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Copy number variants detection by microarray and multiplex ligation-dependent probe amplification in congenital heart diseases

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ABSTRACT

Congenital heart diseases (CHDs) are the most common birth defects among life births, which could be presented as isolated or syndromic with other congenital malformations. The etiology of CHD largely unknown, genetic and environmental factors contribute to the disease. Recurrent copy number variants (CNVs) have been reported in the pathogenesis of CHD. The aim of this study was to evaluate the clinical utility of multiplex ligation-dependent probe amplification (MLPA) and microarray analyses on isolated and syndromic CHD cases and to explore the relationship between identified CNVs and CHD. Eighteen prenatal samples, 16 isolated and 33 syndromic patients with mild to severe CHD phenotype were tested. Prenatal and isolated CHD cases did not show pathogenic CNVs. Clinically significant CNVs were detected in 7/33 (21%) syndromic CHD patients: del 22q11.2 (n = 2), 8p23.1 duplication (n = 2), deletion 5p (n = 1), deletion 6q21q22 (n = 1), unbalanced translocation causing partial deletion of 4q34.3 and duplication of 6q25.1 (n = 1). These genomic imbalances contain genes that has been associated with human CHD before. The present study demonstrates that using microarray and MLPA analysis increase the detection rate of causal CNVs in individuals with syndromic CHD.

1. Introduction

Congenital heart diseases (CHDs) refers to structural anomalies of the heart and blood vessels that arise during cardiac embryogenesis and differ in morphology, physiology, functional and clinical outcomes. Current epidemiological data indicate that CHD is the most common birth defect, affecting 10–12/1000 liveborn infants and one of the major causes of the perinatal morbidity and mortality. The successful medical and surgical management of even severe CHD cases greatly increased the survival of affected individuals and contribute to a large extent to its increased prevalence among older children and adults (Hoffman et al., 2004; van der Linde et al., 2011).

Congenital heart defects represent a broad spectrum of malformations, including septal and valve defects, lesions affecting the outflow tract and ventricules. Simple defects have been defined as atrial septal defect (ASD), ventral septal defect (VSD), patent ductus arteriosus (PDA), pulmonary stenosis (PS), foramen ovale apertum (FoA), and in general they have good prognosis. The complex and more severe forms, that is Tetralogy of Fallot (TOF), univentricular heart, hypoplastic left heart (HLH) most frequently require multiple surgical correction procedures if at all possible, and have uncertain long-term outcomes. Patients can be classified as having apparently isolated CHD without identifiable congenital malformations in other organ system or syndromic CHD with extracardiac manifestations, such as dysmorphic features, growth retardation, developmental delay and major structural malformations (Fahed et al., 2013). As survival of patients with CHD has improved, there has been an increased need to reveal of its etiology, understanding patient-specific risk factors that influence outcomes and comorbidities. CHD pathogenesis is still largely unknown, it is widely accepted that genetic and non-genetic factors play an important role in its etiology. About 80% of CHDs are isolated cases with multi-

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Table 1
Summary of data of prenatal samples.

No.	Gestational age (weeks)	Sex	Prenatal ultrasound	Pathological findings, CHD phenotype
1.	21	M	Transposition of the great arteries, ventricular septal defect	Transposition of the great arteries, ventricular septal defect.
2.	22	F	CHD	Atrioventricular septal defect. Pulmonary artery stenosis. Dextroposition of the aorta. Single umbilical artery.
3.	23	F	CHD	Ventricular septal defect. Hydropericardium. Endomyocardial fibrosis. Pulmonary atresia.
4.	20 + 3	F	CHD	Ventricular septal defect. Coarctation of the aorta.
5.	20	M	Tetralogy of Fallot	Tetralogy of Fallot.
6.	19	M	Multiplex malformation	Spina bifida cystica. Ventricular septal defect. Urethral obstruction. Congenital hydronephrosis. Megaloureter. Omphalocele. Left-sided talipes equinovarus.
7.	22	M	CHD	Transposition of the aorta, ventricular septal defect, hypoplastic aortic arch.
8.	19	F	CHD	Ventricular septal defect, hypoplastic left heart, dilatation of right heart.
9.	21	M	Multiplex malformation	Univentricular heart. The aorta originating ventrally from a common ventricle with the pulmonary artery behind it.
10.	22 + 4	F	Complex CHD	Atresia of the ascending aorta, ventricular septal defect.
11.	19 + 4	M	Atrioventricular septal defect	Atrioventricular septal defect.
12.	20 + 3	F	CHD	Ventricular septal defect, coarctation of the aorta.
13.	20	M	Tetralogy of Fallot	Tetralogy of Fallot.
14.	21	M	CHD	Hypoplastic right heart. Hypoplastic pulmonary artery.
15.	21	F	CHD	Hypoplastic aorta, hypoplastic left heart.
16.	21	F	CHD	Hypoplastic aorta, hypoplastic left heart. Dextroposition of the heart.
17.	22	M	Multiplex malformation	Bilateral hydrothorax. Ascites bilateral. Dilated cardiomyopathy.
18.	19	M	Multiplex malformation	Congenital hygroma colli. Left-sided talipes equinovarus. Ventricular septal defect. Left-sided renal and ureteral agenesis.

Note: M-Male, F-Female, CHD-congenital heart defect

factorial inheritance and 20% has genetic origin. Over the past few decades our knowledge has expanded about the genetic factors that contribute to CHDs (Blue et al., 2012; Pierpont et al., 2007). Chromosomal aneuploidies as the first recognized genetic causes of CHD are continuously the major etiological factors that accounts for 8–19% of cases. CHD is observed in 35–50% of liveborns with trisomy 21, 60–80% of liveborns with trisomy 13 and trisomy 18, and 33% with monosomy X (Hartman et al., 2011). Submicroscopic copy number variations (CNVs) refer to structural aberrations containing deletions and duplications ranging in size between 1 kb to several megabases leading to altered dosage of contiguous genes and produce mainly syndromic CHD. Well-characterized CNV induced syndromes are the DiGeorge/Velocardiofacial syndrome (del 22q11), Williams syndrome (del 7q11) and 8p23.1 deletion syndrome. Recent studies of larger cohorts of patients described new, recurrent CNVs associated with CHD, including 1q21.1, 3p25.1, 16p13.11, 15q11.2 and 2p13.3 (Breckpot et al., 2010; Glessner et al., 2014; Goldmuntz et al., 2011). In addition to specific syndromes, an increased burden of CNVs was also identified in patients with isolated CHD. These studies estimate that 5–10% of sporadic, non-syndromic CHD cases are due to certain rare CNVs (Erdogan et al., 2008; Hanchard et al., 2017; Soemedi et al., 2012). Monogenic causes of CHD was initially investigated in multigeneration pedigrees using linkage analysis. Some of the genes first identified are cardiac transcription factors that include *NKX2.5, GATA4, TBX5, TBX1, MEF2, ZIC3* (Prendiville et al., 2014). In addition to these regulators, genes coding for proteins in Notch and RAS signaling pathway, chromatin remodeling, cilia function, sarcomere structure have been identified in the last decade. Genome-wide and high-throughput sequencing techniques have determined that 10% of CHD is caused by *de novo* coding variants, the proportion being even higher in syndromic forms, accounting for 20% of cases (Homsy et al., 2015; Zaidi and Brueckner, 2017). DNA microarray has been widely used in clinical practice for evaluation of causative genomic imbalances in mental retardation and major congenital abnormalities (Battaglia et al., 2013). It is proved to be a powerful technique for genetic investigation of CHD patients also. Application of array CGH allows genome-wide detection of submicroscopic CNVs involving loci and genes with known roles in cardiac development and may also identify candidate genes related to CHD (Shanshen et al., 2018). Multiplex ligation-dependent probe amplification (MLPA) is a multiplex PCR method, detecting small scale CNVs, partial deletions/duplications of specific genes. It is widely used for the validation of array CGH results and screening CNVs in known genes. Compared to microarray, MLPA has a low cost and advantage of easy to use. The inclusion of MLPA in clinical settings as a complementary or screening method can significantly increase the detection rate of CNVs (Monteiro et al., 2017; Sorensen et al., 2012).

The aim of this study was to use whole-genome array CGH and MLPA analysis to identify rare CNVs responsible for isolated and syndromic CHD and to evaluate the clinical utility of these techniques in prenatal and postnatal CHD cases. To our knowledge this is the first study for investigation of CNVs in CHD using array CGH and MLPA in Hungary.

2. Materials and methods

2.1. Patients

Two independent patient cohorts were obtained for this study. The first cohort consisted of 18 ventricular myocardium samples from aborted fetuses with heart defects. The samples were recruited from the First Department of Obstetrics and Gynecology, Semmelweis University, Budapest. In the prenatal study group, the average gestational age was 20.85 weeks. All of the samples were obtained from terminated pregnancies. The diagnosis was made by ultrasound examination and echocardiography. Each cardiac malformation was confirmed by fetal pathology. The phenotypic details of this cohort are summarized in Table 1. The second cohort contained 33 syndromic and 16 isolated CHD cases. The patients were referred mainly from the Clinical Genetic and Cardiology outpatient clinics at the Department of Pediatrics, University of Debrecen, Debrecen. The ages of patients tested ranged from 2 to 18 years in syndromic CHD patient group and from 10 months to 46 years in the isolated CHD group. Peripheral blood samples were taken from probands after obtaining informed consent from the af-

Table 2
Summary of clinical and genetic data of patients with syndromic CHD.

No.	Age/sex	CHD	Karyotype	Array CGH (size)	MLPA (genes)	CNV	Phenotype (OMIM)
1.	3 years/Male	ASD, PDA	47,XY,r(5),+sSMC	arr[hg19] 5p15.33p13.2(113,576-34,700,951)x1 (34.58 Mb)	not done	del	Cri du chat syndrome (MIM # 123450)
2.	9 months/Male	TOF	46,XY,del(6)(q23q2?5)	arr[hg19] 6q21q23.2(113,913,946-132,667,254)x1 (18.753 kb)	not done	del	6q21q22 deletion syndrome
3.	3.5 years/Female	ASD, situs inversus	46,XX Revised karyotype: 46,XX,der(4)t(4;6)(q34.3;q25.1)	arr[hg19] 4q34.3q35.2(178,807,365-190,957,460)x1 6q25.1q27(150,485,038-167,439,226)x3 (4q: 12.15 Mb) (6q: 16.95 Mb)	not done	del/ dup	4q deletion/ 6q duplication syndrome
4.	4.5 years/Male	CoA	46,XY	arr[hg19] 8p23.1(8,093,065-11,935,465)x3 (3.842 kb)	not done	dup	8p23.1 duplication syndrome
5.	22 months/Female	FoA	46,XX	not done	*PPP1R3B*,MSRA, GATA4 ,	dup	8p23.1 duplication syndrome
6.	16 years/Male	TOF	not done	not done	*CLTCL1, HIRA, CDC45, CLDN5, GP1BB, TBX1, TXNRD2, DGCR8, ZNF74, KLHL22, MED15, SNAP29, LZTR1*	del	DiGeorge syndrome (MIM # 188400)
7.	6 months/Female	TOF	46,XX	not done	*CLTCL1, HIRA, CDC45, CLDN5, GP1BB, TBX1, TXNRD2, DGCR8, ZNF74, KLHL22, MED15, SNAP29, LZTR1*	del	DiGeorge syndrome (MIM # 188400)

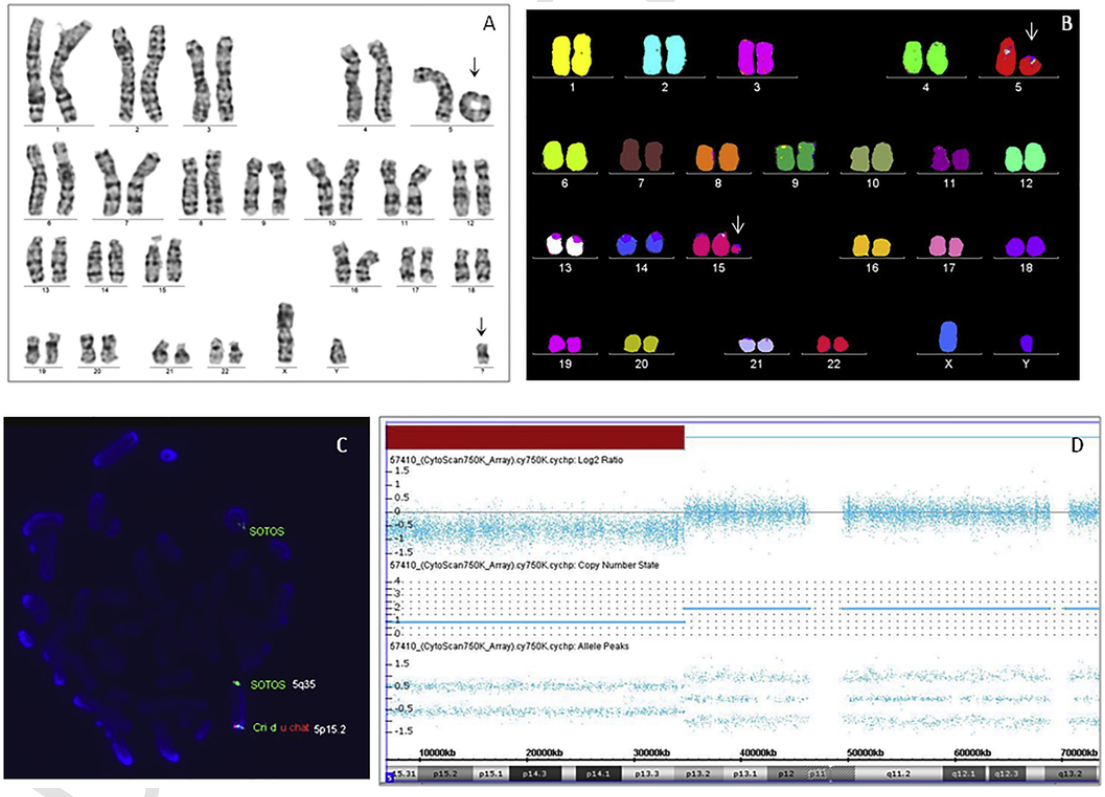

Fig. 1. Genetic results of case No 1. (A) G-banded karyogram of the patient ring chromosome and sSMC are indicated by arrows. (B) Multicolor FISH result showing the ring chromosome originated from chromosome 5 (red) and sSMC derived from chromosome 15 (purple) (C) Metaphase FISH result using locus specific probe combination for Cri du chat (5p15.2) (fusion signal) and SOTOS regions (5q35) (green signal). Cri du chat locus is missing from the ring chromosome 5 (D). Array CGH result of the proband showing deletion of 5p15.33p13.2 with coordinates 113,576-34,700,951. Signal intensity is plotted on a log2 scale, the deleted segment is shown as a red bar. B-allele frequences confirm the deletion (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

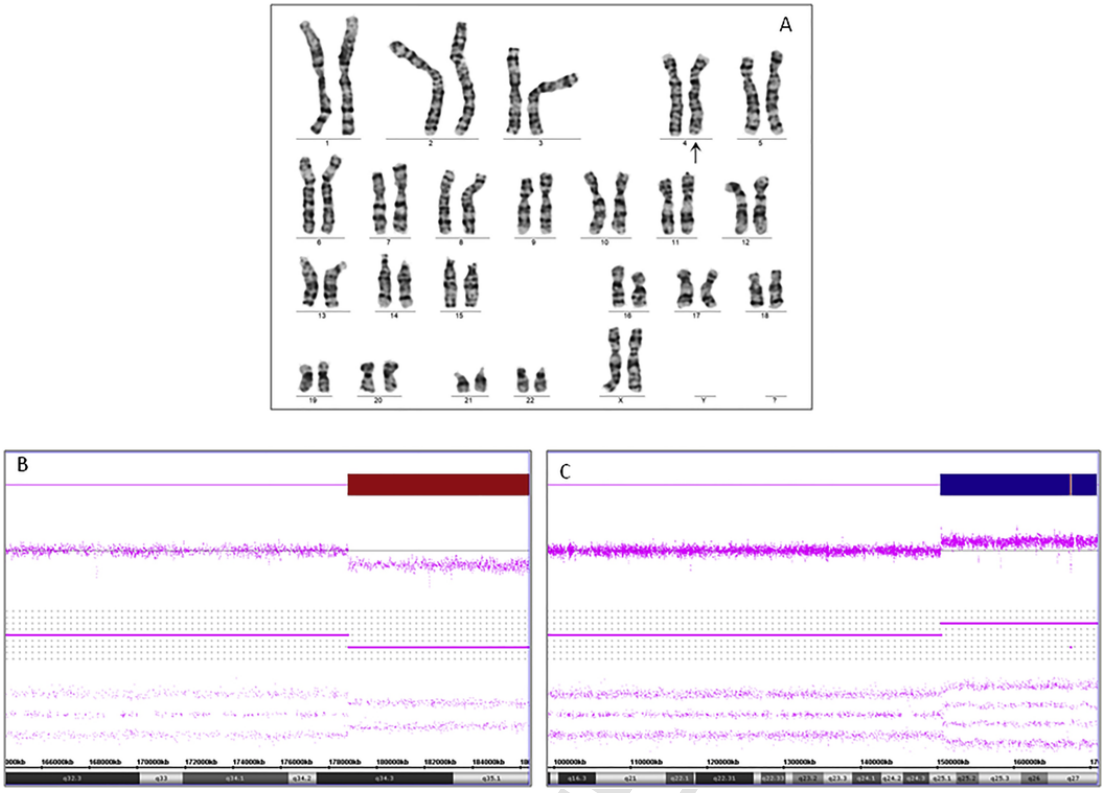

Fig. 2. Cytogenetic and array CGH result of case No 3. (A) G-banded karyogram of the proband. The derivative chromosome 4 is designated by an arrow. The patient's karyotype: 46,XX,der(4)t(4;6)(q34.3;q25.1). (B) Microarray data of the patient. A 12.15 Mb deletion at chromosome 4q34.3q35.2 (178,807,365-190,957,460). (C) A 16.95 Mb duplication at chromosome 6q25.1q27 (150,485,038-167,439,226). Signal intensity is plotted on a log2 scale, the deleted segment is shown as a red bar. B-allele frequences confirm the deletion (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

fected individuals or from their parents according to the national regulations.

2.2. 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, if normal, and there was a suspicion of a common microdeletion, we applied region-specific fluorescence in situ hybridization or MLPA analysis. In case of negative results, array CGH was performed after a careful evaluation and patient selection.

2.3. Cytogenetic analysis

Chromosome analysis was performed on G-banded metaphases prepared from cultured peripheral blood according to standard protocols. The karyotypes were described according to the International System for Human Cytogenetic Nomenclature (ISCN) 2016.

2.4. Fluorescence in situ hybridization (FISH)

FISH was done according to the manufacturer's recommendations using DiGeorge/VCFS TUPLE1 probe, Subtelomer (4p/4q, 6p/6q) specific probes, Cri-du-chat and SOTOS probe combination (Cytocell, Rainbow Scientific Inc., Windsor, CT). For multicolor FISH analysis 24X-Cyte probe was used (MetaSystems, Altlussheim, Germany). Metaphases were analyzed by Zeiss Axioplan 2 fluorescence microscope and Isis software was used for image capturing and processing (MetaSystems, Altlussheim, Germany).

2.5. 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). NanoDrop 2000/2000c UV–vis spectrophotometer (ThermoFisher Scientific, Waltham, Massachusetts, US) was used to determine the quantity and quality of DNA.

2.6. Multiplex ligation-dependent probe amplification (MLPA)

MLPA was performed using SALSA MLPA P250-B2 DiGeorge and SALSA MLPA P311-B1 Congenital Heart Disease probemixes (MRC-Holland, Amsterdam, The Netherlands) according to the manufacturer's protocol. 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 or disorders with phenotypic features of DGS on 22q13 and on chromosomes 4q35, 8p23, 9q34.3, 10p14 and 17p13.3. The P311-B1 kit identifies copy number variations in the following genes and regions previously associated with CHD: *GATA4* (8p23), *TBX5* (12q24), *NKX2.5* (5q35), *BMP4* (14q22), *CRELD1* (3p25) and 22q11.2.

Data were analyzed by Coffalyser Software from MRC-Holland (Amsterdam, The Netherlands). The results were considered abnormal when the ratio was <0.65 for loss or >1.30 for gain of genetic material.

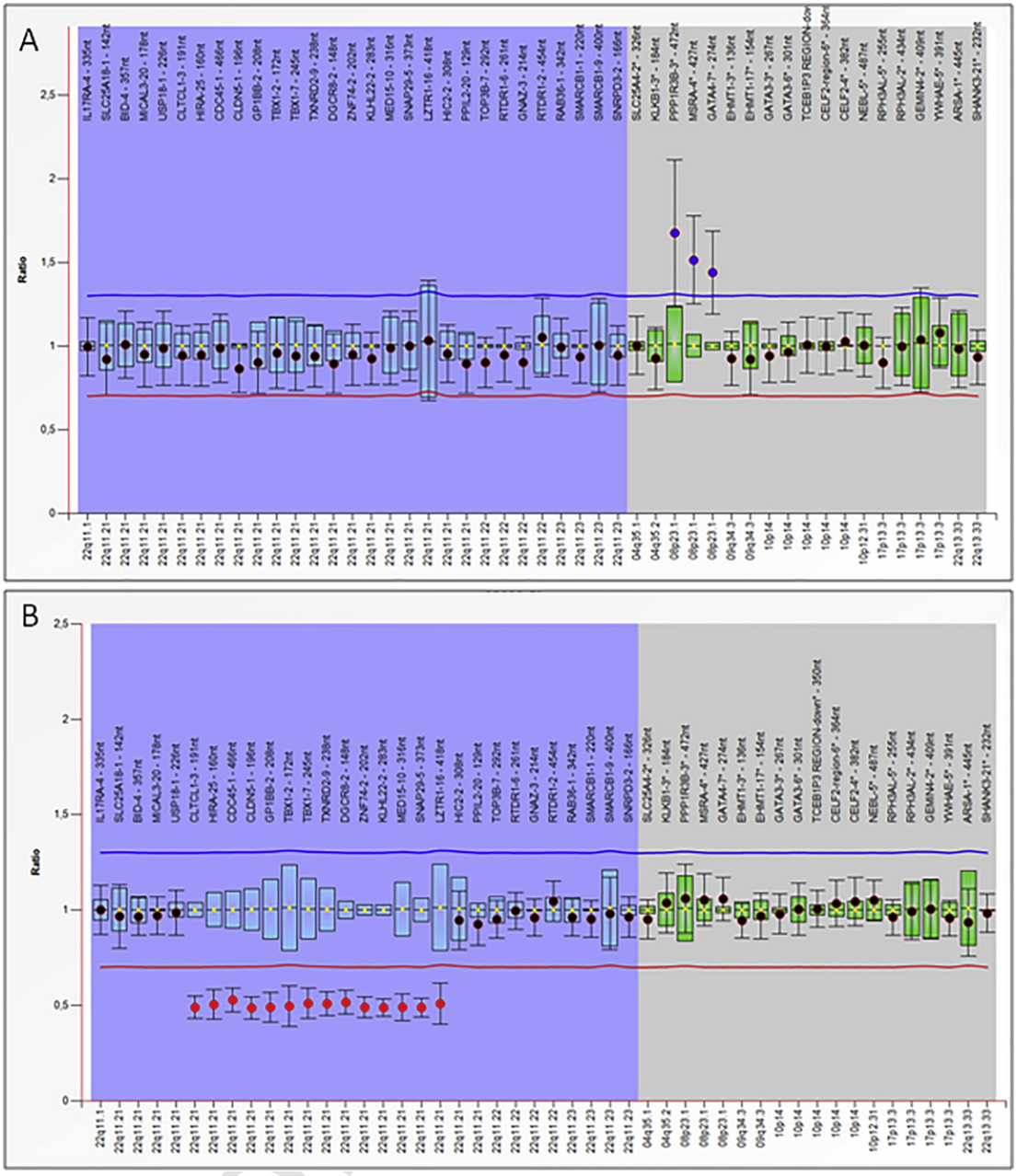

Fig. 3. MLPA results of case No 5. (A) and No 6. (B). Chromosomal locations are displayed on the x-axis and y-axis represent dosage quotient (DQ). Normal copy number is between 0.65 to 1.3. (A) Blue dots show a heterozygous duplication of three genes (*PPP1R3B, MSRA, GATA4*) at chromosome 8p23.1. (B) Red dots represent deletion of the entire 22q11.21 region (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

2.7. Whole genome array comparative genomic hybridization (CGH)

Array CGH was performed using the Affymetrix CytoScan 750 K Array. Genomic DNA samples were digested, ligated, amplified, fragmented, labeled, and hybridized to the CytoScan 750 K Array platform as specified by the manufacturer. The raw data were analyzed by ChAS v2.0 Software (Affymetrix, Thermo Fisher Scientific, Waltham, MA) and CNVs were called and based on human assembly GRCh37 (hg19). FISH and MLPA techniques were applied for validation of the array results.

2.8. Data analysis

We analyzed those CNV calls that involved at least 10 probes for deletion and 20 for duplication. For interpretation of the detected CNV calls we have used freely available websites and databases: Database of Genomic Variants (DGV), UCSC Genome Browser, Database of Chromosomal Imbalance, Phenotype of Humans using Ensemble Resources (DECIPHER), ISCA, PubMed, Online Mendelian Inheritance in Man (OMIM). Identified CNVs were interpreted and classified according to the standards and guidelines of the American College of Medical Genetics (ACMG) (Kearney et al., 2011). Additionally, the loss of heterozygosity (LOH) along the genome was investigated. Those regions that

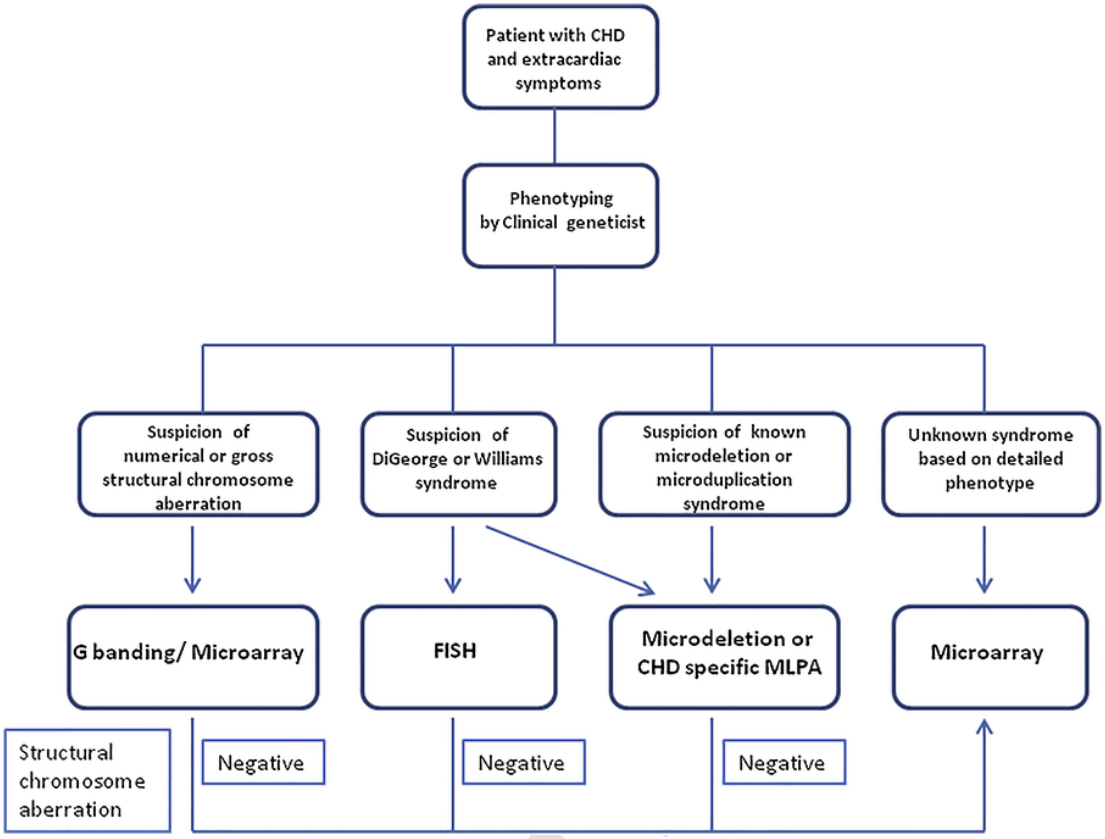

Fig. 4. Diagnostic pathway for determination of CNVs in syndromic congenital heart diseases.

showed ≥10 Mb LOH were analyzed to verify the possible effect of imprinting.

3. Results

3.1. Prenatal cases

The CHD phenotypes of the fetuses were very heterogeneous. Simple ventricular septal defect was determined in only two cases. In 13/18 fetuses the septal defect was associated with other structural malformations of the heart (transposition of great arteries, hypoplasia of the ventricles and/or great arteries, dilatation of ventricules). Two fetuses showed TOF, one case had univentricular heart (Table 1.). All 18 cases were successfully analyzed using P250-B2 and P311-B1 MLPA probemixes. Seventeen samples did not show pathological CNVs in the examined genes and regions. In one case we identified a duplication involving four consecutive probes, specific for exon 1, intron 1, exon 3 and 4 of the *BMP4* gene.

3.2. Syndromic CHD cases

In the syndromic cohort the CHD phenotypes were similarly diverse, ranging from simple to complex: ASD/VSD (n = 12), TOF (n = 8), FoA (n = 3), PDA (n = 1), PS (n = 1), univentricular heart (n = 1), situs inversus totalis (n = 1), other complex (n = 6) cardiac malformations. In this cohort the extracardiac symptoms described by clinical geneticist represented a broad spectrum of malformations associating with the CHD.

A total of 33 affected patients with syndromic CHD were referred for genetic studies. Conventional chromosome analysis was performed in 32/33 cases, 94 percent of analysed cases (30/33) showed normal karyotype, in two patients numerical and structural chromosome aberrations were identified (Case No 1. and 2.). DiGeorge region specific FISH was carried out on 10/33 samples, none of them showed deletion of the 22q11.2 region. Microarray was applied in 25/33 cases. A total of four patients presented pathogenic CNVs, thus the detection rate of array CGH was 16% (4/25). In the remaining 8 cases only MLPA was performed, in 3 out of 8 samples clinically significant CNV was identified. Summary of the patients data and results of genetic tests are shown in Table 2.

Case No 1. had ASD, PDA, microcephaly, hypertelorism, small mouth, retrognathia, low-set ears, laryngeal hypoplasia, syndactyly II-III, axial hypotonia and spasticity of the extremities. GTG-banding revealed a ring chromosome derived from chromosome 5 and a supernumerary small marker chromosome (sSMC). His karyotype was 47,XY,r(5),+sSMC (Fig. 1A). Because of the cat-like cry, Cri du chat syndrome, also known as 5p deletion syndrome was assumed by the clinical geneticist. 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 (Fig. 1B, C). Microarray analysis detected a 34.58 Mb deletion of 5p15.33p13.2 region. (Fig. 1D) 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.

Case No 2. presented with TOF, somatic retardation, hypothyreosis and solitary kidney. Cytogenetic analysis identified an interstitial deletion of the long arm of chromosome 6 with presumed breakpoints at 6q23 and 6q25. Microarray clarified the size (18.753 kb) and the exact breakpoints (6q21 and 6q23.2) of the genomic imbalance.

Case No 3. presented with ASD, situs inversus, macrocephaly, somatic retardation, facial dysmorphism, short and wide neck, brachydactyly, syndactyly III-IV, narrow chest. Her karyotype was normal. Partial deletion and duplication due to unbalanced translocation could be identified by array CGH: a 12.15 Mb deletion of 4q34.3 region con-

taining 21 OMIM genes and a 16.95 Mb duplication of 6q25.1 region involving 49 OMIM genes . The CNVs were confirmed by FISH using 4q and 6q subtelomeric region specific FISH probes. Our patient's karyotype was initially interpreted as normal, however, after microarray 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)[15]mat.
arr[hg19]4q34.3q35.2(178,807,365-190,957,460)x1,
6q25.1q27(150,485,038–167,439,226)x3 (Fig. 2). Genetic counseling was provided on recurrence risk and prenatal genetic testing was recommended in the subsequent pregnancies.

Case No 4. presented with coarctation of the aorta, plagiocephaly, flat occiput, facial dysmorphism: upslanted palpebral fissures, epicanthus, long philtrum, retrognathia, low-set ears, low nasal bridge, hypospadiasis. His karyotype and DiGeorge FISH were normal. Microarray has shown a 3.842 Mb duplication at 8p23.1 region that consisted of 17 OMIM genes. The CNV was confirmed by MLPA. His sister (Case No 5.) was born at 32. weeks of gestation with birth weight of 1900 g (50th percentile). Her clinical features included hypotonia, prominent forehead, broad nasal root, epicanthic fold, motor delay and FoA. In her case only MLPA DiGeorge probemix was used to screen CHD associated CNVs. Similarly to her brother, 8p23.1 duplication was detected (Fig. 3A).

Case No 6. was initially diagnosed with isolated TOF, 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 (Fig. 3B).

Case No 7. was referred to the clinical geneticist based on her congenital heart disease (TOF) and hypoplastic thymus. MLPA analysis using DiGeorge probemix showed heterozygous deletion of all consecutive probes at 22q11.2 region, confirming the diagnosis of DiGeorge syndrome. The result of MLPA analysis of parental samples was negative confirming the *de novo* origin of CNV.

3.3. Isolated CHD cases

The non-syndromic cases (n = 16) had mostly severe, complex CHD: TOF (n = 12), AVSD (n = 1), univentricular heart (n = 1), hypoplastic left heart (n = 1), AVSD + transposition of great arteries (n = 1). There were no CNVs classified as clinically significant in patients with apparently isolated CHD.

4. Discussion

The availability of chromosomal microarray testing as a widely used genetic test has increased the importance of the contribution of CNVs to CHD. Genomic imbalances not only underlie the structural CHD, but also are major contributors to CHD comorbidities such as arrythmia, heart failure, neurocognitive malfunction (Russell et al., 2018).

In the present study we performed genetic investigation of CHD cases to detect causal CNVs using MLPA and microarray techniques. We have analyzed prenatal cases with myocardium samples (n = 18) from foetuses with heterogeneous CHDs by MLPA. No obvious disease causing CNVs were identified in this cohort. In one sample MLPA assay has detected duplication of the entire *BMP4* gene. According to literature data only loss of function of *BMP4* is associated with CHD phenotype (Qian et al., 2014).

In the cohort of isolated CHD patients (n = 16) application of MLPA probemixes did not reveal pathogenic CNVs. This result might be due to the low number of analyzed cases, the generally low frequency (5–10%) of genomic imbalances in isolated cases and targeted analysis of the CHD associated genes (Erdogan et al., 2008; Soemedi et al., 2012). Genome wide investigation of CNVs by microarray is the next step in the genomic evaluation of our prenatal and isolated CHD patient cohort.

Chromosomal screening for deletions/duplications using MLPA and microarray detected imbalances in 21% (7/33) of syndromic CHD patients. Our detection rate is consistent with the reported diagnostic yield of microarray and MLPA in syndromic CHD. Clinical and research-based testing suggests that CNVs contribute to about 3–30% of CHD. Reports vary extensively in the type and sensitivity of the microarray platforms and MLPA kit used, definition of CNV calls and the selection of CHD patients included in the studies. Hightower et al. investigated 173 isolated CHD cases using whole-genome custom-designed oligonucleotide array with >44,000 probes and detected copy number changes in 50/173 (28.9%) patients (Hightower et al., 2015). Richards et al. recruited 20 children with syndromic CHD and compared them with 20 children with isolated CHD. They used high resolution whole genome array containing 385,000 oligonucleotide probes. They detected a 25% rate of CNVs in the population with syndromic CHD but none in the population with isolated CHD (Richards et al., 2008). Kim et al. investigated a prospective cohort of non-syndromic CHD patients (n = 422) and detected a higher burden of potentially pathogenic CNVs compared to pediatric controls (12.1% vs. 5.0%, P = 0.00016) (Kim et al., 2016). Referring to usage of MLPA for detection of CNVs Mutlu et al. screened 45 patients with cardiac septal defects for CNVs using the MLPA P-311 kit and identified three CNVs (n = 3/45, 6.66%) at 22q11.2 region causing DiGeorge syndrome (Mutlu et al., 2018). In an other study, *GATA4* and *GATA3* genes were investigated by SALSA P234-MLPA kit to explore pathogenic CNVs in a non-syndromic CHD cohort (n = 161). The patients did not show any CNV in the examined genes (Guida et al., 2010). Erdogan et al. (Erdogan et al., 2008) reported 17% detection rate for isolated and syndromic CHD, while Breckpot et al. (Breckpot et al., 2010) described 3.6% for isolated and 19% for syndromic CHD. Sorensen et al. (Sorensen et al., 2012) screened 402 patients with CHD using MLPA and identified CNV in 3.2% of them.

In case No 1. array CGH clarified and refined the patient's karyotype, that confirmed the diagnosis of Cri du chat syndrome, also known as 5p deletion syndrome. Ring chromosome 5 is a rare cytogenetic presentation of Cri du chat syndrome, only a few cases have been reported (Basinko et al., 2012). Approximately 15–20% of affected infants have CHD, the most common heart defect is PDA as it was in our patient. Currently there is no clear understanding of the genomic cause of heart defects (Hills et al., 2006). Regarding the sSMC, that originated from chromosome 15 according to multicolor FISH, the microarray analysis did not identify any coding region of it indicating no phenotypic consequences of its presence.

In patient No 2. microarray revealed that the genomic loss of the long arm of chromosome 6 is a large, 18.753 kb interstitial deletion with breakpoints at 6q21 and 6q23.2. This chromosome aberration encompass the 6q21q22 region that is responsible for a rare, unique „6q21q22 deletion syndrome" associated with variable congenital anomalies, intellectual disability (ID), hypotonia, growth retardation and facial dysmorphism. 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). Toschi et al. (Toschi et al., 2012) have reported an overlapping phenotype between patients with acro-cardio-facial syndrome (ACFS) with type B deletion. ACFS syndrome (MIM # 600460) is a condition characterized by CHD, split hand/foot malformations, facial anomalies, cleft lip/palate, genital anomalies and ID. Our patient does not exhibit clinical symptoms of ACFS, only CHD. Concerning cardiac abnormalities the previously reported cases shared the deleted region from 113 to 114.5 Mb including the *MARCKS, HDAC2* and *GJA1* genes having important

role in regulation of cardiac morphogenesis (Rosenfeld et al., 2012). In our case the deleted region includes all three genes explaining the patient's cardiac phenotype.

In case No 3. array CGH revealed partial deletion of 4q35 and duplication of 6q25 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 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 *TLL, HAND2, PDLIM3* and *SORBS2* that are involved in cardiac morphogenesis (Vona et al., 2014). Our patient's phenotype is consistent with the characteristic features of terminal 4q deletion syndrome. The critical region for CHD at 4q35.1 is identical to that in our patient and the deleted region contains all of the aformentioned genes. 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 (from 6q25 to 6qter) can result in additional phenotypic abnormalities. Pivnick E et al. (Pivnick et al., 1990) have summarized the clinical consequences of partial duplication of 6q including craniofacial and cardiac anomalies, joint contractures and webbed-neck.

In two siblings 8p23.1 duplication was detected. In case No 4. the CNV was identified by microarray, in patient No 5. the imbalance was revealed using MLPA. 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 (Barber et al., 2015). The syndrome results from a core 3.68 Mb duplication grouped into telomeric, medial, and centromeric 8p23.1 segments. This interval lies between the olfactory receptor/defensin repeats *REPD* (REPeat Distal) in distal 8p23.1 and *REPP* (REPeat Proximal) in proximal 8p23.1. These repeat sequences predispose to non-allelic homologous recombination and recurrent *de novo* 8p23.1 deletions and duplications. The 8p23.1 duplication syndrome has a variable phenotype with three relatively common features of developmental delay and/or learning difficulties, CHD and a degree of mild dysmorphism that may be minimal (Weber et al., 2014; Yu et al., 2011). Congenital heart disease is found in about 25% of cases. The core region contains 27 genes and 5 microRNAs of which *GATA4, TNKS, SOX7,* and *XKR6* are dosage sensitive genes, their increased expression accounts for some symptoms of the phenotype. *GATA4* in concert with *SOX7* might cause congenital heart disease (Barber et al., 2015). The duplicated region in case No 4. (3.842 kb) covers the previously defined critical interval (3.68 Mb), so are the clinical features overlapping: assymetry of the face and skull, epicanthic fold, deep nasal root, long philtrum, low-set ears, retrognathism. In addition, poor mimics, poorly formed, but not prominent ear lobes and hypospadiasis characterise his clinical appearance. Coarctation of the aorta was discovered and urgently corrected surgically at two weeks of age. Neurologically, the patient is developing well and receives normal education in kindergarten. Case No 5. is the sister of the patient No 4. She was referred to clinical geneticist at the age of 1.5 years because of positive family history, mild dysmorphic features and CHD. Using MLPA analysis the duplication of three genes (*PPP1R3B, MSRA, GATA4*) 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. Although parental samples are not available for genetic investigation, we assume that the genetic imbalance in the siblings is familiar. The increased expression of *GATA4* and *SOX7* genes was sufficient for the CHD in both cases. The lack of apparent additional phenotypic consequences of the duplication may be attributed to the variable penetrance and expressivity of this syndrome. It is possible that microduplication of one or more of the affected genes exert their effects via a network and that variable penetrance reflects compensatory mechanisms found in regulatory transcription factor networks (Weischenfeldt et al., 2013).

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 TOF for 16 years. In case No 7. the thymus hypoplasia with TOF indicated the diagnosis of DiGeorge syndrome at the age of 1 month. 22q11 deletion caused DiGeorge syndrome is the most common microdeletion syndrome, with prevalence of 1:3000-6000 live births, although it is likely to be under recognized because of its clinical variability and heterogeneity (Oskarsdottir et al., 2004). Many patients have CHD, thymic hypoplasia, developmental delay, learning difficulties, psychiatric disorders, facial dysmorphism, renal and eye anomalies, hypoparathyroidism, and skeletal defects. The 22q11.2 region has several blocks of low copy repeats (LCRs) leading to non-allelic homologous recombination with both duplications and deletions. 22q11.2 deletion occurs as a *de novo* 1.5–3 Mb deletion in most individuals, while approximately 5% are inherited (McDonald-McGinn et al., 2001). There are different explanations for the very variable phenotype: 1) the existence of other genetic variations in the genome, in addition to the deletion that act as modifiers, 2) the occurrence of stochastic events during embryogenesis, 3) the existence of environmental exposures in pregnancy (Morrow et al., 2018). 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 array CGH or MLPA can be clinically very useful (Erdogan et al., 2008). Some authors suggest that the array methodology should be used as the first test for newborn carriers of congenital defects, such as CHD (Monteiro et al., 2017).

The advent of contemporary genomic technologies are accelerating of discovery of genetic causes of CHD that is not only a fundamental research, it has a vital role in the healthcare of the affected individuals. Identifying a genetic cause of CHD is relevant for clinicians caring for child or adult with CHD because (1) early diagnosis of syndromic CHD allow early comprehensive care (2) helps assessing risk for neurodevelopmental delays (3) providing more accurate prognosis for the CHD and outcomes of interventions (4) assessing recurrence risk for the offsprings of affected parents and first degree relatives.

This is the first report studying CNVs in a cohort of CHD patients in Hungary. In our study we detected pathogenic genomic imbalances in 21% (7/33) of syndromic CHD patients, this detection rate is similar to those reported in other populations. Moreover, four clinically relevant rare CNV different from the 22q11 deletion were identified among the syndromic patients studied. Rare, pathological CNV with intrafamilial recurrence in one are also reported, supporting expert opinions to apply array CGH as the first–tier test even in apparently mild clinical cases of congenital malformations with or without developmental delay. Searching for point mutations in CHD associated genes using next-generation sequencing is aimed to reveal genetic background of our CNV negative cases. G-banding, FISH and MLPA may still have a role in the diagnostic workflow of CHD cases, requiring a more detailed and careful phenotyping. Depending on the availability of array CGH the most beneficial diagnostic pahway for CHD patients is summarized in Fig. 4.

Our results confirm the previously published data that pathogenic CNVs are more common in syndromic CHD cases compared to isolated ones. Based on our experience the occurence of causative CNVs is higher with complex CHD phenotypes (e.g. tetralogy of Fallot). The re-

ported data emphasize the importance of further clarification of cytogenetic abnormalities by array CGH to explore a more detailed genotype-phenotype correlation. Our results show that using array CGH and MLPA was highly effective in the detection and refinement of clinically significant chromosomal abnormalities in individuals with syndromic CHD.

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