VILNIUS UNIVERSITY

VIOLETA MIKŠTIENĖ

THE GENOMICS OF CONGENITAL / HEREDITARY HEARING LOSS: INFLUENCE ON PATHOGENESIS AND THE PHENOTYPIC MANIFESTATION IN THE LITHUANIAN POPULATION

Summary of a doctoral dissertation

Biomedical Sciences, Medicine (06 B)

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Disertantė VIOLETA MIKŠTIENĖ

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Introduction

"*Blindness separates people from things; deafness separates people from people*." This Helen Keller's (1880-1968) quote illustrates not only the physiologic and psychologic aspects of hearing loss but also the state of social isolation of deaf people. Hearing is one of the five sensory organs, creating our relationship with the world. The relevance of the problem may be demonstrated by its prevalence – congenital hearing loss (CHL) is one of the most common inborn defects diagnosed to 1-2 in a 1000 new-borns [1] and has a tendency to increase up to 3.5 in a 1000 with age [2]. According to the data of SVEIDRA, 1702 (3.26 in 1000) minors with hearing loss were registered in Lithuania in 2015 [3].

Prelingual CHL disturbs the child's development; it is one of the disabling conditions in the present-day environment. It is a highly heterogeneous disorder, with the majority of cases having genetic aetiology. The possibilities of genetic diagnosis, rehabilitation, and possible treatment options are dependent on the search of genes associated with this disorder. The ear is a very complex organ, and the proper development of the tissues in macroscopic, microscopic and molecular levels and function are essential for the perception of sound. These processes are influenced mostly by genetic factors. Recent advances in the gene identification techniques have revolutionized the clinical approach to CHL. More than 400 and 150 genetic loci are associated with syndromic and non-syndromic hearing loss respectively. The high heterogeneity of hearing loss is important in genetic counselling – the disorder may be inherited in the autosomal dominant, autosomal recessive, X recessive and mitochondrial manner.

Despite huge scientific efforts, about 35-40 % of disease causes remain unknown, while the etiologic cure is currently not available and the treatment of HL mostly focuses on rehabilitation with hearing aids and implants. Obviously, not all disease causes are identified. Pathogenic variants in the coding sequences cause 85 % of the monogenic disorders; therefore, results of new generation sequencing may explain a big part of the cases.

This research will significantly contribute to the diagnostic investigation of children with HL in Lithuania as advanced genetic testing was performed on Lithuanian patients and experience in the field of new generation sequencing and an analysis of data were gained. This work aimed to identify new pathogenic alterations of known genes and characterize their phenotypes. An understanding of pathogenesis mechanisms of HL is the basis for developing new treatment strategies. Genotype-phenotype correlation was analyzed in this research to set the principles of predicting the course of disease and the effectiveness of cure measures and to lay the future prospects in treatment and rehabilitation of the patients. The etiological profile of HL in our population was determined and the Lithuanian population burden of HL was assessed. This knowledge, together with genotype/phenotype correlation, aided in developing the recommendations of effective etiologic diagnostics of HL, which are essential in improving the treatment and social integration, predicting the prognosis for the patients and their families. It was the pioneering research in Lithuania of large-scale genome analysis and the evaluation of the etiologic profile of HL and the Lithuanian population burden of HL.

Aim of the study

1. To assess the influence of the genetic factors to pathogenesis and phenotypic manifestation of hearing loss within the Lithuanian population.

Main tasks of the study

1. To form a group of affected participants, characterize it epidemiologically, clinically and genealogically, to perform genetic investigations and an analysis of results evaluating the implication of the genetic alterations to etiopathogenesis of hearing loss;
2. To determine the etiologic structure of congenital/hereditary hearing loss in the Lithuanian group of affected participants;
3. To analyze the impact of genetic variants to hearing loss characteristics;
4. To assess the incidence of autosomal recessive and X recessive hearing loss within the Lithuanian population;

5. To prepare guidelines for the genetic diagnostics of congenital/hereditary hearing loss in Lithuania.

Relevance and novelty of the research

1. A clinical and genetic characterization of the group of affected participants consisting of more than 300 individuals and a determination of the etiologic profile of congenital/hereditary hearing loss within the Lithuanian population was performed for the first time in this study.
2. The new and effective technologies (sequencing of gene panels, whole exome) were applied for the first time in Lithuania to perform comprehensive genetic testing of HL, in such a way allowing to adjust them in research and diagnostics of many disorders. Novel genetic changes were identified and their impact to disease characteristics were delineated.
3. In this study, the incidence of autosomal recessive and X recessive HL was assessed for the first time in Lithuania; recommendations for effective etiologic diagnostics of HL in Lithuania, based on the results of analysis and scientific literature, were prepared.

The description of the new clinical characteristics of the patients with known syndromes, the understanding of the mechanisms of disease pathogenesis and the assessment of the incidence of HL will contribute to the research in the area of hearing loss, as it will serve as a basis for new diagnostics strategies and treatment in the future.

Statements to be defended

1. Genetic factors are the main cause of hearing loss and the Lithuanian population has a distinctive etiologic profile of hearing loss with unique characteristics;
2. Genetic factors contribute to more severe, symmetric hearing loss with positive genealogy;
3. The Lithuanian population has high incidence of autosomal recessive HL;
4. Genetic counselling and testing are keystones in the evaluation and diagnostics of hearing loss.

MATERIALS AND METHODS

A cross-sectional study was performed using data of the two observational projects: DEAFGEN and LITGEN. The scheme of genetic examination was designed to perform a comprehensive analysis of participants (Fig. No. 1).

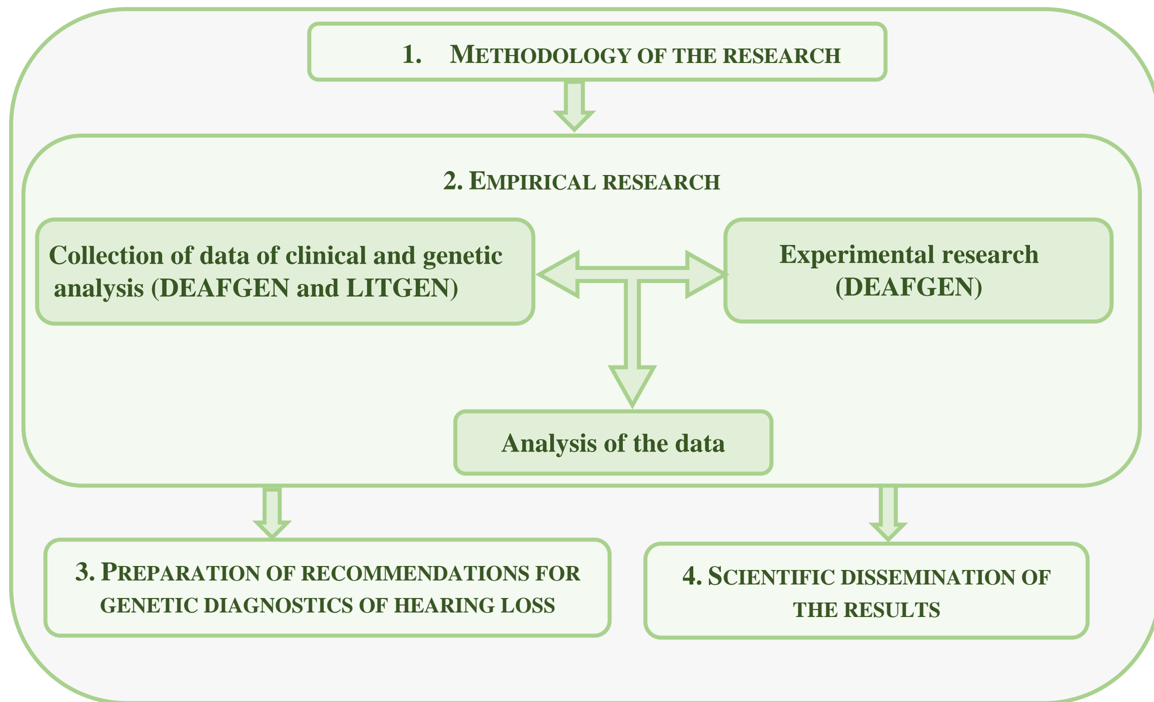

Figure No. 1. Scheme of the research.

The data and venous blood samples of the two groups of participants were collected in the current study for the clinical and genetic analysis (DEAFGEN group: individuals affected with HL and LITGEN group: individuals of ethnic Lithuanian population). The population-specific etiologic profile was determined in syndromic and non-syndromic subgroups of participants, while the genotype-phenotype correlation analysis was performed in a non-syndromic subgroup of participants (DEAFGEN), and the incidence of AR and XR HL within the Lithuanian population was assessed (LITGEN).

The recruitment of participants with HL (DEAFGEN project)

Patients affected with (I) an early onset (before 5 years of age) of non-syndromic hearing loss, (II) syndromic HL, diagnosed at any age or (III) non-syndromic hearing loss with a positive family history diagnosed at any age (except for

presbyacusis) referred to the Center for Medical Genetics and Center of Ear, Nose and Throat at the Vilnius University Hospital Santaros Clinics from 2010 to 2017 were enrolled in this study.

Demographic data and medical records were obtained and physical examinations together with genealogy analyses were performed. In the presence of several affected relatives in the family, only one (randomly chosen) was recruited to the study to avoid bias of analysis.

Clinical evaluation of the group of the affected participants

All participants in the study were assessed in accordance with age-specific, specialized audiological evaluations. Pure-tone audiometry was obtained when possible, with the use of a diagnostic audiometer in a soundproof booth, in accordance with ISO standards. The threshold values in decibels (dB) for 0.5, 1, 2 and 4 kHz were averaged for both ears (pure-tone average PTA). In cases without pure-tone audiometry, the threshold of the wave V of the click-evoked auditory brainstem responses ABR or an auditory steady state response ASSR were used to calculate the hearing level. The definition of the degree and type of HL was based on the most recent audiogram available. The degree of HL was classified according to the PTA (or extrapolated auditory brainstem responses value) as mild (25-40 dB), moderate (41-70 dB), severe (71-90 dB) or profound (>90 dB). The severity of deafness was defined by the degree of hearing loss in the better ear. Asymmetry was defined if the PTA between ears revealed the difference of 15 dB or greater.

Syndromic and non-syndromic (isolated) HL types were defined according to the data of phenotype analysis and instrumental investigation.

The participant was assigned to have positive genealogy if at least one relative with early onset hearing loss was determined within the family or assigned to a negative genealogy subgroup if the case was apparently sporadic.

Venous blood samples and written informed consent forms of affected participants or their parents (in the case of minors under the age of 16 years) were collected for the “Genomics of Congenital/Hereditary Hearing Loss: Implication in Disease

Pathogenesis, Influence to Phenotypic Expression and Treatment Efficiency" (acronym: DEAFGEN) project. The approval to conduct the DEAFGEN project was provided by the Vilnius Regional Research Ethics Committee.

Recruitment of an ethnic Lithuanian population group of healthy individuals (LITGEN project)

The group of healthy participants consisted of 98 unrelated adult individuals. This group represents the pure ethnic Lithuanian population due to the strict criteria of the enrollment conferring the uniqueness of this group: all self-reported healthy study participants indicated at least three generations of Lithuanian ethnicity and residency in the same ethnolinguistic region.

The data, venous blood samples and written informed consent forms were collected from individuals who were invited to the primary healthcare centers in the different regions of Lithuania in the period of 2011-2013 for the "Genetic Diversity of the Population of Lithuania and Changes of Its Genetic Structure Related with Evolution and Common Diseases" (acronym: LITGEN) project. The approval to conduct the LITGEN project was provided by the Vilnius Regional Research Ethics Committee. No follow-up or exposure were performed.

Genetic analysis in the group of affected participants (Fig. No. 2)

Whole genomic DNA was extracted from peripheral blood following the standard phenol-chloroform extraction protocol.

The genetic testing of *GJB2, SLC19A2, SCARF2, MPLKIP, GJA1* genes' coding sequences and *GJB2* gene regulatory sequences was performed by Sanger sequencing. The multiplex PCR assay designed by del Castillo [4] was used to detect the del(*GJB6*-D13S1830) and del(*GJB6*-D13S1854) deletions in the group of affected participants if the *GJB2* pathogenic variants were not identified or only one heterozygous *GJB2* alteration was identified. The analysis of the *MT-RNR1* gene pathogenic variant 1555A>G was made by employing the RFLP method.

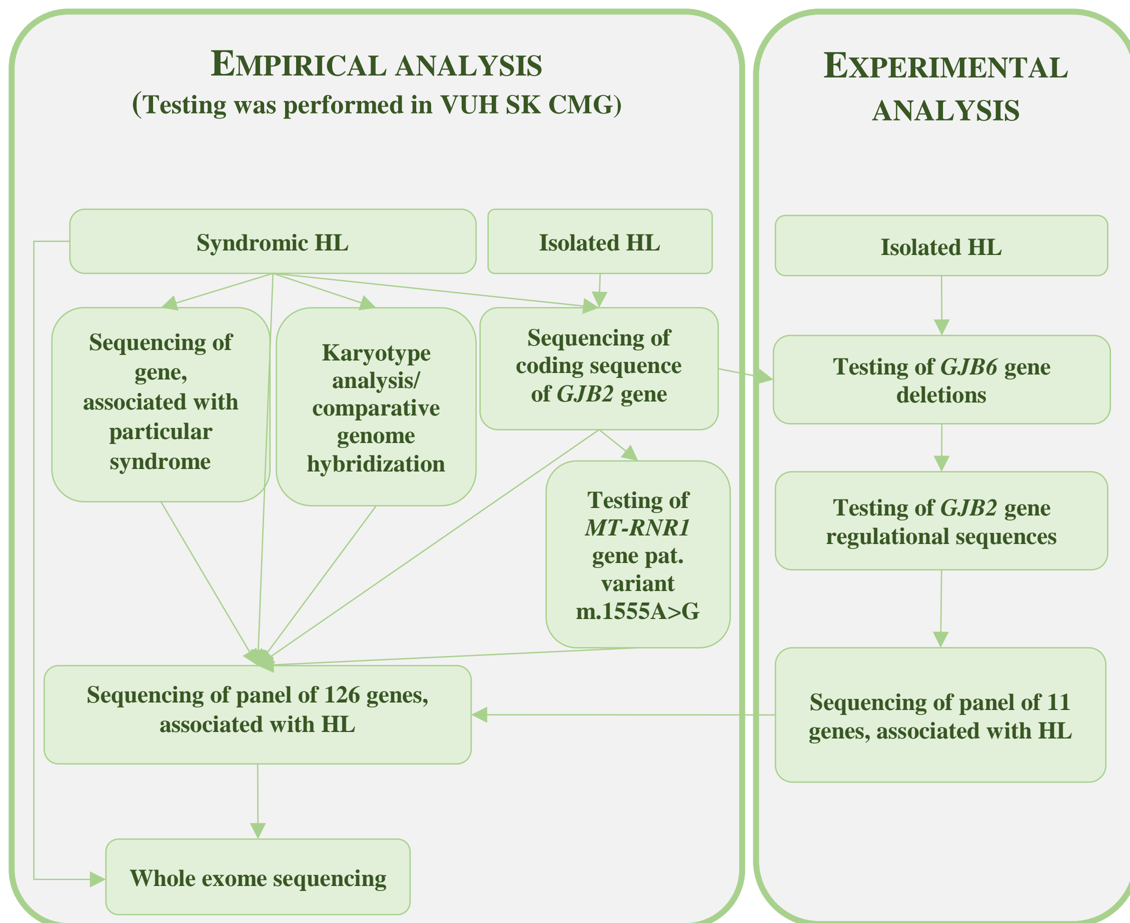

Figure No. 2. Scheme of genetic testing in the group of affected participants.

SNP-CGH was performed using the *HumanCytoSNP-12 BeadChip v2.1* SNP chip (*Illumina* Inc. San Diego, USA). New generation sequencing of 11 genes, associated with autosomal recessive HL, was made using an adapted protocol (*Thermofisher Scientific Baltics*, Lithuania) by employing multiplex PCR and the tagmentation of amplicons to generate a DNA library for new generation sequencing with the MiSeq® (Illumina, Inc., USA) instrument. *Ion AmpliSeq™ Deafness research panel v2* was used to sequence 126 genes associated with HL by the new generation sequencing technology of the Ion PGM™ Sequencer (*Life Technologies*, USA). Whole exome sequencing was performed using *TruSeq Rapid Exome Library Prep kit (8x3plex)* (*Illumina*, *Inc*., USA) and by employing the *HiScanSQ* (*Illumina*, *Inc*., USA) genetic analyzer.

Genetic analysis in the group of ethnic Lithuanian population (LITGEN)

Genomic DNA was extracted from blood using the phenol-chloroform extraction method or automated nucleic acid purification using paramagnetic particles (*TECAN Freedom EVO® 200, Tecan Schweiz AG*, Switzerland). Next-generation exome sequencing after in-solution capture enrichment (*TargetSeq*™, *Life Technologies* or *SureSelect, Agilent*), with an average of a 40-fold coverage, was performed at the Department of Human and Medical Genetics, Faculty of Medicine, Vilnius University with the use of a *5500 SOLiD*™ Sequencer in accordance with the optimized manufacturer's protocols. Sequence alignment and secondary and tertiary analyses were performed using *LifeScope*™ *Genomic Analysis Software v2.5*. The *Genome Analysis Toolkit's (GATK) CombineVariants* tool [5], [6] was used to combine all identified genomic variants from 98 individuals into a single VCF file.

The annotation of new generation sequencing data

(DEAFGEN and LITGEN) was made using the *ANNOVAR* v.2015mar22 [7] program. The pathogenicity of variants was assessed using ACMG criteria [8], taking into account the data provided by the ANNOVAR program, the available databases (*ExAC Browser* [9], *Exome Variant Server* [10], *1000 Genome Project* [11], *NCBI dbSNP* [12], *NCBI dbVar* [13], *HGMD* [14], *NCBI OMIM* [15], *NCBI ClinVAR* [16], *Leiden Open Variation Database* [17], *NCBI Genome* [18], *Deafness Variation Database* [19]) and the relevant scientific literature. The pathogenic/probably pathogenic sequence variants were checked by analyzing individuals' BAM files using the visualization tool Integrative Genomics Viewer (IGV) [20].

Analysis of data

Descriptive and analytical analyses of data of the DEAFGEN and LITGEN projects were made. Categorical variables: gender, type of hearing loss (syndromic/non-syndromic), severity and symmetry of hearing loss, genealogy (positive/negative) were all analyzed.

Hearing loss characteristics (severity and symmetry) and family history were treated as outcome variables in the analysis. The results of genetic testing were treated as predictors.

Categorical variables were expressed as absolute numbers and percentages. The binomial exact test was applied to calculate a confidence interval of 95 % for a proportion. The homogeneity hypothesis between two variables was tested using Pearson's chi-square. Logistic regression analysis was conducted to assess the impact of the genetic changes on non-syndromic HL severity and positive family history. P-values less than 0.05 were considered as statistically significant. The statistical software package *R* (version 3.2.1) was used to obtain the results. *G*Power* (version 3.1) was used for the *post hoc* power analysis of the employed test.

Homogeneity tests were employed to evaluate the impact of the genetic changes on the hearing loss phenotype in the affected group of participants.

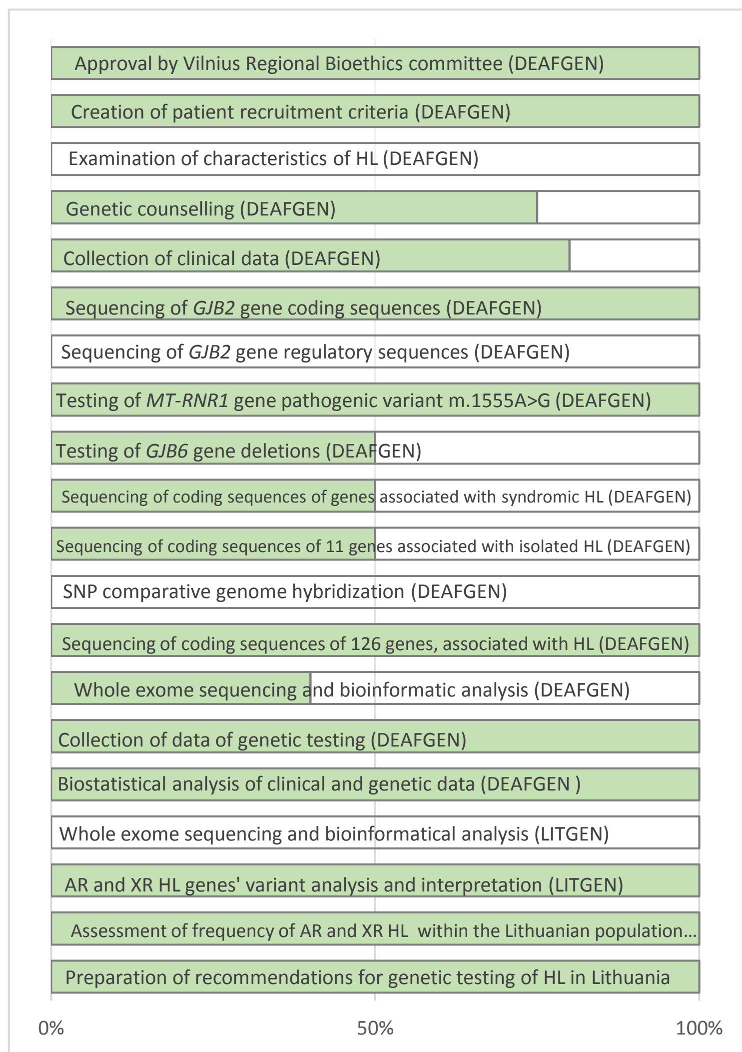

Figure No. 3. Work plan (the contribution of the author indicated in green bars).

The contribution of the author is shown in Fig. No. 3.

Results and discussion

The Lithuanian population group of affected participants

Three hundred and fifteen participants (289 unrelated individuals), 135 (42.9 %) females and 180 males (57.1 %) were enrolled in the Lithuanian population group of affected participants. The subgroup of isolated HL consisted of 241 individuals; syndromic HL was diagnosed to 74 participants (Fig. No. 4.)

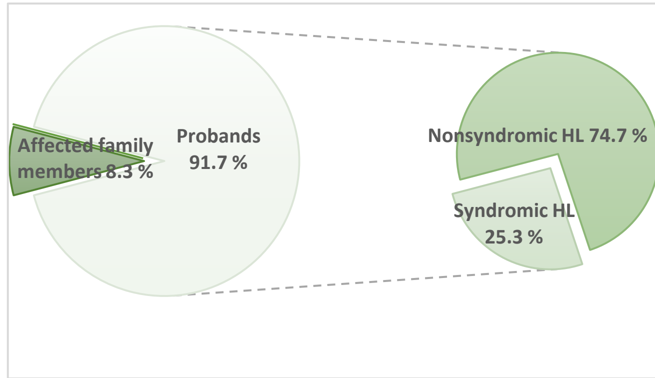

Figure No. 4. Structure of the DEAFGEN subgroup of participants in accordance with the type of HL.

Results of the clinical evaluation in the subgroup of isolated hearing loss

Per clinical evaluation, mild, moderate, severe and profound HL was identified in 28 (13.0 %), 53 (24.5 %), 32 (14.8 %), and 103 (47.7 %) of the affected unrelated participants respectively.

One hundred eighty-eight (87.0 %) individuals suffered from symmetrical hearing loss and 28 (13.3 %) had non-symmetric HL.

The genealogy analysis revealed 88 (40.7 %) unrelated participants with positive family history of hearing loss and 128 (59.3 %) individuals without affected family members (Table No. 1).

The analysis of the course of the disorder showed that seven participants (3.2 %) had a clinically proven, nonhereditary cause of HL, proven by laboratory and / or instrumental methods (Table No. 1).

Table No. 1. Results of the clinical evaluation in the subgroup of isolated HL.

Feature	Type	Counts	%
Severity	Mild	28	13.0
	Moderate	53	24.5
	Severe	32	14.8
	Profound	103	47.7
Symmetry	Symmetric	188	87.0
	Non-symmetric	28	13.0
Genealogy	Positive	88	40.7
	Negative	128	59.3
Nonhereditary cause of HL	Identified	7	3.2
	Not identified	209	96.8

Results of genetic testing in the subgroup of isolated HL

1. The results of sequencing of coding sequence of the *GJB2* gene (NM_004004)

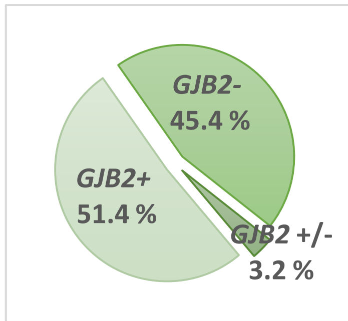

Fig. No. 5. Results of the *GJB2* gene testing in the subgroup of isolated HL.

The sequencing of the *GJB2* gene (NM_004004) was performed to all individuals in the subgroup of isolated HL. The *GJB2* gene coding sequence analysis revealed 2 pathogenic variants in a homozygous or compound heterozygous state in 111 (51.4 %) affected unrelated participants (*GJB2*+), while 7 (3.2 %) individuals had 1 pathogenic variant in a heterozygous state (*GJB2*+/-) and 98 (45.4 %) unrelated participants had no causative *GJB2* gene pathogenic variants (Fig. No. 5).

A total of seven different pathogenic variants (frameshift and missense) were identified in the affected group of participants (Table no. 2).

Table No. 2. Allele frequencies of pathogenic *GJB2* gene variants in the non-syndromic subgroup of unrelated participants.

Pathogenic *GJB2* gene variant	**Count**	**Allele frequency, %**	**Prediction by *in silico* computational analysis**
c.35delG, p.(Gly12Valfs*2), rs80338939	152	66.4	***MutationTaster2*** Disease causing ***HGMD*** ID CD972240
c.313_326del14 p.(Lys105Glyfs*5), rs111033253	53	23.1	***MutationTaster2*** Disease causing ***HGMD*** ID CD991732
c.101T>C p.(Met34Thr), rs35887622	12	5.2	***Sift*** Damaging (score 0.027) ***Polyphen*-2** Benign (score 0.083) ***MutationTaster2*** Disease causing ***HGMD*** ID CM970679
c.109G>A p.(Val37Ile), rs72474224	4	1.7	***Sift*** Tolerated (score 0.717) ***Polyphen*-2** Probably damaging (score 1.0) ***MutationTaster2*** Disease causing ***HGMD*** ID CM000016
c.269T>C p.(Leu90Pro), rs8033894	4	1.7	***Sift*** Damaging (score 0.000) ***Polyphen*-2** Probably damaging (score 1.0) ***MutationTaster2*** Disease causing ***HGMD*** ID CM990691
c.167delT p.(Leu56Argfs*26), rs80338942	3	1.3	***MutationTaster2*** Disease causing ***HGMD*** ID CD972241
c.379C>T p.(Arg127Cys), rs727503066	1	0.4	***Sift*** Damaging (score 0.0) ***Polyphen*-2** Benign (score 0.423) ***MutationTaster*** Disease causing ***HGMD*** ID CM014710
Total	229	100	

The most prevalent *GJB2* gene pathogenic variant in our study group was c.35delG, p.(Gly12Valfs*2) (rs80338939), which accounts for 66.4 % of the pathogenic alleles. (Table No. 2). The second most frequent pathogenic change in the group of affected participants was c.313_326del14, p.(Lys105Glyfs*5) (rs111033253), with a frequency 23.1 % of pathogenic alleles.

Table No. 3. Genotype distribution of pathogenic *GJB2* gene variants in the *GJB2*+ subgroup of participants with isolated HL.

***GJB2* genotype**	**Profound**	**Severe**	**Moderate**	**Mild**	**Total**	**%**
c.35[delG];[delG]	39	8	5	0	52	46.8
c.[35delG];[313_326del14]	21	6	3	1	31	27.9
c.313_326[del14];[del14]	5	1	3	0	9	8.1
c.[35delG];[101T>C]	0	0	2	4	6	5.4
c.[35delG];[269T>C]	1	0	0	2	3	2.7
c.[35delG];[109G>A]	0	0	2	0	2	1.8
c.[c.101T>C];[313_326del14]	0	0	0	2	2	1.8
c.[35delG];[167delT]	0	0	1	0	1	0.9
c.[35delG];[379C>T]	1	0	0	0	1	0.9
c.[269T>C];[313_326del14]	0	0	0	1	1	0.9
c.[167delT];[313_326del14]	0	0	1	0	1	0.9
c.101[T>C];[T>C]	0	0	0	1	1	0.9
c.[101T>C];[109G>A]	0	0	0	1	1	0.9
Total *GJB2* (+)	67	15	17	12	111	100.0

Other pathogenic variants, namely c.269T>C, p.(Leu90Pro) (rs8033894), c.101T>C, p.(Met34Thr) (rs35887622) and c.109G>A, p.(Val37Ile) (rs72474224) were much rarer – each accounted for only up to 5.2 % of pathogenic alleles.

Hearing loss is considered to be a very heterogeneous disorder. Although many genes have been associated with hearing loss, alterations in the *DFNB1* locus are to be the most frequent causes of autosomal recessive hearing loss and routine sequencing of the *GJB2* gene and testing of *GJB6* gene deletions are recommended in the EMQN best practice guidelines [21].

The results show a very high proportion of *GJB2*-positive individuals (51.4 %) in the research subgroup affected with isolated sensorineural HL as compared with other Caucasian populations (from 10 to 40 % of cases [22]) representing an adequate selection of patients for genetic testing by referring physicians and/or a quite high genetic homogeneity in our population.

Though the role of *GJB2* and *GJB6* gene alterations in the pathogenesis of sensorineural HL is undisputed, the structure of pathogenic changes identified in different populations is not uniform. The *GJB2* gene variant c.35delG, p.(Gly12Valfs*2) (rs80338939) is the most frequent in Caucasian populations [23] and accounts for up to 70 % of mutated *GJB2* gene alleles. The most prevalent *GJB2* gene change in the affected group of participants of our study also was c.35delG, p.(Gly12Valfs*2) (rs80338939). Its allele frequency (66.4 %) is consistent with many previously published studies in groups of affected individuals of Caucasian populations.

Figure No. 6. Allele frequencies of c.313_326del14, p.(Lys105Glyfs*5) (rs111033253) mutation in *GJB2*-positive groups of affected individuals in European populations (see references in *Discussion*). Adapted from Europe_political_chart, available under Creative Commons Attribution-Share Alike 3.0 Unported and GNU Free Documentation Licenses.

The c.313_326del14, p.(Lys105Glyfs*5) deletion (rs111033253), formerly called c.310del14, c.312del14, and c.314del14, truncates the *GJB2* gene and consequently interferes with the structural and functional integrity of connexons. To the best of current knowledge, this alteration has been identified previously in many European populations with a frequency of pathogenic alleles in the affected groups of participants from 0.5 % to 7.3 % (the highest allele frequency, 7.3 %, occurs in the Polish population (Fig. No. 6))[24-36].

The high frequency of the c.313_326del14, p.(Lys105Glyfs*5) (rs111033253) (23.1 % of pathogenic alleles) deletion in the subgroup of participants affected with isolated HL was an unexpected finding in this study. The c.[313_326del14]; [313_326del14] genotype was found in 8.1 % of the *GJB2*-positive group of

unrelated affected participants, suggesting not only a high frequency of carriers of this alteration in our population but also its possible origin in Lithuanian ancestors. The high frequency of carriers of the c.313_326del14, p.(Lys105Glyfs*5) (rs111033253) deletion in the entire Lithuanian population is supported by it being identified twice in the ethnic Lithuanian group of healthy participants (a frequency of 2.0 % of carriers in the study group (see Chapter *Results of analysis in research group of ethnic Lithuanian population (LITGEN)*). The frequency of carriers of this deletion, $4.9x10^{-4}$, has been determined in the NHLBI Exome Sequencing Project in a group of European American descent, showing the extreme rarity of this alteration in the healthy population [37]. The pathogenic variant in a homozygous state has been found in 2 out of 12 *GJB2*-positive study participants (16.7 %) of Tatar ethnicity in the Volga-Ural region of Russia [38]. These numbers are too low to make comprehensive conclusions, but homozygosity itself (with the exception of consanguinity) is a marker of a higher carrier rate in that particular population. In light of the close historical relationship between Lithuanians and Tatars during the wars in the 13th-14th centuries, this finding may provide a substantial basis for further analysis or multipopulational research of migration and assimilation processes in Eurasia. Recently, a literature review and a cluster analysis of *GJB2* pathogenic variants were published, in which the Eastern European descent of the alteration was proposed [39]. Pilot genetic screening of hearing impairment in newborns from Grodno oblast (Belarus) revealed c.313_326del14, p.(Lys105Glyfs*5) (rs111033253) allele frequency of 7 % and Polish origin was suggested [40]. From the current analysis, its Lithuanian descent may be presumed. The relatively low frequency of the c.313_326del14, p.(Lys105Glyfs*5) (rs111033253) allele in the Latvian group of affected participants proves that the alteration emerged after the formation of the Baltic tribes. The higher frequencies of the c.313_326del14, p.(Lys105Glyfs*5) (rs111033253) allele amongst neighboring countries (Poland and Grodno oblast of Belarus) may represent the spreading of the deletion due to close interrelationships throughout the history of Lithuania.

The coding sequence analysis of the *GJB2* gene in the subgroup of DEAFGEN individuals revealed pathogenic variants of the *GJB2* gene to be the main cause of isolated HL. The structure of the pathogenic allele frequency is similar when compared with other populations, but a high frequency of c.313_326del14, p.(Lys105Glyfs*5) (rs111033253) deletion encourages to presume its Lithuanian origin, although it could be verified only by additional multipopulational research.

2. <u>Results of analysis of the *GJB2* gene regulatory sequences</u>

Sequencing of the *GJB2* gene regulatory sequences was performed to 105 participants of the isolated HL subgroup. The characteristics of SNP identified are presented in Table No. 4.

Table No. 4. Characteristics of SNP, identified in the *GJB2* gene regulatory sequences.

Identification number	Genomic position	Coding sequence position	Region of gene	MAF (%)[1]	Pathogenicity (ClinVar)
rs138841468	13:g.20193835G>A	c.-1075C>T	5' regul.seq	0.7 (A)	NA
rs9509086	13:g.20193818G>T	c.-1058C>A	5' regul.seq	44.8 (G)	NA
rs9550621	13:g.20193244A>G	c.-484T>C	5' regul.seq	18.3 (A)	NA
rs117685390	13:g.20193170A>G	c.-410T>C	5' regul.seq	21.4 (G)	NA
rs9552101	13:g.20193129T>C	c.-369A>G	5' regul.seq	23.3 (T)	NA
rs3751385	13:g.20188817A>G	c.*84T>C	3' UTR	31.3 (A)	Benign
rs55704559	13:g.20188733T>C	c.*168A>G	3' UTR	2.9 (C)	Benign
rs9237	13:g.20187834C>A	c.*1067G>T	3' UTR	13.0 (C)	Benign
rs7623	13:g.20187749C>T	c.*1152G>A	3' UTR	10.7 (C)	Benign
rs7988691	13:g.20187624A>G	c.*1277T>C	3' UTR	0.3 (A)	Benign

[1] Data of 1000 genomes project;

The genotype and allele frequencies of SNPs identified are presented in Table No. 5.

Table No. 5. Genotype and allele frequencies of SNPs, identified in the *GJB2* gene regulatory sequences.

Identification number	Genotype	Genotype frequency	MAF (%)	H-V equilibrium	
				$\chi2$	*p*
rs138841468	c.-1075[=];[=]	0.97	1.0	0.02	0.882
	c.-1075[C>T];[=]	0.03			
	c.-1075[C>T];[(C>T)]	0			
rs9509086	c.-1058[=];[=]	0.08	22.0	2.37	0.124
	c.-1058[C>A];[=]	0.30			
	c.-1058[C>A];[(C>A)]	0.62			
rs9550621	c.-484[=];[=]	0	0.0	NA	<0.0001
	c.-484[T>C];[=]	0			
	c.-484[T>C];[(T>C)]	1			
rs117685390	c.-410[=];[=]	0.70	15.0	1.12	0.291
	c.-410[T>C];[=]	0.29			
	c.-410[T>C];[(T>C)]	0.01			

Table No. 5 (continuation). Genotype and allele frequencies of SNPs, identified in the *GJB2* gene regulatory sequences.

Identification number	Genotype	Genotype frequency	MAF (%)	H-V equilibrium	
				χ2	*p*
rs9552101	c.-369[=];[=]	0	0.5	0	0.960
	c.-369[A>G];[=]	0.01			
	c.-369[A>G];[(A>G)]	0.99			
rs3751385	c.*84[=];[=]	0.05	16.0	2.61	0.106
	c.*84[T>C];[=]	0.23			
	c.*84[T>C];[(T>C)]	0.72			
rs55704559	c.*168[=];[=]	0.90	5.0	0.32	0.571
	c.*168[A>G];[=]	0.10			
	c.*168[A>G];[(A>G)]	0			
rs9237	c.*1067[=];[=]	0	2.0	0.04	0.842
	c.*1067[G>T];[=]	0.04			
	c.*1067[G>T];[(G>T)]	0.96			
rs7623	c.*1152[=];[=]	0	1.0	0.01	0.922
	c.*1152[G>A];[=]	0.019			
	c.*1152[G>A];[(G>A)]	0.981			
rs7988691	c.*1277[=];[=]	0	1.0	0.01	0.922
	c.*1277[T>C];[=]	0.019			
	c.*1277[T>C];[(T>C)]	0.981			

Results of sequencing of 5′ regulatory region of *GJB2* gene.

An *in silico* analysis with the *MatInspector* tool showed that the 1142 bp 5′ regulatory region has 440 potential binding sites for transcription factors (TF). 45 of them have sequences characteristic to promoter-TF binding matrix models.

In the non-syndromic HL subgroup, five SNPs of the *GJB2* gene 5′ regulatory region were identified, three of them being in promoter sequence. A bioinformatics analysis revealed 22 sequences of potential binding sites for TF that may be disturbed by SNPs identified in *GJB2* gene 5′ regulatory region. Their expression is variable throughout the tissues and the inner ear is not the dominant location. According to MAF and the characteristics of TF, none of the SNPs of the *GJB2* gene 5′ regulatory region could be associated with direct influence on the pathogenesis of hearing loss.

The results of sequencing of *GJB2* gene 3′ UTR region.

Five SNPs were identified in *GJB2* gene 3′ UTR region. A bioinformatics analysis with the *TargetScan* tool did not reveal any conservative binding sites for miRNAs.

Eleven sites with low conservativeness were identified at SNPs rs3751385, rs9237, and rs55704559.

3´ UTR variants may have negative influence on the folding of mRNA and therefore may interfere with its transport to cell cytoplasm and protein translation.

An *in silico* analysis of rare SNPs using the *Mfold* tool demonstrates that rs55704559 rare allele changes the secondary structure of mRNA, but rs7988691 doesn't have any influence (Fig. No. 7).

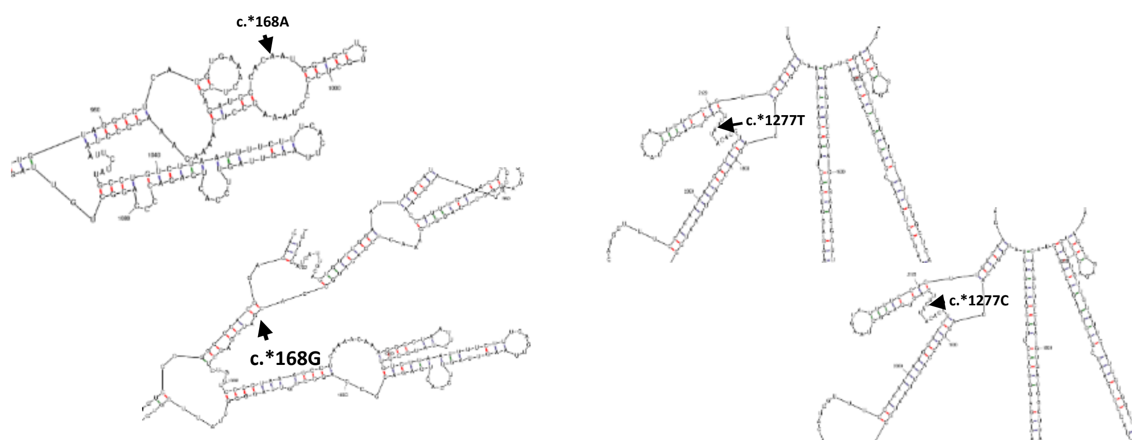

Figure No. 7. Fragments of the secondary structure of *GJB2* mRNA (rs55704559 c.*168A>G and rs7988691 c.*1277T>C).

Although scientific literature explores the possibility of influence of 3´UTR region variant rs55704559 on the posttranscriptional modification of mRNA, the absence of functional analysis deprives from appropriate classification of the variant [41].

3. <u>The results of testing of *MT-RNR1* gene (NR_137294) pathogenic variant m.1555A>G</u>

Testing of the pathogenic variant 1555A>G in *MT-RNR1* gene was performed to 14 participants of isolated HL subgroup with positive HL in maternal genealogy. The pathogenic variant was identified in four members of one family (0.46 % unrelated participants of the isolated HL subgroup). A moderate/severe hearing impairment was diagnosed to the proband in this family; her daughter was noticed to have mild fluctuating hearing loss. The mother and son of the proband currently are non-penetrant carriers.

The *MT-RNR1* gene belongs to the mitochondrial genome, it encodes 12S RNA of mitochondrial ribosomes. The pathogenic variant m.1555A>G is associated with an individual's sensitivity to aminoglycosides. Lower frequency, as compared to other Caucasian populations (where it ranges from 0.6 % to 15 %), may be explained by the low carrier frequency in Lithuanian population, a less frequent administration of aminoglycosides or an incomplete testing in the subgroup of affected participants.

4. <u>The results of testing of the *GJB6* gene (NM_006783) deletions</u>

The genetic testing of the *GJB6* gene deletions del(GJB6-D13S1830) and del(GJB6-D13S1854) was performed to all of the isolated HL subgroup participants. The *GJB6* gene deletions of 232 kb and 309 kb were not identified.

GJB6 gene alterations contribute less to the development of hearing loss, but several variants associated with HL have been described in scientific literature. Gross *DFNB1* locus deletions involving the *GJB6* gene – del(*GJB6*-D13S1830), del(*GJB6*-D13S1854), del(*DFNB1*-131 kb) and del(*DFNB1* >920 kb), which encompass non-translated *GJB6* sequences essential for both *GJB6* and *GJB2* gene transcription, have also been implicated in the pathogenesis of sensorineural HL [42]. In the Lithuanian population subgroup of isolated HL, we have not encountered any affected participant having *GJB6* gene gross deletions, indicating their rarity in our population. These results are similar to the previously published studies in other populations, strengthening the evidence that *GJB6*-related non-syndromic hearing loss is rare worldwide [43].

5. <u>The results of testing of gene panel associated with HL and whole exome sequencing</u>

Eleven genes associated with autosomal recessive HL were chosen for NGS in accordance with scientific literature [44] (Table No. 6).

Table No. 6. Eleven genes associated with AR HL, chosen for NGS.

	Gene	Genetic locus	Transcript	No of amplicons	Amplicon length, bp
1	*CDH23*	10q22.1	NM_022124.5	31	296 – 3834
2	*DFNB59*	2q31.2	NM_001042702.3	2	3000 – 3037
3	*GJB6*	13q12.11	NM_001110219.2	1	1005
4	*MYO15A*	17p11.2	NM_016239	15	345 – 4603
5	*OTOF*	2p23.3	NM_194248	14	300 – 4673
6	*SLC26A4*	7q22.3	NM_000441	8	449 – 4473
7	*TECTA*	11q23.3	NM_005422	15	488 – 3854
8	*TMC1*	9q21.13	NM_138691	17	300 – 1190
9	*TMIE*	3p21.31	NM_147196	3	288 - 744
10	*TMPRSS3*	21q22.3	NM_024022	6	300 – 3964
11	*TRIOBP*	22q13.1	NM_001039141	14	300 – 3554
			TOTAL	126	

NGS of eleven genes associated with HL was performed to 103 unrelated individuals of the isolated HL subgroup. 126 genes were sequenced to 7 unrelated individuals with positive genealogy. Eight previously described pathogenic/probably pathogenic variants in *MYO7A, MYO15A, SLC26A4*, *TMPRSS3, TRIOBP* genes were identified (Table No. 7).

Table No. 7. Previously described pathogenic/probably pathogenic variants.

No.	Variant (identification No dbSNP)	Genotype	Allele frequency in healthy populations	Variant type (according to the Deafness Variation Database[19])	*In silico* evaluation, HGMD identification no
1	*MYO7A* (NM_000260.3): c.1556G>A; p.(Gly519Asp) (rs111033206)	Hetero-zygous	**1000genomes_all:** ND **ExAC_all:** 0,01665	Pathogenic /*Usher* syndrome 1b	***SIFT*** pathogenic (score 0) ***Polyphen-2*** pathogenic (score 1.0) ***LRT*** pathogenic (score 0) ***MutationTaster2*** pathogenic ***GERP++*** conservative (score 5.02) ***HGMD* ID CM004271**
2	*MYO7A* (NM_000260.3): c.3476G>T; p.(Gly1159Val), (rs199897298)	Hetero-zygous	**1000genomes_all:** ND **ExAC_all:** 0.0002	Pathogenic /Usher syndrome 1	***SIFT*** pathogenic (score 0) ***Polyphen-2*** pathogenic (score 1.0) ***LRT*** pathogenic (score 0) ***MutationTaster2*** pathogenic ***GERP++*** conservative (score 5.02) ***HGMD* ID CM114224**
3	*MYO15A* (NM_016239.3): c.8090T>C; p.(Val2697Ala) (rs200451098)	Hetero-zygous	**1000genomes_all:** ND **ExAC_all:** 0.0004	Pathogenic /hereditary hearing loss	***SIFT*** pathogenic (score 0) ***Polyphen-2*** probably pathogenic (score 0.999) ***MutationTaster2*** pathogenic ***PhyloP*** conservative (score 1.952) ***GERP++*** conservative (score 5.19**)**
4	*SLC26A4* (NM_000441.1): c.85G>C; p.(Glu29Gln) (rs111033205)	Hetero-zygous	**1000genomes_all:** ND **ExAC_all:** 0.00005263	Pathogenic/enlarged aqueduct syndrome	***SIFT*** benign (score 0.47) ***Polyphen-2*** probably pathogenic (score 0.662) ***LRT*** pathogenic (score 0.000115) ***MutationTaster2*** pathogenic ***PhyloP*** conservative (score 2.349) ***GERP++*** conservative (score 4.21) ***HGMD* ID CM011487**
5	*SLC26A4* (NM_000441.1): c.304+2T>C (rs746238617)	Hetero-zygous	**1000genomes_all:** ND **ExAC_all:** 0.000008237	Pathogenic /hearing loss	***MutationTaster2*** pathogenic ***PhyloP*** non-conservative (score 2.648) ***GERP++*** conservative (score 3.62) ***Human Splicing Finder*** pathogenic ***HGMD* ID CS093410**

Table No. 7 (continuation). Previously described pathogenic/probably pathogenic variants.

No.	Variant (identification No dbSNP)	Genotype (count)	Allele frequency in healthy populiations	Variant type (according to Deafness Variation Database [19])	*In silico* evaluation, HGMD identification no.
6	*SLC26A4* (NM_000441): c.1246A>C, p.(Thr416Pro) (rs28939086)	Hetero-zygous	**1000genomes_all :** ND **ExAC_all:** 0.0002	Pathogenic /*Pendred* syndrome; hereditary hearing loss	***SIFT*** pathogenic (score 0) ***Polyphen-2*** probably pathogenic (score 1) ***LRT*** pathogenic (score 0) ***MutationTaster2*** pathogenic ***PhyloP*** conservative (score 2.137) ***GERP++*** conservative (score 5.1) ***HGMD* ID CM981505**
7	*SLC26A4* (NM_000441): c.1963A>G p.(Ile655Val) (rs397516424)	Hetero-zygous	**1000genomes_all :** ND **ExAC_all:** 0.00003299	Probably pathogenic / hereditary hearing loss; *Pendred* syndrome	***SIFT*** benign (score 0.56) ***Polyphen-2*** benign (score 0.034) ***LRT*** pathogenic (score 0) ***MutationTaster2*** pathogenic ***PhyloP*** conservative (score 2.183) ***GERP++*** conservative (score 5.74) ***HGMD* ID CM109556**
8	*TMPRSS3* (NM_024022.2): c.208delC; p.(His70 Thrfs*19); (rs727503493)	Hetero-zygous	**1000genomes_all :** ND **ExAC_all:** 0,0004	Pathogenic /hereditary hearing loss	***MutationTaster2*** pathogenic ***HGMD*** ID **CD020353**
9	*TRIOBP* (NM_001039141.2): c.5014G>T; p.(Gly1672Ter) (rs200045032)	Hetero-zygous	**1000genomes_all :** 0,0002 **ExAC_all:** 0,001	Variant of unknown significance	***MutationTaster2*** patogenic ***PhyloP*** conservative (score 1,048) ***GERP++*** conservative (score 1,87) ***PUBMED* ID** 28089734

Table No. 8. Novel, probably pathogenic/uncertain clinical significance variants.

No.	Variant (identification No dbSNP)	Genotype	Allele frequency in healthy populations	*In silico* evaluation, HGMD identification no.	Variant type
1	*CDH23* (NM_022124.5): c.6983A>C; p.(Asn2328Thr) (ND)	Heterozy-gous	**1000genomes_all:** NA **ExAC_all:** NA	***SIFT*** pathogenic (score 0.002) ***Polyphen-2*** pathogenic (score 1) ***MutationTaster2*** pathogenic ***GERP++*** conservative (score 5.53)	Uncertain clinical significance
2	*CDH23* (NM_022124.5): c.9319+1G>A, (ND)	Heterozy-gous	**1000genomes_all:** NA **ExAC_all:** NA	***MutationTaster2*** pathogenic ***GERP++*** conservative (score 3.82) ***Human Splicing Finder*** pathogenic	Probably Pathogenic
3	*MYO6* (NM_004999.3) c.188G>T; p.(Cys63Phe) (ND)	Heterozy-gous	**1000genomes_all:** NA **ExAC_all:** NA	***Polyphen-2*** pathogenic (score 1.000) ***MutationTaster2*** pathogenic ***GERP++*** conservative (score 5.51)	Probably Pathogenic
4	*MYO15A* (NM_016239.3): c.6767_6768delinsT; p.(Gly2256Valfs*13) (ND)	Heterozy-gous	**1000genomes_all:** NA **ExAC_all:** NA	***MutationTaster2*** pathogenic	Probably pathogenic
5	*MYO15A* (NM_016239.3): c.8005dupA; p.(Leu2668fs) (ND)	Heterozy-gous	**1000genomes_all:** NA **ExAC_all:** NA	***MutationTaster2*** pathogenic	Probably pathogenic
6	*MYO15A* (NM_016239.3): c.1894_2541del648; p.(Gln632Leufs*132) (ND)	Heterozy-gous	**1000genomes_all:** NA **ExAC_all:** NA	***MutationTaster2*** pathogenic	Probably pathogenic
7	*OTOF* (NM_194248.2) c.766-1G>A (ND)	Heterozy-gous	**1000genomes_all:** NA **ExAC_all:** NA	***MutationTaster2*** pathogenic ***GERP++*** conservative (score 5.83) ***Human Splicing Finder*** pathogenic	Probably Pathogenic
8	*SLC26A4* (NM_000441.1): c.1766A>C; p.(Gln589Pro) (rs397516422)	Heterozy-gous	**1000genomes_all:** NA **ExAC_all:** NA	***SIFT*** benign (score 0.101) ***Polyphen-2*** probably pathogenic (score 0.937) ***MutationTaster2*** pathogenic ***GERP++*** conservative (score 4.57)	Uncertain clinical significance
9	*TMPRSS3* (NM_024022.2) c.206-2A>C; (ND)	Heterozy-gous	**1000genomes_all:** NA **ExAC_all:** NA	***MutationTaster2*** pathogenic ***Human Splicing Finder*** pathogenic	Probably Pathogenic

Additionally, nine probably pathogenic/uncertain significance variants of *SLC26A4*, *MYO15A*, *MYO6*, *CDH23*, *OTOF*, *TMPRSS3* genes, possibly implicated in pathogenesis of hearing loss, were identified (Table No. 8).

Table No. 9. Etiologic structure of molecular pathology, identified in DEAFGEN study.

Panel	Gene	Count of participants	Frequency in subgroup of isolated HL (%)
Eleven genes	*CDH23*	1	0.46
	MYO15A	2	0.93
	SLC26A4	2	0.93
126 genes	*MYO6*	1	0.46
	MYO7A	1	0.46
	TMPRSS3	1	0.46
	Total	8	3.7

Eight unrelated participants (3.7 % of the subgroup of isolated HL) possess pathogenic/possibly pathogenic variants of *SLC26A4*, *MYO15A*, *CDH23*, *MYO6*, *MYO7A*, *TMPRSS3* genes (Table No. 9).

Table No. 10. Etiologic structure of possible carriers of HL identified in DEAFGEN study.

Gene	Count of participants	Frequency in subgroup of isolated HL (%)
MYO15A	4	1.85
SLC26A4	2	0.93
TRIOBP	1	0.46
Total	7	3.24

Seven participants (3.24 % of the subgroup of isolated HL) were found to be carriers of one heterozygous pathogenic/likely pathogenic variant of autosomal recessive HL (Table No. 10).

The whole exome sequencing was performed on two participants of isolated hearing loss. A list of 10333 genes, associated with hereditary disorders, was chosen for analysis [45]. Unfortunately, no pathogenic/probably pathogenic variants were identified.

Genetic heterogeneity is a common feature of many Mendelian disorders, including hearing loss. The development of the organism in macroscopic, microscopic, molecular levels and its functions are dependent on the coordinated activity of the genes and their products – RNAs and proteins. Therefore, many inherited diseases represent the phenotypic manifestation of pathogenic variants in one of the hundreds or thousands of genes, which are involved in the functioning of a particular organ or tissue. The frequency of one gene alterations could seem negligible in the etiological profile of the disease, but their cumulative impact may

be significant. Genetic testing of such hereditary disorders faces questions regarding the magnitude of analysis. Sanger sequencing of a single or several genes is inadequate in the case of genetically heterogeneous diseases. Next generation sequencing (NGS) technology meets the demands of the high scale and throughput analysis, but the choice of the gene set and methods of DNA library preparation are still facing considerable difficulties. Whole genome sequencing is the widest approach of genetic analysis, but the costs are too high and data management currently is too elaborate to become a routine testing method. Whole exome sequencing is less comprehensive, and it also has the abovementioned limitations. Targeted gene sequencing panels emerged as an option for diagnostic testing of genetically heterogeneous conditions, allowing for a cost-effective and time-saving analysis.

Several strategies for the genetic analysis of HL are implicated in routine testing of HL. According to the European Molecular Genetics Quality Network best practice guidelines, the *GJB2* gene sequencing and testing of common *GJB6* gene deletions is recommended to the patients with non-syndromic sensorineural hearing loss [21]. No universal approach currently exists for the testing of other genes implicated in pathogenesis of HL. Some geneticists prefer the purposeful testing of genes according to the clinical phenotype of the patient, while others use multigene panels with tens or hundreds of genes, associated with hereditary deafness, and analyze them simultaneously. The choice of the genes for inclusion to the targeted gene panel is dependent on the frequency of its alterations in the total etiologic profile of the disorder. Several panels have been designed for sequencing of up to 140 genes (associated with both syndromic and non-syndromic hearing loss) using NGS technologies [46].

Hearing loss with autosomal recessive (AR) inheritance accounts for up to 75 % of all cases. The pathogenic variants of *CDH23*, *DFNB59*, *MYO15A*, *OTOF*, *SLC26A4*, *TECTA*, *TMC1*, *TMIE, TMPRSS3* and *TRIOBP* genes have been listed in the scientific literature among the most frequent causes of AR SNHL [47, 48].

Four *SLC26A4* (NM_000441.1) gene variants (c.85G>C, p.(Glu29Gln) (rs111033205); c.304+2T>C (rs746238617); c.1963A>G, p.(Ile655Val) (rs397516424) and c.1246A>C, p.(Thr416Pro) (rs28939086)), determined in the subgroup of isolated HL, were ascertained as pathogenic/probably pathogenic according to the databases and scientific literature analyzed (Table No. 7). Additionally, one novel *SLC26A4* gene variant of unknown significance c.1766A>C; p.(Gln589Pro) (rs397516422), identified *in trans* with a pathogenic *SLC26A4* gene variant, may be classified as pathogenic (Table No. 8).

The genotype analysis revealed two participants in the subgroup of isolated HL possessing (0.93 %) – two pathogenic/possibly pathogenic variants, both affected with profound, symmetric HL with a negative genealogy. Additionally, heterozygous *SLC26A4* variants were identified in two individuals (0.93 %) The frequency of *SLC26A4*-related, non-syndromic HL is lower in the Lithuanian group of participants as compared to other populations, where it accounts from 2.0 % to 3.5 % [49, 50].

The *MYO15A* gene variant c.8090T>C, p.(Val2697Ala) (rs200451098) has been identified in five out of 8 pathogenic alleles in our study group. This genetic change has been assigned to pathogenic variants in *Deafness Variation Database (*DVD*)* [19, 51], but *ClinVar* classifies it as a variant of unknown significance. Additionally, three novel *MYO15A*, probably pathogenic variants were identified in the subgroup of isolated HL: c.8005dupA, p.(Leu2668fs); c.6767_6768delinsT; p.(G2256Vfs*13); c.1894_2541del648; p.(Gln632Leufs*132), generating a frameshift and premature stop codon. Two participants (0.93 %) possess two pathogenic *MYO15A* gene variants in the DEAFGEN subgroup of non-syndromic HL. The highest frequency of the *MYO15A* gene pathogenic variants was determined in the Eastern population, where it accounts up to 9.5 % [52].

One participant, affected with severe, symmetric, non-syndromic hearing loss with a negative family history, possesses two novel *CDH23* gene variants in a compound heterozygous state; their pathogenicity was estimated using ACMG criteria. One

missense variant (*CDH23* (NM_022124) c.6983A>C, p.(Asn2328Thr)) is located in an extracellular part of the protein and the second splicing variant (*CDH23* (NM022124) c.9319+1G>A) is potentially related to the loss of the terminal part of the protein with a consecutive dysfunction of cadherin 23. Missense *CDH23* gene variants are considered to retain residual protein activity and their association with the milder phenotype without retinal and vestibular manifestation has been elucidated. [53].

The novel *MYO6* gene (NM_001300899) heterozygous variant c.188G>T; p.(Cys63Phe) was determined in one individual suffering from isolated, symmetric, moderate, post lingual HL. According to an *in silico* analysis, the variant is pathogenic, it wasn't identified in healthy populations. Pathogenic changes of the *MYO6* gene are associated with AR and AD progressive hearing loss with a wide spectrum of severity and age of onset, the frequency in Eastern populations reaching 7.5 % within groups of participants with a hearing impairment [54].

The *MYO7A* (NM_000260) gene pathogenic variants c.1556G>A; p.(Gly519Asp) and c.3476G>T; p.(Gly1159Val) were identified in two brothers (0.46 %), affected with isolated, symmetric, profound hearing loss. The *MYO7A* gene is associated with isolated AR or AD HL (up to 3.1 % of cases) [55] and AR *Usher* s. I type. The identified variants were described to patients with *Usher* s., but in our patients, pigmentary retinitis is currently absent; therefore, they were ascertained to the subgroup of isolated HL.

TMPRSS3 gene (NM_024022) variants c.206-2A>C and c.208delC; p.(His70 Thrfs*19) were also determined to two sibs with congenital, symmetric, profound hearing loss. Variant c.208delC; p.(His70fs) generates frameshift, in DVD classified as pathogenic. A novel splice site variant c.206-2A>C has not been described in scientific literature, but its characteristics allow to classify it as a likely pathogenic variant. According to scientific literature, pathogenic alterations of *TMPRSS3* gene confer up to 1.0 % of cases of isolated HL in European populations [56].

The *TRIOBP* (NM_001039141.2) c.5014G>T; p.(Gly1672Ter) (rs200045032) heterozygous variant was identified in one DEAFGEN participant. The change in a heterozygous state is not sufficient to explain the pathogenesis of HL. To date, few dozens of patients suffering with HL due to *TRIOBP* alterations have been identified. Supposedly, in the Lithuanian population, the *TRIOBP*-related HL frequency must be very low.

Whole exome sequencing was performed to triad (two parents affected with isolated HL and their son suffering with retardation of development and HL), pathogenic variants were not revealed. The success of identification of the cause of hereditary HL depends on criteria of recruitment of study participants, mode of inheritance and previously performed testing. The highest probability of identification of pathogenic variants is in consanguineous families and in cases with AR HL (up to 56 %) [57], and it is less in the case of sporadic HL or if gene panel testing was performed prior to WES.

The etiologic structure of HL in the subgroup of non-syndromic (isolated) HL.

Table No. 11. Etiologic structure of isolated HL in the Lithuanian population.

Causes of HL	Count of participants	Frequency in subgroup of isolated HL (%)
Monogenic HL	120	55.6 (48.7 – 62.3)
GJB2+	111	51.4 (44.5 – 58.2)
Other	9	4.2 (1.9 – 7.8)
One heterozygous AR HL variant	14	6.5 (3.6 – 10.6)
Non-hereditary causes	7	3.2 (1.3 – 6.6)
Cause of HL not identified	75	34,7 (28.4 – 41.5)
Total	216	100.0

The analysis of the results of genetic testing in the subgroup of isolated HL revealed that the molecular diagnosis was identified in 120 participants (55.6 %), and that additional 14 individuals (6.5 %) possess one AR HL variant.

Proven nonhereditary causes were implicated in the pathogenesis of HL in 3.2 % of the non-syndromic HL cases (Table No. 11 and Fig. No. 8).

The main causes of non-syndromic HL in the DEAFGEN subgroup were pathogenic variants of the *GJB2* gene (51.4 % (44.5-58.2)). Other genes – *MT-RNR1*, *SLC26A4*, *MYO15A*, *CDH23*, *MYO6*, *MYO7A*, *TMPRSS3* alterations were

less implicated in the pathogenesis of HL (4.2 % of cases) (Table No. 12 and Fig. No. 8).

Table No. 12. Structure of gene variants implicated in pathogenesis of HL in the subgroup of isolated HL.

Gene	**Count of participants**	**Frequency in subgroup of isolated HL, %(95 %CI)**
GJB2 gene (two pathogenic variants)	111	51.4 (44.5 – 58.2)
MYO15A gene (two pathogenic variants)	2	0.93 (0.1 – 3.3)
SLC26A4 gene (two pathogenic variants)	2	0.93 (0.1 – 3.3)
CDH23 gene (two pathogenic variants)	1	0.46 (0.0 – 2.6)
MT-RNR1 m.1555A>G pathogenic variant	1	0.46 (0.0 – 2.6)
MYO6 gene (one probably pathogenic variant)	1	0.46 (0.0 – 2.6)
MYO7A gene (two pathogenic variants)	1	0.46 (0.0 – 2.6)
TMPRSS3 gene (two pathogenic variants)	1	0.46 (0.0 – 2.6)
GJB2 gene (one pathogenic variant)	7	3.2 (1.3 – 6.6)
MYO15A gene (one pathogenic variant)	4	2.78 (0.5 – 4.7)
SLC26A4 gene (one pathogenic variant)	2	0.93 (0.1 – 3.3)
TRIOBP gene (one probably pathogenic variant)	1	0.46 (0.0 – 2.6)

The results show a very high proportion of *GJB2*-positive individuals (51.4 %) in the research subgroup affected with isolated HL as compared to other Caucasian populations representing an adequate selection of patients for genetic testing by referring physicians and/or quite high genetic homogeneity in our population.

The frequency of *MT-RNR1*, *SLC26A4*, *MYO15A*, *CDH23*, *MYO6*, *MYO7A*, and *TMPRSS3* genes-related HL differs among populations. The *MT-RNR1* gene variant 1555A>G – is a very frequent cause of HL in Spain's population, where it accounts for up to 15 % of cases. *SLC26A4* gene variants prevail in Eastern populations (up to 12.6 %) [58]. *MYO15A, MYO6, MYO7A* genes are also frequently listed among the causes of hearing loss [59].

CDH23 gene pathogenic variants are most frequent in Eastern populations (up to 12.5 % of post lingual HL cases) [60]. *TMPRSS3* gene alterations in Caucasian populations are rare causes of disorder (up to 0.38 %) [61], but in populations where consanguineous marriages are common, *TMPRSS3* pathogenic variants are identified more frequently.

6.5 % of participants were found to have only one heterozygous pathogenic/likely pathogenic variant, which is insufficient to explain the disease pathogenesis.

The discussions regarding the potential chances for the carrier of an AR HL heterozygous alteration to develop HL (the effect of modifying genes, digenic mode of inheritance, haploinsufficiency etc.) had ended with lack of evidence. Moreover, often another unrelated hereditary cause of disease is identified to the patient. According to this, these participants are most likely only carriers of the AR HL causing variant. However, without any additional comprehensive testing (quantitative, promoter, other regulatory sequences, epigenetic analysis), the implication of these variants in pathogenesis of HL cannot be completely ruled out.

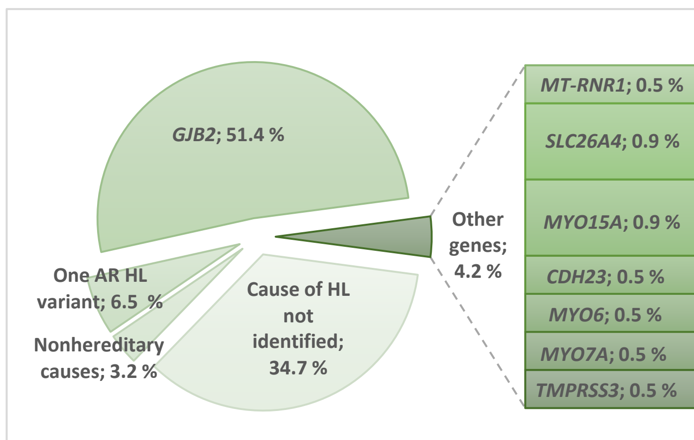

Figure no. 8. Etiologic profile of isolated HL in the Lithuanian population.

Hearing impairment due to environmental factors was determined in 3.2 % of participants. Obviously, the real scale of this cause is much higher, because only life threatening conditions were diagnosed. According to the scientific literature, subclinical CMV infection alone may cause 15-20 % of HL [62].

In the DEAFGEN subgroup of isolated HL, no hereditary/environmental causes were revealed in 34.7 % of the participants. In the Lithuanian population, this group could be even smaller, if the NGS of at least 126 genes panel had been tested in all the participants. Also, the insufficient testing of prenatal CMV could have reduced the part of individuals with no causative factor identified. The diagnostic efficiency in other populations is 40 % on average. However, the results of analysis show that the etiologic profile of isolated HL in the Lithuanian population is similar to other Caucasian populations, although it has distinctive features, particularly revealed when the frequency of alterations is analyzed.

6. <u>The results of genotype-phenotype correlation analysis</u>

To analyze the genotype-phenotype correlation, the subgroup of participants affected with non-syndromic HL was divided into three major subgroups in accordance with the results of genetic testing. The **GEN(+)** subgroup consisted of individuals with pathogenic variants implicated in pathogenesis of HL identified, the **GEN(+/-)** subgroup consisted of individuals with one heterozygous pathogenic variant in autosomal recessive HL gene identified, and **GEN(-)** subgroup consisted of affected participants with no pathogenic variants identified. The **GEN(+)** and **GEN(-)** subgroups were compared with each other to determine the difference in disease severity, symmetry and family history. Data of **GEN(+/-)** subgroup with a single autosomal recessive pathogenic variant were not included in the genotype-phenotype correlation analysis to avoid bias of ascertainment.

The *post hoc* power analysis was performed for all the tests in the study. The calculated empirical effect size ranged from medium to large. The empirical power of the tests was above 0.8. Values for the empirical effect size and power are presented in corresponding Tables.

Homogeneity tests were employed to evaluate the impact of the genetic alterations on the hearing loss phenotype in the subgroup of participants affected with isolated HL. The results of analysis indicate that the severity of hearing loss differs statistically significantly between the GEN(+) and GEN(-) subgroups, $p=5.9x10^{-5}$ (Table No. 13, Fig. No. 9). Profound HL dominates in the GEN(+) subgroup, while moderate and mild HL are more common in the GEN(-) subgroup.

Table No. 13. Distribution of severity of HL in **GEN**+ and **GEN**- groups.

	Profound HL	**Severe HL**	**Moderate HL**	**Mild HL**	**Total**
GEN+	74	15	18	13	120
GEN-	24	14	30	14	82
Total	98	29	48	27	202
Chi-square = 22.2; Degrees of freedom = 3			**p $5.9x10^{-5}$**		
Empirical effect size w=0.7			Empirical power=1.0		

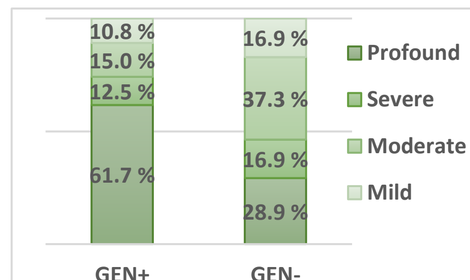

Figure No. 9. Distribution of severity of HL in **GEN**+ and **GEN**- groups.

GJB2+ individuals prevail in the **GEN**+ subgroup. To compare the influence of inactivating (frameshift) and non-inactivating (missense) *GJB2* gene variants on the characteristics of the disease, the *GJB2*(+) subgroup was divided into two classes of genotypes: I and N, where I – two inactivating (frameshift) alteration of the *GJB2* gene identified; N – at least one non-inactivating (missense) variant of the *GJB2* gene in compound heterozygosity identified.

A statistically significant difference in the distribution of HL severity in the classes of the *GJB2(+)* group was observed, p=4.2 x10^{-14} (Table No. 14, Fig. No. 10), with profound HL prevailing in the I subgroup and mild HL in the N subgroup.

Table No. 14. Distribution of severity of HL in I and N subgroups.

***GJB2*+ class**	**Profound HL**	**Severe HL**	**Moderate HL**	**Mild HL**	**Total**
I	66	14	13	1	94
N	2	0	4	11	17
Total	68	14	17	12	111
Chi-square = 65.4; Degrees of freedom = 3		**p 4.2 x10^{-14}**			
Empirical effect size w=2.3		Empirical power = 1.0			

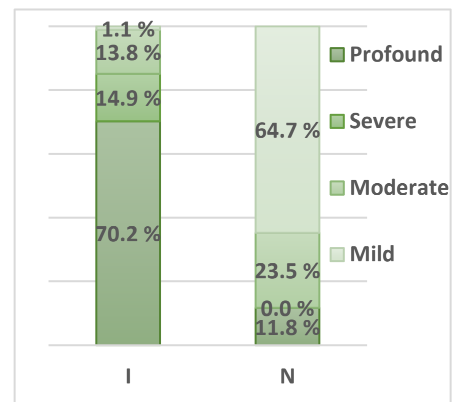

Figure No. 10. Distribution of severity of HL in I and N subgroups.

The influence of gene alterations on symmetry of hearing loss was also analyzed in the subgroup of affected individuals. The difference between **GEN**(+) and **GEN**(-) subgroups was statistically significant, p=1.1x10^{-2} (Table No. 15, Fig. No. 11)

Table No. 15. Distribution of symmetry of HL in **GEN**+ and **GEN**- groups.

	Symmetric HL	Non-symmetric (unilateral) HL	Total
GEN+	**111**	**9**	**120**
GEN-	**66**	**16**	**82**
Total	**177**	**25**	**202**
Chi-square = 6.5; Degrees of freedom = 1		**p=1.1x10^{-2}**	
Empirical effect size w = 0.3		Empirical power = **0.99**	

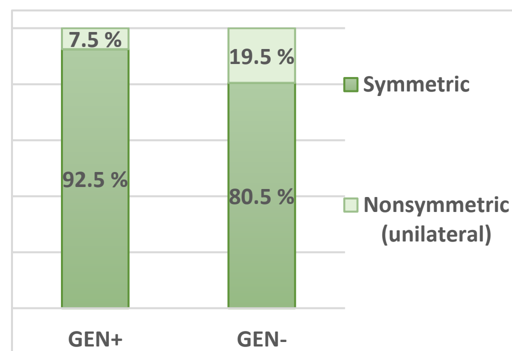

Figure No. 11. Distribution of symmetry of HL in **GEN**(+) and **GEN**(-) groups.

The genealogies of the three generations of the affected group of individuals were analyzed to assess the heredity of hearing loss. The individual was assigned to the subgroup of positive family history when more than one affected individual with early onset hearing loss were present in the family. The comparison of **GEN**(+) and **GEN**(-) subgroups showed a statistically significant difference between the subgroups, $p=1.9x10^{-3}$, indicating a more frequent positive family history in the GEN(+) subgroup (Table No. 16, Fig. No. 12).

Table No. 16. Distribution of types of genealogy in **GEN**+ and **GEN**- groups

	Positive genealogy	Negative genealogy	Total
GEN+	60	60	120
GEN-	23	59	82
Total	83	119	202
Chi-square = 9.7 Degrees of freedom = 1		**p=1.9x10^{-3}**	
Empirical effect size w=0.5		Empirical power =0.99	

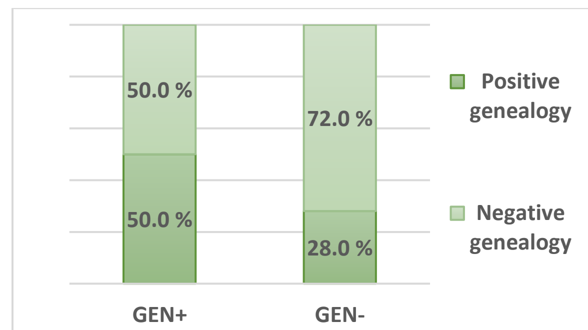

Fig. No. 12. Distribution of types of genealogy in **GEN**(+) and **GEN**(-) groups.

Logistic regression analysis was applied to evaluate the chances of identifying the genetic alterations if profound/severe hearing loss vs. moderate/mild hearing loss was diagnosed and if the patient suffers from symmetric vs. non-symmetric HL.

Table No. 17. Results of logistic regression analysis in the subgroup of isolated HL.

Characteristics of HL	OR (95 % PI)	p	Empirical power
Profound/severe HL ***vs*** **Moderate/mild HL**	3.3 (1.8 – 6.0)	$7.9x10^{-5}$	1.0
Symmetric HL ***vs*** **Nonsymmetric HL**	3.0 (1.3 – 7.1)	$1.4x10^{-2}$	0.99
Positive genealogy ***vs*** **Negative genealogy**	2.6 (1.4 – 4.7)	$2.1x10^{-3}$	0.99

OR 3.3 (95 % CI 1.8 – 6.0; $p=7.9x10^{-5}$) was calculated in our study group of affected individuals, meaning that the probability of having hereditary HL is 3.3 times higher in the case of profound or severe HL. OR 3.0 (95 % CI 1.3 – 7.1 $p=1.4x10^{-2}$) shows a higher probability of identifying hereditary HL in the case of symmetric HL (Table No. 17).

The probability of identifying gene alterations in affected individuals with a positive family history in our population was also estimated. The results indicate that the chances are 2.6 times higher than they are in individuals with a negative family history, $p=2.1x10^{-3}$ (Table No. 17).

Table No. 18. Eesults of logistic regression analysis in the subgroup of isolated HL with negative genealogy.

Characteristics of HL	OR (95 % PI)	p	Empirical power
Profound/severe HL *vs* **Moderate/mild HL**	3.2 (1.5 – 7.0)	$4.0x10^{-3}$	0.99
Symmetric HL *vs* **Non-symmetric HL**	1.9 (0.7 – 5.3)	$2.0x10^{-1}$	0.82

When analyzing the only data of the individuals with negative genealogy, the probability to identify the genetic changes to the patient affected with profound/severe HL is 3.2 times higher (95 % CI 1.5 – 7.0; $p=4.0x10^{-3}$). Whereas in respect of symmetry, the results between **GEN**(+) and **GEN**(-) subgroups did not differ significantly (Table No. 18).

Our study aimed to analyze the impact of genetic factors on the development of early onset hearing loss in an affected group of participants in the Lithuanian population. The subgroup of isolated HL consists of 241 individuals, so the current analysis describes a substantial part (about 2.6 %) of deaf people in Lithuania. The results of genotype-phenotype analysis show the significant impact of genetic alterations on the development of non-syndromic HL in the affected group of Lithuanian origin. Our findings indicate that inactivating *GJB2* gene variants were associated with a more severe phenotype than missense changes – a finding compatible with previous publications [63] and the nature of the variants. Genetic changes also in general lead to more severe (OR 3.3, $p=7.9x10^{-5}$), symmetric HL (OR 3.0, $p=1.4x10^{-2}$) with a positive family history (OR 2.6, $p=2.1x10^{-3}$), compared with the other aetiology of HL in the Lithuanian population. Several studies have made comparisons of the characteristics of HL between *GJB2*-related and *GJB2*-negative groups of affected individuals, and statistically significant differences were determined in genealogy but not in disease severity or other HL characteristics [64].

These observations may be helpful in clinical settings to prognosticate the results of genetic testing and disease course to the patients with HL in the Lithuanian population.

The characterization of subgroup of participants affected with syndromic hearing loss

The subgroup of syndromic HL consisted of 73 unrelated individuals, 23 (31.5 %) females and 50 (68.5 %) males.

The results of clinical evaluation in the subgroup of syndromic hearing loss

According to clinical evaluation, mild, moderate, severe and profound HL was identified in 11 (15.1 %), 30 (41.1 %), 13 (17.8 %) and 19 (26.0 %) of the participants respectively (Table No. 19).

Symmetric HL was diagnosed in 63 (86.3 %) individuals, non-symmetric or unilateral – to 10 (13.7 %) unrelated participants of the syndromic HL subgroup (Table No. 19).

The dysplasia/hypoplasia/aplasia of the outer/middle/inner ear with or without dysmorphias of other organs was diagnosed in 23 (31.5 %) participants with syndromic hearing loss. 50 (68.5 %) individuals had HL without auricular anomalies but with dysmorphias/dysfunction of other organs. (Table No. 19).

Table 19. The results of clinical evaluation in subgroup of syndromic HL.

Feature	Type	Counts	%
Severity	Mild	11	15.1
	Moderate	30	41.1
	Severe	13	17.8
	Profound	19	26.0
Symmetry	Symmetric	63	86.3
	Non-symmetric	10	13.7
External/ middle/ internal ear anomalies	Present	23	31.5
	Absent	50	68.5
Genealogy	Positive	20	27.4
	Negative	53	72.6

The genealogy analysis revealed 20 (27.4 %) unrelated participants with positive family history of syndromic hearing loss and 53 (72.6 %) individuals without any affected family members (Table No. 19).

A clinical diagnosis of specific syndromes with HL was detected in 47 subjects (64.4 %); 26 individuals (35.6 %) remain without a specific pathological pathway leading to a syndromic HL identified.

Monogenic syndromic HL was diagnosed in 30 (63.8 %) participants, 14 individuals (29.8 %) were diagnosed with AD syndromic HL, 13 individuals (27.7 %) – AR syndromic HL, 3 subjects (6.5 %) – XD syndromic HL. The chromosomal origin of HL was determined in six (12.8 %) of the participants, multifactorial / nonhereditary HL was identified in 11 (23.4 %) of the individuals (Fig. No. 13 and Table No. 20).

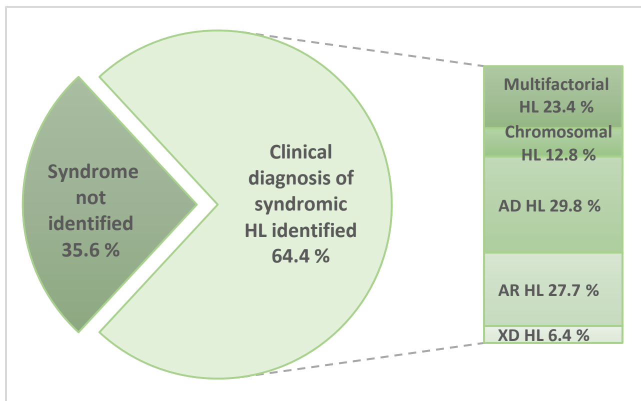

Fig. No. 13. Etiologic structure in the subgroup of participants with syndromic HL.

A genotype-phenotype correlation analysis in the syndromic HL subgroup would not have any biological bases due to the very wide spectrum of pathologies; therefore, it wasn't performed.

Genetic counselling, in the case of syndromic hearing loss, is important both for establishing clinical/molecular diagnosis and foreseeing a further course of illness and prognosis. To date, about 400 syndromes with HL are known (~6 % of rare disorders) [65].

Syndrome recognition depends on many factors, so a multidisciplinary approach is of utmost importance. The experience and intuition of the clinical geneticist helps to identify the rarest disorders. Counselling of other physicians and results of instrumental examinations complement or reject primary diagnosis. Genetic testing (molecular, cytogenetic, biochemical genetic) allows to identify the biological cause of the disorder, sometimes confer the establishment of primary diagnosis (especially in the case of a very rare/unique pathology). In VUH SK Centre for Medical Genetics, large scale, high throughput methods (new generation

sequencing of gene panel, whole exome sequencing, aCGH, SNP-LGH) were started in 2015-2016; therefore, the genetic examination of many patients is not accomplished and molecular diagnoses were established only in 24.7 % of participants.

In different populations, AD syndromic hearing impairment is determined in 15-30% of diagnosed syndromic cases, and *Treacher-Collins* s., *CHARGE* s., branchiootorenal s., *Waardenburg* s. are prevailing disease entities. AR syndromic HL is determined in 30-60 % of syndromes identified, the most common being *Usher* s., *Pendred* s., *Jervel&Lange-Nielsen* s. XR syndromic HL is diagnosed in 5-10 % of cases. Multifactorial HL (mostly *Goldenhar* s.) is diagnosed in 5-15 % patients. Syndromic HL, due to other aetiology (imprinting disorders, mitochondriopathies), is determined in 3-8 % of affected individuals.

The etiologic profile of diagnosed syndromic hearing loss in the Lithuanian population is similar to the structure of other populations, as is similar the spectrum of disease entities identified. Nevertheless, in our group of participants, very rare/unique disorders were determined (*Rogers* s., *ichthyosis – prematurity* s., chromosomal origin syndromic HL due to unbalanced translocation between long arms of 1st and 21st chromosomes), making the structure of syndromic HL in our population distinctive from other nations.

Table No. 20. Clinical diagnoses in the subgroup of syndromic HL.

Type of HL		**Count of participants**	**Clinical diagnose**	**TLK-10-AM**	**Count of participants**	**Pathogenic genetic changes**
Monogenic	AD	14	*Treacher-Collins* s.	Q87.04	5	*TCOF1* (NM_001135243.1) c.[1A>G];[=] (p.[(Met1?)];[=])
						TCOF1 (NM_001135243.1) c.[2103_2106delTGAG(;)4329_4331delGAA]
						TCOF1 (NM_001135243.1) c.[4486_4488delAAG];[=], p.[(Lys1496del)];[(=)]
						TCOF1 (NM_001135243.1) c.[3527C>G];[=], p.[(Pro1176Arg)];[=]
			CHARGE s.	Q87.86	4	*CHD7* (NM_017780.3) c.[5372A>G];[=], p.[(Asp1791Gly)];[(=)]
			Noonan s.	Q87.1	2	-
			Townes-Brocks s.	Q87.89	1	-
			Waardenburg s.	E70.3	1	-
			Congenital malformation with overgrowth	Q87.3	1	-
	AR	13	*Usher* s.	H35.5	7	*MYO7A* (NM_000260.3) c.494C>T(;) 569T>G(;)1969C>T, p.(Thr165Met(;)Leu190Trp(;)Arg657Trp) *GJB2* (NM_004004.5) [35delG];[=], p.[(Gly12Valfs*2)]; [(=)]
						USH2A (NM_206933.2) c.[11864G>A];[(G>A)], p.[(Trp3955Ter)];[(Trp3955Ter)]
			Pendred s.	E07.1	3	*SLC26A4* (NM_000441.1) c.85G>C(;)1246A>C; p.(Glu29Gln(;)Thr416Pro).
						SLC26A4 (NM_000441.1) c.304+2T>C(;)1149+1G>A *GJB2* (NM_004004.5) c.[313_326del14];[=], p.
			Ichthyosis-prematurity s.	-	1	*SLC27A4* (NM_005094.3) c.1528[C>T];[(C>T)], p.(Arg510Cys(;)Arg510Cys)
			Rogers s.	D64.3	1	*SLC19A2* (NM_006996.2) c. 205[C>T];[C>T], p.[(Val69Phe)];[(Val69Phe)] *GJB2* (NM_004004.5) [c313_326del14];[=]
			Jervell&Lange-Nielsen s.	I45.8	1	*KCNQ1* (NM_000218.2) c.[1111G>C];[?], p.[(Ala371Pro)];[?]
	XD	3	*Alport* s.	Q87.81	3	-

Table No. 20 (continuation). Clinical diagnoses in subgroup of syndromic HL.

Type of HL	Count of participants	Clinical diagnose	TLK-10-AM	Count of participants	Pathogenic genetic changes
Chromosomal	6	*Jacobsen* s.	Q93.5	1	46,XY del (11)(q23.3)
		Wolff-Hirschorn s.	Q93.3	1	arr[GRCh37] 4p16.3(48283_3123776)x1
		Duplications and other complex rearrangements (duplications with unbalanced translocations)	Q92.5	4	arr[hg19] 15q11.2q13.1 (22,765,628-28,940,098)x3
					arr[hg19] 1q43q44(240,724,339-249,202,755)x3; 21q22.2q22.3(41,274,744-48,098,824)x1
					arr[GRCh37] 15q13.3 (32018731_32515681)×3
					arr[GRCh37] 3q26.1-qter (166,659,726-197,803,820)x3; 5p13.33-pter (1-33,683,173)x1
Multifactorial inheritance/ nonhereditary	11	*Goldenhar* s.	Q87.08	9	-
		VACTERL association	Q87.27	1	-
		Superficial siderosis	I69.0	1	-
Syndrome not identified	26	Multiple congenital malformations, not classified elsewhere	Q89.7	26	-

The rare (first in Lithuania) case of syndromic hearing loss

1. <u>Rogers (thiamine responsive megaloblastic anaemia (TRMA)) syndrome</u>

Thiamine responsive megaloblastic anaemia syndrome (TRMA s.) [MIM 249270] also known as Rogers' syndrome is a very rare autosomal recessive hereditary disorder. Only ~80 of cases have been described to date mainly in consanguineous families [66]. The number of cases in European countries is even lower – barely few cases have been published [67, 68]. Three main features are characteristic to the disease – megaloblastic anaemia, early onset deafness and non-type I diabetes [69, 70]. Other symptoms – pigmentary retinopathy, short stature, thrombocytopenia, congenital heart defects, seizures, ataxia, developmental delay and cryptorchidism have also been described, but these features were not documented in all the patients, causing difficulty to define the extent of symptoms that can be attributed specifically to TRMA s., especially in the case of consanguinity [70].

A gene *SLC19A2* coding high affinity thiamine transporter (THTR1) mediating vitamin B1 uptake through cell membrane has been identified [71]. THTR1 predominates in inner ear cells, pancreatic islets and hematopoietic stem cells in contrast to THTR2 – a low affinity, high performance, ubiquitously expressed protein. The initial manifestation of the symptoms in presence of thiamine deficiency, due to the decreased activity of THTR1, occurs in most sensitive tissues. The cells lacking thiamine suffer from extensive changes in their metabolism, experience a shortage of energy, an impairment of DNA/RNA biosynthesis and then undergo apoptosis [72-74].

This patient was the first patient with TRMA s. diagnosed in Lithuania.

The patient was referred for genetic consultation at 20 months of age, because profound hearing loss began at 7 months (Fig. No.14).

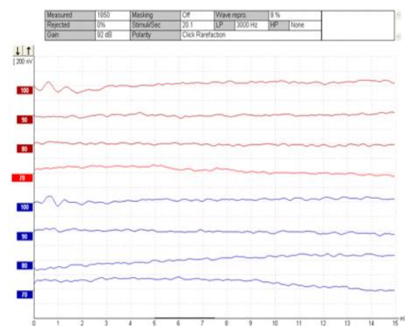

Figure No. 14. Absence of response to100 dB sound (ABR).

Insulin-dependent diabetes and affecto-respiratory spasms developed at 11 months. Despite treatment with an insulin pump, the glycaemia control was not satisfactory – glucose levels in the blood scaled up to 24 mmol/l (Fig. No. 15).

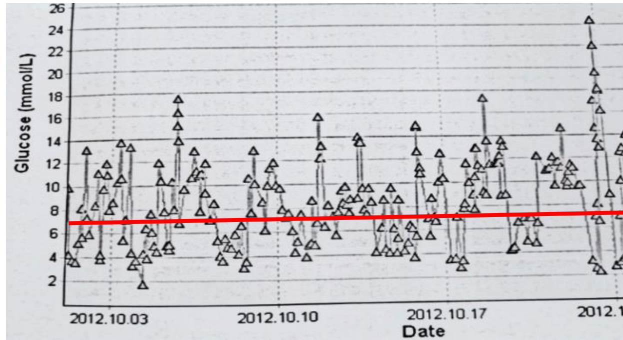

Figure No. 15. Unsatisfactory control of glycemia. The red line indicates upper limit of normal blood glycemia range.

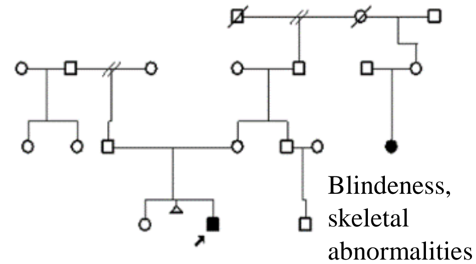

Figure No. 16. Genealogy

A phenotypic evaluation did not reveal any dysmorphic features. A genealogy analysis was uninformative (Fig. No. 16). At the age of 2 years, refractory anaemia was diagnosed; bone marrow aspiration revealed ringed sideroblasts (25 %) (Fig. No. 17). Decreased vision acuity and photophobia was noticed at 2.5 years; subsequently, pigmentary maculopathy was diagnosed as well. At 2 years and 10 months, an acute respiratory infection began: haemorrhagic rash in the skin, severe normochromic anaemia ($2.52x10^{12}$), moderate neutropenia, severe thrombocytopenia (PTL $3x10^{9}/l$); hyperglycaemia (18 – 20 mmol/l) were identified. A TRMA syndrome was suspected and a course of daily thiamine 100 mg doses was initiated.

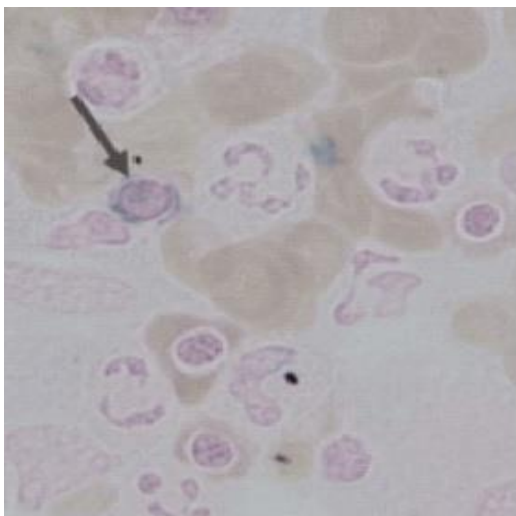

Fig. 15. Bone marrow aspirate. Arrow points to ringed sideroblast.

SLC19A2 gene sequencing was performed and a novel homozygous pathogenic variant c.[205G>T]; p.[(Val69Phe)] was identified. The homozygous change of the first

nucleotide in the second exon of the *SLC19A2* gene potentially results in the replacement of the conserved Val69 with Phe in extracellular loop 1-2 of THTR1 protein or in the loss of splice site acceptor and the subsequent truncation of protein or even nonsense–mediated mRNA decay. The variant has never been published in scientific literature nor submitted in The Human Gene Mutation Database. This novel alteration most probably arose from a common progeny, representing identity by descent. The change was evaluated by *in silico* analysis: ***SIFT*** prediction score 0.003 (damaging), ***Polyphen2*** prediction score 0.998 (probably damaging), ***Mutation taster*** predicted the change to be disease causing. The carrier statuses of the parents and sister of the propositus were confirmed.

Four days after the beginning of thiamine therapy, a marked improvement in haematopoiesis was documented (Hb 94 g/l, PLT – 63 x 10^9/l). After 1.5 months with thiamine treatment, a normal erythrocyte count was observed, but thrombocytosis occurred (RBC – 4.02x10^{12}/l, Hb – 123g/l, WBC – 8.1x10^9/l, PLT – 419x10^9/l).

The control of glycaemia has also improved – it varies in a 4-9mmol/l range; 87 % of the time, normal glycaemia was registered, HbA1c 5.1 % (normal <7.5 %), Insulin requirement decreasing to 0.4 U/kg/day.

Despite the successful control of glycaemia and blood count, the patient developed an allergic reaction after several months of treatment. It manifested with itchy skin rashes and dandruff husk on the scalp. Moreover, the effect of thiamine was not sufficient for a timely mental development and psychological balance. After careful consideration, thiamine was replaced with sulbutiamine 100 mg/day. We observed transitory decrease of haemoglobin to 110 g/l, which resolved within the 1st month of treatment. Blood glycaemia remained stable, with rare elevations due to stress. Four years after CI, speech perception in silence is very good (Speech Discrimination Score is 90 %), language development still being delayed. An allergic reaction of the skin disappeared, making the treatment with sulbuthiamine more convenient to the patient.

Unfortunately, a year of treatment with sulbutiamine did not improve the patient's mental health: emotional outbursts, aggressive behavior, speech delay and inability to address hygiene tasks remain. Also, ophthalmologic features did not ameliorate and clinical signs of pigmentary maculopathy persist.

The three main features of TRMA s. seem to look different in their development and aetiology, so only a multidisciplinary approach can be beneficial in early diagnostics. Several reasons influence the delayed diagnosis: the rarity of the disorder, clinical heterogeneity and reduced penetrance. Any two of the classical triad features presented in the same patient should draw our attention regarding the TRMA syndrome. Only few cases of European descent have been published to date and this is the first TRMA syndrome case in Lithuania.

However, basically, the diagnosis of the TRMA syndrome lies in a thorough clinical analysis and the introducing of empirical daily treatment with pharmacologic doses of thiamine; in most cases, this results in a very rapid amelioration of the patient's condition, which may last until puberty or even longer.

To date, only two compounds – thiamine hydrochloride and S-benzoylthiamine O-monophosphate (benfotiamine) – have been employed in treatment of TRMA s.

The effect of synthetic thiamine's (Th) derivate, O-isobutyrylthiamine disulfide – sulbutiamine (SbTh) has never been described. SbTh, as lipid-soluble Th derivate, possesses good bioavailability and effectively crosses the blood-brain barrier. In contrast to other Th forms, SbTh significantly increases free Th and Th phosphates in the cells. A twice smaller dose of SbTh is needed to raise the levels of intracerebral thiamine phosphate derivatives as compared to benfothiamine [75].

SbTh acts as a regulator of the synaptic transmission of many neurotransmission systems and is used as a psychotropic drug: it is reported that SbTh improves memory in rodents and their cognitive function; also, it has positive influence on the functional asthenias in humans.

However, it currently remains unclear whether the lack of improvement of mental health of the patient was due to late diagnosis and/or any additional possible underlying condition.

The persistence of retinopathy despite treatment could be explained by the high metabolic rates of the retina and the need for the large amounts of Th that could not be provided even by any other up-regulated thiamine transporters (e.g., *SLC19A3*) or passive diffusion [76].

However, basically, the prognosis of the TRMA syndrome lies on the early recognition and lifelong supplementation of derivatives of vitamin B1. In most cases, the treatment results are in very rapid amelioration of condition of the patient, which may last until puberty or even longer. The success of treatment confirms the clinical diagnosis and the genetic testing of the *SLC19A2* gene provides the molecular basis of disease.

Although the TRMA syndrome is very rare, the possibility of successful treatment obliges recognizing it as early as possible.

Analysis results in the research group of the ethnic Lithuanian population (LITGEN)

The group of the ethnic Lithuanian population consisted of 98 unrelated, self-reported healthy individuals (49 female and 49 male participants). In order to assess the carrier frequency and population frequency of hereditary deafness, pathogenic/probably pathogenic variants in genes associated with AR and XR hearing impairment were analyzed.

The *post hoc* power analysis was performed. Assuming medium effect size for the binomial exact test and having a sample size of 98, the calculated power is above 0.8.

<u>Results of analysis of genes associated with AR HL in the group of the ethnic Lithuanian population.</u>

Variants in 83 genes associated with AR hearing loss were filtered (allele frequency ≤2 %) and analyzed. According to ACMG recommendations,

16 pathogenic/likely pathogenic variants (Table No. 21) were identified in the LITGEN group of participants.

Additionally, 144 novel or previously determined SNPs and 9 deletions/duplications were found in coding sequences or splicing sites of the analyzed genes.

The results revealed the heterozygous state of *GJB2* alterations in 7 DNA samples (7.14 %), showing that approximately 1 in 14 individuals in the Lithuanian population is a carrier of the *GJB2* gene pathogenic variants (Table No. 22). Three healthy study participants had the c.101T>C, p.(Met34Thr) (rs35887622) variant (3.1 %), and the deletions c.313_326del14, p.(Lys105Glyfs*5) (rs111033253) and c.35delG, p.(Gly12Valfs*2) (rs80338939) were discovered in a heterozygous state with 2.0 % and 1.0 % carrier frequencies respectively. A novel, previously undescribed truncating change c.206delT (p.Phe69Serfs*13) was identified in one participant (genotype frequency 1.0 %). The high frequency of carriers of the c.313_326del14, p.(Lys105Glyfs*5) (rs111033253) deletion in the entire Lithuanian population is supported by it being identified twice in the ethnic Lithuanian group of healthy participants (a frequency 2.0 % of carriers in the study group). The assumption that there is a high rate of carriers at rate of this alteration in the Lithuanian population may also be supported by the coincidental finding of this change in the patients with the syndromic type of hearing loss – *Rogers* s. and *Usher* s.

Pathogenic variants in *SLC26A4* and *USH2* genes share second and third places in the structure of HL in the LITGEN group. Each heterozygous *SLC26A4* gene variants, determining isolated/syndromic HL c.1003T>C, p.(Phe335Leu) (rs111033212) and c.1963A>G, p.(Ile655Val) (rs397516424), were identified once in the group of the ethnic Lithuanian population (frequency of carriers of *SLC26A4* gene alterations 2.0 %).

Alterations of the *USH2A* gene are associated with most frequent type of Usher s., characterized by mild features of the disorder. The variant c.10073G>A p.(Cys3358Tyr) was determined twice in the LITGEN group (carrier

frequency 2.0 %), whereas its frequency in other populations was lower or the variant was not identified at all (1000genomes_all: ND; ExAC_ALL: 0.0003). The difference may be considered as a feature of the Lithuanian population (the variant originated in the ancient Lithuanian genome, assimilation of populations, migration etc.).

The heterozygous pathogenic variants of *ATP6V1B1* (NM_001692) and *TMPRSS3* (NM_032405.1) genes were determined in 1.0 % of the LITGEN participants. Both alterations were found for a single participant each, so it would be rather complicated to deduce regarding the difference compared with other populations.

In addition to the pathogenic variants described above, likely pathogenic variants of *BDP1*, *MYO15A*, *OTOF*, *OTOG*, *COL4A4*, *HSD17B4*, and *PNPT1* genes were identified in the LITGEN group. These changes haven't been published in scientific literature, were not identified in other populations, but their nature allows to classify them as probably pathogenic.

The alterations of *SLC26A4*, *MYO15A, TMPRSS3, OTOF, USH2A, COL4A4*, and *ATP6V1B1* genes are frequently identified in other groups of affected participants, whereas pathogenic changes in *BDP1, HSD17B4*, and *PNPT1* genes were previously determined only in several affected individuals.

Table No. 21. Pathogenic/probably pathogenic variants implicated in AR HL pathogenesis, identified in the LITGEN group of participants.

No.	Variant (identification No.)	Carrier frequency, %	Allele frequency in other populations	*In silico* analysis	Variant type (HGMD ID)	Disorder associated with gene (MIM)
1	***GJB2*** **(NM_004004.5) c.101T>C, p.(Met34Thr) (rs35887622)**	3.06	**1000genomes_all:** 0.0060 **ExAC_ALL:** 0.0085	***SIFT*** pathogenic (score 0.027) ***Polyphen-2*** benign (score 0.083) ***MutationTaster2*** pathogenic	Pathogenic **(HGMD ID** CM970679)	· Isolated AR HL (220290) · Isolated AD HL (601544) · *Bart-Pumphrey* s. (149200) · Palmoplantar keratoderma (148350) · Ichthyosis with HL (602540) · *Vohwinkel* s. (124500) · KID s. (148210)
2	***GJB2*** **(NM_004004.5) c.313_326del14 p.(Lys105Glyfs*5) (rs111033253)**	2.04	**1000genomes_all:** ND **ExAC_ALL:** 0.00010	***MutationTaster2*** pathogenic	Pathogenic **(HGMD ID** CD982677)	
3	***GJB2*** **(NM_004004.5) c.35delG p.(Gly12Valfs*2) (rs80338939)**	1.02	**1000genomes_all:** 0.0024 **ExAC_ALL:** 0.0060	***MutationTaster2*** pathogenic	Pathogenic **(HGMD ID** CD972240)	
4	***GJB2*** **(NM_004004.5) c.206delT p.(Phe69Serfs*13)**	1.02	**1000genomes_all:** ND **ExAC_ALL:** ND	***MutationTaster2*** pathogenic	Probably pathogenic	
5	***SLC26A4*** **(NM_000441.1) c.1003T>C, p.(Phe335Leu) (rs111033212)**	1.02	**1000genomes_all:** 0.000799 **ExAC_ALL**: 0.0009	***SIFT*** benign (score 0.727) ***Polyphen-2*** probably pathogenic (score 0.997) ***MutationTaster2*** pathogenic ***PhyloP*** conservative (score 1.061) ***GERP++*** conservative (score 5.62)	Pathogenic **(HGMD ID** CM011490)	· Isolated AR HL with enlarged aqueduct (600791) · *Pendred* s. (274600)
6	***SLC26A4*** ***(*****NM_000441.1) c.1963A>G, p.(Ile655Val) (rs397516424)**	1.02	**1000genomes_all:** ND **ExAC_ALL:** 0.000033	***SIFT*** benign (score 0.56) ***Polyphen-2*** benign (score 0.034) ***LRT*** pathogenic (score 0) ***MutationTaster2*** pathogenic ***PhyloP*** conservative (score 2.183) ***GERP++*** conservative (score 5.74)	Pathogenic **(HGMD ID** CM109556)	
7	***USH2A*** **(NM_206933.2) c.10073G>A p.(Cys3358Tyr) (rs148660051)**	2.04	**1000genomes_all:** ND **ExAC_ALL**: 0.0003	***SIFT*** pathogenic (score 0.0) ***Polyphen-2*** probably pathogenic (score 1.0) ***LRT*** pathogenic (score 0.000141) ***MutationTaster2*** pathogenic (score 1.0) ***PhyloP*** non-conservative (score 0.935) ***GERP++*** conservative (score 5.76)	Pathogenic **(HGMD ID** CM104136)	· Isolated AR pigmentary retinitis 39 (613809); · *Usher* s., 2A type) (276901)

Table No. 21 (continuation). Pathogenic/probably pathogenic variants implicated in AR HL pathogenesis, identified in the LITGEN group of participants.

No.	Variant (identification No.)	Carrier frequency, %	Allele frequency in other populations	*In silico* analysis	Variant type (HGMD ID)	Disorder associated with gene (MIM)
8	***ATP6V1B1*** **(NM_001692) c.1149dupC, p.(Tyr383fs) (ND)**	1.02	**1000genomes_all:** ND **ExAC_ALL:** 0.0002	***MutationTaster2*** pathogenic	Pathogenic **(HGMD ID** CI991978)	· Renal tubular acidosis with deafness (267300)
9	***TMPRSS3*** **(NM_032404.2) c.32C>A, p.(Ala11Glu) (rs147231991)**	1.02	**1000genomes_all**: 0.000599 **ExAC_ALL:** 0.0008	***SIFT*** pathogenic (score 0.003) ***Polyphen-2*** probably pathogenic (score 0.973) ***LRT*** neutral (score 0.004687) ***MutationTaster2*** pathogenic (score 0.99) ***PhyloP*** non-conservative (score 0.998) ***GERP++*** conservative (score 4.03)	Pathogenic **(HGMD ID** CM054159)	· Isolated AR HL (601072)
10	***PNPT1*** **(NM_033109.4) c.977-1G>A (ND)**	2.04	**1000genomes_all:** ND **ExAC_ALL**:ND	***MutationTaster2*** pathogenic	Likely pathogenic	· Combined oxidative phosphorylation deficiency 13 (614932); · Isolated AR HL (614934)
11	***BDP1*** **(NM_018429.2) c.1114C>T, p.(Gln372Ter) (ND)**	1.02	**1000genomes_all:** ND **ExAC_ALL:** ND	***MutationTaster2*** pathogenic	Likely pathogenic	· Isolated AR HL
12	***COL4A4*** **(NM_000092.4) c.4775G>A p.(Trp1592Ter) (ND)**	1.02	**1000genomes_all:** ND **ExAC_ALL:** ND	***MutationTaster2*** pathogenic	Likely pathogenic	· AR *Alport* s. (203780)

Table No. 21 (continuation). Pathogenic/probably pathogenic variants implicated in AR HL pathogenesis, identified in the LITGEN group of participants.

No.	Variant (identification No.)	Carrier frequency, %	Allele frequency in other populations	*In silico* analysis	Variant type (HGMD ID)	Disorder associated with gene (MIM)
13	***HSD17B4* (NM_001199292.1) c.295+1G>A (ND)**	1.02	**1000genomes_all :** ND **ExAC_ALL:** ND	***MutationTaster2*** pathogenic ***Human splicing finder*** pathogenic	Likely pathogenic	· D-bifunctional protein deficiency (261515); · Perrault s. (233400)
14	***MYO15A (NM_016239.3)* c.7978C>T, p.(Arg2660Ter) (ND)**	1.02	**1000genomes_all :** ND **ExAC_ALL:** ND	***MutationTaster2*** pathogenic	Likely pathogenic	· Isolated AR HL (600316)
15	***OTOF (NM_194248.2) c.5474C>T, p.(Pro1825Leu)* (ND)**	1.02	**1000genomes_all :** ND **ExAC_ALL:**ND	***SIFT*** pathogenic (score 0.000) ***Polyphen-2*** probably pathogenic (score 1.00) ***MutationTaster2*** pathogenic	Likely pathogenic	· Isolated AR HL (601071); · AR auditory neuropathy (601071)
16	***OTOG* (NM_001277269.1) c.3457C>T, p.(Arg1153Ter) (ND)**	1.02	**1000genomes_all :** ND **ExAC_ALL:**ND	***MutationTaster2*** pathogenic ***LRT*** pathogenic (score $1x10^{-6}$) ***PhyloP*** non-conservative (score 0.852) ***GERPP++*** conservative (score 4.46)	Likely pathogenic	· Isolated AR HL (614945)
	TOTAL	**21.4 (13.8- 30.9)**				

Results of the analysis of genes associated with XR HL in the group of the ethnic Lithuanian population.

Variants in 4 genes associated with XR hearing loss were filtered and analyzed. Variants satisfying ACMG criteria for classifying as pathogenic/probably pathogenic variants were not identified in the LITGEN group of participants. One previously determined SNP of unknown clinical significance was found in coding sequences of the analyzed genes.

These results are similar to the previously published studies in other populations, strengthening the evidence that XR-related, non-syndromic hearing loss is extremely rare worldwide [77].

The prevalence of hereditary hearing loss in the Lithuanian population

One of the tasks of the study was to assess the prevalence of hereditary hearing loss in the Lithuanian population. The LITGEN group represents the pure ethnic population due to the strict criteria for enrolment guaranteeing the uniqueness of this cohort: all 98 self-reported individuals indicated at least three generations of Lithuanian ethnicity and residency in the same ethnolinguistic region. Although a bigger ethnic population group would better reflect the current state of carriers of

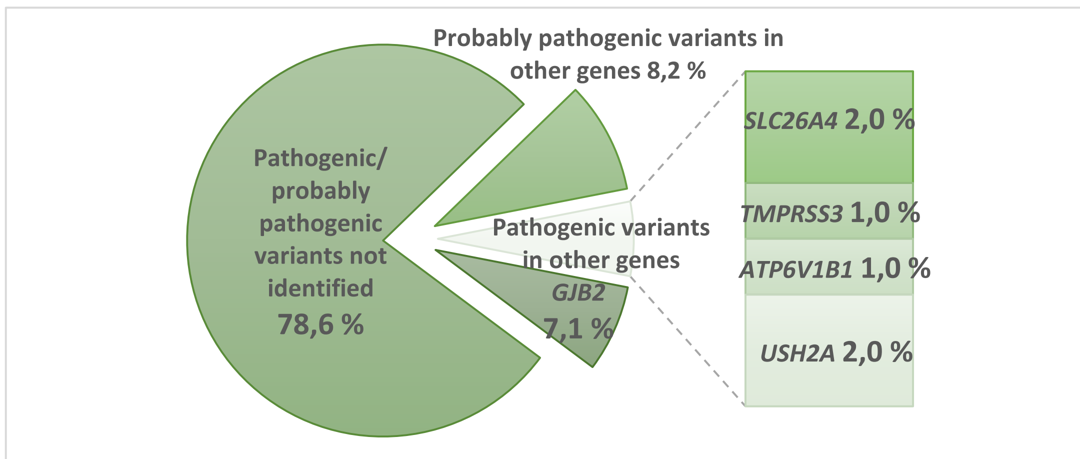

Figure No. 16. Carrier frequency of AR HL in Lithuanian group of ethnic population.

the AR and XR HL alterations, but our results are nevertheless statistically reliable ($p \leq 0.05$).

The overall frequency of carriers of genetic alterations associated with HL in the healthy group of the study amounted to 21.4 % (approx. 1 in 5), allowing us to assess the frequency of AR inherited HL in the Lithuanian population. (Fig. No. 18).

The prevalence of hearing loss of AR inheritance in the Lithuanian population was calculated as well (Table No. 24).

Table No. 22. Prevalence of AR HL in the Lithuanian population.

Gene	The frequency of pathogenic/likely pathogenic variants (95 % CI)	Prevalence in Lithuanian population (%)
GJB2	7.14	1 in 784 (0.1275)
SLC26A4	2.04	1 in 9604 (0.0104)
USH2A	2.04	1 in 9604 (0.0104)
ATP6V1B1	1.02	1 in 38416 (0.0026)
TMPRSS3	1.02	1 in 38416 (0.0026)
PNPT1	2.04	1 in 9604 (0.0104)
BDP1	1.02	1 in 38416 (0.0026)
COL4A4	1.02	1 in 38416 (0.0026)
HSD17B4	1.02	1 in 38416 (0.0026)
MYO15A	1.02	1 in 38416 (0.0026)
OTOF	1.02	1 in 38416 (0.0026)
OTOG	1.02	1 in 38416 (0.0026)
TOTAL	21.4 (13.8 – 30.9)	~1 in 557 (0.1795)

It was estimated to be approximately 1 in 557, if the assortative marriages wouldn't distort this value toward the higher edge. The results of the high frequency of carriers of the alterations of genes associated with AR hearing loss in the ethnic Lithuanian groups of healthy participants demonstrate the significant incidence of AR HL in our population.

Considering that the calculations included only the allele frequencies of pathogenic/likely pathogenic variants in AR HL associated genes, more than 150 VUS were identified in the LITGEN group. Furthermore, variants in genes conferring AD HL were not analyzed. These circumstances allow us to expect the incidence of hereditary HL in Lithuania to be higher than was assessed in this study.

Workflow for genetic testing of congenital/hereditary hearing impairment

The guidelines for genetic testing of the patients affected with HL have been introduced by several leading institutions. The American College of Medical Genetics and Genomics (ACMG) guidelines [78] and the EMQN Best practice guidelines deal with the clinical and laboratory aspects of the issue [21].

The development of new generation technologies has empowered clinicians to achieve the correct diagnosis with maximum effectiveness and minimal time consumption. Still, the stepwise approach remains the most relevant for the identification of genetic alterations to the affected individuals, making the process reasonable and personalized.

The diagnostic workflow begins with genetic counselling (anamnesis, genealogy analysis, clinical examination) (Fig. No. 19), which may give information to the patient about the origin of the disorder, mode of inheritance, prognosis and, sometimes, specific prophylactic measures.

While counselling affected individuals, the clinician needs to deal with some specifics associated with the disorder – the patients or their parents can speak only using sing language; also, deafness is usually their favorable outcome when speaking about the prognosis to their future children. This attitude may aggravate the choice of the diagnostic and therapeutic measures.

The genetic diagnostic workflow usually depends on the correct separation of the syndromic/non-syndromic types of HL (as emphasized in the ACMG guidelines), which sometimes is not so much of a trivial task due to the different time of the onset of symptoms in syndromic HL mimicking isolated HL.

In the case of syndromic HL, genetic testing is performed in accordance with the the suspected syndrome (SNP-CGH, array CGH, sequencing of the particular gene associated with syndrome, biochemical genetic analysis).

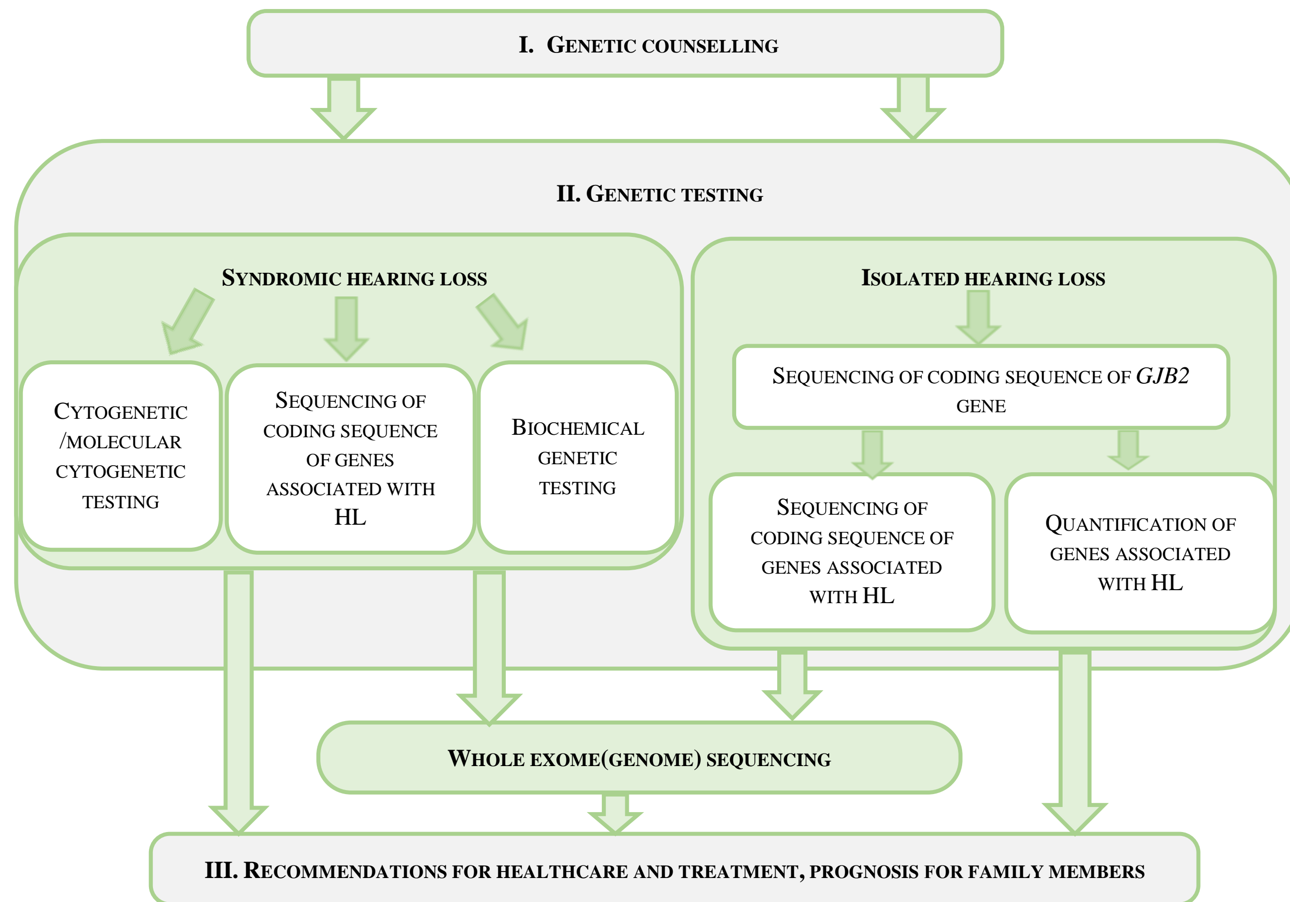

Figure No. 17. Scheme of genetic diagnostic workflow of individuals with HL

If an isolated HL was diagnosed, sequencing of the coding sequence of *GJB2* gene should be the first-tier testing in our population, followed by the *GJB6* gene deletion testing (as is recommended by EMQN). The gene panel and whole exome sequencing should follow if the aetiology was not identified with previously performed tests.

Genetic counselling after genetic testing is an inherent step of workflow, necessary for the correct explanation of the results to the patient. According to the diagnosis, the mode of inheritance and the nature of the alteration as well as the strategy for surveillance are scheduled, the prognosis assessed. Usually, a multidisciplinary approach is the most relevant for the comprehensive health care of the patient. In case of the aetiology/diagnosis of the disorder has not been identified, a repetitive evaluation of the clinical geneticist should be performed every 3 years allowing to observe the subtle symptoms of syndromic HL that were absent earlier and offer additional/new opportunities for genetic testing.

Although the spectrum of genetic testing has expanded over the few years, the diagnostics of hearing loss remain challenging. An early diagnosis is essential for a timely rehabilitation/treatment measures. The ability to determine the molecular cause of the disease will be an important step toward the personalized treatment of hereditary hearing impairment in the future.

CONCLUSIONS

1. Isolated hearing loss is a prevailing type of hearing impairment in the group of affected participants of the Lithuanian population (74.7 % of unrelated individuals);

- Profound and symmetric hearing loss predominates in the subgroup of isolated hearing loss (47.7 % and 87.0 % respectively), with positive genealogy identified in 40.7 % of the unrelated participants;
- Moderate and symmetric hearing impairment with absent malformations of the outer/middle/inner ear were the most common characteristics in the subgroup of syndromic hearing loss (41.1 %, 86.3 %, and 68.5 % respectively), and a positive genealogy was determined in 27.4 % of unrelated participants.

2. The etiologic structure of hearing loss in the Lithuanian population is similar to other European populations:

- The most common cause of isolated hearing impairment are genetic factors (55.6 %) with prevailing (51.4 %) *GJB2* gene pathogenic variants;
- A clinical diagnosis was determined to 64.4 % of the participants in the subgroup of syndromic hearing loss (63.8 % of monogenic and 12.8 % of chromosomal origin);

However, some distinctive characteristics (high allele frequency of *GJB2* c.313_326del14 in the subgroup of isolated hearing loss, extremely rare disorders identified) make the etiologic profile of hearing impairment in the Lithuanian population unique.

3. Genetic factors in general lead to more severe (OR 3.3; $p=7.9x10^{-5}$) and symmetric (OR 3.0; $p=1.4x10^{-2}$) isolated hearing loss with positive family history, (OR 2.6; $p=2.1x10^{-3}$).

4. The high frequency of carriers of pathogenic variants of genes associated with autosomal recessive hearing loss (21.4 % with prevailing *GJB2* gene alterations – 7.1 %) in the group of healthy participants corresponds to the substantial frequency of hereditary hearing impairment in Lithuania (~1 in 557 individuals).

5. The high clinical and genetic heterogeneity of hearing impairment requires a multidisciplinary approach and complex investigation, which makes genetic counselling a cornerstone of an accurate diagnosis.

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1. Beata Aleksiūnienė, Rugilė Matulevičiūtė, Aušra Matulevičienė, Birutė Burnytė, Natalija Krasovskaja, Laima Ambrozaitytė, **Violeta Mikštienė**, Vaidas Dirsė, Algirdas Utkus, Vaidutis Kučinskas. "Opposite Chromosome Constitutions due to a Familial Translocation t(1;21)(q43;q22) in Two Cousins with Development Delay and Congenital Anomalies: A Case Report." *Medicine* (Baltimore). 2017 Apr; 96(16):e6521. **IF 1.803.**

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2. **Mikštienė**, **V.**, Jakaitienė, A., Byčkova, J., Gradauskienė, E., Preikšaitienė, E., Burnytė, B., Tumienė, B., Matulevičienė, A., Ambrozaitytė, L., Kavaliauskienė, I., Domarkienė, I., Rančelis, T., Cimbalistienė, L., Lesinskas, E., Kučinskas, V., Utkus, A. The high frequency of *GJB2* gene mutation c.313_326del14 suggests its possible origin in ancestors of Lithuanian population. 3rd international conference “Evolutionary Medicine: Pre-Existing Mechanisms and Patterns of Current Health Issues.” 2016, Vilnius, Lithuania.
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affected group of patients in Lithuanian population. The 2nd Lithuanian-Polish ENT Congress 2013, Druskininkai, Lithuania.

Poster presentations

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