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An Evaluation of Genomic Factors and their Importance in the Etiopathogenesis of Congenital Heart Diseases

SUMMARY OF DOCTORAL DISSERTATION

Biomedical Sciences,
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This dissertation was prepared at the Department of Human and Medical Genetics of the Institute of Biomedical Sciences, Faculty of Medicine, Vilnius University during 2014–2018.

Scientific supervisor:
Prof. Loreta Cimbalistienė, PhD (Vilnius University, Biomedical Science, Medicine – 06 B)

Scientific consultant:
Prof. Algirdas Utkus, PhD (Vilnius University, Biomedical Science, Medicine – 06 B)

Scientific Council for Medicine:

Chairperson:

Prof. Vytautas Kasiulevičius, PhD (Vilnius University, Biomedical Science, Medicine – 06 B)

Members:

Prof. Aleksandras Laucevičius, PhD (Vilnius University, Biomedical Science, Medicine – 06 B)

Prof. Juozas Raistenskis, PhD (Vilnius University, Biomedical Science, Medicine – 06 B)

Assoc. Prof. Rasa Ugenskienė, PhD (Lithuanian University of Health Sciences, Biomedical Sciences, Medicine – 06 B)

Renata Posmyk, PhD (Medical University of Białystok, Poland, Biomedical Sciences, Medicine – 06 B)

The doctoral dissertation will be defended at the open session of the Council of Medical Sciences at 14:00 on February 15, 2019, in the *Raudonoji auditorija* (Red Hall) of the Santaros Klinikos at Vilnius University Hospital.
Address: 2 Santariškių Str., LT-08661 Vilnius, Lithuania.

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Mokslinė vadovė:
Prof. dr. Loreta Cimbalistienė (Vilniaus universitetas, biomedicinos mokslai, medicina – 06 B)

Mokslinis konsultantas:
Prof. dr. Algirdas Utkus (Vilniaus universitetas, biomedicinos mokslai, medicina – 06 B)

Disertacijos gynimo taryba:

Pirmininkas:

Prof. dr. Vytautas Kasiulevičius (Vilniaus universitetas, biomedicinos mokslai, medicina – 06 B)

Nariai:

Prof. habil. dr. Aleksandras Laucevičius (Vilniaus universitetas, biomedicinos mokslai, medicina – 06 B)

Prof. dr. Juozas Raistenskis (Vilniaus universitetas, biomedicinos mokslai, medicina – 06 B)

Doc. dr. Rasa Ugenskienė (Lietuvos sveikatos mokslų universitetas, biomedicinos mokslai, medicina – 06 B)

Dr. Renata Posmyk (Balstogės medicinos universitetas, Lenkija, biomedicinos mokslai, medicina – 06 B)

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This thesis is dedicated to my Family

CONTENTS

ABBREVIATIONS

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

Abbreviations of proteins

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

GJA5 – gap junction protein alpha 5
GPC3 – glypican 3
HAND2 – heart- and neural crest derivatives-expressed 2
HRAS – HRas proto-oncogene, GTPase
IFT88 – intraflagellar transport 88
IGF1R – insulin like growth factor 1 receptor
IRX4 – iroquois homeobox 4
KCNG2 – potassium voltage-gated channel modifier subfamily G member 2
KCNH2 – potassium voltage-gated channel subfamily H member 2
KRIT1 – ankyrin repeat containing
LMNA – lamin A/C
MAPK12 – mitogen-activated protein kinase 12
MEF2A – myocyte enhancer factor 2A
MEF2C – myocyte enhancer factor 2C
MESP1 – mesoderm posterior basic helix-loop-helix transcription factor 1
MTHFR – methylenetetrahydrofolate reductase
MTR – 5-methyltetrahydrofolate-homocysteine methyltransferase
MTRR – 5-methyltetrahydrofolate-homocysteine methyltransferase reductase
NFATC1 – nuclear factor of activated T cells 1
NIPA1 – NIPA magnesium transporter 1
NIPA2 – NIPA magnesium transporter 2
NOS3 – nitric oxide synthase 3
NPPA – natriuretic peptide A
NPPB – natriuretic peptide B
NR2F2 – nuclear receptor subfamily 2 group F member 2
NRG4 – neuregulin 4
PER2 – period circadian regulator 2
PRKAB2 – protein kinase AMP-activated non-catalytic subunit beta 2
PRKAG2 – protein kinase AMP-activated non-catalytic subunit gamma 2

PRKCI – protein kinase C iota
PRKD1 – protein kinase D1
PTEN – phosphatase and tensin homolog
SEMA3C – semaphorin 3C
SEMA3D – semaphorin 3D
SMAD6 – SMAD family member 6
SMARCD3 – SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily d, member 3
TBX1 – T-box 1
TBX5 – T-box 5
TERT – telomerase reverse transcriptase
TRNAU1AP – tRNA selenocysteine 1 associated protein 1
TRPM8 – transient receptor potential cation channel subfamily M member 8
TXNL4A – thioredoxin like 4A
ZIC3 – Zic family member 3

1. INTRODUCTION

1.1. Research Problem and Its Significance

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

Congenital heart diseases are a group of genetically heterogenous diseases. Heart diseases can manifest themselves in conjunction with additional extracardiac anomalies or may be one of the features of a syndrome; however, they most frequently manifest themselves as isolated defects. Both single nucleotide variants and multiple genome rearrangements may lead to the etiopathogenesis of genetic CHDs. Comparative genome hybridization (CGH) technologies, which were applied in studying CHDs, proved that a copy number variations (CNVs) are significant factors in the aetiology of CHDs, which result in approximately 3–28% of CHDs with extracardiac anomalies and 3–4.3% of isolated CHDs (Geng et al. 2014; Wu et al. 2017). The latest research has determined that rare, large and genic CNVs manifest themselves significantly more frequently in individuals suffering from CHDs. This, in turn, demonstrates that the overall CNV burden may also be an important factor in CHD (Lander and Ware 2014).

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

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

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

1.2. Aim of the Study

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

1.3. Main Tasks of the Study

1. To compose a group of subjects who suffer from congenital heart diseases and to evaluate the group's aetiological structure;
2. To determine the pathogenic copy number variations in the group of subjects who suffer from congenital heart diseases and to evaluate the importance of these variations in the manifestation of congenital heart diseases;

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

1.4. Relevance and Novelty of the Research

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

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

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

1.5. Statements to be Defended

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

2. MATERIALS AND METHODS

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

2.1. Selection of Subjects

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

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

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

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

A group of healthy individuals of ethnic Lithuanian population of LITGEN biomedical research made up a control group for this biomedical study. A control group of 355 healthy individuals was used for comparison of frequency for determining CNV. It was made up of

170 male and 185 female individuals. The individuals from the control group were not diagnosed with CHD or with any other congenital developmental anomaly.

2.2. Genetic Research in the Subject Group

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

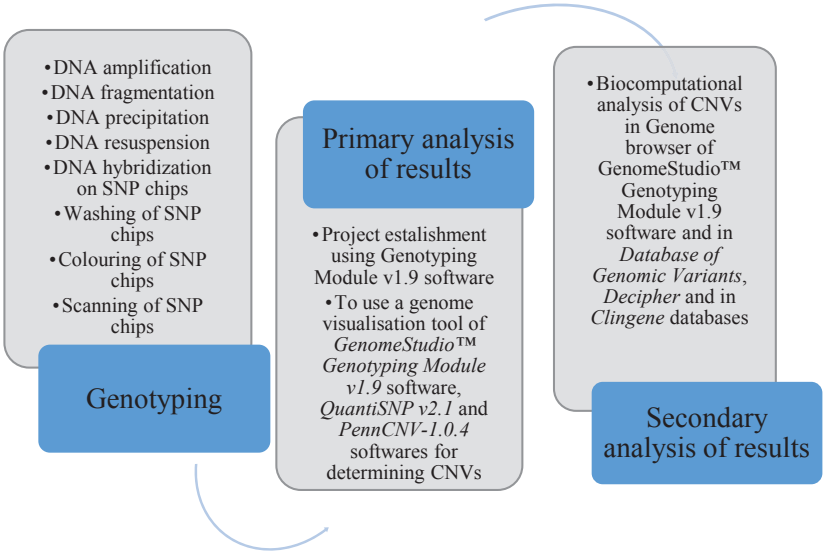

Figure 1. Scheme of the SNP-CGH test.

HumanCytoSNP–12 BeadChip v2.1 chips (Illumina Inc., USA) were used for carrying out a SNP genotyping in accordance with the protocol provided by the manufacturer, Infinium® HD Assay Ultra Manual Experienced User Card. The characteristics of the HumanCytoSNP–12 BeadChip v2.1 chip are as follows: covering 299140 SNP across the genome, a medium resolution of 9,7 kb. The next generation, high-performance genome analysis system Illumina HiScan™SQ (Illumina Inc., USA) was used for genotyping.

GenomeStudio v2011.1 software (Illumina® GenomeStudio 2011, Illumina Inc., USA) and GenomeStudio™ Genotyping Module v1.9 were used for carrying out a primary analysis of the genotyping data and CNV visualization in accordance with the protocol provided by the manufacturer. Data analysis was carried out by assessing the SNP diagrams and the numerical values of the logarithmic estimation ratio (*LogR ratio*, LogRR) of the fluorescence intensity (R) of their parameters (threshold values: – 0.1 and 0.1) and of the B allele frequency (BAF).

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

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

2.3. Interpretation of Results and Biocomputational Analysis

The Database of Genomic Variants (DGV) (MacDonald et al. 2014) was used for carrying out biocomputational data analysis in order to assess the clinical significance of the determined CNVs. In accordance with the ACMG recommendations, the determined CNVs were divided into the following categories (Kearney et al. 2011): 1) Benign

CNVs; 2) Pathogenic CNVs; 3) CNVs of unclear clinical significance, potentially pathogenic CNVs. The test result is described in accordance with the ISCN recommendations (McGowan–Jordan et al. 2016).

In order to compare the features and the frequency of CNVs, the CNVs determined in the research and in the control groups were divided into common CNVs and rare CNVs based on the CNVs overlapping with the common CNVs in a normal population. The CNVs, which overlapped by >70% with at least a single CNV, which was provided in the DGV, were interpreted as **common CNVs**. The CNVs that had partly overlapped (<30%) or failed to overlap with the CNVs, which were provided in the DGV, were interpreted as **rare CNVs**. Rare CNVs were selected for further analysis.

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

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

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

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

2.5. Biostatistical Analysis

Open source software R, version 3.4.3 and MS Excel 2016 calculator were used for carrying out statistical data analysis. When assessing the quantitative indicators, an arithmetic mean $\bar{x}$ was calculated. The c^2 criterion was used for comparing the data of the CHD subjects with normal cardiac segmental connections with that of the subjects with abnormal cardiac segmental connections. An exact Fisher criterion was additionally calculated when the number of observations was low or when at least a single number of observations was lower than five. A Shapiro-Wilk test and certain graphic methods, i.e., a histogram and a rectangular diagram, were used for testing the hypotheses about the normality of variable distribution. The Wilcoxon criterion was used for comparing two dependent samples, whereas the Mann-Whitney-Wilcoxon rank-sum criterion was used for comparing two independent samples. The Friedman criterion was used for comparing three dependent samples, whereas the Kruskal-Wallis rank criterion was used for comparing independent samples.

3. RESULTS AND DISCUSSION

3.1. Distribution of Congenital Heart Defects in the Study Group

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

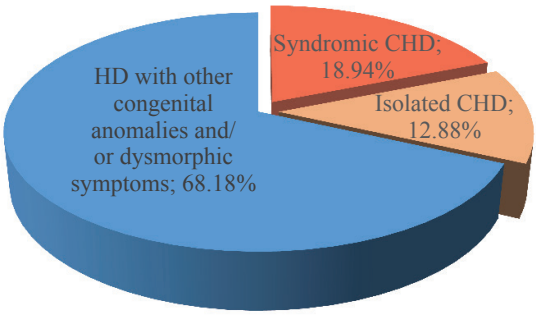

Figure 2. The distribution of subjects in the study group by congenital heart defect.

The group of subjects with isolated CHDs consisted of 17/132 (12.88%) individuals; the group of subjects who had CHDs with other congenital developmental anomalies and/or dysmorphic symptoms consisted of 90/132 (68.18%) individuals; the group of subjects who

had CHDs and a genetic syndrome-specific phenotype consisted of 25/132 (18.94%) individuals (Figure 2).

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

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

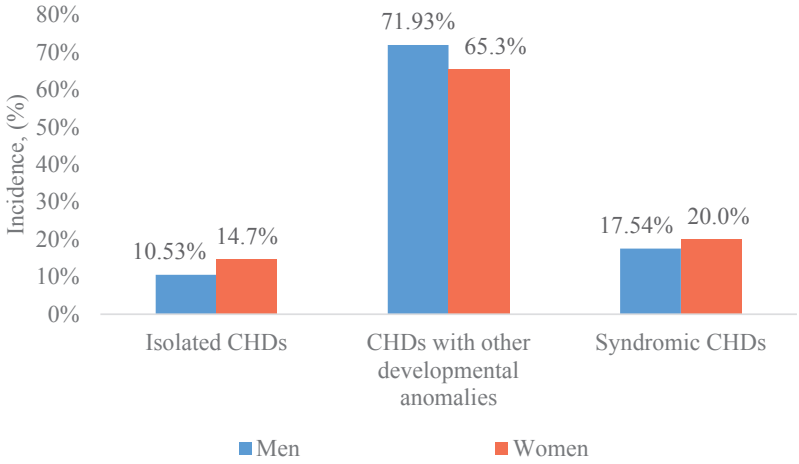

Figure 3. Sex distribution among the subjects in the different CHD groups.

Based on the pathomorphological classification of CHDs (Anderson et al. 2013), the CHDs observed in the study group were divided into four groups by cardiological phenotype, two of which were further divided into smaller subgroups. In total, 189 cases of congenital

heart defects were detected in the study group (39 subjects had complex CHDs) (Table 1). The largest group, 135/189 (71.43%) of all the congenital heart defect cases, had CHDs with normal cardiac segmental connections; CHDs with abnormal cardiac segmental connections comprised 15/189 (7.94%) congenital heart defect cases, major vascular anomalies were seen in 36/189 (19.05%) congenital heart defect cases, and in 3/189 (1.59%) congenital heart defect cases, the congenital heart defect was accompanied by situs anomalies (abnormal cardiac position). Septal defects were the most common cardiac anomaly and were seen in 66/189 cases (34.92%).

Table 1. Incidence distribution among the cardiological phenotypes.

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

An analysis of the connection between sex and CHD manifestation revealed a statistically significant relationship between the incidence of major vascular anomalies and sex ($p=0.029$, $p<\alpha$, $\alpha=0.05$). No other statistically significant dependence on sex was found in the other morphological groups (Figure 4). In comparison with the male group, major vascular anomalies were more frequent in the female group, with 26 (72.22%) cases detected in females as opposed to 10 (27.78%) cases in males.

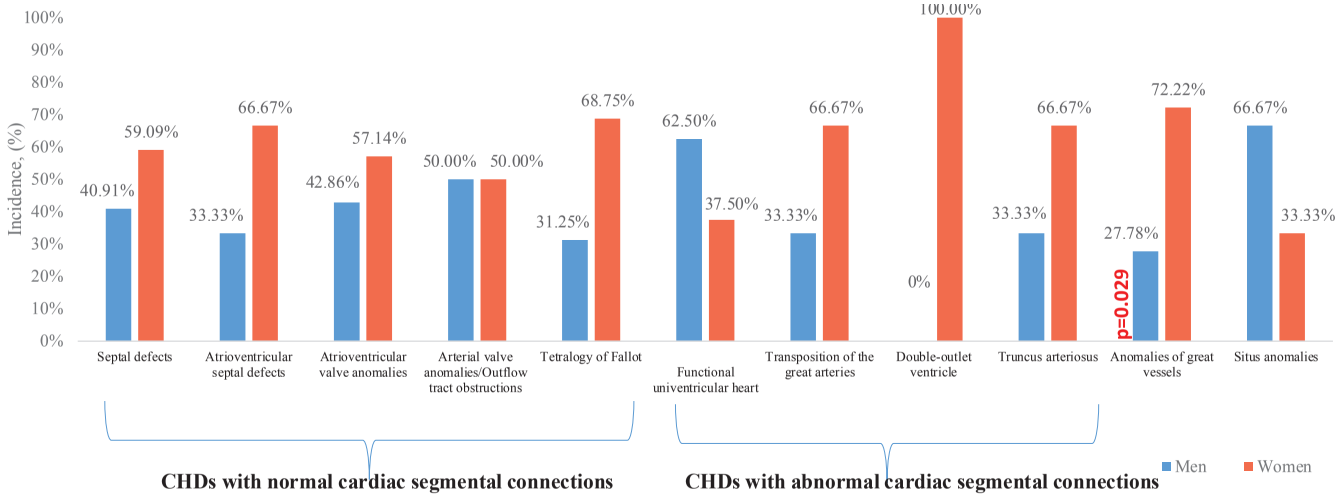

Figure 4. The distribution of cardiological phenotypes by sex.

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

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

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

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

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

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

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

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

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

Extensive genotyping using the SNP-CGH method was carried out for the 132 subjects. A total of 253 CNVs that meet the CNV criteria were identified. In keeping with the ACMG recommendations (Kearney et al. 2011), 202/253 (79.84%) CNVs were classified as benign, 44/253 (17.39%) as pathogenic and 7/253 (2.77%) as potentially pathogenic with an unclear clinical significance (Figure 5).

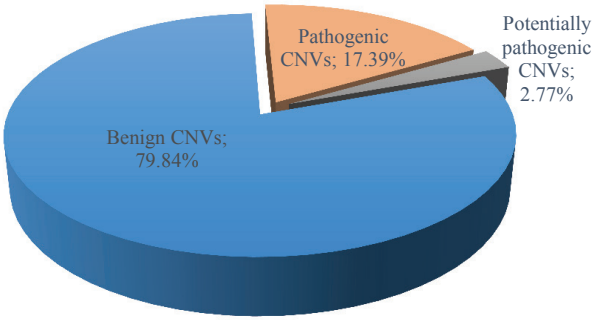

Figure 5. Results of the CNV study in the study group.

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

Table 4. Pathogenic CNVs detected in the study group.

No.	Subject IN	Sex	CNV	Chromosomal locus	Start of alteration*	End of alteration*	CNV size (kb)
1.	CG-007	F	Del	22q11.21	19068642	21339751	2271
2.	CG-010	F	Del	10q22.3q23.33	81388196	95542677	14154
3.	CG-017	F	Dup	2q34	212159787	212766614	607
4.	CG-018	F	Del	4q13.3	72647749	74208199	1560
5.	CG-019	F	Del	5p15.33q13.3	1176601	31541142	30365
6.	CG-037	F	Del	1p36.22p36.32	4236982	12602979	8366

No.	Subject IN	Sex	CNV	Chromosomal locus	Start of alteration*	End of alteration*	CNV size (kb)
7.	CG-039	M	Del	15q11.2	22754322	23066736	312
8.	CG-040	M	Del	22q11.21	19204210	21312429	2108
9.	CG-044	F	Del	18q22.1q23	65094544	78014582	12920
10.	CG-051	F	Del	4p16.3p16.1	85800	6523395	6438
11.	CG-052	M	Del	2q37.1q37.3	234819199	243029573	8210
12.	CG-061	F	Del	16q24.3	89243584	89596063	352
13.	CG-062	F	Del	7q11.23	73172999	74050694	878
14.	CG-067	F	Del	1q22q23.1	155630752	157193893	1563
15.	CG-070	F	Dup	14q23.1	60652103	61536933	885
16.	CG-072	F	Del	1p36.11p35.3	27862451	29004746	1142
17.	CG-074	F	Del	22q13.31q13.33	44286531	51105556	6819
18.	CG-084	F	Del	1q42.3q44	235827443	249202755	13375
19.	CG-089	F	Del	6q27	167615010	171115067	3500
20.	CG-090	M	Del	13q12.11 q12.12	20079051	25441945	5363
21.	CG-092	F	Del	7q11.23	72722981	74138121	1415
22.	CG-093	F	Del	22q11.21	18844632	21462353	2618
23.	CG-097	F	Dup	1q21.1q21.2	146476526	147820342	1344
24.	CG-099	M	Del	15q11.2	22754322	23080702	326
25.	CG-107	M	Dup	7q11.23	72773966	74138121	1364
26.	CG-109	M	Dup	11p15.5	419167	900809	482
			Dup	14q12	29887615	30208568	321
27.	CG-112	F	Del	18q23	76881798	78014582	1133
28.	CG-113	M	Del	4p16.3p16.1	85800	8728783	8643
29.	CG-116	M	Del	7q11.22q11.23	72722981	74138121	1415
30.	CG-117	F	Del	4p16.3p15.2	85800	21866646	21781
31.	CG-118	M	Del	15q11.2	22754322	23140114	386
32.	CG-120	F	Del	1q25.1q31.1	175629207	189971441	14342
33.	CG-124	M	Del	22q11.21	18938367	21462353	2252
34.	CG-126	M	Del	22q11.21	18967371	21462353	2495
35.	CG-132	F	Del	7q34q36.3	141245033	159119486	17874
36.	CG-008	F	Dup	15q21.2q26.3	52649116	99980473	47710
			Del	15q26.3	100052234	102397836	2346
37.	CG-078	F	Dup	3q26.2q29	168175261	197845233	29670
			Del	8p23.3p23.2	176818	3276617	3100
38.	CG-085	F	Del	4q32.3q35.2	167263766	3276617	23617
			Dup	5p15.33p15.2	38139	12392815	12355
39.	CG-106	F	Dup	7q11.21q36.3	64679561	159119486	94440
			Del	Xq25q28	128325352	154880326	25555

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

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

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

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

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

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

The incidence was 29.55% for pathogenic CNVs and 4.55% for potentially pathogenic CNVs with an unclear clinical significance. The overall diagnostic efficiency of the CNV detection in the study group was 34.10%, with the pathogenic CNVs and the potentially pathogenic CNVs with an unknown clinical significance interpreted as positive findings (Figure 6). After the exclusion of the genetic syndromes where known CHD genes had been identified, the diagnostic efficiency, with regard to the detection of new pathogenic CNVs, was found to be as high as 18.94%.

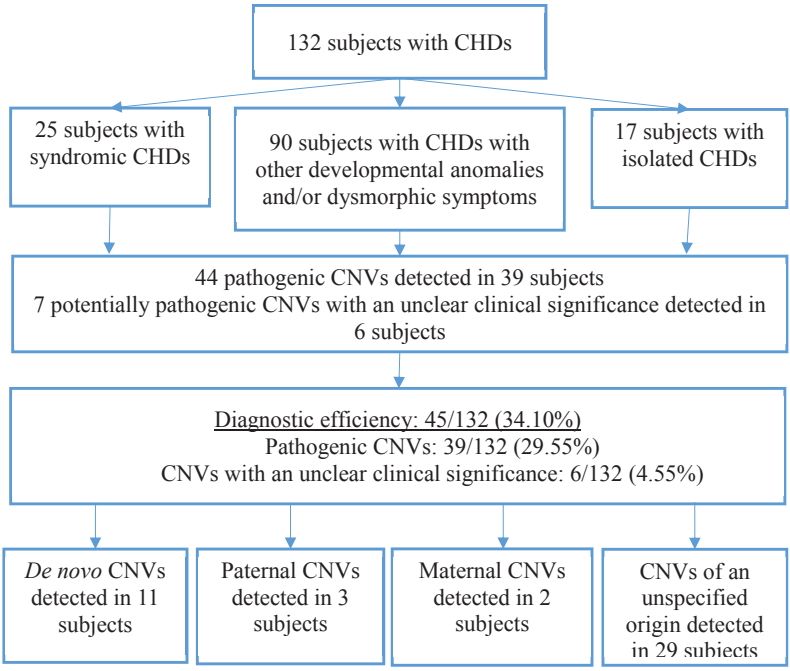

Figure 6. The diagnostic efficiency of the pathogenic CNV detection process.

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

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

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

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

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

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

Known CHD genes are highlighted in bold, * – genes identified through animal studies.

Del – deletion; Dup – duplication; Ao – aorta, AoS – aortic stenosis, RV – right ventricle, MVP – mitral valve prolapse, ASD – atrial septal defect, PVS – pulmonary valve stenosis, VSD – ventricular septal defect, TF – tetralogy of Fallot.

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

Four (4/39) subjects were diagnosed with unbalanced translocations that include known CHD genes; therefore, these CNVs were not included in the subsequent search for CHD genes (Table 6). An unbalanced *de novo* translocation was detected in a female subject (CG-008) with an atrioventricular septal defect accompanied by a massive primary ASD, atrioventricular valve regurgitation and PDA: duplication in the 15q21.2q26.3 locus of 47710 kb in size and deletion in the 15q26.3 locus of 2346 kb in size. The duplication involved the *NR2F2* gene (MIM# 10773) associated with an atrioventricular septal defect (Al Turki et al. 2014) and the CHD gene *SMAD6* (MIM# 602931); three CHD genes, *MAP2K1*/*MEK* (MIM# 176872), *ALDH1A2* (MIM# 603687) and *FBN1* (MIM# 134797), were detected in the locus where the deletion was observed.

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

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

CHD genes are highlighted in bold.

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

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

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

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

3.5. Identification and Characterization of the New CHD Genes

During the initial analysis, 20/253 pathogenic CNV variants with no known CHD genes involved were identified and selected for further analysis and ,search for CHD genes. These pathogenic variants were not found in the control group (355 control subjects with 545 CNVs) and did not overlap with the polymorphic CNV loci described in the DGV database. The scheme for the detection of CNVs and the search for candidate CHD genes is presented in Figure 7.

Out of 44/253 pathogenic CNVs, 16/44 CNVs overlapped with the genomic sequences corresponding to known syndromes and included

known CHD genes. 8/44 CNVs consisted of unbalanced translocations and included known CHD genes. Therefore, an analysis of the genes involved in 20 CNVs was performed to detect new candidate genes for CHD (Table 7).

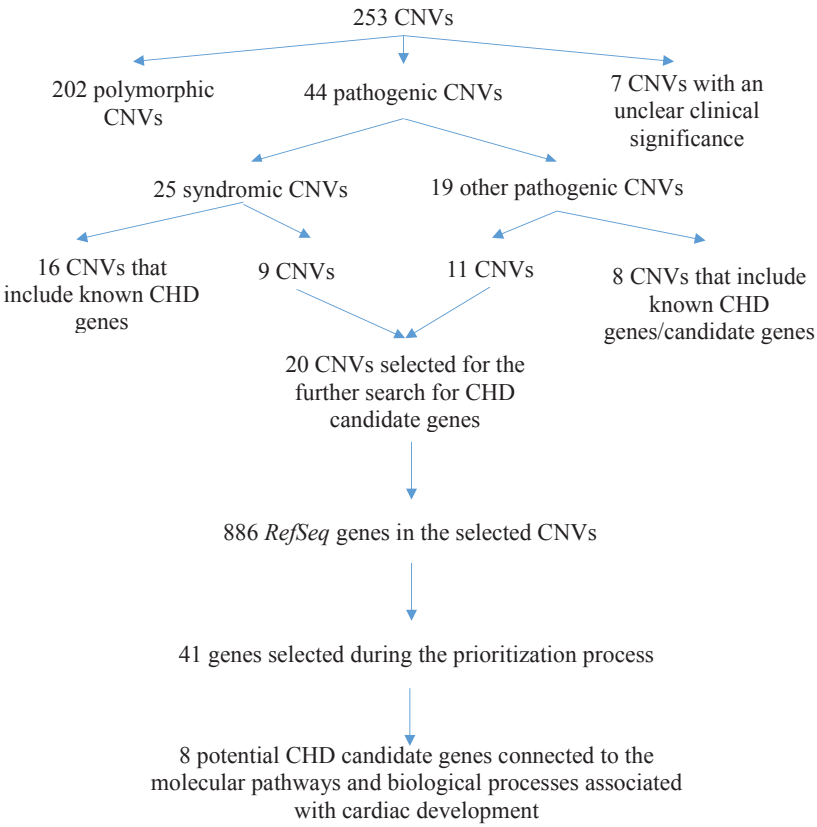

Figure 7. Scheme for the detection of CNVs and search for new CHD candidate genes.

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

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

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

3.5.1. Analysis of the Genes Encompassed by Pathogenic CNVs

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

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

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

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

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

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

During the following stage, the Gene Expression Database (GXD) in the Mouse Genome Informatics (MGI) (http://www.informatics.jax.org/expression.shtml/) database was used to analyze the gene expression patterns peculiar to cardiac embryogenesis in mice. The list of CHD candidate genes was further prioritized depending on gene expressions in the endothelium, the heart and the valves during embryogenesis. It was established that 14 genes were annonated in the MGI database and associated with the manifestation of CHDs in mice, with the following *MGI* terms used: "MP:0005294 *Abnormal heart ventricle morphology*," "MP:0000266 *Abnormal heart morphology*," "MP:0000267 *Abnormal heart development*," "MP:0003921 *Abnormal heart left ventricle morphology*," "MP:0000288 *Abnormal pericardium morphology*" (Table 9).

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

Identification number	Definition	Genes	Gene number	Annotated genes	p value
Molecular function					
GO:0004672	Protein kinase activity	*FGF9, BMPR1A, ERBB4, PRKD1, MAPK12, PRKAG2, MNAT1, KCNH2*	8	649	7.956E-6
GO:0016772	Transferase activity, transferring phosphorus-containing groups	*FGF9, BMPR1A, ERBB4, PRKD1, MAPK12, PRKAG2, MNAT1,KCNH2*	8	1006	1.801E-4
GO:0070851	Growth factor receptor binding	*FGF9, ERBB4, PTEN*	3	143	1.692E-3
Biological process					
GO:0072358	Cardiovascular system development	*ANKRD17, NPPA, FGF9, NPPB, BMPR1A, ERBB4, PRKD1, PTEN, LMNA, NFATC1, IFT88, MNAT1, SHH, SMARCD3, NOS3*	15	1058	2.393E-11
GO:0072359	Circulatory system development	*ANKRD17, NPPA, FGF9, NPPB, BMPR1A, ERBB4, PRKD1, PTEN, LMNA, NFATC1, IFT88, MNAT1, SHH, SMARCD3, NOS3*	15	1058	2.393E-11
GO:0007507	Heart development	*NPPA, FGF9, BMPR1A, ERBB4, PTEN, LMNA, NFATC1, IFT88, MNAT1, SHH, SMARCD3*	11	593	1.477E-9
GO:0048738	Cardiac muscle tissue development	*NPPA, FGF9, ERBB4, PTEN, LMNA, IFT88*	6	215	1.278E-6
GO:0060043	Regulation of cardiac muscle cell proliferation	*FGF9, ERBB4, PTEN*	3	34	2.413E-5
GO:0003007	Heart morphogenesis	*FGF9, BMPR1A, NFATC1, IFT88, SHH, SMARCD3*	6	252	2.116E-6

Identification number	Definition	Genes	Gene number	Annotated genes	p value
Mouse phenotypes					
MP:0005294	Abnormal heart ventricle morphology	*ANKRD17, NPPA, FGF9, ERBB4, PRKD1, PTEN, LMNA, NFATC1, IFT88, NOS3*	10	745	1.479E-5
MP:0000266	Abnormal heart morphology	*ANKRD17, NPPA, FGF9, BMPR1A, ERBB4, PRKD1, PRKAG2, PTEN, LMNA, NFATC1, IFT88, KCNH2, SHH, NOS3*	14	1465	6.869E-6
MP:0000267	Abnormal heart development	*ANKRD17, FGF9, BMPR1A, ERBB4, PTEN, LMNA, NFATC1, IFT88, KCNH2, SHH*	10	388	4.036E-8
MP:0003921	Abnormal heart left ventricle morphology	*NPPA, FGF9, ERBB4, PRKD1, PTEN, LMNA, NFATC1, NOS3*	8	343	2.492E-6
MP:0000288	Abnormal pericardium morphology	*ANKRD17, BMPR1A, PTEN, NFATC1, IFT88, KCNH2, SHH*	7	259	4.586E-6

3.5.2. Characterization of the New CHD Candidate Genes

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

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

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

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

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

The CHD candidate gene *NFATC1* (MIM#600489) was identified in the genomic locus of 18q23. Two female patients with abnormal heart valve formations were discovered to have several deletions in the long arm of chromosome 18. A female patient with pulmonary valve stenosis (CG-044) was found to have a *de novo* deletion in the

18q22.1q23 locus of 12920 kb in size. Another female patient with MVP and abnormal aortic formation (CG-112) had a deletion in the 18q23 locus of 1133 kb in size, whose origin could not be determined.

3.6. Comparison of CNVs in the Study and Control Groups

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

Based on the initial results of this study and the results of similar studies published in the past (Kaminsky et al. 2011; Xie et al. 2017), the hypothesis that rare genic CNVs may be relevant for the manifestation of CHDs was formulated. In order to evaluate the incidence distribution of the rare CNVs in the study and control groups, CNVs were categorized according to the type of chromosomal changes (deletions or duplications) and grouped into five categories: genic CNVs, common CNVs, common genic CNVs, rare CNVs and rare genic CNVs. The CNVs that occurred with a frequency of less than 1% and overlapped with the CNVs provided in the DGV database to a minimal extent (less than 30%) or not at all were regarded as rare. The CNVs that encompassed a *RefSeq* gene sequences were defined as genic. The CNVs that overlapped with at least one of the CNVs provided in the DGV database with a frequency of over 70% were regarded as common. CNV distribution was compared between the study group (132 individuals), where 253 CNVs were detected, and the control group (355 individuals), where 545 CNVs were detected.
Insofar as the incidence distribution of genic CNVs in the study group and control group (Table 10) is concerned, all genic CNVs were 1.3 times more common in subjects with CHDs (77.87% in the study group and 59.82% in the control group), and the difference was statistically significant ($p<0.0001$). Common genic CNVs were also significantly more prevalent in subjects with CHDs ($p=0.0086$). Rare genic CNVs were 2.65 times more common in subjects with CHDs (25.30% in the study group, 9.54% in the control group), and the difference was statistically significant ($p<0.0001$).

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

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

Significant differences in the CNV categories are highlighted in bold.

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

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

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

The significant differences in deletion distribution are highlighted in bold.

Upon analyzing the incidence of rare CNVs in the deletion category, rare deletions were found to be significantl more common (3.26-fold) among subjects with CHDs (46.99% in the study group and 14.40% in the control group) (p<0.0001). Meanwhile, the incidence of common deletions was higher in the control group than in subjects with CHDs, but the difference was not significant. A significant difference (p<0.0001) in the distribution of all genic deletions in the study and control groups

was observed, with these deletions being 1.41 times more common in the study group (78.31% in the study group and 55.60% in the control group). There was no significant difference between the two groups as far as the incidence of common genic deletions was concerned (p=0.1961). However, rare genic deletions were significantly (4.02-fold) more common (p <0.0001) in the study group (43.37% in the study group and 10.80% in the control group) (Table 11).

Upon comparing the incidence distribution of duplications in the two groups, a significant difference was found in the category of rare duplications, which were 1.53 times more common in the study group than in the control group (p=0.0076) (Table 12). The incidence of common duplications was significantly higher in the control group (p=0.0015), but common genic duplications were significantly (1.11-fold) more prevalent in the study group (61.18% in the study group, 54.2% in the control group). Upon assessing the distribution of all genic duplications in the study and control groups, it was discovered that genic duplications were 1.22 times more common in subjects with CHDs (77.65% in the study group and 63.39% in the control group) and the difference was statistically significant (p=0.0001). Rare genic duplications were also significantly (1.94-fold) more common in the study group (16.47% in the study group and 8.47% in the control group) (p=0.0037). There was a statistically significant difference between the study and control groups with regard to the incidence of common genic duplications (p=0.0205) (Table 12).

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

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

The significant differences in duplication distribution are highlighted in bold.

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

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

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

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

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

The significant differences in CNV distribution are highlighted in bold.

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

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

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

The significant differences in deletion distribution are highlighted in bold.

The incidence distribution of the rare genic duplications the size of which was less than or equal to 500 kb was 1.41 times higher in the study group, but the difference was not significant (p=0.2740) (Table 15). No significant difference was found upon comparing the incidence of rare duplications the size of which was less than or equal to 500 kb in the study and control groups (p=0.6257). Common genic duplications of the same size were 1.18 times more common in the

study group, but the difference was statistically negligible (p=0.0721). As far as the incidence of common duplications whose size was less than or equal to 500 kb were more common in the control group, with the difference being insignificant (p=0.6257). Genic duplications were significantly more common in the control group in comparison with the study group (p<0.0001) (Table 15).

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

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

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

The significant differences in duplication distribution are highlighted in bold.

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

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

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

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

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

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

Five hundred forty-five CNVs were detected in the control group, which consisted of 355 subjects. The number of CNVs per subject was 1.54. The size of the CNVs varied from 28 kb to 3941 kb, with an average CNV size of 240 ± 364.75 kb and a median CNV size of 140 kb.

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

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

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

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

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

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

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

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

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

Significant differences in median size are highlighted in bold.

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

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

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

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

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

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

Upon analyzing the differences in the average number of genes encompassed by CNVs in the duplication category, it was found that common duplications encompassed 2.27 times significantly more genes (5.54 ± 6.41 in the study group and 2.44 ± 2.65 in the control group) and the difference was statistically significant ($p <0.001$); rare duplications encompassed 3-times more genes, and the difference was likewise statistically significant ($p=0.0020$) (8.27 ± 7.83 in the study

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

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

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

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

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

The results of the study demonstrated that the genes encompassed by rare CNVs were associated with a lower genes haploinsufficiency (HI) score, which indicates the dose sensitivity of the gene (Huang et al. 2010). Upon comparing the mean value for the number of all CNV-related genes in the study and control groups, it was found that in the

study group, the CNVs encompassed 1.94 times more HI genes, with the difference being statistically significant (p <0.0001) (Table 21). Rare CNVs encompassed 2.45 times more HI genes in subjects with CHDs than in members of the control group, and the difference was statistically significant (p=0.0187). With regard to common CNVs, there was no significant difference between the average number of CNV genes in the study and control groups (p=0.6010) (Table 21).

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

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

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

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

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

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

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

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

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

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

3.8. Discussion of the Results

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

One hundred thirty-two subjects with CHD were examined in this piece of research. The heart diseases of the subjects typically manifested themselves as heterogeneous diseases: 12.88% were isolated CHDs, 18.94% were syndromic CHDs, and 68.18% manifested with other congenital developmental anomalies and/or dysmorphic features. The CHDs of the groups of subjects included four groups of cardiological phenotypes, the biggest group of which (71.43%) were CHDs with normal cardiac segment connections. In terms of complexity, there

were two groups of CHDs – anatomically separate CHDs (49.21%) and complex CHDs (50.79%). Such a diverse manifestation of CHDs shows considerable phenotypic heterogeneity and complexity, which is typical for heart diseases. The frequency distribution of the subjects in terms of gender in all the aforementioned groups, the frequency of male and female were not statistically significantly different in all the groups ($p=0.690$). The assessment of the distribution of phenotype frequency between CHDs determined in a group of subjects, the most frequent CHDs were septal defects, which made up 48.89% of all the cases of CHDs. It coincides with the frequencies of congenital heart diseases in populations, which were described in the literature (Wang et al. 2011; Yang et al. 2012). In accordance with the literature, in terms of the manifestation of CHDs between genders, CHDs of all types manifest more frequently in females (4.55 CHDs in 1 000 females and 3.61 CHDs in 1 000 males; $p<0.0001$) (Marelli et al. 2007). When assessing the dependency of manifestation of CHDs on the gender, no significant dependence was determined ($p>\alpha$, $\alpha=0.05$). A statistically significant dependence was determined only between the anomalies of great vessels and gender ($p=0.029$, $p>\alpha$, $\alpha=0.05$). Women had a larger number of anomalies of great vessels. In accordance with the epidemiological study data, certain types of CHDs manifest themselves by a different manifestation frequency between genders. It was determined that a coarctation of the aorta and the transposition of the great vessels, which are considered as anomalies of great vessels, manifest themselves in males more frequently than in females (Samánek 1994; Digilio et al. 2001b). Females in this group of subjects were diagnosed with anomalies of great vessels 2.6 times more frequently than males. This difference was a consequence of the structure of CHDs in the group of subjects selected.

Large-scale SNP-CGH genotyping was used and 39 (29.55%) subjects with CHDs were diagnosed with 44 pathogenic CNVs, which made up 17.39% of all CNV determined; 6 (4.55%) subjects were diagnosed with potentially pathogenic CNVs of uncertain clinical significance. The diagnostic efficiency of this piece of research was 34.1% and was higher than that in other studies described in literature. In accordance

with the literature, pathogenic and/or potentially pathogenic CNVs are determined in 10–20% of all the cases of CHDs (Carey et al. 2013; Kim et al. 2016). Pathogenic CNVs are most frequently determined in patients with clinically recognizable syndromes, such as DiGeorge syndrome or Williams-Beuren syndrome, and in patients with multiple developmental abnormalities and dysmorphic features. But pathogenic CNVs are significantly more determined in the cases of isolated CHDs than in the population of healthy individuals. Deletions (77.27%) made up a major part of pathogenic CNVs. The results of this piece of research reflect the results of the studies that were published by other authors and confirm that deletions are more linked with clinical phenotypes. The genes, which take part in the processes of cardiac embryogenesis and which are located in the deletion regions, result in CHDs due to an altered expression.

Twenty-five pathogenic CNVs were determined in the group of syndromic CHDs of the group of subjects in the piece of research, 16 pathogenic CNVs of which had a clear cause of pathogenicity because they included the following CHD genes – *TBX1*, *CRKL*, *ELN*, *EVC2*, *EVC*, *MEF2C*, *NIPA1* and *NIPA2*. In accordance with the literature, CNVs result in 17–18% of cases of syndromic CHDs (Thienpont et al. 2007; Breckpot et al. 2010; Geng et al. 2014). But in some distinct studies, the diagnostic efficiency of pathogenic CNVs was 25% and even 52.7% (Richards et al. 2008; Syrmou et al. 2013). At least a single pathogenic change was determined in all the subjects of this study, who were suffering from syndromic CHDs. Including subjects with clinically recognizable genetic syndromes in this study resulted in such a high diagnostic efficiency. The most frequent genomic variant was the 22q11.2 deletion (DiGeorge syndrome), which manifests itself in 0.5–1.9% of all cases of CHDs. In this study, 22q11.21 deletions in the genomic region, which include the *TBX1* gene in all of the cases, were determined in five patients. They made up 3.79% of all the cases of CHDs in the subject group.

Nineteen pathogenic CNVs were determined in 14 subjects in the group of CHDs with other congenital developmental anomalies and/or dysmorphic features, 8 CNVs of which included the following

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

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

The high diagnostic efficiency in the common group of CHDs and in individual groups of CHDs proves that CGH genotyping is a genetic diagnostic method that should be chosen first for the genetic testing of patients with CHDs. This method is recommended for early genetic testing of neonates with CHDs (Bachman et al. 2015) and of children with CHDs, especially with CHDs with other developmental anomalies and/or dysmorphic features (Wu et al. 2017). The diagnostic efficiency of pathogenic CNVs determined in this piece of research confirms the results of other studies.

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

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

This study determined a *de novo* 10q22.3q23.33 deletion of 14154 kb in size, which included candidate genes of CHDs, namely *BMPR1A* and *PTEN*. Large 10q22q23 deletions are linked with manifestation of CHDs. Literature describes patients with large 10q deletions with typical cardiological phenotypes – AVSD, ASD, VSD and tricuspid valve regurgitation (Alliman et al. 2010; Breckpot et al. 2011; van Bon et al. 2011). *BMPR1A* encodes the bone morphogenetic receptor type 1A and participates in the BMP signaling pathway. The BMP signaling

pathway regulates cell proliferation, migration, differentiation and apoptosis. *Bmpr1a* gene deletion in mouse models damages heart morphogenesis in mice and manifests itself with defects in the interventricular septum and in the endocardial cushions (Gaussin et al. 2002). The *PTEN* gene, which is located more distally to the *BMPR1A* gene, is important for tumor suppression. Hamada et al. demonstrated that *PTEN*, which regulates the expression of vascular signaling molecules, is necessary for normal cardiovascular morphogenesis and for postnatal angiogenesis (Hamada et al. 2005).

Deletions in the *de novo* 18q23 region, which were determined in two patients with heart valve development diseases, included another CHD candidate gene. The NFATC1 protein is a part of the Rel/NF–kB family of transcription factors, the members of which regulate the proliferation, differentiation and homeostasis of the majority of cells (Crabtree et al. 2002). The NFATC1 protein is important for the development of heart valves. The expression of *NFATC1* takes part in the primary heart tube of endocardial endothelial cells, and the expression of this gene in the early stages of the formation of endocardial cushions specifically manifests itself in the endothelial cells of the atrioventricular canal and of the outflow tract (de la Pompa et al. 1998). An abnormal expression of the *NFATC1* gene results in atrioventricular and semilunar valve anomalies in mice (de la Pompa et al. 1998; Ranger et al. 1998). Abdul-Sater et al. proved that *NFATC1* gene mutations result in tricuspid valve atresia (Abdul-Sater et al. 2012).

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

A *De novo* 13q12.11q12.12 deletion in a genomic region included the candidate genes of CHDs – *IFT88* and *FGF9.* Both of the genes in mouse models are linked to impaired cardiac morphogenesis. The FGF9 protein belongs to the family of fibroblast growth factors.

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

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

Dosage-sensitive genes may be linked with the manifestation of CHDs in the subjects. Deletions cause a disease phenotype mainly as a result of dosage-sensitive gene haploinsufficiency, whereas duplications may lead to a development of the disease due to triplosensitivity of a gene, a termination of the gene sequence or gene fusion through breakpoints. Another *de novo* interstitial 1q22q23.1 deletion included *LMNA*, which is a candidate gene of CHDs. A 1q22q23.1 microdeletion, which was determined in a patient, is the smallest deletion to be described so far, including the *LMNA* gene (Aleksiūnienė et al. 2018). The *LMNA* gene encodes lamins A and C, which form due to the al-

ternative splicing of transcripts (Depreux et al. 2015). Lamins A and C, which are located in the inner nuclear membrane, interact with the proteins in the nuclear envelope, chromatin and transcription factors (Gupta et al. 2010). Mutations and deletions of the function loss of the *LMNA* gene lead to gene haploinsufficiency and are linked to dilated cardiomyopathy (Zaragoza et al. 2016). The subject of this piece of research (CG-067) and the sibs with 1q21.3q23.3 deletion, described by Quinonez et al., had an identical cardiological phenotype, ASD, PDA, and limb contractures (Quinonez et al. 2012). This suggests that the dosage-sensitive gene haploinsufficiency of the *LMNA* gene could have led to the formation of structural heart defects.

A link between major genomic changes and congenital heart defects was determined in this piece of research. The rare frequency of CNVs (and rare of ≥501 kb) in the subject group, which includes both syndromic CHDs, CHDs with other congenital developmental anomalies and/or dysmorphic features, and isolated CHDs, was significantly higher than the frequency of CNVs in the control group. Rare genetic CNVs manifested themselves in the group of subjects with CHD 2.65 times more frequently, whereas large (of ≥501 kb), rare genetic CNVs were 6.63 times significantly more frequent in subjects with heart diseases. Despite the differences between the design of phenotypic research of CHDs and the resolution of genotyping chips, the majority of research determined a significantly higher frequency in the population of rare, large and/or *de novo* CNVs congenital heart diseases. Silversides et al. found that large, rare CNVs were more frequent in the group of subjects with the Tetralogy of Fallot than in the control group (8.8% in the subject group and 4.33% in the control group, p=0.0117) (Silversides et al. 2012). Other pieces of research revealed 12.7% of *de novo* CNVs, 5.6% of which were significantly linked with CHDs, in comparison with just 2% in the control group (Warburton et al. 2014). Greenway et al. found that *de novo* CNVs resulted in 10% of isolated sporadic TOF in comparison with 4% in the control group (Greenway et al. 2009).

The results of this study revealed that rare genic CNVs, as well as extremely rare genic deletions, which manifested themselves 4.02-times

more frequently in the group of subjects with CHDs, are linked to heart diseases. A significant difference between the average value of a number of genes, which include rare genic CNVs, was determined. It may be concluded that rare deletions and duplications, including a few times genes more on average, lead to a higher risk of CHDs.

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

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

4. CONCLUSIONS

1. Phenotypic heterogeneity and complexity are typical for congenital heart diseases of the subjects:
   - Congenital heart diseases, which manifest themselves with other congenital developmental anomalies and/or dysmorphic features made 68.18%; 18.94% of congenital heart diseases were one of the clinical features of a genetic syndrome, and 12.88% of congenital heart diseases were isolated;
   - Congenital heart diseases with normal cardiac segmental connections, i.e., 71.43% were most frequent, with the biggest part, 34.92%, of septal defects;
   - Anatomically isolated congenital heart diseases made up 49.21%, whereas complex congenital heart diseases made up 50.79% of all congenital heart anomalies;
   - A significant dependence between the manifestation of major vascular anomalies and gender in the female group ($p=0.029$) was determined, but no significant results were determined in separate groups of congenital heart diseases ($p>\alpha$, $\alpha=0.05$).
2. Pathogenic copy number variations are frequent causal variants in the etiology of syndromic congenital heart diseases and of congenital heart diseases, which manifest themselves with other congenital developmental anomalies:
   - Of pathogenic copy number variations, 17.39% were determined, 56.82% of which were in the group of syndromic congenital heart diseases, and 43.18% of which were in the group of congenital heart diseases that had manifested themselves with other congenital developmental anomalies and/or dysmorphic features;
   - Of pathogenic copy number variations, 38.64% included known genes of congenital heart diseases and led to syndromic congenital heart diseases.

3. Eight candidate genes of congenital heart diseases were determined, which were as follows: *FGF9, BMPR1A, PTEN, ANKRD17, NPPA, LMNA, NFATC1* and *IFT88*. Due to an altered expression, the candidate genes that participate in heart development may cause congenital heart diseases.

4. The diagnostic efficiency of the method of large-scale, single-nucleotide polymorphism – a comparative genomic hybridization in the group of congenital heart diseases – was 34.4%. After excluding the cases of the genetic syndromes, where the genes of congenital heart diseases were knowingly determined, the diagnostic efficiency of determining new pathogenic copy number variations amounted to 18.94%.

5. Rare, large copy number variations (rare deletions and duplications), which include a few times more genes on average, may possibly result in a higher risk of congenital heart diseases:
   - Rare genic copy number variations manifested 2.65 times more often in the group of subjects with congenital heart diseases than in the control group ($p<0.0001$);
   - Rare genic copy number variations included 2.45 times more genes with a high degree of haploinsufficiency in the groups of subjects with congenital heart diseases than in the control group ($p=0.0187$).

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LIST OF PUBLICATIONS

Published articles

1. **Aleksiūnienė B**, Matulevičiūtė R, Matulevičienė A, Burnytė B, Krasovskaja N, Ambrozaitytė L, Mikštienė V, Dirsė V, Utkus A, Kučinskas V. Opposite chromosome constitutions due to a familial translocation t(1;21)(q43;q22) in 2 cousins with development delay and congenital anomalies: A case report. *Medicine* (Baltimore). 2017 Apr; 96(16):e6521.
2. **Aleksiūnienė B**, Preiksaitiene E, Morkūnienė A, Ambrozaitytė L, Utkus A. A *de novo* 1q22q23.1 interstitial microdeletion in a girl with intellectual disability and multiple congenital anomalies including congenital heart defect. *Cytogenet Genome Re*s. 2018; 154; 6-11.
3. Vaisvilas M, Dirse V, **Aleksiuniene B**, Tamuliene I, Cimbalistiene L, Utkus A, Rascon J. Acute pre-B lymphoblastic leukemia and congenital anomalies in a child with a de novo 22q11.1q11.22 duplication. *Balkan Journal of Medical Genetics*. 2018; 21(1):87-92.

Spoken presentations

1. Matulevičienė A, **Aleksiūnienė B**, Mikštienė V, Krasovskaja N, Griškevičius L, Utkus A, Kučinskas V. Dup(1)(q43–q44)&del(21)(q22.2–q22.3) characterized by facial dysmorphism, congenital heart defect and mental retardation. The 25th European Dysmorphology Meeting, 2014, Strasbourg, France.
2. **Aleksiūnienė B**, Matulevičiūtė R, Čiuladaitė Ž, Matulevičienė A, Utkus A, Kučinskas V. Coarctation of the aorta with dysmorphic features in a patient with triplication of 15q26.1-q26.3: clinical and molecular analysis. The 26th European Dysmorphology Meeting, 2015, Strasbourg, France.

3. Matulevičienė A, **Aleksiūnienė B**, Tamulienė L, Liubšys A, Čiuladaitė Ž, Utkus A, Kučinskas V. A novel *de novo* del (4)(q28.2-qter)&del(8)(pter-p23.1) due to unbalanced translocation in a girl: clinical and molecular analysis. The 26th European Dysmorphology Meeting, 2015, Strasbourg, France.
4. **Aleksiūnienė B**, Ambrozaitytė L, Matulevičienė A, Krasovskaja N, Utkus A. Comparative whole genome hybridization methods in molecular diagnostics. The XIII Baltic Congress in Laboratory Medicine, 2016, Tartu, Estonia.
5. **Aleksiūnienė B,** Ambrozaitytė L, Dagytė E, Laimutė R, Molytė A, Utkus A. Identification of rare copy number variants in patients with congenital heart diseases. The XIV Baltic Congress Of Laboratory Medicine, 2018, Vilnius, Lithuania.
6. Braždžiūnaitė D, Laimutė R, **Aleksiūnienė B**, Dagytė E, Ambrozaitytė L, Benušienė E, Cimbalistienė L, Utkus A. Molecular karyotyping: clinical utility and practice. The IX International Scientific Conference *Retų ligų gydymo inovacijos* ("Innovations in Treating Rare Diseases"), 2018, Vilnius, Lithuania.

Poster presentations

1. **Aleksiūnienė B**, Matulevičienė B, Benušienė E, Tarutis V. Hypoplastic left heart syndrome in a case with partial 18p monosomy and partial 20q trisomy. European Human Genetics Conference 2015, Glasgow, United Kingdom.
2. Cimbalistienė L, **Aleksiūnienė B**, Dirsė V, Tamulienė I, Utkus A. 6,6 Mb *de novo* 22q11.1-q11.22 duplication in a patient with anomalous pulmonary venous drainage, intestinal malrotation and pre B acute lymphoblastic leukemia. European Human Genetics Conference 2016, Barcelona, Spain.
3. Preikšaitienė E, Maldžienė Ž, **Aleksiūnienė B,** Kučinskas V. Mild intellectual disability, congenital heart defect and skeletal abnormalities in three patients with 4q13.3 microdeletion. European Human Genetics Conference 2016, Barcelona, Spain.

4. **Aleksiūnienė B**, Cimbalistienė L, Dirsė V, Gineikienė E, Marcinkutė R, Utkus A. *De novo* 15q26.2q26.3 duplication and 15q26.3 deletion in a patient with an anomalous parietal sutures. European Human genetics Conference 2016, Barcelona, Spain.
5. **Aleksiūnienė B**, Matulevičienė A, Ambrozaitytė L, Jakutis V, Utkus A. Hypoplastic right ventricle, dysmorphic features and brain structural anomalies in a patient with a *de novo* 1p36.33p36.32 deletion. European Human Genetics Conference 2017, Copenhagen, Denmark.
6. **Aleksiūnienė B**, Tumienė B, Utkus A. *AHDC1* gene truncating 1p36.11p35.3 microdeletion in a patient with developmental delay, dysmorphic features and congenital heart defects. European Human Genetics Conference 2018, Milan, Italy.
7. Blažytė EM, Samsonė VG, Matulevičienė A, **Aleksiūnienė B**, Burnytė B, Dagytė E, Tumienė B, Utkus A. The spectrum of associated congenital malformations in Down syndrome: a retrospective Lithuanian cohort study. European Human Genetics Conference 2018, Milan, Italy.

ABOUT THE AUTHOR

Name:	Beata
Last name:	Aleksiūnienė
Date of birth:	September 5, 1975
Phone number:	+370 685 26535
Email:	beata.aleksiuniene@mf.vu.lt

Academic background:

2014–2018 – Vilnius University, Faculty of Medicine, Institute of Biomedical Sciences, Department of Human and Medical Genetics, PhD student

1998–2000 – Vilnius University, Faculty of Natural Sciences, Genetics study program, Master of Biology

1994–1998 – Vilnius University, Faculty of Natural Sciences, Molecular Biology study program, Bachelor of Biology

1982–1993 – Vievis Gymnasium (formerly the Vievio Secondary School)

Work experience:

2002 – Department of Human and Medical Genetics at the Institute of Biomedical Sciences, Faculty of Medicine, Vilnius University, junior research associate, lector

2000 – Medical Genetics Center at Vilnius University Hospital Santaros Klinikos, medical geneticist

Membership in professional societies:

Lithuanian Society of Human Genetics
European Society of Human Genetics

APIE AUTORĘ

Vardas:	Beata
Pavardė:	Aleksiūnienė
Gimimo data:	1975 m. rugsėjo 5 d.
Telefono numeris	+370 685 26535
El. pašto adresas	beata.aleksiuniene@mf.vu.lt

Išsilavinimas:

2014–2018 – Vilniaus universitetas, Medicinos fakultetas, Biomedicinos mokslų institutas, Žmogaus ir medicininė genetikos katedra, doktorantė

1998–2000 – Vilniaus universitetas, Gamtos mokslų fakultetas, genetikos studijų programa, biologijos magistro kvalifikacinis laipsnis

1994–1998 – Vilniaus universitetas, Gamtos mokslų fakultetas, biologijos studijų programa, biologijos bakalauro kvalifikacinis laipsnis

1982–1993 – Vievio gimnazija (buvusi Vievio vidurinė mokykla)

Darbo patirtis:

2002 – Vilniaus universitetas, Medicinos fakultetas, Biomedicinos mokslų institutas, Žmogaus ir medicininės genetikos katedra, jaunesnioji mokslo darbuotoja, lektorė

2000 – Vilniaus universiteto ligoninės Santaros klinikos Medicininės genetikos centras, medicinos genetikė

Narystė profesinėse organizacijose:

Lietuvos žmogaus genetikos draugijos narė
Europos žmogaus genetikos draugijos narė

Vilniaus universiteto leidykla
Universiteto g. 1, LT-01513 Vilnius
El. p. info@leidykla.vu.lt,
www.leidykla.vu.lt
Tiražas 40 egz.