SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Investigation of pathogenetically significant copy number variants in congenital abnormalities

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INVESTIGATION OF PATHOGENETICALLY SIGNIFICANT COPY NUMBER VARIANTS IN CONGENITAL ABNORMALITIES

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The online Examination will be held at 11:00 a.m., on January 21, 2021.

Head of the **Defense Committee**:	Gábor Méhes, MD, PhD, DSc
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The online PhD Defense will be held at 2:00 p.m., on January 21, 2021.

Live online access will be provided. If you wish to take part in the discussion, please send an e-mail to nagy.orsolya@med.unideb.hu, not later than 12:00 p.m. on the day before the discussion (January 20, 2021).

1. INTRODUCTION

Copy number variants (CNVs) are structural variations of the human genome with intermediate size. The functional consequences of CNVs depend on the size and gene content of the DNA gain or loss caused by them. A significant part of them are benign polymorphisms occuring in the healthy population. Pathogenic CNVs can also be associated with rare congenital abnormalities by deletion or duplication of dosage-sensitive genes.

Congenital heart diseases (CHDs) are the most common developmental anomalies that can be divided into two groups based on their clinical appearance: syndromic (showing symptoms of other developmental disorders) or isolated (affecting only the heart). The etiology of CHD (genetic and environmental factors) is known only in 20–30% of the cases. The identification of syndromic forms and the clarification of the genetic background are essential for the estimation of the prognosis and proper clinical care of the patients. As a result of improved diagnostic and surgical interventions, more than 90% of CHD patients reach adulthood. Determination of the genetic background of the CHDs plays essential role in genetic counseling regarding the assessment of recurrence risk, in reproductive decision-making and in prenatal diagnostics.

In section 5.1. of the thesis, I present the results of cytogenetic microarray and MLPA studies performed in CHD patients. Using these methods, my aim was to identify pathogenic CNVs underlying CHDs and determine genotype-phenotype correlations. I examined the postnatal cases divided into syndromic and isolated groups. The prenatal samples were derived from ventricular tissue samples of aborted fetuses diagnosed with CHD. By testing the effectiveness of the applied methods, I would have liked to work-out an effective diagnostic algorithm to reveal pathogenic CNVs in syndromic CHDs.

Differences of sex developments (DSDs) are rare congenital disorders with highly variable phenotypic abnormalities and difficulties in establishing the clinical diagnosis. In clinical practice, molecular genetic diagnosis is established in less than 20% of the cases, which makes it difficult to choose the adequate treatment (surgical, hormone therapy) and to assess the prognosis of the disease.

In section 5.2. of the thesis, I present a genetic examination of a female child diagnosed with 46,XY DSD. After determination of chromosomal sex it took five years to get a molecular genetic diagnosis explaining her phenotype. In connection to this study, we emphasized the importance of detection small size, intragenic CNVs.

2. OVERVIEW OF THE LITERATURE

2.1. Copy number variants in the human genome and their genetic testing methods

The functional consequences of the intermediate-sized (50 bp – 3 Mb) CNVs depend on the size and gene content of the DNA gain or loss caused by them. The majority of CNVs, which make up approx. 12–16% of the human genome are benign. They represent polymorphic variants present in >1% of the healthy population. Pathogenic CNVs contain developmentally essential, dosage-sensitive, phylogenetically conserved genes whose deletion/duplication can cause rare congenital disorders, autism, psychiatric disorders, common, complex adult diseases or acquired tumors.

One of the oldest method used for detection of submicroscopic (<3 Mb) CNVs is *fluorescent in situ hybridization* (FISH), in which fluorescently labeled DNA probes are hybridized to interphase nuclei or metaphase chromosome preparations to detect deletion, duplication and location of specific genomic region of interest.

Array comparative genomic hybridization (aCGH) can detect CNVs by examining the whole genome in one step. Due to its high resolution, this technique has approx. 15–20% diagnostic efficiancy in identification of CNV-based diseases, therefore it is recommended as a first-line genetic test in case of multiple developmental disorders, somatic and mental retardations of unknown origin, autism and in prenatal cases with fetal malformation confirmed by ultrasound or other screening test.

Using *multiplex ligation-dependent probe amplification* (MLPA), intragenic CNVs can be detected that affect a gene or a few exons in a single gene.

Next-generation *whole genome sequencing* (WGS) is able to detect balanced aberrations in addition to CNVs, along with specifying breakpoints at the sequence level and identifying the orientation of genomic segments.

2.2. Classification, etiology and genetics of congenital heart diseases

CHDs include functional and structural defects of the heart and large vessels. They are the most common birth anomalies and the leading causes of perinatal morbidity and mortality in newborns. Their prevalence is approx. 8/1000 live births, but this number varies by country and continents (2,3–9,3/1000). Thanks to effective treatment and surgical interventions, approx. 90% of patients with CHD reach the childbearing age. Many types of CHD classification system exist. There are simple or complex (multiple vitium together), cyanotic or acyanotic CHDs; based on the involvement of certain parts of the heart we can distinguish the following defects:

right-sided, left-sided, outflow tract or large vessels (conotruncal) and laterality defect (heterotaxia). CHDs can appear as syndromic (showing abnormalities of other organ system as well) or isolated (affecting only the heart). 25–40% of all CHDs are syndromic and 60–75% of them are isolated.

The etiology of CHDs are heterogeneous and is still largely unknown. According to epidemiological data approx. 75% of CHDs have multifactorial origin, i.e. they result from the combined action of environmental factors and multiple minor genes. Teratogenic agents are responsible for approx. 5% of the cases, while 20% has genetic origin.

Based on their size pathogenetic abnormalities underlying CHDs can be chromosomal aneuploidies (trisomy, monosomy), submicroscopic CNVs (microdeletion, microduplication), and small-scale gene mutations. These abnormalities can cause both syndromic (approx. 20%) or isolated CHDs (approx. 80%).

Chromosomal aneuploidies (trisomy/monosomy) as the first recognized genetic causes of CHDs are continuously the major etiological factors accounting for 9–18% of cases. Due to their high gene content, chromosome abnormalities cause almost exclusively multi-organ diseases, syndromic CHDs. Submicroscopic CNVs are detected in 3–10% of isolated, and in 3–25% of syndromic CHDs with extracardiac manifestations. The well-known syndromic CHDs caused by gene mutations are the followings: Alagille (*JAG1*, *NOTCH1*), Holt–Oram (*TBX5*), and Noonan (*PTPN11*, *SOS1*). Mutations underlying isolated CHDs affect genes whose protein products fall into three main groups according to their functions: transcription factors, proteins involved in signal transduction, and structural proteins. Transcription factors regulating gene expressions responsible for normal cardiac development are *GATA*, Homeobox, and T-box factors. Mainly loss-of-function mutations are detected in case of Notch, WNT/BMP, NODAL, and RAS/MAPK signal transduction pathways. Mutations in the genes of sarcomer and extracellular matrix proteins (e.g. *MYH6*, *MYH7*, *MYH11*, *ACTC1*, *ELN*) can be responsible not only for cardiomyopathies but for CHDs also. In addition to the above mentioned genetic alterations, epigenetic factors (DNA and histone methylation, acetylation, miRNA) may also modify the expression of proteins involved in cardiogenesis.

2.3. Classification, etiology and genetics of differences of sex developments

Sexual differentiation begins at 6 weeks of gestation and ends at puberty with the development of reproductive function. The sex chromosome composition of the embryo (XY or XX) determines the differentiation of the bipotential gonad towards to testis or ovary, and

the sex hormones produced by the formed gonads are responsible for the development of the internal and external genitalia.

Sexual differentiation is a complex process, coordinated expression of many genes at the right time and place is necessary for normal development. Chromosome Y, more specifically the sex-determining gene (*SRY*) located on its short arm (Yp11.2), is required to initiate the male-directed differentiation. In the absence of chromosome Y (karyotype XX), female-directed differentiation is initiated. One of the most important function of the *SRY* gene is to induce the expression of the *SOX9* gene, which is responsible for the development of testicular Sertoli cells and production of anti-Mullerian hormone (AMH). As a result, the Mullerian structures regresses and the formation of internal and external male genitalias begins from the Wolffian structures. This process is also supported by androgens produced by testicular Leydig cells. Several other genes (e.g. *GATA4, NR5A1, SOX3, SOX8, SOX10, WT1*) also play important role in testicular development. Their mutations can cause testicular developmental disorders and associated congenital abnormalities. Genes responsible for ovarian differentiation (e.g. *DAX1, FOXL2, RSPO1, WNT4*), in addition to proper follicular differentiation, also inhibit the expression of the male *SOX9* pathway.

DSD refers to chromosomal, gonadal, or phenotypic sex congenital abnormalities. Patients with DSD are clinically very heterogeneous, the incidence varies according to the severity of the phenotype. The spectrum of the clinical picture ranges from the mildest hypospadias (1:250 boy child), through uncertain external genitalia (1:4500) to complete sex reversal (1:20 000). Symptoms appear mainly in infancy, rarely during puberty. A new nomenclature and classification system were introduced in 2006 that is based on the sex chromosome composition. According to this consensus statement three groups of DSDs can be distinguished: (i) sex chromosome DSDs: 45,X; 47,XXY; 45,X/46,XY; 46,XX/46,XY; (ii) 46,XY DSD and (iii) 46,XX DSD. Genetic factors, environmental adverse effects, hormonal abnormalities, and epigenetic changes are all responsible for the development of DSD. Among the genetic abnormalities, the numerical and structural aberrations of sex chromosomes were identified at the earliest, due to their size, these types of alteration result in additional symptoms beside DSD (e.g. Turner, Klinefelter).

Mostly minor deletions, insertions, missense, nonsense, and splicing mutations with loss-of-function have been identified in genes coordinating gender differentiation. In addition to genetic heterogeneity, the highly variable phenotype and expressivity as well as decreased penetrance make more difficult getting genetic diagnosis in DSDs. Relatively little information is available on the pathogenetic role of CNVs in DSD compared to sequence-based mutations.

Large reported CNVs most commonly affect *NR5A1*, *NR0B1*, and *DMRT1* among the known DSD genes.

We present a CNV affecting the *NR5A1* gene – one of the genes responsible for differences of sex development – in a female patient diagnosed with DSD.

The protein encoded by the *NR5A1* (*SF-1*) gene is a nuclear receptor. Expressing in the bipotential gonad, it promotes male-directed differentiation by increasing the expression of *SRY* and *SOX9* genes and stimulates AMH production by Sertoli cells and testosterone synthesis by Leydig cells in the established testis. Among the genes involved in DSD, heterozygous mutations in the *NR5A1* gene are the most common, in case of 46,XY DSD, they occur approx. in 10–20%. Mutation-induced haploinsufficiency results in autosomal dominant inheritance, mostly 46,XY DSD, but it has also been described in 46,XX DSD. The clinical picture caused by mutations of *NR5A1* is very heterogeneous, ranging from hypospadias to uncertain external genitals to complete sex reversal.

2.4. Clinical significance of clarification the genetic background in CHD and DSD

The two groups of congenital abnormalities (CHD and DSD), share common features, namely genetic and clinical heterogeneity and the limited knowledge of the etiological background. Despite the rapid expansion of our knowledge, the proportion of patients with genetic diagnosis is still very low (20–30%).

Adequate clinical care for patients with CHD increasingly requires genetic testing, the results of which are used to answer the following questions: (i) what is the risk of recurrence of CHD, (ii) whether the genetic difference is carried by first-degree relatives, (iii) whether there are (unrecognized until then) extracardiac symptoms, (iv) nerve system developmental disorder as an associated symptom, (v) the prognosis of a given CHD, and the patient's life prospects.

DSD is often associated with ambiguous external genitalia, infertility in adulthood, gender identity problem, and as late consequence, malignant transformation of the dysgenetic gonads. In order to make an accurate diagnosis, to make a decision about the proper sexual development of patients, to plan the corrective surgeries and to make an assessment of the prognosis, it is essential to know the genetic background of DSDs.

3. OBJECTIVES

My goal was to identify pathogenic submicroscopic CNVs in those congenital abnormalities that have unraveled genetic diagnosis in very low proportion of cases. Based on the results, I aimed to describe genotype-phenotype correlations and reveal causative effect of the identified CNVs.

1.) My aim was to identify pathogenic submicroscopic CNVs in patients diagnosed with syndromic CHD using aCGH and MLPA methods. Based on my results, I aimed to work-out an algorithm that can be used to detect pathogenic CNVs of syndromic CHDs in the genetic examination process with high diagnostic efficiency, taking into account the available national opportunities as well.

2.) In my studies, I aimed to perform genetic testing of CNVs in patients diagnosed with an isolated CHD without extracardiac symptoms.

3.) I aimed to detect pathogenic CNVs in ventricular tissue samples from aborted fetuses prenatally diagnosed with isolated and complex CHDs.

4.) Small size CNVs involving one or few exons of genes are very rarely studied mutation types in DSDs. In a case of a female child diagnosed with 46,XY DSD, I aimed to identify the underlying genetic abnormality responsible for the development of the disease by investigating intragenic CNVs in genes involved in sexual differentiation.

4. PATIENTS AND METHODS

4.1. Patients

4.1.1. Groups of CHD patients

4.1.1.1. Patients with syndromic CHD

The samples of patients with syndromic CHD (n=33) were sent from the Clinical Genetic outpatient clinic of the Pediatrics Clinic, Clinical Center, University of Debrecen, the Department of Medical Genetics, Faculty of Medicine, University of Szeged, and the Szent Raphael Hospital in Zalaegerszeg. Patients' phenotypic data were provided by the clinical geneticists caring for them. Patient's age ranged from 2 to 18 years, with a gender distribution of 16 males to 17 females.

4.1.1.2. Patients with isolated CHD

The isolated CHD cases (n=16) were referred from the Cardiology outpatient clinic of the Pediatrics Clinic, Clinical Center, University of Debrecen, and the Department of Medical Genetics, Faculty of Medicine, University of Szeged. Patient's age ranged from 10 months to 46 years, with the gender ratio of 9 males to 7 females.

4.1.1.3. Samples from abortions

The samples were recruited from the First Department of Obstetrics and Gynecology, Semmelweis University. The diagnosis was made by ultrasound examination and echocardiography. All of the samples were obtained from terminated pregnancies. Each cardiac malformation was confirmed by fetal pathology. In the prenatal study group, the average gestational age was 20,85 (19–23) weeks. This cohort consisted of 18 ventricular myocardium samples from aborted fetuses with heart defects.

4.1.1.4. Study design

The aim of the study was to identify pathogenic CNVs in syndromic and isolated CHD cases with unknown etiology and to determine genotype-phenotype correlations. The sequence and priority of the genetic tests applied largely depended on the patients' phenotype and their severity. In general, any patient with a CHD with or without accompanying symptoms (developmental delay, dysmorphism) underwent a G-banded karyotyping. In case of negative

results or when further specification of findings was needed, or a specific microdeletion or microduplication syndrome was suspected based on the symptoms, I carried out targeted FISH or MLPA analysis. In case of negative results, aCGH was performed in the diagnostic process.

4.1.2. Patient with DSD

The proband is the first child of non-consanguineous Caucasian healthy parents. Family history was negative for DSD, premature ovarian failure (POF), fertility problems or any genetic disease. The patient was born after an uneventful pregnancy at 36 weeks of gestation with a birth weight of 3200 g. External genitalia showed female appearance with slight clitoromegaly (Prader stage I). At 9 months of age, the child was admitted to a hospital because of abdominal pain and vomiting. Bilateral inguinal hernias were found with gonads, suspected testes, during transabdominal ultrasonography (US). Upon laparoscopy mixed internal genitalia with a very small uterus, fallopian tubes and epididymis were detected. Biopsy of the gonads identified testicular tissue with no spermatogonia and a small number of Leydig cells. The gonads were placed into the abdomen. At that time G-banded chromosome analysis was performed and a *SRY* positive 46,XY karyotype was revealed. Complete androgen insensitivity syndrome was excluded based on low testosterone level (<0,15 nmol/L). Gonadotropin hormone levels were the following: follicle-stimulating hormone (FSH): 6,4 mIU/L (ref.: 0,1–6 mIU/L), luteinizing hormone (LH): 1,5 mIU/L (ref.: 0,1–4 mIU/L). The patient had normal serum cortisol and adrenocorticotropin hormone values and there was no evidence of adrenal insufficiency. The parents raised their baby as a female and the child was referred to an endocrinologist only at the age of 5 years when the transabdominal US examination failed to show Mullerian structures and magnetic resonance imaging (MRI) could not identify an uterus. AMH level at this time (87,3 pmol/L) was above the female reference range (2–32 pmol/L) and below the male reference range (400–1300 pmol/L), this suggested clinical diagnosis of partial gonadal dysgenesis (GD), a form of 46,XY DSD, consistent with the results of the earlier histology examination of the gonads. At 5,5 years of age, laparoscopy and a urethra-cysto-vaginoscopy was done and gonadectomy was performed to clarify the diagnosis and prevent tumorous transformation of the dysgenic testes. A blinded end vagina was identified, but uterus could not be found. Histological examination showed testicular parenchyma, epididymis and a small part of ductus deferens with a piece of tissue resembling the fallopian tube in the right side gonad, while in the left side gonad testicular and epididymidis tissues were found. The seminiferous tubules showed only Sertoli cells without spermatogonia on both sides.

4.2. Methods

4.2.1. Cytogenetic analysis

Chromosome analysis was performed from peripheral blood anticoagulated with Na-heparin. Phytohemagglutinin stimulated test samples were cultured for 72 hours, chromosome preparation, and banding by Giemsa staining (Merck, Darmstadt, Germany) were performed using the standard protocol. Fifteen metaphase cells per patient were analyzed using LUCIA karyotyping software (LUCIA Cytogenetics, Czech Republic). The karyotype was described according to the current international nomenclature (ISCN).

4.2.2. Fluorescent in situ hybridization

The cell suspension obtained from chromosome preparation was used for FISH assays. The following FISH probes were used in samples from patients with CHD: DiGeorge/VCFS TUPLE1, Subtelomer (4p/4q, 6p/6q), Cri-du-chat, and SOTOS (Cytocell, Rainbow Scientific Inc., Windsor, CT), 24X-Cyte (MetaSystems, Altlussheim, Germany). For the patient with DSD, a probe specific for the *SRY* gene and the centromeric region of the X chromosome (Cytocell, Rainbow Scientific Inc., Windsor, CT) was applied. In all cases, the test was performed according to the protocol provided by the manufacturer. Signal patterns were evaluated using a Zeiss Axioplan 2 fluorescence microscope (Carl Zeiss, Jena, Germany) and ISIS software (MetaSystems, Altlussheim, Germany).

4.2.3. DNA isolation

Genomic DNA was isolated from peripheral blood samples of all patients and from ventricular tissues of abortions using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany).

4.2.4. Multiplex ligation-dependent probe amplification

MLPA was performed using SALSA MLPA P250-B2 DiGeorge and SALSA MLPA P311-B1 CHD probemixes (MRC-Holland, Amsterdam, The Netherlands) according to the manufacturer's protocol in CHDs. The P250-B2 DiGeorge kit includes 48 probes: 29 probes are located in the 22q11.2 region and can be used to distinguish the most common types of deletion and 19 probes are present for relevant regions of DiGeorge syndrome (DGS), DGS type II (10p14) or disorders with phenotypic features of DGS on 22q13 and on chromosomes 4q35, 8p23, 9q34.3 and 17p13.3. The SALSA MLPA P311-B1 kit identifies CNVs in the

following genes and regions previously associated with CHD: *GATA4* (8p23), *TBX5* (12q24), *NKX2.5* (5q35), *BMP4* (14q22), *CRELD1* (3p25) and 22q11.2.

In a patient diagnosed with DSD, SALSA MLPA P185-C2 Intersex probemix was used containing probes for selected exons of the following genes: *NR0B1*/*DAX1*, *CXorf21*, *SOX9*, *SRY*, *ZFY*, *WNT4*, *NR5A1* (MRC-Holland, Amsterdam, The Netherlands).

Amplified products were separated by size on ABI-3130 (Applied Biosystems, Foster City, CA) and data were analyzed by Coffalyser software (MRC-Holland, Amsterdam, The Netherlands). A peak was considered abnormal when the ratio was <0,65 (deletion) or >1,30 (duplication) compared to the peaks of the reference probes.

4.2.5. Whole genome array comparative genome hybridization

Array CGH was performed using the CytoScan 750K Array (Affymetrix, Thermo Fisher Scientific, Waltham, MA). This type of cytogenetic microarray contains 750 436 oligonucleotide probes (550 000 unique, non-polymorphic CNV probes and 200 436 SNP probes). Within the genes covered by the cytogenetic microarray (ISCA, OMIM, RefSeq, X chromosome, and tumor genes), the average resolution was 1737 bp, and the total, intra-, and intergenic mean resolution was 4125 bp.

The raw data were analyzed by Chromosome Analysis Suite (ChAS) v2.0 software and CNVs were called and based on human assembly GRCh37 (hg19). As a first step, we checked the parameters characterizing the quality of the samples (MAPD ≤ 0,25; SNPQC ≥ 15; Waviness-SD ≤ 0,12). We analyzed those CNV calls that involved at least 10 probes for deletion and 20 for duplication. Identified CNVs were interpreted and classified (pathogenic, likely pathogenic, unknown of clinical significance, likely benign, benign) according to the standards and guidelines of the ACMG (American College of Medical Genetics and Genomics). For interpretation of the detected CNV calls we have used freely available websites and databases: DGV, UCSC Genome Browser, DECIPHER, ISCA, OMIM, PubMed. Additionally, the loss of heterozygosity (LOH) along the genome was investigated. Those regions that showed ≥10 Mb LOH were analyzed to verify the possible effect of imprinting.

4.2.6. Whole exome sequencing

Whole exome sequencing was performed using Complete Genomics platform by the Beijing Genomics Institute in Hongkong. The data were evaluated in the Laboratory of Clinical Genetics and Endocrinology of the Semmelweis University of Budapest, Institute of Laboratory Medicine by targeted mutation analysis of genes causing DSD (*LIM1*, *CBX*, *SOX9*, *DHH*, *WT-1*, *SRY*, *AR*, *NR5A1*, *CYP17*, *HSD17B3*, *DMRT1*, *DAX*, *AMH*, *WNT4*, *RSPO1*, *CTNNB1*, *MAP3K1*, *NR0B1*, *AMHR2*, *LHCGR*, *FGF9*) (Genome Analysis Toolkit, GATK software).

4.2.7. Confirmation of CNV detected in the NR5A1 gene by QMPSF method

Deletion of exons 5 and 6 of the *NR5A1* gene was confirmed by QMPSF (Quantitative Multiplex PCR of Short Fluorescent Fragments). For the method, primers designed for the *NR5A1* gene and 2 control genes (*HSD17B3, USH2A*) (0,2 μmol/L) and a primer labeled with a universal 5' fluorescent dye (6FAM-M13: 6FAM-5'-TGTAAAACGACGGCCAGT-3', 0,8 μmol/L) and MyTaq HS Mix (Bioline, London, UK) was used according to a previously reported protocol. Fragments of different lengths were separated on an ABI-310 (Applied Biosystems, Foster City, CA) sequencer. To determine the CNV, the fluorescence intensity of the examined exons was first compared to the fluorescence intensity of the amplified exons of the control genes (intra-sample normalization), and then normalization between samples was performed taking into account simultaneously the fluorescence intensity of the same exons of healthy control samples run in parallel.

5. RESULTS

5.1. Groups of CHD patients

5.1.1. Patients with syndromic CHD

In the syndromic cohort the CHD (n=33) phenotypes were diverse, ranging from simple to complex: ASD and/or VSD (n=12), TOF (n=8), FoA (n=3), PDA (n=1), PS (n=1), UH (n=1), totalis situs inversus (n=1), other complex (n=6) CHDs.

In all of the cases diagnosed with syndromic CHD, genetic tests were performed. As part of a routine diagnostic examination, conventional chromosome analysis was performed in 32 patients, confirming normal karyotype in 30/33 cases (94%). Numerical and structural chromosome aberrations were detected in the sample of two patients (Case No 1. and 2.). DGS-specific FISH analysis was performed in 10/33 cases, deletion of the 22q11.2 region was not

confirmed in any of the samples. A cytogenetic microarray study was carried out on a sample of 25/33 patients, 21 of whom had no detectable pathogenic CNV. A total of four patients presented pathogenic CNVs, thus the detection rate of aCGH was 16% (4/25). In the remaining 8 (8/33) cases only MLPA was performed, in 3 out of 8 samples (Case No 5-7.) clinically significant CNV was identified.

5.1.1.1. Clinical characteristics and genetic results of Case No 1.

Based on anamnestic data, the prenatal ultrasound examination did not show morphological abnormalities in the fetus, and the result of the prenatal genetic test for fetal chromosome aberrations (NIPT) from maternal blood was also negative. ASD and PDA were diagnosed in the newborn after delivery. At the age of two weeks, he was investigated by a clinical geneticist who described multiple developmental anomalies: low-set ears, hypertelorism, hypotonia, small mouth, laryngeal hypoplasia, microcephaly, retrognathia, syndactyly II-III, spasticity of the extremities. Because of the cat-like cry, Cri-du-chat syndrome, also known as 5p deletion syndrome was assumed by the clinical geneticist. Conventional chromosome analysis revealed an abnormal karyotype with a ring chromosome derived from chromosome 5 and a small supernumerary marker chromosome (sSMC): 47,XY,r(5),+sSMC. Application of locus specific FISH probe confirmed the deletion of the 5p15.2 region on the ring chromosome and multicolor FISH classified the sSMC as a derivative chromosome 15. Cytogenetic microarray analysis detected a 34,58 Mb deletion of 5p15.33p13.2 region as a sole imbalance. The deleted segment included 65 OMIM genes. No CNV was detected on chromosome 15 indicating the heterochromatic origin of the sSMC.

5.1.1.2. Clinical characteristics and genetic results of Case No 2.

Case No 2. presented with tetralogy of Fallot, solitary kidney, hypothyreosis and somatic retardation.

Cytogenetic analysis identified an interstitial deletion of the long arm of chromosome 6 with presumed breakpoints at 6q23 and 6q25. Array CGH clarified the size (18,753 kb) with 49 OMIM genes and the exact breakpoints (6q21q23) of the genomic imbalance, which also contained CHD-predisposing genes (*GJA1*, *HDAC2*, *MARCKS*).

5.1.1.3. Clinical characteristics and genetic results of Case No 3.

Case No 3. presented with ASD, situs inversus totalis, facial dysmorphism, brachydactyly, narrow chest, macrocephaly, short and wide neck, syndactyly III-IV, somatic retardation. Her karyotype was normal. Partial deletion and duplication due to unbalanced translocation could be identified by aCGH: a 12,15 Mb deletion of 4q34.3q35.2 region and a 16,95 Mb duplication of 6q25.1q27 region. The CNVs were confirmed by FISH using 4q and 6q subtelomeric region specific FISH probes. Within this chromosomal region the OMIM genes are responsible for CHD formation are the followings: *PDLIM3*, *SLC25A4*, *SORBS2*. Our patient's karyotype was initially interpreted as normal, however, after aCGH results the G-banded metaphases were reanalyzed and the karyotype was revised. Genetic evaluation of parental sample revealed that her mother is carrier of the balanced form of the child's translocation. The patient's final karyotype was thereafter: 46,XX,der(4)t(4;6)(q34.3;q25.1)mat. arr[hg19]4q34.3q35.2(178,807,365-190,957,460)x1, 6q25.1q27(150,485,038-167,439,226)x3. Genetic counseling was provided on recurrence risk and prenatal genetic testing was recommended in the subsequent pregnancies.

5.1.1.4. Clinical characteristics and genetic results of Case No 4.

Case No 4. presented with CoA, plagiocephaly, flat occiput, facial dysmorphism (epicanthus, upslanted palpebral fissures, long philtrum, low nasal bridge, low-set ears, retrognathia) and hypospadiasis. His karyotype and DiGeorge FISH were normal. Array CGH has shown a 3,842 kb duplication at 8p23.1 region. The CNV was confirmed by MLPA. The duplicate region contains 17 OMIM gene (*CLDN23*, *MFHAS1*, *ERI1*, *PPP1R3B*, *TNKS*, *MIR124-1*, *MSRA*, *PRSS55*, *RP1L1*, *SOX7*, *PINX1*, *MTMR9*, *SLC35G5*, *FAM167A*, *BLK*, *GATA4*, *NEIL2*), including genes responsible for CHD formation (e.g. *SOX7*, *GATA4*).

5.1.1.5. Clinical characteristics and genetic results of Case No 5.

His sister (Case No 5.) was born at 32. weeks of gestation with birth weight of 1900 g (50th percentile) and FoA. Her clinical features included prominent forehead, hypotonia, epicanthic fold, broad nasal root and motor delay.

In her case only MLPA was used to screen CHD associated CNVs. Similarity to her brother, 8p23.1 duplication was detected with the involvement of three genes (*GATA4*, *MSRA*, *PPP1R3B*) and confirmed the familiar origin of the CNV.

5.1.1.6. Clinical characteristics and genetic results of Case No 6.

Case No 6. was initially diagnosed with isolated tetralogy of Fallot, therefore MLPA DiGeorge kit was applied as a first-tier test. MLPA identified the deletion of all consecutive probes that represent the 22q11.2 region. The deletion affected 14 genes (*CLTCL1*, *HIRA*, *CDC45*, *CLDN5*, *GP1BB*, *TBX1*, *TXNRD2*, *DGCR8*, *ZNF74*, *KLHL22*, *MED15*, *SNAP29*, *LZTR1*) and corresponded to the most frequently deleted region in DiGeorge syndrome that can be characterized by LCR22-A and LCR22-D (low copy repeat 22-A and -D) breakpoints. The result clearly supported the diagnosis of the syndrome. Parental samples were not tested because they were not available.

5.1.1.7. Clinical characteristics and genetic results of Case No 7.

Case No 7. was referred to the clinical geneticist based on her tetralogy of Fallot and hypoplastic thymus. The result of the cytogenetic study showed a normal female karyotype. MLPA analysis revealed heterozygous deletion of 14 genes in the 22q11.21 region between the LCR22-A and LCR22-D breakpoints, as in case No 6. The result of MLPA analysis of parental samples was negative confirming the *de novo* origin of the CNV.

5.1.2. Diagnostic algorithm for syndromic CHD

Based on our study results, we worked-out a diagnostic algorithm to elucidate the pathogenetic background of CNVs in patients with syndromic CHD. The starting and most important element of the diagnostic pathway is the detailed phenotyping performed by the clinical geneticist that determines basically the order of the tests. If symptoms suggest numerical or structural chromosome aberration, conventional chromosome analysis or cytogenetic microarray test is performed, depending on the availability of the method. Targeted FISH or MLPA testing is recommended for the two most common CHD-associated microdeletion syndromes, DiGeorge and Williams or in other, less common microdeletion or microduplication syndromes. In case of the suspicion of a syndrome of unknown genetic background, cytogenetic microarray is the first applied method. If cytogenetic analysis, targeted FISH, and MLPA tests are negative, the aCGH method is recommended to confirm or exclude pathogenic CNVs.

5.1.3. Patients with isolated CHD

The isolated cases (n=16) had mostly severe, complex CHDs: TOF (n=12), AVSD (n=1), UH (n=1), HLH (n=1), AVSD + TGA (n=1).

Because the extracardiac symptoms associated with these cases were not included in the patients' anamnestic data, a CHD-specific MLPA was used to detect CNVs in known CHD genes. There were no CNVs classified as clinically significant in patients with apparently isolated CHD.

5.1.4. Samples from abortions

The CHD phenotypes of the fetuses were very heterogeneous. Simple VSD was determined in only two cases. In 13/18 fetuses the septal defect was associated with other structural malformations of the heart. TOF in two fetuses and a single ventricle in one fetus were described.

All 18 cases were successfully analyzed using two MLPA probemixes. Seventeen samples did not show pathological CNVs in the examined genes and regions. In one sample (Case No 12.) we identified a duplication involving four consecutive probes, specific for exon 1, intron 1, exon 3 and 4 of the *BMP4* gene in 14q22.2 chromosome region. The limited quantity and quality of DNA samples did not allow further aCGH testing.

5.2. Patient with DSD

Routine cytogenetic studies revealed *SRY*-positive 46,XY karyotype. Targeted gene panel analysis showed no pathogenic variants in DSD related genes. After excluding nucleotide sequence mutations, aCGH was performed to identify copy number alterations. Pathogenic CNV was not detected by the aCGH. Assuming that intragenic CNVs may be responsible for GD, the most commonly affected DSD genes were screened by MLPA to detect changes in the intragenic copy number of known sexual differentiation-determining genes (*NR0B1*/*DAX1*, *SOX9*, *SRY*, *WNT4*, *NR5A1*). A heterozygous deletion encompassing exons 5 and 6 of the *NR5A1* gene was identified. Probes targeting all other exons of the *NR5A1* gene showed normal copy number. QMPSF assay confirmed the two-exon deletion. This small intragenic deletion could not be detected by the formerly used aCGH as exons 5 and 6 were not covered by the CNV probes. The two CNV probes that showed normal copy numbers proximal and distal to the deletion were located in introns 4 and 6 suggesting intronic breakpoints of the deletion. Family studies confirmed the *de novo* origin of the mutation that was undetectable in the genomic DNA of the parents. Diagnosis of 46,XY DSD with partial GD was indicated.

6. DISCUSSION

6.1. Groups of CHD patients, genotype-phenotype correlations

6.1.1. Patients with syndromic CHD

Chromosomal screening for pathogenic CNV using MLPA and cytogenetic microarray detected imbalances in 21% (7/33) of syndromic CHD patients. Our detection rate is consistent with the reported diagnostic yield. Clinical and research-based testing suggests that pathogenic CNVs contribute to about 3–25% of CHD.

Using aCGH, the size of the detected pathogenic CNVs varied between 3,8 and 34,58 Mb. It is a well-known that CNVs responsible for the development of congenital diseases are very heterogeneous in terms of their size and thus their gene content, and the clinical picture they cause is therefore very variable. In the case of CNVs confirmed in the background of congenital heart defects, the incomplete penetrance of the CHD phenotype further complicates the determination of the genotype-phenotype relationship.

In the first syndromic patient, aCGH clarified and refined the results of cytogenetics, FISH, and multicolor FISH tests performed during diagnostic testing and confirmed the diagnosis of Cri-du-chat syndrome (MIM # 123450), which is one of the most common microdeletion syndromes, with an incidence of 1:20 000 to 1:50 000. Ring chromosome 5 is a rare cytogenetic presentation of Cri-du-chat syndrome, only a few cases have been reported. The syndrome is *de novo* in 85% of cases, and the size of deletions affecting the short arm 5 varies between 560 kb and 40 Mb. The heterogeneous phenotype of patients is clearly explained by the difference of the size of the deletions. According to a review on genotype-phenotype correlation, the most characteristic symptoms may be related to the loss of the following chromosomal regions: cat-like crying 5p15.31, speech delay 5p15.32–15.33, facial dysmorphism 5p15.2–15.31. Mental retardation is more severe the more proximal regions (5p14.1–14.2) involved in the deletion. In case of our patient severe mental retardation is expected at a later age based on the identified breakpoints of the deletion. Approximately 15–20% of affected infants have CHD, the most common heart defect is PDA as it was in our patient. The genes responsible for CHD in this region have not yet been identified. Regarding the sSMC, that originated from chromosome 15 according to multicolor FISH, the cytogenetic microarray analysis did not identify any coding region of it indicating no phenotypic consequences of its presence.

In patient No 2. cytogenetic microarray revealed that the genomic loss of the long arm of chromosome 6 is a large, 18,753 kb interstitial deletion with 49 OMIM genes and breakpoints at 6q21 and 6q23.2. This chromosome aberration encompass the 6q21–q22 region that is responsible for a rare, unique „6q21–22 deletion syndrome" associated with variable congenital anomalies, facial dysmorphism, somatic and mental retardation. Three different groups of chromosomal abnormalities have been described as pathogenetic factor: „A" or proximal (6q11–q16); „B" or middle (6q15–q25); „C" or terminal (6q25–qter). Our identified CNV belongs to the „B" deletion group. According to literature data examining genotype-phenotype relationship of interstitial 6q deletion, a common deletion segment of 113–114,5 kb can be detected in cases with CHD, that contains genes critical for cardiac morphogenesis (*GJA1*, *HDAC2*, *MARCKS*). In our case the detected deletion also includes these genes and explains the patient's cardiac phenotype.

In case No 3. aCGH revealed partial deletion of 4q34.3q35 and duplication of 6q25.1q27 regions due to unbalanced translocation. Terminal deletion of chromosome 4q is a rare event, about 10–20% are the unbalanced product of a parental reciprocal translocation with concomitant partial duplication of the partner chromosomal segment. Terminal 4q deletion cases present a broad phenotypic range including intellectual disability, developmental delay, CHD, cleft palate, craniofacial dysmorphism and skeletal abnormalities. CHDs were mapped to two separate regions on 4q35, encompassing causative genes *TLL1*, *HPGD* and *HAND2*, are involved in cardiac morphogenesis. In case of unbalanced translocation status, the patient has both partial deletion and partial duplication due to the mispairing of translocated chromosomes. In our case the duplication of the distal long arm of chromosome 6 (6q25–q27) can result in additional phenotypic abnormalities. The CHD candidate genes in this region are *DLL1*, *QKI*, *RPS6KA2*. Due to the carrier status of balanced translocation, genetic counseling was recommended to the parents and the possibility of prenatal genetic testing in a subsequent pregnancy.

In two siblings (Case No 4. and 5.) 8p23.1 duplication was detected. The 8p23.1 duplication syndrome is an ultra rare recurrent genomic condition, with an estimated prevalence of 1 in 58 000. Twenty-four, molecularly characterized cases have been reported until now, most of them being sporadic. The 8p23.1 duplication syndrome has a variable phenotype with three relatively common features of CHD, developmental delay, learning difficulties (>90%), behavioral disorders and a degree of mild dysmorphism. CHD is found in about 25% of cases. The core region contains 27 genes and 5 microRNAs of which *GATA4*, *TNKS1*, *SOX7*, and *XKR6* are dosage sensitive genes, their increased expression accounts for some symptoms of

the phenotype. *GATA4* in concert with *SOX7* might cause CHD, *TNKS* is responsible for behavioral disorders, while *SOX7* is responsible for developmental delay. The duplicated region in the patient (3842 bp) covers the previously defined critical interval, so are the clinical features overlapping: assymetry of the face and skull, epicanthic fold, deep nasal root, long philtrum, retrognathism, low-set ears.

Case No 5. is the sister of the patient No 4. She was referred to clinical geneticist at the age of 22 months because of positive family history, CHD and mild dysmorphic features. Using targeted MLPA analysis the duplication of three genes (*GATA4*, *MSRA*, *PPP1R3B*) at the 8p23.1 region was detected. These genes are located in the telomeric, medial and centromeric part of the core critical region of the 8p23.1, presuming that the duplicated region is similar to that identified in her brother. The lack of apparent additional phenotypic consequences of the duplication may be attributed to the variable penetrance and expressivity of this syndrome.

DiGeorge syndrome (MIM # 188400) was recognized in two patients (No 6., 7.). In the absence of characteristic clinical symptoms, such as immundeficiency, a correct diagnosis of the DiGeorge syndrome had not been made before the MLPA testing in case No 6. The patient was diagnosed with isolated CHD for 16 years. In case No 7. the thymus hypoplasia with TOF indicated the diagnosis of DGS at the age of 1 month. 22q11.2 deletion caused DGS is the most common microdeletion syndrome, with prevalence of 1:3000 – 6000 live births. Many patients have CHD, thymic hypoplasia, developmental delay, learning difficulties, hypoparathyroidism, facial dysmorphism, skeletal defects, renal and eye anomalies, psychiatric disorders in adulthood. The 22q11.2 chromosome region has several blocks of low copy repeats (LCRs) leading to NAHR with both deletions and duplications. There are different explanations for the very variable phenotype. The variable expressivity of DiGeorge syndrome may explain the misdiagnosis of our patient No 7. Delineating syndromic from non-syndromic CHD can be problematic especially when most of the symptoms may not manifest, therefore genetic testing apparently isolated CHD patients with aCGH or MLPA can be clinically very useful.

6.1.2. Patients with isolated CHD

The cohort with isolated CHD consisted mainly of patients with the following complex CHDs: TOF (n=12), UH (n=1), HLH (n=1), AVSD (n=1), AVSD + TGA (n=1). In their case, we performed targeted MLPA assays for known CHD genes (*GATA4*, *TBX5*, *NKX2.5*, *BMP4*, *CRELD1*) and chromosome regions (4q35, 8p23, 9q34.3, 10p14, 17p13.3, 22q11.2, 22q13) to detect CNVs. Application of MLPA probemixes did not reveal pathogenic deletions or duplications. This result might be due to the low number of analyzed cases, the generally low

frequency of genomic imbalances in isolated cases and targeted analysis of the CHD associated genes.

6.1.3. Samples from abortions

Chromosomal aneuploidies and DiGeorge syndrome can be detected in 18–22% of the prenatally recognized CHDs, other genetic abnormalities (CNV, point mutations) can be assumed in the remaining significant portion of fetuses. CHD-specific MLPA can be used to screen for known CHD-associated microdeletion/microduplication syndromes, but using aCGH allows the detection of other rare CNVs also. Therefore, cytogenetic microarray is now recommended as a first-line genetic test in case of fetal abnormalities. In our studies, we did not identify a deletion or duplication in DGS (22q11.2) and other CHD-specific chromosome regions (4q35, 8p23, 9q34.3, 10p14, 17p13.3, 22q13) in samples of fetuses (n=18) with CHDs. CNV screening of genes with pathogenic roles in CHDs (*GATA4*, *TBX5*, *NKX2.5*, *BMP4*, *CRELD1*) in one sample (Case No 12.) revealed duplication of the *BMP4* gene localized to the 14q22.2 chromosome region. In this case, VSD and CoA were diagnosed by ultrasound. In animal models, the BMP4 protein has been shown to play an important role in the formation of cardiogenesis, septa and conduction system. A loss-of-function mutation in the gene results in severe CHDs. There are no literature data on the functional consequences of gene duplication on cardiac development.

Based on our results, the cytogenetic microarray proved to be an effective method for detection of pathogenic CNVs in syndromic CHD. Identification of mutations underlying the CHD allows the accurate description of genotype-phenotype relationship, prognostic classification of patients and provides early, personalized, preventive and therapeutic care of the affected individuals. Knowledge of genetic abnormalities helps the clinical geneticist in the early recognition of associated symptoms, in estimating the risk of recurrence and screening first-degree relatives. When the affected individual reaches fertile age, having the genetic diagnosis makes the prenatal genetic testing possible for them.

6.2. Patient with DSD

We report a case study of a patient with female external genitalia, clitoromegaly, and mild dysmorphic features. Chromosome analysis revealed *SRY*-positive 46,XY DSD. Subsequent imaging studies revealed an absent uterus, blind-ending vagina and a bilateral inguinal hernia. Histological examination of the latter described dysgenetic gonads with testis, epididymis, tuba

uterina and ductus deferens details. Clarification of the genetic background started with targeted DSD gene panel analysis and whole genome aCGH, both of these tests detected no pathogenic variants. MLPA analysis of DSD related genes (*DAX1*/*NR0B1*, *CXorf21*, *SOX9*, *SRY*, *ZFY*, *WNT4*, *NR5A1*) identified a new partial heterozygous deletion within the *NR5A1* gene including exon 5 and 6. The genetic alteration was confirmed by QMPSF method. To our knowledge, the patient is the first reported case carrying mutation affecting exons 5 and 6 of the *NR5A1* gene.

The NR5A1 protein is a member of a nuclear receptor superfamily. The ligand-binding domain is composed of exon 4 (partial), exon 5, 6 and 7. In our case, the partial deletion affecting exons 5 and 6 results in a truncated *NR5A1* gene. The mutation is out-of frame leading to a stop codon right after the coding part of exon 4, thus either no functional NR5A1 is synthesized from this allele or because of nonsense-mediated mRNA degradation no protein will be synthetized at all. The other allele did not appear affected as exome sequencing did not reveal any sequence mutation in this allele. Based on these results haploinsufficiency of *NR5A1* can cause abnormal sexual development as observed in the proband.

46,XY DSD may include complete or partial GD due to disturbances in testis differentiation or undermasculinization/undervirilization as a result of aberrant androgen synthesis or action. From the genes underlying 46,XY DSD, *NR5A1* was the most studied. The NR5A1 protein is a transcription factor necessary for the expression of key genes involved in male sex differentiation (e.g. *SRY*, *SOX9*) that along with the product of the *WT1* gene regulates expression of AMH by Sertoli cells leading to regression of the Mullerian structures. In testicular Leydig cells, it stimulates the expression of enzymes required for testosterone biosynthesis that are essential for Wolffian duct differentiation and formation of internal and external male genitalias.

In humans, loss-of-function mutations of the *NR5A1* gene are associated with highly variable clinical conditions including male factor infertility, hypospadias, undescended testes, bilateral anorchia, primary ovarian insufficiency (in 46,XX female), GD and in rare cases adrenal insufficiency. To date, more than 40 *NR5A1* heterozygous mutations have been described in 46,XY DSD. In contrast to nucleotide sequence mutations, CNVs are extremely rarely detected in 46,XY DSD individuals. Only a few cases with CNVs involving the *NR5A1* gene have been published. The reported CNVs are heterogeneous in regards to their sizes and clinical phenotypes.

In summary, a new partial deletion including exons 5 and 6 of the *NR5A1* gene was identified by MLPA in a female patient with 46,XY partial GD that represents a novel genetic cause of 46,XY DSD. Our results also emphasize the importance of MLPA suitable for the

detection of small size CNV and intragenic deletions/duplications that can improve the diagnostic yield in routine practice. Molecular diagnosis is highly beneficial for DSD patients as it can help the assessment of adrenal and gonadal functions, determination the risk of malignancy of the gonads, improve the accuracy of genetic counseling and personalized management of the patients. CNVs are extremely rarely detected in individuals with gonadal dysgenesis, investigation of these type of mutations is not part of the genetic diagnostic procedure. Based on our data targeted CNV analysis of DSD related genes including *NR5A1* by MLPA in routine genetic screening of patients with 46,XY DSD with unknown etiology should be considered.

7. SUMMARY

In my scientific work, we investigated the occurrence of pathogenic CNVs in CHD and in a patient diagnosed with DSD using aCGH and MLPA methods.

In the first study, we examined a CHD patient population divided into three subgroups.

In the group of patients with syndromic CHD (n=33), we identified pathogenic CNV with 21% (7/33) diagnostic efficiency, which corresponds to data reported in the literature. Determining the genotype-phenotype relationship, we were able to confirm the pathogenetic role of CNV in all positive cases. Three patients had known microdeletion syndrome (2 DiGeorge, 1 Cri-du-chat), two siblings had an ultra-rare familial 8p23.1 duplication syndrome, one patient had a very rare 6q21–q22 deletion syndrome, and one patient had a previously not reported unbalanced translocation. Based on our results, we worked-out a diagnostic algorithm for the effective detection of CNVs in syndromic CHDs.

In the isolated CHD group (n=16), patients had severe, complex CHDs without extracardiac symptoms. No pathogenic CNV with an etiological role was confirmed by CHD-specific MLPA studies. Possible explanation of our results can be the low number of investigated samples or targeted nature of the study.

In ventricular tissue samples from abortions diagnosed with CHD (n=18) were examined by MLPA method. In this cohort we detected a CNV in one case, a duplication of the *BMP4* gene localized to the 14q22.2 chromosome region. The pathogenetic significance of the increased gene copy number involved in cardiogenesis has not been demonstrated.

In the second study, we performed a genetic examination of a patient diagnosed with DSD of unknown etiology. Female external genitalia, missing uterus and GD have been reported in an *SRY* positive, 46,XY karyotype child raised as a girl. No mutation was detected in DSD-

specific genes by next-generation sequencing, and no pathogenic CNV was revealed by whole genome cytogenetic microarray analysis. Using DSD-specific MLPA method, we identified a novel mutation, a heterozygous deletion of exons 5 and 6 of the *NR5A1* gene, which resulted in the haploinsufficiency of the dosage sensitive *NR5A1* gene and explained the DSD of the patient. The mutation confirmed the diagnosis of 46,XY partial GD. Our results confirmed the pathogenetic role of intragenic CNVs in the development of DSDs and emphasize the clinical significance of their detection. The MLPA method has been shown to be suitable for screening for exonal copy number variants of genes associated with DSDs, and its application may improve the very low diagnostic efficiency of the disease.

8. NEW FINDINGS OF THE THESIS

1.) This is the first report studying pathogenic CNVs in a cohort of CHD patients in Hungary using aCGH and MLPA methods. We identified pathogenic CNVs with 21% diagnostic efficiency in syndromic CHDs. In addition to the most common DiGeorge syndrome, we established a genetic diagnosis of such a rare (prevalence <1:2000) or ultra-rare (prevalence <1:50 000) CNV-induced syndromes as Cri-du-chat, 6q21–q22 deletion, or 8p23.1 duplication syndrome. In one case, a previously has not been described familial unbalanced translocation was identified. Since all diagnosed microdeletion/microduplication syndrome show autosomal dominant inheritance patients at fertile age having the genetic diagnosis are allowed for prenatal genetic testing to prevent recurrence of the disease.

2.) We found that aGGH and MLPA techniques are effective diagnostic methods in identifying CNVs underlying syndromic CHDs. Based on our results, we worked-out a diagnostic algorithm suitable for efficient and cost-effective application of genetic tests in the detection of CNVs.

3.) Based on our results in isolated CHD cases and fetal samples diagnosed prenatally with CHD, we concluded that targeted MLPA assays are not sufficient to identify pathogenic CNVs. Genome-wide CNV screening can reveal CNVs of pathogenetic significance with greater efficiency using cytogenetic microarray technique.

4.) In a patient diagnosed with DSD without genetic background, we identified a new, previously unreported mutation in the *NR5A1* gene by MLPA method. Heterozygous deletion of exons 5 and 6 of this gene is a very rare type of mutation causing DSD. Based on our results, we drew attention to study of intragenic CNVs of genes involved in DSD.

We emphasized the importance of using MLPA method in identification of pathogenic CNVs, which can significantly improve the very low diagnostic efficiency of DSDs.

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List of other publications

3. Ujfalusi, A., **Nagy, O.**, Bessenyei, B., Lente, G., Kántor, I., Borbély, Á. J., Szakszon, K.: 22q13 microduplication syndrome in siblings with mild clinical phenotype: broadening the clinical and behavioral spectrum.
   Mol Syndromol. 11 (3), 146-152, 2020.
   DOI: https://doi.org/10.1159/000507103
   IF: 1.198 (2019)
4. **Nagy, O.**, Baráth, S., Ujfalusi, A.: The role of microRNAs in congenital heart disease.
   EJIFCC. 30 (2), 165-178, 2019.
5. Tóth, N. K., Székely, E. G., Czuriga-Kovács, K. R., Sarkady, F., **Nagy, O.**, Lánczi, L., Berényi, E., Fekete, K., Fekete, I., Csiba, L., Bagoly, Z.: Elevated factor VIII and von Willebrand factor levels predict unfavorable outcome in stroke patients treated with intravenous thrombolysis.
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