *probable* types of Meniere's disease. 3T magnetic resonances images of the temporal bone were evaluated to establish the clinical significance of radiological examination when diagnosing Meniere's disease. Grading system of the endolyphatic hydrops (EH), observed in 3T MR images of the temporal bone, was introduced as a results of the research, its clinical significance and application in clinical practice were assessed to diagnose, differentiate, and predict Meniere's disease. So far, scientific medical literature in Lithuania lacked data about objective tests of the peripheral vestibular system in case of Meniere's disease, as well as information about its classification to different types. 3T MRI of the temporal bone was used for the first time to diagnose and evaluate Meniere's disease in Lithuania.

1.4. Practical value

First of all, a new test – 3T MR imaging of the temporal bone – was used in Lithuania for the first time. Researchers assessed its value when differentiating between central and peripheral vestibular dysfunction. This test is quite new in medical practice, and not performed routinely as it is difficult to technically visualize and evaluate small inner ear structures. During the test, gadolinium-based contrast agent is injected intravenously, after 6 hours, a specific technique of 3T MR is used to scan the temporal bone, and then appropriate sequences are assessed. Only experienced neuroradiologists read the scans. Secondly, researchers proposed recommendations for the grading of the endolymphatic hydrops based on the Swiss grading system published in 2014. These practical directions were introduced in the Clinic of Ear, Nose and Throat at Vilnius University Hospital Santaros Clinics. Implementation of such recommendations enabled to improve complicated diagnosis of MD and prognose the course of the disease.

1.5. Hypothesis

The hypothesis was proposed that results of neuro-otologic tests can be insufficinent and not-specific when diagnosing Meniere's disease, and endolymphatic hydrops obswerved in the delayed contrast enhancement 3T MR images is the most specific instrumental test to diagnose probable and definite Meniere's disease.

1.6. Study programme

Considering the aim and objectives of the study, programme of this scientific work was composed at the beginning of the research:

1. Selection of participants based on the typical diagnostic criteria of Meniere's disease;
2. Allocation of study participants to different groups based on the type of Meniere's disease according to the recommendations proposed in 2015 by the Barany Society – the International Society for Neuro-Otology;
3. Clinical and instrumental examination of peripheral vestibular function and balance;
4. Evaluation of 3T delayed contrast enhancement MR images;
5. Data handling and statistical analysis.

2. Material and methods

This prospective single-center cohort study was performed in Vilnius University Hospital, Santaros Clinics in 2015-2018 after receiving approval from the Vilnius Regional Biomedical Research Ethics Committee (No 158200-17-914-424). All participants signed the informed consent form prior to the inclusion to the study.

2.1. Selection of participants

Initial cohort of the study included 225 patients, who were appointed for a planned consultation of a neuro-otologist due to a suspected diagnosis of Meniere's disease. All ofnthem were examined during the remission. Based on the clinical guidelines proposed by the Barany's International Oto-Neurology Society in 2015, all subjects underwent a basic clinical examination necessary for the diagnosis of MD. It included:

- thorough anamnesis: (a) vertigo – its nature (systemic, non-systemic); duration of a vertigo attack, frequency of epizodes; (b) concomitant symptoms – nausea, vomiting, perspiration, diarrhea; (c) aural symptoms – tinnitus, aural fullness, permanent or intermittent hearing loss; (d) association between vertigo and hearing loss; (e) residua symptoms – absent, hearing loss, instability; (f) duration of the disease; (g) concomitant diseases, medications.
- audiologic examination – otoscopy, impedansometry, pure-tone audiometry. In our study, we classified results of the pure tone audiometry (PTA) as informative (unilateral sensorineural or mixed hearing loss) or non-in-

formative (normal, symmetrical sensorineural or symmetrical mixed hearing loss) audiogram. The affected ear was determined by the hearing loss defined in Barany's criteria. Low frequency sensorineural hearing loss was defined as an increase in pure tone thresholds for bone-conducted sound that were higher in the affected ear than the contralateral ear by at least 30dB HL at each of two contiguous frequencies below 2000Hz (250Hz, 500Hz, and 1000Hz). In case of bilateral low-frequency sensorineural hearing loss, the absolute thresholds for bone-conducted sound were 35 dB HL at each of two contiguous frequencies below 2000 Hz.

After the initial examination, all 225 patients were thoroughly studied if they met the inclusion or exclusion criteria.

Inclusion criteria were:

- vertigo attacks;
- remission of the disease (no signs of acute vertigo);
- dominant hearing symptoms (intermittent or permanent);
- normal otoscopic view.

Exclusion criteria were:

- diseases of the outer and middle ear in the past or present at the time of the study (external otitis, otosclerosis, tympanosclerosis, tubotympanic disease, atticoantral otitis media, trauma of the tympanic membrane, ear surgeries, etc.);
- autoimmune condition;
- neurologic, psychiatric diseases.

A total of 105 patients, who met clinical diagnostic criteria for the diagnosis of the MD as well as inclusion criteria, were included into the further study. Based on the clinical diagnostic criteria adopted by the Barany Society, the symptoms were attributed to either *probable* or *definite* MD. The disease was considered *definite* when the following criteria were met:

- two or more episodes of spontaneous vertigo, each lasting 20 minutes to 12 hours;
- low to medium frequency sensorineural hearing loss in one ear documented by the audiogram, defining the affected ear before, during or after at least one episode of vertigo;
- fluctuating aural symptoms (hearing loss, tinnitus or fullness) in the affected ear;
- not better accounted for by another vestibular diagnosis.

Probable MD was diagnosed in case patient met all the above–mentioned criteria except for the sensorineural hearing loss. All participants were divided into the *definite* (N=55) and *probable* (N=50) MD groups.

All 105 subjects later underwent a complex examination of the peripheral vestibular function and balance:

- examination of the vestibulospinal reflexes – Romberg, stepping and tandem gait tests;
- spontaneous nystagmus (SNy) assessment with video Frenzel goggles;
- videonystagmography (VNG):
  - evaluation of Sny;
  - evaluation of the vestibule-ocular reflex with the head impulse test (HIT);
  - evaluation of head-shaking induced nystagmus and provoked nystagmus;
  - evaluation of Unilateral weakness (UW) and Directional preponderance (DP) indeces;
  - computer posturography – assessment of postural stability and fall risk index (FRI).

2.2. 3T MRI with gadolinium contrast

All participants were referred for a 3T MRI of a temporal bone to assess the labyrinth hydrops.The MRI was performed 4 to 6 hours after an intravenous injection of a single dose (0,1 mmol/kg) of Gd-based contrast agent *Gadobutrol* (*Gadovist*, *Bayer*, Berlin, DE). The delay time was modified due to some logistic issues, but was based on results of the recent scientific studies claiming that the perilymph enhancement after an intravenous administration of Gd-based contrast material did not differ at 3, 4.5, and 6 hours and reached plateau between 4.5 and 6 hours after the contrast administration. 3 tesla MRI system (*Achieva*, *Philips*, Best, NL) with a 16-channel array head coil was used. The im aging protocol consisted of 3D-FLAIR and 3D T2DRIVE sequences, and anatomical 3D T1, T2 and DWI sequences to exclude possible central nervous system pathology. If the patient had undergone the brain MRI during 3 past months, abbreviated imaging protocol (3D-FLAIR and 3D T2DRIVE) was used. Parameters for 3D-FLAIR sequence were: time of repetition 4800 ms, time of echo 283 ms, time of inversion 1650 ms, matrix size 224x224, field-of-view 250 mm, slice thickness 0,7 mm, and acquisition time 9.5 minutes. Images were qualitatively assessed by two radiologists: one experienced neuroradiologist (14 years of experience) and one radiologist in training. T2DRIVE and 3D FLAIR images were reviewed side-by-side using T2DRIVE for bone delineation and 3D FLAIR for perilymphatic fluid visualization. The non-enhanced FLAIR regions appearing bright on T2DRIVE were interpreted as endolymphatic structures. Only the vestibular hydrops was assessed since qualityof cochlea images was worse. Distention of vestibular endolymphatic sacs (one or both) was categorized as none, grade

I (mild) or grade II (significant) as described by Barath and colleagues [Barath et al., 2014]. In the normal vestibule, the added surface areas of the saccule and utricle are less than half the area of the vestibule at the midmodiolar level. Grade I (mild) vestibular hydrops presented as distention of the endolymph space of the saccule or utricle or both, with the perilymphatic space still visible along the periphery of the bony vestibule. In grade II (significant) vestibular hydrops, the bony vestibule was entirely encompassed by the dilated endolymphatic spaces.

2.3. Statistical analysis

Categorical parameters are presented with cross tabulation with absolute and percentage values. Distribution of quantitative parameters are defined using mean, standard deviation, median, 1st and 3rd quartiles, minimum and maximum values. Student-t test was used to evaluate the difference between two independent data sets. The differences between two independent qualitative data groups were evaluated by Fisher exact test. Risk factors for MD and MRI test results were assessed by logistic regression analysis. Factors found to be significant in univariate logistic regression model were entered into multivariate logistic model with stepwise model selection process. A two-tailed p-value less than 0.05 was considered to be significant. Statistical analysis was performed using Statistical Analysis System (SAS) package version 9.2.

3. Results

3.1. Baseline characteristics

During the study period, 105 eligible subjects were analyzed: 75 of them were female (71,4%) and 30 males (28,6%). The age of participants ranged from 23 to 78 years, with an average of 55,8±11,69 years (Table 1, Figure 1). Fifty-five (52.4%) patients were diagnosed with a *definite*, and 50 (47.6%) – with a *probable* Meniere's disease. No significant difference was observed between the gender and type of MD (p=0.667) (Table 1, Figure 1).

Frequency of symptoms, i.e. episodes of vertigo, within the last 6-months period was registered in each case. Two or less episodes of vertigo per 6 months were registered in 46 (43.8%) cases, more than two episodes over half-a-year period – in 59 (56.2%) cases. Patients diagnosed with a *definite* MD suffered significantly more episodes of vertigo over a 6-months period – there were 39 (70.9%) and 20 (40.0%) subjects in a definite and probable DM groups respectively, who had more than two episodes of vertigo in lasr 6 months (p=0.002) (Table 1, Figure 1).

Thirty-three (31.4%) participants had MD for less than 2 years, 39 (37.1%) from 2 to 5 years, and the rest of participants – 33 (31.4%) had MD for more than 5 years. Duration of the disease was statistically significantly sssociated with the type of MD: in majority of cases of *probable* MD (22; 44.0%), the disease lasted less than 2 years, however, nearly half of patients with a *definite* MD (26; 47.3%) suffered disease from 2 to 5 years (p=0.020). important to mention is the fact that the number of subjects whose MD lasted more than 5 years was nearly equal in both *probable* and *definite* MD groups – 18 (32.7%) and 15 (30.0%) subjects respectively (Table 1, Figure 1).

TABLE 1. Baseline characteristics of study participants

		Total N=105 (100%)	Definite MD N=55 (52.4%)	Probable MD N=50 (47.6%)	p-value
Gender	Male	30 (28.6)	17 (30.9)	13 (26.0)	0.667
	Female	75 (71.4)	38 (69.1)	37 (74.0)	
Frequency of symptoms	≤2 ep. / 6 mo.	46 (43.8)	16 (29.1)	30 (60.0)	0.002
	>2 ep. / 6 mo.	59 (56.2)	39 (70.9)	20 (40.0)	
Duration of the disease	<2 years	33 (31.4)	11 (20.0)	22 (44.0)	0.020
	2-5 years	39 (37.1)	26 (47.3)	13 (26.0)	
	>5 years	33 (31.4)	18 (32.7)	15 (30.0)	
Age	Mean ± SD Median Q1-Q3 Min-Max	55.8 ± 11.69 58 49-64 23-78	56.3 ± 12.17 59 49-65 27-78	55.3 ± 11.24 54.5 48-64 23-75	0.685

Notes: MD – Meniere's disease; ep. – episodes; mo. – months; SD – standard deviation; Q1 – 1st quartile (or 25% quantile); Q3 – 3rd quartile (or 75% quantile); min – minimum; max – maximum.

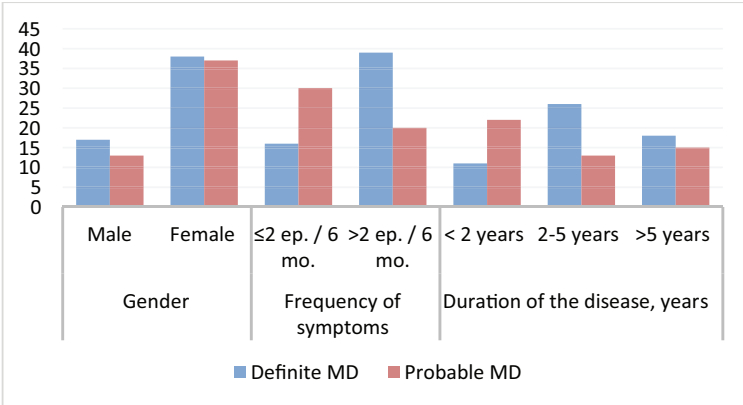


FIGURE 1. Baseline characteristics of participants

Vestibulospinal function (Romberg, tandem gait and Fukuda stepping tests) was normal within all 105 study participants, as well as spontaneous nystagmus under Frenzel goggles absent.

3.2. Results of pure-tone audiometry

Pure-tone audiometry (PTA) was performed for all 105 (100.0%) subjects. There were 72 (68.6%) informative and 33 (31.4%) non-informative audiograms. Informative audiogram was seen twice as much comparing *definite* and *probable* MD groups – there were 50 (90.9%) and 22 (44.0%) informative audiograms in *definite* and *probable* MD groups, respectively ($p < 0.001$). When comparing the distribution of audiograms within the separate study group, there was an extremely high ratio of informative audiogram to non-informative in a *definite* MD group – 50 (90.9%) informative *vs.* 5 (9.1%) non-informative audiograms. On the other hand, audiograms were similarly distributed within the *probable* MD group – informative and non-informative audiograms were observed in 22 (44.0%) and 28 (56.0%) subjects with a *probable* MD, respectively (Table 2).

Distribution of hearing impairment is showed in Figure 2. The highest degree of hearing loss was identified in pacients with a *definite* MD: 38 (69.1%) subjects in a *definite* MD group had moderately severe or severe hearing loss, whereas moderate hearing loss was seen in only 8 (16%) cases in a *probable* MD group ($p<0.001$) (Table 2).

TABLE 2. Results of the pure-tone audiometry results by MD groups

		Total N=105 (100%)	Definite MD N=55 (52.4%)	Probable MD N=50 (47.6%)	p-value
PTA	Informative	72 (68.6)	50 (90.9)	22 (44.0)	<0.001
	Non-informative	33 (31.4)	5 (9.1)	28 (56.0)	
Degree of hearing loss	Normal	45 (42.9)	9 (16.4)	36 (72.0)	<0.001
	Moderate	14 (13.3)	8 (14.5)	6 (12.0)	
	Moderately severe	26 (24.8)	21 (38.2)	5 (10.0)	
	Severe	20 (19.0)	17 (30.9)	3 (6.0)	

Both, PTA and degree of hearing loss, were homogeneously distributed in the groups classified based on the frequency of symptoms ($p=0.298$ and $p=0.195$, respectively). However, informative audiogram was more often seen in patients with more frequent symptoms – there were 29 (63.0%) and 43 (72.9%) patients with two or less and more than two episodes of vertigo per six months, respectively (Table 3).

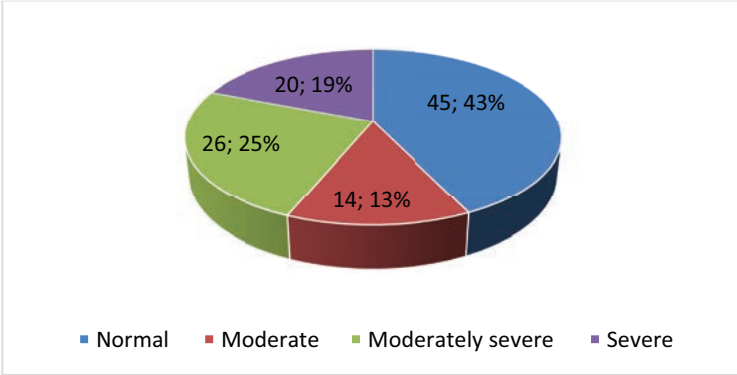


FIGURE 2. Degree of hearing loss

Informative audiogram was observed in about two-thirds of participants at each group classified by the disease duration intervals – there were 21 (63.6%), 30 (76.9%) and 21 (63.6%) subjects with an informative audiogram whose disease lasted less than <2, from 2 to 5, and more than 5 years, respectively. Severe hearing loss was more prominent in cases the disease lasted more than two and less than 5 years – there were 30.8% of patients with the severe hearing loss in this groups of patients, whereas this number decreased to 12.1% and 12.1% in groups of patients whose MD lasted less than 2, or more than 5 years. However, statistical analysis revealed that PTA and degree of hearing loss were both independently distributed within the disease duration intervals: duration of the disease had no impact on the informativeness of the audiogram or on the degree of hearing loss (p=0.374 and p=0.196, respectively) (Table 4).

TABLE 3. Results of the pure-tone audiometry based on the frequency of symptoms

		≤2 ep. / 6 mo. N=46 (43.8%)	>2 ep. / 6 mo. N=59 (56.2%)	p-value
PTA	Informative	29 (63.0)	43 (72.9)	0.298
	Non-informative	17 (37.0)	16 (27.1)	
Degree of hearing loss	Normal	25 (54.4)	20 (33.9)	0.195
	Moderate	5 (10.9)	9 (15.3)	
	Moderately severe	8 (17.4)	18 (30.5)	
	Severe	8 (17.4)	12 (20.3)	

TABLE 4. Results of the pure-tone audiometry based on the duration of the disease

		<2 years N=33 (31.4%)	2-5 years N=39 (37.1%)	>5 years N=33 (31.4%)	p-value
PTA	Informative	21 (63.6)	30 (76.9)	21 (63.6)	0.374
	Non-informative	12 (36.4)	9 (23.1)	12 (36.4)	
Degree of hearing loss	Normal	16 (48.5)	13 (33.3)	16 (48.5)	0.196
	Moderate	7 (21.2)	3 (7.7)	4 (12.1)	
	Moderately severe	6 (18.2)	11 (28.2)	9 (27.3)	
	Severe	4 (12.1)	12 (30.8)	4 (12.1)	

3.3. Results of videonistagmography

Videonistagmography was performed in every case (N=105) – no spontaneous nystagmus was observed, and normal head impuls test (HIT) obtained for all patients. Positional nystagmus was provoked when performing Dix-Hallpike maneuver in five cases (4.8%). The mean age for all five patients with combined MD and benign paroxysmal positional vertigo was 52.4 years, vestibular endolymphatic hydrops on 3T MRI of the temporal bone was observed in all five cases as well. In addition to this, all patie nts suffered MD longer than two, but less than 5 years, and episodes of vertigo repeated more than twice per 6 months.

In more than half of cases (67; 63.8%) caloric test of VNG was altered – both unilateral weaknes (UW) and directional preponderance (DP) were higher than 25%. Altered caloric test results were observed in 42 (76.4%) cases of a *definite* MD, and 25 (50.0%) cases of a *probable* MD (p=0.008). UW dominated in patients with a *definite* type of MD compared to patients with a *probable* MD, however, the difference was not statistically significant: 32 (58.2%) *vs.* 23 (46.0%), respectively (p=0.244). whereas DP differed significantly between MD groups – DP higher than 25% was seen in 18 (32.7%) patients with a *definite* MD, and 6 (12.0%) patients with a *probable* MD (p=0.019) (Table 5). Distribution of UW and DP based on the impairment site are presented in Figure 3.

TABLE 5. Results of the caloric test

		Total N=105 (100%)	Definite MD N=55 (52.4%)	Probable MD N=50 (47.6%)	p-value
Altered caloric test	Yes	67 (63.8)	42 (76.4)	25 (50.0)	0.008
	No	38 (36.2)	13 (23.6)	25 (50.0)	
UW	Yes	55 (52.4)	32 (58.2)	23 (46.0)	0.244
	No	50 (47.6)	23 (41.8)	27 (54.0)	
DP	Yes	24 (22.9)	18 (32.7)	6 (12.0)	0.019
	No	81 (17.1)	37 (67.3)	44 (88.0)	

Alterations in the caloric test were more common in participants who had more than 2 episodes of vertigo within š-months period. In the group of patients with altered caloric test, 43 (72.9%) were registered to suffer more than 2 episodes per half a year, and 24 (52.2%) – two or less episodes per period (p=0.040). However, values of UW and DP were not associated with the frequency of MD symptoms (p=0.698 and p=0.109, respectively) (Table 6).

Alterations in the caloric test were mostly found in case MD lasted longer than 2 and less than 5 years – in 29 cases (74.4%), whereas in the group of patients whose MD lasted more than 5 years, number of altered caloric tests was the least (17 cases; 51.5%). Nevertheless, no significant association was observed between caloric test and duration of the disease (p=0.143) (Table 7). UW and DP were also independent and homogeneously distributed between patients grouped by the duration of the disease (p=0.168 and p=0.591, respectively) (Table 7).

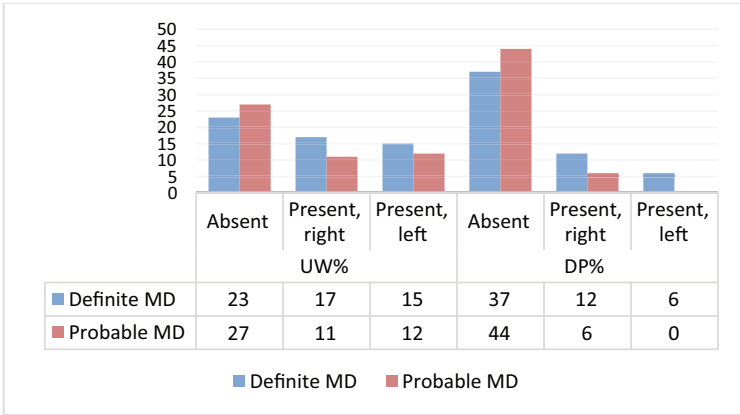


FIGURE 3. Caloric test results

Altered caloric test was more ommon in case of a moderately severe and severe hearing loss – in 50% and 31% of participants, respectively. In addition to this, there was a tendency for the caloric test to remain within normal values in case hearing thresholds were normal. However, no significant differences were found between groups with different caloric test results and the degree of hearing loss (p=0.251) (Figure 4).

TABLE 6. Results of the caloric test based onthe frequency of symptoms

		≤2 ep. / 6 mo. N=46 (43.8%)	>2 ep. / 6 mo. N=59 (56.2%)	p-value
Altered caloric test	Yes	24 (52.2)	43 (72.9)	0.040
	No	22 (47.8)	16 (27.1)	
UW%	Yes	23 (50.0)	32 (54.2)	0.698
	No	23 (50.0)	27 (45.8)	
DP%	Yes	7 (15.2)	17 (28.8)	0.109
	No	39 (84.8)	42 (71.2)	

TABLE 7. Caloric test results based on the duration of the disease

		<2 years N=33 (31.4%)	2-5 years N=39 (37.1%)	>5 years N=33 (31.4%)	p-value
Altered caloric test	Yes	21 (63.6)	29 (74.4)	17 (51.5)	0.143
	No	12 (36.4)	10 (25.6)	16 (48.5)	
UW%	Yes	16 (48.5)	25 (64.1)	14 (42.4)	0.168
	No	17 (51.2)	14 (35.9)	19 (57.6)	
DP%	Yes	6 (18.2)	11 (28.2)	7 (21.2)	0.591
	No	27 (81.8)	28 (71.8)	26 (78.8)	

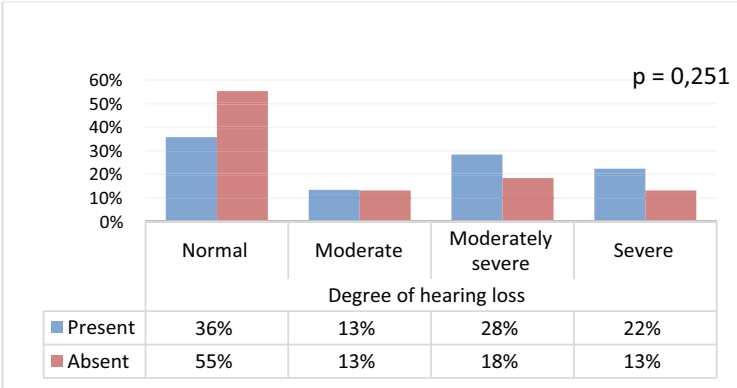


FIGURE 4. Results of the caloric test based on the degree of hearing loss

3.4. Computer posturography

Computer posturography was performed in 94 (89.5%) subjects. Based on the fall risk index counted after the computer posturography, patients were divided into groups of low (20 participants; 21.3%), intermediate (55; 58.5%), and high (19; 20.2%) fall risk. No statistically significant difference was observed between the fall risk index and tyle of MD (p=0.883) (Table 8).

No statistically significant difference was also calculated between the type of MD, frequency of vertigo attacks and duration of the disease duration comparing quantitative results of the computer posturography (Table 9, Figure 5).

TABLE 8. Results of the fall risk index of the computer posturography

		Total N=105 (100%)	Definite MD N=55 (52.4%)	Probable MD N=50 (47.6%)	p-value
Fall risk index	High	19 (20.2)	11 (22.5)	8 (17.8)	0.883
	Medium	55 (58.5)	28 (57.1)	27 (60.0)	
	Low	20 (21.3)	10 (20.4)	10 (22.2)	
	No data	11	6	5	

TABLE 9. Comparisons of quantitative computer posturography fall risk index

		Fall risk index Mean (SD)	p-value
Total		36.9 (27.86)	-
MD group	Definite	37.5 (27.62)	0.827
	Probable	36.2 (28.41)	
Frequency of symptoms	≤2 ep. / 6 mo.	33.0 (24.41)	0.222
	>2 ep. / 6 mo.	40.1 (30.32)	
Duration of MD	< 2 years	43.9 (33.25)	0.215
	2-5 years	34.7 (25.20)	
	>5 years	34.9 (23.64)	

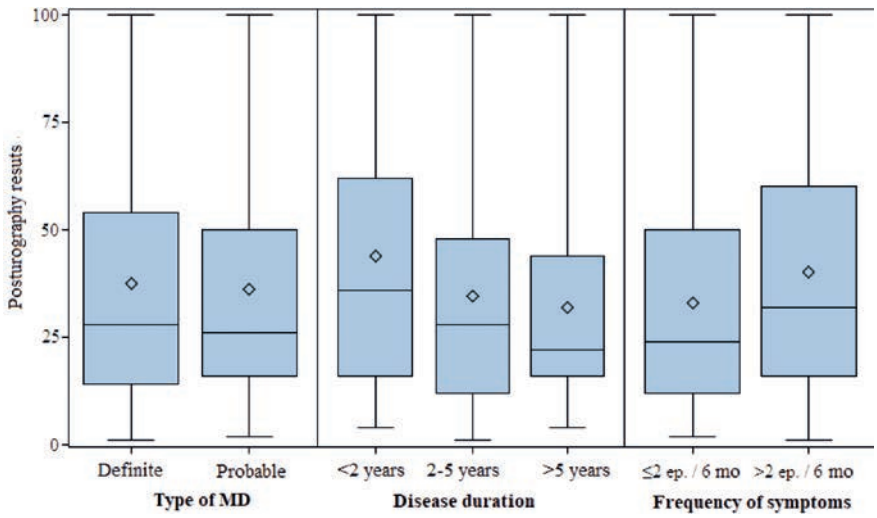


FIGURE 5. Comparisons of quantitative computer posturography fall risk index

3.5. Results of the 3T MR imaging of the temporal bone

Eighty-two (78.1%) subjects had changes observed in MR images (i.e. endolymphatic hydrops, asymmetric contrast enhancement when endolymphatic hydrops was absent). More than 90.0% of patients diagnosed with a *definite* MD had changes in MR images, whereas changes were observed in only 64.0% of patients diagnosed with a *probable* MD (p<0.001) (Table 10, Figure 6).

When talkink about the study sample in general, endolymphatic hydrops (EH) was seen in MR images of 61 (58.1%) participants: 40 (65.6%) subjects were diagnosed with a grade I EH, 21 (34.4%) – with grade II EH. In even 44 (41.9%) cases, EH was not observed on MR images of the temporal bone (Table 10, Figure 6).

EH was found in patients diagnosed with a *definite* MD significantly more compared to patients with a *probable* MD – in 42 (76.4%) and 19 (38.0%) patients respectively (p<0.001). Grade I EH was observed significantly more often in case of a *probable* MD compared to a *definite* – in 17 (89.5%) and 23 (54.8%) cases respectively (p=0.009) (Table 10, Figure 6).

Analysis revealed that the higher degree of EH was associated with a longer duration of the disease (p=0.047). Patients diagnosed with a grade I EH suffered MD for less than 2 years in 13 (32.5%) cases, from 2 to 5 years in 14 (35.0%) cases, and more than 5 years in 13 (32.5%) cases. In case of a grade II EH, 1 (4.8%) patient suffered MD less than 2 years, 11 (52.4%) – from 2 to 5, and 9 (42.9%) – more than 5 years (p=0.047). Frequency of vertigo episodes over the 6-months period correlated strongly with thee degree of EH (p=0.011) – in case of grade I EH, patients distributed evenly based on the frequency of episodes, however, in case of grade II EH, patients tended to suffer more than 2 episodes of vertigo in half a year significantly more (Table 11).

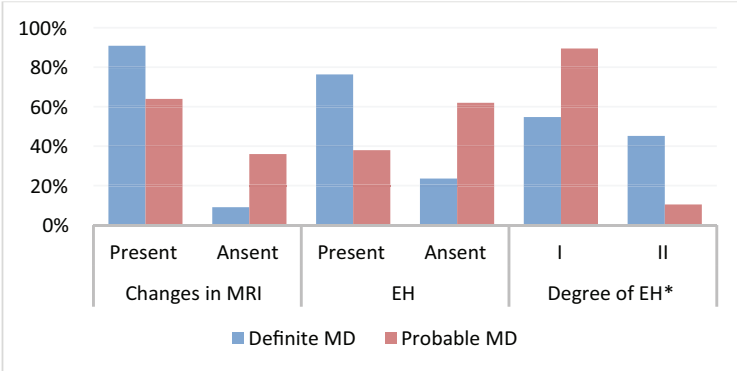


FIGURE 6. Results of the 3T MRI

Notes: *EH – endolymphatic hydrops

TABLE 10. Results of the 3T MRI

		Total N = 105 (100%)	Definite MD N=55 (52.4%)	Probable MD N=50 (47.6%)	p-value
Changes in MRI	Yes	82 (78.1)	50 (90.9)	32 (64.0)	<0.001
	No	23 (21.9)	5 (9.1)	18 (36.0)	
EH	Visible	61 (58.1)	42 (76.4)	19 (38.0)	<0.001
	Non-visible	44 (41.9)	13 (23.6)	31 (62.0)	
Degree of EH*	I	40 (65.6)	23 (54.8)	17 (89.5)	0.009
	II	21 (34.4)	19 (45.2)	2 (10.5)	

Notes: *Percentages are based on subjects with a visible EH; EH – endolymphatic hydrops.

TABLE 11. Distribution of EH based on the frequency of symptoms, duration of the disease, and degree of hearing loss

		Grade I N=40 (65.6%)	Grade II N=21 (34.4%)	p-value
Frequency of symptoms	≤2 ep. / 6 mo.	20 (50.0)	3 (14.3)	0.011
	>2 ep. / 6 mo.	20 (50.0)	18 (85.7)	
Duration of MD	<2 years	13 (32.5)	1 (4.8)	0.047
	2-5 years	14 (35.0)	11 (52.4)	
	>5 years	13 (32.5)	9 (42.9)	
Degree of hearing loss	Normal	17 (42.5)	1 (4.8)	<0.001
	Moderate	8 (20.0)	0 (0.0)	
	Moderately severe	9 (22.5)	10 (47.6)	
	Severe	6 (15.0)	10 (47.6)	

Analysis of data revealed that in case of EH observed on MR images, informative audiogram was present in 45 (42.9%) cases, informative – in 4 (3.8%) cases. What is more, in 41 (39.0%) case, no changes were observed on MR images even though PTA was informative (i.e. changed). There was a strong association between the EH on MR images and informative audiogram, changes in MRI identified correctly the impairment status in 60 (57.0%) cases (Table 12).

Changes in MRI and UW in caloric testing completely corresponded each other in 56 (53.3%) cases. What is more, MRI identified 25 (23.8%) more subjects with anatomical changes although UW test was negative. Changes in MRI and DP in caloric testing completely corresponded each other in 41 (39.0%) cases. Additionally, MRI identified 44 (41.9%) more subjects with anatomical changes although DP test was normal. Significant association was calculated between MRI and UW in caloric testing ($p<0.001$), however, significance relation between MRI and DP test was not found ($p=0.123$) (Table 13).

TABLE 12. Distribution of changes on 3T MR images of the temporal bone based on audiometry results

			EH observed on MR images				p-value
			AD N=27 (25.7%)	AS N=25 (23.8%)	AD/AS N=6 (5.7%)	Normal anatomy N=47 (44.8%)	
Audiometry	Informative	AS	1 (3.2)	16 (51.6)	1 (3.2)	13 (41.9)	<0.001
		AD	21 (51.2)	2 (4.9)	3 (7.3)	15 (36.6)	
	Non-informative	Symmetrical NSP	3 (13.0)	5 (21.7)	2 (8.7)	13 (56.5)	<0.001
		Normal PTA	2 (20.0)	2 (20.0)	0 (0.0)	6 (60.0)	

Notes: AD – right ear; AS – left ear; NSP – sensoneural hearing loss; EH – endolymphatic hydrops; PTA – pure tone audiometry

TABLE 13. Distribution of changes on 3T MR images of the temporal bone based on the calorimetry results

		EH observed on MR images				p-value
		AD N=27 (25.7%)	AS N=25 (23.8%)	AD/AS N=6 (5.7%)	Not found N=47 (44.8%)	
UW	AD	17 (60.7)	0 (0.0)	0 (0.0)	11 (39.3)	<0.001
	AS	0 (0.0)	14 (51.9)	2 (7.4)	11 (40.7)	
	Not found	10 (20.0)	11 (22.0)	4 (8.0)	25 (50.0)	
DP	AD	3 (16.7)	5 (27.8)	2 (11.1)	8 (44.4)	0.123
	AS	1 (16.7)	1 (16.7)	2 (33.3)	2 (33.3)	
	Not found	23 (28.4)	19 (23.5)	2 (2.5)	37 (47.5)	

AD – right ear; AS – left ear; UW – unilateral weakness; DP – directional preponderance; EH – endolymphatic hydrops.

3.6. Prognostic factors for the type of meniere's disease and changes on MRI

Type of MD

Based on the univariate logistic regression analysis, UW was the only factor statistically non-significant for the type of MD. EH observed on MR images and grade II of the EH were the most associated with a *definite* MD – OR (95% CI) was 5.744 (2.458-13.422) (p<0.001) and 7.022 (1.437-34.301) (p=0.016), respectively

(Table 14). ROC analysis identified the presence of EH on MR images and grade II of EH to be the best parameter to classify the type of MD – AUC (95% PI) was 0.702 (0.614-0.790) (p<0.001) and 0.674 (0.570-0.778) (p=0.001), respectively (Figure 7).

However, grade II of the EH was not included in the multivariate logistic analysis model because grade II of the EH was analyzed only in subjects with EH.

Multivariate logistic regression model with a stepwise selection method revealed that only changes on MR images and DP in caloric testing were two independent prognostic factors for the type of MD – OR (95% CI) was 6.603 (2.662-16.377) (p<0.001) and 4.553 (1.453-14.261) (p=0.009), respectively (Table 17). What is more, ROC analysis showed that the same factors (changes on MR images and DP in caloric testing) have better prognostic value for the type of MD when they are together in the logistic model compared to acting separately (p=0.022 and p=0.003, respectively). AUC (95% CI) for a model with two independent factors was 0.747 (0.659-0.838) (p<0.001) (Figure 8).

TABLE 14. Logistic regression estimates for prognostic factors for the type of MD

	Univariate model			Multivariate model	
	OR (95% CI)	p-value		OR (95% CI)	p-value
Changes in MRI	5.625 (1.899-16.657)	0.002		NE	>0.05
Endolymphatic hydrops in MRI	5.744 (2.458-13.422)	<0.001		6.603 (2.662-16.377)	<0.001
Grade II EH*	7.022 (1.437-34.301)	0.016			
Cochler ductus hydrops	3.917 (1.024-14.975)	0.046		NE	>0.05
Altered caloric test	3.231 (1.404-7.433)	0.006		NE	>0.05
Unilateral weakness*	1.633 (0.755-3.535)	0.213			
Direction preponderance	3.568 (1.284-9.915)	0.015		4.553 (1.453-14.261)	0.009
Quantitative posturography risk index	1.002 (0.987-1.016)	0.825			
Low posturography risk index	0.964 (0.346-2.684)	0.945			
High posturography risk index	1.326 (0.463-3.800)	0.600			

Notes: *not included in the multivariate logistic model; NE – not evaluated.

Changes in MRI

Based on the univariate logistic regression analysis, only the type of MD was statistically significant predictor for changes on MR images – OR (95% CI) was 5.625 (1.900-16.657) (p = 0.002) (Table 15).

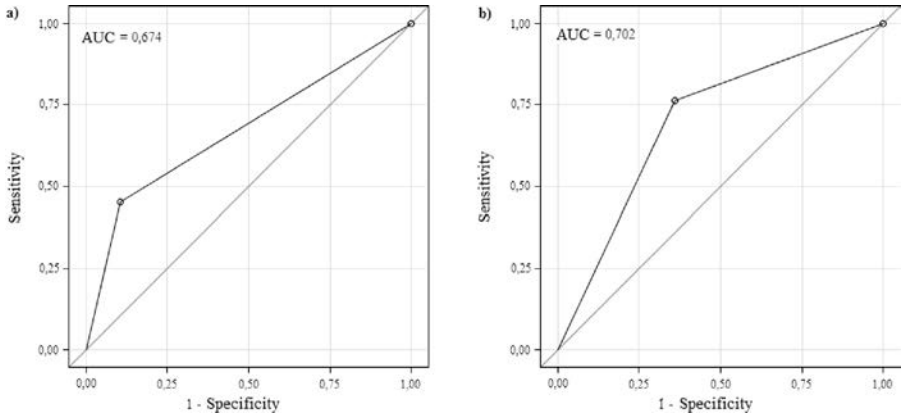


FIGURE 7. ROC curves from the univariate logistic regression: a) endolyphatic hydrops on MR images, b) grade II endolyphatic hydrops

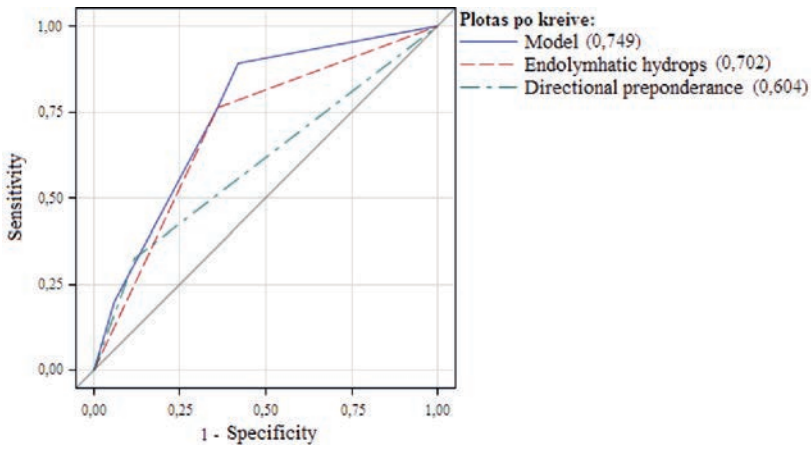


FIGURE 8. ROC curves from the multivariate logistic regression model

TABLE 15. Logistic regression estimates for the prognostic factors of changes on MR images

	Univariate model	
	OR (95% CI)	p-value
Type of MD	5.625 (1.900-16.657)	0.002
Altered calorimetry	1.867 (0.730-4.775)	0.192
Unilateral weakness	1.263 (0.500-3.189)	0.621
Direction preponderance	1.532 (0.466-5.035)	0.483
Quantitative posturography risk index	1.002 (0.985-1.020)	0.792
Low posturography risk index	0.929 (0.283-3.046)	0.903
High posturography risk index	1.651 (0.415-6.569)	0.477

Conclusions

1. Standard clinical neuro-otologic test and computer posturography did not give any specific diagnostic information in cases of different types of Meniere's disease. When performing videonystagmography, altered caloric response was observed more frequently in case of a definite Meniere's disease, and DP index was more significant to diagnose Meniere's disease in general.
2. General changes in 3T MR images of the temporal bone (asymmetric contrast enhancement, endolymphatic hydrops) were more frequently observed in case of a definite Meniere's disease (90.9% compared to 64% in case of a probable Meniere's disease). Endolymphatic hydrops was diagnosed twice as often in case of a definite Meniere's disease (76.4%), and endolymphatic hydrops was more frequently absent than present in case of a probable Meniere's disease.
3. Changes in the endolymphatic system of the temporal bone observed in 3T MR imaging, and Directional preponderance in videonystagmography caloric testing are two independent statistically significant factors when diagnosing Meniere's disease. These independent factors are better predictors of Meniere's disease when they act in a model compared to being separate (p=0.022 and p=0.003, respectively). Changes in the extent of the endolymphatic hydrops observed in MR images depend on the clinical course of the disease. The higher degree of endolymphatic hydrops, the more frequent attacks of vertigo episodes and the longer duration of the disease are (p=0.047). No relation between the clinical course of the disease (duration of the disease and frequency of episodes) and pure-tone audiometry and caloric testing of VNG were calculated.

4. Based on the changes in the 3t mr images of the temporal bone, endolymphatic hydrops observed on images can be classified into two grades. Grade I EH was more frequently observed in case of a probable Meniere's disease, grade II EH was more common in case of a definite Meniere's disease ($p<0.001$). Grade II endolymphatic hydrops observed on 3T MR images of the temporal bones increases the possibility of a definite Meniere's disease diagnosis.
5. Visualization of the endolypmhatic system on the 3T MR images of the temporal bone together with videonystagmography have a bigger significance when diagnosing Meniere's disease, and provide with more specific data compared with other tests of peripheral vestibular system and balance.

Recommendations

Based in the results of the study, its interpretation and comparison with data obtained by other authors, we formulated these practical recommendations:

1. When suspecting Meniere's disease, patient should undergo caloric test of the videonystagmography as it provides with more information about the vestibular function.
2. In case of a Meniere's disease, oto-neurologist should perform provocative manoeuvres and observe for the development of a secondary benign paroxysmal positional vertigo.
3. In case of a suspected Meniere's disease, patient should undergo delayed contrast enhancement 3T MR imaging of the temporal bone with Gd-based contrast agent as it improves diagnosis and differentiation of the disease, and based on the grade of the endolymphatic hydrops enables to establish type and severity of the disease, as well as assess course of the disease.
4. 3T MR imaging of the temporal bone should be performed based on the specific preparation and performance technique:
   a) Dosage of the intravenous Gd-based contrast agent – 0.1 mml/kg;
   b) MR imaging should be performed 4 to 6 hours after an intravenous injection of contrast agent;
   c) Scans should be perfomed using 3 tesla MRI system;
   d) Imaging protocols should include 3D-FLAIR and 3D T2DRIVE sequences, and anatomical 3D T1, T2 and DWI sequences to exclude possible central nervous system pathology;
   e) All images should be assessed by an experienced neuro-radiologist.
5. Based on the MR images, we recommend to classify endolymphatic hydrops of the vestibular space as follows:

a) Normal vestibule – the added surface areas of the saccule and utricle are less than half the area of the vestibule at the midmodiolar level;
b) Grade I vestibular hydrops – distention of the endolymph space of the saccule or utricle or both, with the perilymphatic space still visible along the periphery of the bony vestibule;
c) Grade II vestibular hydrops – the bony vestibule is entirely encompassed by the dilated endolymphatic spaces.

SANTRAUKA

Įvadas

Menjero liga (ML) apibrėžiama kaip lėtinė patologinė vidinės ausies liga, kuriai būdinga tam tikrų simptomų visuma: ūminiai sisteminio pobūdžio galvos svaigimo priepuoliai, ūžesys pažeistoje ausyje (ar ausies užgulimas) ir įvairaus laipsnio klausos pažeidimas. Tikslus šios ligos išsivystymo mechanizmas nėra aiškus, tačiau histopatologinėmis studijomis įrodyta nenuginčijama sąsaja su vidinės ausies plėvinio labirinto (endolimfos) vandene. Plėvinio labirinto vandenė apibūdinama kaip endolimfinės ertmės išsiplėtimas į perilimfinę ertmę [Gürkov ir kt., 2016].

Dažniausiai pažeidžiamos struktūros yra sraigės latakas ir prieangio apvalusis maišelis, tačiau vandenė gali apimti pailgąjį maišelį ir pusratinius kanalus [Merchant, 1995].

Epidemiologinių tyrimų duomenimis, dėl metodologinių šių tyrimų skirtumų, diagnostikos kriterijų kaitos ir skirtingų tirtų populiacijų sergamumo ML ribos yra labai plačios: nuo 34 atvejų iki 190 atvejų šimtui tūkstančių gyventojų [Tyrell, 2014; Shojaku, 2005]. Nors liga nėra dažna, tačiau svarbu gebėti diferencijuoti ją nuo kitų galvos svaigimą ir klausos sutrikimus sukeliančių ligų. 40–60 procentų ambulatoriškai į gydymo įstaigas dėl galvos svaigimo besikreipiančių pacientų nustatomas periferinis vestibulinis pažeidimas. Menjero liga yra antra dažniausia periferinio vestibulinio pažeidimo priežastis ir sukelia apie 10 procentų sisteminio galvos svaigimo atvejų [Jautužis ir kt., 2015]. Ilgą laiką ML diagnozės patvirtinimas daugiausia rėmėsi klinikiniais kriterijais, nes aptikti labirinto vandenę buvo įmanoma tik atlikus pomirtinį ligonio tyrimą. Dabar Menjero ligos diagnozė grindžiama tarptautinės Barany otoneurologų draugijos 2015 m. patobulintais klinikiniais kriterijais [Lopez-Escamez, 2016; Lopez-Escamez, 2015]. Pagal šiuos kriterijus – svaigimo priepuolio pobūdį, kintančius klausos pažeidimo simptomus bei audiometrijos duomenis – skiriama dviejų tipų Menjero liga: tikėtina arba neabejotina. Klausos pažeidimas yra reikšmingas ML požymis, o audiometrija yra vienintelis instrumentinis, bet nespecifinis tyrimas šiai ligai nustatyti. Ligos pradžioje klausos sutrikimas gali būti lengvas, pacientai patys nesupranta ir nenurodo jo kaip nusiskundimo, ypač esant svaigimui ar ūžesiui [Vassiliou, 2011]. Kai ligos simptomai nėra įprasti, ML diagnostika tampa sudėtingesnė ir nuo ligos simptomų pradžios iki ligos patvirtinimo praeina mėnesiai ar net metai [Zhang, 2016]. Taigi, toninė ribinė audiograma (TRA) yra būtinas tyrimas įtariant ML, tačiau jis nėra specifinis ir bet kurio laipsnio sutrikimas, registruojamas

audiogamoje, gali būti ir kitos ligos priežastis. Sutartinai priimtų ML patvirtinančių diagnostinių instrumentinių tyrimų nėra, ir kol kas ML diagnozę yra sunku objektyvuoti. Vis dėlto sparčiai tobulėjant diagnostikai klinikinėje praktikoje taikoma vis daugiau instrumentinių ir vaizdinių tyrimų, kurie leidžia tiksliau nustatyti ligos diagnozę, tačiau stingant tyrimų jie nėra įtraukti į ML patvirtinančius kriterijus. Šio mokslinio darbo tikslas – rasti naujų objektyvių šiuolaikinių Menjero ligos diagnostikos galimybių.

1.1. Darbo tikslas

Nustatyti periferinės vestibulinės sistemos, pusiausvyros ir vaizdinių tyrimų reikšmę diagnozuojant Menjero ligą.

1.2. Darbo uždaviniai

1. Įvertinti kompleksinio (klinikinio ir otoneurologinio instrumentinio) periferinės vestibulinės sistemos tyrimo rezultatus esant skirtingoms Menjero ligos formoms.
2. Įvertinti smilkinkaulių 3T magnetinio rezonanso tomografijos vaizdų pokyčius esant skirtingoms Menjero ligos formoms.
3. Nustatyti periferinės vestibulinės sistemos instrumentinių ir vaizdinių tyrimų prognostinę vertę diagnozuojant Menjero ligą.
4. Remiantis smilkinkaulių 3T magnetinio rezonanso tomografijos vaizdų pokyčių duomenimis, pasiūlyti jų vertinimo sistemą Menjero ligos atveju.
5. Nustatyti tyrimus, kurie turi didesnę reikšmę ir kuriuos tikslinga atlikti diagnozuojant Menjero ligą.

1.3. Mokslinis darbo naujumas

Nustatyti būdingi videonistagmometrijos (VNG) kalorinio mėginio tyrimo rezultatai ir prognostinės reikšmės sergant neabejotina ir tikėtina Menjero liga. Įvertinti vėlyvo kontrastinės medžiagos kaupimo smilkinkaulių 3T magnetinio rezonanso tomografijos (MRT) rezultatai ir nustatyta jų klinikinė reikšmė diagnozuojant Menjero ligą. Pasiūlyta vėlyvo kontrastinės medžiagos kaupimo smilkinkaulių 3T MRT vaizduose matomos vandenės gradavimo sistema, jos reikšmė bei pritaikymas klinikinėje praktikoje diagnozuojant, diferencijuojant ir prognozuojant Menjero ligą.

Iki šiol lietuviškoje mokslinėje medicinos literatūroje duomenų apie objektyvius periferinės vestibulinės sistemos tyrimus ir jų reikšmę esant Menjero ligai nebuvo, kaip ir nebuvo duomenų apie šios ligos diferencijavimą į tipus. Pirmą kartą Lietuvoje Menjero ligos diagnozei nustatyti ir ligai vertinti panaudota vėlyvo kontrastinės medžiagos kaupimo smilkinkaulių 3T MRT bei pasiūlyta labirintų vandenės vertinimo sistema.

1.4. Praktinė darbo reikšmė

Įdiegtas naujas, iki šiol Lietuvoje netaikytas tyrimas – vėlyvo kontrastinės medžiagos kaupimo smilkinkaulių 3T MRT ir įvertinta jo nauda diferencijuojant periferinės vestibulinės funkcijos sutrikimus. Tyrimas yra gana naujas visame pasaulyje, rutiniškai nėra atliekamas, nes techniškai vizualizuoti ir vertinti smulkias vidinės ausies struktūras yra sudėtinga. Atliekant tyrimą į sisteminę kraujotaką suleidžiama gadolinio kontrastinės medžiagos, praėjus 6 val. po suleidimo tiriamasis specialiu režimu skenuojamas 3T MRT aparatu ir vertinamos atitinkamos sekos. Tyrimo vaizdai vertinami tik patyrusių neuroradiologų. Remiantis 2014 m. publikuota šveicarų gradavimo sistema parengtos labirintų vandenės vertinimo rekomendacijos. Praktiniai pasiūlymai įdiegti Vilniaus universiteto ligoninės Santaros klinikų Ausų, nosies, gerklės ligų klinikoje. Rekomendacijų taikymas leidžia pagerinti sudėtingą Menjero ligos diagnostiką ir prognozuoti šios ligos eigą.

1.5. Hipotezė

Iškelta hipotezė, kad vėlyvo kontrastinės medžiagos kaupimo smilkinkaulių 3T MRT matoma vandenė yra labiausiai specifinis instrumentinis tyrimas diagnozuojant tikėtiną ir neabejotiną Menjero ligą.

1.6. Tyrimo programa

Atsižvelgiant į darbo tikslus ir uždavinius sudaryta tyrimo programa, apimanti keletą etapų:

1. Tiriamųjų atranką pagal pasirinktus Menjero ligai būdingus klinikinius diagnostinius kriterijus;
2. Tiriamųjų suskirstymą pagal tarptautinės Barany otoneurologų draugijos 2015 m. rekomenduotas Menjero ligos formas;
3. Tiriamųjų klinikinį ir instrumentinį periferinės vestibulinės funkcijos ir pusiausvyros ištyrimą;
4. Vėlyvo kontrastinės medžiagos kaupimo smilkinkaulių 3T MRT atlikimą ir įvertinimą;
5. Tyrimų rezultatų įvertinimą ir palyginimą.

2. Tyrimo metodika

Mokslinis darbas atliktas 2015–2019 metais Vilniaus universiteto ligoninės Santaros klinikų Ausų, nosies, gerklės ir akių ligų klinikoje. Tyrimui atlikti gautas Vilniaus regiono Biomedicininių tyrimų etikos komiteto leidimas Nr. 158200-17-914-424.

2.1. Tiriamųjų atranka

Buvo tiriami 225 pacientai, kurie planine tvarka atsiųsti į VUL Santaros klinikas įtarus Menjero ligą. Visi pacientai buvo tirti esant ligos remisijai.

Visiems tiriamiesiems pagal 2015 m. tarptautinės Barany otoneurologų draugijos parengtas ML klinikinės diagnostikos rekomendacijas buvo atliekamas pagrindinis klinikinis ligonių tyrimas, kurį sudarė:

- išplėstinė anamnezė (svaigimas: svaigimo pobūdis (sisteminis, nesisteminis), svaigimo priepuolio trukmė, svaigimo priepuolių dažnis; gretutiniai simptomai: pykinimas, vėmimas, prakaitavimas, viduriavimas; klausos simptomai: ūžesys, ausies užgulimas, pilnumo jausmas ausyje, klausos nuolatinis ar kintamas sutrikimas; svaigimo ir klausos sutrikimo ryšys; liekamieji reiškiniai: nėra, klausos sutrikimas, nestabilumas; ligos trukmė; esami sveikatos sutrikimai, gretutinės ligos, vartojami vaistai);
- audiologinis ištyrimas (otoskopija, timpanometrija, toninė ribinė audiometrija). Audiogramoje buvo vertinamas klausos pažeidimo vidurkis, oro-kaulo intervalas ir neurosensorinio prikurtimo laipsnis. Vertinant klausos pažeidimo laipsnį, buvo apskaičiuojama klausos slenksčio vidutinė reikšmė (vertinant tris garso dažnių tyrimo rezultatus – 500, 1000, 2000 Hz). Klausos pažeidimas buvo suskirstytas taip: klausos slenksčiui esant iki 25 dB – klausa normali, 26–40 dB – vidutinis klausos pažeidimas, 41–60 dB – ryškus, 61–80 dB – gilus. Pažeistos ausies audiograma buvo vertinama ir pagal tarptautinės Barany otoneurologų draugijos parengtus Menjero ligos diagnostikos kriterijus: žemo dažnio neurosensorinis prikurtimas nustatytas, kai, toninės slenkstinės audiometrijos duomenimis, kaulinio laidumo klausos slenksčiai pažeistoje ausyje yra didesni daugiau nei 30 dB lyginant su priešingos ausies klausos slenksčiais dviem gretimiems dažniams, esantiems žemiau nei 2000 Hz (250 Hz, 500 Hz, 1000 Hz). Abipusio žemo dažnio neurosensorinio prikurtimo atveju abiejų ausų kaulinio laidumo klausos slenksčiai dviem gretimiems dažniams, esantiems žemiau nei 2000 Hz, buvo 35 dB arba didesni [Lopez-Escamez ir kt., 2017]. Pagal tai audiograma buvo pavadinta informatyvia TRA (vienpusis neurosensorinis ar mišrus klausos pažeidimas, būdingas ML) ir neinformatyvia TRA (normali, simetrinis neurosensorinis ar simetrinis mišrus klausos pažeidimas, nebūdingas ML).

Pagrindiniai tiriamųjų įtraukimo kriterijai:

- bent vienas svaigimo epizodas su klausos sutrikimo simptomais (kintamais, nuolatiniais);
- ligos remisija (nėra ūminio svaigimo);
- vyraujantys klausos simptomai (kintami, nuolatiniai);
- normalus otoskopinis vaizdas.

Pagrindiniai tiriamųjų atmetimo kriterijai:

- esamos ar buvusios išorinės bei vidurinės ausies ligos (išorinis otitas, otosklerozė, timpanosklerozė, tubotimpaninis otitas, atikoantralinis otitas, būgnelio traumos, ausų operacijos ir kt.);

- autoimuninės ligos;
- nervų, psichikos ligos

Į tyrimą buvo įtraukti 105 tiriamieji, kurie atitiko ML klinikinės diagnostikos ir mūsų pasirinktus įtraukimo ir atmetimo kriterijus. Pagal gautus tyrimų rezultatus, remiantis tarptautinės Barany otoneurologų draugijos 2015 m. priimtais klinikinės diagnostikos kriterijais, tiriamieji buvo priskirti tikėtinos arba neabejotinos ML grupei.

Neabejotinos ML grupei priskirti tiriamieji, kurie atitiko šiuos kriterijus:

(a) du ar daugiau spontaninio galvos svaigimo epizodų, trunkančių nuo 20 minučių iki 12 valandų;

(b) žemo vidutinio dažnio neurosensorinis klausos sutrikimas vienoje ausyje, dokumentuotas audiologiškai, atitinkantis pažeistą ausį, prieš, per galvos svaigimo epizodą ar po jo;

(c) fliuktuojantys klausos simptomai (klausos sutrikimas, ūžesys ar pilnumo jausmas) pažeistoje ausyje;

(d) neatitinka kitos pusiausvyros sutrikimo diagnozės.

Tikėtinos ML grupei priskirti tiriamieji, kurie atitiko visus minėtus kriterijus, išskyrus (b).

Neabejotinos ir tikėtinos ML grupių tiriamųjų klinikinis ir instrumentinis periferinės vestibulinės funkcijos ir pusiausvyros ištyrimas:

- Vertinta vestibulospinalinė funkcija (atliekant Rombergo, žingsniavimo bei tandeminės eisenos mėginius);
- Vertintas spontaninis nistagmas (SNy) uždėjus Frenzelio akinius;
- Atlikta instrumentinė videonistagmometrija (VNG):
- vertintas SNy;
- vertintas vestibulookulinis refleksas, atliekant galvos staigaus pasukimo mėginį (HIT);
- vertintas provokacinis nistagmas (Ny), atliekant galvos papurtymo mėginį, ir pozicinis nistagmas, atliekant provokacinius mėginius;
- vertinti vienpusio susilpnėjimo (UW) ir krypties vyravimo (DP) rodikliai atliekant VNG kalorinį mėginį.
- Atlikta kompiuterinė posturometrija tiriamųjų griuvimo rizikos indeksui (GRI) įvertinti.

2.2. Vaizdininių tyrimų atlikimas ir vertinimas

Buvo atliekamas vėlyvo kontrastinės medžiagos kaupimo smilkinkaulių 3T magnetinio rezonanso tomografijos (MRT) tyrimas. Vaizdai vertinti praėjus 4–6 val. po gadolinio pagrindo kontrastinės medžiagos sušvirkštimo į veną (gadobutrolio (*Gadovist, Bayer*, Berlynas, Vokietija) įprasta 0,1 mmol/kg dozė). Buvo lyginami sveikos ir pažeistos ausies MRT vaizdai, kreipiamas dėmesys į labirintų asimetriją, matuojamas endolimfinis tarpas, vertinama labirintų vandenė ir jos laipsniai.

Visi vaizdiniai tyrimai atlikti 3 teslų MRT sistema (*Achieva, Philips*, Bestas, Nyderlandai), naudojant 16 kanalų galvos ritę. Vaizdinimo protokolą sudarė 3D FLAIR ir 3D T2DRIVE sekos, taip pat anatominės 3D T1, T2 ir DWI sekos galimai centrinės nervų sistemos patologijai atmesti. Jeigu tiriamajam neseniai (t. y. trijų mėnesių laikotarpiu) buvo atliktas galvos smegenų MRT tyrimas, taikytas sutrumpintas tyrimo protokolas (3D FLAIR ir 3D T2DRIVE). Parinkti tokie 3D FLAIR sekos parametrai: pakartojimo laikas (TR) 4800 ms, atsako laikas (TE) 283 ms, inversijos laikas (TI) 1650 ms, matricos dydis 224 x 224, apžvalgos laukas (FOV) 250 mm, pjūvio storis 0,7 mm, vaizdinimo laikas 9,5 min. Visi vaizdai kokybiškai vertinti dviejų radiologų: vieno patyrusio neuroradiologo (14 metų darbo stažas) ir vieno radiologijos rezidento. Kiekvienu atveju T2DRIVE ir 3D FLAIR vaizdai vertinti greta, T2DRIVE seka naudota kaulinėms riboms atskirti, 3D FLAIR seka – perilimfai vaizdinti. FLAIR vaizduose nekaupiančios kontrastinės medžiagos prieangio zonos, kurios T2DRIVE vaizduose yra didelio signalo intensyvumo, interpretuotos kaip endolimfos struktūros. Vertinta tik prieangio endolimfos vandenė. Prieangio endolimfinio tarpo (vieno ar abiejų) išsiplėtimas vertintas kaip nesantis, I laipsnio (mažas) ar II laipsnio (didelis), remiantis K. Baratho ir bendraautorių pateikta gradavimo sistema [Barath ir kt., 2014]. Normos atveju suminis apvaliojo ir pailgojo maišelių plotas yra mažesnis nei pusė prieangio ploto ties ausies sraigės ritės (*modiolus*) viduriu. I lapsnio EV nustatyta, kai endolimfinis tarpas pailgojo ar apvaliojo ar abiejų maišelių sąskaita išsiplėtęs, o perilimfinis tarpas dar matomas kaulinio prieangio kanalo periferijoje; II laipsnio EV atveju kaulinis prieangis yra visiškai užimtas išsiplėtusių endolimfinių struktūrų.

3. Tyrimo rezultatai

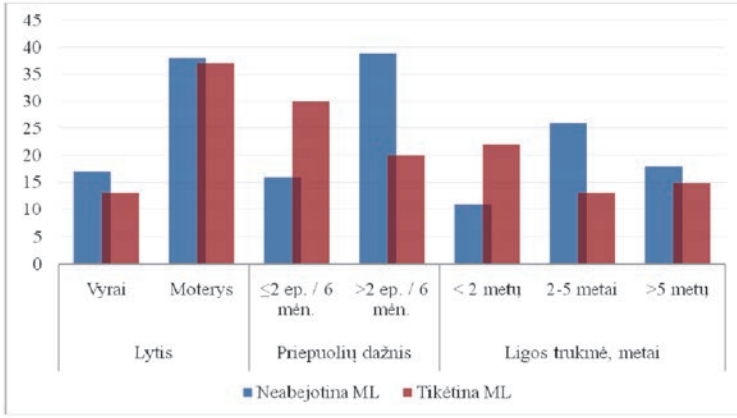
1.1. Bendra tiriamųjų charakteristika

Buvo ištirti 105 tiriamieji, iš jų 75 moterys (71,4 %) ir 30 vyrų (28,6 %). Tiriamųjų amžiaus pasiskirstymas buvo gana platus ir siekė nuo 23 iki 78 metų (vidurkis 55,8 ± 11,69) (1 lentelė, 1 pav.).

Neabejotina ir tikėtina ML nustatyta atitinkamai 55 (52,4 %) ir 50 (47,6 %) tiriamųjų. Abi tiriamųjų grupės buvo homogeniškos pagal lytį (p = 0,667) (1 lentelė, 1 pav.).

Priepuolių dažnis iki 2 epizodų per 6 mėn. buvo nustatytas 46 (43,8 %) tiriamiesiems, daugiau nei 2 epizodai per 6 mėnesius buvo užfiksuoti 59 (56,2 %) tiriamiesiems. Pacientai, sergantys neabejotina ML, patyrė statistiškai reikšmingai daugiau svaigimo priepuolių per šešių mėnesių laikotarpį, t. y. tiriamųjų, kurie patyrė daugiau nei 2 priepuolius per 6 mėnesius, neabejotinos ML ir tikėtinos ML grupėje buvo atitinkamai 39 (70,9 %) ir 20 (40,0 %) (p = 0,002) (1 lentelė, 1 pav.).

Trisdešimt trys (31,4 %) tiriamieji ML sirgo iki 2 metų, 39 (37,1 %) tiriamieji sirgo 2–5 metus ir likę 33 (31,4 %) – daugiau nei 5 metus. Ligos trukmė statistiškai reikšmingai skyrėsi tarp ML grupių. Tikėtinos ML grupėje 22 (44,0 %) tiriamųjų ligos trukmė buvo mažesnė nei 2 metai, o net 26 (47,3 %) neabejotinos ML grupės tiriamųjų ligos trukmė buvo 2–5 metai (p = 0,020). Tiek tikėtinos ML, tiek neabejotinos ML grupėse tiriamųjų, kuriems ligos trukmė buvo daugiau nei 5 metai, buvo beveik vienodai – atitinkamai 18 (32,7 %) ir 15 (30,0 %) (1 lentelė, 1 pav.).



1 PAV. Pagrindinės tiriamųjų charakteristikos pagal ML grupes

1 LENTELĖ. Tikėtinos ir neabejotinos ML grupių pagrindinių tiriamųjų charakteristikų palyginimas

		Bendrai N = 105 (100 %)	Neabejotina ML N = 55 (52,4 %)	Tikėtina ML N = 50 (47,6 %)	P reikšmė
Lytis	Vyrai	30 (28,6)	17 (30,9)	13 (26,0)	0,667
	Moterys	75 (71,4)	38 (69,1)	37 (74,0)	
Priepuolių dažnis	≤2 ep. / 6 mėn.	46 (43,8)	16 (29,1)	30 (60,0)	0,002
	>2 ep. / 6 mėn.	59 (56,2)	39 (70,9)	20 (40,0)	
Ligos trukmė	<2 metai	33 (31,4)	11 (20,0)	22 (44,0)	0,020
	2–5 metai	39 (37,1)	26 (47,3)	13 (26,0)	
	>5 metai	33 (31,4)	18 (32,7)	15 (30,0)	
Amžius	Vidurkis ± SN Mediana Q1–Q3 min–max	55,8 ± 11,69 58 49–64 23–78	56,3 ± 12,17 59 49–65 27–78	55,3 ± 11,24 54,5 48–64 23–75	0,685

ML – Menjero liga; ep. – epizodai; mėn. – mėnesiai; SN – standartinis nuokrypis; Q1 – pirmasis kvartilis (arba 25 % kvantilis); Q3 – trečiasis kvartilis (arba 75 % kvantilis); min – minimali reikšmė; max – maksimali reikšmė

Vestibulospinalinė funkcija (Rombergo, žingsniavimo ir tandeminės eisenos mėginiai) visiems 105 tiriamiesiems buvo nesutrikusi. Negautas ir spontaninis nistagmas vertinant su Frenzelio akiniais. Galvos staigaus pasukimo ir galvos papurtymo mėginiai buvo neigiami. Pozicinis nistagmas atliekant provokacinius Dix–Hallpike mėginius buvo išprovokuotas penkiems tiriamiesiems.

3.2. Audiologiniai rezultatai pagal ML tipus

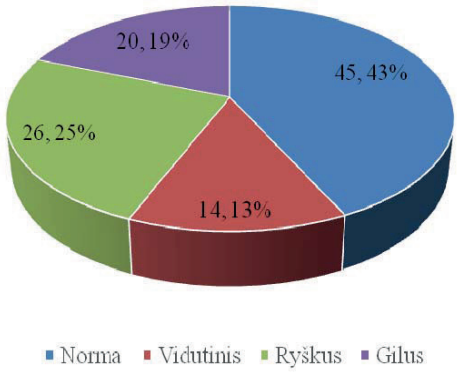
Visiems 105 (100,0 %) tiriamiesiems buvo atliktos toninės ribinės audiogramos (TRA). Iš viso nustatytos 72 (68,6 %) informatyvios TRA ir 33 (31,4 %) neinformatyvios TRA. Informatyvių TRA tiriamiesiems, turintiems neabejotiną ML, buvo du kartus daugiau nei tikėtinos ML grupės tiriamiesiems – atitinkamai 50 (90,9 %) ir 22 (44,0 %) ($p < 0,001$). Tačiau lyginant TRA pasiskirstymą ML grupėse matomas itin didelis informatyvių TRA procentas neabejotinos ML grupėje – 50 (90,9 %), palyginti su 5 (9,1 %) neinformatyvių. Tikėtinos ML grupėje informatyvios ir neinformatyvios TRA pasiskirsčiusios panašiai – atitinkamai 22 (44,0 %) ir 28 (56,0 %) (2 lentelė).

Klausos pažeidimo laipsnio pasiskirstymas vaizduojamas 2 pav. Didžiausias klausos pažeidimo laipsnis buvo nustatytas neabejotinos ML grupės tiriamiesiems: 38 (69,1 %) neabejotinos ML grupės tiriamųjų turėjo ryškų arba gilų klausos pažeidimą, o tikėtinos ML grupėje tokie klausos pažeidimai nustatyti tik 8 (16 %) tiriamiesiems ($p < 0,001$) (2 lentelė).

2 LENTELĖ. Toninės ribinės audiogramos rezultatų palyginimas tarp ML grupių

		Bendrai N = 105 (100 %) N (%)	Neabejotina ML N = 55 (52,4 %) N (%)	Tikėtina ML N = 50 (47,6 %) N (%)	P reikšmė
TRA	Informatyvi	72 (68,6)	50 (90,9)	22 (44,0)	< 0,001
	Neinformatyvi	33 (31,4)	5 (9,1)	28 (56,0)	
Klausos pažeidimo laipsnis	Norma	45 (42,9)	9 (16,4)	36 (72,0)	< 0,001
	Vidutinis	14 (13,3)	8 (14,5)	6 (12,0)	
	Ryškus	26 (24,8)	21 (38,2)	5 (10,0)	
	Gilus	20 (19,0)	17 (30,9)	3 (6,0)	

Tiek TRA, tiek klausos pažeidimo laipsnis tarp priepuolių dažnio grupių yra pasiskirstęs homogeniškai (atitinkamai $p = 0,298$ ir $p = 0,195$). Visgi iš 2 lentelės matyti, kad informatyvią TRA šiek tiek daugiau turi tiriamųjų, kuriems priepuolių dažnis didesnis: atitinkamai 29 (63,0 %) – kai priepuolių dažnis ≤2 epizodai per 6 mėn., ir 43 (72,9 %) – kai šis dažnis >2 epizodai per 6 mėn. (3 lentelė).



2 PAV. Klausos pažeidimo laipsnis visoje tiriamųjų imtyje

3 LENTELĖ. Toninės ribinės audiogramos rezultatų palyginimas tarp priepuolių dažnio

		≤ 2 ep. / 6 mėn. N = 46 (43,8 %) N (%)	> 2 ep. / 6 mėn. N = 59 (56,2 %) N (%)	P reikšmė
TRA	Informatyvi	29 (63,0)	43 (72,9)	0,298
	Neinformatyvi	17 (37,0)	16 (27,1)	
Klausos pažeidimo laipsnis	Norma	25 (54,4)	20 (33,9)	0,195
	Vidutinis	5 (10,9)	9 (15,3)	
	Ryškus	8 (17,4)	18 (30,5)	
	Gilus	8 (17,4)	12 (20,3)	

4 LENTELĖ. Toninės ribinės audiogramos rezultatų palyginimas tarp ligos trukmės grupių

		< 2 metai N = 105 (100%) N (%)	2–5 metai N = 55 (52,4%) N (%)	> 5 metai N = 50 (47,6%) N (%)	P reikšmė
TRA	Informatyvi	21 (63,6)	30 (76,9)	21 (63,6)	0,374
	Neinformatyvi	12 (36,4)	9 (23,1)	12 (36,4)	
Klausos pažeidimo laipsnis	Norma	16 (48,5)	13 (33,3)	16 (48,5)	0,196
	Vidutinis	7 (21,2)	3 (7,7)	4 (12,1)	
	Ryškus	6 (18,2)	11 (28,2)	9 (27,3)	
	Gilus	4 (12,1)	12 (30,8)	4 (12,1)	

Informatyvi TRA sudarė apie du trečdalius lyginant pagal kiekvieną ligos trukmės intervalą: atitinkamai 21 (63,6 %), 30 (76,9 %) ir 21 (63,6 %), kai liga truko <2 metus, 2–5 metus ir >5 metus. Gilus klausos pažeidimas labiau vyravo ligos trukmei esant 2–5 metų: 4 (12,1 %), 12 (30,8 %) ir 4 (12,1 %) gilaus pažeidimo atvejai, kai liga truko atitinkamai <2 metus, 2–5 metus ir >5 metus. Visgi analizė parodė, kad TRA ir klausos pažeidimo laipsnis buvo nepriklausomi nuo ligos trukmės, t. y. ligos trukmė neturėjo įtakos TRA informatyvumui ar klausos pažeidimo laipsniui (atitinkamai p = 0,374 ir p = 0,196) (4 lentelė).

3.3. Videonistagmometrijos rezultatai

Atliekant videonistagmometriją nė vienam iš 105 tiriamųjų negautas spontaninis nistagmas, nebuvo sutrikęs VOR (HIT mėginys), visiems tiriamiesiems buvo neigiami poziciniai bei 5 tiriamiesiems buvo išprovokuotas Dix–Hallpike mėginys.

Daugiau nei pusei tiriamųjų – 67 (63,8 %) – VNG kalorinio mėginio rezultatai buvo pakitę (t. y. UW% ir DP% >25 %). Pakitę šio mėginio rezultatai gauti 42 (76,4 %) tiriamiesiems, sergantiems neabejotina ML, ir 25 (50,0 %) tiriamiesiems, sergantiems tikėtina ML (p = 0,008). Nors neabejotinos ML grupėje, priešingai nei tikėtinos ML grupėje, vyravo vienpusio susilpnėjimo rodikliai (UW%), tačiau statistiškai reikšmingo skirtumo negauta – atitinkamai 32 (58,2 %) ir 23 (46,0 %) (p = 0,244). Krypties vyravimo (DP%) pasiskirstymas tarp ML grupių statistiškai reikšmingai skyrėsi: neabejotinos ML grupėje 18 (32,7 %) turėjo DP% >25 %, o tikėtinos ML grupėje – tik 6 (12,0 %) (p = 0,019) (5 lentelė).

Vienpusio susilpnėjimo bei krypties vyravimo pasiskirstymas pagal pažeidimų puses pavaizduotas 3 paveiksle.

Pakitęs kalorinis mėginys buvo tiriamiesiems, kurių priepuolių dažnis buvo >2 epizodai ir ≤ 2 epizodai per 6 mėnesius, atitinkamai 43 (72,9 %) ir 24 (52,2 %) (p = 0,040). Tačiau

5 LENTELĖ. Kalorinio mėginio rezultatų palyginimas tarp ML grupių

		Bendrai N = 105 (100 %) N (%)	Neabejotina ML N = 55 (52,4 %) N (%)	Tikėtina ML N = 50 (47,6 %) N (%)	P reikšmė
Pakitęs kalorinis mėginys	Taip	67 (63,8)	42 (76,4)	25 (50,0)	0,008
	Ne	38 (36,2)	13 (23,6)	25 (50,0)	
UW%	Taip	55 (52,4)	32 (58,2)	23 (46,0)	0,244
	Ne	50 (47,6)	23 (41,8)	27 (54,0)	
DP%	Taip	24 (22,9)	18 (32,7)	6 (12,0)	0,019
	Ne	81 (17,1)	37 (67,3)	44 (88,0)	

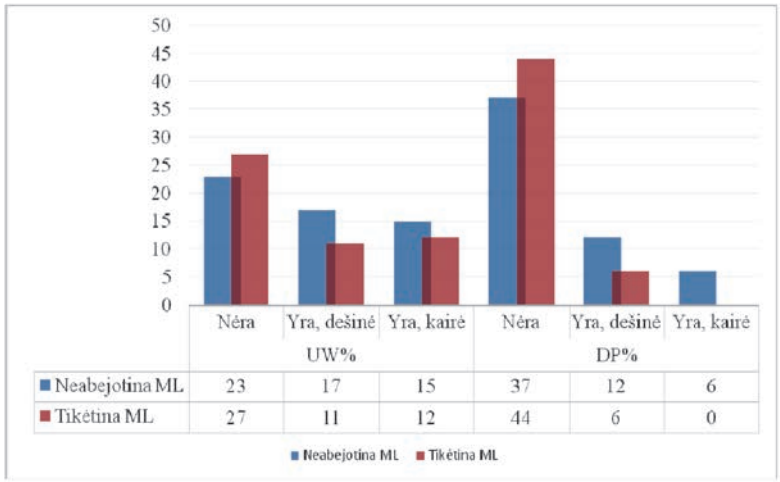
vienpusio susilpnėjimo ir krypties vyravimo rodikliai nepriklausė nuo priepuolių dažnio (atitinkamai p = 0,698 ir p = 0,109) (6 lentelė).

6 LENTELĖ. Svaigimo priepuolių dažnio palyginimas pagal kalorinio mėginio rezultatus

		≤2 ep. / 6 mėn. N=46 (43,8 %) N (%)	>2 ep. / 6 mėn. N=59 (56,2 %) N (%)	P reikšmė
Pakitęs kalorinis mėginys	Taip	24 (52,2)	43 (72,9)	0,040
	Ne	22 (47,8)	16 (27,1)	
UW%	Taip	23 (50,0)	32 (54,2)	0,698
	Ne	23 (50,0)	27 (45,8)	
DP%	Taip	7 (15,2)	17 (28,8)	0,109
	Ne	39 (84,8)	42 (71,2)	

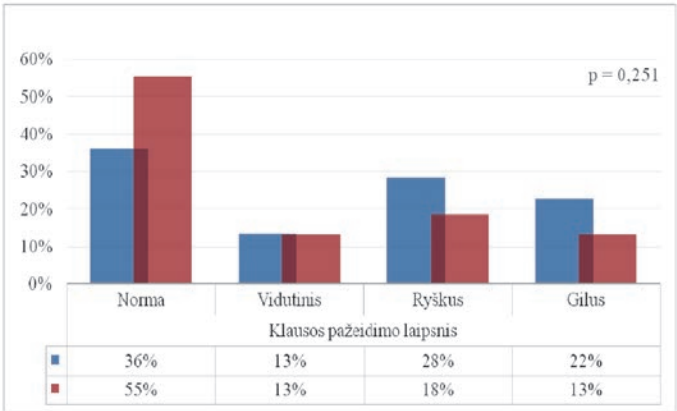
Daugiausia pakitusių kalorinių mėginių rasta esant 2–5 metų ligos trukmei – 29 (74,4 %), mažiausia – 17 (51,5 %), esant > 5 metų ligos trukmei, tačiau statistiškai reikšmingo skirtumo tarp ligos trukmės ir pakitusio kalorinio mėginio rezultatų nenustatyta (p = 0,143) (7 lentelė).

Vienpusio susilpnėjimo ir krypties vyravimo rodikliai taip pat buvo nepriklausomi ir homogeniškai pasiskirstę tarp ligos trukmės grupių (atitinkamai p = 0,168 ir p = 0,591) (7 lentelė).



3 PAV. VNG kalorinio mėginio rezultatai pagal ML grupes

Esant ryškiam arba giliam klausos pažeidimui, dažniau buvo pastebimas pakitęs kalorinis mėginys – atitinkamai 50 % ir 31 %. Be to, matoma tokia tendencija: jei tiriamiesiems klausos pažeidimo laipsnis buvo įvertintas kaip norma, tai jų kalorinio mėginio rezultatas yra linkęs būti nepakitęs. Apibendrinant teigtina, kad nors klausos pažeidimo laipsniai nėra vienodai pasiskirstę pagal kalorinio mėginio rezultatus, visgi statistiškai reikšmingo skirtumo nebuvo rasta (p = 0,251) (4 pav.).



4 PAV. VNG kalorinio mėginio rezultatai pagal klausos pažeidimo laipsnį

7 LENTELĖ. Ligos trukmės palyginimas pagal kalorinio mėginio rezultatus

		< 2 metai N = 105 (100 %) N (%)	2–5 metai N = 55 (52,4 %) N (%)	> 5 metai N = 50 (47,6 %) N (%)	P reikšmė
Pakitęs kalorinis mėginys	Taip	21 (63,6)	29 (74,4)	17 (51,5)	0,143
	Ne	12 (36,4)	10 (25,6)	16 (48,5)	
UW%	Taip	16 (48,5)	25 (64,1)	14 (42,4)	0,168
	Ne	17 (51,2)	14 (35,9)	19 (57,6)	
DP%	Taip	6 (18,2)	11 (28,2)	7 (21,2)	0,591
	Ne	27 (81,8)	28 (71,8)	26 (78,8)	

3.4. Posturometrijos tyrimo rezultatų palyginimas su menjero ligos tipu

Posturometrijos tyrimas buvo atliktas 94 (89,5 %) tiriamiesiems. Vertinant kompiuterinės posturografijos rezultatus gauta, kad 20 (21,3 %) tiriamųjų turi mažą griuvimo rizikos indeksą (GRI), 55 (58,5 %) – vidutinį, 19 (20,2 %) – didelį. Statistiškai reikšmingo skirtumo tarp GRI ir ML grupių nenustatyta (p = 0,883) (8 lentelė).

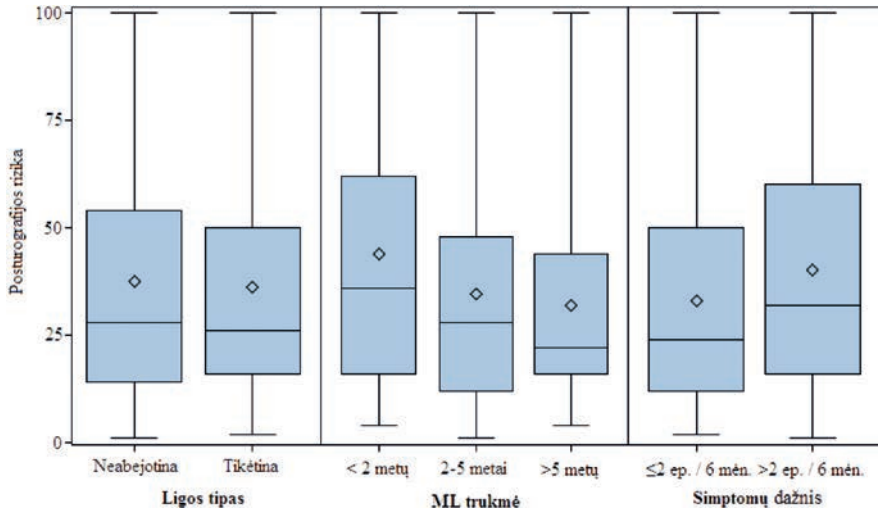
Kiekybiškai vertinant posturografijos rezultatus tarp ML, ML priepuolių dažnio bei ML trukmės grupių, statistiškai reikšmingų skirtumų negauta (9 lentelė, 5 pav.).

8 LENTELĖ. Posturografijos būdu nustatytos griuvimo rizikos palyginimas su ML tipu

		Bendrai N = 105 (100 %) N (%)	Neabejotina ML N = 55 (52,4 %) N (%)	Tikėtina ML N = 50 (47,6 %) N (%)	P reikšmė
Griuvimo rizika	Didelė	19 (20,2)	11 (22,5)	8 (17,8)	0,883
	Vidutinė	55 (58,5)	28 (57,1)	27 (60,0)	
	Maža	20 (21,3)	10 (20,4)	10 (22,2)	
	Nėra duomenų	11	6	5	

9 LENTELĖ. Griuvimo rizikos kiekybinis palyginimas pagal priepuolių dažnį ir ML trukmę

		Griuvimo rizika. Vidurkis (SN)	P reikšmė
Visa imtis		36,9 (27,86)	–
ML grupė	Neabejotina	37,5 (27,62)	0,827
	Tikėtina	36,2 (28,41)	
Priepuolių dažnis	≤ 2 ep. / 6 mėn.	33,0 (24,41)	0,222
	> 2 ep. / 6 mėn.	40,1 (30,32)	
ML trukmė	< 2 metai	43,9 (33,25)	0,215
	2–5 metai	34,7 (25,20)	
	> 5 metai	34,9 (23,64)	



5 PAV. Posturografijos tyrimu nustatytos griuvimo rizikos kiekybinis pasiskirstymas tarp ML, ML priepuolių dažnio ir ML trukmės grupių

3.5. 3T smilkinkaulių mrt rezultatų vertinimas pagal menjero ligos tipus

Vertinant pažeistą ausį, 82 (78,1 %) tiriamųjų nustatyti bendri MRT pokyčiai (įskaitant EV ir asimetrinį kontrastinės medžiagos kaupimą nesant EV). Neabejotinos ML grupės daugiau nei 90 % tiriamųjų fiksuoti bendri MRT pokyčiai, o tikėtinos ML grupės – 64,0 % (p < 0,001) (10 lentelė, 6 pav.).

Bendrai abiejose ML grupėse smilkinkaulių 3T MRT vaizduose EV nustatyta 61 (58,1 %) tiriamajam, iš jų I laipsnio EV – 40 (65,6 %) tiriamųjų, II laipsnio – 21 (34,4 %) tiriamajam, o kitiems 44 (41,9 %) tiriamiesiems EV nebuvo (10 lentelė, 6 pav.).

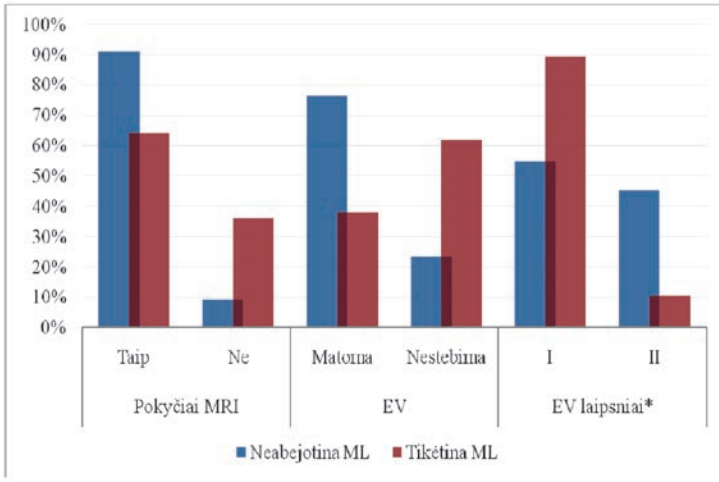
Smilkinkaulių 3T MRT vaizduose EV atvejų rasta statistiškai reikšmingai daugiau neabejotinos ML grupės nei tikėtinos ML grupės tiriamiesiems – atitinkamai 42 (76,4 %) ir 19 (38,0 %) (p < 0,001). I laipsnio EV tikėtinos ML grupės tiriamiesiems nustatyta gerokai dažniau nei abejotinos ML grupės – atitinkamai 17 (89,5 %) ir 23 (54,8 %) (p=0,009) (10 lentelė, 6 pav.).

10 LENTELĖ. Smilkinkaulių 3T MRT rezultatų palyginimas pagal ML tipus

		Bendrai N = 105 (100 %) N (%)	Neabejotina ML N = 55 (52,4 %) N (%)	Tikėtina ML N = 50 (47,6 %) N (%)	P reikšmė
Pokyčiai MRI	Taip	82 (78,1)	50 (90,9)	32 (64,0)	< 0,001
	Ne	23 (21,9)	5 (9,1)	18 (36,0)	
EV	Matoma	61 (58,1)	42 (76,4)	19 (38,0)	< 0,001
	Nestebima	44 (41,9)	13 (23,6)	31 (62,0)	
EV laipsniai*	I	40 (65,6)	23 (54,8)	17 (89,5)	0,009
	II	21 (34,4)	19 (45,2)	2 (10,5)	

*Procentai pateikiami nuo skaičiaus tiriamųjų, kuriems yra EV yra matoma. EV – endolimfos vandenė.

Analizuojant sąsajas tarp EV laipsnio ir ligos sunkumo pastebėta, kad kuo ilgesnė ligos trukmė, tuo didesnio laipsnio EV matoma MRT vaizduose (p=0,047): pacientai, kuriems nustatyta I laipsnio EV, pagal ligos trukmę pasiskirstė daugmaž vienodai, t. y. esant ligos trukmei < 2 metų, 2–5 metų ir > 5 metų tiriamieji pasiskirstė atitinkamai 13 (32,5 %), 14 (35,0 %) ir 13 (32,5 %). Tačiau tiriamųjų, kuriems rasta II laipsnio EV, statistiškai reikšmingai daugiau buvo ligos trukmei esant 2–5 metų – 11 (52,4 %), ar ilgesnei nei 5 metų – 9 (42,9 %) tiriamiesiems (p = 0,047). Svaigimo priepuolių dažnis, vertintas per pusmetį, taip pat buvo labai susijęs su EV laipsniu (p = 0,011): esant I laipsnio EV, galvos svaigimo epizodų dažnis pasiskirstė tolygiai, tačiau tiriamieji, kuriems rasta II laipsnio vandenė, dažniau patyrė > 2 svaigimo priepuolius per pusmetį (85,7 %) (11 lentelė).



6 PAV. Smilkinkaulių 3T MRT rezultatų palyginimas pagal ML grupes

11 LENTELĖ. EV ryšys su Menjero ligos trukme ir ligos sunkumu

		I laipsnio N = 40 (65,6 %) N (%)	II laipsnio N = 21 (34,4 %) N (%)	P reikšmė
Priepuolių dažnis	≤ 2 ep. / 6 mėn.	20 (50,0)	3 (14,3)	0,011
	> 2 ep. / 6 mėn.	20 (50,0)	18 (85,7)	
Ligos trukmė	< 2 metai	13 (32,5)	1 (4,8)	0,047
	2–5 metai	14 (35,0)	11 (52,4)	
	> 5 metai	13 (32,5)	9 (42,9)	
Klausos pažeidimo laipsnis	Norma	17 (42,5)	1 (4,8)	< 0,001
	Vidutinis	8 (20,0)	0 (0,0)	
	Ryškus	9 (22,5)	10 (47,6)	
	Gilus	6 (15,0)	10 (47,6)	

MRT vaizduose fiksuota EV 45 (42,9 %) atvejais atitiko informatyvią TRA, o 4 (3,8 %) atvejais EV pastebėta esant neinformatyviai TRA. Be to, 41 (39,0 %) tiriamojo MRT vaizdai atitiko normą, nors TRA buvo informatyvi. Sąsaja tarp EV MRT vaizduose ir informatyvios TRA buvo statistiškai reikšminga, ir 60 (57,0 %) tiriamųjų MRT metodu buvo tiksliai nustatyta, ar pažeidimas yra ar jo nėra (12 lentelė).

MRT tyrimo rezultatus lyginant su UW% VNG kaloriniuose mėginiuose, 56 (53,3 %) tiriamųjų rezultatai visiškai sutapo. Papildomai MRT leido identifikuoti 25 (23,8 %) tiriamuosius, turinčius MRT pakitimų, nors UW% tyrimas buvo neigiamas. MRT tyrimo rezultatus lyginant su DP% paaiškėjo, kad 41 (39,0 %) tiriamojo rezultatai visiškai sutapo. Papildomai MRT leido identifikuoti 44 (41,9 %) pakitimų atvejus, nors DP% tyrimo rezultatai pateko į normos ribas. Reikšminga atitiktis apskaičiuota tarp EV MRT vaizduose ir vienpusio klausos silpnumo (p < 0,001), tačiau MRT rezultatai ir krypties vyravimas kaloriniuose mėginiuose buvo nesuderinami (13 lentelė).

12 LENTELĖ. Smilkinkaulių 3T MRT vaizdų pasiskirstymas pagal audiometrijos rezultatus

			3T MRT fiksuota EV				P reikšmė
			AD N = 27 (25,7 %)	AS N = 25 (23,8 %)	AD/AS N = 6 (5,7 %)	Nenustatyta N = 47 (44,8 %)	
Audio-grama	Informatyvi	AS	1 (3,2)	16 (51,6)	1 (3,2)	13 (41,9)	< 0,001
		AD	21 (51,2)	2 (4,9)	3 (7,3)	15 (36,6)	
	Neinformatyvi	Simetrinis NSP	3 (13,0)	5 (21,7)	2 (8,7)	13 (56,5)	< 0,001
		Normali TRA	2 (20,0)	2 (20,0)	0 (0,0)	6 (60,0)	

AD – dešinė ausis; AS – kairė ausis; EV – endolimfos vandenė

13 LENTELĖ. Smilkinkaulių 3T MRT vaizdų pasiskirstymas pagal kalorimetrijos rezultatus

		AD N = 27 (25,7 %)	AS N = 25 (23,8 %)	AD/AS N = 6 (5,7 %)	Nenustatyta N = 47 (44,8 %)	P reikšmė
UW	AD	17 (60,7)	0 (0,0)	0 (0,0)	11 (39,3)	<0,001
	AS	0 (0,0)	14 (51,9)	2 (7,4)	11 (40,7)	
	Nenustatyta	10 (20,0)	11 (22,0)	4 (8,0)	25 (50,0)	
DP	AD	3 (16,7)	5 (27,8)	2 (11,1)	8 (44,4)	0,123
	AS	1 (16,7)	1 (16,7)	2 (33,3)	2 (33,3)	
	Nenustatyta	23 (28,4)	19 (23,5)	2 (2,5)	37 (47,5)	

AD – dešinė ausis; AS – kairė ausis; UW – vienpusis susilpnėjimas; DP – krypties vyravimas; EV – endolimfos vandenė

3.6. Prognostiniai veiksniai pagal menjero ligos tipą ir smilkinkaulių pažeidimą 3T MRT vaizduose

Taikant vienmatę logistinės regresijos analizę tik vienpusis susilpnėjimas VNG kalorimetrijoje buvo statistiškai nereikšmingas veiksnys prognozuojant ML tipą. Endolimfos vandenė MTR vaizduose ir II laipsnio EV MRT turėjo didžiausią įtaką neabejotinai ML diagnozuoti: šansų santykis (95 % PI) buvo atitinkamai 5,744 (2,458–13,422) (p <0,001) ir 7,022 (1,437–34,301) (p = 0,016) (14 lentelė). ROC analizė parodė, kad šie veiksniai leidžia tiksliausiai klasifikuoti ML tipą: plotas po kreive (95 % PI) buvo atitinkamai 0,702 (0,614–0,790) (p < 0,001) ir 0,674 (0,570–0,778) (p = 0,001) (7 pav.).

Visgi II laipsnio EV į tolesnės daugiamatės logistinės analizės modelį neįtraukta, nes buvo analizuota tik tiriamiesiems, kuriems buvo pasireiškusi endolimfos vandenė.

Daugiamatė žingsninės logistinės regresijos analizė parodė, kad fiksuotas pažeidimas MRT ir krypties vyravimas VNG kalorimetrijoje buvo nepriklausomi statistiškai reikšmingi veiksniai, nusakantys ML tipą: šansų santykis (95 % PI) – atitinkamai 6,603 (2,662–16,377) (p < 0,001) ir 4,553 (1,453–14,261) (p = 0,009) (14 lentelė). ROC analizės duomenimis, šie nepriklausomi veiksniai (fiksuotas pažeidimas MRT ir krypties vyravimas VNG kalorimetrijoje) veikdami kaip modelis leidžia geriau prognozuoti ML tipą nei tie patys veiksniai atskirai (atitinkamai p = 0,022 ir p = 0,003). Modelio plotas po ROC kreive siekė (95 % PI) 0,747 (0,659–0,838) (p < 0,001) (8 pav.).

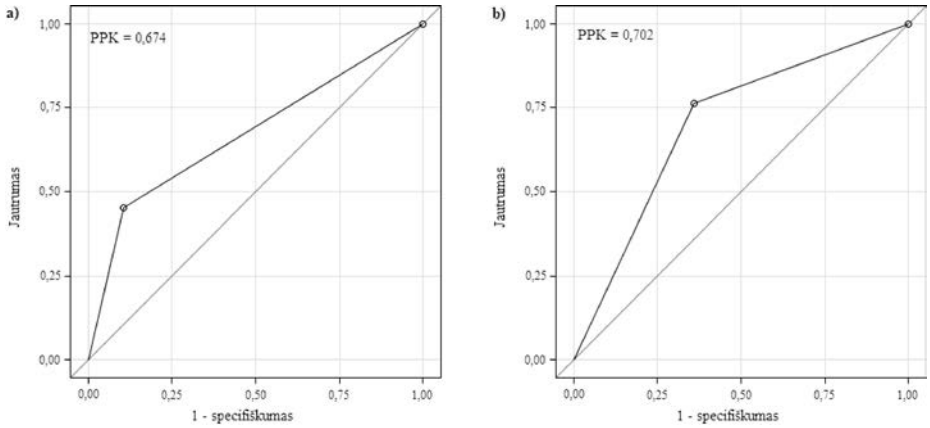
14 LENTELĖ. ML tipų prognostinių veiksnių įverčiai taikant logistinės regresijos analizę

	Vienmatis modelis			Daugiamatis modelis	
	ŠS (95 % PI)	P reikšmė		ŠS (95 % PI)	P reikšmė
Pakitusi MRT	5,625 (1,899–16,657)	0,002		NĮ	> 0,05
Endolimfos vandenė MRT vaizduose	5,744 (2,458–13,422)	< 0,001		6,603 (2,662–16,377)	< 0,001
II laipsnio EV MRT*,**	7,022 (1,437–34,301)	0,016			
Sraigės latako vandenė	3,917 (1,024–14,975)	0,046		NĮ	> 0,05
Pakitęs VNG kalorinis mėginys	3,231 (1,404–7,433)	0,006		NĮ	> 0,05
VNG vienpusis susilpnėjimas**	1,633 (0,755–3,535)	0,213			
VNG krypties vyravimas	3,568 (1,284–9,915)	0,015		4,553 (1,453–14,261)	0,009
Griuvimo rizika	1,002 (0,987–1,016)	0,825			
Pakitusi MRT	0,964 (0,346–2,684)	0,945			
Endolimfos vandenė MRT vaizduose	1,326 (0,463–3,800)	0,600			

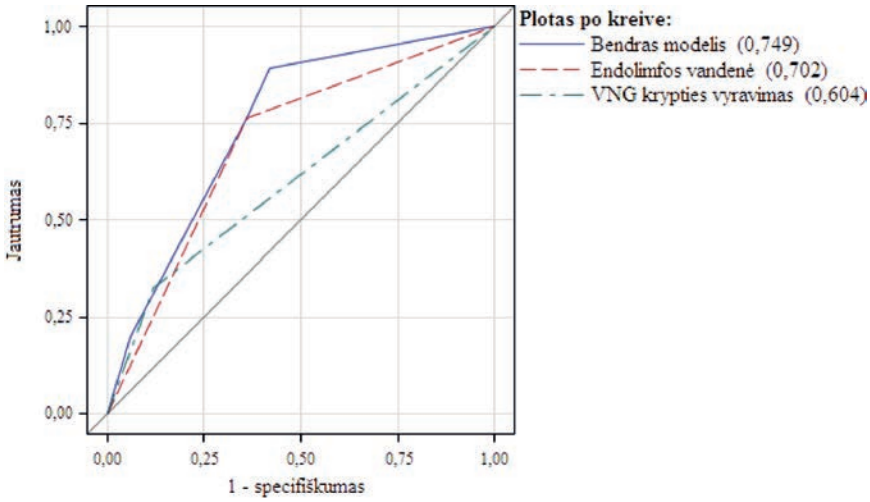
*Neįtraukta į daugiamatės logistinės analizės modelį. NĮ – nėra įvertinta.

3.7. Pakitimai MRT vaizduose

Taikant vienmatę logistinės regresijos analizę tik ML tipas buvo statistiškai reikšmingas veiksnys prognozuojant pakitimus MRT vaizduose: šansų santykis (95 % PI) buvo 5,625 (1,900–16,657) (p = 0,002) (15 lentelė).



7 PAV. Vienmatės logistinės regresijos ROC kreivės: a) endolimfos vandenė, b) EV MRT



8 PAV. Daugiamatės logistinės regresijos ROC kreivės

15 LENTELĖ. MRT rezultatų prognostinių veiksnių įverčiai taikant logistinės regresijos analizę

	Vienmatis modelis	
	ŠS (95% PI)	P reikšmė
ML tipas	5,625 (1,900–16,657)	0,002
Pakitęs VNG kalorinis mėginys	1,867 (0,730–4,775)	0,192
VNG vienpusis susilpnėjimas**	1,263 (0,500–3,189)	0,621
VNG krypties vyravimas	1,532 (0,466–5,035)	0,483
Griuvimo rizika	1,002 (0,985–1,020)	0,792
Maža griuvimo rizika	0,929 (0,283–3,046)	0,903
Didelė griuvimo rizika	1,651 (0,415–6,569)	0,477

Išvados

1. Standartiniai klinikiniai otoneurologiniai testai bei kompiuterinė posturografija esant skirtingoms Menjero ligos formoms specifinės diagnostinės informacijos nesuteikė. Atliekant videonistagmometriją sutrikęs kalorinis atsakas buvo dažnesnis neabejotinos Menjero ligos atvejais, o Menjero ligą statistiškai patikimiau leido diagnozuoti krypties vyravimo (DP%) rodiklis.
2. Smilkinkaulių 3T MRT bendri vaizdų pokyčiai (asimetrinis kontrastinės medžiagos kaupimas, endolimfos vandenė) buvo dažnesni neabejotinos ML grupėje (90,9 %), palyginti su tikėtinos ML grupe (64 %). Endolimfos vandenė nustatyta dvigubai dažniau neabejotinos ML grupės tiriamiesiems (76,4 %), o tikėtinos ML atvejais ji dažniau buvo nematoma nei matoma.
3. Smilkinkaulių endolimfinės sistemos pokyčiai 3T MRT vaizduose ir krypties vyravimas VNG kalorimetrijoje yra nepriklausomi statistiškai reikšmingi veiksniai diagnozuojant ML. Šie nepriklausomi veiksniai, veikdami kaip modelis, leidžia geriau prognozuoti ML nei tie patys veiksniai atskirai (atitinkamai $p = 0,022$ ir $p = 0,003$). Endolimfos vandenės apimties pokyčiai MRT vaizduose priklauso nuo klinikinės ligos eigos. Kuo didesnio laipsnio EV matoma MRT vaizduose, tuo dažnesni priepuoliai ir ilgesnė ligos trukmė ($p = 0,047$). Priklausomybės tarp klinikinės ligos eigos (ligos trukmės ir priepuolių dažnio) ir toninės ribinės audiogramos bei VNG kalorinio mėginio nenustatyta.
4. Pagal smilkinkaulių 3T MRT vaizdų pokyčius EV galima įvertinti laipsniais. I laipsnio EV dažniau nustatyta tikėtinos ML grupės tiriamiesiems, o II laipsnio EV – neabejotinos ML grupės pacientams ($p<0,001$). II laipsnio endolimfos vandenė smilkinkaulių 3T MRT vaizduose padidina neabejotinos ML tikimybę.
5. Smilkinkaulių endolimfinės sistemos 3T MRT vaizdinimas ir VNG kalorinis tyrimas turi didesnę reikšmę diagnozuojant ML ir suteikia specifiškesnių duomenų, palyginti su kitais periferinės vestibulinės sistemos ir pusiausvyros tyrimais.

Rekomendacijos

1. Asmenims, kuriems įtariama ML, tikslinga atlikti VNG kalorinį mėginį, kuris suteikia daugiau informacijos apie vestibulinę funkciją.
2. Asmenims, sergantiems Menjero liga, atlikti provokacinius mėginius ir stebėti dėl galimo antrinio GPPS išsivystymo.
3. Įtariant Menjero ligą, tikslinga atlikti smilkinkaulių vėlyvo kontrastinės medžiagos kaupimo 3T MRT su gadolinio kontrastine medžiaga, nes šis tyrimas padeda diagnozuoti ir diferencijuoti ligą, pagal matomos vandenės laipsnį nustatyti ligos formą bei sunkumą ir vertinti jos dinamiką.
4. Smilkinkaulių 3T MRT rekomenduojama atlikti pagal specialią pasiruošimo ir atlikimo techniką:
   a) intraveninės gadolinio kontrastinės medžiagos dozė – 0,1 mml/kg;
   b) suleidus kontrastinę medžiagą, MRT vaizdinimas turėtų būti atliekamas po 4–6 val.;
   c) vaizdiniai tyrimai turi būti atliekami 3 teslų MRT sistema;
   d) vaizdinimo protokolai turi sudaryti 3D FLAIR ir 3D T2DRIVE bei anatomines 3D T1, T2 ir DWI sekas galimai centrinės nervų sistemos patologijai atmesti;
   e) vaizdai turi būti vertinami patyrusio šios srities radiologo.
5. Pagal MRT vaizdus prieangio endolimfinio tarpo vandenę rekomenduojame vertinti laipsniais:
   a) nėra išsiplėtimo (suminis prieangio apvaliojo ir pailgojo maišelių plotas yra mažesnis nei pusė prieangio ploto ties ausies sraigės ritės viduriu);
   b) I laipsnio EV (endolimfinis tarpas pailgojo ar apvaliojo ar abiejų maišelių sąskaita išsiplėtęs, o perilifinis tarpas dar matomas kaulinio prieangio kanalo periferijoje);
   c) II laipsnio EV (kaulinis prieangis visiškai užimtas išsiplėtusių endolimfinių struktūrų).

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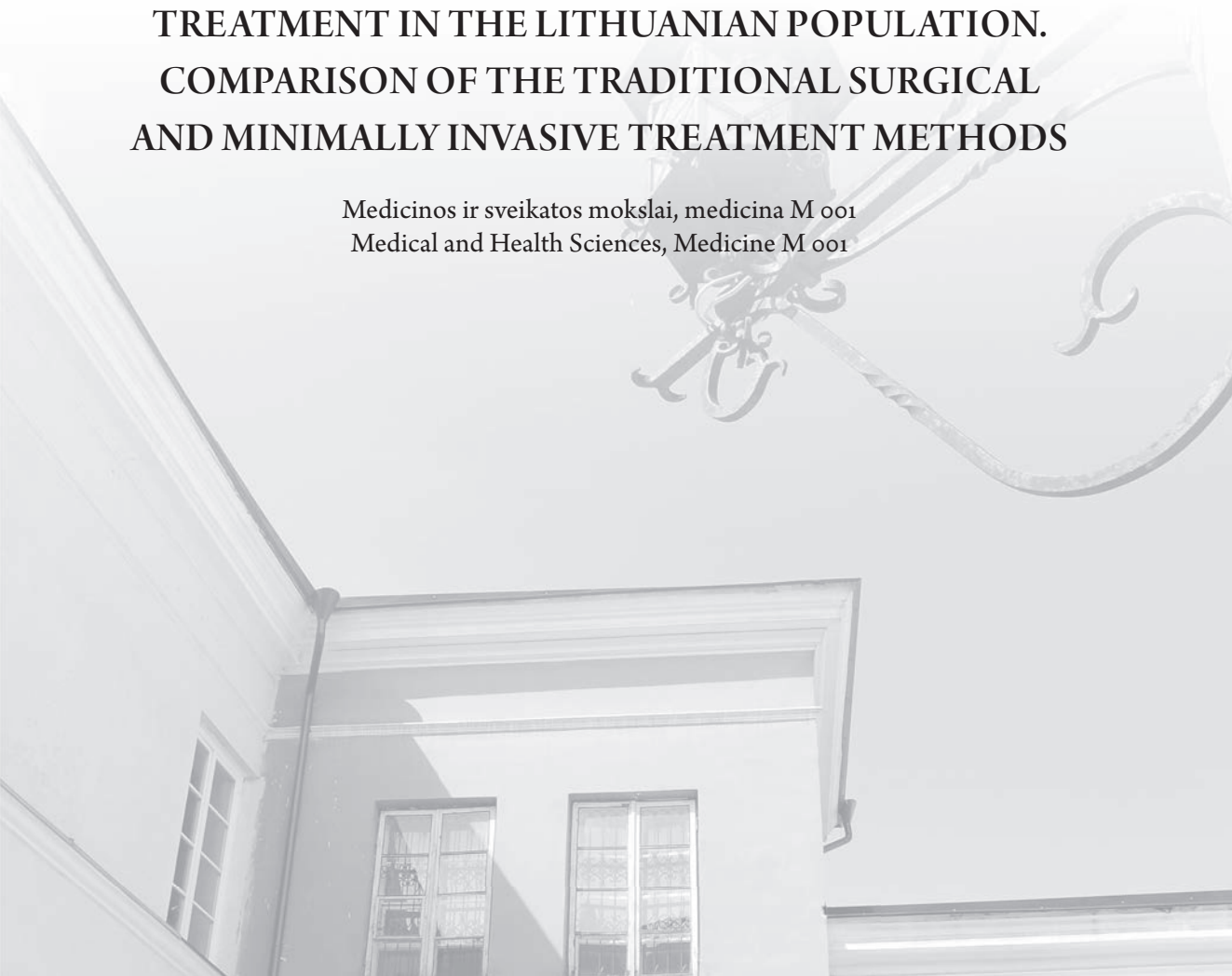
Rimantas Baušys

ANKSTYVO SKRANDŽIO VĖŽIO GYDYMO OPTIMIZAVIMAS LIETUVOS POPULIACIJOJE. TRADICINIO CHIRURGINIO IR MINIMALIAI INVAZYVIŲ GYDYMO METODŲ PALYGINIMAS

OPTIMIZATION OF EARLY GASTRIC CANCER TREATMENT IN THE LITHUANIAN POPULATION. COMPARISON OF THE TRADITIONAL SURGICAL AND MINIMALLY INVASIVE TREATMENT METHODS

Medicinos ir sveikatos mokslai, medicina M 001
Medical and Health Sciences, Medicine M 001



Santrumpos

ASV – ankstyvas skrandžio vėžys
SV – skrandžio vėžys
LMM – limfinių mazgų metastazės
IBL – išgyvenamumas be ligos atkryčio
LSI – ligai-specifiškas išgyvenamumas
BI – bendras išgyvenamumas
ER – endoskopinė rezekcija
RS – rizikos santykis
ŠS – šansų santykis
ESD – endoskopinė submukozinė disekcija
EGR – endoskopinė gleivinės rezekcija
EGDS – Esofagogastroduodenoskopija
KT – kompiuterinė tomografija
pT1 – navikas augantis į *lamina propria, muscularis mucosae*, ar pogleivį
pT2 – navikas augantis į *muscularis propria* sluoksnį
95 % PI – 95 % pasikliautinis intervalas

1. Įvadas

1.1 Literatūros apžvalga

Skrandžio vėžys (SV) yra vienas iš labiausiai paplitusių piktybinių navikų pasaulyje. Chirurginis gydymas išlieka pagrindiniu ir vieninteliu visiško pasveikimo leidžiančiu tikėtis šios ligos gydymo metodu (1). Reikšmingai daliai pacientų sergančių SV jau diagnozės nustatymo metu ar iškart po chirurginio gydymo atlikus histologinį pašalinto preparato vertinimą nustatomos metastazės limfiniuose mazguose (LMM) (2). Žinoma, jog LMM yra susijusios su blogesne pacientų prognoze, o chirurginis gydymas gali būti laikomas radikaliu tik tuo atveju, kai po jo nelieka nei makroskopiškai nei mikroskopiškai matomos ligos, taigi radikalus gydymas įmanomas tik chirurgiškai pašalinant visas LMM (3). Todėl auksiniu bet kurios stadijos rezektabilaus SV gydymo standartu išlieka radikalus chirurginis gydymas – gastrektomija ar skrandžio rezekcija kartu su pakankamos apimties limfonodektomija (4,5).

Ankstyvas skrandžio vėžys (ASV) apibrėžiamas kaip SV, kuriame naviko invazija siekia tik gleivinę ar pogleivį, nepriklausomai nuo to, ar yra LMM (pT1). ASV atveju tradicinis chirurginis gydymas leidžia pasiekti puikius onkologinius rezultatus - 5 metų bendrasis išgyvenamumas (BI) siekia nemažiau 90 % (6,7). Deja, tradicinis chirurginis ASV gydymas yra susijęs su ženkliu pooperacinių komplikacijų dažniu, pooperaciniu mirštamumu bei su suprastėjusia paciento gyvenimo kokybe (8-10). Ilgėjant tikėtinai gyvenimo trukmei bei gerėjant su senatve susijusių gretutinių ligų gydymui SV vis dažniau serga ženkliai vyresnio amžiaus pacientai. Chirurginis SV gydymas tapo standartu gydant geros ar patenkinamos klinikinės būklės

pacientus, tačiau tokio gydymo rezultatai nėra visiškai aiškūs gydant vyresnius pacientus, ypač tuos, kurie pasižymi gausia gretutine patologija (11-13).

Teoriškai SV chirurgijos apimtis galėtų būti reikšmingai sumažinama iki lokalios naviko ekscizijos ar rezekcinės operacijos be limfonodektomijos, jei jau iki operacijos būtų galima patikimai nustatyti ar yra LMM. Deja, visame pasaulyje priešoperaciniam ligos stadijavimui rutiniškai naudojama kompiuterinė tomografija (KT) ar magnetinis branduolinis rezonansas (MBR) nėra pakankamai jautrūs ir specifiški tyrimai identifikuojant LMM (14,15). Po klinikinių pastebėjimų, kad tam tikrais ASV atvejais LMM rizikos nėra ar ji yra itin maža, kaip alternatyva tradiciniam chirurginiam gydymui buvo pasiūlyta lokali endoskopinė naviko rezekcija (ER): endoskopinė gleivinės rezekcija (EGR) ar endoskopinė submukozinė disekcija (ESD). Manoma, jog lokali ER yra pakankama neišplitusio į limfinius mazgus ASV gydymui. Kadangi KT/MBR neleidžia patikimai nustatyti LMM, ASV atvejai turintys minimalią LMM riziką bei tinkami lokaliai ER pradėti nustatyti remiantis histologinėmis ir morfologinėmis pirminio naviko savybėmis. Šiuolaikiniai endoskopinio ASV gydymo kriterijai nurodomi Japonų Skrandžio Vėžio Gydymo Gairėse (16) kaip standartiniai jei tai yra diferencijuota adenokarcinoma be išopėjimo požymių, navikas yra intramukozinis, o jo diametras ≤2 cm. Ši indikacija buvo pirmoji ir ilgą laiką vienintelė kuomet siūlyta taikyti endoskopinį gydymą. Tačiau, bėgant laikui bei Azijos šalyse sukaupus klinikinės patirties, buvo pastebėta, kad ir kai kurie navikai viršijantys šias labai siauras indikacijas galėtų būti gydomi ER. Todėl šiuolaikinės gairės papildytos išplėstiniais kriterijais: 1) diferencijuoto tipo intramukozinė adenokarcinoma be išopėjimo požymių, kurios diametras viršija 2 cm; 2) diferencijuoto tipo intramukozinė adenokarcinoma su išopėjimo požymiais, kurios diametras neviršija 3 cm; 3) nediferencijuoto tipo intramukozinė adenokarcinoma be išopėjimo požymių neviršijanti 2 cm diametro; 4) diferencijuoto tipo pogleivinė (SM1, <500 μm nuo muscularis mucosae) adenokarcinoma neviršijanti 3 cm diametro. Būtina pažymėti, jog išplėstinių kriterijų naudojimas vertinamas kaip eksperimentinis gydymo metodas, todėl kad nėra tvirtų įrodymų apie atokiuosius šio gydymo rezultatus.

Pastaraisiais metais pasirodė keletas studijų pateikiančių atokiuosius chirurginio ir endoskopinio ASV gydymo rezultatus, kurie yra panašūs. Taip pat pažymima, jog pooperacinis komplikacijų dažnis bei mirštamumas yra yra mažesnis po ER, taip pat ši procedūra yra pranašesnė dėl tikėtina geresnės pacientų gyvenimo kokybės (17-24). Visos šios klinikinės studijos, kaip ir pats ER konceptas, buvo sukurtos išskirtinai Azijos šalyse, o šių rezultatų atkartojimas vakarietiškoje populiacijoje šiuo metu kelia aršias diskusijas. Jos kyla dėl kelių svarbių priežasčių. Visų pirma, Vakarų ir Azijos šalyse skiriasi histologiniai SV diagnostikos kriterijai (25), o šie skirtumai yra itin svarbūs ASV atveju. Japonijoje ir kai kuriose kitose Azijos šalyse

histologinė diagnozė yra paremta struktūrinių ir naviko liaukų ląstelių nukrypimo nuo normos laipsniu (25-27) kuomet Vakarų šalyse histologinei SV diagnozei yra būtinas naviko ląstelių invazijos komponentas (25,28). Dėl tokių skirtingų interpretacijų Vakarų šalyse nustatyta aukšto laipsnio displazija Japonijoje jau yra vertinama kaip ASV (25,29). Antra, vakariečiai sergantys SV skiriasi nuo tokių pat pacientų iš Azijos šalių tuo, kad jų tarpe ženkliai dažniau sutinkamas proksimalinės skrandžio dalies vėžys, daugiau difuzinio tipo vėžio, taip pat vakariečiai pasižymi didesniu kūno masės indeksu bei vyresniu amžiumi (30). Esant šiems skirtumams nesutariama ar SV etiopatogenezė šiuose regionuose yra ta pati. Dar daugiau sumaišties į šią diskusiją įnešė dvi nesenai paskelbtos studijos, nurodančios, kad neazijietiška rasė savaime yra LMM rizikos veiksnys (31,32).

Dėl šių išvardintų priežasčių išlieka neaišku ar ASV gydymo ER koncepcija atsiradusi ir išvystyta Azijos šalyse gali būti sėkmingai perkeliama ir taikoma Vakarų šalyse, įskaitant ir Lietuvą.

1.2 Tyrimo hipotezė

ASV gydymas ER yra saugus ir priimtinas Lietuvos populiacijoje.

1.3 Tyrimo tikslas

Nustatyti, ar ASV gydymas ER yra saugus ir priimtinas Lietuvos populiacijoje, kuri gali būti laikoma vakarietiško tipo populiacija.

1.4 Tyrimo uždaviniai

1. Palyginti artimuosius ir atokiuosius pacientų sergančių ASV gydymo rezultatus taikant tradicinį chirurginį gydymą ar ER Lietuvos populiacijoje.
2. Nustatyti LMM rizikos veiksnius pacientams sergantiems ASV Lietuvos populiacijoje.
3. Nustatyti LMM dažnį Lietuvos pacientų sergančių ASV bei atitinkančių standartinius ir išplėstinius endoskopinio gydymo kriterijus grupėje.
4. Nustatyti LMM dažnį Lietuvos pacientų sergančių ASV bei viršijančių standartinius ir išplėstinius endoskopinio gydymo kriterijus grupėje.
5. Nustatyti pooperacinių komplikacijų ir pooperacinio mirštamumo rizikos veiksnius taikant standartinį chirurginį gydymą pacientams sergantiems ankstyvų stadijų SV.

1.5 Tyrimo naujumas

Mūsų žiniomis, tai yra pirmasis tyrimas, lyginantis tradicinio chirurginio ir minimaliai invazyvaus - endoskopinio ASV gydymo rezultatus vakarietiškoje populiacijoje.

2. Metodai

2.1 Etika

Prieš pradedant tyrimą buvo gautas Vilniaus regioninio biomedicininių tyrimų etikos komiteto leidimas.

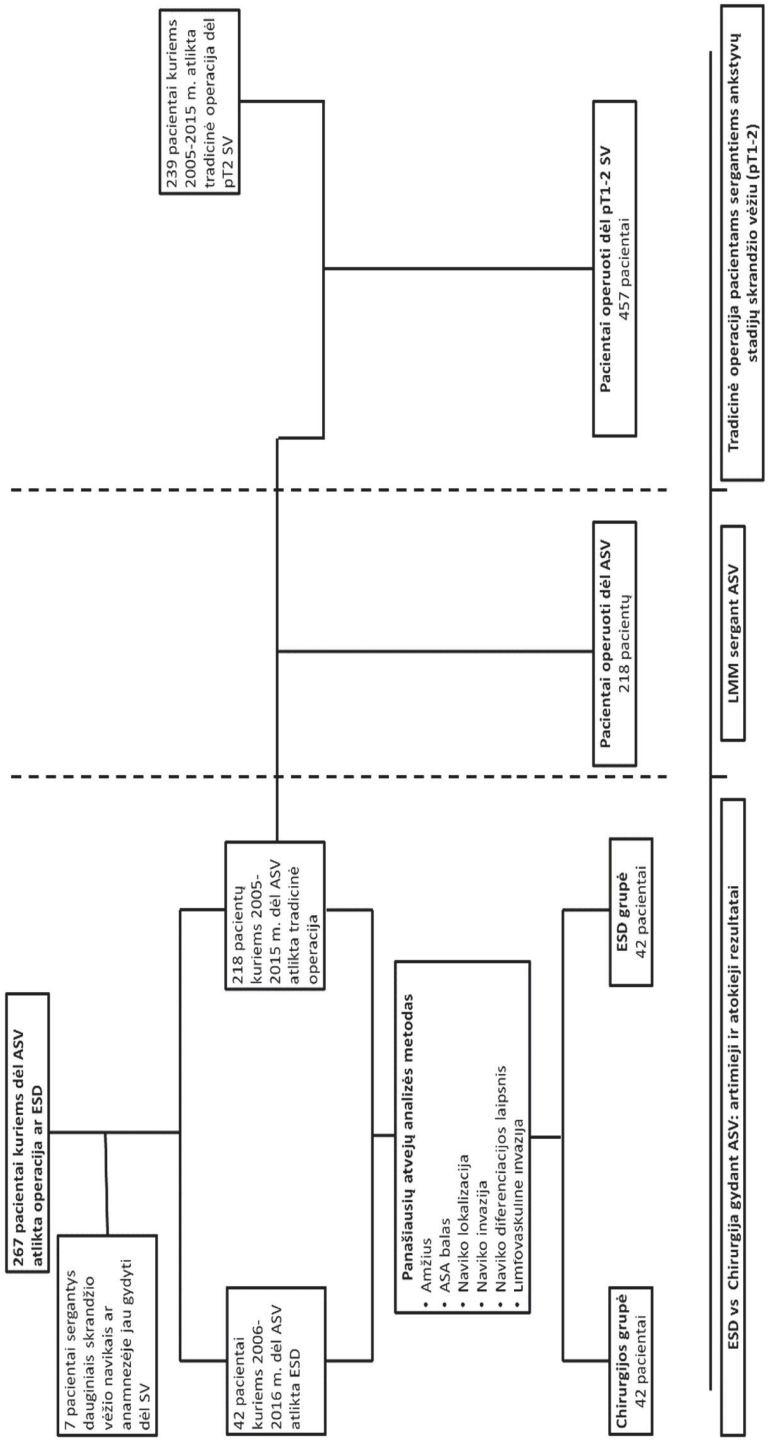
2.2 Pacientai ir tyrimo dizainas

Pacientų įtraukimas į tyrimą pavaizduotas 1-ame paveiksliuke.

2.2.1 ESD ir chirurginio ASV gydymo palyginimas: artimieji ir tolimieji gydymo rezultatai

Į šį retrospektyvaus dizaino tyrimą įtraukti visi pacientai, kurie dėl ASV gydyti Vilniaus Universiteto ligoninės Santaros Klinikose nuo 2006 m. sausio mėn. iki 2016 m. gruodžio mėn. taikant jiems ER ir pacientai, kurie operuoti dėl ASV Nacionaliniame Vėžio Institute nuo 2005 m. sausio mėn. iki 2015 m. gruodžio mėn. ER programa pacientams sergantiems ASV Vilniaus Universiteto ligoninės Santaros Klinikose buvo pradėta 2006 m., todėl nežymiai skiriasi pacientų įtraukimo periodai abiejose institucijose, kurie taip pasirinkti, norint išlaikyti vienodą 10 m. periodą. Pacientai patys rinkosi gydymo centrą. Visi į tyrima įtraukti pacientai buvo europidų rasės. Pacientų neįtraukimo į tyrimą kriterijai: 1) daugybinis arba sinchroninis ASV; 2) ankstesnis SV gydymas, įskaitant ir neoadjuvantinę chemoterapiją.

Taikant įtraukimo ir neįtraukimo į tyrimą kriterijus atrinkta 260 pacientų, kurie suskirstyti į chirurgijos (218 pacientų) ir ESD (42 pacientai) grupes. Abiejų grupių gydymo rezultatai buvo lyginami naudojant panašiausių atvejų analizės metodą (*angl. propensity score matching*). Pirminis šios tyrimo dalies tikslas buvo nustatyti 5 metų išgyvenamumą be ligos atkryčio (IBL) taikant kiekvieną iš šių gydymo metodų. Antriniai tikslai – bendras 5 metų išgyvenamumas (BI), pooperacinių komplikacijų dažnis, operacijos trukmė ir hospitalizacijos laikas. BI buvo skaičiuojamas nuo operacijos datos iki mirties, IBL - nuo operacijos datos iki lokalaus arba atokaus ligos atkryčio.



PAVEIKSLĖLIS 1. Struktūrinė pacientų įtraukimo į tyrimą schema.

2.2.2 LMM pacientų sergančių ASV tarpe

Siekiant nustatyti LMM sergančiųjų ASV tarpe buvo tirti aukščiau minėti 218 pacientų, kuriems dėl ASV atlikta tradicinė operacija kartu su limfonodektomija. Kaip potencialūs rizikos veiksniai buvo vertinami: pacientų amžius, lytis, naviko lokalizacija, naviko invazijos gylis, naviko diferenciacijos laipsnis, limfovaskulinė invazija, naviko tipas pagal Lauren, naviko dydis, naviko išopėjimas ir žiedinių ląstelių komponentas. Nustatytas LMM dažnis tarp pacientų atitinkančių bei viršijančių standartinius/išplėstinius kriterijus ER dėl ASV.

2.2.3 Tradicinis chirurginis pacientų sergančių ankstyvųjų stadijų SV gydymas

Tirti pacientai operuoti dėl ankstyvų stadijų (pT1 (ASV) ir pT2) SV Nacionaliniame Vėžio Institute nuo 2005 m. sausio mėn. iki 2015 m. gruodžio mėn. (Paveikslėlis 1), siekiant nustatyti pooperacinių komplikacijų ir mirštamumo dažnį bei tai lemiančius veiksnius. Operacijos technika operuojant dėl pT1 ir pT2 navikų beveik nesiskiria, todėl siekiant didesnės pacientų imties ir tyrimo galios į šią tyrimo dalį įtraukti ir pacientai su pT2 navikais infiltruojančiais *muscularis propria*. Į šią tyrimo dalį iš viso įtraukti 457 pacientai: 218 pacientų su pT1 (ASV) ir 239 pacientai su pT2 navikais. Siekiant išaiškinti vyresnio amžiaus pacientų gydymo rezultatus pacientai buvo suskirstyti į dvi grupes atsižvelgiant į jų amžių: vyresnieji (V; ≥70 metų) ir jaunesnieji (J; <70 metų) (34).

2.3 Pacientų savybės ir klinikiniai duomenys

Visų pacientų duomenys buvo surinkti iš ligoninėje esančių duomenų bazių kaupiančių medicininę dokumentaciją bei iš perspektyviai surinktos ASV atvejus registruojančios duomenų bazės. SV ligos stadijavimui naudota 7-oji AJCC/UICC TNM sistemos klasifikacija. Vertinti pacientų demografiniai duomenys: lytis ir amžius. Taip pat klinikiniai ir demografiniai duomenys: rūkymas, KMI, gretutiniai susirgimai, fizinė būklė pagal Amerikos Anesteziologų Draugijos (ASA) klasifikaciją, hospitalizacijos trukmė, operacijos ir limfonodektomijos apimtis, operacijos trukmė, operacinė kraujo netektis, naviko lokalizacija, jo dydis, naviko invazijos gylis, naviko diferenciacijos laipsnis, limfovaskulinė invazija, pašalintų ir metastatinių limfinių mazgų skaičius. Pooperacinės komplikacijos klasifikuotos remiantis Clavien-Dindo klasifikacija.

2.4 Priešoperacinis pacientų ištyrimas ir chirurginės procedūros

Iki operacijos/ESD visiems pacientams atlikta ezofagogastroduodeno-skopija (EGDS) su naviko biopsija. Daugumai pacientų prieš ESD papildomai atlikta endoskopinė sonoskopija naviko invazijos gyliui patikslinti. Priešoperaciniam ligos stadijavimui atliktas krūtinės ląstos ir pilvo organų KT tyrimas.

ESD buvo atliekama bendrinėje intraveninėje nejautroje. Procedūra pradedama pažymint naviko ribas, kai į pogleivį suleidžiamas adrenalino ir metileno mėlio tirpalas. Tuomet cirkuliariai apipjaunama gleivinė ir atliekama pogleivio disekcija siekiant pašalinti naviką vienu bloku (*en bloc*). Pašalinus naviką atliekama hemostazė koaguliuojant arba naudojant hemoklipus. Rezekcija buvo laikoma pilnaverte (angl. *complete resection*) tais atvejais, kai navikas pašalintas vienu bloku (*en bloc),* o histologiškai patvirtinama, jog horizontalus ir vertikalus rezekcijos kraštai yra be naviko. Gydomąja rezekcija (angl. *curative resection)* laikyta tuomet, kai atlikta pilnavertė rezekcija (*complete resection),* o pašalintas navikas neviršijo standartinių ar išplėstinių indikacijų ER nurodomų Japonų Skrandžio Vėžio Gydymo Gairėse (16).

Tradicinio chirurginio gydymo apimtis – gastrektomija ar skrandžio rezekcija, buvo pasirenkama atižvelgiant į naviko lokalizaciją skrandyje. Skrandžio rezekcija buvo atliekama, kai buvo įmanoma užtikrinti pakankamą proksimalinį rezekcijos kraštą. Visais kitais atvejais buvo atliekama gastrektomija. Šalinant skrandį ar jo dalį su naviku kartu atliktos D2 apimties limfonodektomijos kaip nurodoma Japonų Skrandžio Vėžio Gydymo Gairėse (16). Kai kuriais atvejais operuojančio chirurgo sprendimu buvo atliekamos mažesnės apimties D1 limfonodektomijos. Visais atvejais atliktos R0 tipo (mikroskopiškai ir makroskopiškai nėra likutinio naviko) operacijos (33).

2.5 Histologinis naviko vertinimas

Patologinis preparato ištyrimas visais atvejais atliktas Valstybiniame patologijos centre, Vilniuje. Naviko histologinis tipas buvo klasifikuojamas pagal Pasaulio sveikatos organizacijos (PSO) ir Lauren klasifikacijas. Mėginiai buvo dažomi hematoksilinu ir eozinu bendram vertinimui. Makroskopiškai tirtas naviko dydis, vertinant didžiausią diametrą. Mikroskopiškai - invazijos gylis, limfovaskulinė invazija bei rezekcijos kraštai. Iš pašalintų chirurginių preparatų buvo išrenkami visi pašalinti limfiniai mazgai, jie paruošti pagal standartinį protokolą ir tirti gydytojo patologo mikroskpiškai, tokiu būdu nustaytos LMM (33).

2.6 Pacientų stebėsena

Sekant pacientus po ESD pirmaisiais metais esofagogastroduodenoskopija (EGDS) buvo atliekama po 3, 6 ir 12 mėn., vėliau kasmet viso penkerius metus. Po standartinių operacijų EGDS buvo atliekama kas 6 mėn. pirmuosius du metus, vėliau kasmet viso penkerius metus. Abiejų grupių pacientams kasmet buvo atliekamas krūtinės ląstos ir pilvo organų KT tyrimas. Jei pacientų stebėsena buvo atliekama kitose gydymo įstaigose nedalyvaujančiose tyrime, duomenys apie sekimo metu atliktų tyrimų rezultatus buvo renkami susisiekiant su pacientu ar jo šeimos gydytoju. Paskutiniai pacientų sekimo duomenys buvo surinkti 2017 m. gruodžio mėn. Du pacientai iš ESD grupės ir vienas iš chirurginės nebuvo sekti (33).

2.7 Statistinė analizė

Visi statistiniai skaičiavimai atlikti naudojantis SPSS 22.0 (SPSS, Čikaga, JAV) programa. Kiekybiniai kintamieji pateikiami kaip vidurkis su standartiniu nuokrypiu arba kaip mediana su interkvartiliu. Kategoriniai kintamieji nurodomi kaip proporcijos.

Siekiant palyginti artimus ir tolimus gydymo rezultatus po ESD ir tradicinio chirurginio gydymo buvo panaudotas panašiausių atvejų analizės metodas, įtraukiant šešis pagrindinius kintamuosius: amžių, fizinę būklę pagal Amerikos anesteziologų draugijos (ASA) klasifikaciją, naviko lokalizaciją ir invazijos gylį, diferenciacijos laipsnį ir limfovaskulinę invaziją. Suskaičiavus panašumo įvertį pacientai iš ESD grupės buvo suporuoti su artimiausią įvertį turinčiu pacientu iš chirurgijos grupės santykiu 1:1. Klinikiniai ir patologiniai rodikliai palyginti naudojant Chi-kvadrato testą, Fišerio testą bei t-testą. BI ir IBL vertinti Kaplano-Mejerio metodu bei palyginti naudojant Log-rank testą. Visuose skaičiavimuose p reikšmė < 0.05 buvo laikoma statistiškai reikšminga (33).

LMM rizikos veiksniai nustatyti naudojant vienpusį ANOVA testą, Chi-kvadrato testą, Fišerio testą ir dvipusį t-testą. Univariacinės analizės metu nustatyti reikšmingi rizikos veiksniai buvo įtraukti į multivariacinę logistinės regresijos analizę leidusią nustatyti nepriklausomus rizikos veiksnius (35).

Pooperacinės komplikacijos ir mirštamumas palygintos naudojant Chi-kvadrato testą, Fišerio testą, t-testą, vienpusės ANOVA testą bei neparametrinius testus (34).

3. Rezultatai

3.1 ESD ir tradicinio chirurginio gydymo palyginimas gydant ASV

3.1.1 Pacientai, jų pagrindinės klinikinės ir patologinės savybės

Palyginus ESD ir chirurginio gydymo grupes iki taikant panašiausių atvejų analizės metodą, jos reikšmingai skyrėsi. Chirurginio gydymo grupėje pacientai buvo jaunesni, turėjo mažiau lydinčių susirgimų. Taip pat šioje grupėje reikšmingai didesnė dalis navikų pasižymėjo invazija į pogleivį, bloga diferenciacija, difuziniu tipu pagal Lauren, išopėjimu bei didesniu naviko dydžiu. ESD grupėje pacientai reikšmingai dažniau sirgo viršutinio skrandžio trečdalio navikais. Pritaikius panašiausių atvejų analizės metodą abi grupės tapo panašios. Vidutinis atvejų panašumo įvertis siekė 0.39±0.22 ir 0.34±0.18 atitinkamai ESD ir chirurgijos grupėje (p=0.290), tačiau net ir pritaikius šį statistinį metodą didesnio nei 2 cm diametro navikai reikšmingai dažniau pasitaikė chirurgijos grupėje (33).

3.1.2 Artimieji ASV gydymo rezultatai taikant tradicinį chirurginį gydymą ir ESD

Chirurgijos grupėje 10 (23.8 %) pacientų atlikta gastrektomija, 32 (76.2 %) pacientams – skrandžio rezekcija. Lyginant gydymo rezultatus tarp chirurgijos ir ESD grupių, nustatyta, kad reikšmingai skyrėsi operacijos trukmė ir hospitalizacijos laikas – jie buvo trumpesni ESD grupėje. Kiekvienoje grupėje 7 (16.7 %) iš 42 pacientų patyrė pooperacinių komplikacijų. ESD grupėje 2 (4.8 %) pacientams stebėtos infekcinės kilmės komplikacijos, 3 (7.1 %) pacientams pooperacinis kraujavimas ir 2 (4.8 %) pacientams skrandžio perforacija. Visais atvejais kraujavimas po ESD sustabdytas taikant endoskopines intervencijas. Abiem perforacijų atvejais reikėjo chirurginės intervencijos – vienu atveju atlikta laparaskopinė, kitu atviro tipo operacija.

Didžioji dalis komplikacijų po chirurginio gydymo buvo klasifikuojamos kaip nedidelės (4 iš 7 ≤II laipsnio pagal Clavien-Dindo klasifikaciją). Du (4.8 %) pacientai buvo operuoti pakartotinai dėl sterkoralinio ir tulžingo peritonito. Vienas pacientas (2.4 %) pooperaciniu periodu mirė dėl ūminio širdies ir kvėpavimo sistemų nepakankamumo.

Visiems pacientams chirurgijos grupėje buvo atlikta radikali (R0) operacija. ESD grupėje *en bloc* ir pilnavertė rezekcija atlikta atitinkamai 38 (92.7 %) ir 32 (78 %) atvejais. Tačiau po galutinio morfologinio ištyrimo tik 30 (71.4 %) atvejų buvo įvertinti kaip gydomoji rezekcija. Dvylika pacientų kuriems atlikta ne-gydomoji rezekcija (angl. *Non-curative resection*) toliau gydyti taip: keturiems pacien-

tams taikytas tradicinis chirurginis gydymas, vienam - pakartotinė ESD, penki pacientai stebėti, vienas pacientas nestebėtas, nes iškrito iš sekimo, o vienam pacientui jį sekant nustatytos atokios metastazės ir tuomet skirtas paliatyvus gydymas (33).

3.1.3 Atokieji ASV gydymo rezultatai taikant tradicinį chirurginį gydymą ir ESD

Vidutinis pacientų stebėsenos laikas po ESD buvo 38±34 mėn., mediana – 28 (Q1:9-Q3:60) mėn., atitinkamai chirurgijos grupėje - 52±36 mėn. ir 42 (Q1:30-Q3:74) mėn. Lyginant atokiuosius gydymo rezultatus pastebėta, kad 5 metų IBL buvo reikšmingai didesnis chirurgijos grupėje - 97.6 % prieš 77.5 %, p=0.002. Į analizę traukiant tik tuos pacientus kuriems ESD metu atlikta gydomoji rezekcija reikšmingo skirtumo tarp grupių nebeliko - 5 metų IBL ESD grupėje siekė 89.7 %, o chirurgijos grupėje 97.6 %, p=0.099. 9 (21.4 %) pacientams ESD grupėje ir vienam (2.4 %) pacientui chirurgijos grupėje (p=0.014) stebėtas skrandžio vėžio ligos atkrytis. 8 (19.0 %) pacientams ESD grupėje ir vienam (2.4 %) pacientui chirurgijos grupėje stebėti lokalūs recidyvai, kurie sėkmingai gydyti pakartotina ESD ar įprasta operacija. Vienam (2.4 %) pacientui ESD grupėje nustatytos atokios metastazės ir karcinomatozė. 8 iš 10 pacientų ligos atkrytis stebėtas per pirmuosius 36 mėnesius nuo gydymo pradžios. ESD grupėje ligos atkrytis reikšmingai rečiau stebėtas tais atvejais kai atlikta gydomoji rezekcija - 10.3 % prieš 45.5 %, p=0.007. Ne-gydomoji rezekcija identifikuota kaip nepriklausomas rizikos veiksnys ligos atkryčiui (RS: 41.79; 95 % PI:7.21 – 242.17)

Nors IBL abiejose grupėse reikšmingai skyrėsi, BI rodiklis nesiskyrė. 5 metų BI chirurgijos ir ESD grupėse atitinkamai siekė 69.0 % ir 73.8 %, p=0.599. 5 metų BI išgyvenamumas reikšmingai nesiskyrė ir lyginant jį ESD grupėje po gydomųjų ir ne-gydomųjų rezekcijų (66.7 % vs. 76.7 %, p=0.581) (33).

3.2 Metastazės limfiniuose mazguose ASV atveju

3.2.1 LMM rizikos veiksniai ASV pacientams

Nustatant LMM rizikos veiksnius į analizę įtraukti 218 pacientų, kuriems atlikta tradicinė operacija kartu su limfonodektomija. Operuoti 117 (53.7 %) vyrų ir 101 (46.3 %) moteris, vidutinis pacientų amžius siekė 65.58±12.33 metus. Gastrektomija atlikta 38 pacientams, skrandžio rezekcija – 180 pacientų. Didžiajai daliai pacientų -195 (89.4 %) atlikta D2 limfonodektomija. Limfonodektomijų metu vidutiniškai pašalinti 19.89±9.69 limfmazgių. LMM nustatytos 43 (19.7 %) pacientams sergantiems ASV. Univariacine analize nustatyti LMM rizikos veiksniai: pogleivinė naviko invazija, limfovaskulinė invazija, bloga naviko diferenciacija, difuzinis arba mišrus naviko tipas pagal Lauren klasifikaciją bei naviko diametras viršijantis 2 cm. Am-

žius, lytis, naviko lokalizacija, išopėjimas ir žiedinių ląstelių komponentas neturėjo reikšmės LMM (35). Multivariacinė analizė parodė, kad naviko invazija į pogleivį, limfovaskulinė invazija ir bloga diferenciacija yra nepriklausomi LMM rizikos veiksniai (35).

3.2.2 LMM dažnis pacientų atitinkančių ar viršijančių standartines/išplėstines indikacijas endoskopiniam ASV gydymui grupėje

Iš 58 ASV atvejų, kurie atitiko išplėstines ESD indikacijas, bet buvo operuoti, 1 (1.7 %) pacientas turėjo metastazes 2-juose iš 22 pašalintų limfinių mazgų. Šiuo atveju navikas buvo intramukozinis, neišopėjęs, vidutiniškai diferencijuotas ir didesnis nei 2 cm diametro (2.2 cm x1.8 cm x1.5 cm). Iš 139 pacientų, kurie sirgo ASV viršijančiu išplėstines indikacijas ER, 42 (30.2 %) pacientams nustatytos LMM (36).

3.3 Tradicinio chirurginio gydymo rezultatai pacientų grupėje, kurie operuoti dėl ankstyvų stadijų skrandžio vėžio

3.3.1 Pacientai ir jų pagrindinės klinikinės ir patologinės savybės

267 iš 457 pacientų (58.4 %) operuotų dėl ankstyvų stadijų skrandžio vėžio (pT1-T2) buvo jaunesni nei 70 metų, 190 (41.6 %) pacientų – vyresni. Jaunesnių ir vyresnių pacientų grupės reikšmingai skyrėsi vertinant pacientų fizinę būklę (ASA balas), naviko histologinį tipą ir diferenciacijos laipsnį. Vyresnių pacientų grupėje didesnė dalis pacientų sirgo sunkiomis gretutinėmis ligomis (ASA III-IV) (61.4 % vs. 31.7 %, p=0.001) ir žarninio tipo vėžiu pagal Lauren klasifikaciją (69.3 % vs. 45.8 %, p=0.001). Jaunesnių pacientų grupėje dažniau nustatytas blogai diferencijuotas vėžys (G3: 64.0 % vs. 42.6 %, p=0.001) (34).

3.3.2 Pooperacinės komplikacijos ir mirštamumas operuojant dėl ankstyvų stadijų skrandžio vėžio

Lyginant chirurginio gydymo rezultatus vyresnių ir jaunesnių pacientų grupėse bendras komplikacijų, sunkių komplikacijų (III-IV laipsnio pagal Clavien-Dindo klasifikaciją) ar chirurginių pooperacinių komplikacijų dažnis reikšmingai nesiskyrė. Vyresnių grupėje stebėtas didesnis nechirurginių komplikacijų dažnis (9.7 % vs. 16.3 %, p=0.035). Pooperacinės komplikacijos sąlygojančios pacientų mirtį stebėtos tik vyresnių grupėje. Intrahospitalinis pooperacinis mirštamumas jaunesnių ir vyresnių pacientų grupėse atitinkamai siekė 0 % ir 5.7 %, p=0.001. Dar didesni pooperacinio mirštamumo skirtumai stebėti, kai buvo palyginti 30 ir 90 dienų pooperacinio mirštamumo rodikliai. 90 dienų mirštamumas jaunesnių grupėje siekė 2.6 %, o vyresnių – 12.6 %, p<0.05 (34).

3.3.3 Pooperacinių komplikacijų rizikos veiksniai jaunesnių ir vyresnių pacientų operuojamų dėl ankstyvų stadijų skrandžio vėžio grupėse

Univariacinės analizės metu nustatyta, kad aukštas ASA (III/IV) įvertis, didesnė operacijos apimtis (gastrektomija) ir multi-organinė rezekcija yra veiksniai susiję su didesniu pooperacinių komplikacijų dažniu vyresnių pacientų grupėje. Multivariacinė analizė parodė, kad tik aukštas ASA įvertis (III/IV) yra nepriklausomas pooperacinių komplikacijų rizikos veiksnys (ŠS=6.47; 95 % PI 2.09-20.06, p=0.021).

Jaunesnių pacientų grupėje nė vienas iš analizuotų faktorių nebuvo statistiškai reikšmingai susijęs su bendru pooperacinių komplikacijų dažniu, tačiau naviko lokalizacija viršutiniame skrandžio trečdalyje buvo susijusi su dažnesnėmis sunkiomis pooperacinėmis komplikacijomis (≥3 laipsnio pagal Clavien-Dindo klasifikaciją) (14.9 % vs. 4.3 %, p=0.014) (34).

3.3.4 Pooperacinio mirštamumo rizikos veiksniai jaunesnių ir vyresnių pacientų operuojamų dėl ankstyvų stadijų skrandžio vėžio grupėse

Kadangi jaunesnių pacientų grupėje per pirmas 30 dienų po operacijos mirusių nebuvo, analizuoti tik 90 dienų mirštamumo veiksniai. Univariacinė analizė parodė, kad aukštas ASA (III/IV) įvertis ir naviko lokalizacija viršutiniame skrandžio trečdalyje yra susijusi su 90 dienų mirštamumu jaunesnių pacientų grupėje.

Vyresnių pacientų grupėje 90 dienų pooperacinis mirštamumas buvo susijęs su aukštu ASA (III/IV) įverčiu ir multiorganine rezekcija. Multivariacinė analizė parodė, kad tik sunkios komplikacijos (≥3 laipsnio pagal Clavien-Dindo klasifikaciją) patirtos hospitalizacijos metu yra nepriklausomas rizikos veiksnys devyniasdešimties dienų pooperaciniam mirštamumui (ŠS=12.82; 95 % PI 1.01-169.21, p=0.049) (34).

4. Diskusija

Pagrindiniai mūsų studijos radiniai buvo tokie: 1) Lietuvos pacientus, sergančius ASV, galima gydyti taikant ESD, nes atokieji gydymo rezultatai yra panašūs į tradicinio chirurginio gydymo rezultatus, tačiau operacijos ir hospitalizacijos laikas yra trumpesni. Tai galioja tais atvejais, kai įmanoma atlikti gydomąją rezekciją; 2) Atlikus negydomąją rezekciją yra didelė ligos atkryčio tikimybė bei tikėtini prasti IBL rezultatai; 3) Naviko invazija į pogleivį, limfovaskulinė invazija ir bloga naviko diferenciacija yra LMM rizikos veiksniai Lietuvos pacientų sergančių ASV populiacijoje; 4) LMM yra nustatomos 1.7 % pacientų, kurie serga ASV atitinkančiu išplėstinius endoskopinio gydymo kriterijus; 5) Taikant tradicinį chirurginį gydymą dėl ankstyvų stadijų SV vyresnis amžius nėra susijęs su didesniu pooperacinių komplikacijų dažniu, tačiau

yra susijęs su išaugusiu pooperaciniu mirštamumu. ASA fizinės būklės vertinimas yra naudingas, prognozuojant vyresnių pacientų chirurginio gydymo išeitis.

LMM yra pagrindinis veiksnys ribojantis endoskopinio gydymo galimybes pacientams sergantiems ASV. Todėl šiuolaikinė endoskopinė ASV gydymo koncepcija remiasi tiksliu tinkamų pacientų (be LMM) atrinkimu atsižvelgiant į klinikines ir patologines naviko savybes. Atlikus išsamią literatūros apžvalgą rasta 17 studijų nagrinėjančių rizikos veiksnius LMM sergant ASV. 6 iš jų atliktos Vakarų šalyse, 11 Azijos šalyse. Studijose iš Azijos LMM dažnis sergant ASV siekia nuo 2.8 % iki 15.5 % kai navikas infiltruoja tik gleivinę (37,44) ir nuo 18.3 % iki 56.2 % kai infiltruoja ir pogleivį (35,37,41). Atitinkamai Vakarų šalyse LMM buvo randamos nuo 1.9 % iki 16.7 % atvejų navikui infiltruojant tik gleivinę ir nuo 18.2 % iki 42.9 % atvejų navikui siekiant ir pogleivį (35,49,50). Mūsų studijos rezultatai yra panašūs, LMM dažnis esant T1a navikams siekia 5.1 %, esant T1b navikams – 22.4 %.

Įvariose studijose nurodomi LMM rizikos veiksniai irgi skiriasi. Azijos šalių studijose dažniausiai minimas invazijos gylis (9 iš 11 studijų), naviko dydis (7 iš 11 studijų) ir limfovaskulinė invazija (7 iš 11 studijų). Vakarų šalių studijose limfovaskulinė invazija kaip rizikos veiksnys LMM minima 5 iš 6 studijų, tą patvirtino ir mūsų studija (35). Naviko invazija į pogleivį kuri nustatyta kaip rizikos veiksnys LMM mūsų studijoje, paminėta 3 iš 6 anksčiau publikuotų studijų. Naviko diferenciacijos laipsnis kaip rizikos veiksnys LMM minimas tik Azijos šalių studijose (44,46,47). Mūsų žiniomis, mūsų studija yra pirmoji Vakarų šalyse, patvirtinusi, kad naviko diferenciacija yra nepriklausomas LMM rizikos faktorius (35).

EGR ir ESD kaip ASV gydymo metodas vis plačiau taikomos ne tik Azijoje, bet ir Vakarų šalyse (53). Deja, tik nedidelė dalis pacientų, sergančių ASV, atitinka standartinius ESD gydymo kriterijus. Remiantis klinikiniais pastebėjimais, kad pernelyg griežtos standartinės indikacijos didina nebūtinų tradicinių operacijų skaičių, buvo pasiūlyti išplėstiniai kriterijai (36,53,54). Gotoda et al., išanalizavęs 5265 operuotų dėl ASV pacientų duomenis, nustatė 4 papildomus navikų tipus, kurie nesant limfovaskulinės invazijos, labai retai metastazuoja į limfinius mazgus (36,55,56). Šie kriterijai Japonų Skrandžio Vėžio Gydymo Gairėse yra apibrėžiami kaip išplėstiniai kriterijai: 1) diferencijuoto tipo intramukozinė adenokarcinoma be išopėjimo požymių, kurios diametras viršija 2 cm; 2) diferencijuoto tipo intramukozinė adenokarcinoma su išopėjimo požymiais, kurios diametras neviršija 3 cm; 3) nediferencijuoto tipo intramukozinė adenokarcinoma be išopėjimo požymių neviršijanti 2 cm diametro; 4) diferencijuoto tipo pogleivinė (SM1, <500 μm nuo *muscularis mucosae*) adenokarcinoma, iki 3 cm diametro (16).

Tinkamai atrinkus pacientus tinkančius endoskopiniam gydymui galima išvengti nebūtinų didelės apimties tradicinių operacijų. Tai leistų pagerinti ASV gydymo rezultatus ypač vyresnio amžiaus pacientų, sergančių gausia gretutine patologija (ASA III/IV), grupėje, kurią mūsų studija identifikavo kaip didesnės rizikos

dėl ženklaus pooperacinio mirštamumo. Tačiau išplėstinių kriterijų ER naudojimas kasdieninėje praktijoje išlieka kontroversiškas, nes nepakanka įrodymų dėl ilgalaikių gydymo rezultatų sekmės (36,57). Tokį nuogastavimą stiprina Jee et al. (58) publikuota studija, nurodanti, kad atlikus tradicinę operaciją su limfonodektomija pacientams sergantiems ASV, kuris atitinka išplėstines indikacijas ER, 2.8 % pacientų nustatomos LMM. Mūsų studija patvirtina tokius radinius, kuomet tokioje pat pacientų grupėje nustatytas LMM dažnis siekia 1.7 %. Įdomu tai, kad Jee et al. (58) nustatė LMM riziką trims iš keturių išplėstinių kriterijų, bet ne diferencijuoto tipo intramukozinei adenokarcinomai be išopėjimo požymių, kurios diametras viršija 2 cm. Todėl iki mūsų studijos ši indikacija laikyta visiškai saugia (58). Mūsų studijoje LMM rastos būtent tokio tipo ASV. Todėl apibendrindami mūsų radinius ir ankstesnių studijų duomenis galime teigti, jog navikai atitinkantys išplėstinius kriterijus visuomet neša metastazių limfiniuose mazguose riziką.

Nors taikant išplėstinius kriterijus LMM rizika ir yra, ji santykinai nedidelė. Ši rizika labai ženkliai išauga, jei yra viršijami išplėstiniai kriterijai. Mūsų studijos duomenimis LMM tuomet randamos iki 30.2 % pacientų (36), todėl tokiems pacientams neabejotinai turėtų būti taikomas tradicinis chirurginis gydymas.

Kai kurie autoriai diskutuoja, kad net ir ne-gydomoji ESD lemia patenkinamus ASV gydymo rezultatus. Hatta et al. (59) savo multicentrinėje studijoje palygino gydymo rezultatus tarp pacientų kurie po ne-gydomosios ESD tik stebėti bei tų, kurie iškart papildomai radikaliai operuoti. Radikaliai operuotų pacientų 3 ir 5 metų BI ir LSI buvo geresnis. Reikia pažymėti, kad LSI skirtumas buvo nedidelis (99.4 % vs. 98.7 %) lyginant su BI skirtumu (96.7 % ir 84.0 %). Nors ligos recidyvo dažnis tarp stebėtų ir iškart operuotų pacientų skyrėsi reikšmingai, tačiau abiejose grupėse jis buvo nedidelis – 1.3 % radikaliai operuotų pacientų grupėje ir 3.1 % stebėtų pacientų grupėje. Vis dėlto gerą LSI rodiklį ir mažą ligos recidyvų skaičių stebimų pacientų grupėje reikėtų vertinti kritiškai dėl tarp tyrimo grupes sudarančių pacientų klinikinių ir patologinių savybių skirtumų. Žinomi LMM rizikos veiksniai (limfovaskulinė invazija, gilesnė invazija į pogleivį) buvo reikšmingai dažniau sutinkami operuotų pacientų grupėje (35,59), todėl grupių nevienalytiškumas galėjo daryti įtaką studijos rezultatams. Suzuki et al. neseniai publikavo panašią studiją, kurioje nurodo, kad pacientai kurie radikaliai operuoti po ne-gydomosios ESD pasiekia geresnius gydymo rezultatus. Pritaikius panašiausių atvejų analizę išaiškėjo, jog operuotų pacientų 5 metų LSI ir BI yra reikšmingai geresni, bei atitinkamai siekia 99.0 % prieš 96.8 % ir 91.0 % prieš 75.5 % (19). Apibendrinant šių dviejų Azijos šalių tyrimų ir mūsų studijos rezultatus galime patvirtinti, kad pacientai, kurie serga ASV viršijančiu išplėstinius ER kriterijus turi būti gydomi tradiciniu chirurginiu būdu iškart arba nedelsiant po to kai išaiškinama, kad atlikta ne-gydomoji ESD, dėl didelės ligos atkryčio ar progresavimo tikimybės (33).

Vertindami ankstyvuosius gydymo rezultatus nustatėme, kad ESD turi pranašumą prieš chirurgiją dėl trumpesnės procedūros trukmės ir trumpesnės hospitalizacijos (33). Vidutinė ESD trukmė buvo 83 minutės ir šis rezultatas yra artimas laikui, skelbiamam didelėse Vakarų ir Rytų šalių studijose (61,62). Chiu et al. (17) studija iš Kinijos retrospektyviai tyrė 74 pacientus, kuriems atlikta ESD dėl aukšto laipsnio displazijos arba ASV bei lygino juos su 40 pacientų, kuriems atlikta tradicinė operacija. Kaip ir mes, šis autorius nurodo, jog ESD grupėje pačios operacijos ir hospitalizacijos trukmė yra trumpesnė. Priešingai nei Chiu et al. studijoje ar kitose panašiose apžvalgose (62), savo studijoje mes nenustatėme, kad ESD grupėje būtų mažiau pooperacinių komplikacijų. Abiejose grupėse pooperacinių komplikacijų dažnis buvo vienodas ir siekė 16.7 %. Pooperacinės komplikacijos buvo panašios ir vertinant jų sunkumą. Medikamentinio gydymo pakako atitinkamai 4/42 (9.5 %) ir 2/42 (4.8 %) pacientams iš chirurgijos ir ESD grupių. Endoskopinės intervencijos buvo reikalingos tik po ESD, kuomet 3 (7.1 %) pacientams atliktas kraujavimo stabdymas. Kraujavimų dažnis po ESD pateikiamas literatūroje yra panašus ir siekia 1.6 % - 7.0 % (61,63,64). 2 (4.8 %) pacientams iš kiekvienos mūsų tyrimo grupės išsivystė komplikacijos, kurių gydymui reikėjo chirurginės intervencijos. Chirurgijos grupėje abu pacientai buvo operuoti dėl peritonito, o ESD grupėje – dėl skrandžio perforacijos. Kitų autorių duomenimis perforacijų dažnis po ESD svyruoja nuo 2.2 % iki 9.7 % Rytų šalyse ir nuo 1.8 % iki 10 % Vakarų šalyse (61-63). Manome, kad skirtingai nuo Azijos studijų, mūsų studijoje bendro pooperacinių komplikacijų skirtumo po ESD ir chirurginio gydymo nebuvimą lėmė santykinai mažas pooperacinių komplikacijų dažnis po tradicinių operacijų ir santykinai didelis nesunkių komplikacijų skaičius (Clavien-Dindo≤2) po ESD (33).

Chirurgijos grupėje po pritaikyto panašiausių atvejų analizės metodo pooperacinis mirštamumas siekė 2.4 %. Ši grupė gerai atspindi visą dėl ASV operuojamų pacientų grupę, nes ankstesniuose darbuose nustatėme, kad mirštamumas po tradicinių chirurginių operacijų dėl ASV siekia 1.8 % (33,35). ESD grupėje mirusių pacientų nebuvo, tačiau reikia įvertinti tai, kad ši grupė buvo santykinai maža todėl galima egzistuojanti rizika neįvertinta. Įvairių autorių duomenimis pooperacinis mirštamumas po ESD gali siekti iki 3.3 % (33,65).

Visiems pacientams, operuotiems dėl ASV, buvo atliktos radikalios operacijos. Azijos šalių studijose pateikiamas radikalių operacijų dažnis yra toks pats – 100 % (33,62). Tačiau ESD grupėje, priešingai nei chirurgijos, *en bloc* rezekcija atlikta tik 38/42 (92.7 %), o pilnavertė rezekcija tik 32/42 (78.0 %) pacientų. Šie rezultatai yra panašūs į literatūroje sutinkamus rezultatus (33,61,63-66). Po pašalinto naviko morfologinio ištyrimo 2 (4.8 %) atvejais nustatyta, jog pašalintas ASV navikas viršija išplėstinius ER kriterijus ir tik 30/42 (71.4 %) ESD atvejų buvo klasifikuoti kaip gydomoji rezekcija. Mūsų tyrimas parodė, jog ne-gydomoji ESD yra rizikos veiksnys ligos atkryčiui. Manome, jog santykinai didelis ne-gydomųjų rezekcijų dažnis ESD

grupėje lėmė tai, kad 5 metų IBL chirurgijos grupėje buvo reikšmingai geresnis (33). Priešingai nei mūsų studijoje, sisteminėje apžvalgoje iš Azijos šalių apimančioje 1331 pacientus toks skirtumas nenustatytas (62). Dėl to palyginome 5 metų IBL rezultatus chirurgijos ir ESD grupėje neįtraukdami į analizę pacientų, kuriems atlikta ne-gydomoji rezekcija. Kaip ir tikėtasi tokiu atveju reikšmingo skirtumo tarp grupių vertinant atokiuosius gydymo rezultatus nebeliko. Nors laikas iki ligos atkryčio statistiškai reikšmingai skyrėsi ir buvo ilgesnis chirurgijos grupėje, BI abiejose grupėse nesiskyrė. Netikėta, bet net ir ne-gydomoji ESD neturėjo įtakos bendram išgyvenamumui. Taip galėjo nutikti dėl kelių skirtingų priežasčių. Pirma, pacientų, kuriems atlikta ne-gydomoji ESD, grupė buvo nedidelė, o 5 iš 12 (41.6 %) šios grupės pacientų taikytas papildomas gydymas atliekant tradicinę operaciją jau per pirmus 6 mėn. po endoskopinio gydymo. Antra, 8 iš 9 pacientų, kuriems nustatytas ligos atkrytis, buvo operuoti arba jiems atlikta pakartotina ESD. Radikalus ligos atkryčio gydymas lėmė gerus gydymo rezultatus, todėl pirmasis nesėkmingas ESD epizodas neturėjo įtakos BI. Trečia, Pasaulinės sveikatos organizacijos 2015 metų duomenimis, tikėtina gyvenimo trukmė Lietuvoje siekė 73.6 metų, o vidutinis mūsų studijos pacientų amžius abiejose grupėse buvo 72 metai. Todėl galime manyti, kad BI sergančių ASV tarpe labiau lemia bendra sveikatos būklė ir natūralus populiacinis mirštamumas, o ne mirštamumas tiesiogiai įtakotas ASV (33).

5. Tyrimo apribojimai

Mūsų studija turi keletą apribojimų, kuriuos būtina paminėti. Pirma, tai retrospektyvinis tyrimas, kuriame pacientai nebuvo randomizuoti į ESD ar chirurgijos grupes. Būtina pabrėžti, kad ESD ir tradicinis chirurginis gydymas niekada nebuvo palyginti prospektyvinėse randomizuotose kontrolinėse studijose, kurios yra laikomos auksiniu standartu lyginant gydymo metodus. Tam yra keletas priežasčių: skirtingo gydymo intervencijos apimtis ir sudėtingumas labai reikšmingai skiriasi, o ESD šiuo metu jau yra plačiai paplitusi ir taikoma kasdienėje praktikoje, taip pat egzistuoja nemažai kitais būdais sukauptų įrodymų apie šio metodo saugumą, todėl randomizuotos kontrolinės studijos pradėjimas šiuo metu keltų didelę etinę dilemą. Taip pat, nors LMM rizika sergant ASV atitinkančiu ER kriterijus egzistuoja, ji yra labai maža, todėl siekiant užtikrinti pakankamą randomizuoto kontrolinio tyrimo galią tektų įtraukti labai didelį pacientų kiekį ir tyrimo kaštai taptų nepakeliami (67). Tam, kad būtų galima sumažinti retrospektyviniams tyrimams lyginantiems du gydymo metodus būdingą pacientų atrankos klaidą mes taikėme panašiausių atvejų analizės metodą (angl. propensity score matching). Ši technika yra laikoma puikia alternatyva tokiais kaip šis atvejais, kai randomizuotas kontrolinis tyrimas yra neįmanomas ir dažnai taikoma chirurginiuose tyrimuose (67). Turime pripažinti, kad

net ir naudojant šį statistinį metodą tarp ESD ir chirurgijos grupių išliko reikšmingas skirtumas, kai didesnio diametro navikai buvo dažnesni chirurgijos grupėje. Išliekantis skirtumas galėjo lemti prastesnius gydymo rezultatus chirurgijos grupėje. Antra, pacientų skaičius, kuriems atlikta ne-gydomoji ESD mūsų studijoje buvo nedidelis, todėl negalėjome palyginti rezultatų taikant papildomą chirurginį gydymą ar tik stebėseną po ne-gydomosios ESD. Trečia, bendra mūsų tyrimo imtis buvo santykinai maža, ypač lyginant su panašiomis studijomis, atliktomis Azijos šalyse. Kadangi ESD dėl ASV Vakarų šalyse atliekama rečiau, o bendras sergamumas ASV Vakaruose taip pat yra ženkliai mažesnis, šį trūkumą būtų galima įveikti tik atliekant didelės imties tarptautines, multicentrines studijas. Nepaisant visų čia išvardintų trūkumų, šioje studijoje mes sugebėjome parodyti ESD ir chirurginio gydymo skirtumus gydant pacientus sergančius ASV. Mūsų žiniomis, tai yra pirmoji pasaulyje studija lyginanti ASV gydymą chirurgija ar ESD vakarietiškoje populiacijoje (33).

6. Išvados

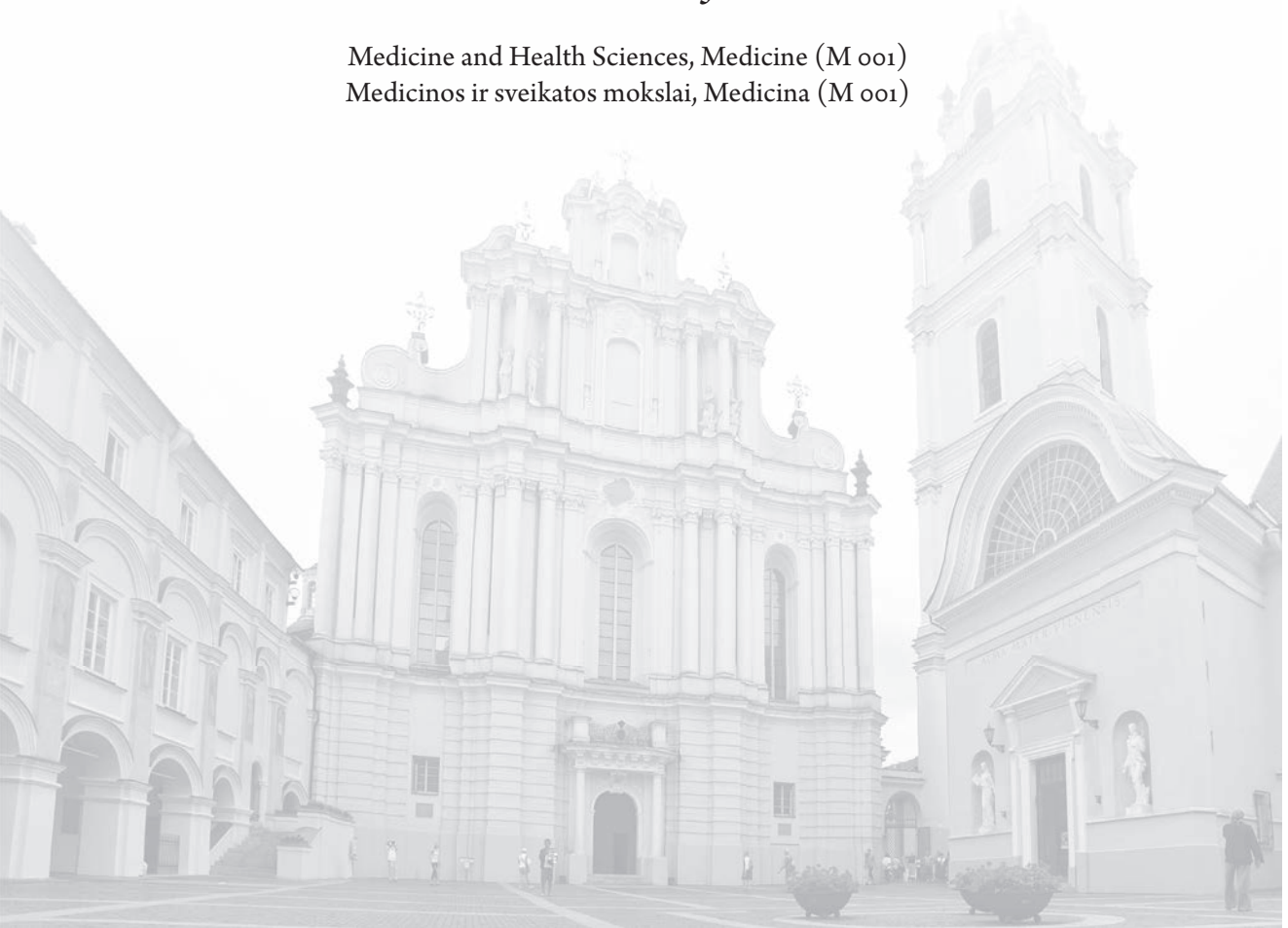
- LMM nustatomos 19.7 % pacientų, sergančių ASV. Naviko invazija į pogleivį, limfovaskulinė invazija ir bloga naviko diferenciacija yra LMM rizikos veiksniai pacientams, sergantiems ASV Lietuvos populiacijoje.
- ESD gali būti puikia alternatyva chirurginiam ASV gydymui Lietuvos populiacijoje dėl trumpesnės operacijos ir hospitalizacijos trukmės bei adekvačių atokių gydymo rezultatų jei pavyksta pasiekti gydomąją rezekciją.
- Ne-gydomoji ESD yra susijusi su didele ligos atkryčio rizika bei nepatenkinamais atokiaisiais rezultatais, todėl po tokių ESD turėtų būti taikomas papildomas tradicinis chirurginis gydymas visais atvejais, kai su juo susijusi rizika yra priimtina.
- Išplėstinių indikacijų endoskopiniam ASV gydymui naudojimas Lietuvos populiacijoje nėra visiškai saugus dėl egzistuojančios nedidelės LMM rizikos. Jei išplėstinės indikacijos endoskopiniam ASV gydymui viršijamos LMM rizika yra didelė, todėl standartinis tokių pacientų gydymas turėtų susidėti iš rezekcinio tipo operacijos kartu atliekant pakankamą limfonodektomiją.
- Pooperacinių komplikacijų skaičius vyresnių ir jaunesnių pacientų grupėje operuojant dėl ankstyvų stadijų SV nesiskiria. Tačiau išsivysčius komplikacijoms vyresnių asmenų grupėje jie dažniau miršta. Aukštesnis ASA balas leidžia prognozuoti, kad vyresnis pacientas operuojamas dėl ankstyvų stadijų SV turi didesnę pooperacinių komplikacijų riziką. Šis rodiklis gali būti naudojamas vertinant su operacija susijusią riziką. Vyresnis amžius neturėtų sulaikyti nuo radikalaus chirurginio gydymo bent su mažesnės apimties limfonodektomija.

Artautas Mickevičius

EFFECT OF CHRONIC PANCREATITIS AND MILD ACUTE PANCREATITIS ON PORTAL VENOUS VELOCITIES

LĖTINIO KASOS UŽDEGIMO IR LENGVO LAIPSNIO ŪMINIO KASOS UŽDEGIMO ĮTAKA VARTŲ VENINĖS SISTEMOS KRAUJOTAKAI

Medicine and Health Sciences, Medicine (M 001)
Medicinos ir sveikatos mokslai, Medicina (M 001)



Introduction

Pancreatitis is one of the most common diseases of the gastrointestinal tract, and the main reason for the hospitalization amongst all gastrointestinal disorders all over the World [Crockett S.D., et al., 2018; Peery A.F., et al., 2015]. The incidence of both acute [Staudacher J.J., et al., 2017; Ikeura T., et al., 2017; Youn G.J., et al., 2017] and chronic [Kleeff J., et al., 2017; Cote G.A., et al., 2011] pancreatitis is increasing every year. Despite accurate incidence of pancreatitis in Lithuania is not known, high alcohol consumption level made acute (AP) or chronic pancreatitis (CP), as well as their complications, one of the most frequent diagnoses among Lithuanians.

Majority of AP cases (about 80 percent) are mild, requiring only symptomatic treatment, and patients become well in one – two weeks. Far more attention is paid to a moderately severe or severe AP that is characterized by the necrosis of the pancreatic parenchyma, edema and / or necrosis of the surrounding tissues, systemic inflammatory response syndrome, and organ dysfunction. In case an organ dysfunction lasts longer than 48 hours, the mortality rate reaches as much as 50 percent [UK Guidelines for the Management of Acute Pancreatitis, 2005], if it lasts longer than 7 days – the mortality increases by 33 percent more [Atasoy C., et al., 2006; Romagnuolo J., et al., 2016]. Because of quick remission, patients with a mild AP are paid little attention to, they are rarely followed afterwards, and changes in case of a mild AP are not that well examined compared to those in case of a severe AP.

Chronic pancreatitis (CP) is still a challenge in clinical practice. Fibrosis of pancreatic parenchyma that develops in case of a chronic process irreversibly damages both exocrine and endocrine pancreatic functions, and consequently health related quality of life. Unfortunately, despite improving diagnostic tools, early stages of CP are still a challenge to diagnose. And only later, stages of the disease have characteristic signs to diagnose a definite CP.

Anatomic localization of pancreas is the reason why its inflammation is associated with various complications, one of which is a disturbance in portal venous system blood flow. For a long time, portal vein thrombosis (PVT) was known to be a complication of CP, especially when acute episodes of CP repeat frequently or cyst in the tail of the pancreas forms. Severe acute pancreatitis, especially necrotizing, is noticed to be associated with acute PVT as well. Recent medical literature presented several cases when even mild acute pancreatitis was associated with a clinically insignificant PVT only visible in radiological images. After symptoms of AP resolved, recanalization of a portal vein lumen was spontaneous. Though information about the PVT pathophysiology was insufficient for a long time, recent studies claim the etiology to be multifactorial: Virchow triad is the key to PVT formation. Despite new conditions associated with a hypercoagulation are being found (as many as 60

percent of all PVT cases are related to myeloproliferative conditions), only few studies paid attention to the other part of Virchow triad – stasis and blood flow changes in the vessel. Portal venous velocities, measured using a Doppler ultrasonography, are well documented among healthy individuals; however, results are not homogeneous and vary a lot. There is much less information about the changes in a blood flow during the AP or CP. Portal venous velocities and their changes during the pancreatic inflammation have not been assessed at all. It remains unclear whether mild acute pancreatitis or chronic pancreatitis (without exacerbation) have any noticeable influence on the blood flow in portal venous system – turbulent blood flow, decrease in portal velocities or formation of thrombi near the vascular wall.

1.1. Aim of the study

The aim of this study is to assess changes in portal venous velocities in case of a chronic pancreatitis and mild acute pancreatitis compared to healthy individuals.

1.2. Objectives of the study

1. To determine parameters of portal venous velocities in healthy adult individuals;
2. To assess parameters of portal venous velocities in adult patients diagnosed with a chronic pancreatitis (not in an acute phase);
3. To assess parameters of portal venous velocities in adult patients diagnosed with a mild acute pancreatitis;
4. To evaluate demographic factors that influence changes in portal venous velocities in case of a pancreatic inflammation;
5. To determine the association between laboratory biochemical and coagulation markers and portal venous velocities in case of a pancreatic inflammation.

1.3. Relevance and scientific novelty of the research

During the research, portal venous velocities of healthy Lithuanians were evaluated, as well as changes in portal venous velocities at the time of a chronic or mild acute pancreatitis.

First of all, normal values of portal venous velocities in Lithuanian population were established for the first time, and results were compared to the ones obtained by foreign authors. Secondly, portal venous velocities have never been assessed during a chronic or mild acute pancreatitis. Many researchers assessed the lumen of the portal vein or formation of a thrombus in case of CP or mild AP; however, subtle or early changes in velocities were never measured. Retrospective and prospective

studies noticed that the risk of portal vein thrombosis in case of a CP increases when acute episodes repeat frequently or cyst in the pancreatic tail forms. This research is the first to measure velocities in the portal system for patients with a CP, and evaluate whether these changes are noticeable and would help to foresee portal vein thrombosis.

Portal venous velocities were measured in the case of a mild AP as well, and it was done for the first time. As with a CP, diameter or formation of thrombus, risk factors for portal vein thrombosis in case of AP were studied in many researches earlier; however, this is the first study to include mild AP cases exclusively and to measure detailed velocities at the time of acute phase and 6 months later to evaluate if AP has any effect on portal venous velocities.

2. Materials and methods

2.1. Plan of the study

Considering the aim and objectives of the study, programme of this scientific work was composed at the beginning of the research:

1. Literature review; research protocol and other documentation required for the research were prepared; approval to perform the research from the Lithuanian Bioethics Committee was received.
2. Selection of participants based on the chosen inclusion criteria, differentiation to the three study groups.
3. Abdominal Doppler ultrasonography was performed to evaluate a blood flow of the portal vein and its branches, hepatic artery and hepatic vein:
   a) Healthy individuals – control group;
   b) Participants with a mild acute pancreatitis;
   c) Participants with a chronic pancreatitis (with no signs and symptoms of an exacerbation).
4. Follow-up abdominal Doppler ultrasonography was performed after three months evaluating blood flow of the portal vein and its branches, hepatic artery and vein.
5. Follow-up abdominal Doppler ultrasonography after six months evaluating blood flow of the portal vein and its branches, hepatic artery and vein.
6. Data handling and statistical analysis; evaluation of factors related to changes in the portal venous velocities.

2.2. Material and methods

This scientific work was conducted since May 2015 to April 2018 in Vilnius University Hospital Santaros Clinics, Centre of Hepatology, Gastroenterology and Dietetics, Department of Hepatology and Gastroenterology. The local Lithuanian Bioethics Committee approved protocol of this study (No 158200-15-774-291) (03 05 2015), an individual informed consent form was read and signed by each of study participants prior to the inclusion to the research. The ethical principles of the Declaration of Helsinki for medical research involving human subjects were fulfilled.

103 individuals participated in the study, data of 71 participants were used in the final analysis, and all of them were examined three times. All participants were divided into three groups: patients diagnosed with a mild acute pancreatitis (50 individuals at the beginning of the study); patients diagnosed with a chronic pancreatitis (35); and healthy individuals (18). Data were analyzed twice: initial analysis included data of 44 participants with an acute pancreatitis, 30 – with a chronic pancreatitis, and 16 healthy participants. Second and final analysis included 29 acute pancreatitis patients, 26 participants with a chronic pancreatitis, and 16 healthy individuals.

2.3. Study groups

All participants met the defined inclusion criteria:

1. Acute pancreatitis group:
   - Acute pancreatitis diagnosed;
   - Mild acute pancreatitis based on the Revised Atlanta Classification;
   - Individuals from 18 to 80 years of age;
   - Patients who agreed to participate in the study and signed the informed consent form;
   - No cardiovascular or coagulation disorders diagnosed earlier based on a medical documentation.
2. Chronic pancreatitis group:
   - Chronic pancreatitis diagnosed;
   - No signs and symptoms of cholangitis and exacerbation of pancreatic inflammation;
   - Patients hospitalized to the Department of Hepatology and Gastroenterology to replace the stent of a common bile duct or a pancreatic duct;
   - Individuals from 18 to 80 years of age;
   - Patients who agreed to participate in the study and signed the informed consent form;
   - No cardiovascular or coagulation disorders diagnosed earlier based on a medical documentation.

3. Control group:
   - Individuals with no signs and symptoms of an acute and chronic pancreatitis;
   - No events of acute pancreatitis in a history;
   - Individuals from 18 to 80 years of age;
   - Patients who agreed to participate in the study and signed the informed consent form;
   - No cardiovascular or coagulation disorders diagnosed earlier based on a medical documentation.

Exclusion criteria:
- Liver disease and/or its complications;
- Myeloproliferative disorder;
- Oncologic condition at the present or earlier;
- Cardiovascular disease;
- Coagulation disorder;
- Individuals younger than 18 and older than 80 years of age.

Acute pancreatitis group. Initially, at the beginning of the study, 50 participants were included in this group. All of them were hospitalized for the thorough diagnostical examination and treatment to the Department of Hepatology and Gastroenterology of Santaros Clinics. During the course of the study, 6 patients were excluded from the initial data analysis because of a liver fibrosis observed on an ultrasound examination; 15 more (21 patients in general) – from the final data analysis as they missed the second or third follow-up visits. Data of 44 patients were used for data analysis, 38 (86,4%) cases of acute pancreatitis were of metabolic (alcohol induced) origin. Based on the criteria established by the International Association of Pancreatology and American Pancreatic Association, acute pancreatitis was diagnosed if it met two out of three criteria:
- Clinical – upper abdominal pain;
- Laboratory parameters – serum lipase or amylase three times greater than the upper limit of normal;
- Characteristic radiologic findings (CT, MRI, ultrasound).

Based on the Revised Atlanta classification, all cases of acute pancreatitis, treated in the Department of Hepatology and Gastroenterology, are classified into three severity levels [Banks P.A., et al., 2013]:
- Mild – absence of organ failure and local or systemic complications;
- Moderately severe – transient organ failure (lasting less than 48 hours) and/or local or systemic complications;
- Severe – persistent organ failure (lasting longer than 48 hours).

This scientific research included only patients diagnosed with a mild acute pancreatitis. Severe acute pancreatitis, especially necrotizing pancreatitis, is a well known risk factor for a portal vein thrombosis, there are a lot of retrospective and prospective studies proving the fact. Nevertheless, mild acute pancreatitis comprises majority of all acute pancreatitis cases. Still, there are no studies assessing whether mild acute pancreatitis influences blood flow in the portal vein and its branches. Therefore, only patients diagnosed with a mild acute pancreatitis and treated in the Department of Gastroenterology and Hepatology in the acute phase of the disease were chosen for the study. All participants were treated in the in-patient department for 5 to 7 days with the fluid therapy (5 ml/kg/per day of intravenous Ringer and / or 0,9 percent sodium chloride solution in the first day of hospitalization (speed of infusion – 200-300 ml per hour), decreasing to 1,5 liters per day at the end of hospitalization), pancreatic enzyme replacement therapy, analgetics (non-steroidal anti-inflammatory drugs), and diet. Generally, anticoagulants are not a part of the treatment algorithm, therefore, none of the participants, diagnosed with an acute pancreatitis received any anticoagulants.

Chronic pancreatitis group. In the beggining of the study, 35 patients, who were hospitalized in Santaros Clinics Department of Hepatology and Gastroenterology for the planned replacement of the stent in a pancreatic or common bile ducts, were included into this study. Patients with any signs or symptoms of an acute disease were excluded from the study. During the research course, 9 patients were excluded from the further examination and data analysis: data of 30 patients were used in the initial diagnosis (due to aliver fibrosis ant breast cancer in anamnesis, 5 patients were excluded from the study in the first stage), later 4 more participants were excluded (ones who missed the second or third appointments). Data of 26 chronic pancreatitis patients were used in the final analysis. Diagnosis of a chronic pancreatitis was established earlier, not when the patient was included into the study or at the time of associated hospitalization. Only patients who were diagnosed with a definite chronic pancreatitis based on the diagnostic radiologic algorithm, proposed by the American Pancreatic Association, were included in the study: pancreatic calcification and/or moderately or severely expressed morphologic pancreatic changes.

Moderately expressed morphologic changes of the pancreas – two or more comfirmed criteria:

- Enlarged main duct (2-4mm);
- Enlarged pancreas;
- Heterogeneous pancreatic parenchyma;
- Irregular pancreatic ducts;
- Increased echogeneity of the main duct wall;

- Pancreatic cavities (<10mm);
- Irregular contour of the head/body of the pancreas.

All patients had symptoms and etiologic factors characteristic of a chronic pancreatitis as well: acute pancreatitis in a history, heavy alcohol consumption in a history, abdominal pain, steatorrhea decreasing after a pancreatic enzyme replacement therapy and others.

Participants were hospitalized in the Department of Hepatology and Gastroenterology from 3 to 5 days. Abdominal utrasound was performed and blood samples taken during the first 24 hours of hospitalization. Planned general anesthesia and endoscopic retrograde cholangiography for the stent replacement procedure was performed on the second day of hospitalization, therefore, did not interferre with the research. Following the procedure, patients were given an intraveous fluid therapy and analgetics if needed.

Control group – healthy participants. 18 volunteers composed the control group. After reading and signing the informed consent form, they were invited to participate in the scientific study. In order to keep with the inclusion and exclusion criteria, all participants were thoroughly examined. Detailed anamnesis was collected and all medical documentation of each participant reviewed, after which one participant was excluded from the research (thyroid cancer in anamnesis). Thorough examination included: measuring arterial blood preasure and heart rate, abdominal palpation, examining leg veins and peripheral edemas. Abdominal ultrasound was performed to measure liver size and structure, size and contour of the gallblader, as well as structure of the pancreas, its size, structure and integrity of the pancreatic duct, abdominal lymphnodes for lymphadenopathy. After abdominal ultrasonography, one patient was excluded from the study due to gallblader stones. Blood samples were taken for biochemical analysis in order to evaluate medical state of volunteers: concentrations of unconjugated and conjugated bilirubin, alanine transaminase, aspartate transaminase, alkaline phosphatase, gama glutamattranspherase and glucose, and lipidogram. Therefore, 16 healthy individuals were finally included into the final examination and data analysis as a control group.

2.4. Study course

All participants were introduced with the course of the research and the concent to participate in the study was received. This study was composed of three stages separated by three months interval each.

During the first examination, venous blood samples were taken for each participant to perform complete blood count, biochemical and coagulation tests. For patients diagnosed with an acute and chronic pancreatitis, starving blood samples were taken in the Department of Hepatology and Gastroenterology during the first

24 hours of hospitalization by the nurse in the blood – sampling room during the venipuncture. Healthy participants' blood samples were taken in the morning in the blood – sampling room in the Centre of Laboratory Medicine of Santaros Clinics. At the same day, participant's height, weight and body mass index, arterial blood pressure and heart rate were measured. Abdominal Doppler ultrasonography was performed for each participant at the same day. Examined in detail were pancreatic parenchyma, liver vessels and their velocities. 90 participants took part in the initial examination, demographic, laboratory and ultrasound results were used in the initial data analysis.

The second examination was performed after three months. Abdominal Doppler ultrasonography was performed. After three more months – thus, six months after the inclusion to the study – participants were examined the third time. Abdominal Doppler ultrasonography was perormed. Neither complete blood count nor biochemical or coagulation tests were repeated during the second and third visits. Data from the second and third examinations were used in the second stage of the analysis. During all the scientific study, Doppler ultrasonography was performed three times for each participant – therefore, 232 times in general.

2.5. Laboratory blood tests

Each participant's blood samples were taken for a complete blood count, coagulation and biochemical blood tests. Blood tests were performed in the Laboratory of Biochemistry in the Centre of Laboratory Medicine in Vilnius University Hospital Santaros Clinics. Venous blood samples of 74 patients with pancreatitis were taken in the in – patient department during the first 24 hours of hospitalization. Venous blood samples of 16 healthy participants, who have starved at least 6 hours, were taken in the out – patient department in the blood – sampling room of the Centre of Laboratory Medicine. Blood samples were collected to the 2,7 and 5 ml tubes with sodum citrate and EDTA during the venipuncture procedure by an experienced nurse. The following blood tests were performed:

- Complete blood count: white blood cell count (WBC), neutrophil count (NEU), platelet count (PLT), hemoglobin concentration (Hgb), hematocrit concentration (Hct), mean platelet volume (MPV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH). Test was performed using the *Coulter DxH 800* analyzer (*Backman Coulter Diagnostics, USA*).
- Coagulation tests: international normalized ratio (INR), activated partial thromboplastin time (APTT), D-dimer, prothrombin time (PT), fibrinogen. Tests were performed using the *STA R Max* analyzer (*Stago Group, France*).
- Biochemical tests: C reactive protein (CRP), sodium concentration (Na),

potassium concentration (K), chlorine concentration (Cl), calcium concentration (Ca), lipase, p-amylase, lactatdehydrogenase (LDH). Tests performed using the *Architect* analyzer (*Abbott, USA*).

2.6. Spectral doppler ultrasound of the abdomen

Abdominal Doppler ultrasonography was performed three times for each participant – at the initial examination, and 3 and 6 months later. Examination was performed in Vilnius University Hospital Santaros Clinis, in the Centre of Hepatology, Gastroenterology and Dietetics. All examinations were performed by the study author, thus all measurements were assessed equally. Ultrasound examinntion was performed using *Toshiba diagnostic ultrasound system* machine, *Model SSA-790A* (*Toshiba medical systems corporation, Japan*) equipped with a *Toshiba PVT-375BT* 3,5MHz transducer probe.

Abdominal ultrasonography was performed for at least 6 hours fasting participants, in the supine position with legs stretched, in the right subcostal region. Participants were examined during a quite inspiration. Vascular liver velocities and indices were measured keeping 30–60° Doppler angle (angle between the vessel and ultrasound wave) in the middle part of the vessel where the laminar blood flow is the fastest. Portal vein was assessed approximately 2 cm towards a liver from the confluence of the splenic and superior messenteric veins. Velocities of the right and left portal veins were measured aproximately 1–2 cm from their branching. Hepatic artery was measured above the head of the pancreas, hepatic veins – approximately 3–6 cm away from the inferior cava vein.

Everytime, head of the pancreas and other abdominal organs were evaluated in order to refute any changes, associated with the exclusion criteria. Main parameters of the portal venous system were assessed:

- Portal veins and their diameter;
- Vmax – time avreaged maximal velocity (automatically counted parameter);
- Vpeak – peak systolic velocity;
- Vmin – minimum velocity;
- Vmean – mean velocity;
- Pulsatility index (PI) – derivate parameter calculated automatically using formula:

$$\frac{\text{Vpeak} - \text{Vmin}}{\text{Vmax}}$$

- Resistivity index (RI) – derivate parameter calculated automatically using formula:

$$\frac{\text{Vpeak} - \text{end diastolic velocity}}{\text{Vpeak}}$$

All portal venous parameters (venous velocities) were recorded three times during each examination, later calculating the average value which was futher used in the data analysis. This was the way to avoid fluctuation in blood flow velocities of portal venous system influenced by the heart cyclic rythm.

2.7. Statistical analysis

Data handling and analysis were performed with the SPSS version 20.0 software (SPSS Inc., Chicago, IL) and Excel program. Quantitative variables are expressed as the mean (± standard deviation), or median and range, and qualitative variables as absolute number and relative frequencies.

Overall statistics for continuous variables were performed using one-way and Welch ANOVA or Kruskal Wallis test depending on the distribution of variable and heterogeneity of the variances. For discrete variables, a Chi-square test was used. To compare the prevalence of risk factors between subgroups, a Fisher's exact test was used for categorical analyses and one-way ANOVA, Kruskal Wallis, Tukey post hoc and the Games-Howell post hoc tests – for continuous variables as applicable. A p value <0.05 was considered to indicate statistical significance. Pearson and Spearman correlation coefficients were used to determine possible associations. Univariate and multivariate binomial logistic regressions were used to find risk factors for the portal vein thrombosis (dependent variables were converted to a nominal measure). Only significant or nearest to significant items on univariate analysis were included in the multivariate analysis. For graphic representation, a pie, stacked bar chart, boxplot and multiple line graphs were drawn.

3. Results

3.1. Comparison between study groups

All in all, 90 individuals participated in the study. 74 (82,2%) of them were diagnosed with pancreatic inflammation – either chronic (n=30; 33,3%) or acute (n=44; 48,9%) pancreatitis; and 16 healthy individuals (17,8%) formed the control group (Fig. 1a). In general, 64 (71,1%) men and 26 (28,9%) women participated in the study. In both acute and chronic pancreatitis groups, majority of participants were men (72,7% and 80,0%, respectively) (Fig. 2), whereas equally men and women composed the control group (8 men and 8 women). Age of participants ranged from 23 to 69 years. All in all, three study groups were statistically homogenous according to the age and individuals' height, but differed significantly comparing weight and body mass index (p=0,039 and p=0,022 respectively) (Fig. 3). Weight and BMI of

patients diagnosed with chronic pancreatitis were significantly lower compared to individuals diagnosed with acute pancreatitis (p=0,015 and p=0,011 respectively). Arterial blood pressure and heart rate have a lot of influence on the blood flow velocities. Mean heart rate of AP patients was significantly higher compared to CP patients or healthy participants: 78 BPM (60–110 BPM) *vs* 74 BPM (62–86 BPM) ir 72 BPM (64–78 BPM), respectively; p=0,004. However, systolic and diastolic blood pressure did not differ significantly comparing groups: mean arterial blood pressure in the AP group was 130/80 mm/Hg; 132/92 mm/Hg in the CP group and 127,5/90 mm/Hg the control group.

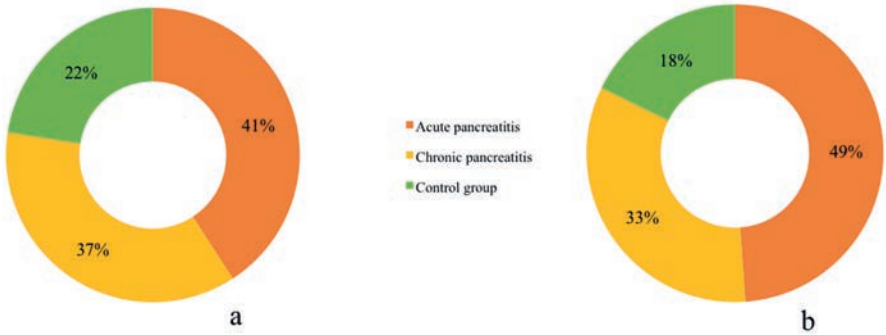


FIGURE 1. Composition of study groups during (a) first examination and (b) after all three examinations

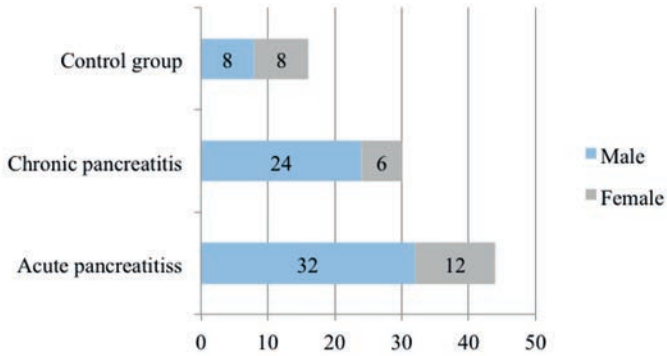


FIGURE 2. Gender distribution between groups

In the second, final, data analysis (after all three examinations), data of 71 participants were used: 26 of them had chronic pancreatitis (36,6%), 29 – mild acute pancreatitis (40,9%) and 16 were healthy controls (22,5%) (Fig.1b). As in the initial data analysis, majority of participants were men: 82,8% in acute pancreatitis and

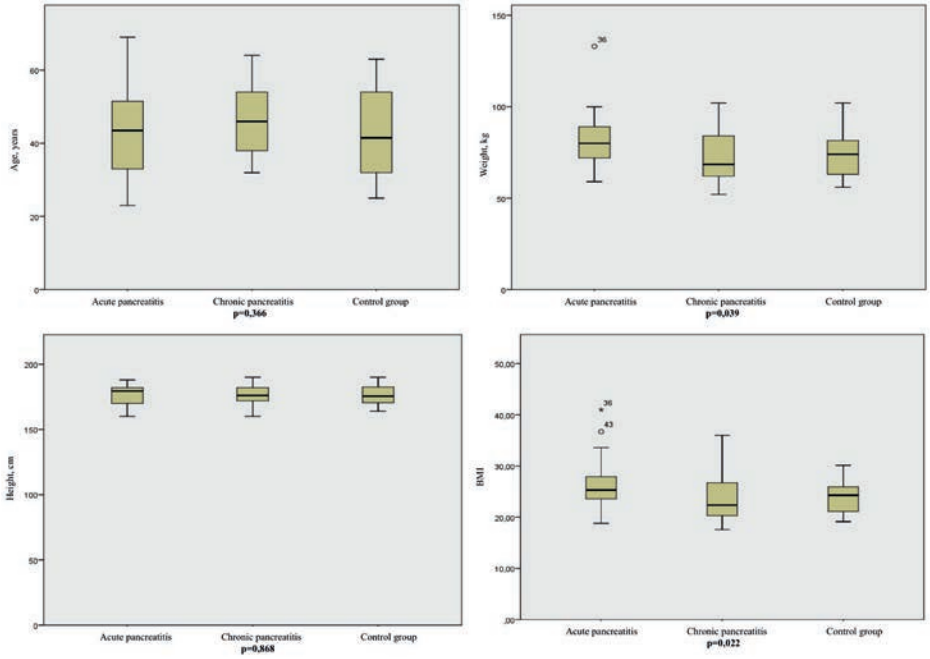


FIGURE 3. Participants' age, height, weight and BMI differences between groups

84,6% in chronic pancreatitis groups; number of participants, as well as their gender distribution, did not change throughout the study. Demographic data of participants in the final analysis did not differ: all groups were homogeneous based on the age, height and gender in patients' groups (Table 1). Despite that, significant difference was observed between acute and chronic pancreatitis groups comparing weight and body mass index: average weight of patients diagnosed with chronic pancreatitis was approximately 10kg lower compared to acute pancreatitis patients (72,6 ± 14,9 kg and 81,9 ± 14,5 kg respectively; p=0,019); BMI in the case of chronic pancreatitis was significantly lower as well (26,5 ± 4,9 in acute pancreatitis and 23,5 ± 4,4 in chronic pancreatitis groups; p=0,011). No significant differences between pancreatitis groups and control group were observed by the weight and BMI.

TABLE 1. Demographic characteristics of participants after the final data analysis

		CP group n=26 (36,6%)	AP group n=29 (48,8%)	Control group n=16 (22,5%)	General p value	p value between groups: CP *vs* AP CP *vs* CG AP *vs* CG
Gender, n (%)	Male	22 (84,6%)	24 (82,8%)	8 (50%)	0,021***	1,000*** 0,032*** 0,037***
	Female	4 (15,4%)	5 (17,2%)	8 (50%)		
Age, Years M, (range)		45,5 (32 – 64)	40,5 (23 – 69)	42,7 (25 – 63)	0,176**	0,109* 0,472* 0,585**
Weight, kg M, (range)		72,6 (52 – 102)	81,9 (59 – 133)	74,8 (56 – 102)	0,056**	**0,019**** 0,640* 0,147**
Height, cm M, (range)		175,9 (160 – 190)	176,2 (160 – 188)	176,7 (164 – 190)	0,942*	0,860* 0,730* 0,845*
BMI, M, (range)		23,5 (17,6 – 36)	26,5 (18,8 – 41)	23,8 (19,2 – 30,1)	**0,032****	**0,011**** 0,783* 0,112**

CP – chronic pancreatitis; AP – acute pancreatitis; CG – control group; M – mean; one-way ANOVA; **Kruskal Wallis; ***Chi-square test. Statistically significant p value (<0,05) in bold.

3.2. Complete blood count results between groups

After analyzing changes in the complete blood count parameters, statistically significant differences were observed between groups in white blood cell count, absolute and relative neutrofil count (p<0,001) and mean corpuscular hemoglobin (p<0,05).

The most prominent elevation in white blood cell count was observed in acute pancreatitis group (11,4 ± 3,9 x 10^9/l). In addition to this, significant differences were observed comparing white blood cell count between acute and chronic pancreatitis groups (p=0,031), between chronic or acute pancreatitis and control groups (p<0,001 and p<0,001 respectively). Significant changes in absolute and relative neutrophil count was observed between all three study groups (p<0,001 and p<0,001 respectively). The highest elevation of neutrophil count was observed in an acute pancreatitis group: absolute neutrophil count was 8,8 ± 3,8 x 10^9/l, relative – 76,8%. Mean neutrophil count values were within normal limits in both chronic pancre-

atitis and control groups. Despite that, both absolute and relative neutrophil count were higher in the CP group compared to healthy individuals (Table 2), therefore, significant differences between changes in absolute and relative neutrophil count were observed comparing acute and chronic pancreatitis groups; acute pancreatitis and control groups; and chronic pancreatitis and control groups ($p \leq 0{,}005$ in all cases) (Table 2).

Less prominent differences were seen between the groups comparing haemoglobin concentration, platelet count and mean corpuscular haemoglobin. Haemoglobin concentration was significantly higher in case of acute pancreatitis compared to chronic (146 ± 15,2 g/l and 136,5 ± 18,1 g/l respectively; p=0,022), however, no significant difference was observed between Hgb comparing patients and healthy individuals' groups. Whereas platelet count was much higher in chronic pancreatitis group compared to patients diagnosed with acute pancreatitis, and the difference was significant (264,3 ± 104,7 x 10^9/l and 205,8 ± 70,2 x 10^9/l respectively, p=0,048). What is more, only in case of chronic pancreatitis, platelet count exceeded the normal limits in some cases – Plt ranged from 120 to 518 x 10^9/l. However, differences between pancreatitis groups and control group by platelet count were not significant. MCH differed significantly comparing acute pancreatitis and control groups as well (31,7 ± 2,2 pg and 29,5 ± 1,2 pg respectively; p=0,015).

3.3. Differences in coagulation parameters between participants

No significant differences were found between groups comparing activated partial thromboplastin time (APTT), prothrombin time (PT) and international normalized ratio (INR) – all values were within normal limits in all – acute and chronic pancreatitis, and control – groups (Table 3).

Significant differences were observed between groups comparing D-dimer and fibrinogen concentrations in blood. In both acute and chronic pancreatitis groups, concentrations of D-dimer and fibrinogen exceeded normal limits (2169,5 ± 2167,0 μg/l and 5,8 ± 1,9 g/l; 886,4 ± 1400,7 μg/l and 4,6 ± 1,3 g/ respectively) and were significantly higher in case of acute pancreatitis compared to chronic ($p<0{,}001$ and p=0,003 respectively). D-dimer and fibrinogen concentrations in blood of healthy controls were within normal limits, therefore, significant difference was observed between healthy individuals and chronic and acute pancreatitis patients comparing the parameters mentioned ($p<0{,}001$ and $p<0{,}001$; p=0,001 and $p<0{,}001$ respectively).

TABLE 2. Comparison between groups by the complete blood count results

Complete blood count parameter	CP group n=30	AP group n=44	Control group n=16	General p value	p value between groups: CP *vs* AP CP *vs* CG AP *vs* CG
White blood cell count, M±SD, x10⁹/l	9,0 ± 3,3	11,4 ± 3,9	5,7 ± 0,8	**<0,001****	**0,031**** **<0,001**** **<0,001****
Absolute neutrophil count, M±SD, x10⁹/l	5,6 ± 2,8	8,8 ± 3,8	3 ± 0,6	**<0,001****	**0,001**** **<0,001**** **<0,001****
Relative neutrophil count, M±SD, %	62,7 ± 12,3	76,8 ± 9,2	52,2 ± 7,4	**<0,001****	**<0,001**** **0,005*** **<0,001****
Mean platelet volume, M±SD, fl	10,3 ± 0,8	10,8 ± 0,8	10,4 ± 1,1	0,205*	0,266 0,571 0,189
Hematocrit, M±SD, l/l	0,4 ± 0,1	0,4 ± 0,1	0,4 ± 0	0,167*	0,198 0,424 0,598
Hemoglobin concentration, M±SD, g/l	136,5 ± 18,1	146 ± 15,2	140,8 ± 10,3	**0,045***	**0,022*** 0,543 0,072
Platelet count, M±SD, x10⁹/l	264,3 ± 104,7	205,8 ± 70,2	234,7 ± 35,9	0,095**	**0,048*** 0,631 0,255
Mean corpuscular volume, M±SD, fl	91,6 ± 6,5	91,2 ± 6,1	89,7 ± 3,7	0,477**	0,730 0,379 0,614
Mean corpuscular hemoglobin, M±SD, pg	30,5 ± 2,2	31,7 ± 2,2	29,5 ± 1,2	**0,041****	0,417 0,182 **0,015****

CP – chronic pancreatitis; AP – acute pancreatitis, CG – control group; M – mean; SD – standard deviation; *one-way ANOVA; **Kruskal Wallis. Significant p value (p<0,05) in bold.

TABLE 3. Comparison between groups by coagulation parameters

Coagulation parameters	CP group n=30	AP group n=44	Control group n=16	General p value	p value between groups: CP *vs* AP CP *vs* CG AP *vs* CG
PT, M±SD, %	93,1 ± 14,1	99,3 ± 15,9	95,4 ± 16,4	0,442	0,217 0,775 0,369
INR, M±SD	1 ± 0,1	1 ± 0,1	1 ± 0,1	0,355	0,273 0,680 0,481
APTT, M±SD, s	36,7 ± 4,4	37,2 ± 5,5	35,4 ± 3,6	0,415	0,492 0,425 0,115
Fibrinogen, M±SD, g/l	4,6 ± 1,3	5,8 ± 1,9	2,8 ± 0,5	**<0,001***	**0,003*** **<0,001*** **<0,001***
D-dimer, M±SD, μg/l	886,4 ± 1400,7	2169,5 ± 2167,0	136,6 ± 58,1	**<0,001***	**<0,001*** **0,001*** **<0,001***

CP – chronic pancreatitis; AP – acute pancreatitis; CG – control group; M – mean; SD – standard deviation; INR – international normalised ratio; APTT – activated partial thromboplastin time; PT – prothrombin time; *Kruskal Wallis. Significant p value (p<0,05) in bold.

3.4. Changes in biochemical blood parameters

Evaluation of changes in the electrolyte concentration revealed that concentrations of sodium, potassium, calcium and chloride in all three groups were within normal limits. Nevertheless, significant differences were still observed between heatlhy individuals and patients with an acute pancreatitis – in the latter group, all electrolyte concentrations were lower compared to healthy controls (p<0,05), the same difference was observed between chronic and acute pancreatitis groups by potassium and calcium concentrations (p<0,05) (Table 4).

Significant differences between groups were observed after assessing changes in enzymes concentration. Concentrations of lipase and p-amylase were several times higher the normal limit in an acute pancreatitis group (3887,5 U/l and 705,4 U/l respectively). Concentration of lipase in the acute pancreatitis group was significantly higher compared to both chronic pancreatitis patients and healthy individuals (p<0,001). Concentration of p-amylase was significantly higher in acute pancreatitis group compared to chronic pancreatitis and control groups as well (p<0,001) (Table 4). Though both lipase and p-amylase concentrations were higher in the blood of patients with chronic pancreatitis compared to healthy individuals (305,4 ± 528,6 U/l

and 126,7 ± 247,4 U/l compared to 45,6 ± 49 U/l and 30,8 ± 18,8 U/l respectively), these differences were not significant (p=0,587 and p=0,078 respectively).

Significant difference was counted comparing all groups on the concentration of lactate dehydrogenase and C-reactive protein. Concentration of CRP increased distinctly only in case of acute pancreatitis (140,2 ± 94,8 mg/l), slightly – in case of chronic pancreatitis, and remained normal in the blood of healthy individuals (38,4 ± 52,9 mg/l and 0,7 ± 0,7 mg/l respectively). Despite the fact, significant difference was observed between acute and chronic pancreatitis groups; between acute pancreatitis and control groups; and between chronic pancreatitis and control groups (p<0,001; p<0,001 ir p<0,001 respectively). Concentration of lactate dehydrogenase was counted to be within normal limits in all three groups (Table 4); however, in both chronic and acute pancreatitis groups, concentrations of LDH varied to the limits higher than normal (145 – 384 U/l and 150 – 1035 U/l respectively). Nevertheless, an average concentration of LDH differed significantly between groups: acute and chronic pancreatitis groups; control and chronic pancreatitis groups; and acute pancreatitis and control groups (p=0,074; p=0,008 and p<0,001 respectively).

3.5. Difference in portal venous velocities between groups

Significant differences in vascular diameter were observed between genders: diameters of the main portal vein, the right and left branches were smaller in the female group compared to males in all three study groups (p=0,000; p=0,022 and p=0,017 respectively). Significantly lower ina female group were pulsatility and resistivity indices (p=0,011 and p=0,006 respectively). Vmin and Vmean were significantly higher between males: p=0,006 and p=0,012 respectively. In addition to this, diameter of the main portal vein was smaller in the control group compared to pancreatitis (acute or chronic) groups (p=0,003); however, it had no significant influence on portal velocities.

No significant differences were found on portal venous velocities by the complete blood count, coagulation and biochemical laboratory parameters in general or in separate study groups (p>0,05). No association was observed between portal vein velocities and arterial blood pressure (systolic and diastolic) and heart rate.

3.5.1. Time averaged maximal velocity

Comparing time averaged maximal velocity (Vmax) in the main portal vein, no significant difference was found between individual study groups (p=0,584). After the initial data analysis, Vmax of the main portal vein was slower in the chronic pancreatitis group compared to acute pancreatitis or control groups; however, the

difference was not significant (Fig. 4). After the second and third examinations, significant difference comparing Vmax between groups dissapeared. In addition to this, Vmax did not differ within each group throughout the first, second and third examinations. Acute pancreatitis had no influence on Vmax – the value was not different compared to healthy individuals during the initial examination, and did not change (neither increased nor decreased) during later visits. Vmax of the right portal vein branch did not differ significantly between groups during every examination, nor it differed within the group throughout all the study. The same observed was after assessing Vmax in the left portal vein branch comparing different groups or different examinations (Table 5).

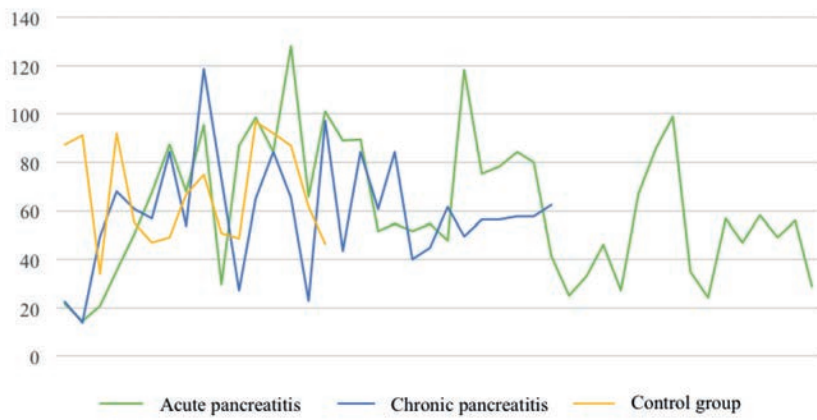


FIGURE 4. Vmax of the main portal vein in different study groups during initial examination (n=90) (p=0,584).

3.5.2. Resistivity index

After assessing changes in resistivity index in the main portal vein, no significant differences were observed comparing RI within individual study groups comparing the first, second and third examinations. What is more, RI in the main portal vein did not differ between groups: it was the same during the first examination (n=90) in all three study groups (p=0,212). During the final examination (6 months later, n=71), RI in all three groups was the same: 0,4±0,2 cm/s in acute pancreatitis, 0,4±0,2 cm/s in chronic pancreatitis, and 0,4±0,2 cm/s in control groups (p=0,929). In addition to this, no significant difference was found measuring RI in the right and left portal vein branches: it did not differ between different study groups. Despite the fact that RI in the right portal vein branch decreased significantly after 6 months

TABLE 4. Differences in biochemical blood parameters between groups

Biochemical blood marker	CP group n=30	AP group n=44	Control group n=16	General p value	p value between groups: CP *vs* AP CP *vs* CG AP *vs* CG
Sodium concentration, M±SD, mmol/l	140,4 ± 4,4	138,8 ± 3,2	141,3 ± 2,1	**0,041***	0,213 0,376 **0,046***
Potassium concentration, M±SD, mmol/l	4,5 ± 0,7	4,2 ± 0,4	4,4 ± 0,5	**0,039***	**0,032*** 0,548 **0,041***
Chloridium concentration, ç, mmol/l	102,6 ± 3,1	102,1 ± 4,9	104,1 ± 1,5	**0,015****	0,329 0,117 **0,027****
Calcium concentration, M±SD, mmol/l	2,2 ± 0,4	2,1 ± 0,4	2,4 ± 0,1	**0,005****	**0,007**** 0,388 **0,033****
Lipase concentration, M±SD, U/l	291,5 ± 627,7	3491,0 ± 5569,9	46,9 ± 57	**<0,001****	**<0,001**** 0,533 **<0,001****
p-amylase concentration, M±SD, U/l	123,0 ± 210,6	708,3 ± 1023,3	30,5 ± 21,1	**<0,001****	**<0,001**** **0,029**** **<0,001****
CRP concentration, M±SD, mg/l	38,4 ± 52,9	140,2 ± 94,8	0,7 ± 0,7	**<0,001****	**<0,001**** **<0,001**** **<0,001****
LDH concentration, M±SD, U/l	202,1 ± 50,5	249,0 ± 140,6	168,5 ± 22,2	**<0,001****	**0,074**** **0,008**** **<0,001****

CP – chronic pancreatitis; AP- acute pancreatitis; CG – control group; M – mean, SD – standard deviation; *one-way ANOVA; **Kruskal Wallis. Significant p value (<0,05) in bold.

in the acute pancreatitis group compared to chronic pancreatitis patients (p=0,003) and healthy individuals (p=0,024), this significant difference was observed in only one branch during one examination (the last one). What is more, RI did not change neither in the right, nor in the left portal vein branches throughout different examinations: thus it did not increase or decrease in acute or chronic pancreatitis group after repeated evaluations (Table 6).

TABLE 5. Changes in Vmax

Portal vein	Examination	CP group	AP group	Control group	General p value	P value between groups: CP *vs* AP CP *vs* CG AP *vs* CG
The main portal vein	First, M±SD, cm/sec	59,4 ± 22,9	61,6 ± 28,1	67,5 ± 21	0,584	0,835 0,313 0,380
	Second, M±SD, cm/sec	61,9 ± 27,4	62,7 ± 19,2	65,7 ± 13,9	0,797	0,993 0,850 0,837
	Third, M±SD, cm/sec	63,7 ± 21,3	68,2 ± 24,1	60,8 ± 20,6	0,496	0,416 0,540 0,496
Right portal vein branch	First, M±SD, cm/sec	39,1 ± 18,1	46,5 ± 24,1	44 ± 23,8	0,603	0,299 0,740 0,740
	Second, M±SD, cm/sec	45,7 ± 19	41,7 ± 14,9	42,8 ± 26,6	0,559	0,440 0,374 0,559
	Third, M±SD, cm/sec	40,4 ± 28,1	42,3 ± 21,8	43 ± 22	0,797	0,515 0,635 0,797
Left portal vein branch	First, M±SD, cm/sec	21,1 ± 8,7	23,4 ± 8,3	20 ± 7,3	0,265	0,151 0,831 0,239
	Second, M±SD, cm/sec	19,9 ± 8,9	17 ± 6,1	22,5 ± 9,6	0,124	0,251 0,342 0,124
	Third, M±SD, cm/sec	18,3 ± 6,2	20,5 ± 10,9	21,2 ± 9,6	0,851	0,942 0,635 0,851

CP – chronic pancreatitis; AP- acute pancreatitis; CG – control group; M – mean, SD – standard deviation.

3.5.3. Mean velocity

After measuring mean velocity in portal veins, no significant differences between groups or between different examinations were observed as well. Vmean in the main portal vein did not differ comparing study groups during the first, second or third examination (p=0,352; p=0,787; and p=0,380 respectively), though, the same as with Vmax, Vmean in the main portal vein was slightly (but not statistically significantly) slower in the chronic pancreatitis group (Fig. 5). What is more,

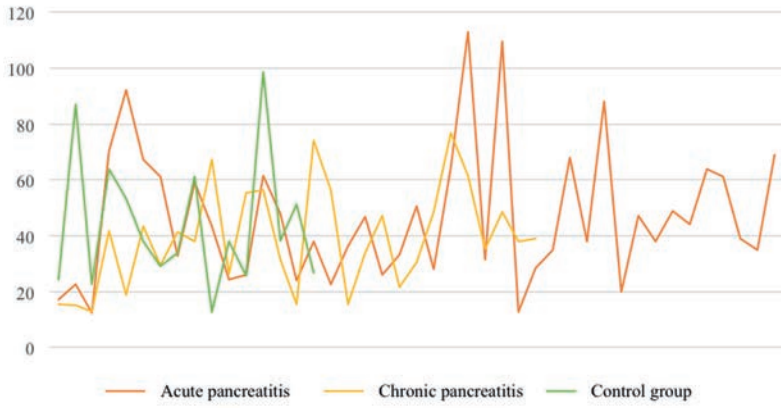


FIGURE 5. Vmean in the main portal vein in different study groups during first examination (n=90) (p=0,352).

Vmean of the main portal vein did not change neither three nor six months later in the acute and chronic pancreatitis groups. The same results were observed comparing Vmean in the right and left portal vein branches: Vmean of those veins did not differ comparing different study groups or within each group comparing different examinations (Table 6).

TABLE 6. Changes in resistivity index and differences between groups

	Examination	CP group n=26	AP group n=29	Control group n=16	General p value	p value between groups: CP *vs* AP CP *vs* CG AP *vs* CG
The main portal vein	First, M±SD	0,4 ± 0,2	0,4 ± 0,1	0,4 ± 0,1	0,212	0,761 0,186 0,077
	Second, M±SD	0,4 ± 0,2	0,4 ± 0,2	0,4 ± 0,1	0,565	0,321 0,416 0,565
	Third, M±SD	0,4 ± 0,2	0,4 ± 0,2	0,4 ± 0,2	0,929	0,828 0,663 0,929

Right portal vein branch	First, M±SD	0,5 ± 0,2	0,4 ± 0,2	0,4 ± 0,1	0,195	0,091 0,344 0,347
	Second, M±SD	0,4 ± 0,2	0,4 ± 0,1	0,5 ± 0,2	0,124	0,427 0,269 0,124
	Third, M±SD	0,5 ± 0,2	0,3 ± 0,1	0,4 ± 0,2	**0,002**	**0,003** 1,000 **0,024**
Left portal vein branch	First, M±SD	0,4 ± 0,1	0,4 ± 0,1	0,3 ± 0,2	0,700	0,919 0,468 0,434
	Second, M±SD	0,4 ± 0,2	0,3 ± 0,2	0,4 ± 0,1	0,461	0,377 0,830 0,461
	Third, M±SD	0,4 ± 0,2	0,4 ± 0,2	0,4 ± 0,2	0,557	0,985 0,691 0,561

CP – chronic pancreatitis; AP – acute pancreatitis; CG – control group; M – mean; SD – standard deviation

TABLE 7. Changes in Vmean between study groups

Portal vein	Examination	CP group n=26	AP group n=29	Control group n=16	General p value	p value between groups: CP *vs* AP CP *vs* CG AP *vs* CG
The main portal vein	First, M±SD, cm/sec	21,2 ± 8,1	21,4 ± 8,1	24,5 ± 8,2	0,352	0,888 0,180 0,192
	Second, M±SD, cm/sec	22,4 ± 8,4	22,3 ± 5,9	25,4 ± 4,5	0,295	0,997 0,377 0,317
	Third, M±SD, cm/sec	23 ± 7,6	24,2 ± 8,9	23,2 ± 7,2	0,883	0,772 0,937 0,883
Right portal vein branch	First, M±SD, cm/sec	14,8 ± 6,7	15,9 ± 7,0	16,4 ± 8,1	0,787	0,509 0,627 0,993
	Second, M±SD, cm/sec	17,6 ± 7,3	17,4 ± 5,8	15,6 ± 7,1	0,622	0,992 0,637 0,687
	Third, M±SD, cm/sec	14,9 ± 9,1	18,2 ± 7,4	16,2 ± 6,9	0,459	0,434 0,874 0,737

Left portal vein branch	First, M±SD, cm/sec	7,1 ± 2,8	8,0 ± 2,9	7,4 ± 2,5	0,380	0,167 0,627 0,547
	Second, M±SD, cm/sec	6,9 ± 2,4	6,4 ± 2,2	8,4 ± 3,4	0,082	0,604 0,118 0,082
	Third, M±SD, cm/sec	6,8 ± 2,3	7,1 ± 3,6	7,9 ± 2,9	0,438	0,800 0,304 0,438

CP – chronic pancreatitis; AP – acute pancreatitis; CG – control group; M – mean; SD – standard deviation

3.6. Correlation between biochemical blood markers and portal venous velocities

No significant association between biochemical blood markers and time averaged maximal velocity and mean portal venous velocity was found.

Though no significant change in Vmax in case of acute and chronic pancreatitis was found during each examination, in addition to this, we assessed association between the biochemical parameters (lipase, D-dimer, CRP and lactate dehydrogenase concentrations) and Vmax of the portal venous system. There were no significant correlations between Vmax and mentioned laboratory markers in the main portal vein and its right branch; however, mild correlation was calculated between lipase

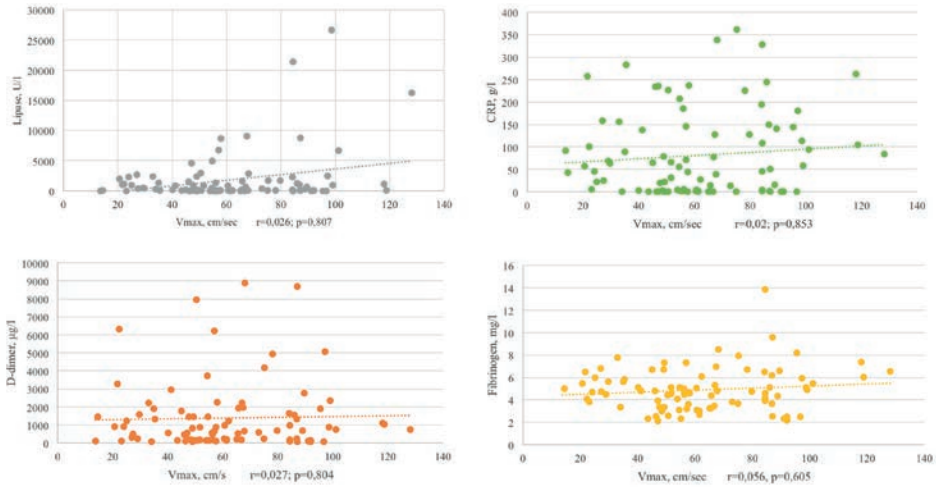


FIGURE 6. Correlation between biochemical blood parameters and Vmax in the main portal vein

concentration and Vmax of the left portal vein branch (r=0,233; p=0,028). Nevertheless, other biochemical parameters did not correlate with the Vmax in the left portal vein branch (p>0,05) (Figure 6).

Evaluated was the relation between biochemical blood parameters and Vmean of the main portal vein as well. Despite concentrations of fibrinogen, D-dimer, CRP and LDH increased significantly in case of pancreatic inflammation, and were significantly higher comparing acute to chronic pancreatitis, however, it had no influence on Vmean in the main portal vein, nor in its right or left branches (p>0,05) (Fig. 7).

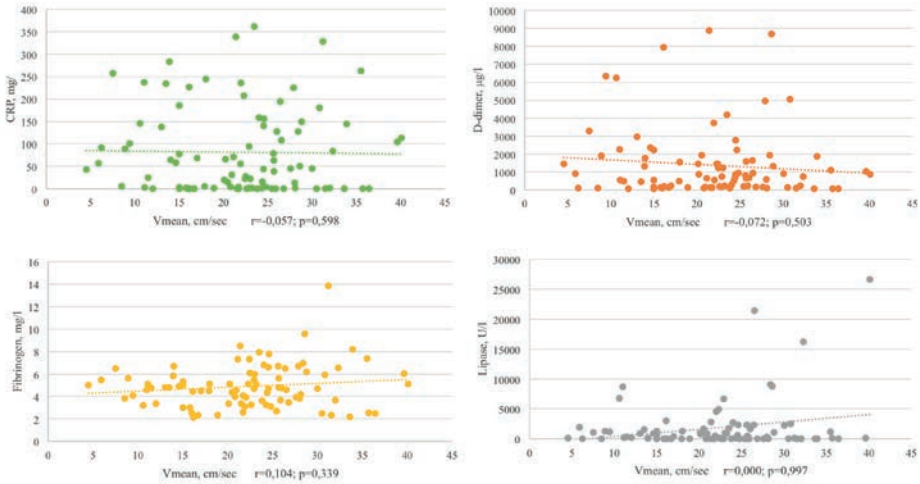


FIGURE 7. Correlation between biochemical blood parameters and Vmean in the main portal vein

Conclusion

1. Estimated mean velocity in the main portal vein of healthy individuals was 24,5 ± 8,2 cm/s, time averaged maximal velocity was 67,5 ± 21 cm/s and did not differ from data in other scientific literature. Venous velocities are approximately 20 cm/s slower in the right portal vein branch and approximately 40 cm/s slower in the let portal vein branch compared to the main portal vein.
2. Portal venous velocities (Vmax and Vmean) are slower in case of chronic pancreatitis (without exacerbation); however, the difference is not significant compared to healthy individuals (p>0,05). During a 6 months follow-

up period, no significant changes in portal venous velocities were observed, thus changes in portal venous velocities in case of chronic stable pancreatitis are very slow.

3. Mild acute pancreatitis has no influence on portal venous velocities in acute phase of the disease – portal vein velocities do not change in case of mild acute pancreatitis compared to healthy individuals (p>0,05). Half year after the onset of mild acute pancreatitis, portal venous velocities do not change significantly.
4. Portal vein velocities do not depend on individual's height, weight or body mass index, and age. Diameter of the main portal vein, its right and left branches is significantly lower between females, whereas Vmean is faster between males.
5. Biochemical and coagulation parameters have no influence on portal venous velocities in case of mild acute pancreatitis or chronic pancreatitis (without exacerbation).

Recommendations

1. Mild acute pancreatitis has little effect on portal venous velocities; therefore, there is no need to follow patients after mild acute pancreatitis for possible portal vein thrombosis.
2. Portal venous velocities are slower in case of chronic pancreatitis; however, this influence is not significant. Observation for the possible portal vein thrombosis in case of chronic pancreatitis is expedient only in case of pancreatic tail cyst and frequent relapses of acute pancreatitis.

SANTRAUKA

Įvadas

Kasa – tai išskirtinis pilvo ertmės organas, liauka, atliekanti tiek egzokrininę, tiek endokrininę funkcijas. Kasos uždegimas – viena dažniausių pilvo organų ligų. Teigiama, kad ūminis pankreatitas (ŪP) yra pagrindinė hospitalizacijos dėl virškinamojo trakto ligų priežastis visame pasaulyje [Crockett S.D., et al., 2018; Peery A.F., et al., 2015]. Sergamumas ir ūminiu [Staudacher J.J., et al., 2017; Ikeura T., et al., 2017; Youn G.J., et al., 2017], ir lėtiniu [Kleeff J., et al., 2017; Cote G.A., et al., 2011] pankreatitu didėja kasmet. Nors tiksliai nėra žinoma, kiek žmonių serga pankreatitu Lietuvoje, dėl piktnaudžiavimo alkoholiu lietuviams dažnai diagnozuojamas ūminis ar lėtinis pankreatitas ir jo sukeltos komplikacijos.

Didžioji dalis visų ŪP (apie 80 proc.) yra lengvos eigos, dažniausiai užtenka simptominio gydymo, pasveikstama per savaitę ar dvi. Kur kas didesnio klinicistų dėmesio sulaukia vidutiniškai sunkios ar sunkios eigos ŪP, šiuo atveju formuojasi kasos nekrozė, aplinkinių audinių edema ir / ar nekrozė, sisteminis uždegiminio atsako sindromas, organų funkcijos nepakankamumas. Jei organų nepakankamumas užsitęsia ilgiau nei 48 valandas, mirtingumas siekia net 50 proc. [UK Guidelines for the Management of Acute Pancreatitis, 2005], jei ilgiau nei 7 dienas – jis padidėja 33 proc. [Atasoy C., et al., 2006; Romagnuolo J., et al., 2016]. Dėl greitos remisijos lengvo laipsnio ŪP sergantys pacientai retai toliau sekami, pokyčiai lengvo laipsnio ligos metu nėra taip detaliai aprašyti kaip sergant sunkiu ūminiu pankreatitu.

Lėtinis pankreatitas (LP) iki šiol kelia daug iššūkių klinikinėje praktikoje. Šio lėtinio proceso metu besivystanti kasos audinio fibrozė negrįžtamai pakenkia tiek egzokrininę, tiek vėliau ir endokrininę kasos funkcijas, ryškiai blogėja su liga susijusi gyvenimo kokybė. Vis dėlto iki šiol, nepaisant tobulėjančių diagnostikos galimybių, ankstyvose stadijose diagnozuoti LP yra sudėtinga. Tik pažengusiose stadijose pakanka įrodymų neabejotinai LP diagnozei.

Dėl savo anatominės lokalizacijos kasos audinio uždegimas siejamas su įvairiomis komplikacijomis, tarp kurių yra ir vartų veninės sistemos kraujotakos sutrikimas. Jau seniai vartų venos trombozė (VVT) yra žinoma kaip lėtinio pankreatito komplikacija, ypač esant dažniems recidyvuojantiems paūmėjimams ar susiformavus kasos uodegos pseudocistai. Sunkios eigos ŪP, ypač nekrotizuojantis, taip pat, pastebėta, dažniau sukelia ūminę VVT. Pastaruoju metu medicininėje literatūroje pasirodė atvejų aprašymų, kuomet net ir lengvos eigos ŪP buvo susijęs su kliniškai nereikšminga, tačiau vaizdinių tyrimų metu stebėta VVT. Praėjus ŪP simptomams vartų venos spindžio rekanalizacija įvyko savaime. Nors ilgą laiką trūko duomenų apie VVT patofiziologiją, tačiau pastarųjų metų darbai teigia, kad priežastis dažniausiai multifaktorinė. Kaip ir kitoms trombozėms, VVT formuotis įtakos turi seniai aprašyta *Virchow* triada. Nors pastaruoju metu randama daug būklių, susijusių su hiperkoaguliacija (net 60 proc. visų VVT siejama su mieloproliferacinėmis būklėmis), mažai autorių vertino kitą *Virchow* triados dalį – stazę ir kraujo tėkmės pokyčius kraujagyslėje. Sveikų žmonių vartų veninės sistemos kraujotakos parametrai, vertinant spalviniu ir spektriniu UG tyrimais su Doplerio režimu, yra gan plačiai aprašyti, tačiau rezultatai labai įvairūs ir nehomogeniški. Tuo tarpu duomenų apie kraujotakos pokyčius ŪP ar LP metu yra kur kas mažiau. Kraujotakos greitis pankreatitu sergantiems asmenims išvis nebuvo vertintas. Lieka neaišku, ar kasos audinio ūminis lengvo laipsnio ar lėtinis (ne paūmėjimo metu) uždegimai turi pastebimos įtakos vartų veninės kraujotakos pokyčiams – turbulencinėms srovėms, kraujo tėkmės greičio sulėtėjimui ar priesieninių trombų formavimuisi.

1.1. Darbo tikslas

Nustatyti, kaip kinta kraujotakos greitis vartų veninėje sistemoje sergant lėtiniu ir ūminiu lengvo laipsnio kasos uždegimu lyginant su sveikais asmenimis.

1.2. Darbo uždavinai

1. Nustatyti vartų veninės sistemos kraujotakos greičio parametrus sveikiems suaugusiems asmenims;
2. Įvertinti vartų veninės sistemos kraujotakos parametrus lėtiniu kasos uždegimu (ne paūmėjimo metu) sergantiems suaugusiems pacientams;
3. Įvertinti vartų veninės sistemos kraujotakos parametrus lengvo laipsnio ūminiu kasos uždegimu sergantiems suaugusiems pacientams;
4. Nustatyti, kokie demografiniai rodikliai turi įtakos vartų veninės sistemos kraujotakos pokyčiams sergant kasos uždegimu;
5. Nustatyti ryšį tarp kraujo biocheminių ir krešėjimo rodiklių bei vartų veninės sistemos kraujotakos pokyčių sergant kasos uždegimu.

1.3. Darbo mokslinis naujumas ir aktualumas

Tyrimo metu nustatyti vartų veninės sistemos kraujotakos parametrai sveikų asmenų populiacijoje ir kraujotakos pokyčiai sergant lėtiniu kasos uždegimu nesant ligos paūmėjimo epizodo ir sergant ūminiu lengvo laipsnio pankreatitu. Kitaip nei anksčiau atliktuose moksliniuose tyrimuose, šiame tyrime vartų veninės sistemos kraujotakos parametrai vertinti tris kartus. Tai leido patikimiau įvertinti sveikų asmenų ir sergančių lėtiniu pankreatitu pacientų kraujotakos greičio vidurkį, taip pat vertinti ūminio pankreatito įtaką kraujotakos greičiui ne tik susirgus, bet ir dinamiškai – praėjus ūmaus pankreatito simptomams ir požymiams.

Literatūroje yra nemažai duomenų apie vartų venos kraujotakos vertinimą ir kraujotakos parametrų variacijas, autorių pateikiami skirtingų šalių gyventojų kraujotakos greičio duomenys. Iki šiol šie rodikliai Lietuvoje sistemingiau nevertinti ir nelyginti su užsienio tyrėjų rezultatais. Šio tyrimo metu įvertintas vartų veninės sistemos kraujotakos greitis, nustatytas jo vidurkis sveikų asmenų populiacijoje Lietuvoje, įvertinta kraujotakos greičio sąsaja su demografiniais rodikliais, palyginti lietuvių duomenys su užsienio tyrėjų pateiktais normos variantais.

Užsienio literatūroje atlikta keletas plačios apimties mokslinių tyrimų, vertinusių kraujotakos pokyčius vartų veninėje sistemoje. Tik keli plačios apimties prospektyviniai tyrimai vertino vartų venos trombozės išsivystymo riziką sergant lėtiniu pankreatitu ir tik nedidelei daliai pacientų išsivystė vartų venos trombozė, tačiau ji buvo susijusi su išsivysčiusiu ūminiu pankreatitu ar kasos uodegos cista. Didesnė dalis atliktų tyrimų yra retrospektyviniai: išsivysčius vartų venos trombozei vertinti galimai įtakos turėję veiksniai, nustatyta LP ir trombozės rizikos sąsaja. Vis dėlto atskirai netirta, ar LP lemia vartų veninės sistemos kraujotakos pokyčius – ar sulėtėjusi kraujotaka, didėjantis rezistentiškumo indeksas gali būti parametrai, leidžiantys nustatyti pacientus, kuriems yra didesnė VVT rizika. Šio tyrimo metu vertintas ne pats trombozės išsivystymas, o ligos įtaka kraujotakos greičio pokyčiams, kurie galėtų turėti įtakos trombozės išsivystymui dinamikoje.

Iki šiol nei Lietuvos, nei užsienio šalių mokslinėje medicinos literatūroje nebuvo duomenų apie vartų veninės sistemos kraujotakos tyrimus sergant ūminiu lengvo laipsnio kasos

uždegimu. Visoje apžvelgtoje literatūroje vartų venos trombozės rizika didėjo sergant ūminiu sunkiu, ypač nekrotizuojančiu, pankreatitu. Vis dėlto komplikuotas kasos uždegimas pasireiškia tik 10–15 proc. atvejų. Didžioji dalis visų ŪP yra lengvo laipsnio, dažnai nereikalaujantys sudėtingo gydymo, tačiau jų įtaka vartų veninės sistemos kraujotakai niekada anksčiau nebuvo tirta. Negana to, autorių, tyrusių lėtinio pankreatito įtaką vartų venos trombozei, duomenimis, kaip tik ūmios ligos epizodai sergant LP lėmė VVT išsivystymą. Todėl šio tyrimo metu vertinta lengvo laipsnio ŪP įtaka vartų veninės sistemos kraujotakai – vertinta, ar kito kraujotakos greitis ūmios ligos epizodu ir vėliau, pranykus ūmios ligos požymiams ir simptomams.

Šio mokslinio tyrimo kryptis taip pat paskatino bendradarbiauti su kitų šalių tyrėjais. Disertacijos autorius dalyvavo dviejose tarptautinėse mokslinėse grupėse: (1) inicijuotas prospektyvinis multicentrinis mokslinis tyrimas – vertinti gyvenimo kokybės pokyčiai sergant LP, vyraujančių uždegiminių rodiklių „fonas“ ir jų įtaka gyvenimo kokybės pokyčiams sergant LP; (2) autorius dalyvauja multicentriniame prospektyviniame kohortiniame tyrime, kurio tikslas sudaryti anksti nustatomą ūminio kasos uždegimo sunkumo vertinimo indeksą (*Early Achievable Severity Index*) – vertinami bendrieji ir biocheminiai kraujo rodikliai, radiologinių (tarp jų ir ultragarsinių) tyrimų duomenys pirmosiomis paromis diagnozavus ŪP siekiant susisteminti duomenis iš skirtingų Europos šalių ir apskaičiuoti galimą komplikuoto ūminio pankreatito išsivystymo riziką remiantis paprastais, kasdienėje klinikinėje praktikoje atliekamais laboratoriniais ir radiologiniais tyrimais.

2. Tyrimo metodika

2.1. Tyrimo programa

Atsižvelgiant į darbo tikslus ir uždavinius, mokslinio darbo pradžioje sudaryta tyrimo programa, apimanti keletą etapų:

1. Literatūros apžvalga; tyrimo protokolo sudarymas ir tirti reikalingų dokumentų rengimas; gautas bioetikos leidimas atlikti tyrimą.
2. Tiriamųjų atranka į tris tiriamąsias grupes pagal pasirinktus įtraukimo kriterijus.
3. Pilvo organų echoskopija, vertinta vartų venos ir jos šakų, kepenų arterijos ir kepenų venų kraujotaka spalviniu Doplerio režimu:
   a. Sveikiems asmenims – kontrolinė grupė;
   b. Ūminiu lengvo laipsnio pankreatitu sergantiems asmenims;
   c. Lėtiniu pankreatitu sergantiems ir ligos paūmėjimo simptomų tyrimo metu neturintiems asmenims.
4. Kraujo paėmimas laboratoriniams tyrimams ir rezultatų vertinimas:
   a. Sveikiems asmenims – kontrolinė grupė;
   b. Ūminiu lengvo laipsnio pankreatitu sergantiems asmenims;
   c. Lėtiniu pankreatitu sergantiems ir ligos paūmėjimo simptomų tyrimo metu neturintiems asmenims.
5. Kontrolinė pilvo organų echoskopija po 3 mėnesių, vertinta vartų venos ir jos šakų, kepenų arterijos ir kepenų venų kraujotaka spalviniu Doplerio režimu.

6. Kontrolinė pilvo organų echoskopija po 6 mėnesių nuo tyrimo pradžios, vertinta vartų venos ir jos šakų, kepenų arterijos ir kepenų venų kraujotaka spalviniu Doplerio režimu.
7. Duomenų suvedimas ir statistinė duomenų analizė, predisponuojančių veiksnių, galinčių turėti įtakos vartų venos kraujotakos pokyčiams, nustatymas.

2.2. Tyrimo medžiaga ir metodai

Mokslinis darbas buvo atliekamas nuo 2015 m. gegužės iki 2018 m. balandžio Vilniaus universiteto ligoninės Santaros klinikų Hepatologijos, gastroenterologijos ir dietologijos centro Hepatologijos ir gastroenterologijos skyriuje. Gautas Biomedicininių tyrimų etikos komiteto leidimas Nr. 158200-15-774-291 (2015 03 03) atlikti tyrimą, visi jame sutikę dalyvauti tiriamieji prieš įsitraukdami į tyrimą turėjo perskaityti informuoto asmens formą ir pasirašyti informuoto asmens sutikimo formą. Atliekant tyrimą paisyta Helsinkio deklaracijoje numatytų mokslinių tyrimų su žmonėmis etikos principų.

Tyrime dalyvavo 103 tiriamieji, į galutinę duomenų analizę įtraukti 71 tiriamieji, visi jie tirti tris kartus. Tiriamieji tyrimo pradžioje suskirstyti į tris grupes: pacientai, sergantys ūminiu lengvo laipsnio pankreatitu (50 asmenų tyrimo pradžioje); pacientai, kuriems diagnozuotas lėtinis pankreatitas (35); ir kontrolinė sveikų tiriamųjų grupė (18). Gauti duomenys apdoroti du kartus: į pirmąją duomenų analizę įtraukti 44 ūminiu pankreatitu sirgę, 29 lėtiniu pankreatitu sirgę ir 16 sveikų tiriamųjų. Į antrąją – galutinę – duomenų analizę įtraukti ir visus tris kartus ištirti 29 pacientai, sergantys ūminiu pankreatitu, 26 pacientai, kuriems diagnozuotas lėtinis pankreatitas, bei 16 sveikų asmenų, sudariusių kontrolinę grupę.

2.3. Tiriamųjų grupės

Moksliniame tyrime dalyvavo tik tie asmenys, kurie atitiko nustatytus įtraukimo kriterijus:

1. Ūminiu pankreatitu sergančių tiriamųjų grupė:
   - Diagnozuotas ūminis pankreatitas;
   - Ūminis lengvo laipsnio pankretitas remiantis atnaujinta Atlantos klasifikacija;
   - 18–80 metų asmenys;
   - Asmenys, sutikę dalyvauti tyrime ir pasirašę informuoto asmens sutikimo formą;
   - Anksčiau mediciniškai nedokumentuoti kraujotakos ir krešėjimo sistemos sutrikimai.
2. Lėtiniu pankreatitu sergančių tiriamųjų grupė:
   - Diagnozuotas lėtinis pankreatitas;
   - Nėra cholangito ar kasos uždegimo paūmėjimo simptomų ir požymių;
   - Į Hepatologijos ir gastroenterologijos skyrių bendrojo tulžies latako ar kasos latako restentavimo procedūrai paguldyti pacientai;
   - 18–80 metų asmenys;

- Asmenys, sutikę dalyvauti tyrime ir pasirašę informuoto asmens sutikimo formą;
- Anksčiau mediciniškai nedokumentuoti kraujotakos ir krešėjimo sistemos sutrikimai.

3. Kontrolinė (sveikų tiriamųjų grupė):
   - Neturintys ūminio ar lėtinio pankreatito požymių ir simptomų asmenys;
   - Asmenys, kuriems niekada anksčiau nebuvo diagnozuotas ūminis pankreatitas;
   - 18–80 metų asmenys;
   - Asmenys, sutikę dalyvauti tyrime ir pasirašę informuoto asmens sutikimo formą;
   - Anksčiau mediciniškai nedokumentuoti kraujotakos ir krešėjimo sistemos sutrikimai.

Į tyrimą neįtraukti asmenys, kuriems tiko bent vienas iš šių kriterijų:

- Diagnozuotos kepenų ligos ir / ar jų sukeltos komplikacijos;
- Diagnozuotos mieloproliferacinės ligos;
- Diagnozuotos ar persirgtos onkologinės ligos;
- Diagnozuotos širdies ir kraujagyslių ligos;
- Diagnozuotos kraujo krešėjimo sistemos ligos;
- Asmenys, jaunesni nei 18 ar vyresni nei 80 metų.

Ūminiu lengvo laipsnio pankreatitu sergančių tiriamųjų grupė. Tyrimo pradžioje į šią tiriamųjų grupę įtraukta 50 tiriamųjų, kurie ištirti ir gydyti buvo hospitalizuoti į Santaros klinikų Hepatologijos ir gastroenterologijos skyrių. Tiriant iš pirmosios (pirmojo etapo) analizės pašalinti 6 pacientai, kuriems UG tyrimo metu nustatyta kepenų audinio fibrozė, dar 15 (iš viso 21 pacientas) – iš galutinės (antrojo etapo) duomenų analizės neatvykus antro arba trečio vizito. Duomenų analizei panaudoti 44 pacientų duomenys, 38 (86,4 proc.) pacientams ūminis pankreatitas buvo metabolinės (alkoholinės) kilmės. Remiantis Tarptautinės kasos ligų asociacijos (*International Association of Pancreatology*) ir Amerikos kasos asociacijos (*American Pancreatic Association*) nustatytais kriterijais, ūminis pankreatitas tiriamiesiems diagnozuotas, kai atitiko bent du iš trijų kriterijų:

- klinikinius simptomus – viršutinės pilvo dalies skausmas;
- laboratorinius požymius – serumo lipazė ar amilazė padidėjusi daugiau nei 3 kartus, lyginant su normos riba;
- specifinius radiologinius vaizdinius (KT, MBR, ultragarsinio tyrimo) radinius.

Hepatologijos ir gastroenterologijos skyriuje gydomų ūminiu pankreatitu sergančių pacientų ligos eiga, remiantis atnaujinta Atlantos klasifikacija, klasifikuojama į tris sunkumo laipsnius [Banks P.A., et al., 2013]:

- lengvas – nėra organų nepakankamumo ir komplikacijų;
- vidutinis sunkus – organų funkcijos nepakankamumas trunka ne ilgiau nei 48 valandas ir / arba diagnozuotos vietinės ar sisteminės komplikacijos;
- sunkus – organų funkcijos nepakankamumas trunka ilgiau nei 48 valandas.

Šiame moksliniame tyrime dalyvavo tik lengvo laipsnio ūminiu pankreatitu sergantys pacientai. Sunkaus laipsnio pankreatitas, ypač nekrozuojantis, yra žinomi kaip aiškūs vartų venos trombozės rizikos veiksniai, atlikta nemažai retrospektyvinių ir prospektyvinių tai pa-

tvirtinančių mokslinių tyrimų. Vis dėlto lengvo laipsnio ūminis pankreatitas sudaro didžiąją dalį visų ūminio pankreatito atvejų, tačiau iki šiol nėra atlikta tyrimų, vertinančių, ar lengvo laipsnio ūminis pankreatitas veikia kepenų vartų venos ir jos šakų kraujotaką. Todėl šio tyrimo metu tirti atrinkti tik lengvo laipsnio ūminiu pankreatitu sergantys ir Gastroenterologijos ir hepatologijos skyriuje ūminiu ligos epizodu gydyti pacientai. Visi tiriamieji skyriuje gydyti 5 – 7 dienas, taikyta skysčių terapija (5 ml/kg/per dieną intraveninių skysčių terapija Ringerio ir / ar 0,9 proc. natrio chlorido tirpalu pirmąją hospitalizacijos dieną (infuzijos greitis 200-300 ml per valandą), mažinant iki 1,5 litro per parą paskutinėmis dienomis), pakaitinė virškinimo fermentų terapija, analgetikai (nesteroidiniai vaistai nuo uždegimo), dieta. Antikoaguliantai įprastai nėra skiriami sergant ŪP, todėl nė vienas šiame tyrime dalyvavęs lengvo laipsnio ŪP sirgęs pacientas nevartojo minėtų medikamentų.

Lėtiniu pankreatitu sergančių tiriamųjų grupė. Tyrimo pradžioje į šią tiriamųjų grupę įtraukti 35 tiriamieji, kurie hospitalizuoti Santaros klinikų Hepatologijos ir gastroenterologijos skyriuje planinėms kasos arba bendrojo tulžies latako restentavimo procedūroms. Į tyrimą įtraukti tik tie lėtiniu pankreatitu sergantys pacientai, kuriems nebuvo jokių ūminės ligos simptomų. Tiriant 9 pacientai pašalinti iš tyrimo ir galutinės duomenų analizės: pirmajai duomenų analizei panaudoti 30 asmenų duomenys (dėl kepenų audinio fibrozės ir krūties vėžio anamnezėje 5 pacientai pašalinti iš tyrimo dar pirmajame etape), vėliau iš tyrimo pašalinti dar 4 asmenys (neatvyko antro ar trečio vizito). Galutinei duomenų analizei panaudoti 26 pacientų duomenys. Lėtinis pankreatitas tiriamiesiems buvo diagnozuotas anksčiau, ne įtraukimo į tyrimą ir su tuo susijusios hospitalizacijos metu. Į tyrimą įtraukti tik tie pacientai, kuriems remiantis radiologiniais vaizdiniais tyrimais anamnezėje patvirtinta neabejotina lėtinio pankreatito diagnozė remiantis Amerikos kasos asociacijos nustatytu diagnostikos algoritmu: vaizdiniais tyrimais matomi kasos audinio kalcifikatai ir / ar vidutiniškai arba stipriai išreikšti morfologiniai kasos pokyčiai.

Vidutiniškai išreikšti morfologiniai kasos pokyčiai – patvirtinti du ar daugiau iš šių kriterijų:

- išsiplėtęs kasos latakas (2–4mm);
- padidėjusi kasa;
- heterogeniška kasos parenchima;
- vingiuoti kasos latakai;
- sustorėjusios kasos latakos sienelės;
- kasos cista (<10 mm);
- netaisyklingas kasos galvos / kūno kontūras.

Stipriai išreikšti morfologiniai kasos pokyčiai – matomi vidutiniškai išreikštiems kasos pokyčiams būdingi požymiai ir papildomai vienas ar daugiau iš išvardytų kriterijų:

- didelės kasos cistos (>10 mm);
- kasos latakų praeinamumo sutrikimas / akmenys;
- kasos latakų obstrukcija ar susiaurėjimas (striktūra).

Visiems pacientams taip pat nustatyti lėtiniam pankreatitui būdingi klinikiniai simptomai ir etiologiniai veiksniai: ūminis pankreatitas anamnezėje, alkoholizmas anamnezėje, pilvo skausmas, steatorėja, mažėjanti vartojant pakaitinius virškinimo fermentus, ir kiti.

Tiriamieji gydyti Gastroenterologijos ir hepatologijos skyriuje 3 – 5 dienas. Pirmąją hospitalizacijos dieną atliktas pilvo organų UG tyrimas, įvertinta vartų venos kraujotaka, atlikti kraujo tyrimai. Numatyta planinė endoskopinė retrogradinė cholangiografija bendrojoje nejautroje atlikta antrą hospitalizacijos dieną, todėl procedūra neturėjo jokios įtakos pirmojo ištyrimo rezultatams. Po procedūros skirta intraveninė skysčių terapija, analgetikai esant skausmui.

Sveikų tiriamųjų – kontrolinė – grupė. Kontrolinę grupę sudarė 18 asmenų, savanoriškai sutikusių dalyvauti šiame moksliniame tyrime. Perskaitę informuoto asmens formą ir pasirašę informuoto asmens sutikimo formą jie pakviesti dalyvauti tyrime. Atliktas išsamus kontrolinę grupę sudariusių asmenų įvertinimas siekiant įsitikinti, kad tiriamieji atitinka visus įtraukimo ir atmetimo kriterijus. Surinkta detali anamnezė ir įvertinti turimi medicininiai asmens duomenys, po to vienas tiriamasis pašalintas iš tyrimo (skydliaukės piktybinio darinio operacija anamnezėje). Atliktas objektyvus ištyrimas: arterinio kraujo spaudimo ir širdies susitraukimo dažnio matavimas, pilvo palpacija, vertinimas dėl periferinių edemų, apžiūrėtos kojų venos. Atlikta pilvo organų echoskopija: vertintas kepenų dydis ir struktūra, tulžies pūslės dydis, kontūras, papildomi dariniai kepenyse ar tulžies pūslėje, taip pat vertinta kasos struktūra, dydis, kasos latako struktūra ir vientisumas, pilvo ertmės limfmazgiai dėl limfadenopatijos. Po pilvo organų echoskopijos vienas asmuo pašalintas iš tolimesnio tyrimo dėl matomų tulžies pūslės akmenų. Paimtas veninis kraujas biocheminiams tyrimams siekiant visapusiškai įvertinti medicininę tiriamųjų būklę: įvertintos bendrojo ir tiesioginio bilirubino, alanininės transaminazės, aspartato transaminazės, šarminės fosfatazės, gama gliutamattransferazės ir gliukozės koncentracijos, lipidograma. Taigi galutinei duomenų analizei panaudoti 16 sveikų asmenų, sudariusių kontrolinę grupę, duomenys.

2.4. Tyrimo eiga

Visi tiriamieji supažindinti su tyrimo eiga ir gautas jų sutikimas dalyvauti tyrime. Tiriamųjų ištyrimą sudarė trys etapai, kiekvieną jų skyrė trijų mėnesių intervalas.

Pirmojo ištyrimo metu visiems tiriamiesiems paimtas veninis kraujas bendrajam kraujo, biocheminiams ir kraujo krešėjimo tyrimams (aprašyti toliau). Ūminiu pankreatitu sergantiems tiriamiesiems veninis kraujas paimtas Hepatologijos ir gastroenterologijos skyriuje nevalgius per pirmas 24 valandas nuo diagnozės nustatymo (ir hospitalizacijos) pradžios, procedūrą atliko slaugytoja procedūriniame kabinete. Lėtiniu pankreatitu sergantiems tiriamiesiems veninis kraujas paimtas Hepatologijos ir gastroenterologijos skyriuje nevalgius per pirmas 24 valandas nuo hospitalizacijos pradžios, procedūrą atliko slaugytoja procedūriniame kabinete. Sveikiems tiriamiesiems veninis kraujas paimtas nevalgius ryte Santaros klinikų Laboratorinės medicinos centro procedūriniame kabinete. Tą pačią dieną išmatuotas tiriamojo ūgis, svoris, apskaičiuotas kūno masės indeksas, pamatuotas arterinis kraujo spaudimas ir širdies susitraukimų dažnis. Visiems tiriamiesiems atliktas pilvo organų ultragarsinis tyrimas su spalviniu Doplerio režimu. Detaliai įvertintas kasos audinys, kepenų kraujagyslės ir jų kraujotaka. Pirmajame ištyrime dalyvavo 89 tiriamieji, gauti demografiniai, laboratori-

nių kraujo tyrimų ir UG tyrimo su Doplerio režimu duomenys panaudoti pirmajai duomenų analizei.

Antrasis ištyrimas atliktas praėjus trims mėnesiams po pirmojo ištyrimo. Aliktas pilvo organų ultragarsinis tyrimas spalviniu Doplerio režimu. Po kitų trijų mėnesių – praėjus pusei metų nuo įtraukimo į tyrimą pradžios – tiriamieji ištirti trečią kartą. Taip pat atliktas pilvo organų echoskopinis tyrimas su spalviniu Doplerio režimu. Nei automatinis, nei biocheminiai ar kraujo krešėjimo tyrimai antrojo ir trečiojo tyrimo metu nekartoti. Gauti duomenys panaudoti antrajame duomenų analizės etape. Per visą tyrimą ultragarsinis tyrimas su spalviniu Doplerio režimu atliktas tris kartus kiekvienam tiriamajam – taigi iš viso atliktas 232 ištyrimai.

2.5. Laboratoriniai kraujo tyrimai

Mokslinio tyrimo metu visiems tiriamiesiems atliktas bendrasis kraujo tyrimas, kraujo krešėjimo ir biocheminiai kraujo tyrimai. Kraujo tyrimai atlikti Vilniaus universiteto ligoninės Santaros klinikų Laboratorinės medicinos centro Biochemijos laboratorijoje. Tyrime dalyvavusiems 73 kasos uždegimu sergantiems pacientams veninis kraujas tyrimams paimtas stacionare pirmąją hospitalizacijos parą. 16 sveikų tiriamųjų veninis kraujas tyrimams paimtas ambulatoriškai Laboratorinės medicinos centro Mokamų paslaugų procedūriniame kabinete, ryte, nevalgius bent 6 valandas. Veninis kraujas surinktas į 2,7 ml ir 5 ml mėgintuvėlius su natrio citratu ir etilendiamintetraacto rūgštimi venos punkcijos, kurią atliko procedūrinio kabineto slaugytoja, metu. Atlikti šie kraujo tyrimai:

- Bendrasis kraujo tyrimas. Vertinti: leukocitų skaičius (WBC), neutrofilų skaičius (NEU), trombocitų skaičius (PLT), hemoglobino kiekis kraujyje (Hgb), hematokrito kieks (Hct), vidutinis trombocitų tūris (MPV), vidutinis eritrocitų tūris (MCV), vidutinis hemoglobino kiekis eritrocituose (MCH). Tyrimas atliktas naudojant *Coulter DxH 800* analizatorių (*Backman Coulter Diagnostics,* JAV).
- Kraujo krešėjimo tyrimai. Vertinti: tarptautinis normalizuotas santykis (INR), aktyvintas dalinis tromboplastino laikas (ADTL), D-dimerai, protrombino laikas (SPA), fibrinogenas. Šie tyrimai atlikti naudojant *STA R Max* analizatorių (*Stago Group,* Prancūzija).
- Biocheminiai kraujo tyrimai. Vertinti: C reaktyvusis baltymas (CRB), natrio koncentracija kraujyje (Na), kalio koncentracija kraujyje (K), chloro koncentracija kraujyje (Cl), kalcio koncentracija kraujyje (Ca), lipazė, p-amilazė, laktatdehidrogenazė (LDH). Tyrimai atlikti naudojant *Architect* analizatorių (*Abbott,* JAV).

2.6. Ultragarsinis pilvo organų tyrimas spalviniu Doplerio režimu

Pilvo organų echoskopija su spalviniu Doplerio režimu tiriamiesiems atlikta tris kartus – per pirmąjį ištyrimą, vėliau po 3 ir po 6 mėnesių. Tyrimas atliktas Vilniaus universiteto ligoninės Santaros klinikų Hepatologijos, gastroenterologijos ir dietologijos centre. Tyrimą visiems tiriamiesiems atliko tyrimo autorius, todėl visų tiriamųjų rezultatai vertinti vienodai. Tyrimo metu naudotas ultragarso aparatas *Toshiba diagnostic ultrasound system, Model SSA-790A* (*Toshiba medical systems corporation,* Japonija) ir *Toshiba PVT-375BT* 3,5 MHz daviklis.

Ultragarsinis pilvo organų tyrimas atliktas bent 6 valandas nevalgiusiems tiriamiesiems gulimoje padėtyje (ant nugaros) dešiniajame pašonkauliniame kampe ištiestomis kojomis. Tiriamieji paprašyti įkvėpti ir sulaikyti kvėpavimą. Kepenų kraujotakos greitis ir indeksai matuoti išlaikant 30–60° Doplerio kampą (kampas tarp kraujagyslės ir ultragarso bangos) ties kraujagyslių vidurine dalimi, kur kraujagyslės spindžio (laminarinis) kraujo tėkmės greitis yra didžiausias. Vartų vena vertinta apie 2 cm link kepenų nuo blužnies ir viršutinės pasaito venos susijungimo vietos. Dešiniosios ir kairiosios vartų venos šakų kraujotaka matuota apie 1–2 cm nuo jų išsišakojimo. Kepenų arterija vertinta virš kasos galvos, kepenų venos – apie 3–6 cm nuo apatinės tuščiosios venos.

Kiekvieno ultragarsinio tyrimo metu vertintas kasos galvos dydis ir kiti pilvo vidaus organai, siekiant išsiaiškinti, ar neatsirado pakitimų, susijusių su atmetimo kriterijais, bei pagrindiniai vartų veninės kraujotakos parametrai:

- Vartų venos ir jos šakų skersmuo.
- Vmax – tai parametras, apskaičiuojamas automatiškai. Jis parodo didžiausią kraujotakos greitį sistolės metu, užfiksuotą per tam tikrą laiko intervalą – šio tyrimo metu per 4–6 sekundes.
- Vpeak – didžiausias sistolinis kraujotakos greitis.
- Vmin – mažiausias kraujotakos greitis diastolės metu.
- Vmean – vidutinis kraujotakos greitis.
- Pulsacijos indeksas (PI) – išvestinis dydis, apskaičiuojamas paties ultragarso aparato pagal formulę:

$$\frac{\text{Vpeak} - \text{Vmin}}{\text{Vmax}}$$

- Rezistentiškumo indeksas (RI) – išvestinis dydis, apskaičiuojamas paties ultragarso aparato pagal formulę:

$$\frac{\text{Vpeak} - \text{end diastolic velocity}}{\text{Vpeak}}$$

Visi vartų venų parametrai (kraujotakos greitis) kiekvieno matavimo metu vertinti tris kartus, vėliau išvedant kiekvieno matavimo vidurkį, kuris ir panaudotas tolimesniems skaičiavimams. Taip stengtasi išvengti širdies ciklinio ritmo nulemtų kraujotakos greičio svyravimų vartų venos sistemoje.

2.7. Statistinė duomenų analizė

Statistinei duomenų analizei naudotos *Microsoft Excel* ir *SPSS (Statistical Package of the Social Sciences) 20.0* programos. Tyrimų rezultatams sisteminti naudota rodiklių aprašomoji statistika: kiekybiniams kintamiesiems absoliutus skaičius, aritmetinis vidurkis su standartiniu nuokrypiu, minimali ir maksimali reikšmės, mediana, moda; kokybiniams kintamiesiems – absoliutus skaičius ir santykinė dalis (procentinė išraiška).

Kiekybinių rodiklų pasiskirstymo normalumas buvo tikrinamas atliekant Kolmogorovo ir Smirnovo testą bei vertinant histogramą. Priklausomai nuo kintamųjų pasiskirstymo

ir dispersijų įvairiarūšiškumo, bendrieji tolydžių kintamųjų palyginimai atlikti naudojant vienos krypties ANOVOS ir *Welch* ANOVOS testus arba *Kruskal-Wallis* testą. Diskretiems kintamiesiems naudotas *Chi-square* testas. Lyginant rizikos veiksnių paplitimą tarp pogrupių, kategoriniams tyrimams taikytas tikslusis (*exact) Fisher* testas; tolydiems kintamiesiems – vienos krypties ANOVA, *Kruskal-Wallis*, *Tukey* post-hoc ir *Games-Howell* post-hoc. Statistiškai reikšminga sąsaja pasirinkta ties $p < 0,05$ riba.

Galimoms asociacijoms nustatyti skaičiuoti *Pearson* ir *Spearman* koreliacijos koeficientai. Vienanarės ir daugianarės binominės logistinės regresijos buvo taikomos vartų venos trombozės rizikos veiksniams nustatyti (priklausomi kintamieji perkoduoti į nominalinę skalę). Į daugianarę analizę įtraukti tik tie rodikliai, kurie buvo reikšmingi arba beveik reikšmingi vienanarėje analizėje. Grafiniam vaizdavimui buvo pasirinkti skritulinis, juostinis stulpelinis, stačiakampis ir sudėtinių linijų grafikai.

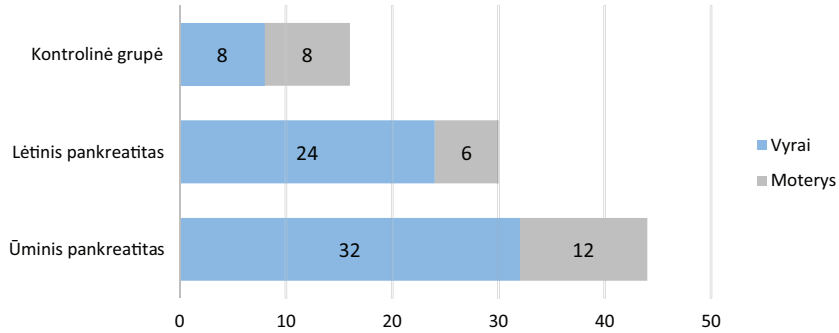
Imties dydis apskaičiuotas naudojant internetines skaičiuokles – Survey System bei Qualtrics. Dėl numatyto nedidelio imties dydžio taikant griežtus atmetimo kriterijus, pasirinktas 95% reikšmingumo lygmuo ir 10 – pasikliautinasis intervalas. Šiuo atveju, turint pakankamai platų diapazoną, padidėja klaidų galimybė, tačiau kartu tikėtina, kad populiacijos rezultatai pilnai į jį pateks. Apskaičiuotas minimalus imties dydis – 38 ūminio ir 28 lėtinio pankreatito grupėms.

3. Darbo rezultatai

3.1. Tiriamieji ir tiriamųjų grupių palyginimas

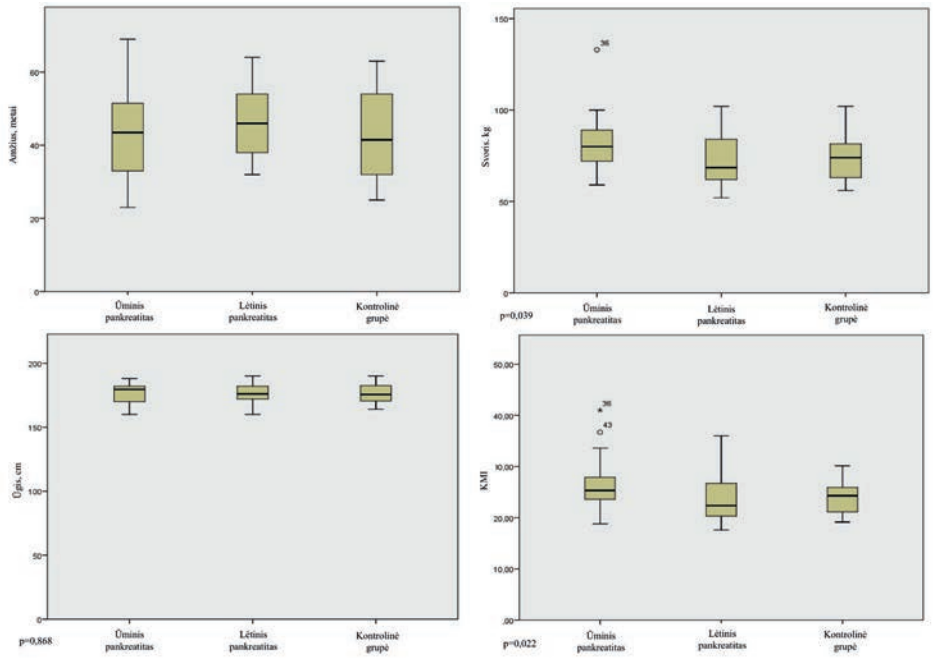
Iš viso tyrime dalyvavo 90 tiriamųjų. Iš jų 74 (82,2 proc.) kasos uždegimu sergantys pacientai, kuriems diagnozuotas lėtinis (n = 30; 33,3 proc.) ir ūminis (n = 44; 48,9 proc.) kasos uždegimas bei 16 sveikų asmenų (17,8 proc.), sudariusių kontrolinę grupę (1a pav.). Tyrime iš viso dalyvavo 64 (71,1 proc.) vyrai ir 26 (28,9 proc.) moterys. Tiek ūminio, tiek lėtinio pankreatito grupėse didžiąją dalį tiriamųjų sudarė vyrai (atitinkamai 72,7 proc. ir 80,0 proc.) (2 pav.), o kontrolinėje grupėje tiriamieji pagal lytį pasiskirstė tolygiai (8 vyrai ir 8 moterys). Tiriamųjų amžius svyravo nuo 23 iki 69 metų. Apskritai visos trys tiriamųjų grupės buvo statistiškai homogeniškos pagal amžių ir tiriamųjų ūgį, tačiau rastas reikšmingas skirtumas grupėse lyginant tiriamųjų svorį ir KMI (atitinkamai p = 0,039 ir p = 0022) (3 pav.). Lėtiniu pankreatitu sergantys buvo statistiškai reikšmingai mažesnio svorio ir kūno masės indekso, lyginant su ūminiu pankreatitu sergančiais (atitinkamai p = 0,015 ir p = 0,011) tiriamaisiais. Didelę įtaką kraujo tėkmės greičiui turi širdies susitraukimų dažnis (ŠSD) ir AKS. Vidutinis ŠSD ŪP grupėje buvo statistiškai reikšmingai didesnis nei LP sergančių ar kontrolinių tiriamųjų (atitinkamai 78 k./min. (60–110 k./min.) *vs* 74 k./min. (62–86 k./min.) ir 72 k./min. (64–78 k./min.); p=0,004). Tuo tarpu, sistolinis ir diastolinis AKS statistiškai reikšmingai nesiskyrė tarp grupių, nors sistolinio AKS ribos buvo platesnės ŪP sergančiųjų grupėje: vidutinis AKS ŪP grupėje buvo 130/80 mm/Hg; 132/92 mm/Hg LP grupėje ir 127,5/90 mm/Hg kontrolinėje grupėje.

1 PAV. Tiriamųjų pasiskirstymas į grupes (a) pirmojo ištyrimo ir pirmosios duomenų analizės metu ir (b) antrosios duomenų analizės metu – po trijų ištyrimų



2 PAV. Tiriamųjų pasiskirstymas pagal lytį

Antrojoje duomenų analizėje (po visų trijų ištyrimų) panaudoti 71 tiriamųjų demografiniai duomenys: iš jų 26 pacientai sirgo lėtiniu pankreatitu (36,6 proc.), 29 – ūminiu (40,9 proc.) ir 16 sudarė kontrolinę grupę (22,5 proc.) (1b pav.). Taip pat kaip ir pirmosios analizės metu didžiąją dalį tiriamųjų sudarė vyrai: 82,8 proc. ūminio pankreatito ir 84,6 proc. lėtinio pankreatito grupėse, kontrolinėje grupėje tiriamųjų skaičius, atitinkamai ir pasiskirstymas pagal lytį viso tyrimo metu nekito. Galutinėje analizėje dalyvavusių demografiniai duomenys nesiskyrė: tiriamųjų grupės buvo homogeniškos pagal amžių, tiriamųjų ūgį bei pagal lytį tiriamosiose grupėse (11 lentelė). Nepaisant to, nustatytas statistiškai reikšmingas skirtumas tarp ūminiu ir lėtiniu pankreatitu sergančių tiriamųjų, lyginant kūno svorį ir kūno masės indeksą: lėtiniu pankreatitu sergančiųjų svorio vidurkis buvo apie 10 kg mažesnis, lyginant su ūminiu pankreatitu sergančiais (atitinkamai 72,6 ± 14,9 kg ir 81,9 ± 14,5 kg; $p = 0,019$), KMI lėtine liga sergančiųjų taip pat buvo reikšmingai mažesnis (26,5 ± 4,9 sergančių ūminiu ir 23,5 ± 4,4 sergančių lėtiniu pankreatitu grupėse; $p = 0,011$). Statistiškai reikšmingo skirtumo lyginant sveikus tiriamuosius su pankreatitu sirgusiais pagal svorį ir KMI nepastebėta.



3 PAV. Tiriamųjų pasiskirstymas pagal amžių, ūgį, svorį ir KMI

1 LENTELĖ. Demografinė galutinėje (antrojoje) duomenų analizėje dalyvavusių tiriamųjų statistika

		LP tiriamieji n = 26 (36,6 %)	ŪP tiriamieji n = 29 (48,8 %)	Sveiki tiriamieji n = 16 (22,5 %)	Bendra p reikšmė	p reikšmė tarp grupių: LP *vs* ŪP LP *vs* sveiki ŪP *vs* sveiki
Lytis, n (%)	Vyrai	22 (84,6 %)	24 (82,8 %)	8 (50 %)	0,021***	1,000*** 0,032*** 0,037***
	Moterys	4 (15,4 %)	5 (17,2 %)	8 (50 %)		
Amžius, metai M, (ribos)		45,5 (32–64)	40,5 (23–69)	42,7 (25–63)	0,176**	0,109* 0,472* 0,585**
Svoris, kg M, (ribos)		72,6 (52–102)	81,9 (59–133)	74,8 (56–102)	0,056**	**0,019**** 0,640* 0,147**
Ūgis, cm M, (ribos)		175,9 (160–190)	176,2 (160–188)	176,7 (164–190)	0,942*	0,860* 0,730* 0,845*
KMI, M, (ribos)		23,5 (17,6–36)	26,5 (18,8–41)	23,8 (19,2–30,1)	**0,032****	**0,011**** 0,783* 0,112**

LP – lėtinis pankreatitas; ŪP – ūminis pankreatitas; M – vidurkis; *one-way ANOVA; **Kruskal Wallis; ***Chi-square testas. Paryškinta statistiškai reikšminga p reikšmė (<0,05).

3.2. Bendrojo kraujo tyrimo rodiklių pokyčiai tarp tiriamųjų

Įvertinus bendrojo kraujo tyrimo rodiklių pokyčius, rasti statistiškai reikšmingi skirtumai tarp grupių pagal leukocitų skaičiaus, neutrofilų absoliutaus ir santykinio kiekio kitimą ($p < 0,001$) ir vidutinę hemoglobinio koncentraciją eritrocituose ($p < 0,05$).

Ryškiausias leukocitų kiekio padidėjimas stebėtas ūminiu kasos uždegimu sergančių pacientų grupėje ($11,4 \pm 3,9 \times 10^9$/l), be to stebėtas statistiškai reikšmingas skirtumas lyginant leukocitų skaičių ūminiu ir lėtiniu pankreatitu sergančių pacientų kraujyje ($p = 0,031$), lėtiniu ar ūminiu pankreatitu sergančių ir sveikų tiriamųjų kraujyje (atitinkamai $p < 0,001$ ir $p < 0,001$). Apskaičiuotas ryškus absoliutaus ir santykinio neutrofilų kiekio pokytis kraujyje lyginant visas tris grupes (atitinkamai $p < 0,001$ ir $p < 0,001$). Daugiausia, lyginant su nustatytomis rodiklio normos ribomis kraujyje, neutrofilų kiekis didėjo ūminiu pankreatitu sergančių tiriamųjų grupėje: absoliutus neutrofilų skaičius kraujyje buvo $8,8 \pm 3,8 \times 10^9$/l, santykinis – 76,8%. O lėtiniu pankreatitu sergančių ir sveikų tiriamųjų kraujyje neutrofilų kiekio vidurkiai atitiko normą. Tačiau ir absoliutus, ir santykinis neutrofilų kiekis sergant lėtiniu pankreatitu vidutiniškai buvo didesnis nei sveikų tiriamųjų (3 lentelė), todėl nustatytas statistiškai patikimas skirtumas tarp neutrofilų absoliutaus ir santykinio kiekio didėjimo lyginant ūminio ir lėtinio pankreatito, ūminio pankreatito kontrolinę ir lėtinio pankreatito bei kontrolinę grupes (visais atvejais $p \leq 0,005$) (2 lentelė).

Kur kas mažiau išreikšti skirtumai tarp tiriamųjų nustatyti lyginant hemoglobino koncentraciją, trombocitų kiekį ir vidutinę hemoglobino koncentraciją eritrocituose. Hemoglobino koncentracija kraujyje buvo statistiškai reikšmingai didesnė ūminiu pankreatitu sergančių tiriamųjų kraujyje lyginant su sergančiais lėtiniu pankreatitu (atitinkamai $146 \pm 15,2$ g/l ir $136,5 \pm 18,1$ g/l; $p = 0,022$), tačiau patikimo skirtumo lyginant sergančius su sveikais tiriamaisiais pagal Hgb koncentraciją nestebėta. O trombocitų skaičius buvo kur kas didesnis lėtiniu pankreatitu sergančių tiriamųjų kraujyje lyginant su ūminiu kasos uždegimu sergančiais tiriamaisiais, ir šis skirtumas buvo statistiškai patikimas (atitinkamai $264,3 \pm 104,7 \times 10^9$/l ir $205,8 \pm 70,2 \times 10^9$/l; $p = 0,048$). Negana to, tik lėtinio pankreatito grupėje trombocitų skaičius kraujyje daliai pacientų viršijo normos ribas – Plt varijavo nuo 120 iki 518×10^9/l. Vis dėlto trombocitų skaičiaus skirtumas tarp kasos uždegimu sergančių ir sveikų tiriamųjų nebuvo statistiškai reikšmingas. MCH statistiškai patikimai skyrėsi lyginant ūminiu pankreatitu sergančius ir sveikus tiriamuosius (atitinkamai $31,7 \pm 2,2$ pg ir $29,5 \pm 1,2$ pg; $p = 0,015$).

3.3. Kraujo krešėjimo rodiklių pokyčiai tarp tiriamųjų

Nerasta jokio statistiškai reikšmingo skirtumo tarp grupių lyginant aktyvuoto dalinio tromboplastino laiką (ADTL), protrombino laiką (SPA) ir tarptautinį normalizuotą santykį (INR) – visų rodiklių vidurkiai atitiko nustatytas normos ribas tiek ūminiu ir lėtiniu pankreatitu sergančių, tiek sveikų tiriamųjų grupėse (3 lentelė).

Vis dėlto apskaičiuoti ryškūs statistiškai reikšmingi skirtumai tarp grupių lyginant D-dimerų ir fibrinogeno koncentracijas kraujyje. Tiek ūminiu, tiek lėtiniu pankreatitu sergančių kraujyje D-dimerų ir fibrinogeno koncentracija viršijo normos ribas (atitinkamai

2169,5 ± 2167,0 µg/l ir 5,8 ± 1,9 g/l; bei 886,4 ± 1400,7 µg/l ir 4,6 ± 1,3 g/l), ir sergančių ūminiu kasos uždegimu grupėje buvo statistiškai reišmingai didesnė lyginant su sergančiais lėtiniu uždegimu (atitinkamai p < 0,001 ir p = 0,003). Sveikų tiriamųjų kraujyje D-dimerų ir fibrinogeno koncentracija atitiko normą, todėl apskaičiuotas statistiškai reikšmingas skirtumas tarp sveikų tiriamųjų ir ūminiu bei lėtiniu pankreatitu sergančių tiriamųjų lyginant grupes pagal minėtus rodiklius (atitinkamai p < 0,001 ir p < 0,001; bei p = 0,001 ir p < 0,001).

2 LENTELĖ. Bendrojo kraujo tyrimo rodiklių pokyčiai ir skirtumai tarp grupių

Kraujo rodiklis	LP tiriamieji n = 30	ŪP tiriamieji n = 44	Sveiki tiriamieji n = 16	Bendra p reikšmė	p reikšmė tarp grupių: LP *vs* ŪP LP *vs* sveiki ŪP *vs* sveiki
Leukocitų skaičius kraujyje, M±SD, x10⁹/l	9,0 ± 3,3	11,4 ± 3,9	5,7 ± 0,8	**<0,001****	**0,031**** **<0,001**** **<0,001****
Absoliutus neutrofilų skaičius kraujyje, M±SD, x10⁹/l	5,6 ± 2,8	8,8 ± 3,8	3 ± 0,6	**<0,001****	**0,001**** **<0,001**** **<0,001****
Santykinis neutrofilų kiekis kraujyje, M±SD, %	62,7 ± 12,3	76,8 ± 9,2	52,2 ± 7,4	**<0,001****	**<0,001**** **0,005*** **<0,001****
Vidutinis trombocitų tūris, M±SD, fl	10,3 ± 0,8	10,8 ± 0,8	10,4 ± 1,1	0,205*	0,266 0,571 0,189
Hematokritas, M±SD, l/l	0,4 ± 0,1	0,4 ± 0,1	0,4 ± 0	0,167*	0,198 0,424 0,598
Hemoglobino koncentracija, M±SD, g/l	136,5 ± 18,1	146 ± 15,2	140,8 ± 10,3	**0,045***	**0,022*** 0,543 0,072
Trombocitų skaičius kraujyje, M±SD, x10⁹/l	264,3 ± 104,7	205,8 ± 70,2	234,7 ± 35,9	0,095**	**0,048*** 0,631 0,255
Vidutinis eritrocitų tūris, M±SD, fl	91,6 ± 6,5	91,2 ± 6,1	89,7 ± 3,7	0,477**	0,730 0,379 0,614
Vidutinė hemoglobino koncentracija eritrocituose, M±SD, pg	30,5 ± 2,2	31,7 ± 2,2	29,5 ± 1,2	**0,041****	0,417 0,182 **0,015****

3.4. Biocheminių kraujo rodiklių pokyčiai tarp tiriamųjų

Įvertinus elektrolitų pokyčius tiriamųjų kraujyje paaiškėjo, jog ir sveikų, ir kasos uždegimu sergančiųjų natrio, kalio, kalcio ir chloro koncentracija atitiko normą, nors apskaičiuoti statistiškai patikimi skirtumai tarp sveikų tiriamųjų ir ūminiu pankreatitu sergančių – pastarojoje grupėje visų elektrolitų koncentracijų vidurkis buvo mažesnis lyginant su sveikais tiriamaisiais ($p<0,05$). Toks pat skirtumas stebėtas lyginant kalio ir kalcio koncentracijos vidurkį lėtiniu ir ūminiu pankreatitu sergančiųjų kraujyje ($p < 0,05$) (4 lentelė).

Įvertinus fermentų koncentracijos pokyčius kraujyje, rasti statistiškai reikšmingi skirtumai tarp grupių. Lipazės ir p-amilazės koncentracijos kraujyje buvo kelis kartus didesnės nei normos riba sergančių ūminiu pankreatitu grupėje (atitinkamai 3887,5 U/l ir 705,4 U/l). Ūminiu pankreatitu sergančių tiriamųjų kraujyje lipazės koncentracija buvo reikšmingai didesnė lyginant ir su lėtiniu pankreatitu sergančiais, ir su sveikais tiriamaisiais ($p < 0,001$). Taip pat ir p-amilazės koncentracijos vidurkis statistiškai patikimai didesnis buvo ūminiu nei lėtiniu pankreatitu sergančių ar sveikų tiriamųjų kraujyje ($p < 0,001$) (4 lentelė). Nors tiek lipazės, tiek p-amilazės koncentracija buvo didesnė lėtiniu pankreatitu sergančiųjų kraujyje lyginant su sveikais tiriamaisiais (atitinkamai 305,4 ± 528,6 U/l ir 126,7 ± 247,4 U/l lyginant

3 LENTELĖ. Kraujo krešėjimo rodiklių skirtumai tarp grupių

Kraujo krešėjimo rodiklis	LP tiriamieji n = 30	ŪP tiriamieji n = 44	Sveiki tiriamieji n = 16	Bendra p reikšmė	p reikšmė tarp grupių: LP *vs* ŪP LP *vs* sveiki ŪP *vs* sveiki
SPA, M±SD, %	93,1 ± 14,1	99,3 ± 15,9	95,4 ± 16,4	0,442	0,217 0,775 0,369
INR, M±SD	1 ± 0,1	1 ± 0,1	1 ± 0,1	0,355	0,273 0,680 0,481
ADTL, M±SD, s	36,7 ± 4,4	37,2 ± 5,5	35,4 ± 3,6	0,415	0,492 0,425 0,115
Fibrinogenas, M±SD, g/l	4,6 ± 1,3	5,8 ± 1,9	2,8 ± 0,5	**<0,001***	**0,003*** **<0,001*** **<0,001***
D-dimerai, M±SD, µg/l	886,4 ± 1400,7	2169,5 ± 2167,0	136,6 ± 58,1	**<0,001***	**<0,001*** **0,001*** **<0,001***

LP – lėtinis pankreatitas; ŪP – ūminis pankreatitas; M – vidurkis; SD – standartinis nuokrypis; INR – tarptautinis normalizuotas santykis, ADLT – aktyvintas dalinis tromboplastino laikas, SPA – protrombino laikas, *Kruskal Wallis. Paryškinta statistiškai reikšminga p reikšmė (<0,05).

su 45,6 ± 49 U/l ir 30,8 ± 18,8 U/l), tačiau šie skirtumai nebuvo statistiškai reikšmingi (atitinkamai p = 0,587 ir p = 0,078).

Statistiškai reikšmingas skirtumas nustatytas tarp visų trijų grupių ir pagal laktatdehidrogenazės ir C-reaktyvaus baltymo koncentraciją kraujyje. CRB koncentracijos vidurkis ryškiai didėjo tik sergančių ūminiu kasos uždegimu grupėje (140,2 ± 94,8 mg/l), šiek tiek padidėjo lėtiniu pankreatitu sergančių ir atitiko normą sveikų tiriamųjų kraujyje (atitinkamai 38,4 ± 52,9 mg/l ir 0,7 ± 0,7 mg/l). Nepaisant to, statistiškai reikšmingas skirtumas stebėtas tiek tarp ūminiu ir lėtiniu pankreatitu sergančių, tiek tarp ūminiu pankreatitu sergančių ir sveikų, tiek tarp lėtiniu pankreatitu sergančių ir sveikų tiriamųjų (atitinkamai p < 0,001; p < 0,001 ir p < 0,001). Apskaičiavus laktatdehidrogenazės koncentracijos vidurkį paaiškėjo, kad visose trijose grupėse jie atitiko normą (14 lentelė), nors tiek lėtiniu, tiek ūminiu pankreatitu sergančiųjų kraujyje LDH koncentracijos svyravo iki ribų, didesnių už numatytąsias normos (atitinkamai 145–384 U/l ir 150–1035 U/l). Vis dėlto LDH koncentracijos vidurkis statistiškai skyrėsi tarp grupių lyginant ūminiu ir lėtiniu pankreatitu sergančius, lėtiniu pankreatitu sergančius ir sveikus, taip pat ūminiu pankreatitu sergančius ir sveikus tiriamuosius (atitinkamai p = 0,074; p = 0,008 ir p < 0,001).

3.5. Vartų venos sistemos kraujotakos rodiklių skirtumai tarp grupių

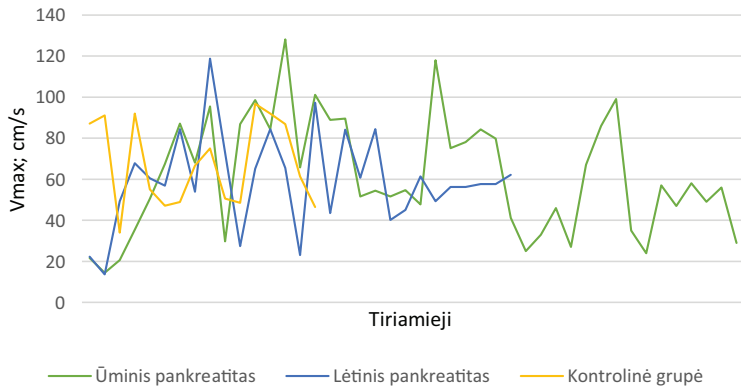
Tyrimo metu nustatyti statistiškai reikšmingi kraujagyslių skersmens skirtumai tarp lyčių. Pagrindinės vartų venos, dešiniosios ir kairiosios jos šakų skersmuo buvo mažesnis tarp moterų, lyginant su vyrais, visose trijose tiriamųjų grupėse (atitinkamai p = 0,000; p = 0,022 ir p = 0,017), reikšmingai mažesnis tarp moterų buvo pulsatiliškumo ir rezistentiškumo indeksas (atitinkamai p = 0,011 ir p = 0,006). Vmin ir Vmean statistiškai reikšmingai didesni buvo tarp vyrų: atitinkamai p = 0,006 ir p = 0,012. Negana to, pagrindinės vartų venos skersmuo taip pat buvo mažesnis sveikų tiriamųjų, kontrolinėje, grupėje lyginant su sergančiais kasos (lėtiniu ar ūminiu) uždegimu (p = 0,003), tačiau tai neturėjo reikšmingos įtakos kraujotakos greičio skirtumams tarp grupių.

Nerasta jokios reikšmingos įtakos kraujotakos greičio parametrams pagal bendrojo kraujo tyrimo, krešėjimo ar biocheminio kraujo tyrimo rodiklius bendrai ar atskirose tiriamųjų grupėse (p > 0,05). Vartų veninės sistemos kraujotakos greitis nebuvo susijęs su AKS ar ŠSD visose trijose tiriamųjų grupėse.

3.5.1. Didžiausias kraujotakos greitis sistolės metu

Lyginant didžiausią kraujotakos greitį sistolės metu per 4–6 s (Vmax) pagrindiniame vartų venos kamiene, neaptikta statistiškai reikšmingo skirtumo lyginant atskiras tiriamųjų grupes (p = 0,584). Vmax pagrindinėje vartų venoje pirmojo ištyrimo (įtraukimo į tyrimą) metu buvo mažesnis lėtinio pankreatito grupėje lyginant su ŪP sergančiais ar sveikais tiriamaisiais, tačiau šis skirtumas nebuvo statistiškai reikšmingas (4 pav.). Per antrą ir trečią vizitus reikšmingo skirtumo tarp Vmax grupėse taip pat neaptikta. Statistiškai reikšmingai Vmax rodiklis nesiskyrė kiekvienoje grupėje lyginant pirmąjį, antrąjį ir trečiąjį etapus. Ūminis lengvo

laipsnio pankreatitas neturėjo reikšmingos įtakos Vmax – rodiklis nebuvo pakitęs lyginant su sveikais asmenimis per pirmą ištyrimą ir nekito (nei mažėjo, nei didėjo) vėliau. Vertinant Vmax dešiniojoje vartų venos šakoje, nerasta statistiškai patikimo skirtumo tarp grupių kiekvieno ultragarsinio tyrimo metu ar atskirose grupėse lyginant pirmąjį, antrąjį ir trečiąjį vizitus. Tokie patys rezultatai gauti ir įvertinus Vmax kairiojoje vartų venoje lyginant atskiras tiriamųjų grupes ar atskirus ištyrimus toje pačioje tiriamųjų grupėje (5 lentelė).



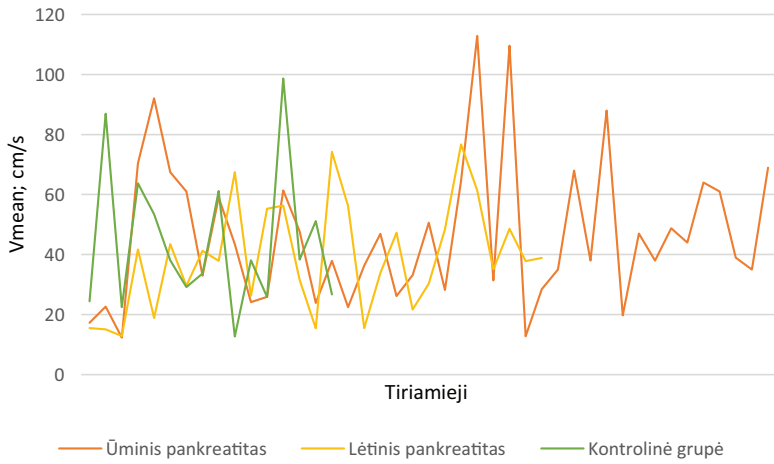
4 PAV. Pagrindinės vartų venos Vmax atskirose tiriamųjų grupėse pirmojo ištyrimo metu (n = 90) (p = 0,584).

3.5.2. Rezistentiškumo indeksas

Įvertinus rezistentiškumo indekso pokyčius bendrame vartų venos kamiene, nerasta statistiškai reikšmingo RI skirtumo atskirose tiriamųjų grupėse pirmo, antro ir trečio ištyrimo metu. Negana to, bendrojo vartų venos kamieno RI statistiškai nesiskyrė ir lyginant skirtingas tiriamųjų grupes: RI buvo vienodas pirmojo ištyrimo metu (n = 90) visose tiriamosiose grupėse (p = 0,212). Per paskutinį ištyrimą (po 6 mėnesių nuo įtraukimo į tyrimą pradžios; n = 71) RI vidurkis visose trijose grupėse taip pat buvo vienodas: 0,4±0,2 cm/s ūminio pankreatito, 0,4±0,2 cm/s lėtinio pankreatito ir 0,4±0,2 cm/s kontrolinėje grupėse (p = 0,929). Taip pat statistiškai reikšmingo skirtumo nerasta skaičiuojant RI dešiniojoje ir kairiojoje vartų venose: rezistentiškumo indeksas reikšmingai nesiskyrė lyginant tiriamųjų grupes. Ir nors RI statistiškai reikšmingai sumažėjo ūminio pankreatito grupėje po pusės metų lyginant tiek su lėtiniu pankreatitu sergančiais tiriamaisiais (p = 0,003), tiek su sveikais tiriamaisiais (p = 0,024) dešiniojoje vartų venoje, tačiau šis reiškmingas skirtumas aptiktas tik vienoje vartų venos sistemos šakoje tik vieno (t. y. paskutinio) ištyrimo metu. Negana to, nei dešiniosios, nei kairiosios vartų venos RI dinamikoje nekito, t. y. nedidėjo ir nemažėjo tiriant ūminiu ar lėtiniu pankreatitu sergančius tirimuosius pakartotinai (6 lentelė).

3.5.3. Vidutinis kraujotakos greitis

Vertinant vidutinį kraujotakos greitį vartų venos sistemos kraujagyslėse taip pat reiškmingų skirtumų tarp grupių ar skirtingų ištyrimų neaptikta. Vidutinis kraujotakos greitis bendrajame vartų venos kamiene statistiškai reikšmingai nesiskyrė tarp tiriamųjų grupių nei pirmo, nei antro ar trečio ištyrimų metu (atitinkamai p = 0,352; p = 0,787; ir p = 0,380), nors, kaip ir Vmax, Vmean vartų venoje buvo šiek tiek (bet ne statistiškai reikšmingai) lėtesnis lėtiniu pankreatitu sergančių tiriamųjų grupėje (5 pav.). Negana to, bendrojo vartų venos kamieno Vmean nekito dinamikoje nei po trijų, nei po šešių mėnesių tarp ūminiu ar lėtiniu pankreatitu sergančių ligonių. Tie patys rezultatai apskaičiuoti lyginant Vmean dešiniojoje ir kairiojoje vartų venose: Vmean nesiskyrė minėtose venose tarp skirtingų tiriamųjų grupių ar atskirose grupėse per pakartotinius ištyrimus (7 lentelė).



5 PAV. Pagrindinės vartų venos Vmean atskirose tiriamųjų grupėse pirmojo ištyrimo metu (n = 90) (p = 0,352).

4 LENTELĖ. Biocheminių kraujo rodiklių pokyčiai tarp tiriamųjų

Biocheminis kraujo rodiklis	LP tiriamieji n = 30	ŪP tiriamieji n = 44	Sveiki tiriamieji n = 16	Bendra p reikšmė	p reikšmė tarp grupių: LP *vs* ŪP LP *vs* sveiki ŪP *vs* sveiki
Natrio koncentracija kraujyje, M±SD, mmol/l	140,4 ± 4,4	138,8 ± 3,2	141,3 ± 2,1	**0,041***	0,213 0,376 **0,046***
Kalio koncentracija kraujyje, M±SD, mmol/l	4,5 ± 0,7	4,2 ± 0,4	4,4 ± 0,5	**0,039***	**0,032*** 0,548 **0,041***

Chloro koncentracija kraujyje, M±SD, mmol/l	102,6 ± 3,1	102,1 ± 4,9	104,1 ± 1,5	**0,015****	0,329 0,117 **0,027****
Kalcio koncentracija kraujyje, M±SD, mmol/l	2,2 ± 0,4	2,1 ± 0,4	2,4 ± 0,1	**0,005****	**0,007**** 0,388 **0,033****
Lipazės koncentracija kraujyje, M±SD, U/l	291,5 ± 627,7	3491,0 ± 5569,9	46,9 ± 57	**<0,001****	**<0,001**** 0,533 **<0,001****
p-amilazės koncentracija kraujyje, M±SD, U/l	123,0 ± 210,6	708,3 ± 1023,3	30,5 ± 21,1	**<0,001****	**<0,001**** **0,029**** **<0,001****
CRB koncentracija kraujyje, M±SD, mg/l	38,4 ± 52,9	140,2 ± 94,8	0,7 ± 0,7	<0,001**	**<0,001**** **<0,001**** **<0,001****
LDH koncentracija kraujyje, M±SD, U/l	202,1 ± 50,5	249,0 ± 140,6	168,5 ± 22,2	**<0,001****	**0,074**** **0,008**** **<0,001****

LP – lėtinis pankreatitas; ŪP – ūminis pankreatitas; M – vidurkis; SD – standartinis nuokrypis; *one-way ANOVA; **Kruskal Wallis. Paryškinta statistiškai reikšminga p reikšmė (<0,05).

5 LENTELĖ. Vmax rodiklio pokyčiai

Vartų sistemos šaka	Ištyrimas	LP tiriamieji	ŪP tiriamieji	Sveiki tiriamieji	Bendra p reikšmė	p reikšmė tarp grupių: LP *vs* ŪP LP *vs* sveiki ŪP *vs* sveiki
Vartų vena	Pirmas, M±SD, cm/s	59,4 ± 22,9	61,6 ± 28,1	67,5 ± 21	0,584	0,835 0,313 0,380
	Antras, M±SD, cm/s	61,9 ± 27,4	62,7 ± 19,2	65,7 ± 13,9	0,797	0,993 0,850 0,837
	Trečias, M±SD, cm/s	63,7 ± 21,3	68,2 ± 24,1	60,8 ± 20,6	0,496	0,416 0,540 0,496
Dešinioji vartų venos šaka	Pirmas, M±SD, cm/s	39,1 ± 18,1	46,5 ± 24,1	44 ± 23,8	0,603	0,299 0,740 0,740
	Antras, M±SD, cm/s	45,7 ± 19	41,7 ± 14,9	42,8 ± 26,6	0,559	0,440 0,374 0,559
	Trečias, M±SD, cm/s	40,4 ± 28,1	42,3 ± 21,8	43 ± 22	0,797	0,515 0,635 0,797

Kairioji vartų venos šaka	Pirmas, M±SD, cm/s	21,1 ± 8,7	23,4 ± 8,3	20 ± 7,3	0,265	0,151 0,831 0,239
	Antras, M±SD, cm/s	19,9 ± 8,9	17 ± 6,1	22,5 ± 9,6	0,124	0,251 0,342 0,124
	Trečias, M±SD, cm/s	18,3 ± 6,2	20,5 ± 10,9	21,2 ± 9,6	0,851	0,942 0,635 0,851

LP – lėtinis pankreatitas; ŪP – ūminis pankreatitas; M – vidurkis; SD – standartinis nuokrypis.

6 LENTELĖ. Rezistentiškumo indekso kitimas ir skirtumai tarp grupių

Vartų sistemos šaka	Ištyrimas	LP tiriamieji n = 26	ŪP tiriamieji n= 29	Sveiki tiriamieji n = 16	Bendra p reikšmė	p reikšmė tarp grupių: LP *vs* ŪP LP *vs* sveiki ŪP *vs* sveiki
Vartų vena	Pirmas, M±SD	0,4 ± 0,2	0,4 ± 0,1	0,4 ± 0,1	0,212	0,761 0,186 0,077
	Antras, M±SD	0,4 ± 0,2	0,4 ± 0,2	0,4 ± 0,1	0,565	0,321 0,416 0,565
	Trečias, M±SD	0,4 ± 0,2	0,4 ± 0,2	0,4 ± 0,2	0,929	0,828 0,663 0,929
Dešinioji vartų venos šaka	Pirmas, M±SD	0,5 ± 0,2	0,4 ± 0,2	0,4 ± 0,1	0,195	0,091 0,344 0,347
	Antras, M±SD	0,4 ± 0,2	0,4 ± 0,1	0,5 ± 0,2	0,124	0,427 0,269 0,124
	Trečias, M±SD	0,5 ± 0,2	0,3 ± 0,1	0,4 ± 0,2	**0,002**	**0,003** 1,000 **0,024**
Kairioji vartų venos šaka	Pirmas, M±SD	0,4 ± 0,1	0,4 ± 0,1	0,3 ± 0,2	0,700	0,919 0,468 0,434
	Antras, M±SD	0,4 ± 0,2	0,3 ± 0,2	0,4 ± 0,1	0,461	0,377 0,830 0,461
	Trečias, M±SD	0,4 ± 0,2	0,4 ± 0,2	0,4 ± 0,2	0,557	0,985 0,691 0,561

7 LENTELĖ. Vidutinio kraujotakos greičio (Vmean) pokyčiai

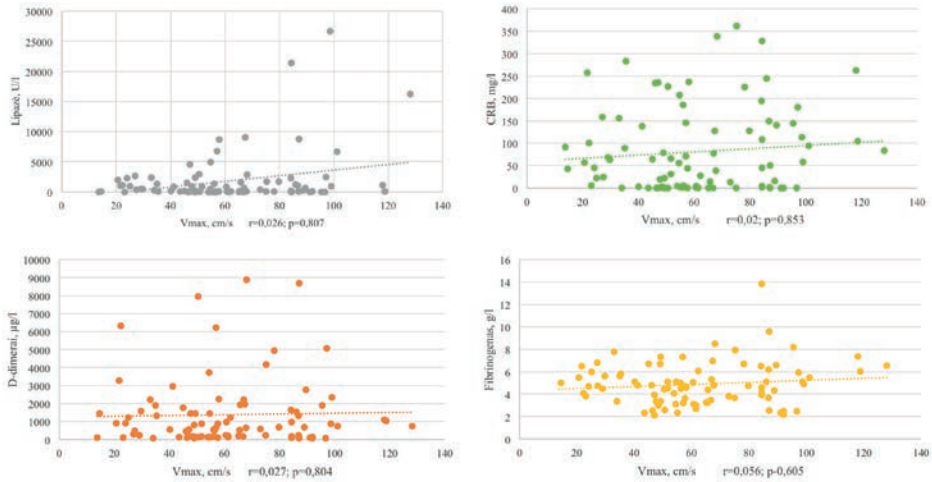
Vartų sistemos šaka	Ištyrimas	LP tiriamieji n= 26	ŪP tiriamieji n = 29	Sveiki tiriamieji n = 16	Bendra p reikšmė	p reikšmė tarp grupių: LP *vs* ŪP LP *vs* sveiki ŪP *vs* sveiki
Vartų vena	Pirmas, M±SD, cm/s	21,2 ± 8,1	21,4 ± 8,1	24,5 ± 8,2	0,352	0,888 0,180 0,192
	Antras, M±SD, cm/s	22,4 ± 8,4	22,3 ± 5,9	25,4 ± 4,5	0,295	0,997 0,377 0,317
	Trečias, M±SD, cm/s	23 ± 7,6	24,2 ± 8,9	23,2 ± 7,2	0,883	0,772 0,937 0,883
Dešinioji vartų venos šaka	Pirmas, M±SD, cm/s	14,8 ± 6,7	15,9 ± 7,0	16,4 ± 8,1	0,787	0,509 0,627 0,993
	Antras, M±SD, cm/s	17,6 ± 7,3	17,4 ± 5,8	15,6 ± 7,1	0,622	0,992 0,637 0,687
	Trečias, M±SD, cm/s	14,9 ± 9,1	18,2 ± 7,4	16,2 ± 6,9	0,459	0,434 0,874 0,737
Kairioji vartų venos šaka	Pirmas, M±SD, cm/s	7,1 ± 2,8	8,0 ± 2,9	7,4 ± 2,5	0,380	0,167 0,627 0,547
	Antras, M±SD, cm/s	6,9 ± 2,4	6,4 ± 2,2	8,4 ± 3,4	0,082	0,604 0,118 0,082
	Trečias, M±SD, cm/s	6,8 ± 2,3	7,1 ± 3,6	7,9 ± 2,9	0,438	0,800 0,304 0,438

3.6. Sąsaja tarp biocheminių kraujo rodiklių ir vartų veninės sistemos kraujotakos greičio

Apskaičiavus sąsają tarp biocheminių kraujo rodiklių ir maksimalaus ir vidutinio kraujotakos greičių vartų venose, statistiškai reikšmingo ryšio neaptikta.

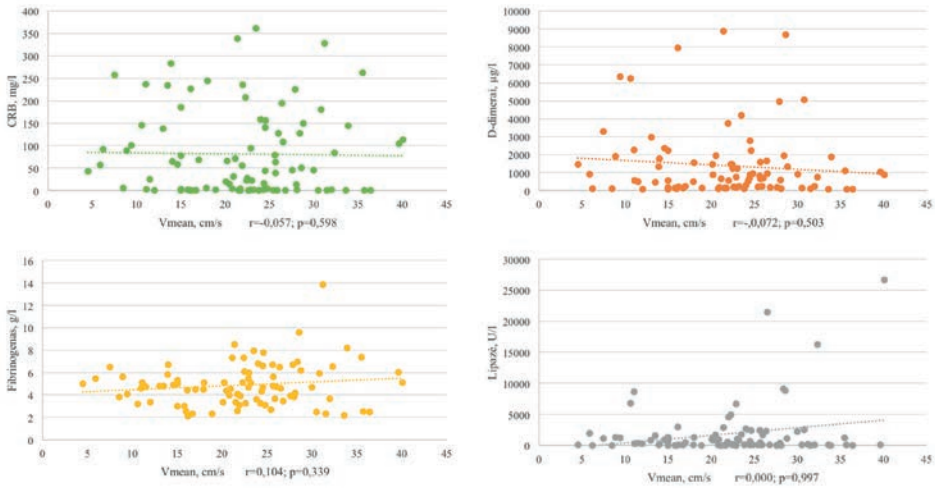
Nors nerasta reikšmingo maksimalaus kraujotakos greičio sistolės metu (Vmax) pokyčio sergant ūminiu ir lėtiniu pankreatitu nė vieno ištyrimo metu, atskirai įvertinta ar biocheminiai pokyčiai (lipazės, fibrinogeno, D-dimerų, CRB ir LDH koncentracija) yra susiję su Vmax vartų veninėje sistemoje. Nerasta statistiškai reikšmingo ryšio tarp Vmax ir minėtų laboratorinių rodiklių pagrindinėje vartų venoje ir dešiniojoje jos šakoje, bet aptikta labai nedidelė koreliacija tarp lipazės koncentracijos ir Vmax kairiojoje vartų venoje (r = 0,233; p =

0,028), tačiau kiti biocheminiai rodikliai neturėjo įtakos Vmax kairiojoje vartų venos šakoje ($p > 0,05$) (6 pav.).



6 PAV. Ryšys tarp biocheminių kraujo rodiklių ir Vmax pagrindinėje vartų venoje

Taip pat apskaičiuotas ryšys tarp biocheminių kraujo rodiklių ir vidutinio kraujotakos greičio vartų veninėje sistemoje. Nors tiek lipazės, tiek fibrinogeno, D-dimerų, CRB ir LDH koncentracija statistiškai reikšmingai didėjo sergant kasos audinio uždegimu, o sergant ūminiu pankreatitu taip pat reikšmingai lenkė lėtiniu pankreatitu sergančiųjų, tai neturėjo jokios įtakos Vmean nei pagrindinėje vartų venoje, nei kairiojoje ar dešiniojoje jos šakose ($p > 0,05$) (7 pav.).



7 PAV. Ryšys tarp biocheminių kraujo rodiklių ir Vmean pagrindinėje vartų venoje.

Išvados

1. Nustatytas vidutinis kraujotakos greitis pagrindinėje vartų venoje sveikų tiriamųjų populiacijoje buvo 24,5 ± 8,2 cm/s, maksimalus kraujotakos greitis sistolės metu per 4–6s buvo 67,5 ± 21 cm/s ir nesiskyrė nuo kitų mokslinėje literatūroje esančių duomenų. Kraujotakos greitis yra vidutiniškai 20 cm/s lėtesnis dešiniojoje vartų venoje ir vidutiniškai 40 cm/s lėtesnis kairiojoje vartų venoje lyginant su pagrindine vartų vena.
2. Kraujotakos greitis (Vmean ir Vmax) vartų veninėje sistemoje yra lėtesnis sergant lėtiniu pankreatitu (kai nėra paūmėjimo), bet šis skirtumas statistiškai nereikšmingas lyginant su sveikais asmenimis ($p > 0,05$). Stebint ligonius pusę metų neaptikta jokių reikšmingų kraujotakos greičio pokyčių vartų veninėje sistemoje, taigi vartų veninės sistemos kraujotakos pokyčiai sergant lėtiniu stabiliu pankreatitu yra lėti.
3. Ūminis lengvo laipsnio pankreatitas neturi įtakos kraujotakai vartų veninėje sistemoje ūmios ligos epizodo metu – kraujotakos greitis reikšmingai nesiskiria lyginant lengvo laipsnio ūminiu pankreatitu sergančius ir sveikus tiriamuosius ($p > 0,05$). Pusę metų po persirgto ūminio lengvo laipsnio pankreatito kraujotakos greitis vartų veninėje sistemoje reikšmingai nekinta.
4. Kraujotakos greitis vartų veninėje sistemoje nepriklauso nuo ūgio, svorio ar kūno masės indekso bei amžiaus. Pagrindinės vartų venos ir jos dešiniosios ir kairiosios šakų skersmuo reikšmingai mažesnis tarp moterų, o Vmean didesnis tarp vyrų.
5. Kraujo biocheminiai ir krešėjimo rodikliai neturi jokios įtakos vartų veninės sistemos kraujotakos pokyčiams sergant ūminiu lengvo laipsnio ar lėtiniu (kai nėra paūmėjimo) pankreatitu.

Rekomendacijos

1. Lengvo laipsnio ūminis pankreatitas turi mažai įtakos kraujotakos pokyčiams vartų veninėje sistemoje, todėl stebėti ligonius dėl galimos vartų venos trombozės išsivystymo nėra tikslinga.
2. Kraujotakos greitis vartų veninėje sistemoje yra lėtesnis sergant lėtiniu pankreatitu, tačiau ligos įtaka nėra reikšminga. Dėl galimos vartų venos trombozės tikslinga sekti lėtiniu pankreatitu sergančius ligonius atsiradus kasos uodegos cistai ar dažniems ūminio pankreatito recidyvams.

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Rima Kavalnienė

EVALUATION OF THE FACTORS THAT AFFECT PATIENTS' SATISFACTION WITH PRIMARY HEALTH CARE SERVICES

VEIKSNIŲ, LEMIANČIŲ PACIENTŲ PASITENKINIMĄ PIRMINĖS SVEIKATOS PRIEŽIŪROS PASLAUGOMIS, ĮVERTINIMAS

Biomedical sciences, Medicine (06 B)
Biomedicinos mokslai, Medicina (06B)

List of Abbreviations

PHC – Primary Healthcare
HC – Healthcare
PSQ-18 – Patients Satisfaction Questionnaire, Short Form
HADS – Hospital Anxiety and Depression Scale
HADS-A – Anxiety Subscale of Hospital Anxiety and Depression Scale
HADS-D – Depression Subscale of Hospital Anxiety and Depression Scale
VAS – Visual Analog Scale
WHO – World Health Organization
GP – General Practitioner
r – Correlation Coefficient
vs. – Versus
CI – Confidence Interval

1. Introduction

1.1. Researched problem and its relevance

The satisfaction of a patient is the cognitive assessment and emotional reaction to their experience of health care services[1]. Their outlook is influenced by various social, demographic factors, illnesses and their own expectations. The Lithuanian health program for 2014 – 2025, which was prepared in accordance with the health policy document of the World Health Organization in the European region "Health 2020: a European policy framework supporting action across government and society for Health and well-being", emphasizes four goals. One of the goals is to ensure effective and quality health care services oriented to the needs of the residents[2]. In order to achieve a patient-oriented healthcare system, the health infrastructure will be developed, and their quality, security and availability will be improved. A general strategic goal is to have healthier residents, to prolong their life expectancy, to improve their health, and to reduce health inequalities by 2025. Therefore, the improvement of residents' health depends on the development of the healthcare and the quality of PHC services. The increased satisfaction with the received services is important as the patients who are satisfied with the consultations are more likely to follow the treatment plan, take a better care of their health, and follow a healthy lifestyle. That conditions better results of the treatment[3].

1.2. Research goal

To identify the factors determining patients satisfaction with primary healthcare services.

1.3. Research objectives

1. To study the relationship between the patients‘ sociodemographic factors and the satisfaction with PHC services.
2. To determine the link between patients‘ satisfaction and the features of chronic diseases, depression and anxiety.
3. To assess the importance of doctor and healthcare institution to patients‘ satisfaction with the services.

1.4. Thesis statements

1. Patients‘ satisfaction with the PHC services is influenced by the sociodemographic factors.
2. The symptoms of depression and anxiety, and the features of chronic diseases affect the patients‘ satisfaction with the services.
3. The level of patients’ satisfaction is modified by age and gender of family doctor, and the type of PHC institution.

1.5. The novelty of the study and its significance

This research is directly related to earlier studies in Lithuania by other scientists. In 2002, E. Žiebienė et al. researched the connection between the patients‘ expectations of the medical consultation, and social and demographic characteristics of patients’ in primary health institutions in Lithuania[4]. Later, R. Aranauskas defended master thesis in M. Romeris University; he investigated the correspondence between the effectiveness of Vilnius University Santaros Clinics family medicine center and patients‘ expectations. In our study, both the satisfaction (PSQ-18), and the depression and anxiety (HADS) scales were used to research the primary link of health. Moreover, the multivariate analysis of satisfaction with the PHC services for the first time evaluates the factors of three important groups: patient (social and demographic characteristics, emotional and physical condition), doctor (certain demographic characteristics), and institution (through the evaluation of its type).

With the aid of this research, the correspondence between the expectations of the depressed, anxious, ill and other investigated patients‘ is evaluated, i.e. the satisfaction with the services received. Following the evaluation and the analysis of the results, the major flaws of the provided services are expected to be revealed of the provided services are expected to be relieved; their correction would allow to improve the quality of the services.

2. Data & methods

The research was approved by "Vilnius Regional Biomedical Research Ethics Committee" in Lithuania (no. 15800-15-795-313).

2.1. Subjects

The patient survey was carried out in PHC institutions from September 2015 to August 2017. The survey took place in 24 PHC institutions in Vilnius, Lithuania. Patients, who visited PHC centers for various reasons and agreed to participate in the study, were asked to fill out an anonymous questionnaire. The main criteria for the participation were age (subjects must be over 18 years old) and the ability to adequately understand and fill out the questionnaire.

2.2. Methods

The patients' satisfaction with the PHC services was evaluated according to the short version of the patients' satisfaction survey with the PHC services (Patient Satisfaction Questionnaire, PSQ-18, Marshall & Hays). The eighteen-statement survey evaluates the medical services according to seven main factors: General Satisfaction, Technical Quality, Interpersonal Manner, Communication, Financial Aspects, Time Spent with Doctor, Accessibility and Convenience. The translation of the PSQ-18 questionnaire from English to Lithuanian was done according to the official recommendations.

The Hospital Anxiety and Depression scale (HADS, Zigmont and Snaith) was used to select the symptoms of anxiety and depression, and to determine the level of their severity.

The respondents were also asked to indicate their gender, age, place of residence (City, District Center, Village), education (Primary, Basic, Secondary, Secondary Special, Higher or Other education), nationality (Lithuanian, Russian, Polish, other), the name of the institution they visit, the gender of their doctor, the approximate age of their doctor (25-35, 36-50, more than 50), their expectations towards the gender and the age of a doctor, to describe their illnesses, to mark their pain level in the visual analog scale (VAS).

The stages of research and the number of subjects are described in Table No. 1. The questionnaire was provided in Lithuanian.

2.3. Statistical Analysis

The descriptive statistics, Chi quadrant (χ^2) method and contingency tables were used in the analysis of the results; the correlations were calculated, the T-test

with two independent samples and single-factor dispersion analysis "One Way ANOVA" test, post hoc multiple comparison procedure were applied.

TABLE 1. The stages of research and the number of subjects

First stage (pilot study): n=598	Second stage: n=887
Questionnaire: • The short version of the patient satisfaction questionnaire (PSQ-18); • The sociodemographic characteristics of patients.	Questionnaire: • The short version of the patient satisfaction questionnaire (PSQ-18); • The sociodemographic characteristics of patients; + The Hospital Anxiety and Depression scale (*HADS*); + The list of chonic illnessess according to the Charlson Comorbidity Index's list of chronic diseases; + Pain Assessment Scale (VAS); + Doctors' age and gender (indicated by 581 patients); + A type of PHC institution.

The polynomial logistic regression was used in order to analyze in what way patients' satisfaction is influenced by different researched factors and how they are distributed among each other, i.e. how they influence each other. The significance level in all cases was 0.05.

The reliability of internal compatibility of each PSQ-18 and HADS subscale was calculated with Cronbach's alpha coefficient. During the second stage, the general PSQ-18 score Cronbach's alpha coefficient was **0.96**; the internal Cronbach's alpha coefficient of separate seven subscales varied **from 0.69 to 0.87**. These ratios were assessed as good.

3. Results

3.1. First stage

3.1.1. General evaluation of the provided services

The majority of the patients (55.5 %) evaluated the services as mediocre; 34 % of the patients evaluated the services as good; the minority (i.e. 10.5 %) evaluated the PHC services as poor. The respondents evaluated the Accessibility and Convenience, and Time Spent with Doctor the poorest; the Interpersonal Manner, and Communication were evaluated the best (Table 2).

TABLE 2. The estimates of PSQ-18 subscales and their arrangement (rating) (n=598)

PSQ categories	Mean estimates and their statutory deviation	Ascending order of estimates
Time Spent with Doctor	2,83 (0,89)	1
Accessibility and Convenience	3,05 (0,89)	2
Financial Aspects	3,17 (1,00)	3
General Satisfaction	3,31 (0,96)	4
Technical Quality	3,37 (0,78)	5
Communication	3,54 (0,89)	6
Interpersonal Manner	3,57 (0,90)	7

3.1.2. The link between the sociodemographic factors and patients' satisfaction

Gender of the patient. The mean of the general PSQ-18 scores between different genders of the respondents did not differ (p=0.105). Meanwhile, the gender of the patient influences the evaluation of the Interpersonal Manner, the Communication; women evaluated these factors better than men (Table 3).

TABLE 3. PSQ-18 estimates according to gender (n=598)

Scale	Gender of patients	Mean	Statutory deviation	95% mean CI		p
				Upper limit	Lower limit	
Interpersonal Manner	Male	3,47	0,92	3,35	3,60	0,035
	Female	3,63	0,89	3,55	3,72	
Communication	Male	3,42	0,92	3,30	3,55	0,023
	Female	3,60	0,87	3,51	3,69	

Age of the patient. The connection between the age of the patient and the satisfaction with the PHC services has not been found; statistically significant correlations have not been established neither with the PSQ-18 scale (p=0.769), neither with the subscales of the questionnaire (the credibility mark is in the diapason from p = 0.183 to p=0.96).

Place of residence. The residents of the district center were the least satisfied with the provided services (the general PSQ-18 score average was 59,99(12,81) in the city, 50,81(13,05) in the district center, and 52,57(11,78) in the village; p<0.001). Inhabitants of different places evaluate the services differently in almost every subscale, except for the Financial Aspects subscale (Table 4).

In nearly every case, the differences were noticed between the estimates of the residents of the city and the residents of the village, and the residents of the city and the residents of the district center. The inhabitants of the city have the greatest estimates, whereas the inhabitants of the city and the district center have the poorest. Statistically significant differences have not been noticed between the residents of the district center and the residents of the village (Table 5).

TABLE 4. PSQ-18 estimates according to the place of residence (n=598)

Scale	Place of residence	Mean	Statutory deviation	95% mean CI		p
				Upper limit	Lower limit	
General Satisfaction	City	3,41	0,95	3,32	3,49	0,000
	District center	2,89	0,80	2,57	3,21	
	Village	2,90	0,91	2,70	3,10	
Technical Quality	City	3,46	0,75	3,39	3,52	0,000
	District center	2,86	0,84	2,53	3,20	
	Village	3,06	0,79	2,88	3,23	
Interpersonal Manner	City	3,66	0,89	3,58	3,74	0,000
	District center	3,15	0,89	2,80	3,50	
	Village	3,21	0,86	3,02	3,40	
Communication	City	3,65	0,86	3,57	3,72	0,000
	District center	2,85	0,85	2,51	3,19	
	Village	3,10	0,87	2,91	3,29	
Time Spent with Doctor	City	2,89	0,88	2,81	2,97	0,000
	District center	2,91	0,84	2,57	3,24	
	Village	2,46	0,83	2,28	2,64	
Accessibility and Convenience	City	3,13	0,89	3,05	3,21	0,000
	District center	2,46	0,98	2,07	2,85	
	Village	2,77	0,70	2,62	2,93	

Education. The mean of the PSQ scale is the highest among the persons with the higher education (Secondary education or 57,06(13,352), Secondary special 55,66(13,417), Higher education 61,60(11,908); $p<0.001$). The differences depending on the education were identified in six out of seven subscales (Table 6). In all cases, patients with higher education evaluated the statements the best, whereas those with special secondary education evaluated them the poorest. The difference

between patients with special secondary education and those with secondary education has not been identified (Table 7).

TABLE 5. Pairwise comparison of PSQ-18 means according to place of residence (n=598)

Scale	p value		
		Village	District center
General PSQ-18 score	City	**0,000**	**0,004**
	District center	0,809	
General Satisfaction	City	**0,000**	**0,008**
	District center	0,999	
Technical Quality	City	**0,000**	**0,004**
	District center	0,540	
Interpersonal Manner	City	**0,000**	**0,017**
	District center	0,951	
Communication	City	**0,000**	**0,000**
	District center	0,408	
Time Spent with Doctor	City	**0,000**	0,993
	District center	0,051	
Accessibility and Convenience	City	**0,000**	**0,005**
	District center	0,292	

Nationality. The connection between the nationality and the general PSQ-18 score estimates has not been established (p=0.345). The influence of the nationality on the separate subscales has not been confirmed as well (the credibility mark is in the diapason from p=0.108 to p=0.721).

TABLE 6. PSQ-18 estimates according to education (n=598)

Scale	Education	Mean	Statutory deviation	95% mean CI		p
				Upper limit	Lower limit	
General Satisfaction	Secondary and lower	3,25	0,95	3,12	3,39	0,001
	Secondary special	3,13	0,98	2,98	3,29	
	Higher	3,48	0,93	3,36	3,60	
Technical Quality	Secondary and lower	3,26	0,82	3,15	3,38	0,000
	Secondary special	3,20	0,79	3,08	3,32	
	Higher	3,57	0,69	3,48	3,66	
Interpersonal Manner	Secondary and lower	3,49	0,91	3,36	3,62	0,000
	Secondary special	3,36	0,93	3,21	3,50	
	Higher	3,79	0,83	3,68	3,90	

Communication	Secondary and lower	3,40	0,97	3,26	3,54	0,000
	Secondary special	3,38	0,91	3,24	3,52	
	Higher	3,76	0,78	3,66	3,86	
Financial Aspects	Secondary and lower	3,10	1.03	2,96	3,25	0,001
	Secondary special	2,99	1.01	2,83	3,15	
	Higher	3,36	0,93	3,24	3,47	
Accessibility and Convenience	Secondary and lower	2,96	0,91	2,83	3,09	0,001
	Secondary special	2,89	0,91	2,75	3,04	
	Higher	3,21	0,84	3,10	3,32	

TABLE 7. Pairwise comparison of PSQ-18 means according to education (n=598)

Scale	p value		
		Secondary and lower	Higher
General PSQ-18 score	Secondary special	0,591	**0,000**
	Higher	**0,001**	
General Satisfaction	Secondary special	0,486	**0,001**
	Higher	**0,032**	
Technical Quality	Secondary special	0,742	**0,000**
	Higher	0,000	
Interpersonal Manner	Secondary special	0,387	**0,000**
	Higher	**0,001**	
Communication	Secondary special	0,978	**0,000**
	Higher	**0,000**	
Financial Aspects	Secondary special	0,551	**0,001**
	Higher	**0,025**	
Accessibility and Convenience	Secondary special	0,768	**0,001**
	Higher	**0,010**	

3.2. Second stage

3.2.1. General evaluation of the provided services

The majority (44%) evaluated the services as good, whilst 38% evaluated them as mediocre, and 18% evaluated the PHC services as poor. Thus, the satisfaction with the researched family medical centers in Vilnius is fair. Similarly to the pilot study, the respondents evaluated the Accessibility and Convenience, as well as Time Spent with Doctor the poorest. The Interpersonal Manner, and Communication were evaluated the best (Table 8).

TABLE 8. PSQ-18 subscale estimates and their arrangement (rating) (n=887)

PSQ-18 categories	Mean estimate and st. deviation	Ascending order of estimates
Accessibility and Convenience	2,97 (0,98)	1
Time Spent with Doctor	3,20 (1,01)	2
General Satisfaction	3,37 (0,98)	3
Financial Aspects	3,40 (0,91)	4
Technical Quality	3,45 (0,84)	5
Communication	3,55 (0,97)	6
Interpersonal Manner	3,59 (0,98)	7

3.2.2. The links between separate factors and the satisfaction with PHC services

Gender of the patient. Women evaluate the PHC services better than men; the mean of the general score was 58,60(15,39) among men, and 60,74(14,07) among women (p=0,033). The differences were noticed in the evaluation of Interpersonal Manner, and Communication. Female patients had a higher average of evaluation in these subscales than men (Table 9).

TABLE 9. PSQ-18 estimates according to gender of a patient (n=887)

Scale	Gender	Mean	Statutory deviation	95% mean CI		p
				Upper limit	Lower limit	
Interpersonal Manner	Male	3,47	1,01	3,36	3,57	0,003
	Female	3,67	0,96	3,59	3,75	
Communication	Male	3,46	1,01	3,35	3,56	0,025
	Female	3,61	0,93	3,53	3,69	
Time Spent with Doctor	Male	3,10	1,03	2,99	3,21	0,017
	Female	3,27	0,99	3,18	3,35	

Age of the patient. Younger patients were more satisfied with the health care (Spearman's r=-0,161; p<0,001). Their satisfaction was evident in all seven subscales (the diapason of Spearman's correlation coefficient was from -0,074 to -0,223; p<0,001) (Table 10).

TABLE 10. Spearman correlation coefficient between age of patients and PSQ-18 estimates (n=887)

PSQ-18 categories	Spearman correlation coefficient
General Satisfaction	-0.155**
Technical Quality	-0.074**
Interpersonal Manner	-0.170**
Communication	-0.153**
Financial Aspects	-0.175**
Time Spent with Doctor	-0.223**
Accessibility and Convenience	-0.171**

**-p<0,001

The place of residence. The residents of the city were more satisfied with the services, whilst the residents of the district center were the least satisfied (the average of the general score in the city was 61,42(14,26), 52,60(13,11) in the district center, and 54,23(15,65) in the village; p<0.001). The residents of the city evaluated the separate subscales describing different quality aspects better than the residents of the district center or the residents of the village (Table 11). The statistical difference between the residents of the city and the residents of the village was found in the pairwise comparisons of almost every subscale. The evaluation between the residents of the district center and the residents of the village differed only in two domains: Financial Aspects and Time Spent with Doctor; they were evaluated better by the residents of the city (Table 12).

TABLE 11. PSQ-18 subscale estimates according to the place of residence (n=887)

Scale	Residence	Mean	St. deviation	95% mean CI		p
				Up. limit	Lower limit	
General Satisfaction	City	3,50	0,94	3,43	3,57	0,000
	District center	2,76	0,88	2,56	2,95	
	Village	2,94	1,01	2,73	3,14	
Technical Quality	City	3,50	0,83	3,44	3,56	0,000
	District center	3,37	0,67	3,22	3,52	
	Village	3,09	0,93	2,90	3,28	
Interpersonal Manner	City	3,67	0,94	3,60	3,74	0,000
	District center	3,24	1,08	2,99	3,48	
	Village	3,24	1,09	3,02	3,47	
Communication	City	3,64	0,94	3,57	3,71	0,000
	District center	3,20	0,93	2,99	3,41	
	Village	3,11	1,01	2,91	3,32	

Scale	Residence	Mean	St. deviation	95% mean CI		p
				Up. limit	Lower limit	
Financial Aspects	City	3,47	0,89	3,40	3,53	0,000
	District center	2,88	0,87	2,69	3,08	
	Village	3,29	1,01	3,08	3,50	
Time Spent with Doctor	City	3,33	0,98	3,26	3,41	0,000
	District center	2,44	0,81	2,25	2,62	
	Village	2,82	1,00	2,61	3,03	
Accessibility and Convenience	City	3,05	0,98	2,97	3,12	0,000
	District center	2,52	0,82	2,34	2,71	
	Village	2,76	0,90	2,58	2,95	

TABLE 12. Pairwise comparison of PSQ-18 means according to the place of residence (n=887)

Scale	p value		
		City	Village
General PSQ-18 score	District center	**0,000**	0,738
	Village	**0,000**	
General Satisfaction	District center	**0,000**	0,426
	Village	**0,000**	
Technical Quality	District center	0,260	0,063
	Village	**0,000**	
Interpersonal Manner	District center	**0,003**	0,999
	Village	**0,001**	
Communication	District center	**0,000**	0,826
	Village	**0,000**	
Financial Aspects	District center	**0,000**	**0,014**
	Village	0,259	
Time Spent with Doctor	District center	**0,000**	**0,017**
	Village	**0,000**	
Accessibility and Convenience	District center	**0,000**	0,162
	Village	**0,015**	

Education. The satisfaction with the PHC services was the poorest among those who have Secondary or lower education (Secondary or lower education 57,84(15,47), Secondary special 61,11(13,36), Higher education 60,55(14,40); p=0,025). Patients with Secondary or lower education evaluated the statements the poorest, however, the statistically significant results were acquired in four subscales (Table 13). Some of the subscales were evaluated the best by patients with Secondary special education; some were evaluated the best by those with Higher education. "General Sat-

isfaction", "Interpersonal Manner" and "Communication" subscales were evaluated poorer by patients with Secondary or lower education than by patients with Higher or Secondary special education. The difference in the "Time Spent with Doctor" subscale was found only between patients with Secondary or lower education and patients with Higher education (Table 14).

TABLE 13. PSQ-18 estimates according to education (n=887)

Scale	Education	Mean	St. deviation	95% mean CI		p
				Upper limit	Lower limit	
General Satisfaction	Secondary and lower	3,21	1,01	3,09	3,33	0,002
	Secondary special	3,51	0,89	3,37	3,66	
	Higher	3,42	0,98	3,33	3,51	
Interpersonal Manner	Secondary and lower	3,40	1,01	3,28	3,52	0,001
	Secondary special	3,71	0,85	3,57	3,84	
	Higher	3,64	1,00	3,55	3,74	
Communication	Secondary and lower	3,38	1,07	3,25	3,50	0,001
	Secondary special	3,70	0,81	3,57	3,83	
	Higher	3,60	0,94	3,51	3,69	
Time Spent with Doctor	Secondary and lower	3,00	1,03	2,88	3,12	0,000
	Secondary special	3,15	0,98	2,99	3,31	
	Higher	3,33	0,98	3,24	3,43	

TABLE 14. Pairwise comparison of PSQ-18 means according to education (n=887)

Scale	p value		
		Secondary and lower	Higher
General Satisfaction	Secondary special	**0,004**	0,542
	Higher	0,015	
Interpersonal Manner	Secondary special	**0,003**	0,725
	Higher	**0,006**	
Communication	Secondary special	**0,001**	0,422
	Higher	**0,013**	
Time Spent with Doctor	Secondary special	0,306	0,126
	Higher	**0,000**	

Nationality. The services were evaluated as more favorable by Lithuanians, and the poorest by Russians; the mean of PSQ-18 score is 61,15(14,17) of Lithuanian patients, 59,00(15,15) of Polish patients and 57,60(15,20) of Russian patients (p=0,013). The differences were found in five evaluation subscales (Table 15).

TABLE 15. PSQ-18 subscale estimates according to nationality (n=887)

Scale	Nationality	Mean	St. de-via-tion	95% mean CI		p
				Upper limit	Lower limit	
General Satisfaction	Lithuanian	3,47	0,94	3,39	3,56	0,003
	Russian	3,21	1,00	3,06	3,36	
	Polish	3,28	1,04	3,13	3,44	
Interpersonal Manner	Lithuanian	3,72	0,95	3,64	3,80	0,000
	Russian	3,36	1,00	3,20	3,51	
	Polish	3,46	1,01	3,31	3,61	
Communication	Lithuanian	3,63	0,92	3,55	3,71	0,004
	Russian	3,35	1,09	3,19	3,52	
	Polish	3,51	0,96	3,37	3,65	
Financial Aspects	Lithuanian	3,46	0,88	3,39	3,54	0,024
	Russian	3,25	0,90	3,12	3,39	
	Polish	3,36	1,01	3,21	3,51	
Time Spent with Doctor	Lithuanian	3,30	1,01	3,21	3,39	0,002
	Russian	3,01	0,99	2,86	3,16	
	Polish	3,13	1,01	2,98	3,28	

TABLE 16. Pairwise comparison of PSQ-18 means according to nationality(n=887)

Scale	p value		
		Lithuanian	Polish
General PSQ-18 score	Russian	**0,020**	0,666
	Polish	0,227	
General Satisfaction	Russian	**0,008**	0,803
	Polish	0,082	
Interpersonal Manner	Russian	**0,000**	0,614
	Polish	**0,008**	
Communication	Russian	**0,008**	0,333
	Polish	0,299	
Financial Aspects	Russian	**0,020**	0,556
	Polish	0,427	
Time Spent with Doctor	Russian	**0,003**	0,520
	Polish	0,129	

The most noticeable difference was observed in the Interpersonal Manner; less noticeable differences were noticed while evaluating the General Satisfaction, Communication, Financial Aspects and Time Spent with Doctor subscales. The subscales with identified statistical difference were evaluated the best by Lithuanians, and the poorest by Russians. The results of the Polish group were better than the Russian results and were nearer the Lithuanians' results. Therefore, the significant difference in the subscales was only noticed between Russian and Lithuanian groups. The difference between the Polish and the Lithuanian groups was noticed only in the evaluation of Interpersonal Manner (Table 16).

Type of PHC institution. The patients of public and private clinics evaluated the provided PHC services differently. The general score was very similar and did not significantly differ (public clinic: 59,60(14,35), private clinic: 60,83(15,59); p=0,295). Some of the subscales were evaluated better by the public clinic patients, and some by the semi-private clinic patients. However, only the satisfaction with the Accessibility and Convenience was evaluated differently: the private clinic patients evaluated that better than public clinic patients (Table 17).

TABLE 17. The influence of a type of institution on the PSQ-18 subscale estimates (n=887)

Scale	Institution	Mean	St. deviation	95% mean CI		p
				Upper limit	Lower limit	
Accessibility and Convenience	Public	2,92	0,96	2,85	2,99	0,008
	Private	3,14	1,02	3,00	3,28	

Chronic illnesses. Patients with chronic illnesses evaluated six out of seven satisfaction with the PHC service factors poorer than other patients. That also had a poorer general PSQ-18 score ("no illnesses" group 62,59(12,88), "one or more illnesses" 57,89(15,53); p<0,001).

The most evident difference has been noticed in the evaluation of the Time Spent with Doctor; ill patients had a significantly poorer mean of the score ("no illnesses" 3,51(0,89), "one or more illnesses" 2,97(1,03); p<0,001). "Technical Quality" was the only subscale to lack statistical significance ("no illnesses" group 3,51(0,82), and "one or more illnesses" 3,40(0,85); p=0,061).

Pain syndrome. Pain intensity correlated with the general PSQ-18 score (r= -0,310; p<0,001). The correlations were also found with all the questionnaire subscales (r diapason varies from -0,226 to -0,326; p<0,001) (Table 19).

TABLE 18. PSQ-18 subscale estimates according to chronic illnesses (n=887)

Scale	Illness	Mean	St. deviation	95% mean CI		p
				Upper limit	Lower limit	
General Satisfaction	No	3,58	0,90	3,49	3,67	0,000
	Yes	3,23	1,00	3,14	3,31	
Interpersonal Manner	No	3,76	0,90	3,67	3,85	0,000
	Yes	3,46	1,02	3,37	3,55	
Communication	No	3,69	0,85	3,61	3,78	0,000
	Yes	3,44	1,04	3,35	3,53	
Financial Aspects	No	3,53	0,83	3,45	3,62	0,000
	Yes	3,30	0,96	3,21	3,38	
Time Spent with Doctor	No	3,51	0,89	3,42	3,60	0,000
	Yes	2,97	1,03	2,88	3,06	
Accessibility and Convenience	No	3,10	0,95	3,00	3,20	0,001
	Yes	2,87	0,98	2,79	2,96	

TABLE 19. Spearman correlation coefficient between VAS for pain and PSQ-18 estimates (n=887)

PSQ-18 categories	Spearman correlation coefficient
General Satisfaction	-0.326**
Technical Quality	-0.226**
Interpersonal Manner	-0.255**
Communication	-0.269**
Financial Aspects	-0.295**
Time Spent with Doctor	-0.284**
Accessibility and Convenience	-0.285**
General PSQ-18 score	-0.310**

**-p<0,001

Gender of the family doctor. The consultations of female doctors were evaluated better by the PSQ-18 score (male doctor group score was 56,25(14,56), female doctor group score was 60,13(14,91); p=0,034). "Interpersonal Manner", "Time Spent with Doctor" and "Accessibility and Convenience" has been evaluated better in the female doctor group than in the male doctor group (Table 20).

The most noticeable difference in the evaluation of the Interpersonal Manner aspect indicates that the patients of male doctors lack the ability to participate in the decision making process, they are less content with the male doctors' ability to hear them out and understand, to politely and respectfully consult.

The gender of patients, who would like to substitute their doctor with the doctor of an opposite sex, does not differ significantly among the female or male doctor

TABLE 20. PSQ-18 subscale estimates according to doctor's gender (n=581)

Scale	Gender	Mean	St. deviation	95% mean CI		p
				Upper limit	Lower limit	
Interpersonal Manner	Male doctor	3,04	0,99	2,81	3,27	0,000
	Female doctor	3,63	1,00	3,54	3,72	
Time Spent with Doctor	Male doctor	2,83	1,01	2,60	3,06	0,004
	Female doctor	3,20	1,03	3,11	3,29	
Accessibility and Convenience	Male doctor	2,71	0,91	2,50	2,92	0,019
	Female doctor	2,99	0,97	2,90	3,07	

groups; such number is small (7,5 % in the female doctor group, 10,5 % in the male doctor group; p=0,654).

We have divided the data into four groups according to the gender of the doctor and the gender of the patient (male doctor and male patient (n=36), male doctor and female patient (n=40), female doctor and male patient (n=195) and female doctor and female patient (n=310)), and we have evaluated the differences of the estimates among these four groups. The estimates of "Interpersonal Manner", and "Time Spent with Doctor" subscales have statistically significant differences in all four groups (Table 21).

TABLE 21. PSQ-18 subscale estimates on the doctor-patient groups according to gender (n=581)

Subscale	Group	Mean	St. deviation	95% mean CI		p
				Up. limit	Lower limit	
Interpersonal Manner	Male doctor – male patient	2,88	0,95	2,55	3,20	0,000
	Male doctor – female patient	3,19	1,01	2,86	3,51	
	Female doctor – male patient	3,56	1,02	3,41	3,70	
	Female doctor – female patient	3,68	0,98	3,57	3,79	
Time Spent with Doctor	Male doctor – male patient	2,72	1,00	2,39	3,06	0,003
	Male doctor – female patient	2,93	1,02	2,60	3,25	
	Female doctor – male patient	3,07	1,04	2,92	3,22	
	Female doctor – female patient	3,28	1,01	3,16	3,39	

The poorest evaluated estimates were in the male doctor and male patient group, slightly better estimates in the male doctor and female patient group, a lot better estimates in the female doctor and male patient group, and the best estimates in the female doctor and female patient group. Significant differences were not proved in all four groups; however, the same difference recur between the female doctor and female patient, male doctor and male patient groups (Table 22).

TABLE 22. Pairwise comparison of PSQ-18 means according to doctor-patient gender groups (n=581)

Scale	p value			
		Female doctor – female patient	Female doctor – male patient	Male doctor – female patient
Interpersonal Manner	Male doctor – male patient	**0,002**	**0,002**	0,511
	Male doctor – female patient	**0,027**	0,166	
	Female doctor – male patient	0,542		
Time Spent with Doctor	Male doctor – male patient	**0,015**	0,232	0,818
	Male doctor – female patient	0,183	0,843	
	Female doctor – male patient	0,129		

Age of the family doctor. The age of the doctor of more than 51 years correlated with the poorer PSQ-18 general score in comparison with the younger age groups (25-35 year group 67,82(10,43), 36-50 year group 64,96(11,90), and older than 51 years 51,58(15,10); p<0,001). The age of the doctors older than 51 years were

TABLE 23. PSQ-18 subscale estimates according to doctors' age groups (n=581)

Scale	Age group	Mean	St. deviation	95% mean CI		p
				Upper limit	Lower limit	
General Satisfaction	25-35	3,93	0,81	3,74	4,12	0,000
	36-50	3,70	0,83	3,60	3,80	
	51and older	2,89	0,96	2,77	3,02	
Technical Quality	25-35	3,73	0,77	3,54	3,91	0,000
	36-50	3,69	0,66	3,61	3,78	
	51 and older	3,15	0,87	3,04	3,25	

Scale	Age group	Mean	St. deviation	95% mean CI		p
				Upper limit	Lower limit	
Interpersonal Manner	25-35	4,04	0,69	3,88	4,20	0,000
	36-50	3,89	0,80	3,80	3,99	
	51 and older	3,05	1,08	2,92	3,19	
Communication	25-35	4,17	0,67	4,01	4,32	0,000
	36-50	3,88	0,76	3,79	3,97	
	51 and older	3,00	1,06	2,87	3,13	
Financial Aspects	25-35	3,69	0,80	3,51	3,88	0,000
	36-50	3,62	0,78	3,53	3,72	
	51 and older	2,95	0,98	2,82	3,07	
Time Spent with Doctor	25-35	3,91	0,58	3,77	4,05	0,000
	36-50	3,46	0,93	3,35	3,57	
	51 and older	2,60	0,96	2,48	2,72	
Accessibility and Convenience	25-35	3,36	0,83	3,16	3,55	0,000
	36-50	3,27	0,90	3,16	3,38	
	51 and older	2,50	0,89	2,39	2,61	

TABLE 24. Pairwise comparison of PSQ-18 means according to doctors' age groups (n=581)

Scale	p value		
		Over 51	36-50
General PSQ-18 score	25-35	**0,000**	0,117
	36-50	**0,000**	
General Satisfaction	25-35	**0,000**	0,088
	36-50	**0,000**	
Technical Quality	25-35	**0,000**	0,948
	36-50	**0,000**	
Interpersonal Manner	25-35	**0,000**	0,262
	36-50	**0,000**	
Communication	25-35	**0,000**	**0,006**
	36-50	**0,000**	
Financial Aspects	25-35	**0,000**	0,784
	36-50	**0,000**	
Time Spent with Doctor	25-35	**0,000**	**0,000**
	36-50	**0,000**	
Accessibility and Convenience	25-35	**0,000**	0,702
	36-50	**0,000**	

evaluated the poorest in all seven subscales (Table 23). The noticed differences are evident in all subscales. The satisfaction with services differs among the youngest and the oldest, and the middle-aged and the oldest doctor groups. The differences among the youngest and the middle-aged doctor groups were noticed only in two ("Communication", and "Time Spent with Doctor") subscales (Table 24).

The patients were asked about their preference of the doctor's age, and we evaluated if that correlates with the age of their doctor. The patients, who have consultations with 36-50 year old doctors, were the most satisfied and wanted to substitute their doctors the least (Table 25).

TABLE 25. Patient expectations of doctors age (n=581)

		Doctor age group			Total
		25-35	36-50	Over 51	
Does the doctor's age meet patients' expectations?	**Yes**	**33a (45,8%)**	**247b(94,3%)**	**25c (10,1%)**	305 (52,5%)
	No	**39a (54,2%)**	**15b (5,7%)**	**222c (89,9%)**	276 (47,5%)
	Total	72 (100%)	262 (100%)	247	581 (100%)

Each subscript letter denotes a subset of "doctor age group" categories whose column proportions do not differ significantly from each other at the 0.05 level.

Patients of doctors who are older than 51 years old were the least satisfied; they wanted to substitute their doctors the most. Out of those patients who would like to substitute their doctor, 93.1% would like to have a doctor of 36-50 years.

Anxiety and depression symptoms of patients. There were 290 (32.7%) patients with any degree of anxiety symptoms, and 193 (21.8 %) patients with any degree depression symptoms. The degree of depression and anxiety symptoms among the subjects is discussed in Table 26.

TABLE 26. The rate of depression and anxiety symptom severity degrees (n=887)

Symptom severity degree	Anxiety symptom scale		Depression symptom scale	
	Number of respondents	%	Number of respondents	%
Normal mood	597	67,3%	694	78,2%
Mild symptoms	146	16,5%	106	12%
Moderate symptoms	127	14,3%	63	7,1%
Severe symptoms	17	1,9%	24	2,7%
Total	887	100%	887	100%

Both anxiety and depression symptom subscales correlated with the general PSQ-18 score (Spearman coefficients -0,446 in HADS-A subscale and -0,536 in HADS-D subscale; $\rho<0,001$). The connections between all the seven PSQ-18 subscales have also been noticed (Table 27 and 28).

TABLE 27. Spearman correlation coefficient between HADS-A and PSQ-18 estimates (n=887)

PSQ-18 categories	Spearman correlation coefficient
General Satisfaction	-0,421**
Technical Quality	-0,368**
Interpersonal Manner	-0,356**
Communication	-0,390**
Financial Aspects	-0,432**
Time Spent with Doctor	-0,390**
Accessibility and Convenience	-0,446**
General PSQ-18 score	-0,462**

**-p<0,001

TABLE 28. Spearman correlation coefficient between HADS-D and PSQ-18 estimates (n=887)

PSQ-18 categories	Spearman correlation coefficient
General Satisfaction	-0,470**
Technical Quality	-0,430**
Interpersonal Manner	-0,405**
Communication	-0,453**
Financial Aspects	-0,490**
Time Spent with Doctor	-0,467**
Accessibility and Convenience	-0,521**
General PSQ-18 score	-0,536**

**-p<0,001

3.2.3. General impact of the researched factors on the satisfaction with PHC services

If the sociodemographic factors of patients, HADS and VAS pain scale estimates, sickness rate and demographic factors of doctors were included to the model of regression analysis, 79.1% of cases would be evaluated as good, 45.3% of cases would be evaluated as mediocre, 78.7% of cases would be evaluated as poor. The general percentage of correctly classified respondents of the sample is 67.5%. Nagelkerke pseudo coefficient is 0,609. The Chi quadrant statistics for the highest probability ratio of the model x2 is = 431,523, p<0,001. The statistically significant variables in the table of maximum likelihood ratio Chi quadrant criterion results for each regression are the severity of patients' depression symptoms, place of residence, education, doctor's age and chronic illnesses (Table 29).

After the application of Wald criterion, we have found that the factors which increase a possibility of poor evaluation are stronger depression symptoms, place of residence in the village (in comparison with the district center), Secondary education, chronic illnesses and older doctor age (over 51 years) (Table 30).

TABLE 29. The table of the maximum likelihood ratio chi quadrant criterion results for each regression (with demographic factors of patients, HADS and VAS for pain scales, chronic illnesses and demographic factors of doctors) (n=581)

	Model Fitting Criteria	Likelihood Ratio Tests		
Effect	-2 Log Likelihood of reduced model	Chi-Square	Degree of Freedom	p
Intercept	760,999	0,000	0	
Patient's age	763,599	2,600	2	0,273
Anxiety symptoms	766,085	5,086	2	0,079
Depression symptoms	**810,783**	**49,785**	**2**	**0,000**
VAS for pain estimate	762,520	1,521	2	0,467
Patient's gender	761,299	0,300	2	0,861
Place of residence	**797,922**	**36,924**	**4**	**0,000**
Education	**772,291**	**11,292**	**4**	**0,023**
Nationality	767,389	6,390	4	0,172
Type of institution	761,993	0,994	2	0,608
Doctor's age	**803,369**	**42,371**	**4**	**0,000**
Doctor's gender	763,063	2,065	2	0,356
Chronic illnesses	**776,751**	**15,753**	**2**	**0,000**

While evaluating the regression coefficients, we have also observed that the higher estimates of anxiety symptoms could influence the evaluation of the services, as the regression coefficient of this variable in the "good vs. poor" evaluation submodel is -0,167, and p=0,026. However, we should exclude this variable as it was statistically insignificant in the table of the highest likelihood ratio Chi quadrant criterion results for every regression (p=0,079).

Limitations of the study. This study has a few important limitations which should be considered. Respondents were from one city and district around the city, and the opinion of respondents from other cities could be different. The questionnaire was presented in the official state language of Lithuania, and the ethnic minorities, who do not speak the language, could not participate in the study; their opinion could differ from those, who understand the official language of Lithuania. This study surveyed only those residents of the district center and village who visit PHC centers in the city; the opinion of those who go to the village and district center clinics could differ. The evaluation of doctors' age was a bit subjective, as the patients themselves had to guess and indicate the age of their doctor. Nonetheless, they had wide intervals to select their age.

TABLE 30. The polynomial logistic regression indicator variables in the evaluation of the services, and the patients' sociodemographic factors, HADS and VAS for pain subscales, chronic illnesses and doctors' demographic factors (Nagelkerke = 0,609) (Extract from the full model) (n=581)

Evaluation of services	Factors	B	Wald	p	Odds ratio
Mediocre vs. poor	**Depression symptoms**	**-0.142**	**5.308**	**0.021**	**0.868**
	Place of residence: -city	-0.004	0.000	0.993	0.996
	-district center	**2.030**	**12.682**	**0.000**	**7.612**
	-village	0[b]			
	Education: **-Secondary and lower**	**-1.125**	**8.441**	**0.004**	**0.325**
	-Secondary special	-0.187	0.170	0.680	0.829
	-Higher	0[b]			
	Doctor's age: -25-35	1.513	3.156	0.076	4.540
	-36-50	**1.170**	**8.790**	**0.003**	**3.221**
	-over 51	0[b]			
	Chronic illnesses: **-none**	**1.808**	**9.881**	**0.002**	**6.097**
	-yes	0[b]			
Good vs. poor	**Depression symptoms**	**-0.432**	**35.720**	**0.000**	**0.649**
	Doctor's age: -25-35	**3.112**	**13.118**	**0.000**	**22.462**
	-36-50	**2.178**	**25.978**	**0.000**	**8.832**
	-over 51	0[b]			

b. Comparative variable

4. Conclusions

1. The poorer satisfaction with the PHC services was determined by such sociodemographic factors of patients as masculine gender, older age, lower education, Russian nationality, place of residence out of the city.
2. Severe anxiety and depression symptoms, chronic illnesses and pain conditions poorer patient satisfaction with the services received.
3. Masculine gender of the doctor and older age negatively affect the satisfaction with services. Patients of private clinics evaluated the accessibility of the services better.

Recommendations

The evaluation of the PHC services is closely related to various social, cultural and biopsychological factors of the patients, which must be taken into consideration when the labor in family centers is being organized. Discontent with the services of family centers is determined by several core matters: the unchanging health care system and the administrative gap, inability and unwillingness to organize the medical labor appropriately, to adapt to the changing needs of the clients and their dynamics. It is suggested:

- To use the PSQ-18 questionnaire in the healthcare institutions regularly to evaluate the quality of the institution.
- To organize the courses of basic communication, provision of emotional support and successful communication for the physicians.
- To give more attention and spend more time during the consultations with the elderly, less educated people with several illnesses who might have symptoms of anxiety and depression and have the pain syndrome.
- The ministry of health should legalize the longer duration of consultations and include additional qualified PHC specialists (health management, information and psychological-social help provision, and others) into the care of the patients officially.

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Presentations:

1. Gaidamovič (Kavalnienė) R, Kasiulevičius V, Šapoka V, Deksnytė A, Aranauskas R. The influence of social and demographic factors on patient satisfaction with primary care. 9-th Baltic Family Medicine Conference. Vilnius, 2015.

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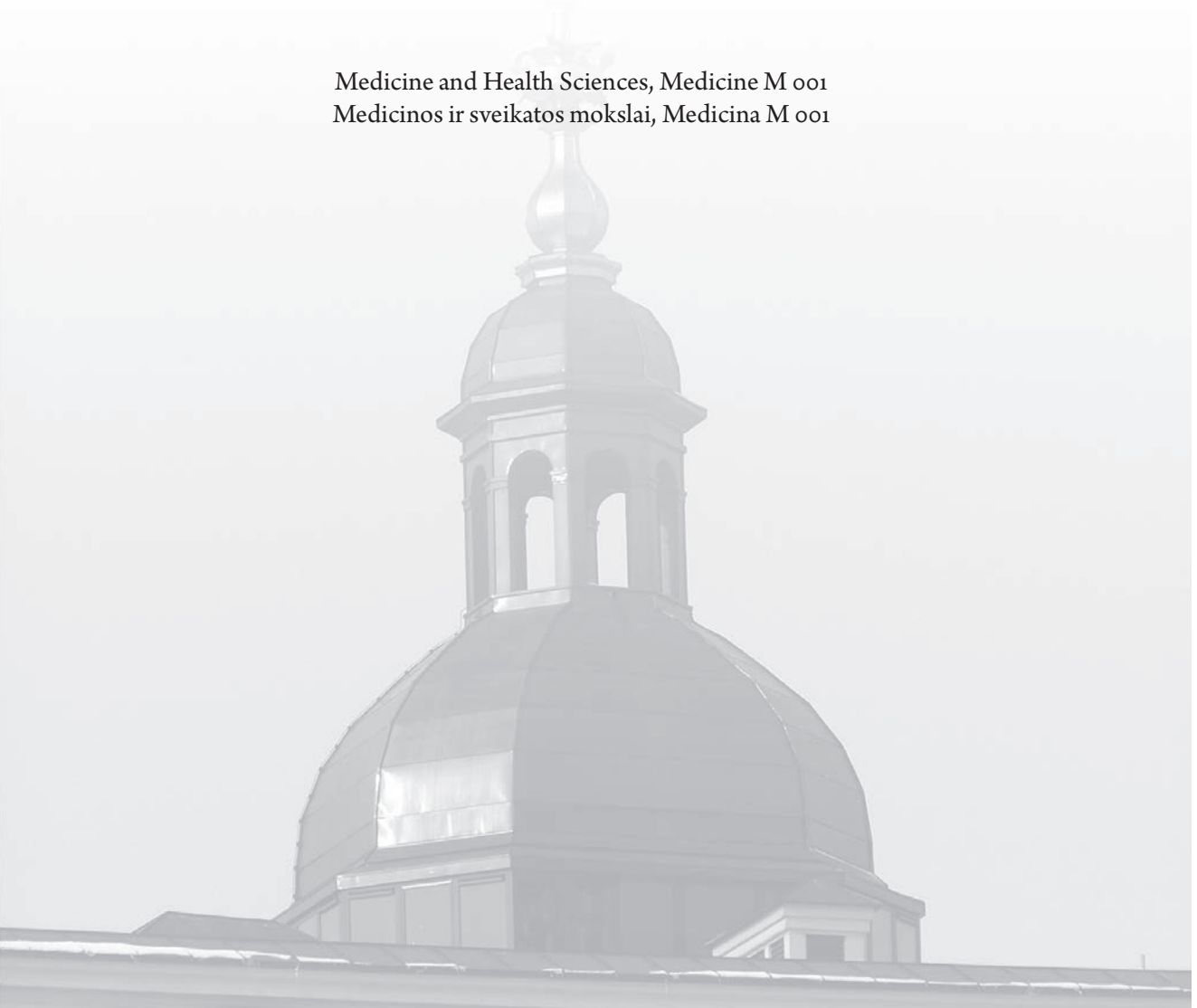
Eglė Sakalauskaitė-Juodeikienė

NERVOUS SYSTEM DISEASES IN THE FIRST HALF OF THE 19TH CENTURY IN VILNIUS

NERVŲ LIGOS VILNIUJE XIX-OJO AMŽIAUS PIRMOJE PUSĖJE

Medicine and Health Sciences, Medicine M 001
Medicinos ir sveikatos mokslai, Medicina M 001



Abbreviations

CCA	common carotid artery
GDL	Grand Duchy of Lithuania
NS	nervous system
PLC	Polish–Lithuanian Commonwealth
VMS	Vilnius Medical Society
VU	Imperial University of Vilnius

Santrumpos

ATR	Abiejų Tautų Respublika
BMA	bendroji miego arterija
LDK	Lietuvos Didžioji Kunigaikštystė
NS	nervų sistema
VMD	Vilniaus medicinos draugija
VU	Vilniaus imperatoriškasis universitetas

Introduction

Vilnius University (VU) was founded in 1579 by Jesuits in the multinational and multiconfessional city of Vilnius in the Grand Duchy of Lithuania (GDL). VU is one of the oldest universities in Central and Eastern Europe and its founders were strongly influenced by Renaissance, Reformation, and Counter–Reformation notions and ideas. Modelled after the Jesuit College in Rome, VU originally had two faculties: Philosophy and Theology.

After the abolition of the Society of Jesus in 1773, with the papal bull of Clement XIV (1705–1774), VU came under the jurisdiction of the Educational Commission of the Polish–Lithuanian Commonwealth (PLC), which promoted a new curriculum of civic education and secularism. The Faculty of Medicine was founded in 1781, and special attention was paid to the promotion of natural sciences in accordance with the ideals of the Age of Enlightenment. Following the abolition of the PLC and the annexation of the GDL by the Russian Empire, VU was able to maintain the same rapid pace of intellectual life, continuing to stimulate new ideas, especially in the natural sciences.

Little can be stated about courses involving the nervous system (NS) and its diseases or about how systems of medicine were taught in Vilnius before the 19th century. What is known is that Jakob Marquart (1583–1658), a doctor of theol-

ogy, had introduced the basics of human anatomy to his students during the 17th century using Andreas Vesalius' (1514–1564) *De humani corporis fabrica* ('On the Fabric of the Human Body'). After the Faculty of Medicine was founded in 1781, Stephanus Bisius (Stephanus Bisio, Stefano Lorenzo Bisio; 1724–1790?), a physician of Italian descent, began to lecture on anatomy and physiology, including not only osteology, splanchnology, angiology, and myology but also what would become neurology, with this happening between 1781 and 1787. S. Bisius also might have been the first physician in Vilnius to publish an original study on NS and mental diseases, naming his work *Responsum Stephani Bisii Philosophiae et Medicinae Doctoris ad Amicum Philosophum De Melancholia, Mania et Plica Polonica sciscitantem* ('A Reply of the Doctor of Philosophy and Medicine Stephanus Bisius to the Question from the Philosophical Society about Melancholia, Mania and Plica Polonica'). Additionally, as revealed in the VU curriculum of 1783 to 1784, Professor of Surgery Jacques Briôtet (1746–1819) lectured on external head wounds, concussions, subdural and intracerebral extravasations, and trepanation. Separately, Professor of Anatomy Joannes Andreas Loebenwein (1758–1820) lectured on the theory of *sensibilitas* (sensibility). An Irish Professor of Therapy, John O'Connor (1760–1801), additionally lectured through the academic year of 1800 to 1801 on inflammatory diseases, including 'inflammations of the cerebral membranes'. J. A. Loebenwein had a rich collection of skulls of suicide victims and dead criminals in his anatomical cabinet and used them for teaching Franz Joseph Gall's (1758–1828) cranioscopic doctrine to his students.

By the early 19th century, VU had become the largest such institution in the Russian Empire, based on student numbers and university departments. In 1803, certain reforms were enacted by Czar Alexander I (1777–1825), a supporter of limited liberalism, who wanted to transform Vilnius with its university into a gateway from the Russian Empire to an enlightened Europe. The number of departments was doubled and foreign professors were invited to lecture at the university. Prominent scholars and scientists from Austria, Germany, Italy, England, and France began to travel in increasing numbers to Vilnius. Joseph Frank (1771–1842), who had become a Professor of Special Therapy and Clinical Medicine at VU, wrote in his *Mémoires* in 1808: 'the Emperor gave the students at the Institute of Medicine everything I asked for' and 'the money (...) was to be paid (...) in silver'.

VU remained a centre of scientific thought and political freedom until it was closed by Russian imperial authorities in 1832, following the suppression of a Polish and Lithuanian national uprising in 1831. It was during the early 19th century, which was marked by an increased interest in new biological theories and clinical medicine, that Johann Peter Frank (1745–1821) and his son, Joseph Frank, both Professors of Clinical Medicine, as well as Andrew Sniadecki (Jędrzej Śniadecki, An-

drzej Śniadecki; 1768–1838), Professor of Natural Sciences, and Ludwig Heinrich Bojanus (1776–1827), Professor of Zoology and Comparative Anatomy, among many other famous scientists, lectured at VU; established the VU Therapy clinic (in 1805), Vilnius Medical Society (VMS, in 1805), Outpatients' Clinic (in 1807), and VU Surgery clinic (in 1808); edited science and culture journals; introduced new ideas to Vilnius students and physicians; and published treatises and textbooks on physiology of the human NS and its diseases. Vilnius at that time was a progressive city, a place where the natural sciences were supported and where new theories and explorations from the scientific centres of Western Europe reached not only scientists and medical professionals but also the general public. The lack of research on the origins of clinical neurology and neurosurgery in the first half of the 19th century in Vilnius indicated the need for this historical study.

The aim of this study

The aim of the present study is to reveal the perception, diagnosis, and treatment methods of NS diseases in the first half of the 19th century in Vilnius.

Objectives

1. To reveal the basic medical systems (doctrines, theories) and their influence on the perception of NS diseases and treatment options.
2. To reveal the principles of the human cognition research.
3. To evaluate the perception of aetiology, pathogenesis, and symptoms, to reveal the prophylaxis and treatment methods of NS diseases.
4. To discover the origins of neurosurgery.
5. To evaluate the association between the phenomenon of *plica polonica* and NS diseases.

Scientific novelty and the significance of this study

There appear to be no studies analysing the origins of clinical neurology and neurosurgery in the 19th–century Vilnius published in Lithuania or worldwide. The present study is special due to its interdisciplinary approach (it considered the history of neurology and neurosurgery, including some aspects of psychiatry, public health, the humanities, and philosophy) and multilingualism (it relied on primary sources written in languages such as Latin, Polish, Russian, French, and English).

This study is also important considering its didactics and practical significance. The results will be adapted into the teaching courses of students of medicine and other specialties and could help to develop students' interdisciplinary approaches using historical examples and cases.

Defensive statements

1. The origins of clinical neurology and neurosurgery in Vilnius go back to the beginning of the 19th century.
2. VU, Vilnius clinics, and the Vilnius Medical Society significantly influenced the development of clinical neurology and neurosurgery in Vilnius.
3. The level of diagnostics and treatment methods of NS diseases in the first half of the 19th century in Vilnius corresponded to the scientific level of Western Europe.

Materials and methods

We first analysed 25 doctoral dissertations written in Latin on the subject of NS diseases and defended at VU in the first half of the 19th century. Then, we turned to *Consessus Caesareae Societatis Medicae Vilnensis* ('The Protocols of Vilnius Medical Society'), written in Latin and Polish, and analysed the clinical reports recorded during the period of 1806 to 1842. J. Frank's *Acta Instituti Clinici Caesareae Universitatis Vilnensis* ('Proceedings of Vilnius University Clinic'), published in 1808 and 1812 in Leipzig; his textbook *Praxeos medicae universae praecepta partis secundae volumen primum sectio prima, continens doctrinam de morbis systematis nervosi in genere et de iis cerebri in specie* ('Practical Textbook of General Medicine... Containing Doctrine of Nervous System Diseases and the Special Diseases of Cerebrum'), written in Latin in Vilnius and published in

1818 in Leipzig; and his *Mémoires Biographiques de Jean-Pierre Frank et de Joseph Frank son fils* ('Biographical Memoirs of Johann Peter Frank and his Son Joseph Frank'), written in French were reviewed. Investigating the principles of the human cognition research, we studied *Filozofia umysłu ludzkiego czyli rozwazny wywód sit, i dziatań umysłowych* ('The Philosophy of the Human Mind'), written in Polish by VU Professor Jan Śniadecki (1756–1830). A leading tractate in the philosophy of animal and human nature in Europe, *Teorya jestestw organicznych* ('Theory of Organic Beings') by A. Sniadecki, published in Polish in 1804 (first volume), 1811 (second volume), and 1838 (third volume), was also included in this study. Lastly, we analysed articles written in Polish and published in the following Vilnius science and cultural journals: *Dziennik Wileński* ('Vilnius Daily'), *Pamiętnik Magnetyczny Wileński* ('Vilnius Magnetism Diary'), and *Wiadomości Brukowe* ('Pavement News'), among the other primary sources.

This study was conducted in the vein of a historical–medical analysis and synthesis of primary sources as well as by using comparative analysis, analogy, descrip-

tive methods, and the method of retrospective diagnosis. The chronological boundaries of this study were 1803 to 1842, which corresponds to the period of existence of the Imperial University of Vilnius (1803–1832) and the Imperial Medical–Surgical Academy (1832–1842). In order to explain the long–term processes, we went beyond the chronological limits. Studies characterizing the 1803 to 1842 period, published after 1842, were considered as relevant literature. Medical terms, pathophysiological theories, and diagnostic and treatment methods in this study were perceived and analysed in the context of the level of knowledge of the described historical period.

Results

1. Medical systems

Humoralism was the most popular system of medicine in the first half of the 19th century in Vilnius. During this time, antiphlogistic drugs and treatment methods were widely used at the VU clinics. Prophylaxes of NS diseases mainly included the principles of recreation and moderation. Bloodletting, the use of purgatives, leeches, cupping therapy, and diet adjustments were frequently employed as treatment options for patients with apoplexy, myelitis, St. Vitus' dance, epilepsy, encephalitis, tetanus, hydrophobia, somnambulism, and other NS diseases. Although the main cause of NS diseases at the beginning of the 19th century in Vilnius was believed to be in the solid parts of the human body, and no longer recognised as being an imbalance of the four humours, therapeutic options of bloodletting, purging, and the promotion of sweating were still proposed as interventions that formerly served to correct these humoral imbalances.

VU professor J. Frank stated that he had chosen vitalism for his approach of diseases and treatment strategies, which was based on the medical systems of Georg Ernst Stahl (1659–1734), Friedrich Hoffmann (1660–1742), William Cullen (1710–1790), John Brown (1735–1788), and Christoph Wilhelm Hufeland (1762–1836). However, noted J. Frank, a physician should never forget that the human body is also dependent on mechanical and chemical laws. Professor warned his Vilnius students that it is dangerous to rely on any medical system in practice and that all theories should vanish at the bedside of the sick. Although the doctrine of vitalism and Brunionian medicine was well known in Vilnius, NS diseases were more frequently treated using more aggressive, antiphlogistic treatment methods. When the condition of the patient was extremely severe and physicians decided that the disease or its complication was incurable, it was recommended to refrain from administering active treatment.

After becoming a popular treatment method at the end of the 18th and beginning of the 19th centuries in Western Europe, animal magnetism (mesmerism) had also attracted the attention of VU professors, Vilnius physicians, and especially local quacks and charlatans. Mesmerism was studied by A. Sniadecki and J. Frank, who postulated that animal magnetism is a very important pathological phenomenon and involves a phenomenon wherein the nerves are extremely sensitive to the internal and external stimuli that cause illusions and delusions. A special newspaper, *Pamiętnik Magnetyczny Wileński* ('Vilnius Magnetism Diary'), was devoted to the topic of animal magnetism and was published in Vilnius between 1816 and 1818. However, *Pamiętnik Magnetyczny Wileński* was criticized and satirized in the newspaper *Wiadomości Brukowe* ('Pavement News'). The history of animal magnetism was also presented in three issues of *Dziennik Wileński* ('Vilnius Daily') in 1816.

Franz Joseph Gall's theory of organology with its primary method of cranioscopy became well–known and was generally accepted at the beginning of the 19th century in Vilnius and at its University (Fig. 1). However, J. Frank considered the idea that organology leads to absurdity and uselessness. With his decidedly negative bias and, unlike A. Sniadecki, he neither taught F. J. Gall's system to his students nor presented it to the public in Vilnius. On the contrary, A. Sniadecki was intrigued and

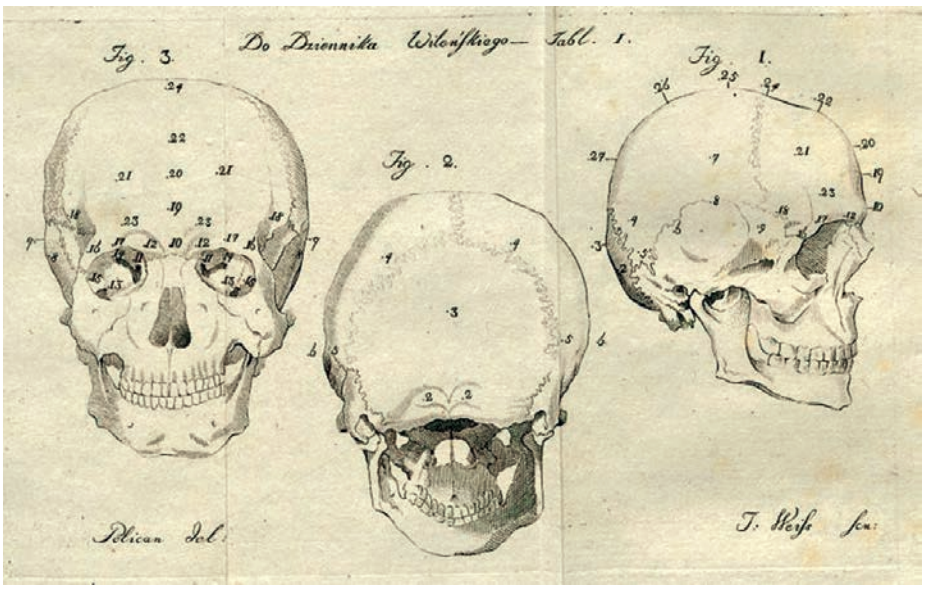


FIG. 1. The illustration of cortical organs by F. J. Gall presented in A. Sniadecki's article (*Dziennik Wileński*, 1805). Vilnius University Library, Rare book department. Used with permission.

for the most part had positive feelings about F. J. Gall's theory, although he was convinced that the cortex is anatomically and structurally a solid and integral substance. Professor questioned whether it really could consist of so many independent organs. His cautious attitude was consistent with what he would derive from his observations and would express in his own theorizing, as can be seen in his *opus magnum*, *Teorya Jestestw Organicznych*, where he wrote about the human brain operating like a single organ. A. Sniadecki stated that the grounds on which F. J. Gall's theory stands have the accuracy that every science should have.

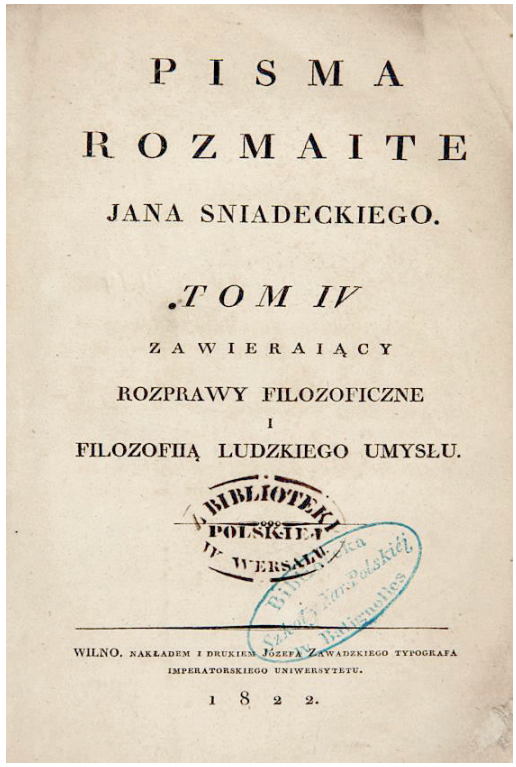
Ludwig Heinrich Bojanus, while practicing at Vienna's General Hospital in 1797 to 1798, also attended some of F. J. Gall's public lectures and, in 1801 and 1802, became one of the first physicians in Europe to provide detailed reports on organology in French and English. Although L. H. Bojanus considered the human mind to be indivisible and did not entirely agree with F. J. Gall's assumption that the brain consists of a number of independent organs, he defended the founder of organology against the charge that his sort of thinking would lead to materialism and predicted that the new system would be fruitful for developing and stimulating new research about the brain and mind.

2. The human cognition

One of the most important philosophers in VU was Jan Sniadecki, a graduate of the University of Cracow and a mathematician, astronomer, philosopher, and Rector of VU (1807–1815). J. Sniadecki was an author of various treatises on algebra, trigonometry, geography, and philosophy. In his *Filozofia ludzkiego umysłu* (Fig. 2), published in 1822 in Vilnius, J. Sniadecki was probably the first to broadly analysed the functions of the human mind and clearly associated it with the activity and physiology of the NS.

Professor J. Sniadecki reasoned that all operations in the human soul correspond to changes in the NS, while changes in the NS result in alterations in sensation and reasoning. He defined the human cognition as an organic process, dependent upon the structure of NS and its physiology, and perceived the phenomena of human ideas and concepts as a result of material interactions.

J. Sniadecki introduced the following fundamental principles of the human cognition: sensation (the beginning and primary condition of all human concepts and ideas); reasoning (the most prominent, noblest human ability that enables one to distinguish truth from falseness); and volition (which comes from human freedom and free choice). Attention was defined as one of the most important powers in the process of human cognition. Memory was perceived as the ability to maintain; keep; and, if necessary, present the acquired concepts and mind articles. J. Sniadecki



PISMA

ROZMAITE

JANA SNIADECKIEGO.

TOM IV

ZAWIERAIĄCY

ROZPRAWY FILOZOFICZNE

I

FILOZOFIIĄ LUDZKIEGO UMYSŁU.

WILNO. NAKŁADEM I DRUKIEM JÓZEFA ZAWADZKIEGO TYPOGRAFA IMPERATORSKIEGO UNIWERSYTETU.

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FIG. 2. The title page of the fourth volume of J. Sniadecki's scriptures, where his *Filozofia ludzkiego umysłu* was published in 1822. Vilnius University Library, Rare book department. Used with permission.

suggested the existence of two types of memory, depending on the duration of attention: reminiscence and recognition. Professor J. Sniadecki described changes of memory during aging: as sensation and perception weaken, the memory and other intellectual capacities, which are the noblest parts of the human cognition, gradually decline.

Even though Auguste Comte (1798–1857), considered the founder of positivism, presented his main works in the middle of the 19th century, J. Sniadecki had already introduced the first concepts of prepositivism, criticised idealism, and metaphysics by that point. VU philosopher postulated that the truth in natural sciences can be shown using the two basic methods of observation and experimentation.

J. Sniadecki was the first at VU to extensively study the human mind and cognition and associate such with the physiology of the NS. Based on the concepts that came from Greco–Roman times as well as the Renaissance, Baroque, and Enlight-

enment periods, J. Sniadecki proposed that human cognition is an organic process, dependent upon the structure and physiology of the NS. Distanced from metaphysics, idealism, and romanticism, VU professor stated that philosophy cannot explain what exceeds human understanding.

3. The perception of nervous system diseases

The terminology of NS diseases in the 19th–century Vilnius was the same that was used in other European cities. Some names of NS diseases remain unchanged today [e.g., encephalitis, hydrocephaly (Fig. 3), myelitis, somnambulism, and others]. Encephalitis was differentiated from inflammation of the cerebral membranes (arachnoiditis). Physicians from Vilnius clinics diagnosed not only diseases of the cerebrum but also those of the spinal cord. NS diseases in most cases were considered to be biological and pathological phenomena, while metaphysical interpretations and folk superstitions were definitely neglected.

NS diseases were initially assumed to be a result of the imbalance of the four humours. It was thought that the NS diseases were caused by the physical, psychological, and biological factors that increase blood flow to the brain. Dizziness, vomiting, and seizures were considered to be symptoms of other NS diseases, rather than separate entities. Special attention was paid to the cerebral cortex, its functions, and focal neurological signs. The brainstem was perceived as a structure, responsible for the state of consciousness and vital functions. However, the symptoms of NS diseases were still linked to an excess of blood in the brain and spinal cord.

For example, J. Frank defined apoplexy as a sudden weakening, interruption, or extinction of sensation and the patient's ability to move, although respiration and heart activity were usually unaffected. Face flushing, double vision, amaurosis, tinnitus, yawning, hiccups, formication, slurred speech, and abnormal movements of the extremities were believed to be prodromal signs of apoplexy. Slow, deep breathing; a slow, full, hard, irregular pulse; paraplegia, hemiplegia; urinary bladder paralysis; and impaired vision, hearing, and memory were assumed to be the principal symptoms of apoplexy.

J. Frank distinguished three causes of apoplexy: predisposing, promotional, and cardinal. Predisposing causes included male gender, obesity, a luxurious and sedentary lifestyle, worrying, low atmospheric pressure, cold and humid weather, time of the equinox, and summer heat. Promotional causes involved dancing; going to bed shortly after meals; hot baths; diving in cold water; satiety; intoxication; drug use (including opium and nicotine); strong odours; and emotions such as joy, horror, anger, and resentment. Lastly, the cardinal cause of apoplexy was the brain, *cerebellum*, or *medulla* compression by way of an effect of venous or arterial blood, serum, tumour, or concretion.

Headache; nausea and vomiting; disturbances of consciousness; and focal neurological signs such as diplopia, mydriasis, eyelid ptosis, limb paralysis, sensory disturbances, and seizures were indicated as the main signs of encephalitis and arachnoiditis. The importance of brain localization was emphasized in VU clinics, associating cortical lesions with seizures and paralysis. The elements of bulbar palsy (e.g., dysarthria, dysphagia) were associated with lesions in the brainstem.

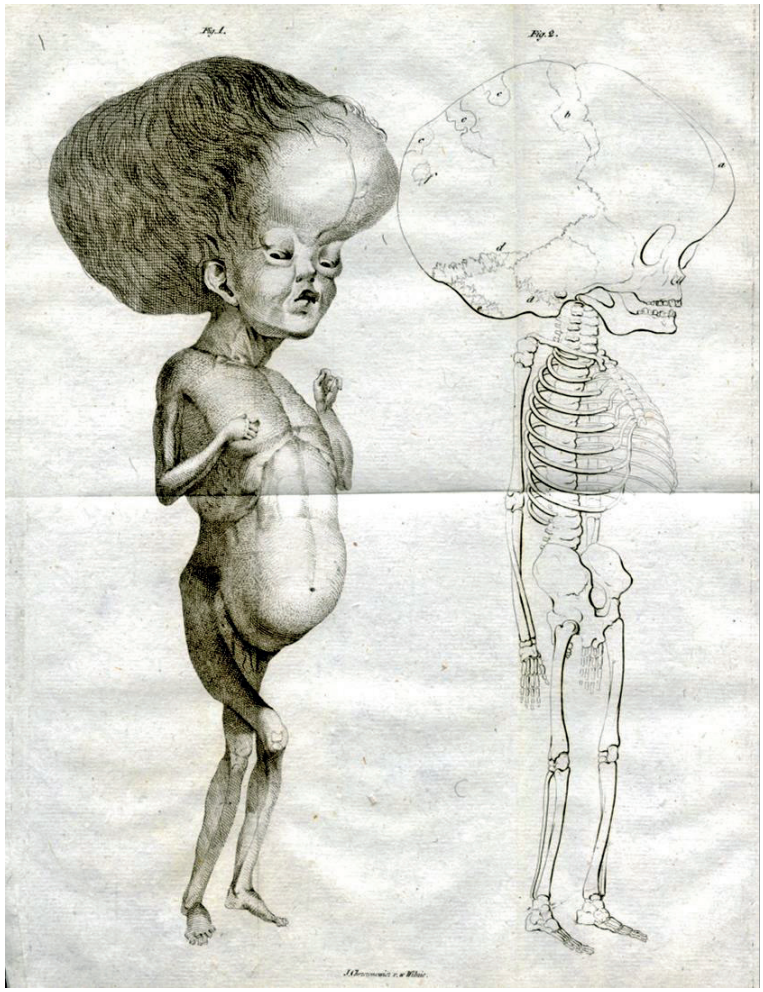


FIG. 3. The morphology of two deceased patients with chronic hydrocephalus. From *Derszkoff F. Dissertatio inauguralis medico— practica de hydrocephalo chronico binas observationes medico- practicas continens. Vilnae: typis A. Marcinowski; 1819.* Vilnius University Library, Rare book department. Used with permission.

Typical focal motor seizures, evolving to bilateral convulsive seizures, with foaming at the mouth, involuntary urination, and postictal somnolence were described in a doctoral dissertation on puerperal seizures, written by Paulus Mokrzycki. Although the seizure semiotics for the determination of the epileptogenic focus had not yet been considered, the onset of puerperal seizures was closely monitored and thoroughly described. It was thought that a plethoric patient (one presenting as robust, red–faced, and suffering from frequent headaches, with a full and hard pulse) had a higher risk of convulsions and, therefore, antiphlogistic treatment (especially bloodletting) was considered the best choice.

Physicians, professors, and students in VU clinics distinguished primary from secondary insomnia. They believed that young patients with insomnia should be observed for encephalitis, epilepsy, and hydrocephalus; adult patients for mania, hypochondria, and melancholy; and elderly individuals for apoplexy, respectively. It was stated that heavy snoring predisposed patients to various NS diseases, especially apoplexy and headaches.

Anthropometric, morphometric, somatic, and primitive neurological examinations were performed in VU clinics. Vilnius physicians and professors of clinical medicine as well as medical students evaluated pupillary light reflex, patients' responses to painful stimuli, mental conditions, verbal responses, paroxysmal movements, state of certain sensory modalities (e.g., touch, vision, audition, smell, taste), limb positioning, weakness of limb movements, dysfunction of the pelvic organs, and other signs and symptoms. It was recognized that sleep disturbances caused sleepiness during the daytime and that patients with sleep disorders had a higher risk for other chronic diseases.

In Vilnius, as well as in other European clinics, the causes of diseases were sought in the internal organs (the influence of the solidism theory), but autopsy findings usually revealed brain and spinal cord congestion with blood, confirming the inflammation of the nervous system (or humoralism theory).

For example, autopsy findings of patients with apoplexy revealed a pathology of cerebral blood vessels (both veins and arteries); lesions in the cerebrum, brainstem, and spinal cord; and inflammatory changes in the meninges. J. Frank described the dilation of blood vessels in the *dura mater* and *pia mater,* blood in the brain surface, softening of the brain and thalamus, or the presence of blood in the thalamic area. J. Frank also noted tubercles in the *corpus striatum*; pathological cavities in the medullar substance of hemispheres; compressed nearby ventricles; red, inflamed *plexus chorioideus*; a watery, bloody *cerebellum*; and *medulla oblongata*. Furthermore, J. Frank described carotid artery obstruction, aneurysms, rupture of the cerebral arteries, blood in the spinal canal, *aorta* defects, and *vena cava* compression in the chest wall with tumour as other possible causes of apoplexy.

A good prognosis was given to patients presenting with mild symptoms and relatively benign conditions (e.g., headaches, dizziness, somnambulism, symptomatic seizures) in VU clinics. A worse prognosis was predicted for those with severe NS diseases (e.g., apoplexy, encephalitis, arachnoiditis, tetanus, hydrophobia, myelitis). The state of the patient's consciousness, neurological signs and symptoms helped the physician to determine their prognosis more accurately.

For example, the worst prognosis, according to J. Frank, was associated with an observed severe condition of the patient. Foaming at the mouth, an elongated and cyanotic face, difficulty swallowing, wheezing respiration, paraplegia, palpitations, cold sweats, and involuntary urination and defecation were associated with the worst prognosis for a patient with apoplexy. On the other hand, a favourable prognosis was given if warm sweating, profuse urination, menstruation, diarrhoea, and improvement after bloodletting were observed. The prognosis was believed to be poor if the NS disease affected the *medulla oblongata* and if bloody fluid was localised in the skull base, especially around the glossopharyngeal, vagus, and hypoglossal nerves.

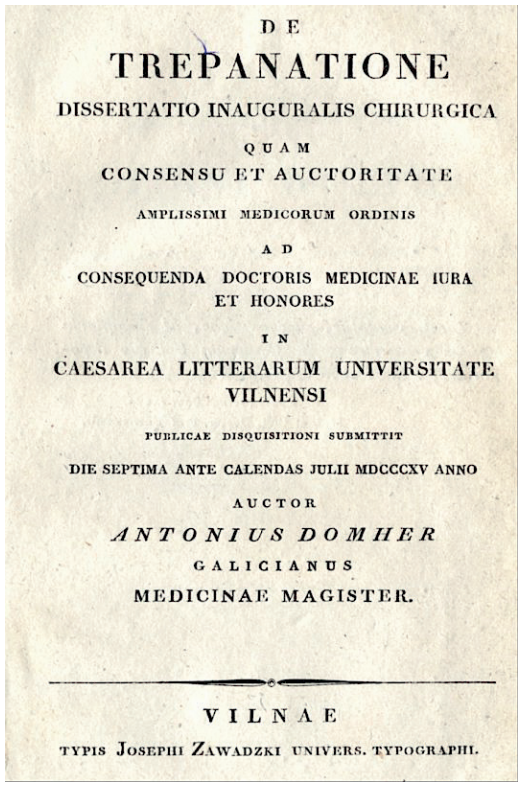
Bloodletting for patients presenting with acute NS diseases was considered a life–saving procedure. Other antiphlogistic treatment options were also frequently used, such as leech therapy, emetics, purgatives, diuretics, and blistering medicines. Opium tincture was used as an analgesic and hypnotic. For patients with acute NS disease, strict diet and liquids were recommended. Calomel (mercury chloride) was used as a purgative and anti–inflammatory drug. Peruvian bark (source of quinine) was prescribed as an anti–inflammatory drug for treating NS diseases and various fevers. In the cases of traumatic tetany and traumatic arachnoiditis, surgical wound treatment was recommended in order to stop the spreading of the inflammation. The use of *acidum borussicum* (hydrocyanic acid) in patients with hydrophobia represents an example of a desperate, 'heroic' medicine while treating a fatal illness. Sleep hygiene and lifestyle recommendations (e.g., maintaining a safe environment for sleep, performing gymnastics, taking fast walks, and avoiding long periods of sitting and sleeping during the daytime) were suggested for patients with sleep disorders.

Prophylaxes for apoplexy mainly included the principles of recreation and moderation. Following the ancient tradition, it was suggested that periodic bloodletting and purgatives should be used. Bloodletting (phlebotomies of the veins), leeches on the head, and cupping therapy on the dorsum were also popular treatment methods in Vilnius. Diet was an essential part of treatment. J. Frank recommended vegetables; fat–free food; sour drinks; dishes with boletus, egg yolks, rice, or ginger; and broths of veal, chicken, fish, frogs, turtle, and poultry. If a patient became weak, an infusion of Arabic coffee, raspberry water, and white wine with lemon juice and

sugar was prescribed. Chemicals such as various purgatives, potassium nitrate, and physical treatment (i.e., rubbing the limbs with wool cloth) were also employed in patients with apoplexy.

4. Origins of neurosurgery

Prior to the end of the 19th century, neurosurgical operations were rarely performed in European university clinics and city hospitals. The development of neurosurgery was not possible without major medical discoveries like anaesthetics and antiseptics, effective haemostasis and atraumatic surgical techniques. However, the origins of neurosurgery in Vilnius go back to the beginning of the 19th century. Various operations, including trepanation (Fig. 4), evacuation of epidural or subdural hematomas, ligation of the common carotid artery (CCA), and excision or ligation of the surface head tumours were performed in the VU Surgery clinic.



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VILNAE

TYPIS JOSEPHI ZAWADZKI UNIVERS. TYPOGRAPHI.

FIG. 4. The title page of a doctoral thesis on skull trepanation. From *Domher A. De trepanatione dissertatio inauguralis chirurgica. Vilnae: typis Josephi Zawadzki Univers. typographi; 1825*. Vilnius University Library, Rare book department. Used with permission.

Evaluating patients with external head injuries, University professors, surgeons, and students performed a primitive neurological examination and estimated signs of brain compression. Dizziness, somnolence and stupor, stertorous breathing, headache, and weakness of voluntary movements were evaluated, considering the side of hemiplegia as one of the most important signs. If the patient presented with head trauma causing brain compression, but no external head injuries or signs of skull fractures were observed, then the side of limb paralysis was evaluated and trepanation was performed contralaterally to the paralysis. *Post mortem* examinations of deceased patients in the VU Surgery clinic were also performed to confirm the clinical diagnosis.

A patient who underwent successful trepanation was described in a doctoral thesis by Basilius Woyciechowski, defended in 1825 at VU. A four–year–old noble boy fell out of the window, struck his head, was suddenly paralysed, and fell asleep. These symptoms were considered as signs of blood congestion in the brain due to head trauma. The trepanation was performed and described briefly, without any mention of how the surgeons prepared the operating field, what instruments were used, or whether anaesthesia was performed, i.e.,

'the trepanation was performed, bone fractures removed, and subdural extravasate released'. Subsequently, the wound did not heal well, with a red, solid nodule observed to be growing from the brain after a few months, overcoming the skull bones and reaching the size of the goose egg. 'Initially, we tried to remove the tumour with a knife, but this caused severe bleeding, then we decided to ligate the base of the excrescence'. Using the ligature, the tumour was separated from the head in 10 days, at which point, the patient presented with transient trismus and tetanus. 'After applying a metal plate to protect the brain from the external injuries, dry wound treatment, and balsams locally, the boy fully recovered (*puer pristinam valetudinem recuperavit*)'.

5. *Plica polonica* and neurology

In many cultures, hair plays an important role in the development of social constructs about the body. Hairstyles convey powerful messages about a person's beliefs; morality; gender roles; sexual orientation; and even their religion, political views, and socioeconomic status. The symbolic power of the human hair could be traced back to Biblical times: Samson, mentioned in the Book of Judges, had superhuman abilities to kill a lion, to slay an entire army, or to destroy a temple with his bare hands; however, after his hair was cut off, Samson's powers were gone and he was imprisoned by the Philistines. Being forced to cut one's hair symbolizes a defeat, humiliation, or punishment: for example, Nazi Germany's forced cutting of the hair of Jews represented the government's control of this population.

Plica polonica, a tuft of matted, felted, and filthy hair (Fig. 5), is a phenomenon that was often considered an affliction exclusively characteristic for PLC; however, a number of publications on *plica* appear to have come mostly from central European regions. Approximately 900 articles on *plica polonica* were published up to the 19th century. Almost all authors emphasized the devastating signs and symptoms of *plica* such as irreversible plaiting of the hair accompanied by lice, headache, mutilating arthritis, scoliosis, and onychogryphosis. *Plica polonica* had been thought to be the effect of a spell cast by a witch, suggesting that occurrences of *plica* were caused by a supernatural influence or a punishment from God and constituting a disease that could not be simply disposed of by cutting off one's hair, as this would incur serious complications and even a patient's death. Moreover, *plica* was believed to break the patient, causing ulcerations, apoplexy, seizures, headaches, insomnia,

FIG. 5. Depiction of *plica polonica* in H. Dobrzycki's tractate *O kołtunie pospolicie 'plica polonica' zwanym* ('On Plait, Commonly Called Plica Polonica'), published in 1877 in Warsaw. Vilnius University Library, Rare book department. Used with permission.

blindness, deafness, and a myriad of other aches and diseases. This was because the disease was assumed to circulate in the blood and thus exist systemically throughout the body.

Plica polonica, called a national plague, a result of chronic contagions and local conditions, was discussed as a unique disease in J. Frank's *Mémoires* and in his textbook. The disease, according to J. Frank, is disastrous for the current population; furthermore, it would harm the future generations. J. Frank assumed that *plica* involves not only the hair but also other parts of the body: carcinomatous ulcers erode the patient's skin; bones decay; noses bend; eyes and ears begin to 'fear' light and sounds; insomnia lasts for months, exacerbating patients' torments; and, finally, convulsions begin, a patient becomes delirious, several years pass, and death comes, with rare exceptions.

Plica, as described in the doctoral dissertation by Carolus Kaczkowski (Karol Maciej Kaczkowski; 1797–1867) in 1821, was associated with various integumentary and NS diseases. The author noted that patients often complained of the specific smell of their sweat; uncombed hair plaits in the scalp and other body parts (e.g., armpit, groin, chest); and rough, bent and nodulated nails. Patients with *plica* were often diagnosed with impetigo, erysipelas, and carcinomatous ulcers. Furthermore, they complained of headaches and experienced vertigo and epileptic seizures. Syncope, sleep disorders, diseases of the spinal cord, and paralysis were also diagnosed in affected individuals. These patients complained of hallucinations and experienced melancholy, mania, and hypochondria.

Ludovicus Knothe, a graduate of VU, published another doctoral dissertation on *plica polonica* in 1830. The author described predisposing factors and causes that had a major influence on the development of *plica polonica*. For example, individuals from certain age groups (maturity, senescence) were considered to be more prone to *plica* entanglement, while those from some social classes (villagers, beggars) and Jews were believed to have a higher chance of developing *plica polonica*. Unhealthy environmental conditions (swampy or flooded lands, contact with sulphur vapours and metals) were also believed to provoke *plica*. Sadness, anxiety, resentment, and horror were assumed to predispose patients to *plica polonica* onset. L. Knothe also discussed whether *plica* was a contagious or congenital disease and disputed *plica's* association with syphilis, leprosy, arthritis, and lymphatic system diseases.

To avoid or eliminate *plica*, L. Knothe firstly recommended building a special hospital for patients suffering from the disease, prohibiting marriages among the affected, improving living conditions of the servants, prohibiting persons with *plica* from entering public bathhouses, and forbidding the sale of old clothes. L. Knothe stated that, firstly, the cause of *plica* should be treated and, secondly, doctors should promote the entanglement of *plica*, especially in the scalp region, at which point, the

mature *plica* could be cut off. However, various eye diseases, limb contractures, urinary retention, and even madness can occur if *plica* is cut off too early, he warned. L. Knothe noted that mercury preparations are 'very efficacious' for treating ulcers, impetigo, chronic inflammations, and NS diseases related to *plica polonica*. Mature *plica* (which is almost naturally separated from the scalp when signs of new hair growth are seen) can be cut off without any harm to the patient.

Until the end of the 19th century in Vilnius and other European cities, *plica polonica* was believed to be a multiorgan disease, involving the skin and its appendages and associated with a number of chronic, including NS, diseases. Additional and more careful studies of *plica polonica* cases can provide valuable information about the morbidity of some integumentary, NS, and mental diseases up to the second half of the 19th century in Europe. However, today we cannot find *plica polonica* in the International classification of diseases (ICD–10), and the phenomenon of *plica* could be explained simply perhaps by a lack of hygiene, pediculosis, and pyoderma.

Limitations

This study has some limitations. We analyzed the perception of NS diseases only in the small European region and during a brief period of time, focusing on VU and VMD in the first half of the 19th century. Therefore our findings cannot generalize the situation in the other regions. Secondly, our results are based only on the official viewpoint of Vilnius physicians and VU scientists, documented in the scientific literature, and we do not know much how the NS diseases were perceived by the traditional healers, barbers, herbalists, clergymen, wise women, charlatans, and even the patients themselves, for it is well known that patients often took care of themselves before consulting the professional physician. Last but not least, we used the method of retrospective diagnosis occasionally, although this method has been criticized by some science historians of today.

Conclusions

1. The origin of clinical neurology and neurosurgery in Vilnius goes back to the beginning of the 19th century: patients with apoplexy, encephalitis, arachnoiditis, myelitis, hydrophobia, tetanus, sleep disorders, St. Vitus' dance, acute and chronic hydrocephalus, and other nervous system diseases were examined and treated in Vilnius university Therapy clinic; skull trepanations were performed, subdural and epidural hematomas were removed, ligation of the common ca-

rotid artery, and excision or ligation of the surface head tumours were completed in the Surgery clinic.

2. Vilnius university, Vilnius clinics, and Vilnius Medical Society significantly influenced the development of clinical neurology and neurosurgery in the Vilnius region: although the principles of the nervous system anatomy and physiology were taught in the Baroque epoch, after the reorganization of Vilnius university in 1803, and the opening of the Therapy clinic in 1805 and then the Surgery clinic in 1808, the diagnosis and treatment of nervous system diseases improved markedly; medical students acquired practical skills at the bedside of the sick, participated in neurosurgical operations and autopsies of deceased patients, and 25 doctoral dissertations on nervous diseases were defended; during the meetings of the Vilnius Medical Society, Vilnius physicians demonstrated patients presenting with neurological disorders, discussed treatment options, innovations, and used the autopsy findings as an essential part of the anatomo–clinical method.
3. Humoralism was the most popular system of medicine in the first half of the 19th century in Vilnius, antiphlogistic drugs and treatment methods were widely used at the Vilnius university clinics; solidism theory was essential for developing anatomo– clinical method; Brunonian system of medicine was more criticized than practiced; animal magnetism had attracted the attention of Vilnius university professors, Vilnius physicians, and even local charlatans; Franz Joseph Gall's theory of organology was well known and was generally accepted in the beginning of the 19th century in Vilnius and at its University.
4. The level of diagnostics and treatment of nervous system diseases in Vilnius corresponded to the level of Western Europe in the first half of the 19th century: the terminology of nervous system diseases, pathogenesis, and treatment methods in Vilnius were the same as that which were used in the other European cities; Vilnius physicians and professors of clinical medicine as well as medical students evaluated patients' pupillary light reflex, responses to painful stimuli, mental conditions, verbal responses, paroxysmal movements, some sensory modalities (e.g., touch, vision, audition, smell, taste), limb position, weakness of limb movements, dysfunction of the pelvic organs, and other signs and symptoms; human cognition at Vilnius university was assumed to be an organic process, dependent on the structure and physiology of the nervous system; the causes of neurological disorders were sought in the cerebrum and spinal cord, using autopsy findings as an essential part of the anatomo–clinical method.
5. The side of limb paralysis was evaluated and trepanation was performed contralaterally to paralysis, evacuating epidural or subdural hematomas for the patients presenting with head trauma in Vilnius clinics, even though cerebral localization

theory would be generally accepted not until the second half of the 19th century in Europe.

6. Until the end of the 19th century in Vilnius, as well as in the other European cities, *plica polonica* was believed to be an independent, multiorgan disease associated with a number of chronic conditions including nervous system diseases.

Further directions

We plan to analyse the development of neurology and neurosurgery in the first half of the 20th–century Lithuania.

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SANTRAUKA

Įvadas

Vilniaus universiteto (VU) kūrimąsi daugiausia lėmė Reformacijos ir Kontrreformacijos konkurencija: Vilniaus kolegijos steigimo idėja jėzuitams kilo dar 1565 m., mat protestantų siekiai Lietuvos Didžiojoje Kunigaikštystėje (LDK) įkurti savo kolegiją vertė jėzuitus paskubėti. Popiežiui Klemensui XIV (1705–1774) 1773 m. panaikinus Jėzuitų ordiną, tais pačiais metais Edukacinė komisija pradėjo VU reorganizavimą, kurios metu 1781 m. įsteigtas Medicinos fakultetas, ypatingą dėmesį skirtant gamtos mokslų bei gamtotyros idėjų skleidimui. VU organizaciniu dinamizmu pralenkė ilgą laiką vyravusį Krokuvos universitetą, VU XIX a. pradžioje studentų ir katedrų skaičiumi buvo didžiausias Rusijos imperijoje bei išsaugojo savo autonomijos statusą. Universiteto statutas 1803 m. taip pat reglamentavo tam tikrą čia dirbusių mokslininkų minties laisvę. Beveik ištisą trisdešimtmetį iki uždarymo 1832 m. VU, iškilęs ant LDK tradicijų pamato, nepaisant carinės valdžios daromo spaudimo, buvo tuometinės mokslinės minties bei politinių laisvių skelbėjas, elitinės kultūros centras ir Lietuvos krašte, ir visose buvusios Abiejų Tautų Respublikos (ATR) žemėse.

Nedaug žinoma apie nervų sistemos (NS) anatomijos, fiziologijos ir nervų ligų dėstymą VU. Profesorius Steponas Laurynas Bizijus (Stephanus Bisio, Stefano Lorenzo Bisio; 1724–1790?) 1781–1787 m. VU dėstė anatomijos ir fiziologijos pagrindus: osteologiją, splanchnologiją, angiologiją, miologiją, adenologiją ir tai, kas vėliau bus vadinama neurologija. Kitas anatomijos profesorius, Jonas Lobenveinas (Johannes Andreas Loebenwein; 1758–1820), 1787–1820 m. dėstęs anatomiją, osteologijos kurse aiškino apie kūnų judėjimo teoriją, splanchnologijoje – atskirų organų veiklą, neurologijos kurse – apie smegenis ir iš jų po visą kūną einantį nervų išsišakojimą, jutimo (*sensibilitas*) teoriją. Chirurgijos programos aprašyme Žakas Briote (Jack, Jacques Briotet, 1746–1819) 1783–1784 m. nurodė, kad daug dėmesio skirs galvos žaizdoms, smegenų sutrenkimui, kaukolės trepanacijai, nurodė, kad aiškins apie kiaušo ištinimą ir atsidarymą, jo žaizdas, sutraiškymus, įvairias ekstravazacijas, esančias po kietuoju smegenų dangalu ir pačiose smegenyse; apie smegenų sutrenkimą ir įvairius įlūžimus, taip pat apie trepano panaudojimo būdą. Terapijos kurso programoje praktinės medicinos profesorius Džonas O'Konoras (John O'Connor; 1760–1801) numatė studentams dėstyti ir apie uždegimines ligas: ne tik akių, įvairių rūšių gerklės, krūtinės pleuros, plaučių, bet ir „smegenų plėvių" uždegimus.

Ligoninių būklė Vilniuje iki XIX a. pr. buvo sunki. VU klinikinės medicinos profesorius Jozefas Frankas (Joseph Frank; 1771–1842) minėjo Gailestingųjų seserų ligoninę: šioje įstaigoje vienai lovai tekdavo 2 ligoniai, kuriuos dėl vietų stokos guldydavo ant smėliu barstomų grindų, oras buvo troškus, vaistus vienuolės gamindavosi pačios, daktaras ligoninėje kartais nesilankydavo kelias savaites. Dirbti tokiomis sąlygomis, vesti medicinos studentams praktikos darbus atrodė neįmanoma, galbūt dėl šių priežasčių vienas pirmųjų profesoriaus Johano Pėterio Franko (Johann Peter Frank; 1745–1821), 1804 m. su sūnumi J. Franku atvykusio į Vilnių, darbų buvo Terapijos klinikos, priklausančios universitetui, įkūrimas 1805 m. Po metų prie VU buvo įkurtas Medicinos institutas, kuris nemokamai rengė gydytojus Rusijos imperijos kariuomenei ir per savo gyvavimo laikotarpį parengė apie 1500 gydytojų. Kiek vėliau, 1808 m. buvo įkurta ir VU Chirurgijos klinika.

Vilniaus universitetui XIX a. pirmoji pusė buvo aukso amžius. VU buvo viena didžiausių ir svarbiausių aukštojo mokslo įstaigų Rytų Europoje: su keturiais fakultetais, įsteigtomis universiteto klinikomis, augančiu mediciną studijuojančių studentų skaičiumi, teorines ir praktines disciplinas dėsčiusiais aukščiausio lygio specialistais, pakviestais iš Vakarų Europos, stabilia finansine padėtimi, su įkurtu Medicinos institutu, kuriame medicinos nemokamai mokėsi gabūs studentai. Nors XIX a. pradžioje neurologija kaip atskira medicinos šaka dar nebuvo susiformavusi, tačiau susidomėjimas NS ligomis buvo didelis. To laikotarpio VU darbuose jau skelbta, kad svarbiausia sielos gebėjimų vieta, išorinių jutimų centras, pirminė judesių pradžia yra galvos smegenys, vadintos kilniausiu žmogaus kūno organu (*humanae nobilissimum organon*). Todėl ypatingas dėmesys, skirtas NS ligų diagnostikai ir gydymui, manome, buvo ne tik neurologijos mokslo ir praktikos XIX a. pr. Vilniuje priešaušris, bet ir svarbi jos raidos dalis, kurią šioje disertacijoje panagrinėsime plačiau.

Darbo tikslas

Šio darbo tikslas – aptarti nervų ligų suvokimą, diagnostiką ir gydymą Vilniuje XIX a. pirmoje pusėje.

Darbo uždaviniai

1. Išnagrinėti, kaip pagrindinės medicinos sistemos (doktrinos, teorijos) paveikė nervų ligų etiopatogenezės sampratą, nervų ligų diagnostiką ir gydymą Vilniuje.
2. Apžvelgti pažinimo principų tyrimus Vilniuje.
3. Ištirti, kaip minėtu laikotarpiu Vilniuje buvo suvokiama nervų ligų etiologija, patogenezė, simptomatika, kokie buvo taikyti diagnostikos, profilaktikos ir gydymo metodai.
4. Įvertinti neurochirurgijos mokslo ir praktikos ypatumus VU Chirurgijos klinikoje.
5. Įvertinti kaltūno (*plica polonica*) fenomeno sąsajas su nervų sistemos ligomis.

Darbo naujumas ir praktinė reikšmė

Nervų ligų samprata, diagnostika ir gydymas, neurologijos, neurochirurgijos mokslo ištakos ir raida XIX a. pirmojoje pusėje Vilniuje – iki šiol netyrinėta sritis. Darbas išskirtinis tarpdisciplinišku mu (įtraukti ne tik neurologijos ir neurochirurgijos, bet ir psichiatrijos, visuomenės sveikatos, humanitarinių mokslų, filosofijos tyrinėjimai), daugiakalbiškumu (nagrinėti iki šiol mažai tyrinėti arba visai netyrinėti šaltiniai, parengti lotynų, lenkų, rusų, prancūzų, anglų kalbomis). Darbas taip pat svarbus ir didaktine, praktine prasme: darbo rezultatai yra ir ateityje toliau bus pritaikomi medicinos ir kitų specialybių studentų mokymams, sprendžiant klinikinės medicinos problemas, siekiant istoriniais pavyzdžiais ugdyti studento tarpdisciplinį požiūrį.

Ginamieji disertacijos teiginiai

1. Klinikinės neurologijos ir neurochirurgijos ištakos Vilniuje siekia XIX a. pradžią.
2. Vilniaus imperatoriškasis universitetas, Vilniaus universiteto klinikos, Vilniaus medicinos draugija turėjo lemiamą reikšmę neurologijos ir neurochirurgijos vystymuisi Vilniaus krašte.
3. Nervų ligų diagnostikos ir gydymo Vilniuje lygis atitiko to meto Vakarų Europos lygį.

Darbo medžiaga ir metodai

Didžiausią nagrinėtų šaltinių dalį sudarė lotynų k. parengtos ir VU 1803–1832 m. laikotarpiu apgintos medicinos daktarų disertacijos (į darbą įtrauktos 25 disertacijos), skirtos NS ligų analizei, bei lotynų ir lenkų k. parašyti Vilniaus medicinos draugijos (VMD) protokolai (nagrinėtas 1806–1842 m. laikotarpis). Trečiasis šaltinis – VU klinikos darbai (*Acta Instituti Clinici Caesareae Universitatis Vilnensis*), profesoriaus J. Franko parengti lotynų k., išleisti 1808 m. ir 1812 m. Leipcige. Taip pat nagrinėtas J. Franko vadovėlio *Praxeos medicae universae praecepta* („Praktinės medicinos nurodymai“) antrosios dalies pirmasis tomas, skiras NS ligoms, parengtas dirbant Vilniuje ir išleistas 1818 Leipcige. Išanalizuoti profesoriaus J. Franko *Mémoires Biographiques de Jean-Pierre Frank et de Joseph Frank son fils* („Johano Pėterio Franko ir jo sūnaus Jozefo Franko biografiniai atsiminimai”). Kitas svarbus šaltinis – Jano Sniadeckio (Jan Śniadecki; 1756–1830) „Žmogaus intelekto filosofija”, 1822 m. publikuota J. Sniadeckio raštų rinkinyje *Pisma rozmaite Jana Sniadeckiego*. Remtasi ir Andriejaus Sniadeckio (Jędrzej Śniadecki, Andrzej Śniadecki; 1768–1838) monografija *Teorya jestestw organicznych* („Organinių būtybių teorija“), kurios I tomas buvo išleistas 1804 m., II tomas – 1811 m., III tomas – ir 1838 m. Analizuoti Vilniaus periodiniai leidiniai *Dziennik Wileński* („Vilniaus laikraštis“), *Pamiętnik Magnetyczny Wileński* („Vilniaus magnetizmo dienoraštis”), *Wiadomości Brukowe* („Gatvės žinios“) ir kiti pirminiai šaltiniai.

Atsižvelgiant į darbo tarpdisciplinišku mą, tyrimas buvo atliekamas remiantis istorine ir medicinine pirminių šaltinių analize bei sinteze, lyginamosios analizės, analogijos ir aprašomuoju metodais. Šiame darbe kartais naudojome ir retrospektyvios diagnostikos metodą.

Darbo chronologinės ribos apima 1803–1842 m.:

1. Vilniaus imperatoriškojo universiteto (1803–1832) laikotarpį,

2. Imperatoriškosios medicinos – chirurgijos akademijos (1832–1842) gyvavimo laikotarpį.

Siekdami paaiškinti tam tikrus ilgalaikius procesus, išėjome už minėtų chronologinių ribų. Šaltinių ir literatūros skirtimi laikytos 1803–1842 m. laikotarpio ribos. Visus tyrimus, charakterizuojančius 1803–1842 m. arba ankstesnį laikotarpį, paskelbtus po 1842 m., laikėme literatūra, o į minėtas chronologines ribas patenkančius darbus – pirminais šaltiniais. Darbe stengtasi vartoti XIX a. pr. paplitusius medicinos terminus ir gydymo metodus. Taip pat buvo atsižvelgiama į medicinos istorijos kontekstą ir tuometines ligų gydymo indikacijas.

Darbo rezultatai

1. Medicinos sistemos

Antiflogistiniai vaistai ir gydymo metodai, remiantis Hipokrato keturių organizmo skysčių teorija, buvo dažniausiai taikomi VU Terapijos klinikoje. Daugelio nervų ligų (apopleksijos, mielito, Šv. Vito chorėjos, epilepsijos, encefalito, tetanijos, hidrofobijos, somnambulizmo ir kitų) gydymui XIX a. pirmoje pusėje skirti kraujo nuleidimai, gydyta taurėmis, dėlėmis, klizmomis, viduriavimą, vėmimą, prakaitavimą skaitinančiais vaistais, pūsles odoje sukeliančiais tepalais ir pleistrais, siekiant koreguoti organizmo skysčių pusiausvyros sutrikimą, pašalinti „žalingojo“ kraujo perteklių galvos ir nugaros smegenyse.

Pavyzdžiui, VU auklėtinis Mykolas Homolickis 1815 m. apgintoje disertacijoje postulavo, kad kraujagyslių būklė turi didelę reikšmę ligonio sveikatai. Pritardamas humoralinei teorijai, daktaras teigė: „Kraujagyslėmis nešamas didžiulis skysčio kiekis, reikalingas įvairių kūno dalių mitybai, tačiau skysčio perteklius taip pat keičia nervų sistemos funkcijas“. Kadangi „svarbiausia sielos gebėjimų vieta, išorinių jutimų centras, pirminė judesių pradžia yra galvos smegenys, kurias krauju maitina slankstelinės ir miego arterijos“, „nenuostabu, kad susirgimai, kurių metu kraujo kiekis padidėja, arba [kuomet yra] sutrikdomas kraujo nutekėjimas venomis (...), pasireiškia kilnios galvos smegenų veiklos sutrikimu“. M. Homolickis, prisimindamas Antikos mąstytojus, tvirtino, kad traukuliai dažniausiai pranašauja kraujo išsiliejimą galvos smegenyse, be to, ir „dieviškasis Hipokratas“ tvirtinęs, kad, nenuleidus kraujo ar jo nuleidus nepakankamai, dažnai išsivysto epilepsija. Daktaras disertacijos pabaigoje priėjo prie išvados, kad gydymas dėlėmis, kraujo nuleidimais bei kitais laiku panaudotais antiflogistiniais metodais gali išgelbėti ligoniui gyvybę.

Solidizmo doktrina Vilniaus klinikose buvo pripažįstama ir šios sistemos principais („ligos priežasčių derėtų ieškoti vidaus organuose“) buvo remiamasi, atliekant autopsijas ir klinikinę diagnozę patvirtinant po ligonio mirties. Pvz., VU auklėtinis lietuvis Vincentas Vladislovas Herberskis 1812 m. apgintoje disertacijoje apie stuburo kanalo vandenę taip aprašė VU anatomijos profesoriaus J. Lobenveino pristatytą naujagimio *spina bifida* atvejį:

„Žydė moteris, 25 m. amžiaus (...), vidutinio ūgio, liekno kūno sudėjimo, sveika ir ištekėjusi už sveiko vyro, trijų vaikų motina, kuriai kartais patindavo kaklo ir pažandinės liaukos, 4–ą nėštumo mėnesį stipriai išgąsdinta, pajuto didelį nuovargį (...), o paskutinėmis nėštumo dienomis – susilpnėjusius vaisiaus judesius.

1810 m. lapkričio 11 d. pagimdė kūdikį be matomų lytinių organų, be išangės, kirkšnyse stebėti atsivėrę šlapimtakiai (...), kryžkaulio srityje stebėtas žąsies kiaušinio dydžio tumoras, minkštas, elastingas, fliuktuojantis, neskausmingas, beveik nemažėjantis suspaudus, be odos spalvos pokyčių. Kūdikio apatinės galūnės buvo paralyžiuotos, jis vangiai žindo krūtį, nyko, [ir galiausiai] mirė“

Atlikta kūdikio autopsija: galvos smegenys ir smegenėlės be patologijos, skilveliai laisvi, neišsipūtę, smegenų skystis perėjo į kryžmens srities tumorą. Suskaičiuoti 23 slanksteliai, iš jų keturi juosmeniniai. Krūtininės ir juosmeninės dalies slanksteliai buvo pakrypę į kairę pusę, jų keterinės ataugos – be matomos patologijos. Rastas plyšys trijuose kryžmeniniuose slanksteliuose, per kuriuos ir išsiveržė tumoras, sudarytas iš nugros smegenų, *cauda equina* bei nugaros smegenų skysčio.

Tiek J. Frankas, tiek A. Sniadeckis pritarė vitalizmui. A. Sniadeckis šią medicinos sistemą išplėtojo „Organinių būtybių teorijoje“. Pasak profesoriaus, gyvuose organizmuose esanti organinė jėga pradeda gyvybę, ją palaiko ir tobulina, organinė jėga funkcionuoja medžiagų apykaitoje. Nors vitalizmo doktrina buvo žinoma ir pripažįstama, VU klinikose NS ligos dažniau gydytos, remiantis aktyvesniais, agresyvesniais gydymo metodais. Kuomet ligonio būklė būdavo kraštutinai sunki ir gydytojai nuspręsdavo, kad liga ar ligos komplikacija yra nepagydoma, rekomenduota susilaikyti nuo aktyvaus gydymo.

Braunizmo teorija Vilniuje (ypač akademiniuose sluoksniuose) nebuvo populiari, dažniau kritikuota nei praktikuota, tam didelę reikšmę turėjo J. Franko pažiūrų evoliucija ir braunizmo doktrinos atmetimas. VU moksliniuose darbuose, VU Terapijos klinikos protokoluose pirmiausia buvo pabrėžiama antiflogistinio gydymo svarba. Kita vertus, gydymas opijumi, stimuliuojančiais vaistais ar vynu kai kuriose disertacijose yra paminėtas, tačiau tik kaip pagalbinis metodas.

Vitalinis magnetizmas Vilniuje vertintas kaip nepaprastos svarbos patologinis NS reiškinys, tačiau, patekęs į mėgėjų ir šarlatanų rankas, dažnai tapdavo neprofesionalaus gydymo metodu. Vitalinis magnetizmas XIX a. pr. Vilniuje buvo praktikuotas tiek VU profesorių, tiek nelegaliai medicinos praktika besiverčiančių „aiškiaregių“, tradicinės medicinos atstovų. „Vilniaus magnetizmo dienoraštyje” magnetizmo praktika skleista ir propaguota, „Vilniaus laikraštyje” siekta santūriai ir kritiškai papasakoti vitalinio magnetizmo istoriją, „Gatvės žiniose“ magnetizmo praktika užsiimantys asmenys buvo pašiepiami, kritikuoti įvairūs metafiziniai potyriai ir aiškiaregystės.

Viena pirmųjų galvos smegenų žievės lokalizacijos teorijų, prieštaringai vertinta Franco Jozefo Galo (Franz Joseph Gall; 1758–1828) organologija, XIX a. pradžioje buvo puikiai žinoma ne tik VU profesoriams, studentams bei miesto gydytojams, bet, daugiausia A. Sniadeckio dėka, ir plačiajai Vilniaus visuomenei. A. Sniadeckis pripažino, kad F. J. Galo orga-

nologijos mokslas, nepaisant kai kurių prieštaringumų, yra vertas Vilniaus skaitytojų dėmesio, nes „pagrindai, ant kurių stovi šis mokslas, turi tokį tikslumą, kokį turi turėti gerai paremti mokslai". Dar daugiau: „Galo darbas yra vertas pagarbos, nes mus užveda ant panašių tyrimų kelio ir jo spėjimai kai kuriais atvejais gali mus ženkliai priartinti prie tiesos". A. Sniadeckis manė, kad organologijos ateitis – lyginamosios anatomijos ir tarpdisciplinių mokslų srityse: „Ilgainiui daugelio mokslininkų darbai šioje srityje bus sujungti; nuolatiniai ir dėmesingi anatominiai smegenų tyrimai, daugiausia gyvūnų, mus nušvies, [ir į klausimus], kaip smegenų masė įvairiose gyvūnų klasėse ir rūšyse tobulėja, kokius talentus ir polinkius nulemia, [bus atsakyta]".

2. Žmogiškasis pažinimas

Janas Sniadeckis veikiausiai buvo pirmasis XIX a. pr. VU mokslininkas, išsamiai nagrinėjęs žmogaus protines, pažintines funkcijas ir jas siejęs su nervų sistemos veikla. Filosofas, remdamasis Antikos, Renesanso, Baroko ir Apšvietos mąstytojų darbais, taip pat brolio A. Sniadeckio „Organinių būtybių teorija", priėjo prie išvados, kad žmogaus mąstymas yra organinis procesas – toks, kurio egzistavimas, veiksena ir įvairovė priklauso nuo nervų sandaros ir jaudinimo.

J. Sniadeckis apibrėžė žmogiško pažinimo pamatinius principus: žmogus junta, mąsto ir nori. Būtent jutimu, mąstymu ir norais pasireiškia žmogaus siela. Jutimai (*sensatio*), pasak profesoriaus, būdingi ne tik žmogui, bet ir visam gyvūnijos pasauliui, tačiau aukštieji sielos gebėjimai žmogų daro protaujančiu gyvūnu, o troškimų ir norų sutvarkymas – moraliu kūriniu. Jutimai, kilę iš materialaus pasaulio, pasak mąstytojo, yra visų mūsų sąvokų ir idėjų pradžia. Materialaus pasaulio pažinimas veda žmogų į sielos gebėjimų pažinimą. Kūnas ir siela yra susiję: kūno ligos sukelia minties nusilpimą, nereikalingas ir besaikis nugrimzdimas į mąstymą naikina kūną ir daro jį ligotą.

Atmintį J. Sniadeckis apibūdino kaip svarbiausią proto galią. Atmintis – tai gebėjimas išlaikyti, saugoti ir prireikus pateikti jau įgytas sąvokas ir proto dirbinius. Profesorius skyrė dvi atminties rūšis, kurias siejo su dėmesio trukme: prisiminimą, *reminiscentia* (tai atmintis, susieta su trumpalaikiu dėmesiu) ir atpažinimą, *recordatio* (atmintis, susieta su ilgalaikiu ir ištvermingesniu dėmesiu). J.

Sniadeckis įvardijo tris atminties elementus: tai lengvas įsiminimas (1), tvirtas išlaikymas (2) ir pasirengimas atkurti (3) reiškinius ir sąvokas. Atmintis tobulėja dažnai prisimenant, atsimenant, pasakojant ir užrašant įgytas žinias, atminčiai būdinga ir tai, kad kuo jautresni ir gyvesni pojūčiai, kuo stipresni ir gausesni įspūdžiai, tuo geriau ir ilgiau bus įsimintas reiškinys. Štai kodėl, pasak filosofo, seni žmonės atsimena ir pasakoja jaunystėje girdėtus pasakojimus, o pamiršta nesenus įvykius. Pasak J. Sniadeckio, senų žmonių atminties sutrikimą gali lemti ir jutimo sutrikimai, nes vyresniame amžiuje dėl „atbukusio juslumo" jiems mažai kas rūpi.

Pasak J. Sniadeckio (kuris buvo ir prepozityvzmo pradininkas VU), gamtos moksluose tiesą galima parodyti ir suprasti dviem pagrindiniais metodais – stebėjimu ir eksperimentu.

Stebėjimas, pasak mąstytojo, yra tai, „kas vyksta gamtoje, paimtoje savaime, suvokimas“, o eksperimentas – „gamtos tyrimas tokiame būvyje ir padėtyje, kokią mes patys suteikiame“.

Kūno – sielos, materijos – idėjos dualumui ir sąveikai pagrįsti filosofas pasitelkė nervų sistemą – tą vietą žmogaus organizme, kurioje išorinis materialus pasaulis susilieja su dvasiniu, idėjų pasauliu. Nervus profesorius apibūdino kaip jutimo ir mąstymo organus teigdamas, kad kiekvieną sielos operaciją atitinka tam tikras judėjimas ir pakitimas nervuose, o pakitimus nervuose lydi jutimo ir mąstymo pakitimai. Toks požiūris į sielos veikseną, pripažino J. Sniadeckis, yra fiziologinis, ir yra ypač svarbus medicinoje.

3. Nervų sistemos ligų suvokimas

Išanalizavę VU apgintas daktarų disertacijas, VMD protokolus, VU klinikos raštus, Vilniuje parengtą J. Franko vadovėlį, pastebėjome, kad VU klinikose XIX a. pirmojoje pusėje vartoti nervų ligų terminai atitiko to meto Europoje vartotus, kai kurie išliko nepakitę iki šių dienų (pvz., encefalitas, hidrocefalija, mielitas, somnambulizmas). Pradėta skirti encefalitą nuo galvos smegenų dangalų uždegimo, diagnozuotos ne tik galvos, bet ir nugaros smegenų ligos. NS ligos daugeliu atvejų vertintos visų pirma kaip biologinis, patologinis fenomenai, atsiribojant nuo metafizinių aiškinimų ir liaudies prietarų. Kita vertus, miego sutrikimų vertinimas XIX a. pr. vis dar buvo veikiamas mitologinių ir demonologinių aiškinimų.

Analizuojant daugelio nervų ligų priežastis, XIX a. pirmojoje pusėje Vilniuje dažniausiai remtasi keturių organizmo skysčių teorija. Manyta, kad NS ligas sukelia visi fiziniai, psichiniai ir biologiniai veiksniai, didinantys kraujo pritekėjimą į smegenis. Galvos svaigimas, vėmimas ir traukuliai laikyti ne savarankiškomis ligomis, o kitų ligų simptomais. Kaltūno liga ar nesubrendusio kaltūno nukirpimas XIX a. pr. Vilniuje vis dar laikytas dažna NS ligų priežastimi. Nors VU klinikose atkreiptas dėmesys į galvos smegenų žievę ir jos funkcijas, bendrasmegeninę ir židininę neurologinę simptomatiką, galvos smegenų kamieno reikšmę ligonio sąmonės būklei ir gyvybinėms funkcijoms, daugelio NS ligų simptomai vis dar sieti su kraujo pertekliumi galvos smegenyse.

Marcelijus Oktavijus Malevičiaus 1829 m. apgintoje disertacijoje apie VU Terapijos klinikoje stebėtas nervų ligas teisingai nurodė pagrindinę bendrasmegeninę (galvos skausmą, pykinimą ir vėmimą, sąmonės sutrikimą) ir židininę (dvejinimąsi, midriazę, viršutinio voko ptozę, galūnių paralyžius, jutimų sutrikimus, traukulių priepuolius) encefalitu sergančio ligonio neurologinę simptomatiką, nustatė galvos smegenų kamieno reikšmę ligonio sąmonės būklei, gyvybinėms funkcijoms. Vincentas Juchnevičiaus 1825 m. apgintoje disertacijoje apie arachnoiditą pabrėžė galvos smegenų lokalizacijos reikšmę, užsiminė apie žievės pažeidimo sąsajas su traukuliniu sindromu, paralyžiais, kamieno pažeidimus siejo su bulbarinio sindromo elementais. Galbūt ne visiškai tiksliai, tačiau V. Juchnevičiaus disertacijoje taip pat bandyta suprasti kodėl, vienoje pusėje išsiplėtus galvos smegenų skilveliui, nusilpsta priešingos pusės akies voko raumenys.

Pauliaus Mokžickio 1824 m. apgintoje disertacijoje apie gimdyvių traukulius išvardyti būdingi židininiai motoriniai su arba be išplitimo į galvos smegenų pusrutulius (antrine ge-

neralizacija) traukulių priepuolių simptomai, teisingai pastebėta, kad priepuoliams būdinga ne tik putos iš burnos, bet ir nevalingas pasišlapinimas, popriepuolinis mieguistumas. Nors XIX a. pr. traukulių semiologijos reikšmė epileptogeninio židinio nustatymui dar nebuvo svarstoma, VU Akušerijos klinikoje įvykę gimdyvių traukuliai (ypač – traukulių pradžia) buvo atidžiai stebėta ir kruopščiai aprašyta. Manyta, kad pilnakraujės ligonės (tvirtos, paraudusio veido, kurių pulsas buvo kietas, gero prisipildymo, jei sergančiąsias vargino dažni galvos skausmai) turėjo didelę konvulsijų riziką, todėl pagrindiniai taikyti traukulių profilaktikos ir gydymo metodai buvo antiflogistiniai.

VU Terapijos klinikoje vertinti antropometriniai, morfometriniai duomenys, somatinė ligonio būklė bei atliekama tai, ką galėtume pavadinti neurologinės apžiūros ištakomis (vertinta vyzdžių reakcija į šviesą, ligonio reakcija į skausmą, protinė būklė, kalba, paroksizminiai judesių sutrikimai, bendrieji ir specialieji jutimai – paviršinis jutimas, rega, klausa, uoslė, skonis; taip pat galūnių padėtis, raumenų būklė, dubens organų funkcijos). Jono Adomo Šliocerio 1816 m. VU apgintame darbe apie somnambulizmą paliestos ir platesnės miego sutrikimų sukeliamos problemos kasdieniam ligonio gyvenimui: paminėta, kad miego sutrikimai naktį sukelia mieguistumą dienos metu, nuolat blogai išsimiegantis ligonis yra linkęs sirgti kitomis lėtinėmis ligomis.

Vilniuje, kaip ir daugelyje Vakarų Europos klinikų, ligos priežasčių ieškota vidaus organuose (solidizmo teorijos įtaka), tačiau ligonių autopsijos ir makroskopiniai tyrimai, kurių dažniausi radiniai buvo kraujo priplūdimas galvos ir nugaros smegenyse, toliau patvirtino keturių organizmo skysčių pusiausvyros sutrikimo, uždegiminę NS ligų patogenezės teoriją.

Gera ligos prognozė būdavo skirta lengvesnėmis NS ligomis sergantiems arba ligų simptomams (galvos skausmui, svaigimui, somnambulizmui, simptominiams traukuliams), bloga – sunkiomis NS ligoms sergantiems (apopleksija, encefalitu, arachnoiditu, tetanija, hidrofobija, mielitu). Stebėta neurologinė simptomatika ir ligonio sąmonės būklė padėdavo gydytojui tiksliau nustatyti ligos prognozę.

Pagrindinis VU klinikose taikytas NS ligų gydymo metodas buvo antiflogistinis. Kraujo nuleidimas buvo laikytas gyvybę gelbstinčia procedūra. Dažnai skirti emetikai, viduriavimą, šlapinimąsi, prakaitavimą skatinantys vaistai. Opijus daugeliu atvejų vartotas ne kaip stimuliantas, bet kaip analgetikas ir hipnotikas. Dieta taip pat turėjo svarbią reikšmę: sergant sunkiais uždegimais, rekomenduotas lengvas, gerai virškinamas maistas, skysčiai. Kalomelis (gyvsidabrio chloridas) NS ligų gydymui naudotas kaip laksantas ir rezorbciją gerinantis vaistas. VU klinikose gan populiari buvo ir chininmedžio (kinmedžio) žievė, kurios nuoviras vartotas kaip priešuždegiminis vaistas. Sergant traumine tetanija arba trauminiu arachnoiditu, rekomenduota chirurgiškai sutvarkyti žaizdą, stabdyti uždegimo plitimą. Hidrofobija sirgusių ligonių gydymas prūsų rūgštimi (ciano vandeniliu) – tai desperatiškas, „herojinės“ medicinos pavyzdys, gydant mirtiną ligą. Mokėta teikti pirmąją pagalbą, ištikus traukulių priepuoliui. Ligoniams, sergantiems miego sutrikimais, teiktos miego higienos, gyvenimo būdo rekomendacijos (pvz., rekomenduota gimnastika, energingi pasivaikščiojimai gryname ore, drausti ilgi sėdėjimai, gulėjimai lovoje dienos metu, neleista vartoti alkoholinių gėrimų, kavos, arbatos prieš miegą ir kit.).

4. Operacinio nervų ligų gydymo ištakos

VU Chirurgijos klinikoje atliktos trepanacijos, subdurinės ir epidurinės hematomų pašalinimo, bendrosios miego arterijos (BMA) perrišimo bei paviršinių galvos darinių ekscizijos ir ligavimo operacijos.

VU auklėtinio Bazilijaus Voiciechovskio disertacijoje 1825 m. aprašytą, VU Chirurgijos klinikoje atliktą trepanacijos operaciją veikiausiai galime laikyti pirmąja VU moksliniuose darbuose paminėta sėkminga trepanacijos operacija: „Ketverių metų amžiaus kilmingas berniukas dėl nepriežiūros per langą iškrito laukan, stipriai susitrenkė galvą, ligoniui lūžo kaukolės skliautas". Vaiką bemat suparalyžiavo ir jis užmigo. Šie simptomai vertinti kaip kraujo išsiliejimo galvos smegenyse požymiai. Trepanacijos procedūra aprašyta trumpai, nepaminėjus, kaip buvo paruoštas operacinis laukas, kokiais instrumentais naudotasi, ar buvo taikytas nuskausminimas: „Vaikui atlikta kaukolės trepanacija, išimtas lūžgalis, išleistas po kietuoju dangalu susikaupęs ekstravazatas". Keletą mėnesių laukta operacinės žaizdos gijimo, tačiau iš smegenų pradėjo kilti raudonas, kietas mazgelis, per kelias dienas peraugęs kaukolės kaulus, padidėjęs iki žąsies kiaušinio dydžio. „Iš pradžių bandyta tumorą pašalinti peiliu, tačiau prasidėjo sunkiai sustabdomas kraujavimas, tad nutarta darinio pamatą perrišti". Per 10 dienų ligatūra užveržė darinį, jam nukritus, pasireiškė trizmas ir tetanija. „Uždėjus metalinę plokštelę, apsaugančią smegenis nuo išorinių sužalojimų, taikytas vietinis sausinantis žaizdos gydymas, tepti balzamai. Berniukas pasveiko (*puer pristinam valetudinem recuperavit*)".

Antano Domherio 1825 m. apgintoje disertacijoje siūlytos trepanacijos indikacijos buvo racionalios ir logiškos, neurologinė ligonio būklė – pagrindinis lemiantis veiksnys operacinės gydymo taktikos pasirinkimui. Nustačius, kad po galvos smegenų dangalais išsiliejęs kraujas spaudžia smegenis (ligonis, patyręs galvos traumą, guli „be gyvybės ženklų", „be proto, kalbos ir jutimų"), rekomenduota nedelsiant trepanuoti. Vilniaus chirurgai taip pat žinojo pagrindines struktūras (veninius sinusus, vidurinę smegenų dangalų arteriją), kurių pažeidimas operacijos metu galėjo sukelti mirtinas komplikacijas.

Operacijų metu VU Chirurgijos klinikoje nebuvo taikomi aseptikos ir antiseptikos principai. Trepanacijos operacijoje dalyvavo 4 žmonės: chirurgas ir jo padėjėjas, du asistentai laikydavo ligonio galvą ir galūnes, kad nesiblaškytų, nuskausminimas neminėtas. Pagrindiniai kraujavimo stabdymo metodai, aprašyti A. Domherio darbe – kompresija, cheminis prideginimas ir šaltis – veikiausiai nebuvo efektyvūs nenuskausmintam, judančiam ligoniui, o kraujagyslės perrišimas – vienintelė efektyvios hemostazės galimybė. Todėl suprantama, kodėl vengta operuoti giliau – po voratinkliniu dangalu, smegenų parenchimoje, ar net ties kaukolės pamatu.

Bandydami atsakyti į klausimą, kurioje galvos pusėje reikėtų dėti trepaną, jei nestebima jokių išorinių galvos sužalojimų požymių, tačiau įtariama kraujosruva, VU klinikos chirurgai susiejo ligonio simptomus (vienpusį paralyžių) su tikėtina galvos smegenų pažeidimo (kontralateralinio paralyžiui) lokalizacija. Tikėtina, kad VU Chirurgijos klinikoje galvos smegenų lokalizacijos teorija buvo atrasta empiriškai ir taikyta praktiškai.

Tirdami ligonio, patyrusio galvos traumą, smegenis *post mortem*, bet nestebėdami aiškių makroskopinių pakitimų, Vilniaus klinikų chirurgai neatmesdavo, kad mirtis ligonį ga-

lėjusi ištikti būtent dėl smegenų sukrėtimo. Tokius grėsmingus kaip priekinės ir vidurinės kaukolės daubos lūžiams būdingus požymius (kraujavimą iš nosies ir ausų, speninės ataugos hematomą) XIX a. pr. Vilniaus chirurgai laikė geros prognozės požymiu. Remdamiesi keturių organizmo skysčių teorija, gydytojai manė, kad per nosį, ausis ar kitas kaukolės ertmes pasišalinus „žalingam" kraujui, jo mažiau liks smegenyse ir šis „kilnusis organas" bus mažiau spaudžiamas.

Kito VU auklėtinio Jozefo Domherio 1825 m. apgintame darbe nurodytos BMA perrišimo indikacijos. BMA perrišama, jei nustatoma: 1) miego arterijos aneurizma; 2) komplikuota miego arterijos ar jos stambiųjų šakų žaizda, kurios jokiais kitais chirurginiais būdais neįmanoma sutvarkyti; 3) kraujagyslinis grybas (*fungus haemathodes*), esantis virš miego arterijos; 4) patologinės degeneracijos, susijusios su jungo vena, miego arterija ar klajokliu nervu, kurias pašalinus, yra didelė nukraujavimo rizika; 5) egzostozės, osteosarkomos, augliai, augantys iš apatinio žandikaulio, taip pat, jei planuojama chirurgiškai pašalinti paausinę, pažandinę liaukas, šalia augančius auglius. BMA perrišimas buvo tiek BMA aneurizmos gydymo operacija, tiek efektyvus hemostazės metodas, atliekant auglių šalinimą ar chirurginį žaizdų gydymą galvos srityje.

Dažniausiai taikytas encefalocelės gydymas VU Chirurgijos klinikoje buvo galvos smegenų išvaržos kompresija, encefalocelę palaipsniui spaudžiant metalinėmis (aukso ar sidabro) plokštelėmis, siekiant grąžinti išsiveržusias smegenis atgal į kaukolės ertmę. Teigta, kad kaustikos taikymas yra kenksmingas, nes pažeidžiami smegenis gaubiantys dangalai, jie tampa uždegimiški ir supūliuoja, o vietinis gydymas (emolietai, įvairūs tirpalai) pūliavimo procesą tik pagreitina. Perspėta, kad chirurginis gydymas (encefalocelės rezekcija) beveik visada mirtinas, todėl nerekomenduotinas.

5. Kaltūno fenomenas ir neurologija

Visose kultūrose, civilizacijose, įvairiose epochose plaukai turėjo didelę reikšmę kuriant žmogaus kūno įvaizdį. Plaukų būklė, spalva, ilgis, įvairios šukuosenos suteikdavo informacijos apie asmens socialinį ir ekonominį statusą, politines pažiūras, religiją, moralę bei seksualinę orientaciją. Tikėjimas žmogaus plaukų galia siekia biblinius laikus: Samsonas, paminėtas Senojo testamento Teisėjų knygoje, buvo antžmogiškai stipri būtybė, galėjusi perplėšti liūtą tarytum ožiuką, su asilo žandikauliu užmušti tūkstantį filistinų ar plikomis rankomis sugriauti miesto vartų stulpus. Nukirpus Samsono plaukus, jis neteko galios, buvo nugalėtas ir įkalintas filistinų. Plaukų nukirpimas simbolizuoja ne tik fizinės galios netekimą ir pralaimėjimą. Nacistinėje Vokietijoje prievartinis plaukų nukirpimas žydams, patekusiems į koncentracijos stovyklas, reiškė ir socialinę vyriausybės kontrolę žydų atžvilgiu, ir asmens bei tautos pažeminimą, bausmę. Taigi plaukai – ne tik žmogaus kūno dalis, anatomiškai priklausanti odos priedams, bet ir fenomenas, turintis socialinę, religinę bei asmeninę reikšmę.

Kaltūnas (lotynų k. *plica polonica, plica polonica judaica, trichoma, lues sarmatica*) šiandien yra suvokiamas kaip susivėlusių, neiššukuojamų ir nešvarių plaukų darinys, susiformavęs dėl higienos, plaukų ir odos priežiūros stokos bei utelėtumo, tačiau ilgus amžius laikytas

savarankiška, sunkia, recidyvuojančia viso organizmo liga. Nuo XVI a. iki XIX a. antrosios pusės Europoje publiktuota daugiau nei 900 disertacijų, traktatų ir straipsnių, skirtų kaltūno ligos aiškinimui. Kaltūnas kaip reiškinys ATR buvo taip paplitęs, jog bet kokio paaiškinimo paieškos tapo svarbia užduotimi, patikėta to meto gydytojui.

Išnagrinėję dvi VU apgintas disertacijas apie kaltūną, pastebėjome, kad kaltūnu sergančiam ligoniui XIX a. pradžioje buvo nustatomi ne tik odos ir jos priedų, bet ir įvairių organų ir organizmo sistemų pažeidimai (dažniausiai nespecifiniai), kurie buvo aktyviai gydyti gana agresyviais medikamentais. Kaltūnuotiems dažnai nustatyti galvos, nugaros smegenų, jų rezginių ir nervų pažeidimai, diagnozuoti galvos skausmai, svaigimas, nuomaris, alpimai, nemiga, naktiniai košmarai, haliucinacijos, melancholija, manija, hipochondrija, stuburo kanalo pažeidimai, nugaros skausmai, įvairūs paralyžiai, pilvinio rezginio ligos. Tvirtinta, kad kaltūnas pažeidžia jutimo organus: regą (ligonį vargina ašarojimas, akių skausmas, junginės venų varikozė, ragenos pažeidimai, hipopija, katarakta, fotopsija, diplopija, didelio laipsnio amaurozė, akį judinančių raumenų nejudrumas), klausą (pasireiškia zvimbimas, ūžimas ausyse, klausos haliucinacijos), uoslę (ji silpnėja dėl lėtinio rinito), sutrinka paviršiniai jutimai (pasireiškia galvos bei įvairių kūno dalių odos nejautra arba sustiprėję jutimai, parestezijos).

Nors konstatuota, kad pakitimai mirusiųjų kūnuose gali būti būdingi tiek kaltūnui, tiek kitoms ligoms, kaltūnas laikytas savarankiška sistemine viso organizmo liga. Paradoksalu, bet tikėta, kad kaltūnas turi ir gydomąjį poveikį: skatintas kaltūno susivėlimas galvos plaukuose tikintis, kad ligonis pasveiks nuo lėtinių ligų, kaltūno ligos simptomai palengvės. Siūlytas įvairus simptominis gydymas, pavyzdžiui, galvos skausmai gydyti burgundiškos eglės dervos pleistrais, sutirštintomis paprastosios durnaropės sultimis, sumaišytomis su siera. Kaltūnui gydyti buvo naudoti net gyvsidabrio preparatai. Liudvikas Knotė 1830 m. apgintoje disertacijoje apie kaltūną teigė, kad gyvsidabris yra veiksmingas gydant kaltūninius išopėjimus, pūlinėlinę bei su kaltūnu susijusias NS ligas. Kaltūno gydymui naudotas ir stimuliantas kamparas, kuris, pasak L. Knotės, veiksmingai gelbsti gydant NS ligas, taip pat skiriamas, jei kaltūnuotam ligoniui pabąla veidas, išsausėja oda, netenkama jėgų.

Galiausiai tik XIX a. antrojoje pusėje Vilniaus krašte pripažinta, kad kaltūnas nėra liga, o žmonių tamsumo, prietarų ir higienos stokos rezultatas. Henrikas Dobžickis (Henryk Dobrzycki; 1841–1914), parengęs veikalą apie kaltūną, dalyvavo jau keletą dešimtmečių organizuojamame VMD konkurse ir 1876 m. gavo šios draugijos apdovanojimą už geriausią darbą, parašytą kaltūno tema.

Darbo trūkumai

Nagrinėjome NS ligų suvokimą mažo Europos regiono viename mieste, sutelkdami dėmesį į VU ir VMD mokslininkų veiklą konkrečiu laiko periodu – XIX a. pirmojoje pusėje, todėl gautos išvados negali apibendrinti situacijos visose buvusiose LDK žemėse ar kituose Rusijos imperijos regionuose. Mūsų išvados yra pagrįstos vien oficialiu, mokslinėje literatūroje dokumentuotu Vilniaus gydytojų bei VU mokslininkų požiūriu, todėl nedaug žinome, kaip NS ligos buvo aiškinamos liaudies medikų, žolininkų, barzdaskučių, slaugytojų, dvasi-

ninkų, aiškiaregių, pačių ligonių ir jų artimųjų, galiausiai – kaip NS susirgimai buvo gydomi dar prieš kreipiantis į gydytoją. Šiame darbe kartais naudojome ir retrospektyvios diagnostikos metodą, nors toks diagnozių nustatymas ligoniams, gydytiems prieš keletą šimtmečių, žinant tik kai kurias nusiskundimų, anamnezės ir ligos eigos detales, šiandien kai kurių mokslo istorikų yra kritikuojamas.

Išvados

1. Klinikinės neurologijos ir neurochirurgijos ištakos Vilniuje siekia XIX–ojo amžiaus pradžią: Vilniaus imperatoriškojo universiteto Terapijos klinikoje diagnozuota ir gydyta apopleksija, encefalitas, arachnoiditas, mielitas, hidrofobija, tetanija, miego sutrikimai, Šv. Vito chorėja, ūminė ir lėtinė hidrocefalija, nervų sistemos sklaidos ydos ir kitos nervų ligos; Vilniaus imperatoriškojo universiteto Chirurgijos klinikoje atliktos trepanacijos, subdurinės ir epidurinės hematomų pašalinimo, bendrosios miego arterijos perrišimo bei paviršinių galvos darinių išpjovimo arba perrišimo operacijos.

2. Vilniaus imperatoriškasis universitetas, Vilniaus universiteto klinikos bei Vilniaus medicinos draugija turėjo lemiamą reikšmę neurologijos plėtotei Vilniaus krašte: 1803 m. reorganizavus universitetą, 1805 m. atidarius Terapijos, 1808 m. – Chirurgijos kliniką, pagerėjo nervų ligų diagnostika ir gydymas, padaugėjo mokslo darbų nervų ligų tematika (apgintos 25 disertacijos), medicinos studentai buvo mokomi praktinių įgūdžių prie ligonio lovos, dalyvaudavo neurochirurginėse operacijose, mirusių ligonių autopsijose; Vilniaus medicinos draugijos susirinkimų metu universiteto profesoriai ir Vilniaus gydytojai demonstruodavo nervų ligomis sirgusius ligonius, aptardavo jų gydymą, ligoniui mirus, kartu išanalizuodavo autopsijos radinius.

3. Vilniaus universiteto klinikose gydant nervų sistemos ligas dažniausiai vadovautasi humoraline doktrina; patvirtinant klinikinę diagnozę po ligonio mirties – solidizmo teorija; braunizmas, kaip ir visoje Europoje, taip ir XIX a. pr. Vilniuje buvo labiau kritikuojamas nei praktikuojamas; vitalinį magnetizmą pripažino ne tik Vilniaus gydytojai, bet ir nelegaliai medicinos praktika besiverčiantys „aiškiaregiai“; organologija, viena pirmųjų galvos smegenų lokalizacijos teorijų, XIX–ojo amžiaus pradžioje taip pat buvo puikiai žinoma universiteto profesoriams, studentams bei Vilniaus miesto gydytojams.

4. Nervų ligų diagnostikos ir gydymo Vilniuje lygis atitiko to meto Vakarų Europos lygį: Vilniaus klinikose XIX–ojo amžiaus pirmojoje pusėje naudoti nervų ligų terminai, patogenezės teorijos ir gydymo metodai atitiko to meto Vakarų Europoje naudotus; Vilniaus klinikose buvo įvertinami antropometriniai duomenys, somatinė ligonio būklė bei atliekama tai, ką galėtume pavadinti neurologinės apžiūros ištakomis (vertinta vyzdžių reakcija į šviesą, ligonio reakcija į skausmą, protinė būklė, kalba, paroksizminiai judesių sutrikimai, bendrieji ir specialieji jutimai, galūnių padėtis, raumenų būklė, dubens organų funkcijos); žmogaus pažinimo ir atminties procesai vertinti visų pirma kaip nervų sistemos fiziologijos reiškiniai; Vilniaus klinikose buvo naudotas anatominis – klinikinis metodas, atliekant mirusių ligonių galvos ir nugaros smegenų autopsijas, klinikinę diagnozę patvirtinant remiantis *post mortem* radiniais.

5. Vilniaus klinikų chirurgai, spręsdami, kurioje galvos pusėje reikėtų dėti trepaną, ligonio vienpusį paralyžių po patirtos traumos siejo su tikėtino galvos smegenų pažeidimo, kontralateralinio paralyžiui, lokalizacija.

6. Kaltūnas Vilniaus krašte, kaip ir kituose Vakarų Europos miestuose, XIX–ojo amžiaus pirmoje pusėje vis dar buvo įvardijamas kaip savarankiška viso organizmo liga ir vienas iš nervų ligų etiologinių veiksnių.

Darbo tąsos kryptys

Planuojama apžvelgti tolesnę neurologijos ir neurochirurgijos raidą XX a. pirmojoje pusėje Lietuvoje.

Daiva Valadkevičienė

THE BIOPSYCHOSOCIAL ASPECTS OF DISABILITY ASSESSMENT IN PATIENTS WITH MULTIPLE SCLEROSIS

NEGALIOS VERTINIMO SERGANTIEMS IŠSĖTINE SKLEROZE BIOPSICHOSOCIALINIAI ASPEKTAIS

Medicine and Health Sciences, Medicine M 001
Medicinos ir sveikatos mokslai, Medicinos mokslo kryptis M 001

Abbreviations

BICAMS – Brief International Cognitive Assessment for Multiple Sclerosis

BVMT R – Brief Visuospatial Memory Test Revised

CHIIS – Compulsory Health Insurance Information System "Sveidra"

CVLT II – California Verbal Learning Test II ed.

DWCAO – the Disability and Working Capacity Assessment Office under the Ministry of Social Security and Labour of the Republic of Lithuania

EDSS – Expanded Disability Status Scale

FDS – Fatigue Descriptive Scale

HADS – Hospital Anxiety and Depression Scale

ICF – the International Classification of Functioning, Disability and Health

MS – multiple sclerosis

SDMT – Symbol Digit Modalities Test

SF36 – Short Form 36 Medical Outcomes Study Questionnaire

WCL – working capacity level

WHO – the World Health Organization

Introduction

Fifteen percent of the world's population – almost 1 000 000 000 people – are disabled.[1] In order to meet the needs of people with disabilities and enforce their rights, health and social security agencies require holistic descriptions for assessing an individual's activity and disability. Contemporary society finds it important to ensure that people remain capable at work, independent, universally active, and taking part in all areas of their life and the life of society as long as possible; therefore, it is crucial to make use of all possibilities provided by medical care and medical and professional rehabilitation in order to overcome disease-related obstacles in an individual's life and postpone disability. When health disorders become long-term and disability is established, the question of medical and social assistance becomes even more relevant.

The World Health Organization (WHO) recommends assessing the severity of disability according to the International Classification of Functioning, Disability and Health (ICF). The basis of ICF is a biopsychosocial model, whereby an individual's health, disability, and functioning are established taking into account their interaction with the physical, social, and psychological environment. The Disability Strategy for 2017–2023 of the Council of Europe also states that

> disability is the result of interaction between individual impairments and existing attitudinal and environmental barriers. Disability may hinder the full enjoyment of human

1 WHO. No Title. World Report on Disability, 2011, 1–23.

rights and fundamental freedoms and prevent persons with disabilities from participating effectively and equally in the society. Persons with multiple, complex and intersecting impairments face additional barriers and are at higher risk of institutionalisation, exclusion and poverty. Measures to prevent or remove existing barriers are necessary investments for sustainable development and improved accessibility.[2]

To address the issue of the comprehensive assessment of disability, a solution is offered – using international instruments intended for the establishment of disorders of an individual's body structure and functions and of unfavorable environmental factors. WHO notes that the provision of assistance to patients should take into account not only the causes of health disorders but also the consequences of diseases, which can be reduced by adapting the environment, using technical means and attempting to affect the patients' behavior.

However, the National Program for Social Integration of the Disabled for 2013–2019, approved by the Government of the Republic of Lithuania, takes a narrower approach toward this issue.[3] The National Program has an objective of ensuring the satisfaction of the special needs of the disabled with different disabilities by providing social integration services (in the areas of social security, health care and education). A more complete picture, not only of medical but also of social factors of disability (following the implementation of ICF), would reduce the gap between the practice of disability assessment in Lithuania and recommended international standards, encourage relevant experts and agencies to focus on the often-disregarded biopsychosocial factors of disability and highlight the needs of a particular patient and their everyday obstacles, which could lead to concrete measures ensuring better quality of life for the disabled.

Relevance of the Study

Even though the biopsychosocial aspects of the life of the disabled attract researchers' attention, studies are usually fragmentary. They are not sufficiently focused on comprehensive prevention, diagnostics and disability assessment, do not always show a proper understanding of the needs of persons with chronic diseases and do not provide an adequate assessment of their possibilities to function in society.

After a change of attitude toward an individual's health, it is now being understood not as the absence of a disease but as comprehensive physical, spiritual, and social well-being, the understanding of disability has changed as well. On May 22,

2 *Human Rights: A Reality for All*, 2017.

3 Lietuvos Respublikos Vyriausybė. *Dėl nacionalinės neįgaliųjų socialinės integracijos 2013–2019 metų programos patvirtinimo nutarimas*, Vilnius, 2012.

2001, WHO approved the implementation of ICF into practice and the assessment of consequences of diseases and health disorders based on the following three aspects: changes in organs' functions and structure, disruptions of activity and participation, and changes in environmental and personality factors. The application of this methodology is already gaining momentum in Germany, France, Spain, Switzerland, and Italy, whereas in Lithuania, we only have isolated attempts to apply the principles of this methodology in medical rehabilitation. In his study on the biopsychosocial aspects of disability, Z. Skvarciany assessed the disability of working-age persons with malignant tumours and their health-related quality of life.[4]

The paper is even more relevant due to a lack of statistical data on psychosocial disability, incidence rates, the prevalence of diseases, the establishment of working capacity, and the absence of any scientific foundation for drafting laws and regulations on the establishment of disability. Currently, the working capacity level is established only on the basis of the degree of severity of the consequences of a disease established by the patient's doctor, the so-called basic working capacity level, and an assessment of the questionnaire which is common to all the disabled irrespective of the nature of their disability, activity, and participation.

Neurodegenerative and inflammatory diseases of the nervous system are one of the most important global and national challenges; the prevalence of these diseases and their burden on society are continually on the increase.

Multiple sclerosis (MS) is a frequent and critical autoimmune inflammatory demyelinating neurodegenerative disease of the central nervous system prevalent in Lithuania. Previous studies conducted in Lithuania (by R. Kizlaitienė, N. Giedraitienė, L. Malcienė, R. Balnytė, R. Leonavičius, B. Daškevičienė) have been focused on various aspects of MS's development, its clinical manifestation and diagnostics; however, the aspects of MS's incidence rates and disability assessment have not been examined so far. It is noteworthy that MS is generally diagnosed to young people. Even though treatment modifying MS development has advanced considerably in the 21st century in terms of controlling the course of the disease, MS is known to usually progress over time and cause long-term consequences that are treated as disabilities. The most frequent final consequence of MS is an established disability that brings long-term social and financial consequences on a national scale. Besides, certain data suggest that the recent MS incidence rate has been growing in other countries; however, we did not have the data on MS incidence trends in Lithuania before.

The working capacity level for MS patients is established using the Kurtzke Expanded Disability Status Scale and the said questionnaire, which is common to all

4 Skvarciany Z. *The Disability and Health-Related Quality of Life of Working Age Persons Suffering From Malignant Cancer Tumors*, 2012, 1–17.

and assesses educational background, profession, work experience, age, adaptation of the work environment and a brief activity and participation questionnaire. However, account is not taken of any specific changes in the cognitive functions of MS patients, their fatigue, anxiety, and depression, which significantly aggravate the functioning of these people and disturb their activity and participation in the life of society. Physical disability shows itself in more advanced stages of the disease; therefore, the usual establishment of disability is delayed and should be deemed discriminatory.

The results of this dissertation are also important to practitioners. A comprehensive personal disability assessment based on ICF would let them know an individual's needs and obstacles in everyday life, which would enable a targeted planning of social and health promotion services, rehabilitation means required by the individual, and the assessment of treatment and rehabilitation efficiency. And if, from the point of view of the final consumer, meeting individual needs would determine their higher satisfaction with the received services and quality of life, then, on a macro-level, such a change would contribute to the optimization of the state's financial expenditure and rational distribution of human resources.

Aim of the Study

To analyze the biopsychosocial aspects of disability assessments in people with multiple sclerosis.

Objectives of the Study

1. To analyze the dynamics of the incidence of MS in the Lithuanian population.
2. To analyze the dynamics of the working capacity level of MS patients in relation with sociodemographic data, employment status, and life expectancy.
3. To analyze the relationship between the working capacity level and the different parameters of physical and mental health (memory, fatigue, anxiety, depression, cognitive functions, physical and mental quality of life, the results of Brief ICF Core Set for Multiple Sclerosis).

Statements to be Defended

1. The incidence of MS is constantly increasing; therefore, it is necessary to strengthen the early diagnosis of this disease and provide the patients with the needed assistance during its initial stages.
2. A longitudinal connection between MS's severity and an individual's working capacity is not linear: in the early stages of the disease, the working capacity level significantly decreases, whereas later on, it stabilizes.

3. Psychosocial factors predict the working capacity level of MS patients; thus, their inclusion into the disability establishment procedure would ensure a more objective assessment of disability.

Scientific Novelty

This paper takes a comprehensive approach toward MS, a frequent neurological disease causing significant disability. For the first time in Lithuania, country-wide MS incidence data for the last 15 years have been provided and changes in the incidence rate have been calculated, which makes it possible to predict MS's incidence trends.

The use of ten-years worth of data on a country-wide level, provided by the Disability and Working Capacity Assessment Office under the Ministry of Social Security and Labour of the Republic of Lithuania (hereinafter referred to as DWCAO), made it possible to conduct the first ever analysis of the changing trends in the working capacity level of MS patients during repeat assessments of disability; employment and life expectancy predictions have also been evaluated.

Based on modern diagnostic tests, fatigue, anxiety, and depression experienced by 184 MS patients were thoroughly examined; their cognitive functions and quality of life were assessed as well. In addition, it was the first time that a disability assessment used an ICF questionnaire for MS, including such factors as changes in body functions and structure, restrictions of activity and participation, and the impact of environmental factors. The results of this comprehensive study made it possible to record links between psychosocial factors and not only the assessment of the initial working capacity level but also the annual change in this assessment.

There had been no comprehensive MS disability studies such as this in Lithuania before. It is our hope that the results of the study will help with a timely and more objective establishment of disability of MS patients, while the ICF, used for the assessment of biopsychosocial factors, will open new possibilities in assessing the working capacity level.

Structure of the Paper

According to the complexity of the materials of the analyzed phenomenon, the results are presented in three different levels (Figure 1): epidemiological, the working capacity level, and the employability of patients and the psychosocial factors of MS.

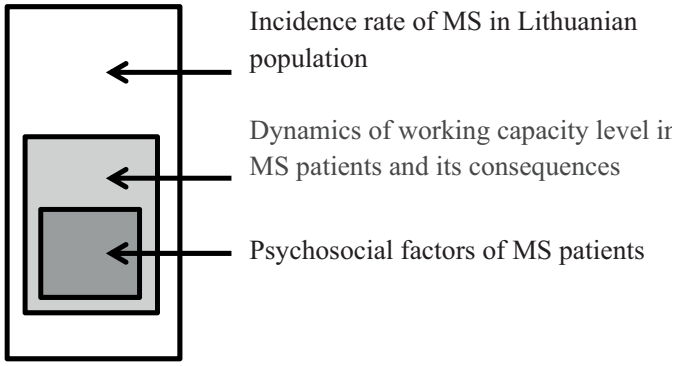


FIGURE 1. Levels of analysis and research stages

Methods

In different stages of research, different data were gathered, and different analysis procedures were applied. Thus, we introduce each research stage separately.

Research Stage 1: Incidence Rate of Multiple Sclerosis in Lithuania

With the help of the Compulsory Health Insurance Information System "Sveidra" (CHIIS) held by the National Health Insurance Fund, the data about patients who were diagnosed with code G35 (Multiple Sclerosis) of ICD-10-CM during ambulatory check-ups and stationary visits from 2001 to 2015 were analyzed. CHIIS data provide a possibility to analyze information on an individual level, eliminate duplicate diagnoses and calculate health indicators according to a patient's residency.

The residents' number in sex and age group categories was obtained from Statistics Lithuania (a national agency that develops, produces, and disseminates official statistics). The crude incidence rates (CIR – ratio between individuals with newly diagnosed MS in ambulatory service) were calculated.

In order to evaluate the influence of resident structure changes on the incidence of MS during the last 15 years, the direct method of standardization using the European standard was applied, and standardized incidence rates (SIR) were calculated. Ninety-five percent confidence intervals were estimated for the averages. The data were processed using the Minitab set to estimate a linear trend model for the temporal changes of 16 parameters. For the prediction of incidence, a simple trend extrapolation method was used from the linear regression line.

Research Stage 2: Dynamics of the Working Capacity Level and Its Relationship with the Employment Status and Life Expectancy in MS Patients

This part of the study was conducted after signing a contract No. F5-39/BS-150000-1123-2015-05-12 between Vilnius University and DWCAO on April 23, 2015. The DWCAO agreed to provide personal data for the biomedical study "Biopsychosocial Aspects of Disability Assessment in People with Multiple Sclerosis," and Vilnius University agreed to analyze depersonalized data and ensure ethical standards of research.

Enrolled in the study were 2 072 people with an MS diagnosis who visited the DWCAO during the period of 2006–2015. Of them, 1 341 were women, and 731 – men; average age during the first data collection was 42.49 years. It is important to note that for 1 182 patients, the first data collection was not during their first visit to the DWCAO.

The MS diagnosis for all patients was confirmed by a third-level neurology health care service using the McDonald criteria (2010 revision). All patients arrived at the DWCAO during remission, with stable long-term physical and mental dysfunctions that had affected their daily functioning and quality of life.

In all documents presented to the DWCAO, the main diagnosis was multiple sclerosis (code G35 in ICD-10-CM). If the diagnosis code and the main diagnosis had been changed during the next visit to the DWCAO, the person was excluded from the study. If the diagnosis code had been changed during the next visit, but the main diagnosis remained the same (multiple sclerosis; G35), the data from these patients were further analyzed.

Evaluations performed during the second stage of the study:

Working capacity level – the ability of a person to implement a professional competency he/she gained earlier, to acquire a new professional competency, or to perform jobs that require less professional competency. The working capacity level is established for people over 18 years of age until they reach the age of eligibility for pension in a complex way, by assessing not only medical but also functional, professional, and other criteria that limit a person's capacity for work.

The working capacity level is set using 5% intervals, in accordance with legal regulations on the process of setting the working capacity level. A range of 0–25% working capacity indicates that an individual cannot work or has the ability to work only in the environment with adaptations to the nature of disability. A range of 30–55% indicates that an individual has a reduced level of working capacity, meaning that an individual is able to work in an environment with adaptations or no adaptations. A range of 60–100% indicates that a person has full working capacity. It is

noteworthy that a different working capacity range is related to different monetary allowances for the patients. For the purposes of this study, the working capacity level data set was divided into 5 groups of individuals with the following intervals of working capacity: 0–10%, 15–25%, 30–40%, 45–55%, and 60–100%.

According to legal regulations, the validity of the working capacity level may last for 1 or 2 years or, if there are no indicators for possible improvement, can be permanent (until the end of life). Nevertheless, patients with a permanent validity of working capacity may arrive at the DWCAO for an additional assessment if they believe their disease condition has changed. While analyzing the data, it was assumed that if the patient was alive, and if the most recent validity of the working capacity level was permanent, the capacity level and employment status were the same until the end of the study.

Periodicity of assessment: the primary evaluation is conducted during the first visit and when the decision on the level is done. A repeat evaluation of the working capacity level is conducted at the end of the validity of the primary evaluation or due to any changes in the individual's health condition, changes of reasons for the working capacity level, or if a person or the funding institution do not accept the results of the DWCAO's evaluation. There were also inquiries about possible deaths of patients who did not return to repeat evaluations.

Sociodemographic data (gender, age during the first visit), employment status and its changes during re-evaluations: sociodemographic and employment data were collected after patient visits to the DWCAO and transferred to local research spreadsheets from the DWCAO database for further analysis.

Research Stage 3: Psychosocial Factors of MS Patients

Research was conducted at Vilnius University Hospital Santaros Klinikos (inpatient department of neurological disorders and the MS service in an out-patient clinic) and the DWCAO during 2014–2018. The study received an approval (No.158200-14-753-271) from the Lithuanian Bioethics Committee on December 9, 2014. The State Data Protection Inspectorate approved the Vilnius University's Plan for Legal Protection of Personal Data while conducting this study (04–20–2015, No.2R-2270(2.6-1). Each participant signed an informed consent and an agreement regarding personal data usage.

The cross-sectional study involved 184 people with MS. One hundred fifty-seven people visited the DWCAO to determine their working capacity levels (study group), and 27 people with MS did not visit the DWCAO (control group).

Inclusion criteria:

1) Age over 18 years;
2) MS diagnosis confirmed using the McDonald criteria revised in 2010;
3) MS remission stage and a stable neurological condition;
4) No other diseases related to the central or peripheral nervous system, metabolic, or other significant diseases that could affect the working capacity level;
5) Fluent Lithuanian language;
6) Voluntary consent to take part in the study or voluntary consent to take part in the study and the signature of an informed consent form including an agreement regarding personal data usage.

Exclusion criteria:

For people with MS, those were any findings contradicting the inclusion criteria.

One person was excluded from the study due to an insufficient fluency of Lithuanian language, and one study participant refused to continue the participation in the study after failure in conducting the California verbal learning test. For the control group, the data about the disease were not collected.

Assessment instruments and data collection

1. **Expanded Disability Status Scale,** EDSS. The EDSS scale ranges from 0 to 10 in 0.5 unit increments that represent higher levels of disability. 0 – normal neurologic exam, 10 – Death due to MS.
2. **Working capacity level** – assessment using 5% intervals, as described in Research Stage 2.
3. **Fatigue self-assessment** – 10-point Likert type scale measuring fatigue during the study; 0 – no fatigue, 10 – very high fatigue.
4. **Fatigue Descriptive Scale,** FDS, including subscales of Initiative, Modality, Frequency, Severity and the Uhthoff's effect. Total score range is 0-17; 0 – no fatigue, 17 – strong fatigue.
5. **Hospital Anxiety and Depression Scale,** HADS. Fourteen item scale with scores between 0 and 21; 0 – no symptoms of anxiety or depression, 21 – heavy symptoms of anxiety or depression.
6. **Memory self-assessment** – 10-point Likert type scale measuring one's own memory; 0 – very bad, 10 – very good.
7. **Brief International Cognitive Assessment for Multiple Sclerosis**, BICAMS, which consists of:
   - Symbol Digit Modalities Test, SDMT; Performance is defined by total number of correct pairings within 90 seconds.

- Brief Visuospatial Memory Test-Revised, BVMT-R (3 sets); Score range for each set is 0-12.
- California verbal learning test II ed., CVLT-II, Lithuanian version (5 attempts). Total score is range is 0-80; 0 – no recall of words, 80 – full recall of words.

8. **Short Form 36 Medical Outcomes Study Questionnaire**, with subscales of Physical functioning, Role limitations due to physical health, Pain, General health, Physical component summary, Energy/fatigue, Social functioning, Role limitations due to emotional problems, Emotional well-being, Mental component summary; SF-36. Each subscale score range is 0-100; 0 – worst health, 100 – best health.
9. **Brief ICF Core Set for Multiple Sclerosis** including data about: b130 – Energy and drive functions, b152 – Emotional functions, b164 – Higher-level cognitive functions, b210 – Seeing functions, b280 – Sensation of pain, b620 – Urination functions, b730 – Muscle power functions, b770 – Gait pattern functions, s110 – Structure of the brain, s120 – Spinal cord and related structures, d175 – Solving problems, d230 – Carrying out one's daily routine, d450 – Walking, d455 – Moving around, d760 – Family relationships, d850 – Remunerative employment, e310 – Immediate family, e355 – Health professionals, e410 – Individual attitudes of immediate family members, e580 – Health services, systems and policies. The level of impairment or restriction qualified for all components by a qualifier scale (0 = no impairment/restriction to 4 = complete impairment/restriction). For 'Environmental factors' a comparable 0–4 scale is applied, where categories can be either a facilitator or a barrier.
10. **Disease data** – MS type, time from the first MS symptoms in calendar years, time from MS diagnosis in calendar years.
11. **Sociodemographic data** – gender, age in years, education, employment status, possession of profession.

The working capacity level and EDSS information were collected twice: at the beginning of the study and after 1 year. All other variables were measured only at the beginning of the study.

Statistical analysis was performed using the statistical software package SPSS 17.0 (version for MS Windows). Descriptive statistics for quantitative variables are presented using mean (M) and standard deviations (SD), and for qualitative (discrete) variables – using the absolute value and percentage of the analyzed sample group (%). The Student t test was used to compare the means of two independent groups of quantitative variables. The normality of variables was assessed using a Kolmogorov-Smirnov test. If a given comparison consisted of more than 2 groups,

an analysis of variance ANOVA was conducted (if there were no equal variances, Welch's ANOVA was applied). Group comparisons were made using a Tukey HSD Post Hoc test. To compare discrete (qualitative) variables, a Chi-square ($\chi 2$) independence criterion was used and, due to the small sample size, the exact Fisher test was conducted. The Pearson correlation coefficient was used to determine the relationship between variables. A logistic regression analysis (Forward Wald) was used to predict the worsening of health state in one year. A stepwise selection of independent variables was conducted. A variable was included in the model if $p < 0.05$ and was excluded from the model if $p > 0.1$. An ROC analysis was conducted to get the critical values for predicting change in employment status, the worsening of health condition's or death. The level of significance was $\alpha = 0.05$.

Results

I. The incidence of MS in Lithuanian population in 2001–2015

The first objective of this study was to analyze the dynamics of the incidence of MS in Lithuanian population. This was done in 3 steps:

1) The crude incidence rates of MS and incidence rates standardized by age were calculated;
2) A prediction of the incidence of MS in the entire population was conducted;
3) A prediction of the incidence of MS according to gender was performed.

The data from CHIIS showed that the number of newly diagnosed MS cases was increasing in Lithuania every year. In 2001, MS was diagnosed to 162 new individuals (61 males and 101 females), whereas 343 new cases of MS (101 males and 242 females) were diagnosed in 2015. The CIR and SIR of MS are presented in Table 1. During 2001–2015, the incidence of MS was on average 6.5 (95% CI 5.7–7.3) cases per 100 000 residents, 4.9 (95% CI 4.5–5.3) and 8.1 (95% CI 6.9–9.3) for 100 000 males and females, respectively. These rates had been fluctuating over the past 15 years – the difference between the highest and the lowest incidence rates was 5.7 times. The incidence difference among males was 2.9 times, and among females – 8.9 times.

In 2015, 2 526 persons had the diagnosis of MS, so the prevalence was 87 cases per 100 000 residents of Lithuania.

In Lithuania, the overall incidence rate tended to increase during the period of 2001–2015 (Fig. 2). However, an increase in the incidence rate of MS in 2011 and 2013 could be related to changes of the disease registration system.

During the period of 2001 to 2015, the overall incidence rate was increasing on average by 3.5 cases per 100 000 persons yearly. If the tendencies prevail, the overall incidence rate of MS is estimated to reach 13 cases per 100 000 persons in 2020.

The incidence of MS among males showed only a slight increase until 2012 and a sharp increase beginning with 2013 (Fig. 3). The incidence rate in males was increasing by 3.1 cases per 100 000 yearly. If the tendencies remain, incidence is estimated to reach 9 cases per 100 000 males in 2020. During 2001–2015, incidence increased by 3.8 cases per 100 000 females yearly. If the tendencies remain, the MS incidence among females in Lithuania is estimated to reach 16 new cases per 100 000 females in 2020 (Fig. 4).

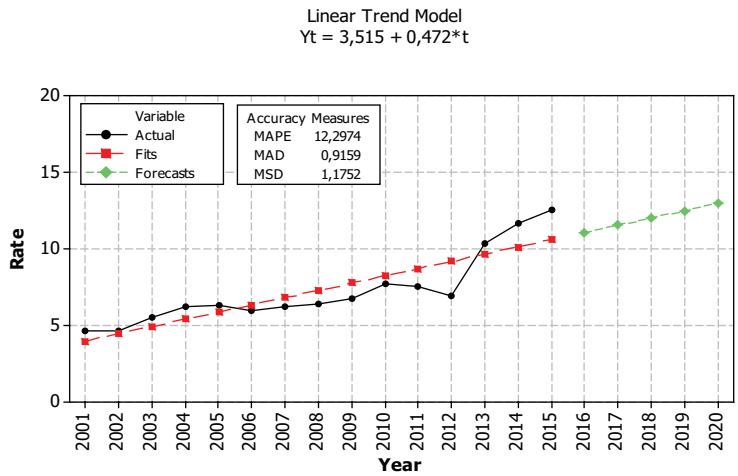


FIGURE 2. The incidence of MS in the Lithuanian population per 100 000 residents from 2001 to 2015, and estimated results in 2020

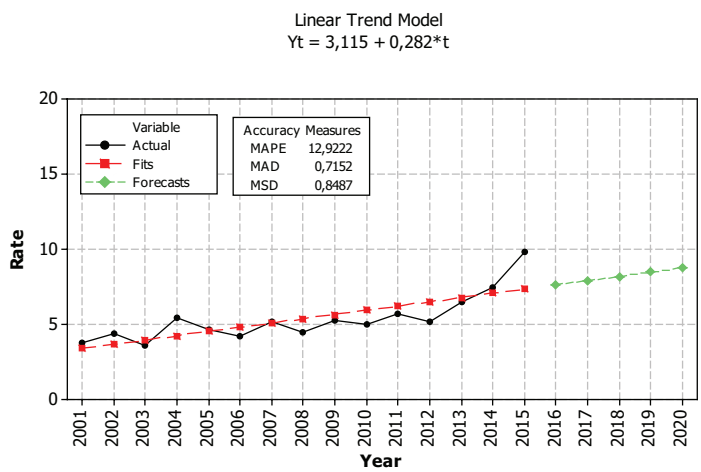


FIGURE 3. The incidence of MS in males in Lithuania per 100 000 males from 2001 to 2015, and estimated results in 2020

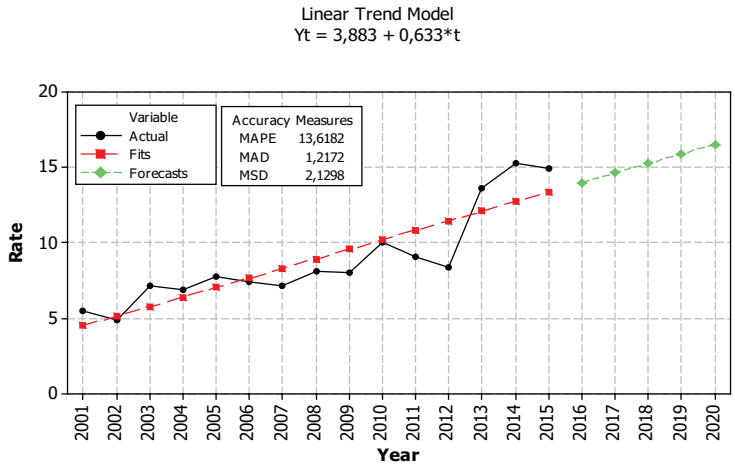


FIGURE 4. The incidence of MS in females in Lithuania per 100 000 females from 2001 to 2015, and estimated results in 2020

The female-to-male sex ratio in the incidence rate in MS in Lithuania showed a tendency to increase over the period of 2001 to 2015. Currently, females are affected from 1.5 to 2 times more often than males. If this tendency remains, in 2020, females are expected to be diagnosed with MS two times more often than males.

II. Dynamics of the Working Capacity Level of MS until a Repeated Visit to the DWCAO

The second objective of the study was to analyze the dynamics of the working capacity level of MS patients in relation with sociodemographic data, employment status and life expectancy. This was done in 4 steps:

1) The evaluation of the dynamics of the working capacity level until a repeat visit to the DWCAO;
2) The analysis of the structure of the working capacity level depending on age and gender of the patients;
3) The dynamics of employment status among the patients;
4) The relationship between the death of patients, their working capacity level, and age.

As mentioned before, the working capacity level is usually set for a limited period of time (6 months, 1 year, 2 years) unless a change in health state or vocational or functional factors is permanent; then, the working capacity level is set until reaching pension. During the period of 2006–2015, 2 072 people visited the DWCAO in order to have their working capacity level estimated. Of these patients, 451 (21.7%)

visited the DWCAO twice, 291 (13.9%) – three times, 323 (15.4%) – four times, 256 (12.2%) – five times, and 170 (8.1%) patients – six times and more. While comparing the working capacity level during the first and second visits, it was noted that the result was the same among 52.5% of patients, with the working capacity increasing among 8.3% of patients and decreasing among 39.2% of patients (average time until the second visit was 1.5 years). The dynamics of MS did not depend on the gender of patients (p = .882).

Similar proportions of the working capacity level are seen when analyzing the data from the second and the third visits (11.9%, 54.7%, and 33.4%), and there is no difference between genders either (p = .540) (average time from the second to the third visit was 1.8 years). However, there are changes seen when we analyze the proportions of the third and fourth visits: the working capacity level had increased among 12.2% of patients, remained the same among 31.3%, and decreased among 56.5% (the average time from the second to the third visits was 3.0 years or on average 6.3 years since the first visit). While comparing the results of the fourth visit between genders, there were no differences found (p = .454). It proves that visiting

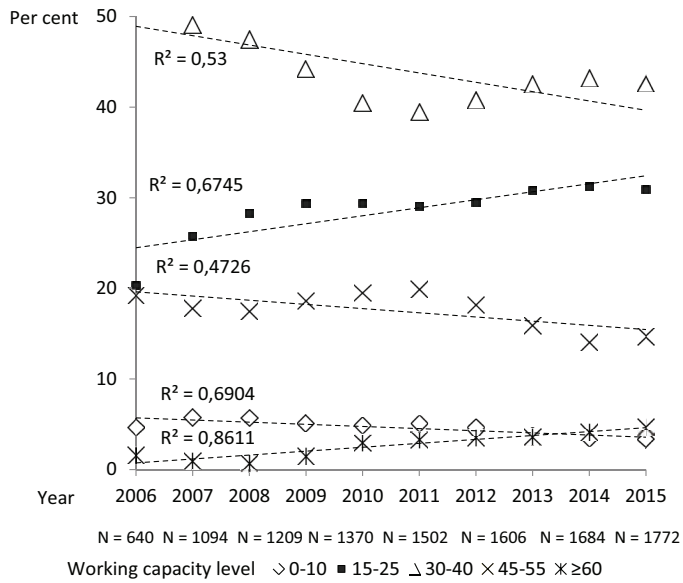


FIGURE 5. The proportion of different working capacity levels established by the DWCAO among MS patients in 2006–2015.

Notes: Data are taken on the last day of each year. If the patient had not visited the DWCAO during the analyzed year and their working capacity level was valid, it was assumed it was the same as during the previous visit.

the DWCAO repeatedly is related not only to the validity of the working capacity level but to the worsening condition of the patients as well.

Additionally, we have analyzed how many patients had MS diagnosis each year and what was their working capacity level. The data sample was N = 14 291. As it was mentioned before, the validity of the working capacity level may differ from 1 or 2 years to an unterminated duration. Thus, the gaps between repeated visits were filled with data from previous visits. The results are presented in Figure 5. It is seen that during the analyzed period, the proportion of lower working capacity level (15–25) patients present in the sample had been increasing each year (linear regression R^2 = .67). The same could be said for the proportion of the highest working capacity level (≥60) group in the sample (linear regression R^2 = .86).

Knowing that the incidence rate between men and women is different and that MS progresses with time, it was important to compare the working capacity level among different age and gender groups. The study population was divided into five groups: 25 years of age and younger, 26–35, 36–45, 46–55, and over 55. The data presented in Figure 6 show that the older the patient becomes, the lower is their working capacity level. The Pearson correlation coefficient between age and the working capacity level is r = -.313, p < .001. No statistically significant difference was found between gender groups (Chi-square = 2.785, p = .733).

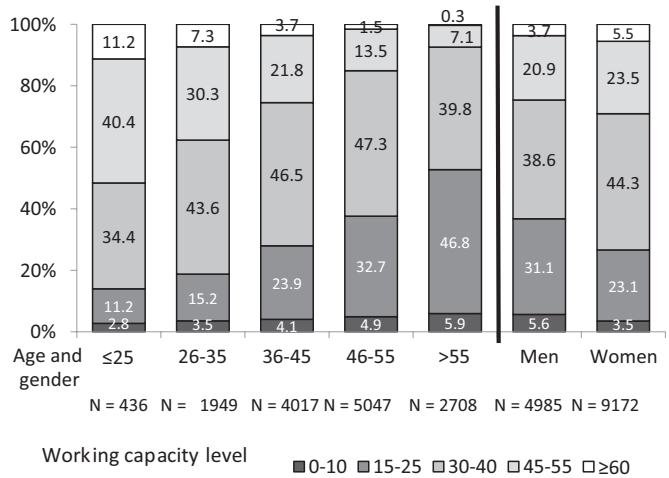
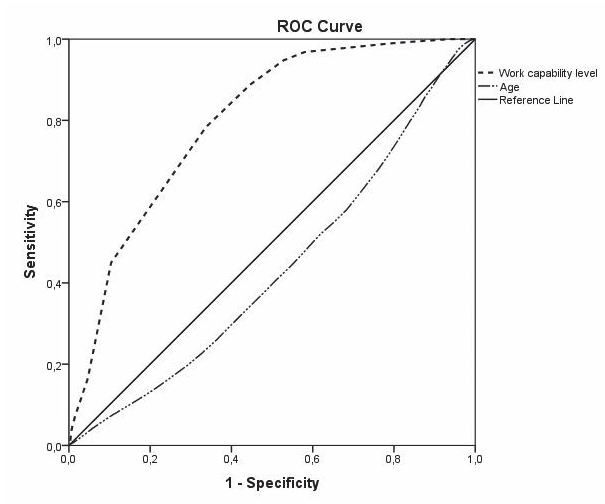


FIGURE 6. The proportion of different working capacity levels established by the DWCAO among MS patients of different age and gender groups.

Note: During the study, data on some MS patients were taken ten times, while on others just once, but it is rational to include all the data into the study because the patients were getting older (they changed their age groups), and their working capacity levels were changing as well.

The next step of our study was to analyze how the employment statuses of patients were related with their working capacities and age. A comparison of the results of patients who visited the DWCAO at least twice (N = 1 491) revealed that out of 688 (46.1%) patients who were working at the beginning of the study, 212 (30.8%) did not work during the second visit, compared to 52 (6.5%) patients who changed their employment status in the non-working group (N = 803) ($p < .001$). The total sample of working patients during the second visit was 528 (35.4%).

a)



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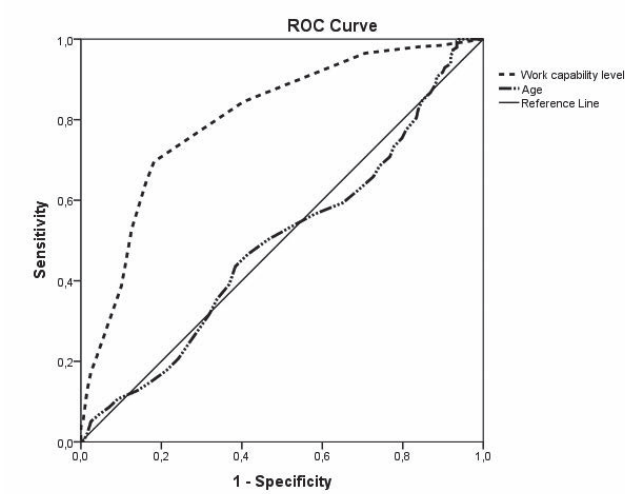

FIGURE 7. ROC curve in predicting (a) change in employment status and (b) death by working capacity level and age

In order to determine the critical value of the working capacity level when patients change their employment status from working to non-working, we conducted an ROC curve analysis (Figure 4). It was established that when sensitivity was .783, specificity was .663, and the area under the curve (AUC) (95% CI) was .797 (.789 - .804), $p < .001$, whereas the critical value of the working capacity level was 37.5.

While analyzing what is the critical value of age for the change of an employment status from working to non-working, AUC (95% CI) was .433 (.423 - .443), $p < .001$ and did not fit the minimal requirements for further analysis.

To conclude, it could be stated that a change of employment status in patients with MS is more related with the severity of the disease than with the age of a patient.

Finally, we analyzed the relationship between patient deaths, their working capacity levels and age. During the study, 198 (9.6%) patients died because of various reasons (the exact death cause was not analyzed). In order to determine the critical value of the working capacity level when patients die, we conducted an ROC curve analysis (Figure 5). The data sample was N = 2 072; that is the final status of each patient at the end of the study. It was established that when sensitivity was .696, specificity was .838, and the area under the curve (AUC) (95% CI) was .803 (.769 - .836), $p < .001$, while the critical value of the working capacity level was 22.5. While analyzing what is the critical value of age for the prediction of death, AUC (95% CI) was .495 (.453 - .537), $p = .806$, which shows no relationship between these two variables. Thus, the deaths of MS patients should be more related with the disease rather than age parameters.

Psychosocial factors of MS patients

The third objective of the study was to analyze the relationship between the working capacity level and different parameters of physical and mental health. This was done in 8 steps:

1) The relationship between the working capacity level, sociodemographic and clinical characteristics was assessed;
2) The relationship between the working capacity level and quality of life was evaluated;
3) The relationship between the working capacity level and cognitive functions was calculated;
4) A comparison of fatigue between different working capacity groups was conducted;
5) A comparison of anxiety and depression between different working capacity groups was done;

6) The results of ICF between different working capacity groups were analyzed;
7) Correlations of EDSS with all variables of the study were calculated;
8) Factors predicting a worsening of the working capacity level were identified.

Relationship between the Working Capacity Level, Sociodemographic and Clinical Characteristics

The cross-sectional study involved 184 people with MS that had different working capacity levels and visited the DWCAO a different number of times. During the first research stage, it was proved that the incidence rate in women was higher; thus, it was important to ensure that the results of that study stage could be analyzed in the whole sample. We conducted a comparison of sociodemographic and clinical characteristics between gender groups (Table 1).

TABLE 1. Comparison of sociodemographic and clinical characteristics between gender groups.

		Men N = 65	Women N = 119	p
Age		4.5 ± 11.7	41.4 ± 1.9	.604
Time from symptoms		11.0 ± 7.8	9.6 ± 8.3	.266
Time from diagnosis		3.6 ± 4.9	3.2 ± 5.2	.869
Starting level of EDSS		3.9 ± 1.4	3.7 ± 1.3	.182
Education	Basic	1 (1.5%)	3 (2.5%)	.569
	Secondary	19 (29.2%)	22 (18.5%)	
	Vocational	10 (15.4%)	20 (16.8%)	
	College	9 (13.8%)	18 (15.1%)	
	Higher	26 (4.0%)	56 (47.1%)	
Profession	(has)	50 (76.9%)	103 (86.6%)	.073
Participation in labour market	(yes)	42 (66.7%)	79 (66.4%)	.553
Type of disease	SPMS	8 (12.3%)	11 (9.2%)	.713
	PPMS	2 (3.1%)	2 (1.7%)	
	PRMS	0 (.0%)	1 (.8%)	
	RRMS	55 (84.6%)	105 (88.2%)	

SPMS – secondary-progressive multiple sclerosis, PPMS – primary-progressive multiple sclerosis, PRMS – progressive-relapsing multiple sclerosis, RRMS – relapsing-remitting multiple sclerosis.

The analysis of the data presented in Table 1 shows that both men and women were of similar age and educational background and had a similar EDSS starting level, that the duration from symptoms and disease diagnosis in both groups does not differ either, and that there were no differences between the type of disease. Thus, a further analysis of the data in the entire group is correct.

Knowing that the working capacity level is divided into 4 groups, which differ by the severity of disease, it was reasonable to analyze all these groups without joining them into some larger units.

Table 2 presents data about the relationship between the working capacity level and sociodemographic and clinical characteristics. Their analysis reveals that a lower working capacity is related to older age and longer tenure of the disease. Those

TABLE 2. Relationship between the working capacity level, sociodemographic and clinical characteristics.

		WCL 0–25 N = 30	WCL 30–40 N = 100	WCL 45–55 N = 21	WCL 60–100 N = 6	WCL unknown N = 27	p
Age		48.3 ± 1.3	42.8 ± 1.6	35.3 ± 8.0	36.0 ± 8.3	32.3 ± 9.3	**<.001** ab
Time from symptoms		14.7 ± 1.2	1.1 ± 8.1	9.2 ± 4.8	9.3 ± 8.3	6.1 ± 5.2	**.002** cd
Time from diagnosis		4.7 ± 7.5	3.1 ± 4.5	3.6 ± 4.8	4.5 ± 4.5	2.0 ± 3.4	.316
Starting EDSS level		5.7 ± .8	4.1 ± .8	2.7 ± .7	2.1 ± .5	2.1 ± .7	<.001 ef
Gender	Men	14 (46.7%)	33 (33.0%)	4 (19.0%)	1 (16.7%)	13 (48.1%)	.125
Education	Basic	2 (6.7%)	1 (1.0%)	0 (0%)	1 (16.7%)	0 (.0%)	.149
	Secondary	8 (26.7%)	18 (18.0%)	4 (19.0%)	2 (33.3%)	9 (33.3%)	
	Vocational	7 (23.3%)	17 (17.0%)	4 (19.0%)	1 (16.7%)	1 (3.7%)	
	College	4 (13.3%)	16 (16.0%)	4 (19.0%)	1 (16.7%)	2 (7.4%)	
	Higher	9 (3.0%)	48 (48.0%)	9 (42.9%)	1 (16.7%)	15 (55.6%)	
Profession	(has)	23 (76.7%)	86 (86.0%)	19 (9.5%)	3 (5.0%)	22 (81.5%)	.134
Participation in labor market	(yes)	9 (3.0%)	67 (68.4%)	14 (66.7%)	5 (83.3%)	26 (96.3%)	**<.001** ag
Type of disease	SPMS	10 (33.3%)	9 (9.0%)	0 (0%)	0 (0%)	0 (0%)	**.009**
	PPMS	1 (3.3%)	3 (3.0%)	0 (0%)	0 (0%)	0 (0%)	
	PRMS	0 (.0%)	1 (1.0%)	0 (0%)	0 (0%)	0 (0%)	
	RRMS	19 (63.3%)	87 (87.0%)	21 (100%)	6 (100%)	27 (100%)	

SPMS – secondary-progressive multiple sclerosis, PPMS – primary-progressive multiple sclerosis, PRMS – progressive-relapsing multiple sclerosis, RRMS – relapsing-remitting multiple sclerosis.

Post hoc: a - 1<2,3,4,5, b - 2>3,5, c - 1>2,3,5, d - 2>5, e - 3>5, f - 1>2>3,4,5, g - 2,3<4,5

with the lowest working capacity level had higher EDSS scores and were employed more seldom. However, we should see the positive side of the data as well: 9 out of 30 patients with working capacity of 0–25% were working. Thus, it could not be said that the most severe form of MS eliminates these people from active participation in the labor market.

Relationship between the Working Capacity Level and Quality of Life

In the second step of analysis, we compared the results of the Short Form 36 Medical Outcomes Study Questionnaire in different working capacity groups (Table 3).

TABLE 3. Relationship between the working capacity level and health-related life quality (SF-36).

	WCL 0–25 N = 30	WCL 30–40 N = 100	WCL 45–55 N = 21	WCL 60–100 N = 6	WCL unknown N = 27	p
Physical functioning	16.5 ± 18.7	37.0 ± 21.0	46.7 ± 28.5	53.3 ± 17.5	85.4 ± 14.4	**<.001**[a]
Role limitations due to physical health	3.3 ± 8.6	18.3 ± 28.4	27.4 ± 35.3	20.8 ± 18.8	72.2 ± 32.8	**<.001**[d]
Pain	45.9 ± 28.6	50.1 ± 27.3	55.6 ± 21.4	59.3 ± 32.7	71.6 ± 26.0	**.003**[b]
General health	22.7 ± 12.2	27.1 ± 14.4	30.2 ± 17.5	27.5 ± 27.0	49.8 ± 17.7	**<.001**[c]
Physical component summary	88.4 ± 42.1	132.4 ± 64.5	159.8 ± 77.1	160.9 ± 68.0	279.0 ± 71.6	**<.001**[a]
Energy/fatigue	32.3 ± 17.7	36.5 ± 17.2	39.3 ± 16.7	42.5 ± 23.6	64.1 ± 16.2	**<.001**[c]
Social functioning	25.9 ± 19.7	39.4 ± 18.6	38.6 ± 20.7	40.7 ± 23.0	63.0 ± 21.8	**<.001**[d]
Role limitations due to emotional problems	15.6 ± 32.4	37.0 ± 42.1	42.9 ± 41.0	22.2 ± 17.2	80.3 ± 33.7	**<.001**[d]
Emotional well-being	45.6 ± 20.9	53.6 ± 19.6	48.8 ± 20.7	47.3 ± 26.5	71.7 ± 14.1	**<.001**[c]
Mental component summary	119.4 ± 68.8	166.5 ± 77.0	169.5 ± 82.3	152.8 ± 79.5	279.0 ± 71.5	**<.001**[d]

Post hoc: a - 1<2,3,4<5, b - 1,2,3<5, c - 1,2,3,4<5, d - 1<2,3<5 and 4<5.

The data presented in Table 3 show that people with an unknown working capacity level had higher scores of SF-36 in all subscales comparing to those who already know their working capacity level. The analysis only of those 157 with known working capacity level demonstrates that:

- Patients with a working capacity of 0–25% had lower results in Physical functioning, Role limitations due to physical health, Physical component summary; Social functioning, Role limitations due to emotional problems and Mental component summary subscales comparing to those with working capacities of 30–40% or 45–55%.

- There is no difference between 0–25%, 30–40% and 45–55% working capacity groups in the subscales of Pain, General health, Energy/fatigue and Emotional well-being.

Relationship between the Working Capacity Level and Cognitive Functions

In the third step of analysis, we compared cognitive functions between groups (Table 4).

TABLE 4. Relationship between the working capacity level and cognitive functions.

	WCL 0–25 N = 30	WCL 30–40 N = 100	WCL 45–55 N = 21	WCL 60–100 N = 6	WCL unknown N = 27	p
Memory self-assessed	5.9 ± 2.5	5.9 ± 2.0	6.7 ± 2.4	7.0 ± 1.8	8.3 ± 1.3	**<.001** b
SDMT	30.9 ± 16.2	40.5 ± 11.7	45.4 ± 13.3	51.0 ± 15.0	50.3 ± 12.2	**<.001** ac
CVLT-II 1 attempt	5.4 ± 2.8	6.2 ± 2.0	6.5 ± 2.1	6.5 ± 2.2	6.3 ± 2.0	.390
CVLT-II 2 attempt	7.1 ± 2.9	8.5 ± 2.4	8.5 ± 2.7	9.3 ± 2.2	9.1 ± 2.5	**.038** f
CVLT-II 3 attempt	8.1 ± 3.0	9.6 ± 2.5	10.4 ± 2.5	10.7 ± 1.6	10.8 ± 2.6	.001 ac
CVLT-II 4 attempt	8.3 ± 3.3	9.9 ± 2.4	10.4 ± 2.5	12.0 ± 2.4	11.5 ± 2.3	**<.001** ac
CVLT-II 5 attempt	9.0 ± 4.0	10.4 ± 2.8	11.0 ± 2.9	11.2 ± 2.0	12.4 ± 2.7	**.001** bc
CVLT-II sum	38.0 ± 15.2	44.6 ± 10.5	46.7 ± 11.3	49.7 ± 5.1	50.0 ± 10.3	**.002** ac
BVMT-R 1 set	3.2 ± 2.2	3.9 ± 2.0	3.9 ± 2.2	5.2 ± 2.0	4.9 ± 2.6	**.039** hc
BVMT-R 2 set	4.5 ± 2.7	5.9 ± 2.5	6.4 ± 2.8	8.2 ± 1.7	7.9 ± 2.9	**<.001** ade
BVMT-R 3 set	4.7 ± 2.8	6.9 ± 2.7	7.1 ± 2.8	9.0 ± 2.5	9.0 ± 2.5	**<.001** ag
BVMT-R sum	12.4 ± 7.3	16.7 ± 6.4	17.2 ± 7.2	22.3 ± 6.0	21.7 ± 7.0	**<.001** ade

Post hoc: a - 1<2,3,4,5, b - 1,2,3<5, c- 2<5, d - 2<4,5, e - 3<5, f - 1<2,5, g - 2,3<5, h - 1<4,5

The data presented in Table 4 show that people with unknown working capacity levels had higher scores in all subscales, with the exception of CVLT-II 1 attempt. The analysis only of those with known working capacity levels demonstrates that:

- Patients with a working capacity of 0–25% had lower results in SDMT, CVLT-II 3, 4 and 5 attempts and CVTL-II sum, BVMT-R 2 and 3 sets and BVMT-R sum comparing to those with working capacities of 30–40%, 45–55% or 60–100%.

- Patients with a working capacity of 30–40% had statistically lower results in BVMT-R 2 set and in BVMT-R sum comparing to those with working capacity of 60–100%.
- There were no differences between groups in memory self-assessment and CVLT-II 1 attempt.

Relationship between the Working Capacity Level and Fatigue

In the fourth step of analysis, we compared fatigue results between groups (Table 5).

TABLE 5. Relationship between the working capacity level and fatigue

	WCL 0–25 N = 30	WCL 30–40 N = 100	WCL 45–55 N = 21	WCL 60–100 N = 6	WCL unknown N = 27	p
Fatigue self-assessed	6.6 ± 2.1	6.1 ± 2.0	5.6 ± 2.4	5.8 ± 2.7	3.8 ± 2.7	**<.001** a
FDS-Initiative	1.7 ± 0.5	1.6 ± 0.5	1.7 ± 0.6	1.5 ± 0.6	1.2 ± 0.7	**.007** b
FDS-Modality	1.6 ± 0.6	1.0 ± 0.8	1.1 ± 0.8	0.8 ± 1.0	0.4 ± 0.8	**<.001** ce
FDS-Frequency	2.1 ± 0.7	1.9 ± 0.7	1.5 ± 0.9	1.8 ± 1.0	1.1 ± 1.0	**<.001** fg
FDS-Severity	2.4 ± 0.8	2.0 ± 0.8	1.6 ± 0.9	1.8 ± 0.8	0.6 ± 0.8	**<.001** hjk
FDS-Uhthoff's effect	0.9 ± 0.3	0.9 ± 0.2	0.9 ± 0.4	1.0 ± 0.0	0.3 ± 0.5	**<.001** a
FDS sum	11.4 ± 4.3	8.8 ± 3.9	8.8 ± 4.4	7.5 ± 2.4	3.7 ± 4.3	**<.001** d

Post hoc: a - 1,2,3,4>5, b - 1,2,3>5, c- 1>2,3,4,5, d - 1>2,3,4>5, e - 2,3>5, f - 1>3,5, g - 2,4>5, h - 1>2,3,5, j - 2>3,5, k - 3,4>5

The data presented in Table 5 show that people with unknown working capacity feel less fatigue. The analysis only of those with known working capacity levels demonstrates that:

- Patients with working capacity of 0–25% had higher results in fatigue modality, severity and FDS sum comparing to those with working capacities of 30–40% and 45–55%.
- The comparison of the groups by frequency of fatigue shows that those with a working capacity of 30–40% seldom feel fatigue comparing to those with a working capacity of 0–25%.
- The groups do not differ by fatigue self-assessment, FDS initiative and FDS Uhthoff's score.

Relationship between the Working Capacity Level, Anxiety and Depression

In the fifth step of analysis, we compared the levels of anxiety and depression in different working capacity groups (Table 6).

TABLE 6. Relationship between the working capacity level, anxiety and depression.

		WCL 0–25 N = 30	WCL 30–40 N = 100	WCL 45–55 N = 21	WCL 60–100 N = 6	WCL unknown N = 27	p
HAD Anxiety		8.4 ± 4.9	7.2 ± 3.7	7.3 ± 4.2	6.3 ± 4.8	3.3 ± 4.3	**<.001** [a]
HAD Depression		11.0 ± 5.3	9.1 ± 3.8	1.4 ± 4.1	12.2 ± 6.2	6.2 ± 4.1	**<.001** [b]
HAD Anxiety	1	13 (43.3%)	51 (51.0%)	9 (42.9%)	4 (66.7%)	26 (96.3%)	**.019**
	2	9 (3.0%)	31 (31.0%)	8 (38.1%)	1 (16.7%)	1 (3.7%)	
	3	6 (2.0%)	15 (15.0%)	3 (14.3%)	1 (16.7%)	0 (0%)	
	4	2 (6.7%)	3 (3.0%)	1 (4.8%)	0 (0%)	0 (0%)	
HAD Depression	1	7 (23.3%)	37 (37.0%)	5 (23.8%)	2 (33.3%)	17 (63.0%)	**<.001**
	2	4 (13.3%)	23 (23.0%)	8 (38.1%)	0 (0%)	7 (25.9%)	
	3	11 (36.7%)	34 (34.0%)	3 (14.3%)	1 (16.7%)	2 (7.4%)	
	4	8 (26.7%)	6 (6.0%)	5 (23.8%)	3 (5.0%)	1 (3.7%)	

1 – normal, 2 – mild, 3 – moderate, 4 – severe level of depression or anxiety.
Post hoc: a - 1,2,3>5, b - 1,2,3,4>5 and 1>2

The analysis of the data presented in Table 6 shows that those with an unknown working capacity had fewer anxiety and depression symptoms. Comparing the groups with known working capacities, it is seen that those with a working capacity of -25% have more severe depression than those with working capacity of 30–40%. There were no other differences between groups.

Relationship between the Working Capacity Level and the Results of the Brief ICF Core Set for Multiple Sclerosis

In the sixth step of analysis, the results of the results of the Brief ICF Core Set for Multiple Sclerosis were compared between groups. First of all, we analyzed the distribution of different parameters in our sample (Figure 8 and Figure 9). The data revealed that 66% of the study's participants indicated that d455 – Moving around was a complete difficulty. The second biggest difficulty is d850 – Remunerative employment; this was indicated by 32% of the study's participants.

On the other hand, as many as 81% of the respondents said that b164 – Higher-level cognitive functions were not impaired. For 63% of the study's participants,

there were no problems with d230 – Carrying out daily routine, and 57% had no issues with d175 – Solving problems.

The analysis of the environmental factors (Figure 9) shows that only 7–10% of the study's participants experience negative attitudes from e310 – Immediate family or e355 – Health professionals and that it is a barrier for them. But most often, immediate family is the strongest facilitator helping to manage difficulties related to the disease.

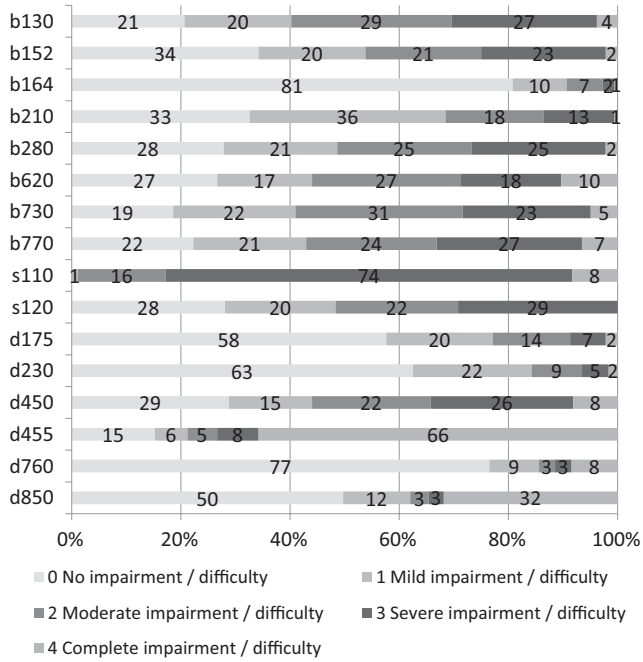

FIGURE 8. An evaluation of ICF body functions, body structure and activity & participation in percent.

Note: b130 – Energy and drive functions, b152 – Emotional functions, b164 – Higher-level cognitive functions, b210 – Seeing functions, b280 – Sensation of pain, b620 – Urination functions, b730 – Muscle power functions, b770 – Gait pattern functions, s110 – Structure of brain, s120 – Spinal cord and related structures, d175 – Solving problems, d230 – Carrying out daily routine, d450 – Walking, d455 –Moving around, d760 – Family relationships, d850 – Remunerative employment.

There was an assumption that patients with different working capacities faced different difficulties and barriers. Thus, the results were compared between groups (Table 7).

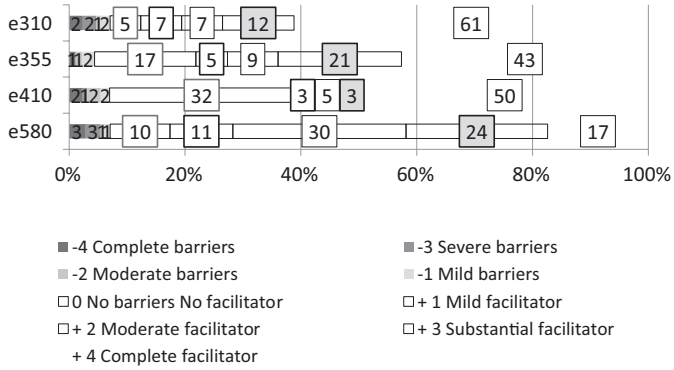

FIGURE 9. An evaluation of ICF environmental factors in percent.

Note: e310 – Immediate family, e355 – Health professionals, e410 – Individual attitudes of immediate family members, e580 – Health services, systems and policies.

This time, as in previous steps of analysis, the results of those with unknown working capacities were better than in other groups. The analysis only of the groups with known working capacities demonstrates that:

- The groups do not differ by their results of b152 – Emotional functions, b164 – Higher-level cognitive functions, b280 – Sensation of pain, s120 – Spinal cord and related structures, d760 – Family relationships, e310 – Immediate family, e355 – Health professionals, e410 – Individual attitudes of immediate family members, e580 – Health services, systems and policies.

TABLE 7. The scores of ICF between different working capacity groups.

	WCL 0–25 N = 30	WCL 30–40 N = 100	WCL 45–55 N = 21	WCL 60–100 N = 6	WCL unknown N = 27	p	Post hoc
b130	2.2 ± 1.0	1.9 ± 1.1	1.9 ± 1.3	1.2 ± 1.2	0.5 ± 0.7	**<.001**	1,2,3>5 1>4
b152	1.9 ± 1.3	1.5 ± 1.2	1.3 ± 1.3	1.7 ± 1.6	0.3 ± 0.5	**<.001**	1,2,3,4>5
b164	0.5 ± 1.0	0.3 ± 0.7	0.2 ± 0.5	0.8 ± 1.6	0.0 ± 0.2	.063	
b210	1.5 ± 1.1	1.3 ± 1.0	0.7 ± 0.7	0.7 ± 0.5	0.6 ± 0.9	**<.001**	12,>3,5
b280	1.7 ± 1.3	1.8 ± 1.2	1.4 ± 0.9	1.3 ± 1.2	0.6 ± 0.8	**<.001**	1,2,3>5
b620	2.9 ± 1.0	1.8 ± 1.2	1.2 ± 1.2	1.5 ± 1.1	0.3 ± 0.6	**<.001**	1>2,3,4,5 2>3,5 3,4>5
b730	2.8 ± 0.9	2.0 ± 1.0	1.1 ± 1.0	1.3 ± 0.8	0.3 ± 0.5	**<.001**	1>2,3,4,5 2>3,5 3,4>5

	WCL 0–25 N = 30	WCL 30–40 N = 100	WCL 45–55 N = 21	WCL 60–100 N = 6	WCL unknown N = 27	p	Post hoc
b770	2.8 ± 1.0	2.0 ± 1.0	1.2 ± 1.0	1.0 ± 0.6	0.1 ± 0.3	**<.001**	1>2,3,4,5 2> 4,5 3,4>5
s110	3.1 ± 0.6	2.9 ± 0.5	2.8 ± 0.4	2.5 ± 0.6	2.7 ± 0.5	**.002**	1>3,4,5 2>4,5
*(17/53/8/3/9) s120	1.6 ± 1.3	1.5 ± 1.2	1.5 ± 1.1	1.0 ± 1.7	1.8 ± 1.2	.891	
d175	1.3 ± 1.4	0.8 ± 1.0	0.9 ± 1.1	0.3 ± 0.5	0.0 ± 0.2	**<.001**	1>2,4,5 2,3>5
d230	1.0 ± 1.1	0.7 ± 1.0	0.5 ± 0.8	0.8 ± 1.0	0.0 ± 0.2	**.002**	1>2,5 2>5
d450	3.2 ± 0.7	1.9 ± 1.2	1.1 ± 1.1	1.2 ± 0.8	0.0 ± 0.2	**<.001**	1>2,3,4,5 2>3,5; 3>5
d455	3.8 ± 0.8	3.6 ± 1.0	3.0 ± 1.4	2.8 ± 1.3	0.2 ± 0.5	**<.001**	1>3,45 2>3,5 3,4>5
*(27/89/18/5/27) d760	0.6 ± 1.2	0.7 ± 1.4	0.4 ± 1.0	1.0 ± 1.7	0.0 ± 0.2	.111	
d850	2.8 ± 1.7	1.6 ± 1.8	1.4 ± 1.9	1.5 ± 2.0	0.0 ± 0.2	**<.001**	1>3,5 2,3,4>5
*(28/91/18/6/27) e310	7.5 ± 1.6	6.9 ± 2.0	7.1 ± 1.9	7.0 ± 1.6	5.7 ± 2.0	**.019**	1,2,3>5
e355	6.5 ± 2.1	6.6 ± 1.8	6.6 ± 1.7	6.7 ± 1.8	6.1 ± 1.6	.832	
*(21/64/13/5/26) e410	7.0 ± 1.9	6.6 ± 2.2	5.8 ± 2.5	5.8 ± 2.7	4.0 ± 0.5	**<.001**	1,2,3>5
e580	6.1 ± 1.0	6.4 ± 1.3	6.1 ± 1.7	5.8 ± 3.1	3.7 ± 2.5	**<.001**	1,2,3>5

* during the study, information was not gathered from all patients; thus, the sample size of each group is presented.

b130 –Energy and drive functions, b152 – Emotional functions, b164 – Higher-level cognitive functions, b210 – Seeing functions, b280 – Sensation of pain, b620 – Urination functions, b730 – Muscle power functions, b770 – Gait pattern functions, s110 – Structure of brain, s120 – Spinal cord and related structures, d175 – Solving problems, d230 – Carrying out daily routine, d450 – Walking, d455 – Moving around, d760 – Family relationships, d850 – Remunerative employment, e310 – Immediate family, e355 – Health professionals, e410 – Individual attitudes of immediate family members, e580 – Health services, systems and policies.

- Those with a working capacity of 0–25% have more problems in b620 – Urination functions, b730 – Muscle power functions and b770 – Gait pattern functions comparing to those with working capacity of 30–40%, 45–55% or 60–100%.

- Those with working capacity of 0–25% have more severe impairments in s110 – Structure of brain and more difficulties in d455 – Moving around comparing to those with working capacity of 45–55% or 60–100%.
- Those with working capacity of 0–25% have more difficulties in d230 – Carrying out daily routine or d175 – Solving problems comparing to those with working capacity of 30–40%; they have more difficulties related to 850 - Remunerative employment and their b210 – Seeing functions are more impaired comparing to those with working capacity of 45–55%; they also have more difficulties related to b130 – Energy and drive functions comparing to those with working capacity of 60–100%.
- Those with working capacity of 30–40% have more difficulties related with b210 – Seeing functions, b620 – Urination functions, b730 – Muscle power functions and d455 – Moving around comparing to those with working capacity of 45–55%.
- Those with working capacity of 30–40% have impaired s110 – Structure of brain and have more problems related to b770 – Gait pattern functions comparing to those with working capacity of 60–100%.

The analysis of the dynamics of different categories during the worsening of working capacity demonstrates that people in the 0–25% category could be in a lower position by more than 1 row score comparing to those with higher working capacity. For example, those with a working capacity of 0–25% indicated that d450 – Walking function was a severe difficulty, while those with the working capacity of 30–40% indicated that it was a moderate difficulty (M = 3.2 and M = 1.9 respectively). The same tendency is seen with b620 – Urination functions (M =2.9 in the 0–25% working capacity group and M = 1.8 in the 30–40% working capacity group) or with d850 – Remunerative employment (M = 2.8 and M = 1.6, respectively).

Additionally, a bigger difference than by 1 row score is seen between those with the working capacity of 60–100% and those with an unknown working capacity. That is seen in b152 – Emotional functions (M = 1.7 and M = 0.3, respectively), b620 – Urination functions (M = 1.5 and M = 0.3, respectively), d450 – Walking (M = 1.2 and M = 0.04, respectively), d455 – Moving around (M = 2.8 and M = 0.2, respectively) and d850 – Remunerative employment (M = 1.5 and M = 0.04, respectively).

To conclude, the cascaded worsening of different functions could be related with the working capacity level.

Relationship of EDSS with Life Quality, Cognitive Functions, Fatigue, Anxiety, Depression and Results of ICF

Currently, EDSS is the main instrument for the evaluation of a person's functionality in primary care. Thus, it was important to know which parameters it was related to the most. Correlations are presented in Table 8.

TABLE 8. Correlations of EDSS with research variables.

Variable	r	Variable	r	Variable	r	Variable	r	Variable	r
Physical functioning	-.628**	CVLT-II 1 attempt	-.061	Memory self-assessed	-.227**	b130	.285**	d175	.264**
Role limitations due to physical health	-.445**	CVLT-II 2 attempt	-.195**	SDMT	-.361**	b152	.229**	d230	.252**
Pain	-.244**	CVLT-II 3 attempt	-.257**	Fatigue self-assessed	.346**	b164	.016	d450	.692**
General health	-.331**	CVLT-II 4 attempt	-.342**	FDS-_initiative	.165*	b210	.233**	d455	.555**
Physical component summary	-.538**	CVLT-II 5 attempt	-.298**	FDS-_modality	.353**	b280	.229**	d760	.174*
Energy/fatigue	-.372**	CVLT-II sum	-.272**	FDS-_frequency	.370**	b620	.551**	d850	.340**
Social functioning	-.368**	BVMT-R 1 set	-.167*	FDS-_severity	.490**	b730	.648**	e310	.170*
Role limitations due to emotional problems	-.292**	BVMT-R 2 set	-.322**	FDS- Uhthoff's effect	.361**	b770	.672**	e355	-.036
Emotional well-being	-.162*	BVMT-R 3 set	-.368**	FDS sum	.428**	s110	.012	e410	.325**
Mental component summary	-.357**	BVMT-R sum	-.319**	HAD Anxiety	.281**	s120	-.353**	e580	.235**
* p < 0,05, ** p< 0,01				HAD Depression	.122				

EDSS – Expanded Disability Status Scale, CVLT – California verbal learning test II ed., BVMT–R – Brief Visuospatial Memory Test-Revised, SDMT – Symbol Digit Modalities Test, FDS – Fatigue Descriptive Scale, HAD – Hospital Anxiety and Depression Scale, b130 – Energy and drive functions, b152 – Emotional functions, b164 – Higher-level cognitive functions, b210 – Seeing functions, b280 – Sensation of pain, b620 – Urination functions, b730 – Muscle power functions, b770 – Gait pattern functions, s110 – Structure of brain, s120 – Spinal cord and related structures, d175 – Solving problems, d230 - Carrying out daily routine, d450 – Walking, d455 – Moving around, d760 – Family relationships, d850 – Remunerative employment, e310 – Immediate family, e355 – Health professionals, e410 – Individual attitudes of immediate family members, e580 – Health services, systems and policies.

Table 8 shows that the strongest EDSS correlation is r = .692, p < .01 with d450 – Walking: the greater the EDDS score, the more this function is impaired. High correlations were found with b770 – Gait pattern functions (r = .672, p < .001) and b730 – Muscle power functions (r = .648, p < 0.001). As for SF-36, the highest correlation was with Physical functioning (r = -.628, p < .001): the higher the EDSS score, the lower Physical functioning was.

Interestingly enough, there were no statistically significant correlations only with the 1st attempt of CVLT-II 1, the HAD depression scale, b164 – Higher-level cognitive functions, s110 – Structure of brain and e355 – Attitudes of health professionals.

Relationship between a Worsening Working Capacity and Life Quality, Cognitive Functions, Fatigue, Anxiety, Depression and the Results of ICF

The last step in this analysis was to assess a worsening working capacity (or EDSS) in relation with different parameters of physical and mental health. The analysis was conducted only among those study participants whose diagnoses had been made during the last five years, because scientific literature indicated that after such a period the disease becomes stabilized.[5]

There were 72 study participants who fit this criterion. The change of their condition was analyzed one year after initial assessment. A worsening of the condition was indicated if the participant had a higher EDSS score comparing to the starting one at the end of a study year – in total, 21 study participants fell within that category. Another group included those whose conditions had not change or had improved; it consisted of 51 study participants. Improvement was noted among 9 study participants, but usually that was improvement in the EDSS scale by 0.5 point.

In order to ensure that differences between groups are not affected by the initial characteristics of different group participants, the first step in analysis was group comparison based on sociodemographic and clinical parameters. Results are presented in Table 9.

The data presented in Table 9 show that groups do not differ by initial parameters, with the exception of rehabilitation. Thus, the further analysis, discovering the variables related with a worsening of the condition, was correct.

The comparison of SF-36 results between these two groups revealed that those whose condition had worsened had lower results in Physical functioning (33.6 ± 21.2 vs 53.0 ± 28.0, p= .006), Physical component summary (122.4 ± 69,8 vs 171.444 ± 89.6, p = .016) and Mental component summary (145.6 ± 88.1 vs. 193.7 ± 81.0, p = .039) comparing to those whose condition had not changed or had improved.

5 Confavreux C, Vukusic S, Moreau T, Adeleine P. "Relapses and Progression of Disability in Multiple Sclerosis." *N Engl J Med*. 2000 Nov 16;343(20):1430–8.

TABLE 9. A comparison of patients whose conditions had worsened in one year and whose conditions had not change/had improved.

		Condition worsened N = 21	Condition had not changed/ had improved N = 51	p
Age		43.5 ± 9.6	38.6 ± 12.45	.110
Time from symptoms		5.3 ± 4.0	5.3 ± 5.6	.947
Time from diagnosis		3.7 ± 4,0	3.3 ± 5.6	.733
Starting EDSS level		3.6 ± 1.1	3.3 ± 1.2	.321
Starting working capacity level		39.0 ± 9.7	39.6 ± 14.5	.858
Gender	Men	5 (23.8%)	19 (37.3%)	.206
Education	Basic	0 (.0%)	1 (2.0%)	.975
	Secondary	5 (23.8%)	13 (25.5%)	
	Vocational	4 (19.0%)	9 (17.6%)	
	College	4 (19.0%)	10 (19.6%)	
	Higher	8 (38.1%)	18 (35.3%)	
Profession	(has)	17 (81.0%)	41 (7.7%)	.617
Participation in the labor market	(yes)	14 (66.7%)	40 (8.0%)	.184
Type of disease	SPMS	2 (9.5%)	2 (3.9%)	.284
	PPMS	0 (.0%)	1 (2.0%)	
	PRMS	1 (4.8%)	0 (.0%)	
	RRMS	18 (85.7%)	48 (94.1%)	

SPMS – secondary-progressive multiple sclerosis, PPMS – primary-progressive multiple sclerosis, PRMS – progressive-relapsing multiple sclerosis, RRMS – relapsing-remitting multiple sclerosis.

The analysis of cognitive functions related with the change in condition found that Visuospatial Memory results were statistically different between groups: for those whose conditions had worsened, the sum of BVMT-R was 14.8, while for those whose conditions had not changed or had improved, this sum was 19.0 (p = .016)

The comparison of fatigue results between groups also revealed differences. Both using self-assessment and the FDS scale, it was discovered that those whose conditions had worsened felt more intense fatigue (6.3 ± 1.9 vs. 4.9 ± 2,2, p = .007), it was felt more frequently (1.9 ± 0.7 vs 1.5 ± 0.8, p =.032), the fatigue limited their functioning in different areas of life (2.2 ± 0.8 vs 1.4 ± 1.0, p = .002), and it was more often related with Uhthoff's effect (1.0 ± 0 vs .7 ± .5, p = .005) comparing to those whose conditions had not changed or had improved.

There were no differences found between groups while analyzing their emotional states (anxiety and depression).

Finally, the results of ICF were compared between those whose conditions had worsened and those whose conditions had not changed or had improved. In the group with worsened conditions, the following functions and structures were more impaired or caused more difficulties:

- Energy and drive functions (2.0 ± 1.0 vs 1.4 ± 1.1, p = .040);
- Emotional functions (1.8 ± 1.2 vs. 1.1 ± 1.1, p =.047);
- Higher-level cognitive functions (.6 ± .8 vs. .1 ± .3, p = .001);
- Walking (1.8 ± 1.2 vs. 1.1 ± 1.3, p = .048);
- Moving around (3.3 ± 1.4vs. 2.2 ± 1.8, p = .018);
- Family relationships (1.0 ± 1.7 vs. .3 ± .9, p = .048);
- Remunerative employment (2.2 ± 1.8 vs. 1.0 ± 1.6, p = .006).

In order to analyze the factors having the greatest predicting value for condition worsening (coded 1) comparing to the stability or improvement of the condition (coded 0), a logistic predictive analysis (Forward Wald) was conducted. To avoid multicollinearity, the following regressors were included into the analysis: Physical functioning, Physical component summary, Mental component summary, BVMT-R sum, FDS sum, b130 – Energy and drive functions, b152 – Emotional functions, b164 – Higher-level cognitive functions, d450 – Walking, d455 – Moving around and d850 – Remunerative employment.

The results showed that only 2 regressors – FDS sum and b164 – Higher-level cognitive functions – predicted a worsening of the condition (Model Fit $\chi 2 = 18.7$ $p < .001$; Hosmer and Lemeshow $\chi 2 = 11{,}369$ $p = .123$). With their help, 74.3% of the data can be classified correctly and the 33.2% of result dispersion could be explained (the determinant coefficient of Negelkerke).

TABLE 10. Regressors predicting a worsening of the condition in patients with MS in 1 year.

Regressors	Regression coefficient (B) (standard error)	Wald statistics	p	Exp (B)
FDS sum	.20 (.08)	6.17	.013	1.22
b164 – Higher-level cognitive functions	1.39 (.55)	6.45	.011	4.00
Constant	–2.98 (.83)	13.00	<.001	.05

Knowing that the FDS sum dispersion was equal from 0 to 17, it was reasonable to establish its critical value for predicting a condition worsening after 1 year. An ROC analysis was conducted. Its results are presented in Figure 10. The area under the curve is .728 (95% CI 0.614–0.843), p =.002. With sensitivity of .762 and specificity of .627, the critical value is 8.5; when it is exceeded, the chances of worsening increase.

The dispersion of b164 – Higher-level cognitive functions was too small; thus, an ROC analysis was not performed.

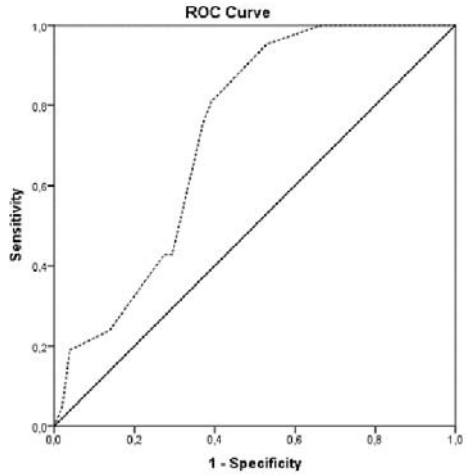

FIGURE 10. An ROC analysis predicting worsening of MS condition in 1 year by results of FDS sum

Conclusions

1. In 2000–2015, the incidence rate of Multiple Sclerosis in Lithuania was increasing (3.5 cases per 100 000 people), among women in particular. Females are affected on average 1.7 times more frequently than males, and this ratio was growing in 15 years. Should such a tendency remain, women are expected to be diagnosed with MS two times more often than men in Lithuania in 2020.
2. In time, advancing MS has impact on a person's working capacity. Before their repeat visit to the DWCAO, the MS patients' health conditions worsen and their working capacity levels decrease; however, the actual course of the disease gradually stabilizes. The critical value of working capacity predicting the individual's participation in the labor market is equal to 37.5 percent. The working capacity level decreases with increasing age; no difference between genders was identified.
3. For less than five years, a worsening of the condition of MS patients in one year is best predicted by fatigue and impairment of higher-level cognitive functions. The higher the working capacity level of the individual, the fewer the impairment specified by patients in the assessment by ICF categories.

For MS patients, Moving Around and Remunerative Employment causes the largest difficulty in activity and participation, and the brain is most damaged compared with other body structures. Inclusion of the ICF category sets into the criteria for the establishment of the working capacity level would ensure a disability assessment that is individual and comprehensive, i.e., biopsychosocial.

Practical proposals and recommendations

1. Considering that MS causes irreversible disability early on, it is crucial that medical doctors provide a timely referral to MS patients for the establishment of their working capacity levels, following a prior clinical assessment of all their impaired functions focusing on cognitive functions and fatigue, because these are the best predictors of decreasing working capacity. Poor indicators of the latter functions are the first to hinder the patients from remaining in employment.
2. We recommend using the ICF category sets in health care institutions, rehabilitation units, where teams of professionals would provide a detailed multidisciplinary description of impaired functions as well as conduct the assessment of functioning that a person with a disability can achieve with the help of auxiliary means. Such a thorough examination of patients would also enable an objective establishment of disability.
3. We recommend integrating ICF provisions into the disability establishment criteria and ensuring more objective comprehensive procedures for an individual's evaluation, including not only medical factors but also individual social factors limiting the person's activity and participation, such as social relationships and circumstances.
4. The ICF should become a widely-used practical instrument encompassing the spectrum of disease symptoms as well as functionality restrictions, taking into consideration the factors of the environment where people live; such factors influence physical and social exclusion as well as the attitude toward the disabled. For this purpose, we recommend continuing with research, since the ICF is a comprehensive classification based on a universal language understood by health professionals, investigators, policy developers, patients, and organizations of the disabled around the world.
6. We recommend adopting the universal ICF system in Lithuania; this system is applied globally by various professionals based on various parameters and for various assessments.

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SANTRAUKA

15 procentų pasaulio gyventojų – beveik 1 000 000 000 žmonių, – yra neįgalūs. Siekiant patenkinti žmonių su negalia poreikius ir teises sveikatos ir socialinės apsaugos tarnyboms reikalingi holistiniai žmogaus veiklos ir negalios vertinimo aprašai. Šiuolaikinei visuomenei svarbu, kad asmenys kuo ilgiau išliktų darbingi, savarankiški, visapusiškai aktyvūs, dalyvaujantys visose savo gyvenimo ir visuomenės srityse, todėl būtina išnaudoti visas medicininės priežiūros ir medicininės bei profesinės reabilitacijos galimybes įveikti ligos sukeltas kliūtis

asmens gyvenime ir atitolinti negalią. Kai sveikatos sutrikimai tampa ilgalaikiai ir nustatoma negalia, medicininės ir socialinės paramos klausimas tampa dar aktualesnis.

Pasaulio sveikatos organizacija (PSO) rekomenduoja negalios sunkumą vertinti remiantis Tarptautine funkcionavimo, negalios ir sveikatos klasifikacija (TFK). TFK pagrindas yra biopsichosocialinis modelis, kuriame žmogaus sveikata, negalia ir funkcionavimas nustatomas atsižvelgiant į asmens sąveiką su fizine, socialine ir psichologine aplinka. Kad negalia nėra tik asmens sveikatos problema konstatuojama ir Europos Tarybos 2017–2023 m. strategijoje dėl negalios. Joje minima, kad „negalia yra atskirų sutrikimų ir egzistuojančio neigiamo požiūrio bei aplinkos kliūčių sąveikos rezultatas. Negalia gali trukdyti neįgaliesiems naudotis visomis žmogaus teisėmis pagrindinėmis laisvėmis ir neleisti jiems veiksmingai ir lygiai su kitais dalyvauti visuomenės gyvenime. Asmenys, turintys daugybinių, sudėtingų ir kompleksinių sutrikimų, susiduria su papildomomis kliūtimis, taip pat gresia didesnis nepalankių įpročių visuomenėje įsitvirtinimas, atskirties ir skurdo rizika". Todėl kaip sprendimas siūlomas tarptautinių instrumentų, skirtų asmens kūno sandaros ir funkcijų sutrikimams bei nepalankių aplinkos veiksnių nustatymui, naudojimas. PSO atkreipia dėmesį, kad teikiant pagalbą ligoniams turi būti vertinamos ne tik sveikatos sutrikimų priežastys, bet ir ligų pasekmės, kurias galima sumažinti pritaikant aplinką, naudojant technines priemones, stengiantis paveikti ligonių elgseną.

Visgi, Lietuvos Respublikos Vyriausybės patvirtintoje Nacionalinėje neįgaliųjų socialinės integracijos 2013–2019 metų programoje į šią problemą žvelgiama šiek tiek siauriau. Joje keliamas tikslas – užtikrinti skirtingas negalias turinčių neįgaliųjų specialiųjų poreikių tenkinimą socialinės integracijos paslaugomis (socialinės apsaugos, sveikatos priežiūros, švietimo srityse). Pilnesnis vaizdas (įdiegus TFK) apie ne tik medicininius, bet ir socialinius negalios veiksnius priartintų mūsų šalyje taikomą negalios vertinimo praktiką prie rekomenduojamų tarptautinių standartų, skatintų atkreipti didesnį atsakingų specialistų ir tarnybų dėmesį į iki šiol neretai ignoruojamus negalios biopsichosocialinius veiksnius, išryškintų konkretaus paciento poreikius ir kliūtis kasdieniniame gyvenime, o tuomet jau galima tikėtis ir konkrečių priemonių, padedančių geresnei neįgaliųjų gyvenimo kokybei.

Nors biopsichosocialiniai neįgaliųjų gyvenimo aspektai sulaukia tyrėjų dėmesio, dažniausiai tyrimai yra fragmentiški. Juose skiriamas nepakankamas dėmesys kompleksinei prevencijai, diagnostikai ir negalios vertinimui, ne visada tinkamai suprantami lėtiniais susirgimais sergančių asmenų poreikiai, netinkamai įvertinamos jų galimybės funkcionuoti visuomenėje.

Pasikeitus požiūriui į asmens sveikatą ir suprantant ją ne kaip ligos nebuvimą, o kaip visavertę fizinę, dvasinę ir socialinė gerovę, pakito ir negalios samprata. Dar 2001 m. gegužės 22 d. PSO pritarė nuostatai įdiegti į praktiką TFK, ir ligų sukeltas pasekmes bei sveikatos sutrikimus vertinti trimis aspektais: organų funkcijos ir struktūros pokyčiai, veiklos bei dalyvumo sutrikimai, aplinkos ir asmenybės veiksnių kaita. Šios metodikos taikymas Vokietijoje, Prancūzijoje, Ispanijoje, Šveicarijoje, Italijoje jau įgauna pagreitį, tuo tarpu Lietuvoje turime tik pavienius bandymus taikyti šios metodikos principus medicininėje reabilitacijoje. Žinomas negalios biopsichosocialinius aspektus nagrinėjęs mokslinis tyrimas. Jame Z. Skvarciany

tyrė darbingo amžiaus asmenų, sergančiųjų piktybiniais navikais, negalumą bei su sveikata susijusią gyvenimo kokybę. Jis pabrėžė, kad nustatant neįgalumą būtina atsižvelgti į biopsichosocialinį aspektą ir vertinant sveikatos būklę sergantiems piktybiniais navikais naudoti gyvenimo kokybės klausimyną (EQ–5D–SL) bei TFK, kadangi ji atspindi pagrindines gyvenimo veiklos funkcijas ir sritis.

Darbo aktualumą didina ir tai, kad trūksta statistinių duomenų apie psichosocialinę negalią, sergamumą, ligų paplitimą, darbingumo nustatymą, nėra mokslinio pagrindimo rengiant negalios nustatymo teisės aktus. Šiandien darbingumo lygis nustatomas, vadovaujantis tik gydančio gydytojo nustatytu ligos padarinių sunkumo laipsniu, taip vadinamu baziniu darbingumo lygiu, ir bendru visiems neįgaliesiems, nepriklausomai nuo negalios pobūdžio, veiklos ir dalyvumo klausimyno įvertinimu.

Neurodegeneracinės ir uždegiminės nervų sistemos ligos – vienas reikšmingiausių ir nacionalinių iššūkių, šių ligų paplitimo mastas ir našta visuomenei nuolatos auga. Išsėtinė sklerozė – dažna ir labai svarbi autoimuninė uždegiminė demielinizuojanti neurodegeneracinė nervų sistemos liga, paplitusi Lietuvoje. Ankstesniuose Lietuvoje atliktuose moksliniuose darbuose (R. Kizlaitienė, N. Giedraitienė, L. Malcienė, R. Balnytė, R. Leonavičius, B. Daškevičienė) buvo tiriami įvairūs IS eigos, klinikinio pasireiškimo, diagnostikos aspektai, tačiau iki šiol nenagrinėti IS sergamumo ir negalios vertinimo aspektai. Verta pabrėžti, kad išsėtine skleroze (IS) dažniausiai suserga jauno amžiaus žmonės. Nors IS eigą modifikuojantis gydymas XXI amžiuje padarė didelę pažangą kontroliuojant ligos eigą, visgi bėgant laikui IS paprastai progresuoja bei sukelia ilgalaikius padarinius, kurie traktuojami kaip negalia. Dažniausia galutinė IS pasekmė yra nustatoma negalia, kuri valstybės mastu turi ilgalaikius socialinius ir finansinius padarinius. Be to, yra duomenų, kad IS sergamumas pastaruoju metu kitose šalyse dažnėja, tačiau duomenų apie IS sergamumo tendencijas Lietuvoje iki šiol neturėjome.

Sergantiems išsėtine skleroze darbingumo lygis nustatomas Kurtzke išplėstinės neįgalumo būklės įvertinimo skale bei aukščiau minėtu visiems bendru klausimynu, vertinančiu išsilavinimą, profesiją, darbo patirtį, amžių, darbinės aplinkos pritaikymą bei trumpą veiklos ir dalyvavimo klausimyną. Tačiau nėra atsižvelgiama į išsėtine skleroze sergančių specifinius pažintinių funkcijų pokyčius, jų patiriamą nuovargį, nerimą, depresiją, kas ženkliai apsunkina šių žmonių funkcionavimą, trikdo asmens veiklą ir dalyvavimą visuomeniniame gyvenime. Fizinė negalia išryškėja jau ligai progresuojant, todėl įprastinis negalios nustatymas tampa pavėluotu ir laikytinas diskriminaciniu.

Šios disertacijos rezultatai yra svarbūs ir praktikams. Atlikus asmens kompleksinį negalios vertinimą naudojant TFK, būtų žinomi asmens poreikiai ir kliūtys kasdieniniame gyvenime, kas leistų tikslingai planuoti jam reikalingas socialines ir sveikatinimo paslaugas, reabilitacijos priemones, vertinti gydymo ir reabilitacijos efektyvumą. Ir jei, žvelgiant iš galutinio vartotojo perspektyvos, individualių poreikių atliepimas lemtų didesnį asmens pasitenkinimą gaunamomis paslaugomos ir gyvenimo kokybe, tai makro lygiu šis pokytis prisidėtų prie valstybės finansinių išlaidų optimizavimo ir racionalaus žmogiškųjų išteklių paskirstymo.

Darbo tikslas

Išanalizuoti negalios vertinimo sergantiems išsėtine skleroze biopsichosocialinius aspektus.

Darbo uždaviniai

1. Įvertinti Lietuvos populiacijos sergamumo IS dinamiką.
2. Įvertinti sergančių IS darbingumo lygio dinamikos sąsajas su sociodemografiniais rodikliais, užimtumo statusu ir gyvenimo trukme.
3. Įvertinti, kokios yra asmens darbingumo lygio sąsajos su skirtingais asmens fizinės ir psichinės sveikatos rodikliais (atmintimi, nuovargiu, nerimu, depresija, pažinimo funkcijomis, fizine ir psichine sveikata, Tarptautinės funkcionavimo, negalumo ir sveikatos klasifikacijos kategorijų rinkinio rezultatais).

Ginamieji teiginiai

1. Sergamumas IS nuolat didėja, todėl būtina stiprinti šia liga sergančių ankstyvą diagnostiką ir pagalbos teikimą pradinėse ligos stadijose.
2. IS ligos sunkumo ir asmens darbingumo longitudinė sąsaja nėra linijinė: pradiniame ligos etape darbingumo lygis ženkliai mažėja, o vėliau stabilizuojasi.
3. Psichosocialiniai veiksniai prognozuoja sergančiųjų IS darbingumo lygį, todėl jų įtraukimas į neįgalumo nustatymo procedūrą užtikrintų objektyvesnį negalios vertinimą.

Darbo naujumas

Šiame darbe kompleksiškai pažvelgta į dažną ir reikšmingą negalią sukeliančią neurologinę ligą – išsėtinę sklerozę. Pirmą kartą šalyje populiaciniame lygmenyje pateikiami paskutiniųjų penkiolikos metų IS sergamumo duomenys ir apskaičiuotas sergamumo kitimas, kas leidžia prognozuoti IS sergančiųjų paplitimo tendencijas.

Panaudojant visos Lietuvos NDNT dešimties metų duomenis, pirmą kartą išanalizuotas sergančiųjų IS darbingumo lygio kitimo tendencijos pakartotinų DL vertinimų metu, įvertintos užimtumo ir gyvenimo trukmės prognozės.

Remiantis šiuolaikiniais diagnostiniais tyrimais detaliai ištirtas 184 asmenų, sergančių IS, patiriamas nuovargis, nerimas bei depresija, įvertintos pažintinės funkcijos, gyvenimo kokybė. Be to pirmą kartą asmenų su IS kūno funkcijos ir struktūros pokyčiai, veiklos ir dalyvumo apribojimai bei aplinkos veiksnių poveikis negaliai įvertinti panaudojus TFK klausimyną skirtą IS. Visapusiško tyrimo rezultatai leido fiksuoti psichosocialinių veiksnių sąsajas ne tik su pradiniu darbingumo lygio įvertinimu, bet ir šio įvertinimo pokyčiu per metus.

Taip visapusiškai IS šalyje nebuvo tyrinėta. Tikimės, kad tyrimo rezultatai padės laiku ir objektyviau nustatyti negalią, o biopsichosocialinių veiksnių įvertinimui panaudota TFK atvers naujas galimybes vertinant darbingumo lygį.

Išvados

1. 2000–2015 m. sergamumas išsėtine skleroze Lietuvoje didėjo (3,5 atvejo 100 000 gyventojų kasmet), labiau moterų tarpe. Moterys išsėtine skleroze serga vidutiniškai 1,7

karto dažniau už vyrus, ir šis santykis per 15 metų didėjo. Išliekant šioms tendencijoms, tikėtina, kad 2020 metais Lietuvoje moterys sirgs 2 kartus dažniau už vyrus.

2. Laikui bėgant progresuojanti IS paveikia asmens darbingumą. Asmenų, sergančių IS, iki pakartotino apsilankymo NDNT sveikatos būklė blogėja ir darbingumo lygis mažėja, tačiau ilgainiui ligos eiga stabilizuojasi. Kritinė darbingumo lygio vertė, prognozuojanti ar asmuo išliks darbo rinkoje, yra 37,5 proc. Darbingumo lygis mažėja didėjant amžiui, skirtumo tarp lyčių nenustatyta.
3. Mažiau nei penkerius metus IS sergančių pacientų būklės pablogėjimą per vienerius metus geriausiai prognozuoja nuovargis ir aukštesniosios pažinimo funkcijos sutrikimas. Kuo didesnis asmens darbingumo lygis, tuo mažiau kliuvinių TFK kategorijų vertinime nurodo sergantieji. Sergantiems IS didžiausi veiklos ir dalyvumo sunkumai buvo ėjimas kitais nei ėjimas būdais ir mokamas darbas, iš kūno struktūrų labiausiai pažeistos galvos smegenys. TFK kategorijų rinkinių įtraukimas į darbingumo lygio nustatymo kriterijus užtikrintų individualų, visapusišką, t.y. biopsichosocialinį, negalios vertinimą.

Praktiniai pasiūlymai ir rekomendacijos

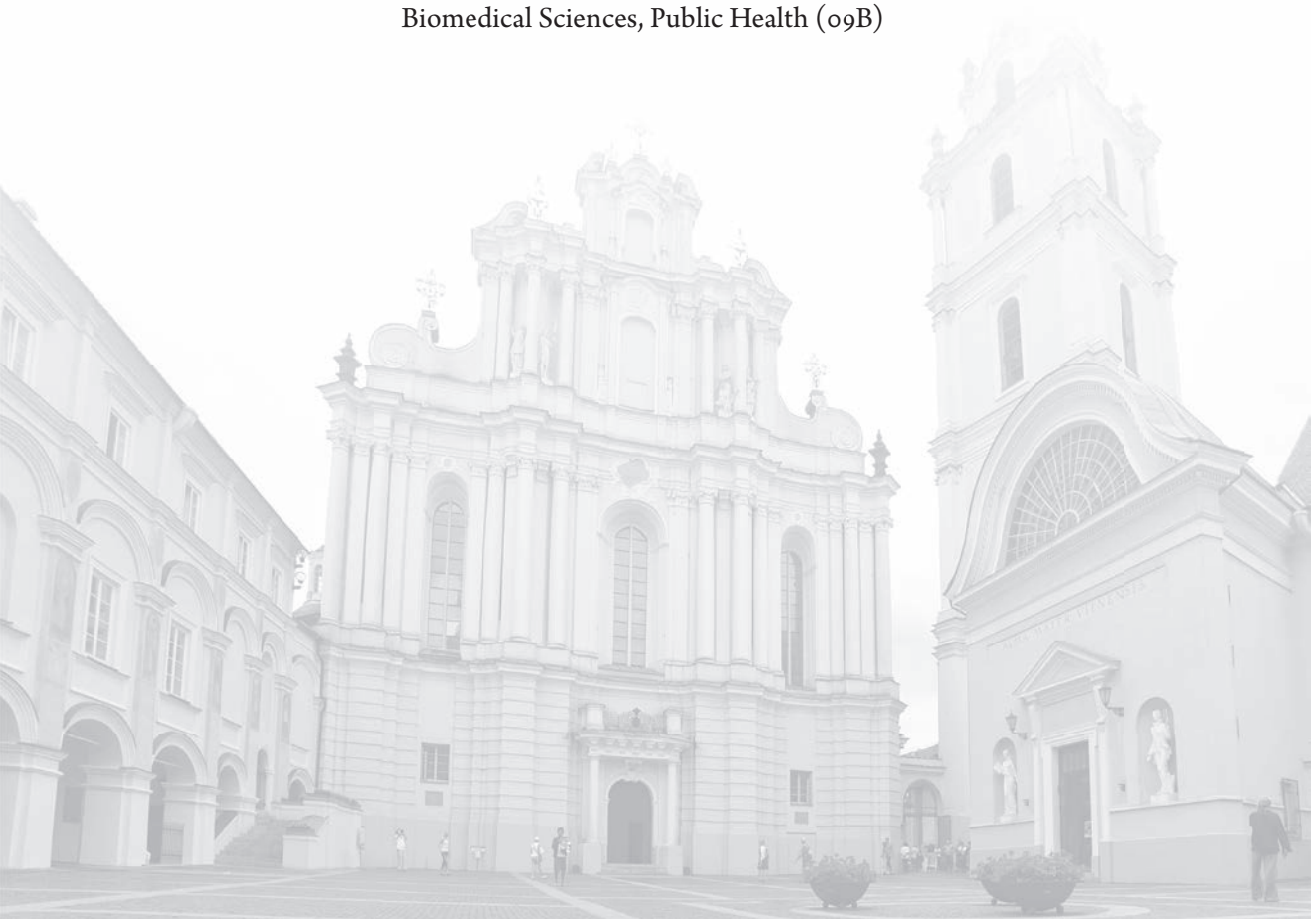
1. Kadangi IS anksti sukelia negrįžtamą negalią, svarbu, kad gydytojai sergančiuosius laiku nukreiptų darbingumo lygiui nustatyti, prieš tai kliniškai įvertinę visas sutrikusias funkcijas, atkreipiant dėmesį į pažinimo funkcijas ir nuovargį, nes tai geriausiai prognozuoja darbingumo mažėjimą. Pastarųjų funkcijų prasti rodikliai greičiausiai užkerta kelią dirbti.
2. Rekomenduojame TFK kategorijų rinkinius naudoti gydymo įstaigose, reabilitacijos skyriuose, kur specialistų komanda užtikrintų išsamų daugiadisciplininį sutrikusių funkcijų aprašymą ir apibūdinimą bei funkcionavimo, kurį asmuo su negalia gali pasiekti panaudojus pagalbines priemones, įvertinimą. Toks išsamus ligonių ištyrimas leistų ir objektyviai nustatyti negalią.
3. Rekomenduojame TFK nuostatas integruoti į neįgalumo nustatymo kriterijus, užtikrinti objektyvesnes kompleksines asmens vertinimo procedūras, kurių metu įvertinami ne tik medicininiai faktoriai, bet ir individualūs asmens veiklą ir dalyvumą ribojantys socialiniai faktoriai: socialiniai ryšiai bei aplinkybės.
4. TFK turi tapti plačiai naudojamu praktiniu įrankiu, kuris apima ir ligos simptomų spektrą ir funkcionavimo apribojimus, atsižvelgiant į aplinkos, kurioje žmonės gyvena, veiksnius, kurie įtakoja fizinę, socialinę atskirtį ir požiūrį į neįgaliuosius. Šiuo tikslu rekomenduojame tęsti mokslinius tyrimus, nes TFK yra visapusiška klasifikacija, kuri pagrįsta universalia kalba, kurią supranta sveikatos specialistai, tyrėjai, politikos kūrėjai, pacientai ir neįgaliųjų organizacijos.

Laura Steponavičienė

ATRANKINĖ MAMOGRAFINĖ PATIKRA LIETUVOJE: PROGRAMOS EIGA IR JOS ĮTAKA KRŪTIES VĖŽIO EPIDEMIOLOGINĖS SITUACIJOS POKYČIAMS

MAMMOGRAPHY SCREENING IN LITHUANIA: PROGRAM'S COURSE AND ITS INFLUENCE ON THE BREAST CANCER EPIDEMIOLOGICAL SITUATION

Biomedicinos mokslai, visuomenės sveikata (09B)
Biomedical Sciences, Public Health (09B)



Abbreviations

APC– Anual percentage change
BIRADS – Breast Imaging Reporting and Data System
BRCA1 – oncogene Breast Cancer Type 1
BRCA2 – oncogene Breast Cancer Type 2
BSE – Breast self-exam
CBE – Clinical breast examination
EU – European Union
ME – Mammography exam
MH – Ministry of Health
MRI – Magnetic resonance imaging
MS – Mammography screening
MSP – Mammography screening program
NCI – National Cancer Institute
NHIF – National Health Insurance Fund under the Ministry of Health
WHO – World Health Organization

1. Introduction

1.1. Revalence of the study

The number of achievements of contemporary medicine in the development of various means meant to avoid cancer, its treatment and cure are growing steadily, however oncological diseases remain a critical problem of public health. On a global scale, oncological diseases are one of the leading causes of mortality and disability.

Breast cancer is among the most relevant problems of female health in the world and in Lithuania also. It is the second most common malignant disease after lung cancer and the most common malignant disease of women in the entire world. Each year, more than 1.5 million new cases of breast cancer are diagnosed. In 2012, there were approximately 1.7 million (25 % of all cancer cases) new cases [http://globocan.iarc.fr]. In Lithuania, like in the rest of the world, breast cancer is the most common malignant disease of women. According to the latest data of Cancer Registry of Lithuania, 1526 breast cancer cases were identified in Lithuania (18 % of all malignant tumors) [http://www.nvi.lt/index.php?1014526156]. Compared to other states of the European Union (EU), rate of the standardized incidence of breast cancer among women in Lithuania is by half smaller (65,2 cases/100 000 residents) than in France (121 cases/100 000 residents), Holland (131 cases/100 000 residents), Finland (121,0 cases/100 000 residents), but mortality from the breast cancer in Lithuania and the said countries is nearly the same (23,4; 23,7; 26,0; 19,7 cases/100 000 residents correspondingly) [http://eco.iarc.fr/eucan/CancerOne.aspx?Cancer=46&Gender=2].

Incidence of breast cancer can be reduced through the application of the primary measures of cancer prophylaxis. The objective of the primary prophylaxis is

to reduce the incidence by affecting the factors that determine it. In pursuit of the objective to reduce the economic and social burden due to mortality from breast cancer, methods to reduce the mortality are sought systematically. Mortality from breast cancer can be reduced by applying the measures of secondary prophylaxis. Screenings of large groups of residents, comprised of individuals who do not feel any symptoms of the disease and consider themselves healthy, to detect cancer or precancerous alterations, are called screenings. Screening examination for breast cancer does not protect from the development of breast cancer but allows to identify it earlier, when the effective treatment is possible and less aggressive treatment methods can be applied. Thus the survival duration of the disease-stricken individuals is prolonged and better life quality assured. In the currently performed women's screening for breast cancer, it is recommended to use an x-ray examination of breasts – mammography as an examination distinguished for most prominent sensitivity and specificity [Greif JM. 2010].

The effectiveness of the screening mammography was evaluated in randomized clinical trials. The first trial started in the USA in 1963 [Shapiro S. 1997]. Later, similar trials were conducted in other countries. Results obtained allowed to determine how effective mammography screening (MS) is in the reduction of mortality from breast cancer. According to data from different authors, the performance of MS can reduce mortality by 20–35 % [Marmot MG, et al. 2013; Gotzsche PC, et al. 2011]. Results of randomized clinical trials had demonstrated that mammography screening could significantly reduce mortality from breast cancer; therefore, screening mammographies began in many European countries on a national or regional level. In 2012, after reviewing of 20 trials, Njor and co-authors had established that MS has an influence on reduced mortality in European countries and that mortality of women summoned to take part in MS dropped by 26% [Njor Set al. 2012]. Broeders and co-authors conducted a comprehensive review of all observationals studies in which an influence of MS in reduced mortality from breast cancer was assessed, and established that mammography screening conducted in Europe reduces mortality among women summoned to take part in screening by 25–31 %, and by 38–48 among women who took part in the screening [Broeders M, et al. 2012].

The efficiency of MS conducted in general population may differ from the efficiency established during clinical trials [10]. Differences may occur due to varying professionalism of staff taking part in MS, due to different characteristics of women taking part in MS, due to a different technique of mammography, etc. [Gabe R, et al. 2005; van Schoor G, et al. 2011]. It is noteworthy that mammography is just one element of the whole sequence of events which allows diagnosing cancer or ensures that there is no cancer. When performance of MS began in general population, and upon realization that it is a integrated and complicated process with undesirable

effects (hyperdiagnostics, X-ray radiation, pain experienced during the procedure, additional diagnostic procedures, and the effect of the psychological stress), as well as upon realizing, how important it is to ensure the appropriate quality of screening, efforts were made to develop the standards for quality assurance. Back in 1989, Day with co-authors announced the most significant efficiency measurement indicators for correctly organized MS, which are mandatory to ensure reduced mortality from breast cancer [Day NE, et al. 1989]. Therefore, already in the eighties, the need for mammography screening programs (MSP) has been realized, which will allow performing mammography screening in an organized manner, by following specific standards of quality. In 2006, upon summarizing data from numerous clinical trials and experience of various countries in performance of MS, based on guidelines previously published and reviewed in the European Union, the fourth comprehensive multidisciplinary recommendations were published, in which measures assuring the quality of the performed MSP are indicated [Perry N, et al. 2006]. In this document, the course of MSP, necessary conditions for its efficient performance is discussed at length. In each part of the recommendations, indicators (their minimum and desirable values) are pointed out, monitoring of which guarantee a smooth course of the program and good quality, and allows to expect that such program will be effective, i.e., the reduced mortality rate from breast cancer will be achieved. Performance of the MSP is assessed according to numerous criteria, which could be distributed into 2 main groups: program performance indicators necessary to assess and ensure that the program is executed correctly according to the existing recommendations, and influence indicators of the program, reflecting the influence of SMP implementation on changes of epidemiologic indicators of cancer. In Lithuania, implementation od SMP began back in 2005, however, neither the course of the program nor its effectiveness was not researched thus far.

1.2. Aim of the study

To assess the course of the mammography screening program in Lithuania and its influence on changes in the epidemiologic situation of breast cancer in Lithuania.

1.3. Tasks of the study

1. To assess the performance indicators of mammography screening program in Lithuania.
2. To assess incidence and mortality, as well as changes in early and advanced stages of breast cancer before the start of the mammography screening program and during its implementation.
3. To establish the differences of pathological characteristics of breast cancer in

groups of women who did and did not participate in mammography screening program.
4. To assess the ratio of interval cancers.
5. To establish the sensitivity of the screening mammography program.

1.4. Statements to be defended

1. Mammography screening program implemented in an organized manner can reduce mortality from breast cancer. To achieve this objective, constant monitoring of mammography screening program implementation is necessary.
2. Its implementation (participation rate, rate of repeated mammographies due to technical reasons, rate of additional summonings) and effect (reduced mortality, interval cancer rate, stages of cancers) are critically important for monitoring of the mammography screening program.
3. Aiming to assess the efficiency and sensitivity of the screening mammography program, monitoring of interval cancers is a necessary process.

1.5. Scientific novelty of the study

In Lithuania, MSP is being implemented since 2005; however, comprehensive and consistent assessment of program implementation and its influence on the epidemiological situation of breast cancer has not been executed thus far.

In works performed in Lithuania, only the quality of radiologists of the MSP and survival of women diagnosed with breast cancer, ethical aspects of MS or costs due to breast cancer were analyzed in Kaunas city region only. No assessment research, encompassing both and MSP implementention process nationwide in Lithuania, and MSP influence indicators assessment research in which contemporary cancer epidemiology methods are employed has not been performed until now.

For the first time since the start of the MSP for cancer detection, the relative interval cancer rate and sensitivity of MSP implemented in Lithuania was assessed. For the first time, an influence of the MSP on the incidence of breast cancer, distribution of diagnosed disease stages, and changes in mortality in Lithuania has been assessed. Differences of pathological characteristics of breast tumors in groups of women who did and did not participate in the MSP were assessed for the first time.

2. Material and methods

The research was conducted from 2015 to 2019 at the National Cancer Institute (NCI). The research protocol was assessed and approved by the council of the National Cancer Institute (September 28, 2016, protocol No. A37-5). Permission to carry out the biomedical research named "Analysis of the course of programs of mammographic screening and early prostate diagnostics and impact on the oncological situation in the country" was approved by Vilnius Regional Biomedical Research Ethics Committee (December 13, 2016, Protocol No. 158200-16-879-388). Permission for processing of data and database creation was received from the State Data Protection Inspectorate (April 05, 2017, protocol No. 2R-2129).

2.1. Sources of data

The primary sources of data were Cancer Registry data on newly diagnosed breast cancers and deaths caused by them in Lithuania, National Health Insurance Fund (NHIF) data about provided services within limits of the mammography screening program, and MS information system of the NCI.

Cancer Registry of Lithuania is a cancer registry of population in which personal and demographic information (place of residence, gender, date of birth and vital status), as well as information about diagnosis (cancer localization, histology, date of diagnosis and cancer identification method), and the death of the patient (date of death and cause of death). Main information sources about cancer are primary, secondary, and tertiary healthcare institutions, responsible for submission of reports when a malignant disease is newly diagnosed. All doctors, hospitals, and other institutions of the state must send a notification to the Cancer Registry of Lithuania in every case when cancer is diagnosed. In the formation of the database, other data sources are also used, based on which the currently possessed data is specified, corrected, and missing cases of oncological diseases are registered. Death certificates are one of the most critical data sources for supplementation of the incidence database and control of the quality of registration of oncological diseases, as well as for identification of the cause of death, date, and face in case of already registered oncological disease. This database contains information about cases of cancer, diagnosed in Lithuania since 1978. From 1988, data of the register were included into "Cancer Incidence in Five Continents," a central publication of the International Agency for Research on Cancer.

Data about participation in MSP of Lithunia was obtained from the NHIF. This database was created in 1999 aiming to compensate from the Social Insurance Fund for healthcare services provided to healthcare institutions. The system is used to

manage, accumulate, exchange, analyze and provide reports on healthcare services provided by healthcare institutions. In the national database, an information regarding information provided to women on MS, mammographies performed and their assessment.

Data on organizing MS and its implementation was obtained from information system of the National Cancer Institute. At the Institute, all data of women who performed a prophylactic mammogram – date of mammogram, assessment results and recommendations of two radiologists are accumulated in MSP information system. If a woman was summoned to perform additional tests, data about additional visit time, tests performed and established diagnosis are also accumulated in this system.

2.2. Fregments of the study

Several phases of study were distinguished. First of all, MSP implementation indicators and changes since its beginning until 2016 were assessed. Later, assessment of MSP influence was carried out by calculating parameters of incidence, mortality, and other surrogate indicators of MSP influence. MSP implementation and fundamental indicators were separately examined in a specialized center of screening mammography. NCI, as one of the largest centers providing services according to the MSP and has an MS information system, was selected as such center.

2.2.1. Assessment of the organization and implementation of the screening mammography program

Organization and implementation data of MSP were obtained from NHIF about provided services (informing about prophylaxis of malignant breast tumors and referral to perform mammography, service of mammography performed, and mammography assessment service (BIRAD system – Breast Imaging Reporting and Data System), and dates when these services were provided). Organizational aspects of the MSP assessed: what is the age of women participating in the SM; where from do they get information about SM; how is the response received. Dynamics of women's screening according to MSP during the period from 2006 to 2017 was assessed. The number and rate of tumors annually diagnosed by MSP in 10 000 screened women was calculated, and a portion from all diagnosed tumors in a group of women aged 50 to 69 y.o. in the period from 2006 to 2012.

During the implementation of the MSP, the rate of screen-detected cancers was calculated according to the following formula:

$$\text{Rate of screen detected cancers} = \frac{\text{Number of cances diagnosed during MSP}}{\text{Number of women screened}} \times 10{,}000$$

Screen-detected cancers were considered tumors which were histologically confirmed after BIRADS 4, BIRADS 5, and BIRADS 0 assessment categories. Only invasive breast carcinoma cases were included in the analysis because data on carcinoma in situ is not systemically accumulated in cancer register.

2.2.2. Assessment of the influence of the screening mammography program

Aiming to assess the influence of the MSP, a database was compiled by connecting data about services provided to the data of the Cancer Registry about malignant breast tumors diagnosed in the period from 2005 to 2012. During the assessment of the influence of the MSP, the distribution of screen-detected cancers according to stages of the disease has been calculated, trends in the incidence of breast cancer, changes in stages and mortality before the start of the MSP and during its implementation were assessed. An analysis of pathological characteristics of breast tumors in the groups of women who have and have not participated in MSP was performed. The interval cancer rate was established and program sensitivity was assessed. Also, differences in interval cancers from screen-detected cancers were scrutinized. Only cases of invasive breast carcinoma were included in the analysis because data on carcinoma in situ is not systemically accumulated in Cancer register.

Analysis of incidence and changes in stages included the period from 1998 to 2012, aiming to assess the trends in changes before the start of MS implementation and during its course. A period until 2012 was selected for analysis because the data of later years in Cancer Registry is not complete. Primary data accumulated in Cancer Registry database about diagnosed breast tumors during the asses period – dissemination of the disease, stage, histological form, also demographic characteristics of those sick with the disease was used. Women were divided into 3 age groups: from 0 to 49 y.o., 50 to 69 y.o. (target population summoned to take part in MS); 70 y.o. and older, aiming to assess the influence of the SM within the target population. The research period was divided into 2 periods: a period before MS (from 1998 to 2005) and a period of MS implementation (from 2006 to 2012). Breast tumors less than 20 mm in size with no identified metastases in lymph nodes were considered stage I cancer. Tumors of advanced stages (II+) included tumors T2, T2, and T4 tumors, and all those with metastases in lymph nodes. Tumors, stage of which is not indicated in Cancer Registry are separated into a separate category. To assess changes, an age standardized incidence was calculated (the direct standardization method was applied). Standardized incidence (mortality) rate (IR) was calculated from age-specific incidence (mortality) rate (wi), and coefficient indicating which standard part of the population is comprised of that age group (wi):

Standardized rate = $\Sigma(ri\ wi)/\Sigma(wi) \times 10{,}000$
ri – incidence (mortality) rate in a specific age group i,
wi – part of standard population in a specific age group i.

Analysis of changes in mortality included the period from 1998 to 2017. The aim was to assess the trends of changes before the start of MS implementation and during its course. Just as in case of calculation of changes in incidence, women were divided into 3 age groups: from 0 to 49 y.o.; from 50 to 69 y.o. (target population summoned to take part in MS); 70 y.o. and older, aiming to assess the influence of the MS on mortality in the target population. The period of research was divided into 2 periods: a period before MS (1998 to 2005) and a period of MS implementation (from 2006 to 2017). Age standardized mortality rates were calculated for assessment of changes (the direct standardization method was applied).

Analysis on breast cancer incidence, changes in the division of stages of the disease, and mortality were performed by using segmented regression model, in which trend is explained by a specific number of linear segments and joinpoints in which the change is established. This regression model was also used for identification of number and location of joinpoints, and calculation of annual average changes. The largest number of joinpoints was three in every age category. Location of best joinpoint of each model (i.e., year and their 95 % of confidence intervals) was identified by using the grid search method algorithm. Directions of changes were assessed by presuming that data is approximated by Poisson distribution (which is used in cancer epidemiology, since it's considered that the cancer is a rare random event and presumption about normality is not made), and by using log-linear regression equation $(y) = b + xb + a$, where x is an independent variable year, y is incidence or mortality rate, b is a coefficient of regression, a is an error (assessed by making presumption about equality of dispersions). The logarithm of incidence or mortality in the model is a linear function of time (year), which shows internal annual percentage change (APC) of the rate in percentage when antilogarithmized.

Aiming to compare the main characteristics of tumors diagnosed to women who have ever participated and have not participated in the MSP, two groups of women were compiled: women who were diagnosed with breast cancer after 2006 and who participated in MSP (the cancer was diagnosed not necessarily during MS), and women to whom breast cancer was identified after 2006, and those who never participated in the MSP. Differences were assessed according to age age at the diagnosis, histologic type, stage of the disease, size of the tumor, presence of metastases in lymph nodes, presence of distant metastases and the degree of differentiation of a tumor. The same parameters were also analyzed when comparing screen-detected cancers with interval breast cancers.

Interval cancer rate was calculated according to the formula:

$$\text{Interval cancer rate} = \frac{\text{Number of interval cancers}}{\text{Number of women screened}} \times 10{,}000$$

Interval cancers were considered malignant breast tumors, identified in the period of 90–730 days (period of one screening cycle) after performed mammography if no changes were seen in the mammography, or if the changes were assessed as non-malignant (BIRADS 1, 2 and 3).

The ratio of interval cancers was calculated according to the methodology proposed by Andersen and co-authors:

$$\text{Interval cancers ratio} = \frac{\text{Interval cancers}}{\text{Interval cancers} + \text{ screen} - \text{detected cancers}}$$

Sensitivity of the program was calculated according to formula:

$$\text{MSP sensitivity} = \frac{\text{Screen-detected cancers}}{\text{"Screen-detected cancers" } + \text{all interval cancers}}$$

The sensitivity of the program was calculated dependingly from age and the histological variant of breast cancer. Women were divided into 5 age groups: 50 to 54 y.o.; 55 to 59 y.o.; 60 to 64 y.o.; 65 to 69 y.o.; 70 y.o. and older. For assessment of the program sensitivity according to the histological variant of cancer, 2 groups were separated: lobular carcinoma of the breast and ductal and carcinoma of the breast.

2.3. Statistical data analysis

For assesment of qualitative attributes, rates (n) of values and percentages of relative rates (%) were calculated. The sensitivity of the program is presented in percentage by providing 95 % confidence intervals. Comparison between groups was performed by using t criterion for the significance of the difference of averages, and by using Chi-square criterion for the difference of proportions. Differences between groups were considered statistically significant if p value was less than 0.05.

Statistical analysis was performed by using computer statistical software package SPSS 17.0 and Microsoft Excel software. Analysis of breast cancer incidence, changes in the distribution of disease stages and mortality was carried out by using JOINPOINT software (version 4.3.1.0), APC considered significant if p value was less than 0.05.

The graphic depiction was implemented by using Microsoft Excel software.

3. Results

3.1. Organization of the screening mammography program in Lithuania

Services of the elective screening mammography program are implemented in Lithuania since October 2005. According to the order of the Minister of Health of the Republic of Lithuania No., V-729, issued on September 23, 2005, "Regarding Funding Approval of the Mammography Screening Program For Breast Cancer", preventive services of three types are provided: informing about prophylaxis of malignant breast tumors; performing mammograms; assesment of mammograms. The methodology of implementation of program services is regulated in the order of the Minister of Health of the Republic of Lithuania No., V-901, issued on December 10, 2004, "Regarding Approval of Methodology of Mammography Screening for Women." According to the approved methodology, the patient receives information about the screening and referral for examination from the family doctor. After receiving the referral, women go to the certified center where mammography is performed and assessed. Mammograms are done in two directions (craniocaudal and mediolateral). Mammography can be both film-screen and digital or fully digital. Two independent radiologists evaluate performed mammograms under the BIRAD (Breast Imaging-Reporting and Data System) system, ACR (American College of Radiologists) system is used to assess breast tissue density. The reply is sent within two weeks to the family doctor who issued a referral. In Lithuania, mammograms according to MS, is performed every 2 years. The target population: women aged 50 to 69 years old. 19 certified centers were registered at the beginning of the program, in 2016, 31 such centers were registered. Some centers provide only mammography performance service, others provide both performance and assessment services. Only 5 of these centers in Lithuania perform further testing of the identified changes. Specialized treatment is also available in these centers. Additional summoning to specialized centers is usually performed when opinions of two radiologists do not match or when suspicious changes are identified. Only in specialized centers such procedures like a magnification of the suspicious trouble-spots, mammogram by using additional breast compression, breast tomosynthesis, ultrasound examination, core needle biopsy or vacuum-assisted biopsy, and breast MRI, or excisional biopsy are performed.

According to the order on program services implementation methodology, only a few indicators are assessed: number of women invited to take part in the program, number of perfomed and assessed mammograms, and funding of a program.

3.2. Indicators of screening mammography program implementation

According to NHIF data, the number of screened women from the target population is continuously increasing. This number has increased from 47 440 women in 2006 up to 106 403 women in 2017. In 2006, 14% of women from the target population were screened, and in 2017, there was 24.3%. The number of target population and women screened is presented in table 1.

TABLE 1. Participation in MSP in Lithuania from 2006 to 2017

Year	Target population	Women screened	Part from the target population, %
2006	347487	48540	14.0
2007	351518	47396	13.5
2008	356621	52670	14.8
2009	365987	59150	16.2
2010	379063	61537	16.2
2011	399730	76902	19.2
2012	406323	83278	20.5
2013	405494	81227	20.0
2014	434969	93372	21.5
2015	438196	97072	22.2
2016	435580	100101	23.0
2017	437441	106403	24.3

TABLE 2. Breast cancer cases in Lithuania from 2006 to 2012

Year	All diagnosed breast tumors	Breast cancers in age group 50 to 69 y.o.	Screen-detected cancers	Number of cancers diagnosed during MSP/10000 women screened	% of the cases diagnosed in the age group 50 to 69 y.o, %
2006	1436	765	114	23.5	14.9
2007	1378	653	195	41.1	29.9
2008	1534	742	187	35.5	25.2
2009	1527	733	190	32.1	25.9
2010	1490	735	180	29.3	24.5
2011	1544	798	206	26.8	25.8
2012	1531	784	225	27.0	28.7

The number of new cases of breast cancer in Lithuania is growing every year. Approximately 1500 new cases of breast cancer are diagnosed in Lithuania each

year; however, breast tumors identified during MS comprise just a small portion of all tumors, diagnosed among women aged from 50 to 69 y.o. (table 2).

In 2006, 114 breast cancer cases were diagnosed during MS, in 2012 – 225 new cases of breast cancer. During the implementation of MS in the analyzed period, 1297 breast tumors were identified in total. The most prominent frequency of tumors identified during SM was in 2007 – 41.1 case in 10 000 women screened. During the implementation of the MS, the frequency of identified tumors was 30.2 cases in 10 000 women screened (1297/429 473). The part of tumors identified during the MS is increasing; however, only approximately a quarter of tumors, diagnosed in the target population are identified during MS.

3.3. Indicators of the impact of the screening mammography program

Distribution of screen-detected cancers by stage

In the period from 2006 to 2012, during MS, 632 I stage tumors were identified (48.7%), 441 stage II tumors (34.0%), 163 stage III tumors (12.6%), and 35 stage IV tumors (2.7%). Majority of tumors identified during the implementation of the program were of I stage (48.7%), however, even 15% of tumors identified during the the program were of the advanced (III od IV) stage. Distribution of tumors identified during MS according to stage of the disease is presented in figure 1.

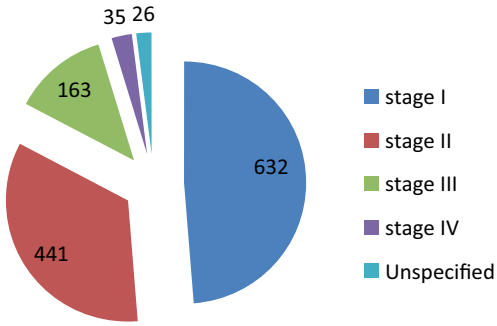


FIGURE 1. Distribution of screen-detected cancers by stage, in Lithuania from 2006 to 2012

Changes in breast cancer incidence before the start of MSP implementation and during its course

From 1998 to 2012, 13 874 cases of invasive breast cancer were diagnosed in Lithuania in total. During the research, the standardized incidence rate increased from 56.8 cases/100 000 women to 71.7 cases/100 000 women. Breast cancer inci-

dence increased by 1.6% each year (95 % CI=1.1;2.1). (figure 2). Breast cancer incidence rates and changes in separate age groups are presented in table 3 and figure 3.

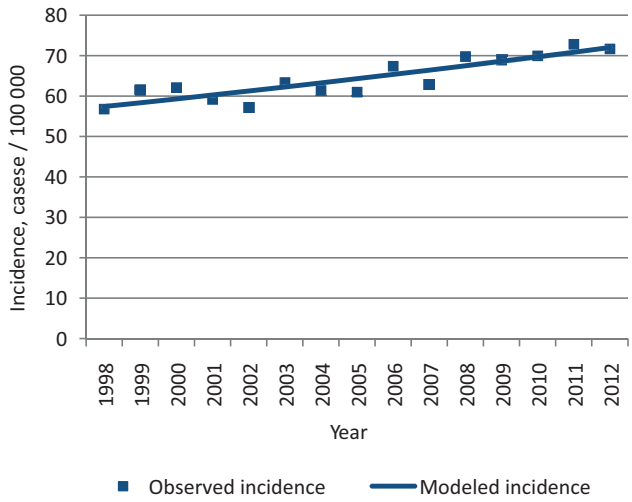


FIGURE 2. Trends in breast cancer incidence in Lithuania from 1998 to 2012

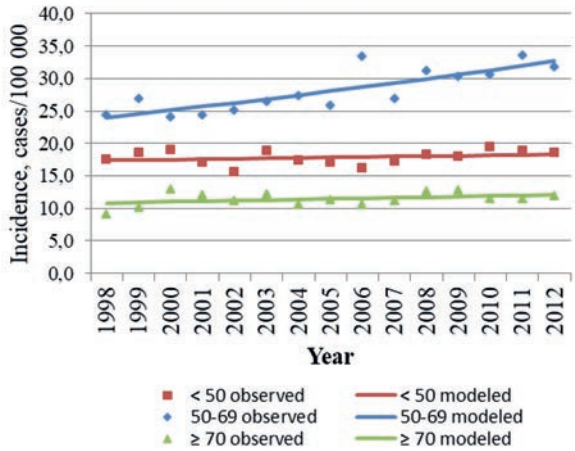


FIGURE 3. Age standardized incdidence rates by age groups in Lithuania from 1998 to 2012

TABLE 3. Age standartized breast cancer incidence rates and changes in age groups in Lithuania from 1998 to 2012

Age group	1998		2012		APC	95 % CI
	Newly diagnosed cases	Incidence rate	Newly diagnosed cases	Incidence rate		
< 50	310	17.5	285	18.5	0.4	[-0.4;1.1]
50–69	591	24.4	788	31.8	2.2	[1.3;3.1]
≥ 70	266	9.1	461	11.9	0.8	[-0.4;1.9]
In total	1167	56.8	1534	71.7	1.6	[1.1;2.1]

Changes of incidence of early and advanced stages of breast cancer before the start of the program implementation and during its course

Main characteristics of malignant breast tumors are presented in table 4. More tumors of early stages were identified during MSP implementation period (29.1%) that before SMP (18.7%), meanwhile the part of advanced tumors decreased from 78.4 % during the period before start of MSP down to 63.7 % during MSP implementation period.

TABLE 4. Characteristics of the newly diagnosed breast cancer cases before MSP implementation (from 1998 to 2005) and during its course (from 2006 to 2012)

	1998–2005		**2006–2012**		**P value**
	Newly diagnosed tumors		Newly diagnosed tumors		
	Cases	Percent	Cases	Percent	
Breast tumors	10252	100	10492	100	-
Age group					<0.001
< 50	2551	24.9	2214	21.1	
50–69	4828	47.1	5239	49.9	
≥ 70	2873	28.0	3039	29.0	
TNM Stage					<0.001
Stage I	1915	18.7	3054	29.1	
Stage II +	8033	78.4	6687	63.7	
Unspecified	304	3.0	751	7.2	

In all age groups, the incidence of stage I breast cancer constantly grew, meanwhile, the incidence of advanced breast cancer decreased (figure 4). It was established that the incidence of stage I breast cancer increased by 7.9 % each year. From 1998 to 2012, the increasing incidence was observed in all age groups – in the age group under 50 y.o. by 3.6 %, in target population by 10.2 %, and in age group older

than 70 y.o. by 6.2 %. The incidence of of advanced breast cancer significantly decreased by 1.1 %, 1.2 %, 1.1 %, and 1.6 %, in all age groups, in the group under 50 y.o, in the group of 50 to 69 y.o, and in the group of 70 y.o. and older, respectively.

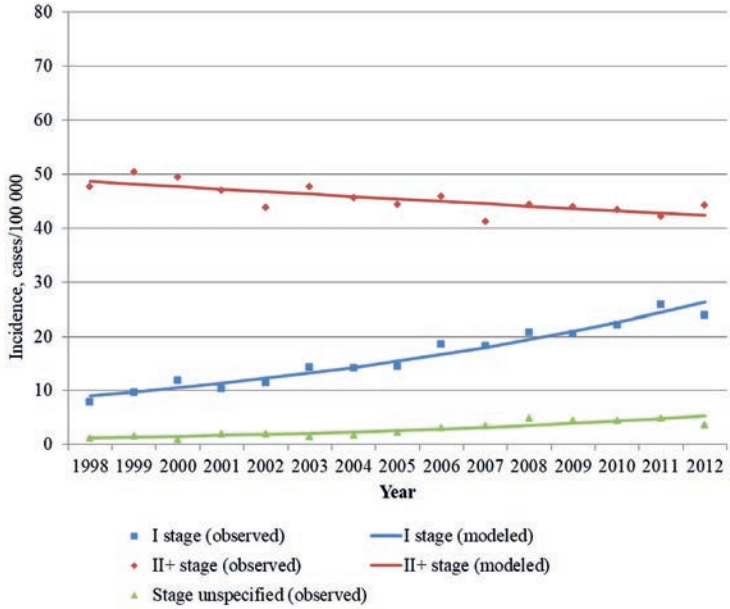


FIGURE 4. Breast cancer incidence rates in Lithuania according to the stage of the disease from 1998 to 2012

Changes of incidence rates based on stages of the disease and age groups are presented in figure 5.

Changes of incidence rates based on stages of the disease and period in age groups (before the start of MS and during its implementation) are presented in table 5.

During the study period, the incidence in the target population increased from 3.3/100 000 in 1998 up to 12.2/100 000 in 2012. Increasing incidence of stage I breast cancer was more substantial during the period before MS start than during its implementation period (from 2006 to 2012) (10.3 % and 5.7 % respectively). Throughout the entire period a small but statistically significant decrease of incidence of advanced stages of breast cancer was monitored (-1.1% each year), however, in the period of MS implementation, no significant changes were found in the incidence of advanced stage breast cancers. Similar changes were observed in the age group older than 70 y.o. Incidence of stage I breast cancer significantly increased by 13.4 % each year during the period before the MS start and increased statistically insignificantly

TABLE 5. Changes in breast cancer incidence before the start of MS (1998 to 2005), and during its implementation (2006 to 2012) according to age and cancer stage in Lithuania

Incidence based on age groups	1998		2012		AMP	95 % CI	1998–2005		2006–2012	
	Newly diagnosed cases	Incidence rate	Newly diagnosed cases	Incidence rate			APC	95 % CI	APC	95 % CI
All age groups	1167	56.8	1534	71.7	1.6	[1.1;2.1]	0.6	[-0.9;2.1]	1.7	[0.1;3.3]
Stage I	162	7.9	475	23.9	7.9	[6.8;9.0]	8.1	[4.4;11.9]	5.6	[2.8;8.5]
Stage II+	978	47.7	958	44.3	-1.0	[-1.5;-0.5]	-1.4	[-2.8;0.0]	-0.3	[-2.1;1.5]
Unspecified	27	1.2	101	3.6	11.0	[7.5;14.5]	7.6	[-0.9;16.8]	3.6	[-5.0;13.0]
< 50 y.o.	310	17.5	285	18.5	0.4	[-0.4;1.1]	-0.7	[-3.2;1.8]	2.2	[0.4;4.0]
Stage I	49	2.7	93	6.1	3.6	[2.2;5.0]	3.2	[-2.8;9.6]	4.1	[1.5;6.9]
Stage II+	256	14.5	180	11.7	-1.2	[-2.2;-0.3]	-2.2	[-4.9;0.6]	1.2	[-1.8;4.3]
Unspecified	5	0.3	12	0.8	8.5	[4.3;12.9]	4.6	[-11.3;23.3]	4.8	[-5.1;15.6]
50–69 y.o.	591	24.4	788	31.8	2.2	[1.3;3.1]	0.9	[-0.8;2.7]	0.8	[-2.8;4.6]
Stage I	86	3.3	297	12.2	10.2	[8.4;12.0]	10.3	[5.6;15.2]	5.7	[1.6;9.9]
Stage II+	493	20.8	465	18.6	-1.1	[-1.9;-0.3]	-1.5	[-3.4;0.4]	-1.9	[-5.7;2.0]
Unspecified	12	0.3	26	1.0	9.6	[4.7;14.8]	8.4	[-6.0;24.9]	0.4	[-12.0;14.6]
≥ 70 y.o.	266	9.1	461	11.9	0.8	[-0.4;1.9]	1.4	[-3.0;6.0]	1.0	[-2.3;4.5]
Stage I	27	0.9	85	2.3	6.2	[4.0;8.5]	13.4	[8.2;18.9]	4.7	[-0.8;10.6]
Stage II+	229	7.9	313	8.1	-1.6	[-3.0;-0.2]	-0.8	[-6.1;4.8]	-1.2	[-4.9;2.6]
Unspecified	10	0.3	63	1.4	13.1	[8.7;17.8]	10.6	[-0.1;22.5]	6.0	[-8.4;22.6]

by 4.7 % during the period of MS implementation. Meanwhile, in the age group up to 50 y.o., the incidence of stage I breast cancer significantly increased during the period of MS implementation. No significant changes in the incidence of advanced breast cancer stages in groups up to 50 y.o. and older than 70 y.o. have been identified during the period of MS implementation (the period from 2006 to 2012).

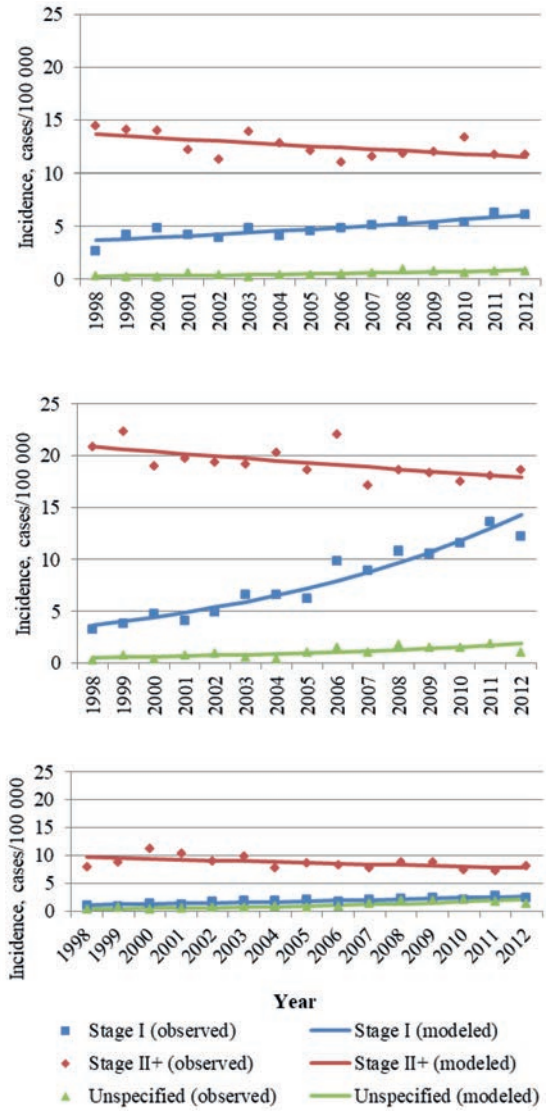


FIGURE 5. Breast cancer incidence rates according to the disease stages in groups under 50 y.o. (a), 50 to 69 y.o. (b), and older than 70 y.o. (c) from 1998 to 2012 in Lithuania

Changes in breast cancer mortality before the start of the program and during its course

The age standardized mortality rate in 1998 was 25.1/100 000, and in 2017, it was 18.6/100 000. During the study period, mortality was statistically decreasing by -1.1% each year (95% CI=-1.3;-0.9). The decrease in mortality was also observed in all age groups, but in the group older than 70 y.o. it has not been statistically significant. Mortality rates and their changes are presented in table 6.

TABLE 6. Breast cancer mortality rates and changes in age groups in Lithuania from 1998 to 2017

Age group	1998		2017		APC	95 % CI
	Number of deaths due to breast cancer	Mortality rate	Number of deaths due to breast cancer	Mortality rate		
< 50	103	5.8	42	3.0	-2.1	[-2.5.;-18]
50–69	266	13.6	189	9.4	-1.4	[-1.6;-1.2]
≥ 70	166	5.7	258	6.2	0.2	[-0.2;0.5]
In total	535	25.1	489	18.6	-1.1	[-1.3;-0.9]

Changes in mortality in all age groups and according to age groups are presented in figures 6 and 7.

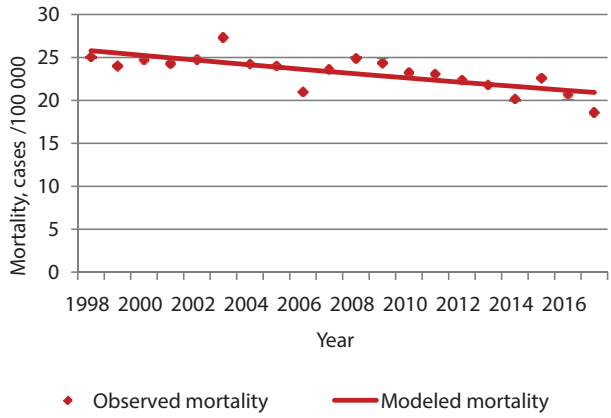


FIGURE 6. Age standardized mortality rates in Lithuania in the period from 1998 to 2017

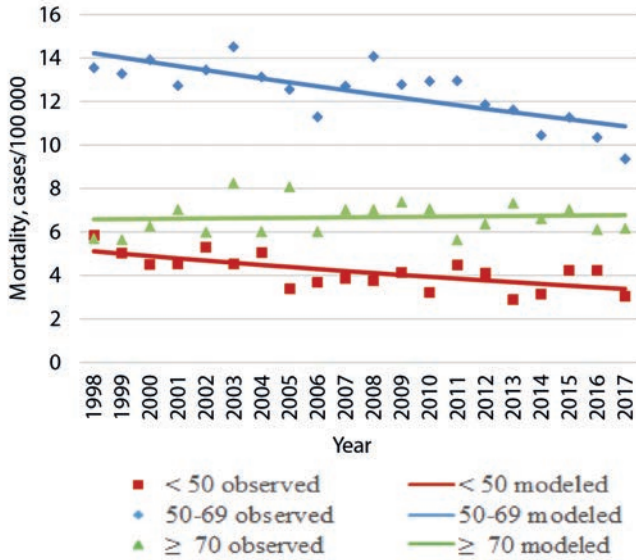


FIGURE 7. Age standardized mortality rates by age groups in Lithuania in the period from 1998 to 2017

Differences in pathological characteristics of breast tumors in groups of women who have and have not participated in the mammography screening

After comparing the main characteristics of tumors diagnosed to women who have ever participated in MSP and women who have not, it was established that tumors of smaller size were diagnosed to women who took part in MSP. T1 tumors (<2 cm) in the group of women who participated in MSP comprised 60.8% of all diagnosed tumors, meanwhile in the group of women who have not participated in MSP these tumors comprised only 31.4%. In the group of women who have not participated in MSP, a much larger number of T4 tumors (with skin lesion) was identified: 13.7% in comparison to 3.0% in the group of women who took part in MSP. In the group of women who took part in MSP, well-differentiated tumors (13.8%) were far more often diagnosed than in the group of women who were never screened (7.5%). Metastases in regional lymph nodes were more often identified in the group of women who never took part in MSP (46.0% in comparison to 35%). Women who took part in MSP were twice more often diagnosed with stage I tumors (46.3%) than women who have never participated in MSP (21.4%). The main characteristics of tumors diagnosed from 2006 to 2012 to women who have ever participated in MSP and to those who have not are presented in table 7.

TABLE 7. Pathological characteristics of breast tumors diagnosed to women who have and have not participated in MS

Characteristic	Have participated in MSP		Have not participated in MSP		P value
	Cases	%	Cases	%	
Overall number of tumors	2053	44.2	2592	55.8	-
Histological type					<0.001
Ductal carcinoma	1495	72.8	1676	64.7	
Lobular carcinoma	208	10.1	181	7.0	
Other	280	13.6	465	17.9	
Unspecified	70	3.4	270	10.4	
Size of a tumor (according to TNM)					<0.001
T1	1249	60.8	813	31.4	
T2	594	28.9	897	34.6	
T3	45	2.2	137	5.3	
T4	61	3.0	356	13.7	
Unspecified	104	5.1	389	15.0	
Metastases in lymph nodes					<0.001
Present	718	35.0	1193	46.0	
None	1156	56.3	872	33.6	
Unspecified	179	8.7	527	20.3	
Distant mestastases					<0.001
Present	56	2.7	309	11.9	
None	1736	84.6	1605	61.9	
Unspecified	261	12.7	678	26.2	
Stage					<0.001
I	951	46.3	555	21.4	
II	702	34.2	953	36.8	
III	289	14.1	555	21.4	
IV	61	3.0	348	13.4	
Unspecified	50	2.4	181	7.0	
Degree of differentiation					<0.001
G1	284	13.8	194	7.5	
G2	681	33.2	823	31.8	
G3	386	18.8	437	16.9	
G4	0	0.0	0	0.0	
Unspecified	702	34.2	1138	43.9	

Interval cancers and sensitivity of mammography screening program

Throughout the entire period of the study, 431 cases of interval cancers were diagnosed among women to whom mammography was performed according to MS, and no signs of tumors were visible (assessment according to BIRAD system: BIRADS1, BIRADS2, BIRADS3). Interval cancer rate is presented in table 8.

TABLE 8. Interval breast cancer rate in Lithuania from 2006 to 2012

Year	Interval cancers	Interval cancer rate /10,000 womn screened	Interval cancer ratio
2006	40	8.24	0.26
2007	64	13.50	0.25
2008	74	14.05	0.28
2009	84	14.20	0.31
2010	89	14.46	0.33
2011	62	8.06	0.23
2012	18	2.16	0.07

Interval cancer ratio fluctuated from 0.23 to 0.33. Interval cancer rate of the year 2012 is low because these women were observed for only 1 year after mammography was performed. Overall relative interval tumor rate was 0.25 (431/431+1279).

Overall sensitivity of MSP in Lithuania reached 75.1% (95% CI =71.1;79.3). The sensitivity of a program according to age and histological type of tumor is presented in tables 9 and 10. Age and histological type of the tumor had an impact on the sensitivity of the program. Lesser sensitivity of the program was observed in the age group 50 to 54 y.o. (69.0%; 95% CI=54.5;87.4), especially among women diagnosed with lobular tumor (60.0%; 95%. CI =19.4;186.0).

TABLE 9. MSP sensitivity in age groups in Lithuania from 2006 to 2012

Age group	Screen-detected cancers		Interval cancers		Sensitivity of the program, % [95% CI]
	Cases	%	Cases	%	
50–54	69	5.3	31	7.2	69.0 [54.5;87.4]
55–59	368	28.4	121	28.1	75.3 [67.9;83.4]
60–64	442	34.1	140	32.5	75.9 [69.2;83.4]
65–69	405	31.2	129	29.9	75.8 [68.8;83.6]
In total	1297	100.0	431	100.0	75.1 [71.1;79.3]

TABLE 10. Sensitivity of MSP according to histological tumor type and age in Lithuania from 2006 to 2012

Age groups	Screen-detected cancers		Interval cancers		Sensitivity of a program, % [95% CI]
	Cases	%	Cases	%	
Lobular carcinoma					
50–54	3	2.4	2	3.7	60.0 [19.4;186.0]
55–59	41	33.3	11	20.4	78.8 [58.1;107.1]
60–64	42	34.1	23	42.6	64.6 [47.8;87.4]
65–69	37	30.1	18	33.3	67.3 [48.7;92.8]
Viso	123	100.0	54	100.0	69.5 [58.2;82.9]
Ductal carcinoma					
50–54	56	5.8	23	7.7	70.9 [54.6;92.1]
55–59	268	27.7	82	27.3	76.6 [67.9;86.3]
60–64	331	34.2	95	31.7	77.7 [69.8;86.5]
65–69	305	31.5	91	30.3	77.0 [68.8;86.2]
In total	969	100.0	300	100.0	76.4 [71.7;81.3]

Upon examination of the main characteristics of tumors identified during SM and interval cancers, during the study it was established that interval cancers were larger, with more frequent metastases in lymph nodes, more often of lobular type than tumors identified during MSP, however, these differences were not statistically significant (table 11).

TABLE 11. Differences of pathological characteristics of screen-detected cancers and interval cancers in Lithuania from 2006 to 2012

Characteristic	Screen-detected cancers		Interval cancers		P value
	Cases	%	Cases	%	
Total number of tumors	1297	75.1	431	24.9	-
Histological type					0.10
Ductal carcinoma	969	74.7	300	69.6	
Lobular carcinoma	123	9.5	54	12.5	
Other	161	12.4	65	15.1	
Unspecified	44	3.4	12	2.8	
Tumor size (according to TNM)					0.40
T1	823	63.5	257	59.6	
T2	350	27.0	132	30.6	
T3	24	1.9	11	2.6	
T4	35	2.7	8	1.9	
Unspecified	65	5.0	23	5.3	

Characteristic	Screen-detected cancers		Interval cancers		P value
	Cases	%	Cases	%	
Metastases in lymph nodes					0.48
Present	432	33.3	157	36.4	
None	747	57.6	235	54.5	
Unspecified	118	9.1	39	9.0	
Distant metastases					0.24
Present	31	2.4	14	3.2	
None	1083	83.5	368	85.4	
Unspecified	183	14.1	49	11.4	
Stage (according to TNM)					0.12
I	632	48.7	191	44.3	
II	441	34.0	142	32.9	
III	163	12.6	67	15.5	
IV	35	2.7	17	3.9	
Unspecified	26	2.0	14	3.2	

4. Conclusions

1. Only about half the number of women to whom it is recommended to take part in mammography screening program in Lithuania take part in it. Breast tumors, diagnosed during mammography screening, comprise only 25% of all breast tumors diagnosed in women aged 50 to 69 y.o., and stage I breast tumors comprise only 49% of all diagnosed tumors.
2. In Lithuania, increased breast cancer incidence and a small decrease in mortality due to this disease is observed. The number of breast tumors diagnosed in early stages is increasing, and the number of tumors diagnosed in late stages is decreasing; however, the influence of the mammography screening program on these changes was not established. Changes established during the study period could reflect increasing awareness about breast cancer, improvement of diagnostics, and increased influence of options for effective treatment.
3. Tumors diagnosed in women who have ever participated in mammography screening program were smaller in size, better differentiated, of earlier stages, with less frequent metastases in lymph nodes.
4. Interval cancer ratio in Lithuania from 2006 to 2012 was 0.25. This ratio does not fundamentally differ from data published by other countries.
5. The overall sensitivity of the mammography screening program implement-

ed in Lithuania reaches 75% and is similar to that of other countries. The most substantial sensitivity of the screening mammography program was monitored among the oldest participants of the program and in cases of ductal histology of the tumor.

5. Practical recommendations

1. It is necessary to develop a centralized system of invitations to take part in screening mammography program, which would allow to significantly increase the number of women participating in the program, and that, in turn, would allow expecting an increase in the incidence of early breast cancer stages and decreased mortality due to breast cancer.
2. Continuous monitoring and evaluation of the implementation of the mammography screening program must be carried out. In order to accomplish this task, it is necessary to create an information system of a mammography screening program that collects data on the course and execution of the program and then analyzes the data collected.
3. Continuous monitoring and evaluation of the impact of the mammography screening program must be carried out. To achieve this goal, it is necessary to develop a monitoring system for the impact indicators of the mammography screening program to assess changes in incidence, stage distribution, mortality, interval tumors, and program sensitivity.
4. Continuous monitoring and assessment of the mammography screening program must be performed, and, depending on the results obtained, the program must be corrected and improved.

List of scientific publications and presentations on the topic of the dissertation

Scientific publications

1. Steponaviciene L, Briediene R, Vanseviciute R, Smailyte G. Trends in Breast Cancer Incidence and Stage Distribution Before and During the Introduction of the Mammography Screening Program in Lithuania. Cancer Control. 2019;26:1–7.

2. Steponaviciene L, Vincerzevskiene I, Briediene R, Smailyte G. Breast cancer screening program in Lithuania: interval cancers and program sensitivity after seven years of mammography screening. Submited

3. Gudavičienė D, Steponavičienė L, Lachej N. Breast cancer in Lithuania (in Lithuanian). Acta Medica Lituanica 2015; 3(22):150–60.

4. Steponavičienė L, Vansevičiūtė R. Breast cancer risk factors and diagnostics (in Lithuanian). Lietuvos akušerija ir ginekologija. 2017; 2(20):160–5.

5. Steponavičienė L, Briedienė R, Šenbergė S, Gudavičienė D, Smailytė G. Mammography screening program. Experience at the National Cancer Institute (in Lithuanian). Sveikatos mokslai. 2017;27(6):161–8.

6. Steponavičienė L, Gudavičienė D, Smailytė G. Interval cancers and their impact on breast cancer screening program sensitivity. Review of the literature (in Lithuanian). Visuomenės sveikata. 2018;1(80):11–21.

7. Steponavičienė L, Vincerževskienė I, Vansevičiūtė-Petkevičienė R, Smailytė G. Implementation of mammography screening program in 2006-2017 in Lithuania (in Lithuanian). Visuomenės sveikata. 2019; 1(84):39–46.

Scientific presentations

1. Steponaviciene L, Briediene R, Grigiene R, Cechanoviciene I. Breast Cancer Services in Lithuania. Plenary of the European Commission Initiative on Breast Cancer (ECIBC). December 9–11, 2016. Baveno, Italy.

2. Steponaviciene L, Smailyte G, Briediene R, Gudaviciene D. Opportunistic screening does not improve early breast cancer (BC) detection and does not reduce BC mortality. 15 th St. Gallen International Breast Cancer Conference March 15–18 2017. Vienna, Austria.

3. Steponaviciene L. Breast cancer in Lithuania. Trends in incidence and mortality. Annual Conference of the Lithuanian Senology Society "Trends in Modern Breast Cancer Treatment". May 19, 2017. Vilnius, Lithuania.

4. Steponaviciene L, Smailyte G, Vincerzevskiene I, Gudaviciene D. Breast cancer screening in Lithuania: trends in incidence and stage distribution. International Conference of the Cancer registries „Ensuring quality and use of data from cancer registries in the 21 th century". October 17–19, 2017. Utrecht, The Netherlands.

5. Steponaviciene L, Vanseviciute R, Zabuliene L, Jasilionis D, Smailyte G. Reproductive factors and breast cancer risk in Lithuanian women: A population based cohort study. 11 th European Breast Cancer Conference. March 21–23, 2018. Barcelona, Spain.

6. Steponaviciene L. Screening for breast cancer. Experience at the National Cancer Institute. 1st International Doctoral Students' Conference „Science for Health 2018". April 13, 2018. Kaunas, Lithuania.

7. Steponaviciene L, Vincerzevskiene I, Gudaviciene D, Briediene R, Smailyte G. Breast cancer screening in Lithuania: interval breast cancers and sensitivity of the programme. 11th European Public Health Conference „Winds of change: towards new ways of improving public health in Europe". November 28–December 1, 2018. Liubliana, Slovenia.

8. Steponaviciene L, Vincerzevskiene I, Smailyte G. Overall and breast cancer mortality in breast cancer patients by participation in mammography screening program in Lithuania. 16th St.Gallen International Breast Cancer Conference. March 20–23, 2019. Vienna, Austria.

9. Steponaviciene L, Vincerzevskiene I, Smailyte G. Breast cancer screening program implementation in Lithuania. International Cancer Screening Conference. June 3–5, 2019. Rotterdam, The Netherlands.

SANTRAUKA

Darbo aktualumas

Nuolat daugėja šiuolaikinės medicinos laimėjimų kuriant įvairias priemones, skirtas išvengti vėžio, jį gydyti ir išgydyti, tačiau onkologinės ligos išlieka itin aktuali visuomenės sveikatos problema. Pasaulyje onkologinės ligos – viena pagrindinių mirčių ir neįgalumo priežasčių.

Krūties vėžys yra tarp aktualiausių moterų sveikatos problemų pasaulyje ir Lietuvoje. Tai antra pagal dažnį piktybinė liga po plaučių vėžio ir dažniausia moterų piktybinė liga visame pasaulyje. Kasmet pasaulyje diagnozuojama daugiau kaip 1,5 mln. naujų krūties vėžio atvejų. 2012 metais naujų atvejų buvo apie 1,7 mln. (25 proc. visų vėžio atvejų) [http://globocan.iarc.fr]. Lietuvoje, kaip ir visame pasaulyje, krūties vėžys yra dažniausia piktybinė moterų liga. Paskutiniais Lietuvos vėžio registro duomenimis, 2012 m. Lietuvoje nustatyti 1526 krūties vėžio atvejai (18 proc. visų moterų piktybinių navikų) [http://www.nvi.lt/index.php?1014526156]. Palyginti su kitomis Europos Sąjungos (ES) šalimis, Lietuvos moterų standartizuotas sergamumo krūties vėžiu rodiklis yra maždaug perpus mažesnis (65,2 atv./100 000 gyv.) nei Prancūzijoje (118,6 atv./100 000 gyv.), Olandijoje (131,3 atv./100 000 gyv.), Suomijoje (121,0 atv./100 000 gyv.), bet mirtingumas nuo krūties vėžio Lietuvoje ir šiose šalyse yra beveik vienodas (23,4; 23,7; 26,0; 19,7 atv./100 000 gyv. atitinkamai) [http://eco.iarc.fr/eucan/CancerOne.aspx?Cancer=46&Gender=2].

Sergamumą krūties vėžiu galima sumažinti taikant pirminės vėžio profilaktikos priemones. Pirminės profilaktikos tikslas – sumažinti sergamumą veikiant jį lemiančius veiksnius. Siekiant sumažinti mirtingumo dėl krūties vėžio ekonominę bei socialinę naštą, sistemingai ieškoma būdų mirtingumui mažinti. Mirtingumą nuo krūties vėžio galima sumažinti taikant antrinės profilaktikos priemones. Didelių gyventojų grupių, kurias sudarantys žmonės nejaučia jokių ligos simptomų ir laiko save sveikais, tyrimai siekiant aptikti vėžį ar ikivėžinius pokyčius vadinami atrankinės patikros tyrimais. Atrankinės patikros tyrimas dėl krūties vėžio neapsaugo nuo krūties vėžio išsivystymo, bet leidžia nustatyti krūties vėžį anksčiau, kai yra galimas efektyvus gydymas bei galima taikyti mažiau agresyvias gydymo priemones. Taip prailginama susirgusiųjų išgyvenimo trukmė bei užtikrinama geresnė jų gyvenimo kokybė. Šiuo metu atliekant moterų atrankinę patikrą dėl krūties vėžio rekomenduojama naudoti rentgeninį krūtų tyrimą – mamografiją, kaip tyrimą, pasižymintį didžiausiu jautrumu ir specifiškumu [Greif JM, 2010].

Mamografinės patikros veiksmingumas buvo įvertintas randomizuotuose kontroliuojamuose tyrimuose. Pirmasis tyrimas pradėtas JAV 1963 m. [Shapiro S, 1997]. Vėliau panašūs tyrimai atlikti kitose šalyse. Gauti rezultatai leido nustatyti, kiek mamografinė patikra efektyvi mažinant mirtingumą nuo krūties vėžio. Skirtingų autorių duomenimis, mirtingumas vykdant mamografinę patikrą gali sumažėti 20–35 proc. [Marmot MG, et al. 2013; Gotzsche PC, et al. 2011]. Randomizuotų kontroliuojamų tyrimų rezultatai parodė, kad vykdoma mamografinė patikra gali reikšmingai sumažinti mirtingumą nuo krūties vėžio, todėl daugelyje

Europos šalių nacionaliniu arba regioniniu lygmeniu buvo pradėtos atrankinės mamografinės patikros (angl. *screening mammography*) (AMP). Njor su bendraautoriais atlikę 20 tyrimų apžvalgą 2012 m. nustatė, jog AMP turi įtakos mirtingumo sumažėjimui Europos šalyse ir kad moterų, pakviestų dalyvauti AMP, mirtingumas sumažėjo 26 proc. [Njor S, et al. 2012]. Broeders su bendraautoriais atliko išsamią visų stebėjimo tyrimų, kuriuose vertinta AMP įtaka mirtingumo nuo krūties vėžio sumažėjimui, apžvalgą ir nustatė, kad Europoje vykdoma AMP sumažina mirtingumą 25–31 proc. tarp moterų, pakviestų dalyvauti atrankinėje patikroje, bei 38–48 proc. tarp moterų, dalyvavusių patikroje [Broeders M, et al. 2012].

AMP, vykdomos bendroje populiacijoje, efektyvumas gali skirtis nuo veiksmingumo, nustatyto klinikinių tyrimų metu [10]. Skirtumai gali atsirasti dėl skirtingo personalo, dalyvaujančio AMP vykdyme profesionalumo, dėl AMP dalyvaujančių moterų skirtingų charakteristikų, dėl skirtingos mamogafinės technikos ir kt. [Gabe R, et al. 2005; van Schoor G, et al. 2011]. Pažymėtina, kad mamografija yra tik vienas elementas visos įvykių sekos, kuri leidžia diagnozuoti vėžį arba užtikrina, kad vėžio nėra. Pradėjus vykdyti AMP bendrose populiacijose, supratus, kad tai sudėtingas kompleksinis procesas, turintis ir nepageidaujamų poveikių (hiperdiagnostika, rentgeno spinduliuotės, skausmo patiriamo tyrimo metu, papildomų diagnostinių procedūrų bei psichologinio streso poveikis), bei supratus, kaip svarbu užtikrinti tinkamą atrankinių tyrimų kokybę buvo dedamos pastangos sukurti kokybės užtikrinimo standartus. Dar 1989 metais Day su bendraautoriais paskelbė svarbiausius teisingai organizuotos AMP efektyvumo matavimo rodiklius, kurie yra būtini norint užtikrinti mirtingumo nuo krūties vėžio sumažėjimą [Day NE, et al. 1989]. Taigi jau 9-ajame dešimtmetyje buvo suprasta, kad reikalingos atrankinės mamografinės patikros programos (AMPP), kurios leis vykdyti atrankinę mamografinę patikrą organizuotai, laikantis tam tikrų kokybės standartų. Apibendrinus daugelio klinikinių tyrimų duomenis bei įvairių šalių patirtį vykdant AMP, 2006 m. Europos Sąjungoje anksčiau skelbtų ir peržiūrėtų gairių pagrindu buvo išleistos ketvirtosios išsamios multidisciplininės rekomendacijos, kuriose nurodomos priemonės užtikrinančios vykdomos AMPP kokybę [Perry N, et al. 2006]. Šiame dokumente labai išsamiai aptariama AMPP eiga, būtinos sąlygos, kad AMPP būtų vykdoma efektyviai. Kiekvienoje rekomendacijų dalyje labai aiškiai nurodomi rodikliai (jų minimalios bei pageidautinos reikšmės), kurių stebėsena garantuoja sklandžią programos eigą ir gerą kokybę bei leidžia tikėtis, kad tokia programa bus efektyvi, t.y. bus pasiektas mirtingumo nuo krūties vėžio sumažėjimas. AMPP vykdymas vertinamas pagal daug kriterijų, kuriuos galima būtų suskirstyti į 2 pagrindines grupes: programos vykdymo rodikliai, kurie būtini siekiant įvertinti ir užtikrinti, kad programa vykdoma teisingai, pagal esamas rekomendacijas, bei programos įtakos rodikliai, kurie atspindi AMPP vykdymo įtaką krūties vėžio epidemiologinių rodiklių pokyčiams. Lietuvoje AMPP pradėta vykdyti dar 2005 metais, tačiau nei programos eiga, nei jos efektyvumas iki šiol nebuvo tirti.

Darbo tikslas

Įvertinti Lietuvoje vykdomos Atrankinės mamografinės patikros programos eigą ir jos įtaką krūties vėžio epidemiologinės situacijos pokyčiams Lietuvoje.

Darbo uždaviniai

1. Įvertinti atrankinės mamografinės patikros programos Lietuvoje vykdymo rodiklius.
2. Įvertinti sergamumo ir mirtingumo bei sergamumo ankstyvųjų ir paženguslųjų stadijų krūties vėžiu pokyčius iki atrankinės mamografinės patikros programos vykdymo pradžios ir jos vykdymo metu.
3. Nustatyti krūties navikų patologinių charakteristikų skirtumus atrankinėje mamografinės patikros programoje dalyvavusių ir nedalyvavusių moterų grupėse.
4. Įvertinti intervalinių navikų santykinį dažnį.
5. Nustatyti atrankinės mamografinės patikros programos jautrumą.

Ginamieji teiginiai

1. Organizuotai vykdoma atrankinė mamografinės patikros programa gali sumažinti mirtingumą nuo krūties vėžio. Tam kad šis tikslas būtų pasiektas, būtina nuolatinė atrankinės mamografinės patikros programos vykdymo stebėsena.
2. Atrankinės mamografinės patikros programos stebėsenai svarbiausi yra jos vykdymo (dalyvavimo dažnis, mamogramų kartojimo dėl techninių priežasčių dažnis, papildomų iškvietimų dažnis ir kt.) bei poveikio (mirtingumo mažėjimas, intervalinių navikų dažnis, navikų stadijos ir kt.) rodikliai.
3. Intervalinių navikų stebėjimas yra būtinas procesas, siekiant vertinti atrankinės mamografinės patikros programos efektyvumą bei jautrumą.

Darbo mokslinis naujumas

Lietuvoje AMPP vykdoma nuo 2005 metų, tačiau iki šiol nebuvo atliktas išsamus bei nuoseklus programos vykdymo ir jos įtakos krūties vėžio epidemiologinei situacijai vertinimas.

Lietuvoje atliktuose darbuose analizuota tik Kauno regione vykdomos AMPP radiologų darbo kokybė bei pacienčių, kurioms nustatytas krūties vėžys, išgyvenamumas, etiniai AMPP aspektai arba kaštai dėl krūties vėžio. Iki šiol apimančio visoje Lietuvoje vykdomos AMPP tiek proceso, tiek poveikio rodiklių vertinimo tyrimo, kuriame panaudojami šiuolaikiniai vėžio epidemiologijos metodai, atlikta nebuvo.

Pirmą kartą nuo pat AMPP vykdymo Lietuvoje pradžios apskaičiuotas intervalinių krūties navikų santykinis dažnis bei Lietuvoje vykdomos AMPP jautrumas. Pirmą kartą įvertinta AMPP įtaka sergamumo krūties vėžiu, nustatomos ligos stadijų pasiskirstymo ir mirtingumo pokyčiams Lietuvoje. Pirmą kartą nustatyti krūties navikų patologinių charakteristikų skirtumai AMPP dalyvavusių ir nedalyvavusių moterų grupėse.

Išvados

1. Atrankinėje mamografinės patikros programoje Lietuvoje dalyvauja tik apie pusė rekomenduojamo dalyvauti moterų skaičius. Krūties navikai, nustatyti vykdant atrankinę mamografinę patikrą, sudaro tik 25 proc. visų krūties navikų, diagnozuotų

50–69 metų amžiaus moterims, o I-os stadijos krūties navikai sudaro tik 49 proc. visų nustatytų navikų.

2. Lietuvoje stebimas sergamumo krūties vėžiu didėjimas bei nežymus mirtingumo nuo šios ligos mažėjimas. Didėja krūties navikų skaičius, diagnozuotų ankstyvosiose stadijose bei mažėja vėlyvosiose stadijose, tačiau atrankinės mamografinės patikros programos įtaka šiems pokyčiams nebuvo nustatyta. Tiriamuoju laikotarpiu nustatyti pokyčiai gali atspindėti žinomumo apie krūties vėžį didėjimo, diagnostikos tobulėjimo ir efektyvaus gydymo galimybių padidėjimo įtaką.
3. Moterims, kada nors dalyvavusioms atrankinėje mamografinės patikros programoje, diagnozuoti navikai buvo mažesni, geriau diferencijuoti, ankstyvesnių stadijų, rečiau su metastazėmis limfmazgiuose.
4. Intervalinių navikų santykinis dažnis Lietuvoje 2006–2012 metais buvo 0,25. Šis dažnis iš esmės nesiskiria nuo kitų šalių publikuotų duomenų.
5. Bendras Lietuvoje vykdomos atrankinės mamografinės patikros programos jautrumas siekia 75 proc. ir yra panašus kaip ir kitose Europos šalyse. Didžiausias atrankinės mamografinės patikros programos jautrumas stebėtas tarp vyriausių programos dalyvių bei esant duktalinei naviko histologijai.

Nadežda Lachej

CLINICAL SIGNIFICANCE OF THE NOTCH SIGNALING PATHWAY IN UTERINE CANCER

NOTCH SIGNALINIO KELIO KLINIKINĖ SVARBA GIMDOS KŪNO VĖŽIO ATVEJU

Medical and Health Sciences, Medicine M 001
Medicinos ir sveikatos mokslai, medicina M 001

Abbreviations

ADAM – Disintegrin and metalloproteinase domain-containing protein

AXIN2 – Axin-like protein 2 gene

BMI – Body mass index

cDNA – Copy DNA

CTNNB1 – β-catenin

DLL – Notch ligand (Delta-like ligand)

DNA – Deoxyribonucleic acid

FIGO – International Federation of Gynecology and Obstetrics

FZD – Frizzled receptor

G – Grade of tumor differentiation

HES1 – Gene of hairy and enhancer of split-1, Notch pathway component/target, suppressor of transcription preferentially binding to promoter non-canonical N-box

HEY1 – Gene of hairy and enhancer of split-1, Notch pathway component/target, suppressor of transcription preferentially binding to promoter canonical E-box

Hh – Hedgehog signaling pathway

HPRT1 – Hypoxanthine phosphoribosyltransferase gene

JAG – Notch ligand (Jagged)

mRNA – Messenger RNA

Notch – Notch signaling pathway

PSEN – Presenilin

PSENEN – Presenilin enhancer 2 homolog

RNA – Ribonucleic acid

p – Level of significance

PCR – Polymerase chain reaction

Wnt – Wnt (Wingless and Int-1) signaling pathway

1. Introduction

Uterine cancer is the sixth most common cancer among women worldwide. In 2018, over 380,000 new cases of uterine cancer were diagnosed [Bray F. et al., 2018]. The highest incidence rate is recorded in North America and Europe, while the lowest – in Africa and Asia. Uterine cancer is the most common malignant tumor of female genital tract in Western countries, with approximately 170,000 new cases diagnosed each year [Torre L.A. et al., 2015]. The incidence of uterine cancer is increasing. This is thought to be related to the current epidemic of obesity, increased life expectancy, reduced fertility, hormone replacement therapy, especially without progestin [Kitchener H.C. et al., 2009; Lortet-Tieulent J. et al. 2018].

Approximately 600–700 cases of uterine cancer are diagnosed in Lithuania every year. It is the third most common malignancy in women after skin (except melanoma) and breast cancer. The incidence of uterine cancer in Lithuania is also increasing [https://www.nvi.lt/uploads/pdf/Vezio%20registras/Vezys_lietuvoje_2012.pdf; https://ecis.jrc.ec.europa.eu].

The relapse and progression of uterine cancer lead to poor prognosis, and the side effects of modern cytostatics often result in discontinuation of treatment. In ad-

dition, resistance to conventional treatment is observed in cases of later stages of the disease and in tumor recurrences. There are no approved biological therapies for the treatment of uterine cancer. These factors explain why much focus is recently laid on molecular genetic research in an attempt to identify the genetic disorders affecting the carcinogenesis of the uterine, and which markers can help to predict the course of the disease and to choose individual treatment.

In recent years, studies linking the formation of tumor cells with dysregulation of signaling pathways in that cells leading to malignancy have intensified. Notch signaling is one of such pathways. The signal transmitted through the Notch signaling pathway receptors in combination with various cellular components influences cell differentiation, proliferation and apoptosis [Morgan T.H., 1917; Lai E.C., 2004; Gordon W.R. et al., 2008; Kopan R. et al., 2009]. Although the function of many newly identified proteins in the Notch signaling pathway is not clear enough, the proteins that form the basis of this signaling pathway are receptors and ligands. Notch receptors are the transmembrane proteins that interacts with adjacent cells by receiving a signal and then transmitting it to the nucleus. Mammals have been found to have four Notch receptors (NOTCH1–4). Notch ligands are also the transmembrane proteins involved in cell interactions that send a signal to the receptor. Mammals have five NOTCH ligands – DLL1, DLL3, DLL4 (delta-like) and JAG1, JAG2 (jagged). Notch targets include the best characterised families of transcriptional repressors *HES* and *HEY* genes. The Notch signaling pathway is proved to be closely related to other oncogenic signaling pathways, including Wnt (a name derived from the names of the *Wingless* and *Int-1*), whose components (CTNNB1, AXIN2, etc.) play an important role in clonogenicity of tumor cells [Bray S.J., 2006; Fiuza U.M. et al., 2007; Gordon W.R. et al., 2008; Kopan R. et al., 2009; Ranganathan P. et al., 2011].

A partial loss of Notch function or abnormal activation of the Notch signal has been shown to be associated with various human developmental disorders and diseases. Mutations in the genes encoding components of the Notch signaling pathway lead to congenital disorders [Meester J.A.N., 2019].

The evidence that the Notch signaling pathway is involved in carcinogenesis is growing. Notch signaling pathway can play a dual role in cancer development: as an oncogene and as a tumor suppressor [Leong K.G. et al., 2006]. The effect of the Notch signal depends on the tumor cell and the type of tissue. Altered expression of certain components of the Notch signaling pathway correlates with poorer prognosis and shorter survival rates in cases of patients with breast, pancreatic, ovarian, prostate, and other cancers [Santagata S. et al., 2004; Doucas H. et al., 2008; Yuan X. et al., 2015; Chen C. et al., 2017; Pancewicz-Wojtkiewicz J. et al., 2017; Wang J.W. et al., 2018; Wang M. et al., 2018; Zou B. et al., 2018].

Changes in the Notch signaling pathway expression in uterine cancer have received little scholarly attention. Individual literature data indicate that Notch signaling pathway components are involved in the development of uterine cancer [Suzuki T. et al., 2000; Cobellis L. et al., 2008], but research in this area is fragmented as only data concerning isolated components of this signaling pathway have been published. The relationship between the Notch signaling pathway components and the clinical-pathological characteristics of the tumor and the organism has also been scarcely investigated. Further research in this area is needed to reveal the significance of the components of the Notch signaling pathway in the development and progression of uterine cancer, as well as to determine their potential implications for the survival of patients.

1.1 Aim of the study

To determine the expression of the Notch signaling pathway components (receptors, ligands and targets) in tumor and adjacent normal uterine tissue and to evaluate their importance for the survival of uterine cancer patients.

1.2 Objectives of the study

1. To compare the expression of the Notch signaling pathway components at the level of mRNA and protein in tumor and normal uterine tissues.
2. To determine the expression of the Notch signaling pathway components at the level of mRNA and protein at different stages of uterine cancer, degree of tumor differentiation, histologic type, lymphovascular invasion and myometrial invasion.
3. To evaluate the influence of the Notch signaling pathway components on survival of patients with uterine cancer.
4. To carry out multivariate analysis and identify statistically significant prognostic factors.

1.3 Statements to be defended

1. The expression of the Notch signaling pathway components is different in tumor and normal uterine tissues.
2. The expression of the Notch signaling pathway components is related to the clinical-pathological characteristics and survival of patients with uterine cancer.

1.4 Scientific novelty and practical relevance of the study

1. The analysis of the expression of the Notch signaling pathway components was performed by comparing the tumor and normal uterine tissues of the same patient, which is important in order to individually evaluate the changes in the expression of these pathway components and their importance for survival of uterine cancer patients.
2. The study identified a prognostic significance of the Notch signaling pathway components for survival of patients with uterine cancer.
3. Less extensive inhibition of *NOTCH2* gene expression is a negative prognostic factor and can be used in predicting prognosis of the disease in patients with uterine cancer.

2. Subjects and study methods

2.1 Subjects and their characteristics

This prospective observational study enrolled 109 patients who were diagnosed with uterine cancer from 2010 through 2016.

Prior to carrying out the study, the permission was obtained from the Vilnius Regional Biomedical Research Ethics Committee (Protocol No. I-2010-1, issue No. 158200-05-180-43). All samples were collected with the patients' written consent to participate in the study.

The study used operative material obtained at the clinic of Vilnius University Institute of Oncology (now The National Cancer Institute) from patients with stage I–IV uterine cancer. At the time of removal of the uterus, 109 samples of malignancies and 109 samples of normal (surrounding) uterine tissues from the same patients were obtained and histologically examined.

The mean age of the women included in the study was 65.2±9.2 years (range 43–81). The majority of patients were 60–69 years old and 70–79 years old – 33.9% of each group (n = 37); the age group 50–59 – 23.9% (n = 26). The smallest proportion of subjects were women aged 40–49 (3.7%, n = 4) and 80–89 years (4.6%, n = 5).

The majority of patients were postmenopausal patients (92.7%, n = 101), only 7.3% (n = 8) of them were premenopausal subjects.

The mean body mass index (BMI) of the subjects was 33.8±7.2 kg/m^2 (19.4 to 62.4 kg/m^2). Most patients – 72.5% (n = 79) were obese women (BMI 30.0 kg/m^2 and above), 19.3% (n = 21) of subjects were overweight (BMI 25.0–29.9 kg/m^2) and only 8.2% of women (n = 9) were of normal body weight (BMI 18.5–24.9 kg/ m^2).

TABLE 1. Clinical-pathological data of patients participating in the study.

Characteristics	Number of patients (%)
FIGO stage	
IA	51 (46.8)
IB	33 (30.3)
II	8 (7.3)
IIIA	1 (0.9)
IIIB	1 (0.9)
IIIC	9 (8.3)
IVB	6 (5.5)
Histologic type of tumor	
Endometrioid adenocarcinoma	90 (82.6)
Serous adenocarcinoma	9 (8.3)
Carcinosarcoma	10 (9.2)
Tumor differentiation grade	
G1	38 (34.9)
G2	38 (34.9)
G3	28 (25.7)
Undetermined grade	5 (4.6)
Metastasis to regional lymph nodes	
Yes	11 (10.1)
No	98 (89.9)
Lymphovascular invasion	
Yes	18 (16.5)
No	91 (83.5)
Myometrial invasion	
< 1/2	51 (46.8)
≥ 1/2	58 (53.2)
Menopausal status	
Premenopausal	8 (7.3)
Postmenopausal	101 (92.7)
Body mass index	
18.5–24.9	9 (8.3)
25.0–29.9	21 (19.3)
≥30	79 (72.5)

FIGO, International Federation of Gynecology and Obstetrics; IA, tumor limited to the endometrium or invades less than half of the myometrium; IB, tumor invades half or more of the myometrium; II, tumor has spread from the body of the uterus and is growing into the supporting connective tissue of the cervix, but it has not spread outside the uterus; IIIA, tumor involves the serosa of the corpus uteri and/or adnexa of the uterus; IIIB, The cancer has spread to the vagina or to the tissues around the uterus (the parametrium); IIIC, metastasis to pelvic and/or para-aortic lymph nodes; IVB, distant metastasis; G1, well-differentiated tumor; G2, moderately differentiated tumor; G3, poorly differentiated tumor; < 1/2, tumor invades less than half of the myometrium; ≥ 1/2, tumor invades half or more of the myometrium.

The majority of subjects were diagnosed with primary arterial hypertension (68.8%, n = 75). Only a small proportion of patients had diabetes (8.3%, n = 9).

All the patients in the study underwent surgical treatment: removal of the uterus (hysterectomy), including adnexa of the uterus, and pelvic and para-aortic lymphadenectomy according to indications. The stage of the disease was determined by the International Federation of Gynecology and Obstetrics (FIGO 2009) and by the Tumor, Nodes, Metastasis (TNM) staging system (2010). The majority of women had endometrioid adenocarcinoma of 82.6% (n = 90), other morphologic variants of uterine cancer were less frequently diagnosed – serous adenocarcinoma (8.3%, n = 9) and carcinosarcoma (9.2%, n = 10). In all, 84 (77.1%) patients were diagnosed with cancer of stage I, 8 (7.3%) – stage II, 11 (10.1%) – stage III and 6 (5.5%) – stage IV. Clinical-pathological characteristics of patients are presented in Table 1.

Depending on the stage of the disease and the degree of tumor differentiation, some patients (n = 56) underwent postoperative adjuvant radiotherapy according to the National Cancer Institute standards of treatment: some with vaginal brachytherapy (n = 28), others with combined radiation therapy, i.e. pelvic external-beam radiation therapy along with vaginal brachytherapy (n = 28). A single dose (SD) for pelvic external-beam radiation therapy was administered – 1.8–2.0 Gy in 23–28 fractions five days per week, total dose (TD) 46.0-50.4 Gy. Vaginal (intracavitary) brachytherapy was applied once a week, with 5 Gy (SD) at 0.5 cm depth in the vaginal wall during each brachytherapy procedure, using an iridium-192 source. There were three procedures combined with external-beam radiation therapy (TD – 15 Gy) and four procedures with brachytherapy alone (TD – 20 Gy).

Chemotherapy with cisplatin (50mg/m^2) or carboplatin dose of AUC 5 (area under the curve) in combination with doxorubicin (50mg/m^2) was administered to 16 patients: five post-operative patients received palliative chemotherapy alone for distant metastases, while the remaining patients underwent chemotherapy before or after radiation therapy.

2.2 Study methods

2.2.1 Anthropometric measurements

Body mass index was calculated for all subjects by dividing body mass by height squared (m^2). According to WHO recommendations, patients are normal in body mass with a BMI between 18.5 and 24.9 kg/ m^2, overweight with a BMI of 25.0 to 29.9 kg/ m^2, and obese if the BMI is 30 kg/ m^2 or more [http://www.euro.who.int/en/health-topics/disease-prevention/nutrition/a-healthy-lifestyle/body-mass-index-bmi].

2.2.2. Used equipment

- Homogenizer Mikro-Dismembrator S (Sartorius);
- Centrifuge 5804 (Eppendorf, USA);
- NanoDrop 2000 spectrophotometer (Thermo Scientific, USA);
- Shaker MS 3 basic (IKA®, Germany);
- Thermocycler – Mastercycler (Eppendorf, Germany);
- Thermomixer – Termomixer comfort (Eppendorf, Germany);
- Quantitative PCR system – StepOnePlus™ Thermocycler (Applied Biosystem, USA) or Mastercycler ep realplex (Eppendorf, Germany).

2.2.3 Isolation of RNA

Samples of normal and pathological uterine tissues were frozen in liquid nitrogen and stored at -80 °C temperature. Part of the tissues frozen in nitrogen was crushed in a Mikro-Dismembrator S (Sartorius) homogenizer according to the manufacturer's recommendations, the rest – manually in a chilled ceramic crucible. Homogenized tissues were lysed with cell lysis buffer. About half of the lysate was left for protein extraction and the remainder for RNA extraction according to the manufacturer's recommendations (PARIS™ Kit, Ambion). DNase (Thermo Fisher Scientific) was used to remove DNA impurities in purified RNA samples. The resulting RNA solutions were frozen at -80°C. The RNA concentration and purity were determined spectrophotometrically using $OD_{260/280}$ (optical density) and $OD_{260/240}$ ratios. The concentration of patient samples for quantitative PCR testing was standardized prior to carrying out copy DNA synthesis.

2.2.4 Copy DNA (cDNA) synthesis

DNA copy was synthesized using the High Capacity RNA to cDNA Master Mix (Life Technologies) and Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo scientific) according to the manufacturer's recommendations. A similar amount of total RNA from each sample was used to synthesize copy DNA. The synthesized cDNA is stored at -20 ° C temperature until use.

2.2.5 Quantitative PCR

The gene expression research was performed using oligonucleotides Gene expression assays (Table 2) and TaqMan Gene Expression Master Mix (Life Technologies) of PCR. The composition of 20 µl chain reaction mix: 9 µl of five-fold diluted cDNA, 1 µl of oligonucleotides and 10 µl of PCR mix. The reaction temperature mode: initial denaturation at 95 ° C for 10 min.; the following steps repeated 45

times: 95 °C for 15 sec. and 60 °C for 1 min. The cDNA level of the tested gene was normalized according to two housekeeping genes *HPRT1* (the hypoxanthine phosphoribosyltransferase 1 gene) and *B2M* (the beta-2-microglobulin gene). On the basis of laboratory experience and the results of the first experimental studies, it was found that the expression of these genes is constant.

The relative change in gene expression (compared to control) was calculated by the Comparative C_t method, i.e. the fold change of gene expression equal to $2^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t$ is:

$$(C_{t\ \text{test gene}} - C_{t\ \text{housekeeping gene}})_{\text{tumor tissue}} - (C_{t\ \text{test gene}} - C_{t\ \text{housekeeping gene}})_{\text{normal tissue}}.$$

The results are presented as fold change of gene expression normalized to housekeeping genes expression, average of three measurements. The quantitative PCR system was used in the study – thermal cycler StepOnePlus™ (Applied Biosystem, USA) or Mastercycler ep realplex (Eppendorf, Germany).

TABLE 2. Oligonucleotide primers used for quantitative PCR.

Gene	Number	Number of mRNA numeris
HPRT1	Hs02800695_m1	NM_005524.3
B2M	Hs00984230_m1	NM_004048.2
DLL1	Hs00194509_m1	NM_005618.3
JAG1	Hs 01070032_m1	NM_000214.2
JAG2	Hs 00171432_m1	NM_002226.3
NOTCH1	Hs 01062014_m1	NM_017617.3
NOTCH2	Hs 01050702_m1	NM_001200001.1
NOTCH3	Hs 01128541_m1	NM_000435.2
NOTCH4	Hs 00965889_m1	NM_004557.3
HEY1	Hs0114113_m1	NM_001040708.1
HES1	Hs00172878_m1	NM_005524.3
AXIN2	Hs00610344_m1	NM_004655.3
CTNNB1	Hs00355045_m1	NM_001904.3

2.2.6 Quantitative PCR using arrays

The Human Notch signaling pathway RT² Profiler PCR Array (SABiosciences) was used in the study. In all, 84 tested genes were analyzed. Five housekeeping genes – *B2M, HPRT1, RPL13A* (ribosomal protein L13a gene), *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase gene), *ACTB* (beta cytoskeletal actin gene) were used for comparison. Positive and negative controls were performed in the analysis.

cDNA was synthesized from 1 μg of isolated RNA using RT^2 First strand Kit (SA-Biosciences) according to the manufacturer's recommendations. Quantitative PCR was performed using the fluorescent dye SYBR Green Mastermix (SABiosciences). The relative change in gene expression was calculated using the manufacturer's recommended analysis software (PCRArrayDataanalysis V3.3 Sofware, SABiosciences) based on five housekeeping genes mentioned above.

2.2.7 Western blot analysis

Protein concentration was determined by bisinchoninic acid (BCA) method. 30 μg of protein was used for the experiment. Proteins were fractionated in 12 percent NDS-polyacrylamide gel under denaturing conditions. Electrophoresis was performed at 80 V, a current of 150 mA for 1 hour, and at 120 V, a current of 150 mA for 3 hours. A semi-dry transfer apparatus V20-SDB was used to transfer the proteins onto the nitrocellulose membrane (0.45 μm). The transfer was carried out for 1 h at a current of 1 mA/cm2. The membrane was then incubated with rabbit monoclonal antibodies anti-NOTCH1 (1:200 dilution; sc-9170, Santa Cruz Biotechnology, or 4147S, Cell Signaling), anti-NOTCH4 (1:200 dilution; sc-5594, Santa Cruz Biotechnology), anti-HES1 (PA5-28802, Thermo Scientific), anti-JAG2 (1:300; # 2210, Cell Signaling), with anti-non-phospho β-catenin antibodies (8814S, Cell Signaling), and mouse monoclonal anti-β-actin antibodies (1:1000; sc-8432, Santa Cruz Biotechnology, or MA5-15739, Thermo Scientific) overnight at +4°C temperature. These antibodies were the only choice at the start of the study, while others such as anti-NOTCH2 were not available at the time.

The membranes were further incubated for 2 hours with alkaline phosphatase-conjugated secondary antibodies against rabbit or mouse IgG (1:1000 dilution; # 18-732-292604, GenWay Biotech or sc-2008, Santa Cruz Biotechnology, respectively). Proteins were expressed using a mixture of NBT (nitromel tetrazole) and BCIP-T (5-brome-4-chlore-3-indolyl phosphate, p-toluidine salt). Membrane-bound primary antibodies NICD1 (NOTCH intracellular domain1), HES1 (hairy and enhancer of split-1) and β-catenin were detected using horseradish peroxidase-conjugated anti-rabbit IgG secondary antibodies (31460, Thermo Scientific). β-actin antibodies were detected using horseradish peroxidase-conjugated anti-mouse IgG secondary antibodies (31430, Thermo Scientific). The NOTCH protein content was normalized to β-actin content. The relative amount of NOTCH protein is the ratio of the amount of protein in tumor tissue to the amount of protein in normal tissue.

2.2.8 Evaluation of protein expression

The ImageJ program evaluated the protein strip area and color intensity. The relative amount of test protein was normalized to the relative amount of β-actin ($\frac{\text{the intensity of the specific protein band of the sample}}{\text{the intensity of the } \beta - \text{actin band of the same sample}}$).

The change in protein expression is expressed multiplied by times, comparing the expression of the test protein in tumor and normal tissues.

2.2.9 Statistical analysis of the data

Calculation of the sample. It is estimated that 84 clinical samples are required to detect a significant correlation (if any) with a maximum low correlation coefficient (0.3) and choosing a criterion power of 0.8 and a significance level of 0.05. A power criterion of 0.8 and a significance level of 0.05 were also used to calculate the sample needed for the analysis of variance; it is estimated that if the difference between the mean values of the samples is equal to or greater than 1, with a standard deviation of 0.5, at least 7 samples from each group will be required.

To evaluate the clinically meaningful effect on patient survival, a total of 92 samples (46 in each group) were prognozed, where 30% difference between survival rates was considered clinically significant along with power criterion 0.8 and significance level 0.05.

The sample size was also calculated taking into account that about 140 new cases of uterine cancer are diagnosed at the National Cancer Institute (former Vilnius University Institute of Oncology) per year. Planning that about 65 percent of patients will be excluded from the study for a variety of reasons (e.g., failure to meet inclusion criteria, insufficient volume of tumor and normal uterine tissue, surgical treatment) and taking into account that up to 10% extreme values can be included in the study, it was foreseen that the study shall include about 25 percent of the patients who visited the National Cancer Institute during the study period, i.e. about 35 patients a year.

The sample size was calculated so that, at a significance level of 0.05, the power of the criterion was sufficient to detect clinically significant changes. When the *P* value was between 0.05 and 0.1, the differences were considered to be tendentious. Data normality was assessed using the Shapiro–Wilk W test. The Mann–Whitney U test was used to compare two independent groups, and Kruskal–Wallis dispersion analysis was used to compare several independent groups. The correlation analysis was performed using Spearmen correlation coefficient. All experimental data were used for statistical analysis, but extremes are not presented in graphical representa-

tions. The extremes accounted for less than 10% of the total data. One-factor survival analysis was performed using the Log-rank test. COX regression analysis was used in conducting the multivariate survival analysis.

When the *P* value was 0.05 to 0.1, the differences between the groups were considered to be trendy, and when the *P* value was less than 0.05, the differences were considered statistically significant. Statistically significant results were visualised using box-plots and survival curves in the summary of doctoral dissertation.

SigmaPlot 13.0 and Statistica Basic Academic 13 were used for data analysis.

Most of the experiments were performed at the Life Sciences Center of Vilnius University under the supervision of dr. Violeta Jonušienė (quantitative PCR), dr. Aušra Sasnauskienė (Western blot immunoassay), dr. Daiva Dabkevičienė (statistical data analysis). The author is grateful for their valuable contribution.

3. Results

3.1 Study plan

Tumor and normal uterine tissue samples from 109 patients obtained after surgery were used for research. The expression of Notch signaling pathway receptors (*NOTCH1-4*), ligands (*JAG1, JAG2, DLL1*) and targets (*HES1, HEY1*) genes was assessed by quantitative PCR.

In the analysed samples, the expression of NOTCH1 (n = 70), NOTCH4 (n = 75), and HES1 (n = 41) was assessed at protein level. In the samples of some patients (n = 59), the gene expression of the regulatory factor *AXIN2* (n = 59) and the transduction factor *CTNNB1* (n = 59), as well as CTNNB1 (n = 32) protein level of the components of the WNT pathway closely related to the Notch signaling pathway, were also evaluated. The study of the Wnt signaling pathway components was purely exploratory and of limited scope, therefore it is excluded from the aim of the dissertation, its objectives, titles of the sections and conclusions, however, we hope that the obtained results will be useful for further work in this direction.

The expression of the above Notch and Wnt signaling pathway components was not investigated in all samples due to the insufficient amount of available tumor and normal (control) tissue required to obtain reliable results.

Subsequently, 10 patient samples of tumor and normal tissues were selected because of they were of sufficient size to allow more extensive analysis. In these samples, 84 genes were analyzed by quantitative PCR.

The results of mRNA assays are presented *as fold changes in gene expression*, normalized to housekeeping gene expression (detailed in the Study methods sec-

tion). It is important to note that for the descriptive statistics, the mean values of the study data series – the medians – were used.

3.2 Changes in the expression of components of the Notch signaling pathway depending on the stage of the disease

The study found that the expression of *NOTCH1-4* genes in the tumor tissue was decreased compared to normal uterine tissue in the same patient, median values < 0 in all cases. The Kruskal–Wallis test was used to compare the expression of Notch signaling pathway receptors genes between stages I and II–IV, regardless of the histologic type of tumors, i.e. all malignancy histologic types were included. There is no statistically significant difference in the expression of *NOTCH1–4* genes, in all cases $p > 0.05$.

The study found that the expression in Notch signaling pathway ligands *JAG1, JAG2, DLL1*, targets *HES1, HEY1* genes and the expression of regulatory factor *AXIN2* and transduction factor *CTNNB1* of the WNT signaling pathway in tumor tissues compared normal tissues from the same patient is also reduced in most cases, with median values < 0 in all cases. The Mann–Whitney test was used for comparison of the expression of ligands *JAG1, JAG2, DLL1,* targets *HES1, HEY1* genes, and regulatory factor *AXIN2* and transduction factor *CTNNB1* of the WNT signaling pathway, between stages I and II–IV, irrespective of tumor histology, i.e. all tumor histologic types were included. The results showed that there was no statistically significant difference in the expression of the mentioned genes, in all cases $p > 0.05$.

Changes in the expression of receptors *NOTCH1–4*, ligands *JAG1, JAG2, DLL1*, targets *HES1, HEY1* genes, *AXIN2, CTNNB1* components of the Wnt pathway were evaluated according to the histologic type of malignancies separately in each case of endometrioid adenocarcinoma, carcinosarcoma, serous adenocarcinoma. The data analysis did not show statistically significant differences in the gene expression between the various stages of these histologic types of tumors. In the case of carcinosarcoma and serous adenocarcinoma, the absence of statistically significant differences may be explained by a small number of subjects.

Although no significant changes in the expression of *NOTCH1-4, JAG1, JAG2, DLL1, HES1, HEY1, AXIN2, CTNNB1* genes were observed depending on the stage of the disease, however, the majority of the samples corresponded to stages IA and IB (77.1%), therefore an assumption was made that the gene expression may differ between stages IA and IB and additionally only stages IA and IB were compared.

Comparing the mRNA expression between stages IA and IB in all histologic types, there was a statistically significant difference in *NOTCH4* gene expression between samples at stages IA and IB (Figure 1A). There was also a significant dif-

ference in *HEY1* gene expression (Figure 1B). The results show that the expression of *NOTCH4* and *HEY1* genes at the level of stage IB is lower than at stage IA, i.e. in a more advanced disease, the expression of *NOTCH4* and *HEY1* genes at the transcript level is suppressed.

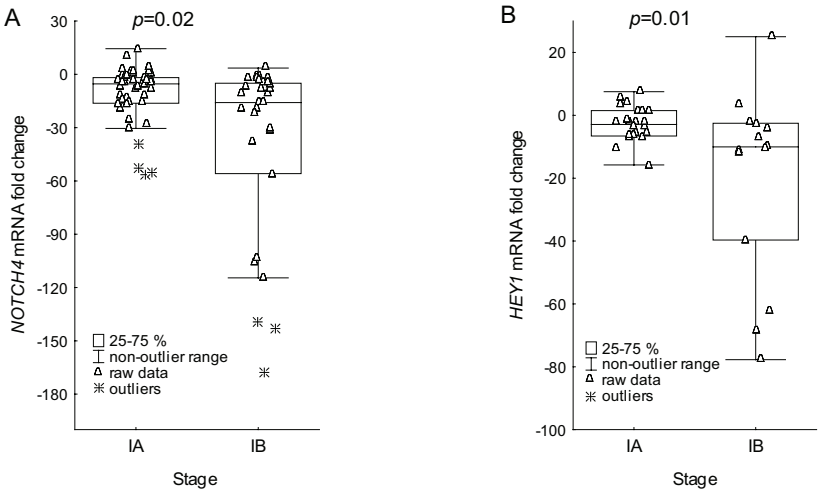


FIGURE 1. Changes in *NOTCH4* (A) and *HEY1* (B) genes expression in tumor tissue compared to normal tissue regardless of the histologic type of tumor at stage I

The study also evaluated fold changes of the expression of *NOTCH1-4*, *JAG1*, *JAG2*, *DLL1*, *HES1*, *HEY1*, *AXIN2*, *CTNNB1* genes between stages IA and IB for each of the three histologic types separately.

The largest group of patients (n = 90) consisted of women diagnosed with endometrioid adenocarcinoma, including 70 cases with stage I. These patients were divided into two groups: stage IA (41 patients) and stage IB (29 patients). The statistical analysis revealed that the *NOTCH4* gene expression was significantly lower in the histologic tissue of the examined tumor in stage IB patients than in the corresponding tumor tissues of stage IA (Figure 2A). In endometrioid adenocarcinoma, statistically significant differences in *HEY1* gene expression between stage IA and IB were also found (Figure 2B). In stage IB, the gene expression in tumor tissues was more decreased when compared to normal tissues in stage IA.

In serous adenocarcinoma, there was a trend for *NOTCH1* gene expression to be lower in stage IB than in stage IA (p=0.08).

After the changes in the gene expression of the components of Notch and Wnt signaling pathways had been examined, further research focused on changes at the protein level.

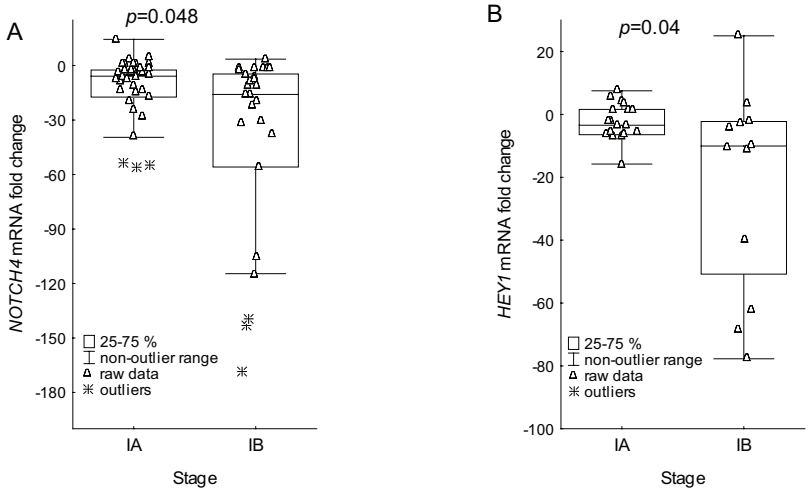


FIGURE 2. Changes in *NOTCH4* (A) and *HEY1* (B) genes expression in tumor tissue compared to normal tissue in stage I endometrioid adenocarcinoma.

After carrying out the Western blot analysis, changes in NOTCH1 and NOTCH4 expression at the protein level were assessed in all the samples of tumor and normal uterine tissues. HES1 and transduction factor CTNNB1 protein levels were detected in the later enrolled patients (n = 59). Protein levels were normalized to β-actin. A significant change is determined where more than a double increase or decrease in the protein level is observed.

The data of our study revealed that NOTCH1, NOTCH4, HES1 and CTNNB1 protein levels in tumor tissues varied within a relatively small interquartile range: medians (interquartile range) were 1.15 (2.04), 1.03 (2.96), 0.4 (2.25) and 1.03 (2.96), respectively, compared to normal tissues. However, the full range of meanings was quite wide: 17, 11, 33, 55, respectively. It is important to note that NOTCH1 protein level tended to increase with the increasing stage of the disease, while NOTCH4 tended to decrease with the increasing stage of the disease, particularly in IA transition to IB. The Mann–Whitney test

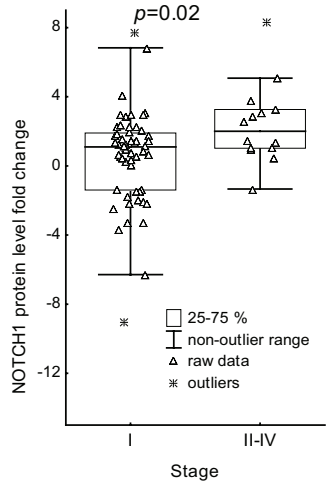


FIGURE 3. Change in NOTCH1 protein level depending on the disease stage.

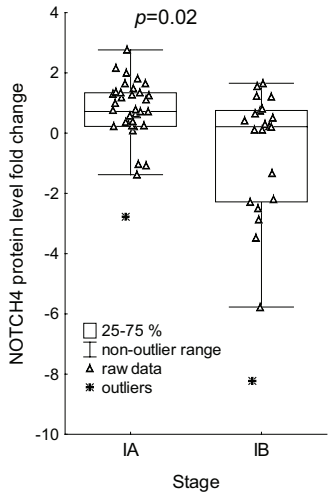


FIGURE 4. Change in NOTCH4 protein level regardless of the histologic type of tumor at stage I.

was used to compare the level of NOTCH1 protein between stages I and II–IV, irrespective of the histologic type of tumor (Figure 3). The results revealed that the level of NOTCH1 protein is significantly higher in stages II–IV compared to protein levels observed in I stage samples (p=0.02).

As with the identification of gene expression, the differences in the protein levels of the receptors NOTCH1 and NOTCH4, the target HES1 and the transduction factor CTNNB1 as the component of the Wnt signaling pathway were evaluated separately in samples from patients with stage IA and IB uterine cancer. NOTCH4 protein level was found to be significantly lower in stage IB compared to protein level found in IA stage samples (Figure 4).

A separate analysis of different histologic types of tumors showed a statistically significant decrease in the level of receptor NOTCH4 protein at stage IB compared to that of stage IA (p=0.01).

The evaluation of the levels of other proteins (HES1 and CTNNB1) did not detect a statistically significant difference between the various stages of uterine cancer.

We further analyzed the expression changes of the components of Notch and Wnt signaling pathways at mRNA and protein levels with respect to other clinical-pathological characteristics – grade of tumor differentiation, histologic type, lymphovascular and myometrial invasion.

3.3 Changes in the expression of components of the Notch signaling pathway depending on grade of tumor differentiation

The results of this study fragment showed that, depending on the degree of differentiation, a statistically significant difference in the target *HEY1* and Wnt pathway component *AXIN2* gene expression was found – the expression of poorly differentiated tumors is significantly lower compared to well- and moderately differentiated tumors (Figure 5).

There is a trend for *NOTCH4* gene expression to be lower in moderately and poorly differentiated tumors compared to well- differentiated ones (Figure 6A). Similar changes observed when analyzing NOTCH4 protein expression are given in Figure 6B.

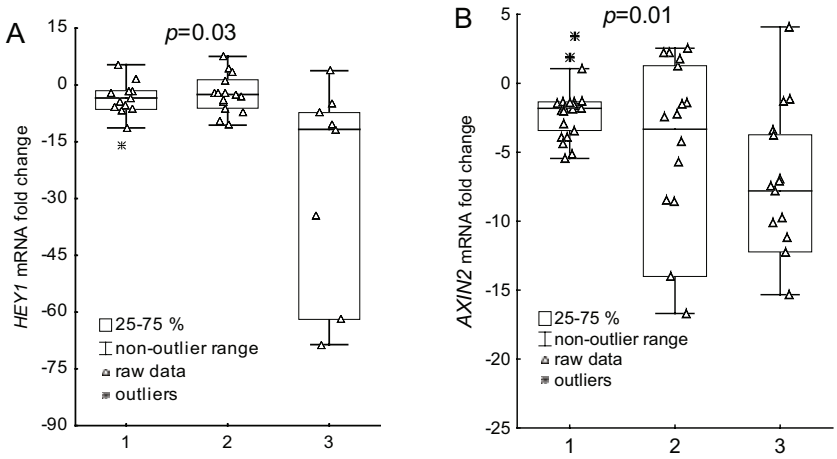


FIGURE 5. Changes of *HEY1* (A) and *AXIN2* (B) genes expression in tumor tissue compared to normal tissue depending on the degree of tumor differentiation (1 – well-differentiated tumor; 2 – moderately differentiated tumor; 3 – poorly differentiated tumor).

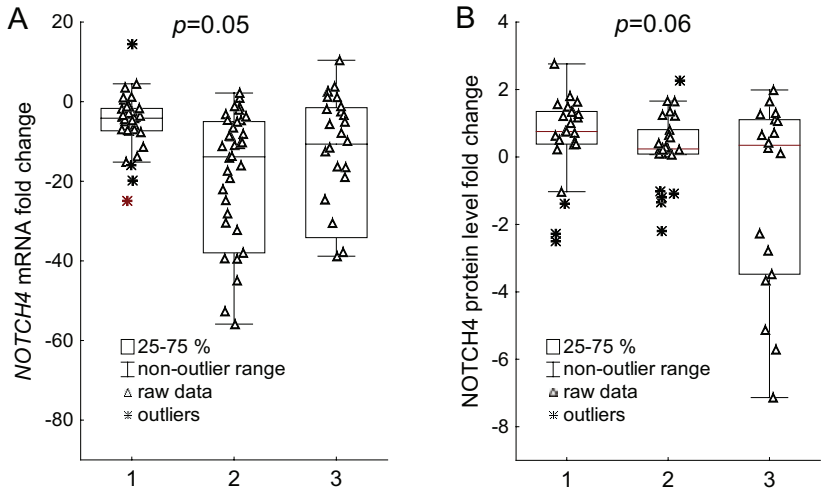


FIGURE 6. Changes of *NOTCH4* (A) gene expression and NOTCH4 protein level (B) in the tumor tissue compared to normal tissue depending on the degree of tumor differentiation (1 – well-differentiated tumor; 2 – moderately differentiated tumor; 3 – poorly differentiated tumor).

The analysis showed that there is a trend of *DLL1* gene expression to decrease in poorly differentiated uterine tumors compared to well-differentiated ones (p=0.09).

3.4 Changes in the expression of components of the Notch signaling pathway depending on tumor histologic type

The research into this fragment disclosed a trend for *NOTCH2* gene expression to be lower in endometrioid adenocarcinoma than in carcinosarcoma or serous adenocarcinoma samples (p=0.09). The analysis of the Wnt signaling pathway component *AXIN2* gene expression the showed that the expression is the lowest in serous adenocarcinoma samples, but in this case the sample size is too small to state significant changes.

Thus, it is clear from the results presented that no statistically significant differences in the expression of gene and the levels of protein were observed between the histologic types of tumors. This may have been influenced by insufficient carcinosarcoma and serous adenocarcinoma samples.

3.5 Changes in the expression of components of the Notch signaling pathway depending on lymphovascular and myometrial invasion

The research into this fragment did not reveal any statistically significant differences depending on whether lymphovascular invasion was present in the tumor or not, but a trend was observed that NOTCH1 protein level (p=0.1), *JAG1* (p=0.08) and *DLL1* (p=0.10) genes expression in the presence of tumor lympho-

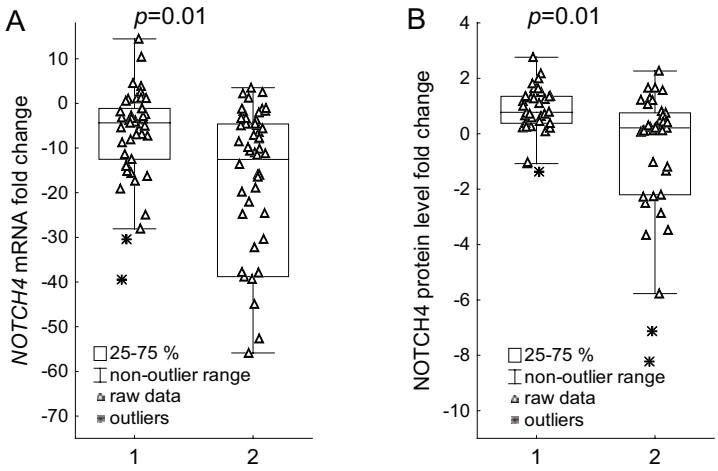


FIGURE 7. Changes of *NOTCH4* gene expression (A) and NOTCH4 protein level (B) depending on the myometrial invasion (1 – < 1/2 myometrium; 2 – = or > 1/2 myometrium).

vascular invasion is higher compared to samples where lymphovascular invasion was not determined.

Changes in *NOTCH4* gene expression and NOTCH4 protein level were statistically significant, depending on tumor invasion of the myometrium – if the tumor infiltrates half or more than half myometrium, the expression of *NOTCH4* gene and NOTCH4 protein level in uterine tissue is lower than when the tumor infiltrates less than half myometrium. The results obtained are shown in Figure 7.

3.6 Changes in the expression of the Notch signaling pathway components depending on patient age and the evaluation of correlation between the investigated parameters

On the one hand, no statistically significant differences were found between the results obtained in different age groups, and on the other hand, a trend was observed for *JAG2* ligand gene expression to be lower in tumors of after-surgery patients over 65 years of age compared to younger patients (Figure 8).

The study also evaluated the correlation between gene expression of the Notch and Wnt signaling pathways and protein levels in the samples tested. Using the Spearmen correlation coefficient, a positive correlation was found between many genes and proteins. Correlation coefficients were significant at $p < 0.05$ as shown in Table 3. A statistically significant correlation was found between different Notch signaling pathway receptors, between receptors and ligands, and between components of the Notch and Wnt signaling pathways. After evaluating the correlation between clinical-pathological data and the test molecules, a positive correlation was found between *AXIN2* gene expression and body weight (correlation coefficient 0.31, $p<0.05$).

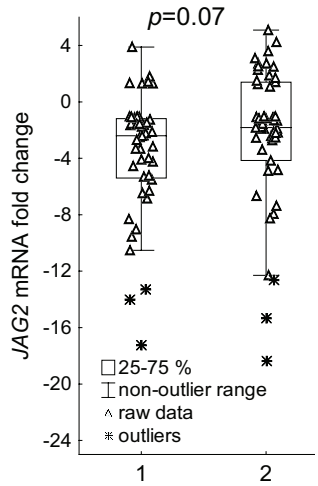


FIGURE 8. Change of *JAG2* gene expression in tumor tissue compared to normal tissue with respect to patient age (1 – to 65 years, inclusive; 2 – over 65 years).

TABLE 3. Evaluation of the correlation between the components of the Notch and Wnt signalling pathways. Only statistically significant ($p < 0.05$) correlation coefficients differing from 0 are presented in the table. Proteins (NOTCH4, HES1, CTNNB1) that did not correlate with all investigated components are not included in the table.

	NOTCH1 mRNA	NOTCH1 protein	*NOTCH2* mRNA	*NOTCH3* mRNA	*NOTCH4* mRNA	*JAG1* mRNA	*JAG2* mRNA	*DLL1m-*RNA	*HES1* mRNA	*HEY1* mRNA	*AXIN2* mRNA	*CTNNB1* mRNA
***NOTCH1* mRNA**			0.75	0.75	0.75	0.75	0.79	0.77	0.77	0.55	0.48	0.40
***NOTCH1* protein**							-0.28		-0.30			
***NOTCH2* mRNA**	0.75			0.76	0.74	0.71	0.64	0.76	0.73	0.51	0.42	0.61
***NOTCH3* mRNA**	0.75		0.76		0.66	0.72	0.66	0.68	0.71	0.50	0.43	0.54
***NOTCH4* mRNA**	0.75		0.74	0.66		0.67	0.65	0.76	0.63	0.48	0.45	0.37
***JAG1* mRNA**	0.75		0.71	0.72	0.67		0.74	0.67	0.73	0.65		0.38
***JAG2* mRNA**	0.79	-0.28	0.64	0.66	0.65	0.74		0.70	0.67	0.65		
***DLL1* mRNA**	0.77		0.76	0.68	0.76	0.67	0.70		0.71	0.58	0.57	0.34
***HES1* mRNA**	0.77	-0.30	0.73	0.71	0.63	0.73	0.67	0.71		0.64	0.32	0.33
***HEY1* mRNA**	0.55		0.51	0.50	0.48	0.65	0.65	0.58	0.64			
***AXIN2* mRNA**	0.48		0.42	0.43	0.45			0.57	0.32			0.30
***CTNNB1* mRNA**	0.40		0,61	0.54	0.37	0.38		0.34	0.33		0.30	

3.7 Changes in gene expression of the Notch signaling pathway components in tumor tissue as determined by PCR arrays

The samples of the ten patients. with sufficient amount of tumor and normal tissues were analysed using Human Notch signaling pathway RT² Profiler PCR Array. The changes in Notch signaling pathway and related gene expression were assessed using the Human Notch signaling pathway RT² Profiler PCR Array. Five samples of endometrioid adenocarcinomas and five samples of carcinosarcoma and their corresponding normal tissue samples were examined. The changes in the expression of some genes involved in maturation of Notch receptors, as well as WNT and Hedgehog signaling pathways in stage I malignant uterine cells are shown in Figure 9. The results show that expression of genes involved in Notch receptor's maturation in tumor tissues is reduced: *ADAM10* (ADAM metalopeptidase 10 domain), *PSEN1* (presenilin 1), *PSEN2* (presenilin 2), *PSENEN* (presenilin enhancer) (Figure 9A). The study also found that the expression of components of the Hedgehog (Hh) signaling pathway is reduced or remains unchanged: expression of SHH ligand, transcription factor GLI1 (GLI family zinc finger 1), SUFU (suppressor of fused homolog)

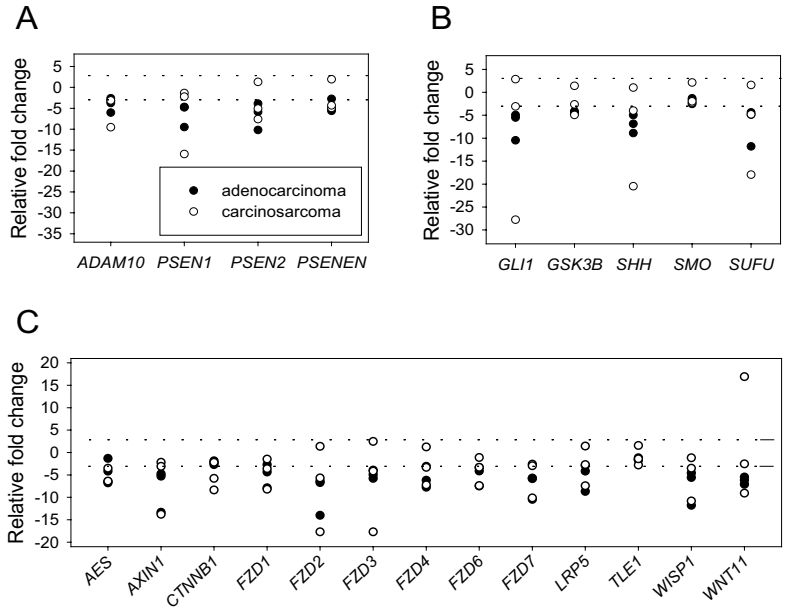


FIGURE 9. Amounts of transcripts of different signaling pathways (Notch signaling pathway (A), Hh pathway components (B) and Wnt signaling pathway (C)) components. Hollow characters – carcinosarcoma, filled characters – adenocarcinoma. The expression was considered to be significantly altered when it was reduced or increased more than three-fold (dashed line).

was reduced in tumor tissue, compared to normal tissue, whereas the expression of SMO (smoothened, frizzled family receptor) and GSK3B (glycogen synthase kinase 3 beta) remained unchanged (Figure 9B). Reduced levels of the mRNA of the following proteins were observed in tumor uterine tissue samples: Wnt signaling pathway receptor *FZD1* (frizzled family receptor 1), *FZD2, FZD3, FZD4, FZD6, FZD7* and other components' AES (amino-terminal enhancer of split), *AXIN1*, *CTNNB1* (catenin beta 1), *LRP5* (low density lipoprotein receptor-related protein 5), *WISP1* (WNT1 inducible signaling pathway), *WNT11* (Wingless-type MMTV integration site family, member 11). The expression of the *TLE1* (transducin-like enhancer of split 1) remained unchanged (Figure 9C).

The observed differences in gene expression of components of these signaling pathways in tumor tissues and normal tissues indicate the relevance of further studies into these signaling pathways in order to identify new molecular markers of uterine cancer.

3.8 Patient survival depending on clinical-pathological characteristics and expression of the Notch signaling pathway components

Patients were enrolled in the study between 2010 and 2016 and monitored. Censorship date is 1 March 2019.

The analysis of the data showed that in the studied group of women with uterine cancer, females who, when diagnosed, were 65 years of age or younger had a significantly longer survival rate than older patients (Figure 10).

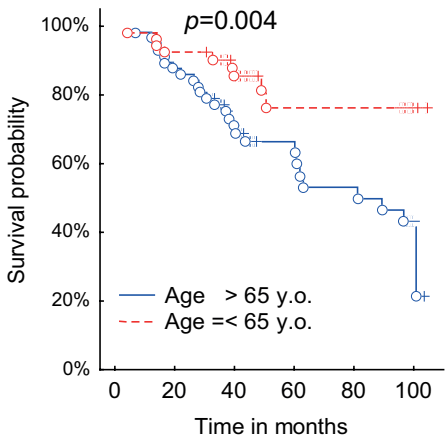


FIGURE 10. Age-related survival of patients with uterine cancer; o – fatal cases, + censored cases.

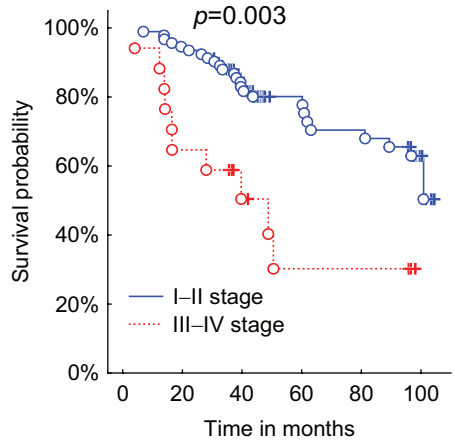


FIGURE 11. Survival rates in patients with uterine cancer depending on the stage of the disease; o – fatal cases, + censored cases.

As expected, the disease stage significantly influenced the survival rate – with locally advanced or metastatic tumor of the uterus (stage II–IV of the disease), the survival was worse than with stage IA–IB, where the tumor did not spread beyond the uterine body (Figure 11).

Patients with histologically confirmed endometrioid adenocarcinoma had a better survival rate than those with other histologic forms – carcinosarcoma and serous adenocarcinoma (Figure 12).

The degree of tumor differentiation was a significant determinant for survival – with the lowest degree of tumor differentiation (G3, poorly differentiated tumor) the worst survival results were obtained (Figure 13).

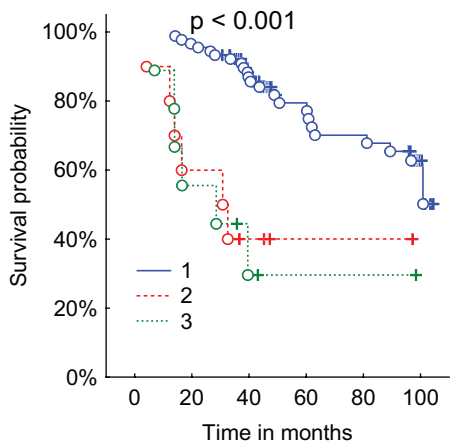


FIGURE 12. Survival rate in patients with uterine cancer depending on the histologic type of tumor: 1 – endometrioid adenocarcinoma; 2 –carcinosarcoma; 3 – serous adenocarcinoma; o – fatal cases, + censored cases.

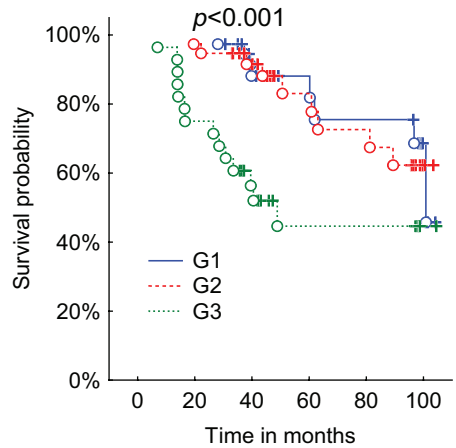


FIGURE 13. Survival rates of uterine cancer patients depending on the degree of tumor differentiation (G1, well-differentiated tumor; G2, moderately differentiated tumor; G3, poorly differentiated tumor); o – fatal cases, + censored cases.

The survival rates also statistically significantly depended on tumor lymphovascular invasion – in the absence of lymphovascular invasion the survival results were better compared to the patients with lymphovascular invasion (Figure 14).

In our group of patients with uterine cancer, myometrial invasion had no significant effect on patient survival rates (p=0.83).

Patients who underwent surgery alone had significantly better survival rates than those receiving post-operative radiation therapy or/and chemotherapy (Figure 15).

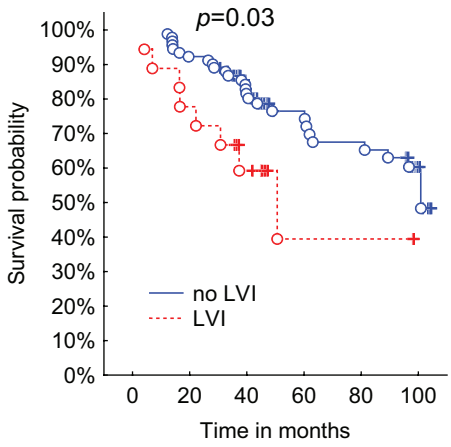
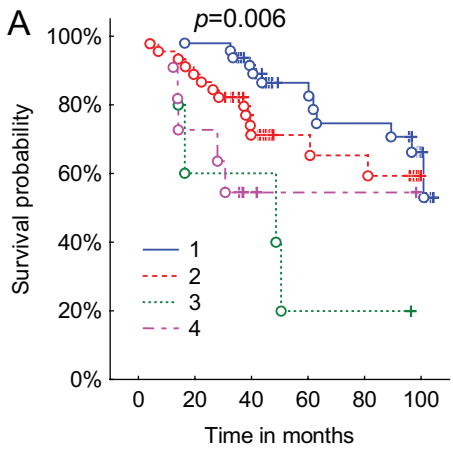


FIGURE 14. Survival rates in patients with uterine cancer depending on lympho-vascular invasion (LVI); o – fatal cases, + censored cases.



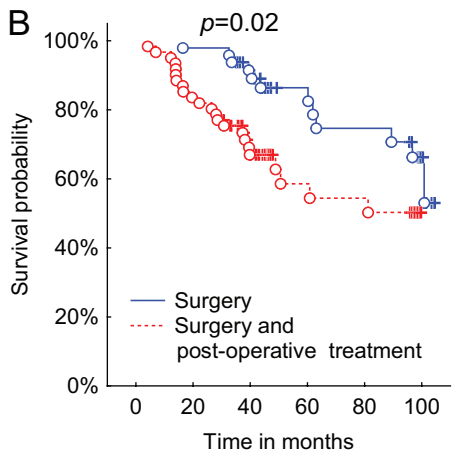


FIGURE 15. Survival rates of patients with uterine cancer depending on the treatment used (1 – surgery; 2 – surgery followed by radiation therapy; 3 – surgery followed by chemotherapy; 4 – surgery followed by radiation therapy and chemotherapy); o – fatal cases, + censored cases.

The data showed that BMI had no statistically significant effect on patient survival rates (p=0.36).

Thus, the analysis of the data obtained showed that, as expected, the survival rate of patients with uterine cancer is influenced by clinical-pathological characteristics such as stage of disease, histologic type of tumor, degree of tumor differentiation, lymphovascular invasion. The study found that patient survival was undoubtedly dependent on the treatment they underwent, with the best prognosis being in the group of patients who underwent only surgery.

The study evaluated the potential influence of the investigated Notch signaling pathway components on survival rates in patients with uterine cancer. The *P* values are shown in Table 4.

TABLE 4. Influence of the Notch signaling pathway components on survival rates in patients with uterine cancer.

Notch signaling pathway component tested	*P* value
NOTCH1 receptor gene	0.59
NOTCH1 receptor protein	0.23
NOTCH2 receptor gene	***0.002***
NOTCH3 receptor gene	0.33
NOTCH4 receptor gene	0.64
NOTCH4 receptor protein	0.998
JAG1 ligand gene	0.66
JAG2 ligand gene	0.98
DLL1 ligand gene	0.078
HES1 target gene	0.556
HES1 target protein	0.295
HEY1 target gene	0.31

The analysis of the obtained data showed that there is a statistically significant association between the survival rates of uterine cancer patients and the expression of NOTCH2 gene (Figure 16). In case of higher the *NOTCH2* gene expression, the disease prognosis is worse. There was also a trend for *DLL1* ligand to influence the survival rates (p=0.08).

The possible influence of Wnt pathway components closely related to the Notch signaling pathway on the survival rate of patients with uterine cancer has also been identified (Table 5). There is a trend observed from these results that *AXIN2* may

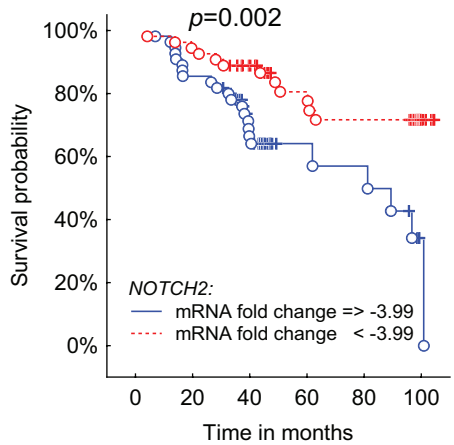


FIGURE 16. Survival rate of uterine cancer patients depending on the fold change of the *NOTCH2* gene expression; o – fatal cases, + censored cases.

influence the survival (p=0.08). Apparently, in order to get reliable results, a larger sample and/or a longer patient monitoring period is needed.

TABLE 5. Influence of Wnt signaling pathway components on survival rates of patients with uterine cancer.

Wnt signaling pathway component tested	*P* value
AXIN2 gene	0.08208
CTNNB1 gene	0.81667
CTNNB1 protein	0.57615

Multivariate Cox regression analysis was performed to evaluate the significance of clinical-pathological characteristics and their interaction on patient survival rates. Factors that were significant in the one-way analysis were included in the model: disease stage, histologic type, degree of differentiation, lymphovascular invasion, treatment methods, as well as changes in *NOTCH2* receptor and *DLL1* ligand genes expression.

Multivariate Cox regression analysis revealed that histologic type, stage of the disease and changes of *NOTCH2* gene expression were among major determinants of patient survival. The coefficients (Hazard Ratio) of other factors selected in the one-factor survival analysis did not show a statistically significant difference from 1 in the Cox model (Table 6).

TABLE 6. Results of multivariate analysis of patients with uterine cancer (only statistically significant results are presented).

Characteristics	*P* value	Hazard ratio	95,0% CI* for Hazard ratio
Fold change of *NOTCH2* expression: ≥ -3.99 < -3.99 (reference group)	 0.004 –	 3.17 1	 1.46–6.89 –
Histologic tumor type: Serous adenocarcinoma/ carcinosarcoma Endometrioid adenocarcinoma (reference group)	 <0.001 –	 4.35 1	 2.03–9.31 –
FIGO stage: III–IV I–II (reference group)	 <0.001 –	 4.31 1	 1.98–9.38 –

* CI is the confidence interval

The data presented indicate that *NOTCH2* gene expression, along with disease stage and tumor histologic type, are important prognostic factors in uterine cancer.

4. Discussion of the results

Endometrial cells undergo intense expression of the Notch signaling pathway receptors and ligands. This pathway is thought to be important for the endometrial change processes [Mitsuhashi Y. et al., 2012]. NOTCH1 receptor expression in the proliferating endometrium is known to be higher than in other phases, and in the menopausal endometrium, where glandular volume is reduced, mucosa is thinned, protein levels of the Notch signaling pathway are decreased. These findings have led to the theory that the Notch signaling pathway is important for endometrial cell proliferation and the formation of uterine cancer [Cobellis L. et al., 2008].

It was determined that the NOTCH4 receptor is involved in endometrial turnover processes and is important for carcinogenesis [Suzuki T. et al., 2000]. It was also proved that NOTCH1 and JAG1 protein levels are higher in the secretion stage than in proliferation, while NOTCH4 shows reversed results. With reference to literature, an increase in NOTCH1 protein level and a decrease in NOTCH4 and JAG1 protein levels have been observed in tumor cells [Cobellis L. et al., 2008]. However, other authors suggest that the levels of NOTCH1, NOTCH3, DLL4, and JAG1 proteins are the same in both secretory and proliferating endometrium, and therefore they are not important for cell proliferation and differentiation. Elevated levels of these

proteins have been detected in tumor cells [Groeneweg J.W. et al., 2014]. It should be noted that all of the above results were obtained by immunostaining of tissues, also by comparing them to the tissues of healthy subjects and cancer patients, and a group of healthy subjects also included younger women.

Our study compared the expression of Notch signaling pathway components in tumor and normal tissues of the same patient's uterine. To our knowledge, this was the first study of the kind at the beginning of our work. It is noteworthy that almost all women in the study were in postmenopause, so menopausal status was not evaluated by statistical analysis. The majority of the samples (82.6%) consisted of samples of endometrioid adenocarcinoma type I. A relatively small number of samples of other histologic types – of type II cancer (serous adenocarcinoma, carcinosarcoma) and especially a low number of samples of later stages (II–IV) made it difficult to obtain statistically reliable results for these uterine tissues.

Based on our findings, the expression of the Notch signaling pathway components (*NOTCH1–4, JAG1, JAG2, DLL1, HES1, HEY1*) in tumor was decreased compared to their expression in normal uterine tissue of the same patient.

Comparing tumor samples from different histologic types, we found a statistically significant difference in expression between *NOTCH4* receptor and *HEY1* target genes between stages IA and IB, i.e. their expression in stage IB uterine cancer was significantly lower than in stage IA tissues. This was confirmed by analyzing samples of endometrioid adenocarcinomas separately. The data obtained show that the expression of *NOTCH4* and *HEY1* at stage IB is lower than at stage IA, i.e. in a more advanced disease, the expression of *NOTCH4* and *HEY1* genes is suppressed. A significantly reduced *NOTCH4* expression at IB stage of uterine cancer suggests that this gene may be mutated in uterine tumor cells. Mutations in the genes *NOTCH1, NOTCH2, NOTCH3* are found in the head and neck carcinoma cells [Agrawal N. et al., 2011; Stransky N. et al., 2011].

The evaluation of the differences in the expression of the Notch signaling pathway components depending on the clinical-pathological factor such as the degree of tumor differentiation revealed that poorly differentiated tumors have lower levels of *HEY1* gene expression compared to well- and moderately differentiated tumors. A similar trend is observed for *NOTCH4* and *DLL1* expression. The obtained results support the notion that the NOTCH signaling pathway is important for cell differentiation and is involved in carcinogenesis – the reduction of expression of the mentioned Notch pathway components is favorable for carcinogenesis.

The study found a trend for *NOTCH2* receptor gene expression to be lower in endometrioid adenocarcinoma than in samples of carcinosarcoma or serous adenocarcinoma. Taking into account that the prognosis is better in endometrioid adenocarcinoma than in other histologic types, it is possible that the *NOTCH2* gene

is less suppressed in tumors with a worse prognosis. In order to obtain statistically significant data, the sample size for carcinosarcomas and serous adenocarcinomas has to be increased.

Another prognostic factor, lymphovascular invasion, is likely to be related to components of the Notch signaling pathway, such as ligands *JAG1* and *DLL1*, but further research is needed in this area.

A significant factor correlating with invasion of the uterine tumor into myometrium was found to be *NOTCH4* gene expression and NOTCH4 protein level. The results obtained from the study confirm the already described scholarly data that the expression of *NOTCH4* is different between tumor samples corresponding to stage IA and IB. Thus, the results of the study confirm the findings of other authors that the Notch signaling pathway may play an important role in the development of uterine cancer and reveal that the expression of relevant Notch signaling pathway genes and protein level may be influential contributors to the development of a particular disease state.

The study has also evaluated the changes in the expression of some components of the Wnt signaling pathway closely related to the Notch signaling pathway, namely, regulatory factor *AXIN2* and transduction factor *CTNNB1*, and their impact on uterine cancer. The analysis of the data obtained revealed that the expression of *AXIN2* and *CTNNB1* in uterine tumor tissue is decreased as compared to normal Notch signaling components in the same patient. In our study we found a statistically significant difference in *AXIN2* gene expression depending on the degree of tumor differentiation, the expression in poorly differentiated tumors is lower compared to well and moderately differentiated tumors. A positive correlation between expression of *AXIN2* and body weight was found after evaluating the correlation between clinical-pathological data and the molecules tested. These results may lead to the development of further studies on the components of the Wnt signaling pathway.

The present study used PCR arrays to reveal that the amount of mRNA encoding proteins (*ADAM10, PSEN1, PSEN2, PSENEN*) related to Notch receptor maturation in tumor tissue was reduced. The mRNA levels in the Notch signaling pathway targets *HES1* and *HEY1* also decreased in tumor tissue compared to normal tissue. Members of the *HES1* and *HEY1* gene families are transcriptional inhibitors and are primary targets of the Notch signaling pathway, and their reduction in expression reveals that the Notch signaling pathway in tumor tissue is inhibited from the very first components of the signaling pathway.

The expression of some genes in other Wnt and Hedgehog signaling pathways related to the Notch signaling pathway was also investigated. Wnt and Hedgehog signaling pathways are important in embryogenesis and changes in them occur at the presence of various oncological diseases. An abnormal activation of the Hedge-

hog signaling pathway is important for the proliferation of endometrial carcinoma cells [Hecht J.L. et al., 2006]. The results of our study using PCR arrays also confirm the associations between Notch and other signaling pathways, and the observed differences in gene expression of these signaling pathway components in tumor and normal tissues suggest the relevance of further studies into these pathways in order to identify new molecular markers of uterine cancer.

One of the aims of this study was to evaluate the influence of the Notch signaling pathway components on the survival of uterine cancer patients. Literature data on this topic are very limited. One study [Groeneweg J.W. et al., 2014] found that the prognosis of the disease was influenced by changes in NOTCH1 and JAG1 proteins levels, while another noted that the Notch signaling pathway ligand DLL3 protein may be an independent marker predicting poorer survival rate in patients with uterine cancer [Wang J. et al., 2018]. Our study did not examine the DLL3 ligand, so this type of study could be one aspect of our future work.

This study aimed at the evaluation of the impact of the Notch and WNT signaling pathway components on patient survival. The influence of clinical-pathological factors on disease prognosis was evaluated as well. As expected, the analysis of the data obtained showed that the survival rate of patients with uterine cancer is affected by the stage of the disease, histologic type of tumor, degree of tumor differentiation, and lymphovascular invasion. In our study, myometrial invasion was not a statistically significant factor influencing patient survival. Patient survival was also found to be dependent on the treatment applied – the best prognosis was found in the group of patients who had received surgery only. This is explained by the fact that additional postoperative treatment (radiation therapy, chemotherapy) is administered in the presence of unfavorable prognosis factors.

The examination of the influence of Notch and Wnt signaling pathway components on patient survival revealed a statistically significant association between the uterine cancer survival rate and *NOTCH2* gene expression: if the *NOTCH2* gene expression is higher, the prognosis of the disease is worse. The weakness of our dissertation is that this study did not investigate NOTCH2 protein level, which should be one of the priority objectives for further research. We also had few cases of serous adenocarcinoma and carcinosarcoma, which did not allow statistically significant evaluation of the *NOTCH2* expression dependence on histologic type. As mentioned above, the results of our study suggest the hypothesis that the Notch signaling pathway is increasingly suppressed in the progression of uterine cancer. Although more detailed studies are needed to explain this phenomenon, the findings of our study indicate the potential prognostic importance of *NOTCH2* expression.

The data of our study suggest that Notch signaling pathway ligand *DLL1* and Wnt pathway component *AXIN2* may also be important to the survival of patients

with uterine cancer, however, due to an insufficient sample of subjects, it was impossible to prove their statistical significance. Therefore, future in-depth research could prove their impact on patient survival.

There is limited evidence in the literature that NOTCH2 receptor is an important prognostic factor. In a recent publication, it has been reported that high levels of *NOTCH2* expression show a statistically significant correlation with poorer survival in ovarian cancer patients [Chen C. et al., 2017]. In 2018, a study was published in which NOTCH2 protein is one of the factors contributing to the prognosis of endometrial cancer [Polychronidou G. et al., 2018]. Thus, our findings that *NOTCH2* gene expression is an important factor in predicting prognosis of the disease in patients with uterine cancer, are relatively recent, and confirm the scientific and practical significance of the studies performed.

5. Conclusions

1. The Notch signaling pathway is suppressed: expression of components of this pathway in tumor uterine tissue is lower than in normal tissue of the same patient.
2. The expression of Notch signaling pathway components is related to the clinical-pathological characteristics of patients with uterine cancer:
   - for stage IB, the expression of the *NOTCH4* at mRNA and protein level and the expression of the *HEY1* gene are significantly lower than their expression in stage IA;
   - the expression of *HEY1* gene is significantly lower in poorly differentiated tumors as compared to its expression in well- and moderately differentiated tumors;
   - if the tumor has infiltrated half or more than half of the myometrium, the expression of the *NOTCH4* at mRNA and protein level is significantly lower than when the tumor has infiltrated less than half myometrium.
3. A statistically significant association between patient survival and the degree of suppression of *NOTCH2* gene expression was found in uterine cancer survival outcomes: the prognosis is worse with less suppressed *NOTCH2* gene expression in tumor tissue as compared to its expression in normal tissue.
4. Statistically significant independent prognostic factors are *NOTCH2* gene expression, histologic type of uterine tumor, and stage of disease.

6. Future studies

1. In future studies, it is necessary to increase sample size of patients with II–IV stages of uterine cancer and to expand the range of histologic types of tumors.
2. Taking into account the predictive significance of *NOTCH2* gene expression in the survival rate of uterine cancer in the present study, it is appropriate to further investigate changes in the protein content of this gene and to evaluate its significance as a circulating marker.
3. The trend (apparently due to insufficient number of analyzed cases) of the changes of the Notch signaling pathway ligand *DLL1* and the Wnt pathway, closely related Notch signaling pathway (both pathways important for cell differentiation and proliferation), component *AXIN2* genes expression, was identified to influence the survival rate of uterine cancer which is an encouragement to pursue research in this direction.

List of scientific publications and presentations on the topic of the dissertation

Scientific publications

1. Jonušienė V, Bielskienė K, Lachej N, Kazbarienė B, Mickė I, Didžiapetrienė J. Notch signalinis kelias ir jo įtaka ląstelės likimui. Laboratorinė medicina. 2011; 13(4):199-204.

2. Lachej N, Didžiapetrienė J, Kazbarienė B, Kanopienė D, Jonušienė V. Association between Notch signaling pathway and cancer. Acta Medica Lituanica. 2012; 19(4):427–37.

3. Jonusiene V, Sasnauskiene A, Lachej N, Kanopiene D, Dabkeviciene D, Sasnauskiene S, Kazbariene B, Didziapetriene J. Down-regulated expression of Notch signaling molecules in human endometrial cancer. Med Oncol. 2013; 30(1):438.

4. Lachej N, Dabkevičienė D, Sasnauskienė A, Trimonytė RM, Kanopienė D, Kazbarienė B, Didžiapetrienė J. NOTCH signalinio kelio ir ginekologinių piktybinių navikų sąsaja. Acta Medica Lituanica. 2017; 24(1):35–43.

5. Lachej N, Jonušienė V, Gasianec A, Sasnauskienė A, Dabkevičienė D, Šimienė J, Sužiedėlis K, Didžiapetrienė J. Changes of Notch and Wnt signaling molecules expression in human endometrial cancer. Acta Medica Lituanica. 2019; 3 (*submitted*).

Scientific presentations

1. Lachej N, Jonusiene V, Sasnauskiene A, Dabkeviciene D, Kanopiene D, Didziapetriene J. Down-regulated expression of Notch signalling molecules has prognostic value in human endometrial cancer. The 1st International Doctoral Students' Conference „Science for Health". Kaunas (Lithuania), April 13, 2018.

2. Lachej N, Jonusiene V, Sasnauskiene A, Dabkeviciene D, Gasianec A, Didziapetriene J. The activity of Notch signalling pathway in human endometrial cancer. 14th Baltic Congress of Laboratory Medicine. Vilnius (Lithuania), May 10-12, 2018.

SANTRAUKA

Pasaulyje gimdos kūno vėžys tarp piktybinių moters navikų užima šeštąją vietą. 2018 metais iš viso diagnozuota daugiau nei 380 tūkst. naujų gimdos kūno vėžio atvejų [Bray F. ir kt., 2018]. Didžiausias sergamumas yra Šiaurės Amerikoje ir Europoje, mažiausias – Afrikos ir Azijos šalyse. Gimdos kūno vėžys yra dažniausias moterų lyties organų piktybinis navikas Vakarų šalyse, kur kasmet diagnozuojama apie 170 tūkst. naujų atvejų [Torre L. A. ir kt., 2015]. Sergamumas gimdos kūno vėžiu didėja. Manoma, kad tai susiję su dabartine nutukimo epidemija, ilgėjančia gyvenimo trukme, vaisingumo sumažėjimu ir hormonų pakaitine terapija, ypač preparatų be progestinų [Kitchener H. C. ir kt., 2009; Lortet-Tieulent J. ir kt., 2018].

Lietuvoje per metus diagnozuojama apie 600–700 gimdos kūno vėžio atvejų. Tai trečias pagal dažnį moterų piktybinis navikas po odos (išskyrus melanomą) ir krūties vėžio. Sergamumas gimdos kūno vėžiu Lietuvoje taip pat didėja [https://www.nvi.lt/uploads/pdf/Vezio%20registras/Vezys_lietuvoje_2012.pdf; https://ecis.jrc.ec.europa.eu].

Gimdos kūno vėžio atkryčiai bei metastazavimas sąlygoja blogą prognozę, o nepageidaujamas šiuolaikinių citostatinių preparatų poveikis organizmui dažnai tampa gydymo nutraukimo priežastimi. Be to, esant vėlesnei ligos stadijai ir naviko recidyvams, stebimas atsparumas tradiciniam gydymui. Registruotų biologinės terapijos preparatų gimdos kūno vėžiui gydyti nėra. Minėti veiksniai paaiškina, kodėl pastaraisiais metais skiriama daug dėmesio molekuliniams genetiniams tyrimams siekiant išsiaiškinti, kokie genetiniai sutrikimai turi įtakos gimdos kūno kancerogenezei, kokie žymenys gali padėti prognozuoti ligos eigą bei parinkti individualų gydymą.

Pastaraisiais metais daugėja tyrimų, siejančių piktybinių ląstelių atsiradimą su signalinių kelių reguliacijos sutrikimais tose ląstelėse. Vienas iš tokių kelių yra Notch signalinis kelias. Signalas, perduodamas per Notch signalinio kelio receptorius ir veikdamas kartu su įvairiais ląstelės komponentais, daro įtaką ląstelės diferenciacijos, proliferacijos ir apoptozės procesams [Morgan T. H., 1917; Lai E. C., 2004; Gordon W. R. ir kt., 2008; Kopan R. ir kt., 2009]. Nors daugelio naujai identifikuotų baltymų funkcijos Notch signaliniame kelyje nėra visiškai aiškios, galima išskirti baltymus, kurie sudaro šio signalinio kelio pagrindą – tai receptoriai ir ligandai. Notch receptorius yra transmembraninis baltymas, kuris dalyvauja greta esančių ląstelių tarpusavio sąveikoje priimdamas signalą ir toliau perduodamas jį į branduolį. Nustatyta, kad žinduoliai turi keturis Notch receptorius (NOTCH1–4). Notch ligandas taip pat yra transmembraninis baltymas, dalyvaujantis ląstelių sąveikoje ir siunčiantis signalą receptoriui. Žinduoliai turi penkis Notch ligandus – DLL1, DLL3, DLL4 (angl. *delta-like ligand*) ir JAG1, JAG2 (angl. *jagged*). Tarp Notch taikinių yra geriausiai apibūdintos transkripcijos slopiklių *HES* ir *HEY* genų šeimos. Nustatyta, jog Notch signalinis kelias glaudžiai susijęs su kitais onkogeniniais signaliniais keliais, tarp jų ir su Wnt (pavadinimas kilęs iš *Wingless* (liet. besparnis fenotipas) baltymo/integrazės-1), kurio komponentai (CTNNB1, AXIN2 ir kt.) yra svarbūs naviko ląstelių kamieniškumui [Bray S.J., 2006; Fiuza U.M. ir kt., 2007; Gordon W.R. ir kt., 2008; Kopan R. ir kt., 2009; Ranganathan P. ir kt., 2011].

Nustatyta, kad dalinis Notch funkcijos praradimas arba nenormalus Notch signalo aktyvinimas yra susijęs su įvairiais žmogaus vystymosi sutrikimais ir ligomis. Genų, koduojančių Notch signalinio kelio komponentus, mutacijos sukelia įgimtus sutrikimus [Meester J.A.N. ir kt., 2019].

Daugėja įrodymų, kad Notch signalinis kelias dalyvauja kancerogenezėje. Notch signalas, vystantis navikui, gali veikti dvejopai: kaip onkogenas ir kaip naviko augimo slopiklis [Leong K.G. ir kt., 2006]. Notch signalo poveikis priklauso nuo naviko ląstelės ir audinio tipo. Pakitusi tam tikrų Notch signalinio kelio komponentų raiška koreliuoja su blogesne ligos prognoze ir trumpesniu išgyvenamumu krūties, kasos, kiaušidžių, priešinės liaukos ir kitų lokalizacijų navikų atveju [Santagata S. ir kt., 2004; Doucas H. ir kt., 2008; Yuan X. ir kt., 2015; Chen C. ir kt., 2017; Pancewicz-Wojtkiewicz J. ir kt., 2017; Wang J.W. ir kt., 2018; Wang M. ir kt., 2018; Zou B. ir kt., 2018].

Gimdos kūno vėžio atveju Notch signalinio kelio raiškos pokyčiai yra mažai tirti. Pavieniai literatūros duomenys rodo, kad Notch signalinio kelio komponentai dalyvauja formuojantis gimdos kūno vėžiui [Suzuki T. ir kt., 2000; Cobellis L. ir kt., 2008], tačiau tyrimai šioje srityje yra fragmentiški – paskelbti duomenys tik apie pavienius šio signalinio kelio komponentus. Sąsaja tarp Notch signalinio kelio komponentų ir naviko bei organizmo klinikinių-patologinių charakteristikų taip pat mažai tirta. Reikalingi tolesni šios srities moksliniai tyrimai siekiant patikslinti Notch signalinio kelio komponentų reikšmę formuojantis bei progresuojant gimdos kūno vėžiui, taip pat nustatyti jų galimą reikšmę sergančiųjų išgyvenamumui.

Darbo tikslas

Nustatyti Notch signalinio kelio komponentų (receptorių, ligandų ir taikinių) raišką navikiniame ir sveikame gimdos kūno audinyje bei įvertinti jų svarbą sergančiųjų gimdos kūno vėžiu išgyvenamumui.

Darbo uždaviniai

1. Palyginti Notch signalinio kelio komponentų raišką iRNR ir baltymo lygiais navikiniame ir sveikame gimdos kūno audinyje.
2. Įvertinti Notch signalinio kelio komponentų raiškos iRNR ir baltymo lygiais skirtumus esant skirtingoms gimdos kūno vėžio stadijoms, naviko diferenciacijos laipsniui, histologiniam tipui, limfovaskulinei invazijai bei invazijai į miometriumą.
3. Įvertinti Notch signalinio kelio komponentų raiškos pokyčių įtaką sergančiųjų gimdos kūno vėžiu išgyvenamumui.
4. Atlikti daugiaveiksnę analizę ir nustatyti statistiškai reikšmingus prognozinius veiksnius.

Ginamieji teiginiai

1. Notch signalinio kelio komponentų raiška skirtinga navikiniame ir sveikame gimdos kūno audinyje.
2. Notch signalinio kelio komponentų raiška susijusi su pacienčių, sergančių gimdos kūno vėžiu, klinikinėmis-patologinėmis charakteristikomis ir išgyvenamumu.

Darbo mokslinis naujumas ir praktinė reikšmė

1. Notch signalinio kelio komponentų raiška analizuota lyginant tos pačios pacientės navikinį ir sveiką gimdos kūno audinį, kas svarbu siekiant individualizuotai vertinti tirtųjų šio kelio komponentų raiškos pokyčius ir jų svarbą sergančiųjų gimdos kūno vėžiu išgyvenamumui.
2. Nustatyta Notch signalinio kelio komponentų prognozinė reikšmė sergančiųjų gimdos kūno vėžiu išgyvenamumui.
3. Mažiau slopinama *NOTCH2* geno raiška yra neigiamas prognozinis veiksnys ir gali būti pritaikytas kaip prognozinis žymuo parenkant pacientei gydymo ir stebėjimo taktiką.

Išvados

1. Notch signalinis kelias yra slopinamas: šio kelio komponentų raiška gimdos kūno navikiniame audinyje yra mažesnė, palyginti su jų raiška sveikame tos pačios pacientės audinyje.
2. Notch signalinio kelio komponentų raiška yra susijusi su pacienčių, sergančių gimdos kūno vėžiu, klinikinėmis-patologinėmis charakteristikomis:
   - IB stadijos atveju *NOTCH4* raiška iRNR ir baltymo lygiais bei *HEY1* geno raiška yra statistiškai reikšmingai mažesnė, palyginti su jų raiška IA stadijos atveju;
   - *HEY1* geno raiška blogos diferenciacijos navikuose yra statistiškai reikšmingai mažesnė, palyginti su jo raiška vidutiniškai ir gerai diferencijuotuose navikuose;
   - jei navikas yra infiltravęs pusę ar daugiau kaip pusę miometriumo, *NOTCH4* raiška iRNR ir baltymo lygiais yra statistiškai reikšmingai mažesnė, nei tuo atveju, kai navikas infiltravęs mažiau negu pusę miometriumo.
3. Įvertinus sergančiųjų gimdos kūno vėžiu išgyvenamumo rezultatus priklausomai nuo Notch signalinio kelio komponentų raiškos, nustatyta statistiškai reikšminga sąsaja tarp pacienčių išgyvenamumo ir *NOTCH2* geno raiškos slopinimo laipsnio: esant mažiau slopinamai *NOTCH2* geno raiškai navikiniame audinyje, palyginti su sveiku audiniu, prognozė yra blogesnė.
4. Statistiškai reikšmingi nepriklausomi prognoziniai veiksniai yra *NOTCH2* geno raiška, gimdos naviko histologinis tipas ir ligos stadija.

Vilniaus universiteto medicinos fakulteto disertacijos, 2019 / Vilnius university Faculty of Medicine dissertations, 2019. – Vilnius : Vilniaus universiteto leidykla, 2020. – 872 p.: iliustr.

ISSN 2538-8495 (spausdinta knyga)

ISSN 2538-8517 (skaitmeninis PDF)

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Tiražas 75 egz. 47,9 aut. l.