GENETIC EXAMINATION OF CONGENITAL HEART DEFECTS AND NON-SYNDROMIC INTELLECTUAL DISABILITY

Ph.D. Dissertation

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Contents

APPENDIX

ABBREVIATIONS

ABR	Active BCR-Related
ACMG	American College of Medical Genetics
AID	Autoinhibitory domain
AoS	Congenital stenosis of the aorta
array-CGH	Array-comparative genomic hybridization
ASD	Atrial septal defect
ASVD	Atrioventricular septal defect
ATP	Adenosine triphosphate
CHD	Congenital heart defect
CMA	Chromosomal microarray analysis
CNV	Copy number variation
CoA	Coarctation of the aorta
DD	Developmental delay
ddPCR	Digital droplet polymerase chain reaction
DEL	Deletion
DGS	DiGeorge Syndrome
DNA	Deoxyribonucleic acid
DORV	Double outlet right ventricle
DUPL	Duplication
GOR	Gastro-oesophageal reflux
GTP	Guanosine triphosphate
HLHS	Hypoplastic left heart syndrome
IAA	Interrupted aortic arch
ID	Intellectual disability
Kbp	Kilobase pair
LCRs	Low copy repeats

Mb	Megabase
MLPA	Multiplex ligation-dependent probe amplification
NS-XLID	Non-syndromic X-linked intellectual disability
PA	Pulmonary atresia
PAKs	p21-activated kinases
PBD	p21 binding domain
PCR	Polymerase chain reaction
PDA	Patent ductus arteriosus
RD	Rare disease
RGD	Rare genetic disease
SNV	Single nucleotide variant
TAC	Persistent truncus arteriosus communis
TBX1	T-box transcription factor 1 gene
TGA	Transposition of the great arteries
TOF	Tetralogy of Fallot
TOP3B	Topoisomerase III Beta gene
TPAVD	Total anomalous pulmonary venous drainage
TPAVR/PPAVR	Total/Partial anomalous pulmonary venous return
TUSC5	Tumour suppressor candidate 5 gene
VSD	Ventricular septal defect
VUS	Variant of unknown significance
WT	Wild type
XLID	X-linked intellectual disability
YWHAE	Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein Epsilon gene

PUBLICATIONS RELATED TO THE THESIS

I. **Gloria Kafui Esi Zodanu**, Mónika Oszlánczi, Kálmán Havasi, Anita Kalapos, Gergely Rácz, Márta Katona, Anikó Ujfalusi, Orsolya Nagy, Márta Széll, Dóra Nagy.
Systemic screening for 22q11.2 copy number variations in Hungarian pediatric and adult patients with congenital heart diseases identified rare pathogenic patterns in the region.
Front. Genet. 12:635480. doi: 10.3389/fgene.2021.635480 IF:3.258

II. Dóra Nagy, Katalin Farkas, Luís Armengol, Maria Segura, **Gloria Kafui Esi Zodanu**, Bernadett Csányi, Alíz Zimmermann, Barbara Vámos, Márta Széll. Further delineation of the phenotype of PAK3-associated X-linked intellectual disability: identification of a novel missense mutation.
Eur J Med Genet. 63(4):103800. 2020.
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III. Dóra Nagy, **Gloria Kafui Esi Zodanu**, Kálmán Havasi, Anita Kalapos, Mónika Oszlánczi, Márta Katona, Márta Széll.
A 22q11 kromoszomális régió kópiaszám eltéréseinek széles klinikai spektruma congenitalis vitiumos betegekben; Wide clinical spectrum of chromosome 22q11 copy number variations in patients with congenital heart defects.
CARDIOLOGIA HUNGARICA 49 (SupplA) pp. C113-C113 2019.
Journal Article/Abstract /Scientific [30711379].

1 INTRODUCTION

1.1 Rare diseases

Rare disease (RD) is generally defined as a condition that affects ≤1 in 2000 people. However, this definition is highly debated and various definitions are used in different contexts (Richter et al., 2015). For example, the prevalence rate, the severity of the condition and the mode of classification of the disorder are also used to determine whether a condition fits this classification.

Individually, RDs affect only a small number of people in the entire population, but collectively these diseases affect a sizeable portion of our population making it an important public health issue. In general, there are more than thousands of rare diseases and to date, about 30 million people in Europe are diagnosed with a debilitating form of RD (Cannizzo et al., 2018). RDs often represent chronic and progressive conditions with no therapeutic options or no effective management and thus, serve as major medical concerns. In most cases, early diagnosis could provide suitable treatment and medical care to improve the life quality of these individuals (Nguyen, 2018). However, rare disease patients face on average 5-30 years to get a diagnosis (Vinkšel et al., 2021).

Rare genetic diseases (RGDs) usually exhibit Mendelian or monogenic patterns of inheritance. Research continues to expand our knowledge of the phenotypic and genetic diversity of these RGDs, however, establishing the number of RGDs is challenging since many of them are yet to be discovered and characterized (Boycott et al., 2017; Harel et al., 2015). RGDs could be the result of single-nucleotide variants (SNV), small insertions or deletions, trinucleotide or hexanucleotide repeat expansions, transcriptional, translational alterations of the gene products (Iafrate et al., 2004) or deletions and duplications, collectively referred to as copy number variations (CNVs) (Blue et al., 2012; Redon et al., 2006).

CNVs have been linked to many diseases by affecting a single gene or series of contiguous genes (Henrichsen et al., 2009). They may extend from kilobases (kb) to megabases (Mb) in size. The majority of these CNVs occur sporadically, but in 10-20% may be familial and follow an autosomal dominant pattern of inheritance (Yamagishi, 2002).

Technological advances and innovations in molecular genetic techniques of the last decade, such as high throughput sequencing or comparative genome hybridisation arrays, have provided new opportunities in understanding the underlying molecular mechanisms of RDs

and significantly improved their diagnostic rate. The rate of novel disease-causing genes has also increased accordingly (Durmaz et al., 2015). By implementing these new methods in the routine diagnostics, we may improve the diagnostic rate and also the time for the diagnosis of RDs, thus, providing a better care for patients and their families (Le Scouarnec & Gribble, 2012). Rare diseases are present in all fields of medicine. RDs may affect an organ or multiple organs which may present as intellectual disability with unclear aetiology, developmental delay, muscular alterations, errors of metabolism or as congenital developmental anomalies. Some disease groups, such as intellectual disabilities, neurological and neurodegenerative diseases are more in focus than others, like congenital heart defects.

In this study our research group focused mainly on identifying the genetic background of congenital heart defects and intellectual disability in patients presenting at the University of Szeged, Szent-Györgyi Albert Clinical Centre.

1.2 Congenital heart defects

Congenital heart defect (CHD) refers to a problem in the structure or function of the heart that is present at birth. Different types of CHDs affect only a small portion of the population, however collectively, they represent the most common form of birth defects, affecting ~1% of live births each year (Zhang et al., 2017). Most cases of CHDs have no known cause. They could occur alone (isolated CHDs) or in association with other anatomical defects, as part of a syndrome (syndromic CHDs) (Fahed et al., 2013).

CHDs are classified in various ways depending on their anatomy and pathophysiology. The disease can be grouped as the following: CHD with or without shunt between systemic and pulmonary circulation, left heart CHD, right heart CHD, or as CHD with anomalous origin of great arteries and miscellanea. However, CHD is generally simplified and categorized into more than 20 different types based on the specific anatomic or hemodynamic lesions. They include ventricular septal defect (VSD), atrial septal defect (ASD), patent ductus arteriosus (PDA), atrioventricular septal defect (AVSD), double outlet right ventricle (DORV), Tetralogy of Fallot (TOF), persistent truncus arteriosus communis (TAC), coarctation of the aorta (CoA), coronary artery anomalies, valvular pulmonary stenosis (PS), pulmonary atresia (PA), total or partial anomalous pulmonary venous return (TPAVR, PPAVR), interrupted aortic arch, transposition of the great arteries (TGA), and hypoplastic left heart syndrome (HLHS) (Micheletti, 2019; Pan et al., 2015).

CHDs develop during the embryonic period. Environmental factors are for long time known to contribute to their development, such as maternal diabetes, phenylketonuria or alcohol, drug or medicament consumption during the pregnancy. In the last couple of decades, the influence of genetic factors behind CHDs have been intensively studied (Nagy & Széll, 2018). The majority of CHDs are isolated and not accompanied by any other health issue. However, it is only about 10-12% of these cases can a genetic diagnosis be set up. Approximately, 30-40% of CHDs are syndromic, associated with other malformation and caused by genetic factors such as chromosome aneuploidies (trisomy 21, 18 13 or monosomy X), a single gene mutation (CHARGE syndrome, Noonan syndrome, Adams-Oliver syndrome) or copy number variants (Williams-Beuren syndrome, DiGeorge syndrome). The most common human CNVs affect chromosomal region 22q11.2 (Digilio & Marino, 2016; Fahed et al., 2013; Nagy & Széll, 2018).

1.2.1 Chromosome 22q11.2 region

Human chromosome 22 is acrocentric and contains about 51 million base pairs. Though it is the second smallest chromosome, a study of the entire human genome has revealed a high number of variabilities within it. This variability could be a loss or gain of certain segments. Some are commonly present in healthy individuals as well and some tend to cause health problems (Yu et al., 2012).

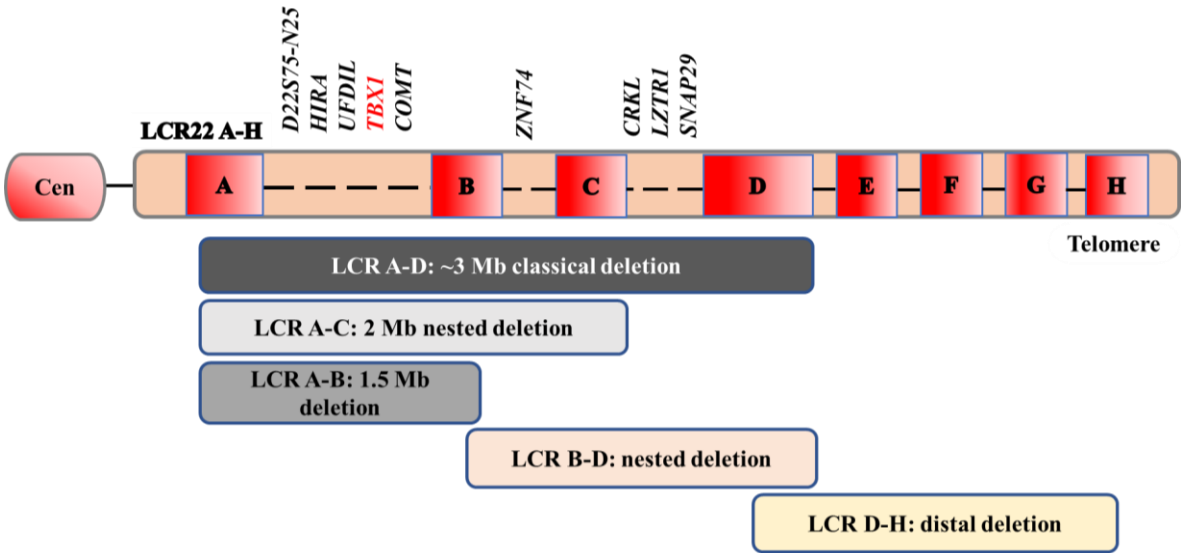
Low copy repeats (LCRs) have been reported as the main factor in the formation of CNVs. LCRs consist of repetitive blocks of sequences that provide the structural basis for a wide range of genomic variation. They are often ~5-10 kb long in size with ~95%-97% similarity to each other. The proximal region of chromosome 22q11 contains eight LCRs (LCR22-A to LCR22-H) (Figure 1) (Burnside, 2015; Kruszka et al., 2017). This region is highly susceptible to copy number errors that might occur during crossing-over in meiosis (Demaerel et al., 2019). These non-allelic homologous rearrangements may result in translocations, inversions, deletions and duplications. Proximal microdeletions of 1.5-3Mb in this chromosomal region typically include the sequence between LCR A and B or LCR A and D. These microdeletions often result in the classical phenotype of DiGeorge syndrome (DGS, 22q11 deletion syndrome).

One of the main genes in this region implicated for pathogenicity is T-box transcription factor 1 gene (*TBX1*). It is a transcription factor that plays a vital role during embryogenesis. Studies in mouse models have reported that haploinsufficiency of *TBX1* gene may generate craniofacial, thymic and parathyroid anomalies, similar to what has been observed in humans

with DGS, while homozygous mutations of *TBX1* most often resulted in severe developmental defects of the second pharyngeal arch, and the third and fourth pharyngeal pouches or even death in mouse. (Epstein, 2001; Lindsay et al., 2001).

Central deletions, occurring at LCR22 B-D, LCR22 C-D or distal deletions at LCR22 D-H may cause different and markedly variable phenotypes with growth restriction, developmental delay and intellectual disability (Burnside, 2015; Kruszka et al., 2017). 85-90% of individuals with 22q11 microdeletion syndrome have the classical ~2.5-3-Mb large LCR22 A-D deletion, whereas 8-10% have a nested ~1.5-Mb LCR 22A-B deletion (Hacıhamdioğlu et al., 2015; McDonald-McGinn et al., 2015).

Figure 1 Schematic overview of LCR in 22q11.2 region on chromosome 22 that facilitates genomic instability



Cen: centromere. A-H red boxes: low copy repeat regions (LCR) on chromosome in 22q11.2 region. Horizontal bars beneath the map illustrates the most common deletions at the 22q11.2 region. Figure reproduced after McDonald-McGinn et al, 2015.

1.2.2 The 22q11 deletion syndrome

22q11 deletion syndrome (22q11 DS, OMIM: #188400), also known as DiGeorge syndrome or velocardiofacial syndrome is the most frequent chromosomal microdeletion syndrome. Because the symptoms of 22q11.2 DS are very diverse, they were once described as separate conditions hence with different names. It is estimated to have an incidence rate of 1 in 4000 to

1 in 6000 live births per year and is responsible for approximately 5% of all CHDs occurring at birth (Shi & Wang, 2018).

Important characteristics of the syndrome is its varied phenotypic expressions resulting in some patients being mildly affected while others have severe forms of malformations, cognitive or psychiatric problems. However, the main features of the syndrome include CHDs, particularly conotruncal malformations (Tetralogy of Fallot, ventricular septal defect, interrupted aortic arch, and truncus arteriosus), palatal abnormalities (clefting, nasopharyngeal insufficiency, nasal voice), immune deficiency as a result of thymus aplasia or hypoplasia, characteristic facial features, learning difficulties, hearing loss, gastrointestinal, ophthalmologic, central nervous system, skeletal, and genitourinary anomalies, psychiatric illness and autoimmune disorders (Scambler, 2000; Torres-Juan et al., 2007).

Duplications have also been identified in this chromosomal region but are associated with a more significant phenotypic variability than deletions resulting in a different entity (Wentzel et al., 2008). Regarding the large phenotypic spectrum of 22q11.2, CNVs from almost asymptomatic appearance to multiple defects, the clinical diagnosis may be challenging and significantly delayed (van Engelen et al., 2010). Previous studies have drawn attention to the importance of routine screening for 22q11.2 CNVs in patients with congenital heart defects, especially with conotruncal anomalies (Goldmuntz, 2020; Huber et al., 2014; Wozniak et al., 2010).

1.3 Non-syndromic X-linked intellectual disability

Intellectual disability (ID) refers to a significantly reduced ability in understanding information and applying skills. The onset of ID is usually before 18 years of age. ID can be categorized as syndromic or non-syndromic. Syndromic ID occurs mostly in association with other signs and symptoms such as dysmorphic, neurological or systemic features. On the other hand, non-syndromic ID is characterized by intellectual disability as the only feature (Tejada & Ibarluzea, 2020). It could occur due to genetic or environmental factors, yet, in about 60% of the cases, no known cause has been identified. Environmental factors such as exposure to teratogens during pregnancy, viruses, radiation, and intense head trauma are some of the known causes. Nevertheless, genetic factors are assumed the cause of 25-50% of the cases (Muthusamy et al., 2017).

The X-chromosome is enriched with genes that are involved in the development and maturation of the nervous system, hence, a mutation occurring in these genes may cause either a syndromic or a non-syndromic form of X-linked intellectual disability. This accounts for 5-16% of all intellectual disabilities (ID) in males. Till now, more than 700 genes have been reported to be involved in ID and ~10-12% of these genes are on the X-chromosome (Kaufman et al., 2010; Magini et al., 2014; Vissers et al., 2016).

Non-syndromic X-linked intellectual disabilities (NS-XLID) may have a wide range of features. The p21-activated kinase 3 (*PAK3)* gene, for example, was the fourth gene to be associated with non-syndromic X-linked intellectual disability (OMIM:#300558) (Allen et al., 1998).

1.3.1 The *PAK3* protein and gene

The p21-activated kinase proteins (PAK1-PAK6) are classified into two groups. PAK1, PAK2 and PAK3 belong to group I, while PAK4, PAK5 and PAK6 belong to group II. Group I PAK-kinases include the p21-binding domain (PBD) which overlaps with an autoinhibitory domain (AID) at the N-terminus, and one serine/threonine kinase domain at the C-terminus (Kumar et al., 2017; Rousseau et al., 2003; Zhang et al., 2017).

The most studied PAK-kinase is PAK3. PAK3 function and regulation is complex; it is involved in different signalling pathways that regulate the cytoskeletal organization and cell cycle (Bienvenu et al., 2000).

PAK3 is a serine/threonine kinase that can only be activated by the binding of small GTPases: Rac1 and Cdc42. PBD acts as the binding site of active Rac1 and Cdc42, while AID binds to the kinase domain of another PAK3-molecule to form an inactive homodimer (Kumar et al., 2017). The homodimer gets dissociated once the GTPases are activated by binding to PBD. GTPase activation results in a conformational change and subsequent autophosphorylation on various serine-threonine sites. This autophosphorylation in the kinase domain allows the binding of PAK3 to the substrate thereby using its catalytic role as a monomer (Bokoch, 2003; Kreis et al., 2007; Kumar et al., 2017; Rousseau et al., 2003; Zhang et al., 2017).

The cytoskeleton is a complex network of filaments that aids in the formation of cell structure as well as cell dynamics, including cell cycle regulation and apoptosis. Neurons are in connection with each other through synapses. For these synapses the formation of structurally normal dendritic spines in adequate number, is essential. Functional studies have proven that

PAK3-kinase plays a crucial role in this process. The intact PAK3 function contributes to the fine-wiring of the synaptic network in the brain. Therefore, loss of function mutations in the *PAK3* gene are believed to lead to a dysregulated actin cytoskeleton, abnormal dendritic spine morphology, density and stability, changes in the dendritic currents, decreased neural plasticity (Kreis et al., 2007; Dubos et al., 2012; Thévenot et al., 2011) and ultimately cognitive impairment without major structural brain abnormalities, also referred to as synaptopathies (Horvath et al., 2018).

1.4 AIMS

In light of the diagnostic challenges with 22q11.2 CNVs and considering the relatively low referral number of patients with 22q11.2 CNV to the Department of Medical Genetics, University of Szeged in the last decade, we hypothesized that some patients with 22q11.2 CNVs — especially in the adult population — may have remained undiagnosed. Therefore, we aimed to investigate the 22q11.2 CNVs and *TBX1* gene variants in the paediatric and adult CHD patients in the Southern-Hungarian region, to perform an in-depth genotype-phenotype comparison and to carry out variant segregation analysis in the positive cases.

The second major aim of our study was to investigate the role of a novel *PAK3*-mutation in a male patient with non-syndromic intellectual disability.

2 PATIENTS AND METHODS

2.1 Patients

2.1.1 Patients with congenital heart defects

The study was conducted at the University of Szeged between 2016 and 2019. All individuals recruited in this study were previously cardiologically diagnosed with non-syndromic congenital heart defects. 212 unrelated patients consisting of 110 females and 112 males with an average mean age of 26.9 years and age range between 2 months to 74 years were recruited in the study. The DNA of 211 Hungarian individuals with no CHDs (confirmed with cardiological examination), and with no family history of CHD (144 females, 67 males, mean age: 37 years, age range: 8–73 years) was used as controls for the comparative analyses. In positive cases, genetic testing was offered to all first-degree family members.

2.1.2 Patient with non-syndromic X-linked intellectual disability

We report the case of a 14-years-old boy visiting the genetic counselling unit presenting with intellectual disability, autistic characteristics and behavioural problems.
He was born at term by spontaneous delivery following a normal pregnancy with a normal birth weight and length as a first child of Caucasian non-consanguineous parents.
Autistic characteristics and delayed psychomotor development were first noted at the age of 3 years (Brunet-Lézine test: gross motor skills: 65; fine motor skills: 61; language skills: 58; sociability: 52; overall developmental quotient of 59). Special training was initiated. He started to speak and maintain eye contact at 4 years and let his mouth hang open with constant drooling until the age of 4.5 years. He had three generalized tonic seizure episodes with fever in early childhood and experienced short absence-like episodes and unusual grimacing in the 1.5 year previous to examination. Baseline and sleep-deprived EEGs were repeatedly normal and brain MRI detected no abnormality. Neurological examination revealed small muscle bulk in the limb-girdle muscles with normal tone and strength, mild postural and intentional tremor, symmetric brisk reflexes without spasticity and no gait disturbance. Neuropsychological assessment showed mild-to-moderate intellectual disability with moderate impairment of visuo-spatial, reading, writing, comprehension and counting skills and severe attention deficit,

mood imbalance, anxiety and autistic traits (Woodcock-Johnson and Snijders-Oomen nonverbal intelligence tests: age equivalent of 5;2 and 5;3, respectively).

On examination, microcephaly, mild thoracic kyphosis, dorsolumbar scoliosis, ankle valgus, pectus carinatum, wide-spaced nipples and spina bifida occulta with a sacral dimple were noted. His facial features included low forehead, downslanting palpebral fissures, thin upper lip and high-arched palate.

Quantitative and qualitative blood count, serum electrolytes, lactate, carbamide, uric acid, creatinine, creatine kinase and liver enzyme levels, inflammatory and autoinflammatory parameters, serum amino acid and acyl-carnitine profile, serum and urine dopamine and serotonin levels showed no marked discrepancy. Audiology detected mild sensorineural hearing loss, though the examination was inconclusive due to lack of cooperation. On nephrological examination, underactive bladder function was detected. Abdominal ultrasound echocardiology, ECG and ophthalmology showed no abnormality.

Karyotyping on G-banded chromosomes using standard procedures detected no major aberration and testing for Fragile-X syndrome showed no triplet repeat expansion in *FMR1*.

Written informed consent was obtained from all participants and/or legal guardians/parents before enrolment in the study. Investigations in this study were performed according to the Helsinki Declaration 2008 and approved by the National Medical Research Council (No CHD-01/2016 – IF-6299-8/2016) and the Local Ethical Committee of the University of Szeged (No 105/2016-SZTE).

2.2 METHODS

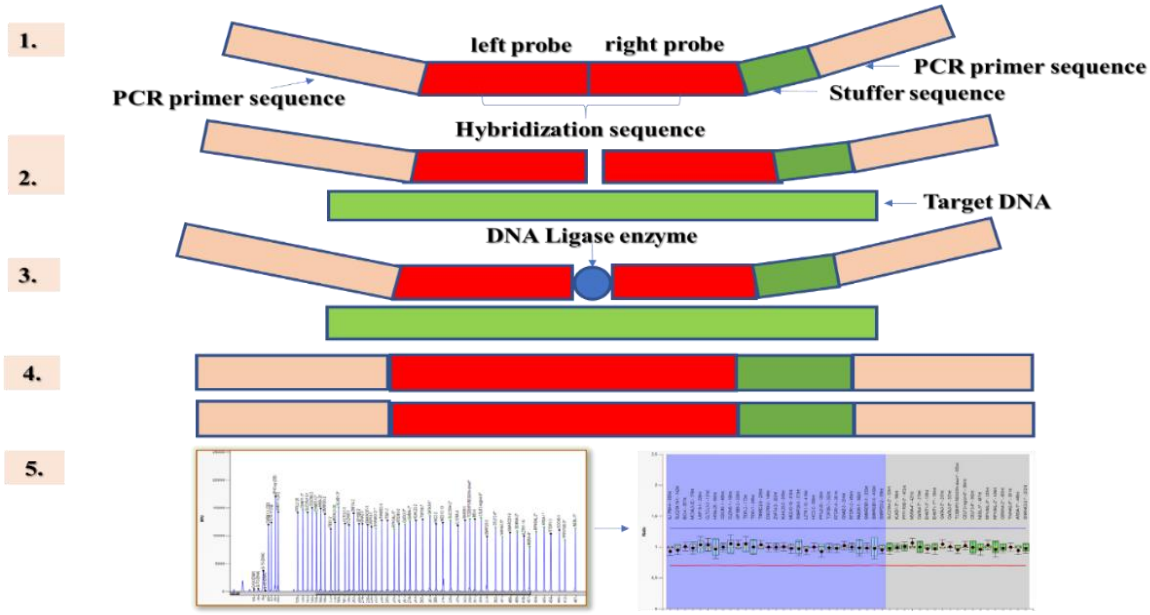
2.2.1 Sample preparation

Peripheral blood samples from all subjects were collected into EDTA tubes. DNA was extracted from the peripheral blood with QIAamp DNA Blood Mini kit (QIAGEN, Gödöllő, Hungary).

2.2.2 Multiplex ligation-dependent probe amplification

To identify CNVs in the 22q11.2 locus in a large number of samples, the multiplex ligation-dependent probe amplification (MLPA) technique was used (Figure 2). This technique involves probes that are designed to bind specifically to genomic regions of interest to detect CNVs in that region in 5 main steps as illustrated in Figure 2

Figure 2 Steps of multiplex ligation-dependent probe amplification (MLPA)



Each probe in the MLPA kit contains two oligos, complementary to one specific chromosomal region, universal primer sequences and stuffer sequences, which allow the separation of the PCR products in step 5.

1. Step: Denaturation of the double-stranded target DNA
2. Step: Hybridization of the MLPA probes to the complementary DNA-sequence of the template
3. Step: Ligation of the two perfectly hybridized parts of the MLPA probes
4. Step: Amplification of the complete MLPA probes with a universal PCR reaction
5. Step: Separation of the amplified probes, identification of the fragments by using capillary electrophoresis and data analysis by Coffalyser.net software

All samples were screened using P250-B2 DiGeorge SALSA MLPA Probemix (IVD, MRC-Holland, Amsterdam). This MLPA kit is suitable for the detection of deletions or duplications in the 22q11.2 region, including the region of Cat-Eye syndrome, proximal, central and distal regions of 22q11.2 microdeletions. Additionally, it contains probes for the detection of copy number status on 4q, 8p, 9q, 10p (DiGeorge syndrome type II), and 17p regions, which may cause similar phenotypic features as DGS, if deleted.

Polymerase chain reaction (PCR) products generated in the fourth step were loaded onto a capillary electrophoresis device, ABI 3500 Genetic Analyzer (ThermoFisher Scientific, Waltham, MA) for fragment separation by length.

Copy numbers were then determined by comparing the target probes' peak heights of patient sample to the peak heights of reference regions and of reference samples with normal copy number using Coffalyser.net software (MRC-Holland, Amsterdam). According to these, dosage quotient (DQ) for each probe was calculated.

Samples with a DQ of 0.75-1.3 have normal two copies of the examined chromosomal region, while samples with a DQ of 0.4-0.7 carry only one copy (heterozygous deletion) and samples with a DQ 1.3-1.7 three copies (heterozygous duplication).

2.2.3 FISH and chromosomal microarray

MLPA was repeated for all samples in which copy number variants were identified. Deletions and duplications were confirmed with an independent method, including FISH (Vysis DiGeorge Region LSI N25 SO/ARSA SGn Probes, Abbott Molecular Inc., Des Plaines, IL, USA, and SureFISH 22q11.21 CRKL, Agilent Technologies, Cedar Creek, TX, USA), a supplementary MLPA kit (P372-SALSA MLPA Microdeletions 6, MRC-Holland, Amsterdam, Netherlands) or chromosomal microarray analysis (CMA, Affymetrix, CytoScan 750 K, Thermo Fisher Scientific, Waltham, MA, USA). CMA was performed as described by Nagy and colleagues (2019). Chromosomal microarray analysis was performed at the Department of Laboratory Medicine, Faculty of Medicine, University of Debrecen, Hungary.

In cases where one probe was deleted, the probe region was sequenced with bidirectional capillary sequencing to exclude MLPA-interfering single-nucleotide polymorphisms (SNPs) in the sample DNA. These validation methods were confirmed results that were detected by MLPA (i.e., no false positives).

2.2.4 Droplet digital PCR

A droplet digital polymerase chain reaction (ddPCR) technique was designed for the confirmation of the recurrent single-probe CNVs in the Topoisomerase III Beta gene (*TOP3B*) gene from the CHD patient samples and also to determine the frequency of *TOP3B* CNVs in the control cohort.

The analysis was performed on the QX100 Droplet Digital PCR system (Bio-Rad Laboratories, Hercules, CA, USA), according to the manufacturer's instruction.

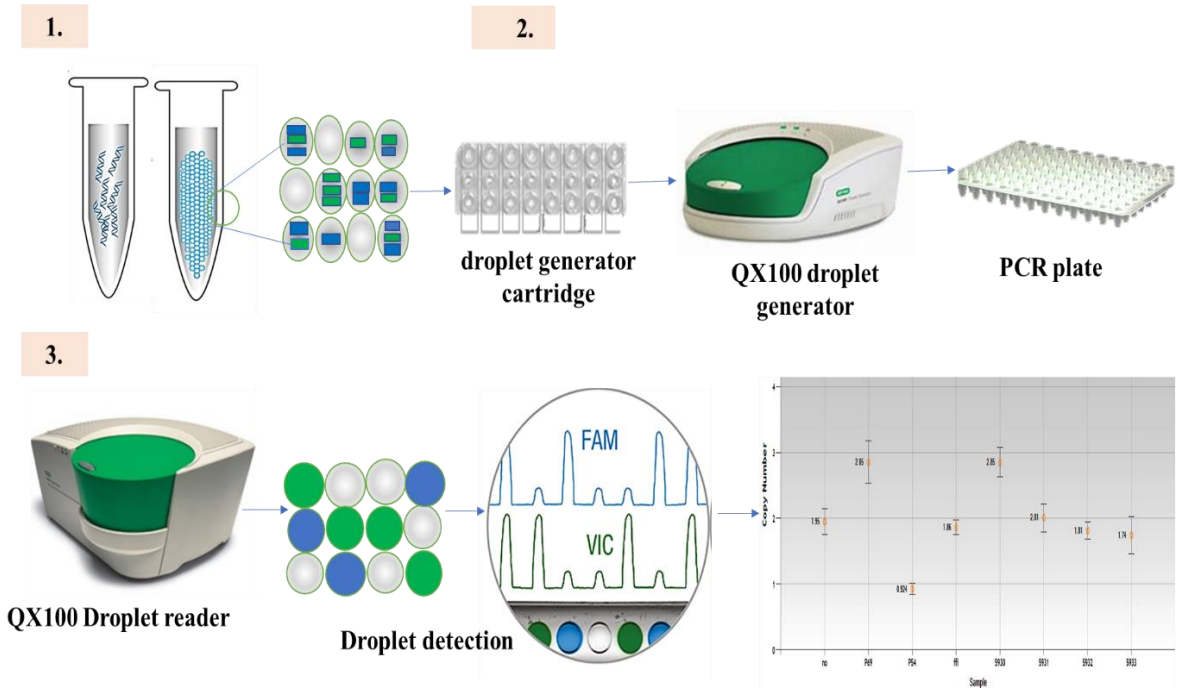
In ddPCR around 20 000 droplets were generated simultaneously. A droplet contained the sample DNA-fragment (or none), the designed primers, the fluorescently labelled probes and the ddPCR master-mix. The primers and fluorescently labelled probes were designed for *TOP3B* exon 7 (HEX-labelled) and for the *PRDM15* gene (FAM-labelled) as reference region on chromosome 21 (Table 1). The target *TOP3B*-DNA as well as the reference *PRDM15*-DNA was amplified with a PCR reaction in separate emulsion droplets simultaneously. During the amplification reaction the fluorescent tags detached from the oligonucleotides and were scanned with the QX100 Droplet reader (Figure 3). Based on the intensity of the read signals from the target *TOB3B* and reference *PRDM15*, the copy number status of the sample was calculated by QuantaSoft Software.

TOP3B CNVs found in the controls with ddPCR were confirmed with MLPA.

Table 1 Primers and probes used in ddPCR

TOP3B	**(NM_001282112.2: Chr22q11) -** exon 7
Forward primer	5' TCTCTCATCTCCTTTGGGCC 3'
Reverse primer	5' CTCTCAGAATCCATGCCCCA 3'
Probe	5' /HEX/ACCTTGGCCTGCAGCACCCA/BHQ/ 3'
PRDM15	**(NM_001040424.3: Chr21) –** intron 6-7
Forward primer	5' ATGTTTCGCCAACTTCTGAG 3'
Reverse primer	5' AGAGCTATGGCACAAACCTG 3'
Probe	5' /FAM/AGGATTTGG/ZEN/GGCTGCGC 3'

Figure 3 Digital droplet PCR technique



1. DNA sample, primers, probes and ddPCR MasterMix
2. Prepared samples are loaded into individual wells of a droplet generator cartridge. A droplet generation oil is then added to the channels of the cartridge and placed in a QX100 Droplet Generator. Samples & oils are combined to create an emulsion for each sample. Emulsified samples are transferred onto a PCR plate for amplification.
3. PCR amplified products are placed onto a QX100 Droplet reader. Positive & negative droplets in each sample are read. Concentrations are then analyzed with QuantaSoft software.

Target DNA Reference DNA

2.2.5 Sequencing of *TBX1* gene

The entire coding region and the flanking introns of the *TBX1* gene were analysed. The nucleotide sequences of the PCR amplified exons were determined using bidirectional Sanger sequencing. PCR was performed using Dream Taq Green PCR Master Mix according to the manufacturer's protocol and run on VWR UNO^{96} and Doppio Thermal cyclers (Germany). The PCR products were run by gel electrophoresis, stained with Gel red and visualized on a Gel Doc EZ Imaging system (Bio-Rad Laboratories Inc.) Sequencing of the PCR products was performed on an ABI 3500 Genetic Analyser and compared to the human reference sequence (Ensembl: NM_080647).

Table 2 Primers for *TBX1* gene

TBX1 Primer	5'-3' sequence
Exon 2-forward	GTTCAGCATCGCCTCTCTG
Exon 2-reverse	CTACCAAGAGCTGCCTCCAC
Exon 3-forward	ATCTCCGCCGTGTCCAG
Exon 3-reverse	CGGCGGAGGATAGGTGTTAG
Exon 4-forward	CCCCAGGCAGGTCAAGG
Exon 4-reverse	GACCCGCCACTTTCCAG
Exon 5-forward	AAGGCCCTCTGGGTTCAC
Exon 5-reverse	ACAGGCCTCTTAGGGACAGG
Exon 6-forward	CTCCCACCCCAGATCCTC
Exon 6-reverse	TTACACCCGCTTTTCCAGAG
Exon 7/8-forward	CTTGGTGCGCTTCTCCTAAC
Exon 7/8-reverse	GAACCCGGATCCCACGAC
Exon 9-forward	ACTTGGGGTCTCGGGCAC
Exon 9-reverse	GAACTTCGGGGCTGTGCAG

2.2.6 Clinical exome analysis for X-linked non-syndromic intellectual disability

Clinical exome analysis was performed on whole-exome sequence data using Illumina NextSeq500 sequencer after library preparation with Roche KAPA HyperPrep library kit and SeqCap EZ MedExome capture kit. The mean average depth of on-target coverage in the sequenced exome was 69x (target bases at 10x coverage: 96%; at 20x coverage: 93%; at 30x coverage: 86%). Reads were then aligned to the human reference genome (GRCh37) using BWA (v.0.7.12). The library preparation, sequencing and related bioinformatics were carried out in the QGenomics Laboratories, Barcelona, Spain.

Among 120 469 variants, deleterious ones were prioritized on the basis of the functional relevance of genes, inheritance models and minor allele frequency (MAF) in the general population (GnomAD and in-house databases). As a result of the filtering, a novel variant in the *PAK3* gene was identified as the most probable pathogenic variant. The variant was confirmed by bidirectional Sanger sequencing.

PyMOL Molecular Graphics System (version 2.0 Schrödinger, LLC) was used to evaluate *in silico* the changes in the mutant PAK3 protein structure. The wild-type three-dimensional protein structure has been obtained from RCSB Protein Data Bank (ID: 6fd3) and submitted to PyMOL's Wizard/Mutagenesis on protein application to create and visualize the specific mutant PAK3 protein.

2.2.7 CNV and SNV interpretation

Identified CNVs and SNVs were classified according to the standards and guidelines of the American College of Medical Genetics (Richards et al., 2015; Riggs et al., 2020).
The following websites and databases were used for CNV interpretation: Database of Chromosomal Imbalance, Phenotype of Humans using Ensemble Resources (DECIPHER, (Firth et al., 2009), Database of Genomic Variation (DGV) (MacDonald et al., 2014), PubMed and GeneReviews (McDonald-McGinn et al., 1993). For SNV interpretation, VarSome (Kopanos et al., 2019), ClinVar (Landrum et al., 2020) and Genome Aggregation Database (GnomAD, (Karczewski et al., 2020) databases were used.

2.2.8 Statistical Analysis

GraphPad Prism (GraphPad Software, San Diego, California, USA) version 9.00 for Windows, was used for statistical analysis. The frequency of *TOP3B* CNVs and *TBX1* variants in the patient cohort was compared with the frequency in the control cohort and also with the frequency in the global dataset of GnomAD using Fisher exact test and χ^2 test. $P<0.05$ was considered to be statistically significant.

3 RESULTS

3.1 CHD

3.1.1 Distribution of CHDs in the patient cohort

In our CHD cohort, eighteen different types of CHDs were identified. The four most common CHD types (higher than 10%) were ventricular septal defect (VSD), atrial septal defect (ASD), congenital aorta stenosis (AoS) and tetralogy of Fallot (TOF). In 81% of the patients, only one cardiac entity was diagnosed, while in 19% of the patients, two or more CHDs occurred together. The distribution of the different CHDs among the South-Hungarian Registry patients corresponded well with the frequency described in the literature (van der Linde et al, 2011) (Table 3).

Table 3 Distribution of congenital heart defects among patients (N=212)

TYPE OF CONGENITAL HEART DEFECT	NUMBER OF PATIENTS (%)
Ventricular septal defect	**36 (16.9%)**
VSD alone	25
VSD + ASD + PDA	5
VSD + ASD	2
VSD + PDA	2
VSD + PS	2
Atrial septal defect	**35 (16.5%)**
ASD alone	27
ASD + PDA	3
ASD + VSD	3
ASD + PS	2
Congenital aorta stenosis	**31 (14.6%)**
AoS alone	17
AoS + bicuspid aortic valve	13
AoS + ASD	1
Fallot IV	**30 (14.1%)**
TGA	**21 (9.9%)**

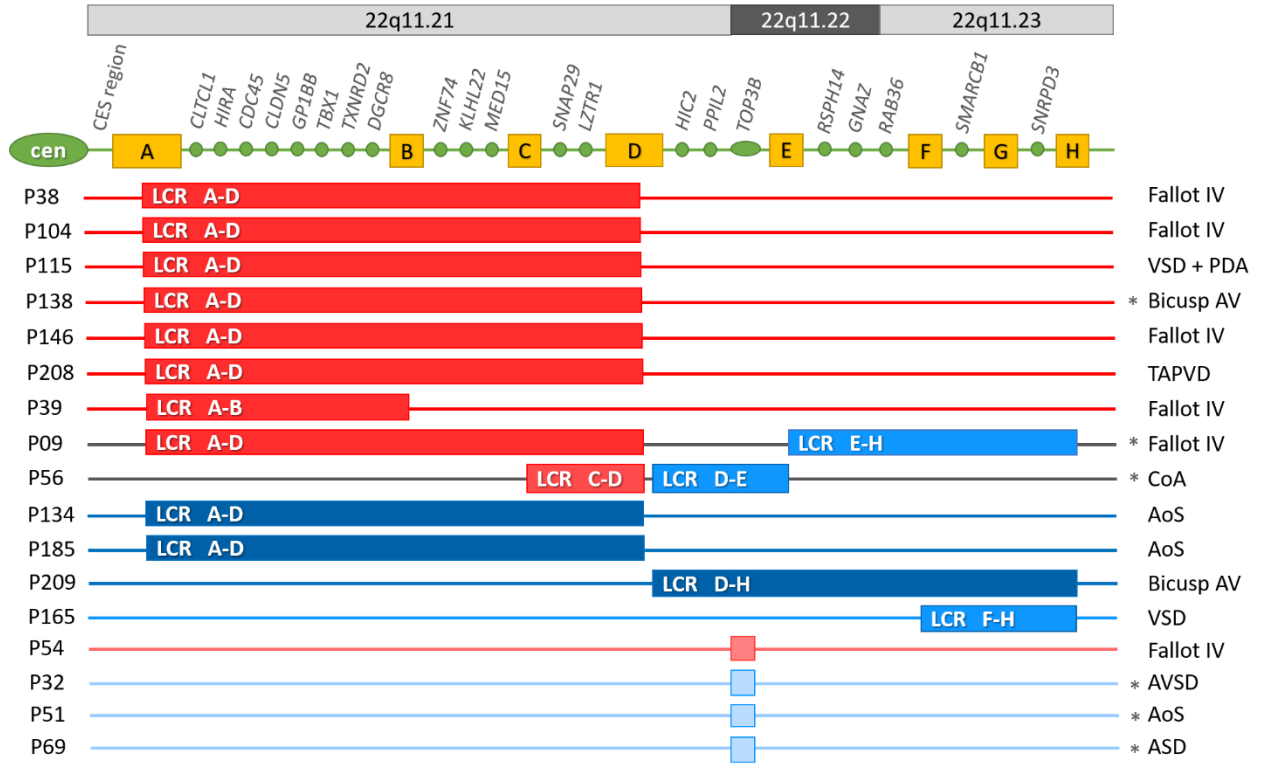
Bicuspid aortic valve	**19 (8.9%)**
Coarctation of the aorta	**17 (8%)**
CoA alone	10
CoA + bicuspid aortic valve	3
CoA + VSD + PDA	4
Atrioventricular septal defect	**5 (2.4%)**
Anomalous pulmonary venous drainage	**4 (2%)**
TAPVR alone	2
TAPVR + PA + VSD	1
PPAVR	1
Pulmonary stenosis (congenital)	4 (2%)
Univentricular heart	3 (1.4%)
Hypoplastic left heart syndrome	2 (0.9%)
Pulmonary atresia	2 (0.9%)
Truncus arteriosus communis	1 (0.5%)
Double outlet right ventricle	1 (0.5%)
Ebstein anomaly	1 (0.5%)

AoS: congenital aorta stenosis; ASD: atrial septal defect; CoA: coarctation of the aorta; Fallot IV: Tetralogy of Fallot; PA: pulmonary atresia; PDA: patent ductus arteriosus; PPAVR: partial pulmonary anomalous venous return; PS: pulmonary stenosis; TGA: transposition of the great arteries; TAPVR: total anomalous pulmonary venous return; VSD: ventricular septal defect

3.1.2 Distribution of positive MLPA results and classification of the detected CNVs

In 17 cases out of the 212 patients (8%), previously diagnosed with non-syndromic CHD, putative genetic variants were detected by MLPA. After evaluation, 11 (5.2%) of these copy number changes were interpreted as pathogenic variant, two as variant of unknown significance (VUS, 0.9%) and four (1.8%) as benign (Figure 4).

Figure 4 **Pathogenic, VUS and benign 22q11.2 copy number variations in the CHD cohort**



Cen, centromere; CES, Cat eye syndrome region; A-H yellow boxes: low copy repeat regions in locus 22q11.2. Genes indicated between the LCR regions, are the ones that have corresponding probes in the P250-B2 DiGeorge SALSA MLPA Probemix. LCR: low copy repeat region. AoS, congenital aorta stenosis; AVSD, atrioventricular septal defect; bicuspid AV: bicuspid aortic valve; CoA, coarctation of the aorta; Fallot IV, tetralogy of Fallot; PDA, patent ductus arteriosus; TAPVD, total anomalous pulmonary venous drainage; VSD, ventricular septal defect. Dark red: pathogenic microdeletion; Dark blue: pathogenic microduplication; Middle red: microdeletion of uncertain significance; Middle blue: microduplication of uncertain significance; Light red: benign microdeletion; Light blue: benign microduplication.
LCR A-D: the typical _2.5–3 Mb microdeletion in region 22q11.21; LCR A-B: _1.5 Mb proximal microdeletion in region 22q11.21; LCR C-D: _0.5 Mb central microdeletion in region 22q11.21; LCR D-E: _1.2 Mb central microduplication in region 22q11.21q11.22; LCR D-H: _3.1–3.5 Mb central-distal microduplication in region 22q11.21q23; LCR E-H: _1.55–2 Mb distal microduplication in region 22q11.22q11.23; LCR F-H: _1–1.2 Mb distal microduplication in region 22q11.23; Asterix denotes familial CNVs.

The most frequent CNVs of the positive MLPA results were microdeletions (8/17); however, microduplications (7/17) and a combination of deletions and duplications (2/17) were also observed (Figure 5).

Among pathogenic CNVs, 7 microdeletions, 3 duplications and 1 combination of a deletion and a duplication, whereas among the VUS one duplication and one combined CNV was detected. Also, within the benign variants one deletion and three duplications were found (Figure 5).

Figure 5 Positive MLPA results visualized with ratio charts by Coffalyser Software

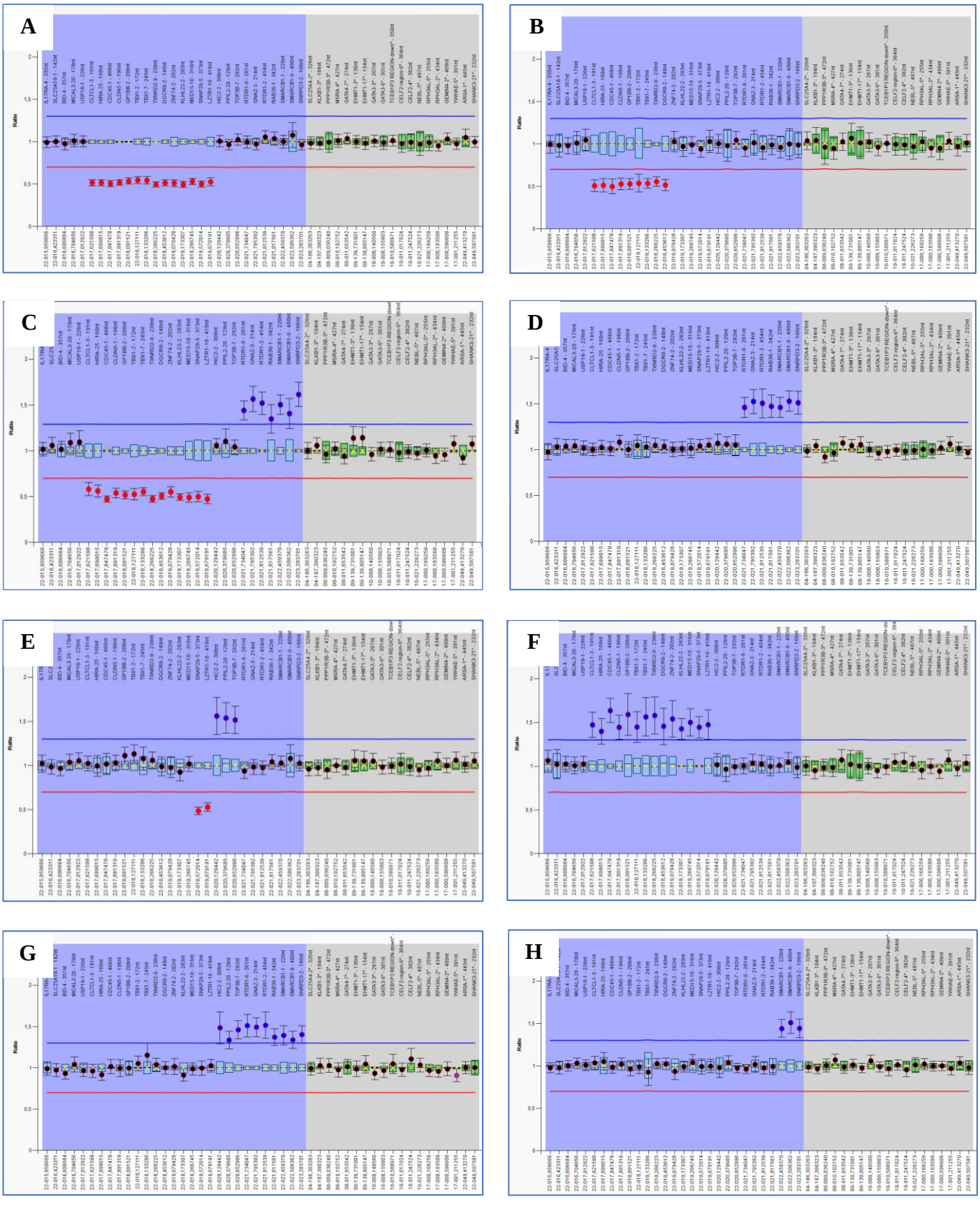
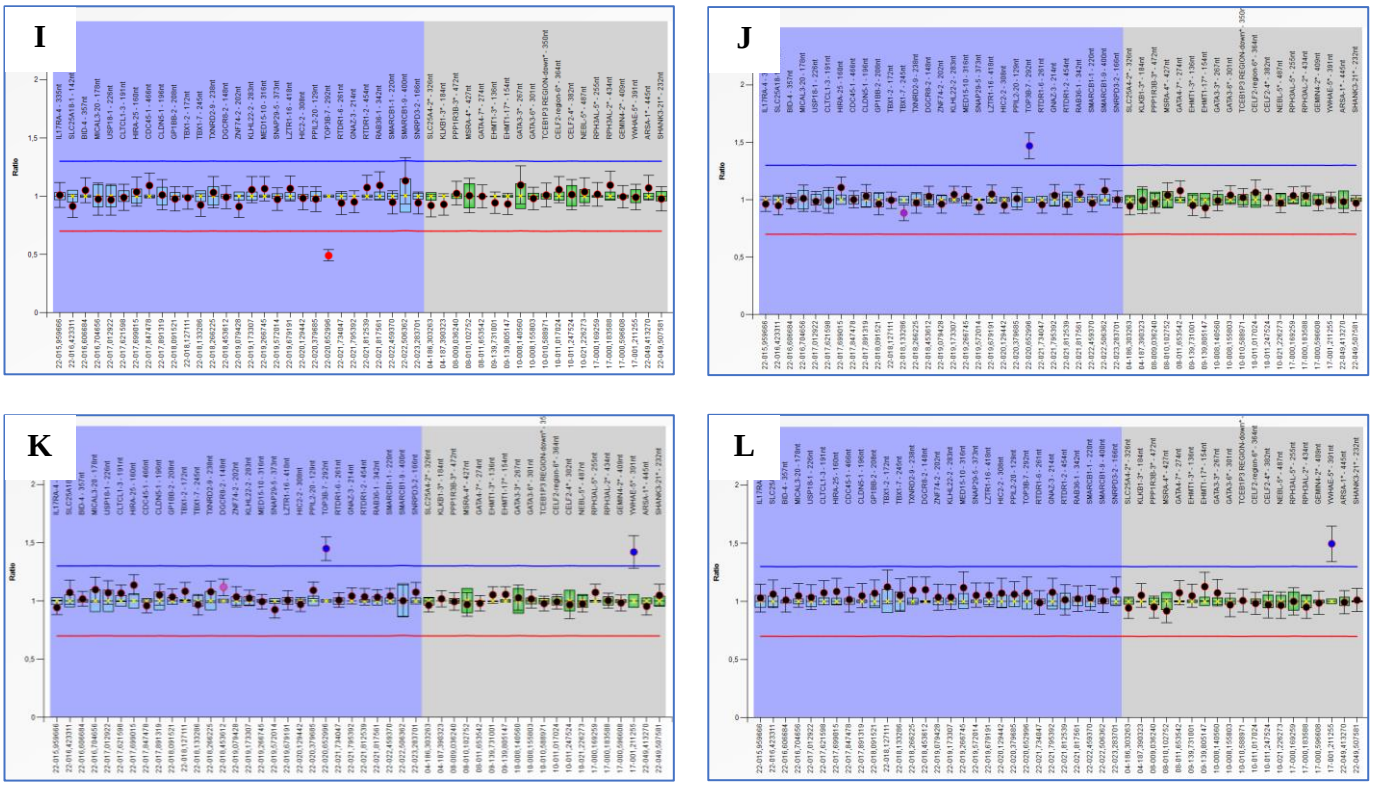


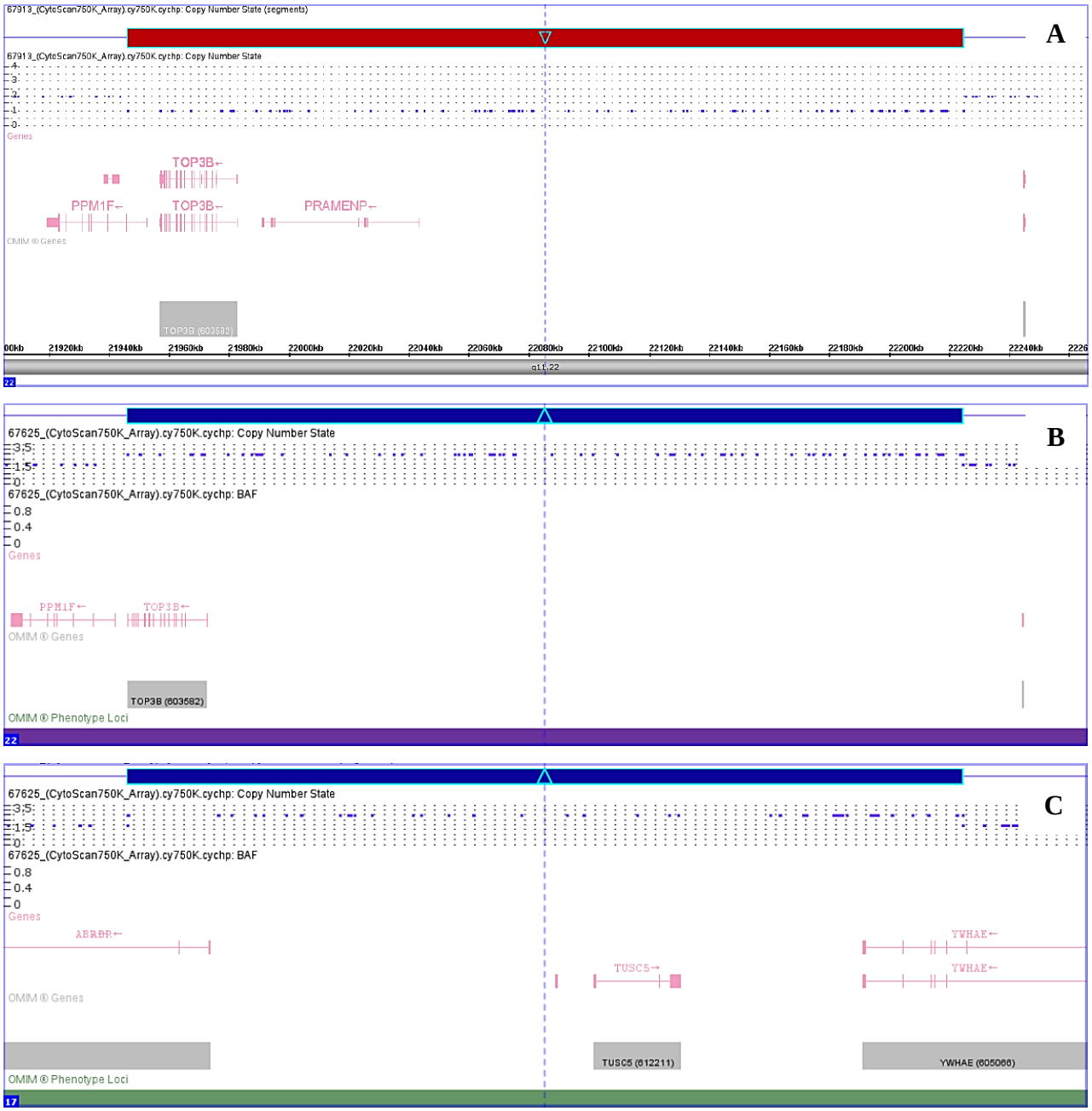
Figure 5 continued



A: Classical LCR A-D deletion in probands P38, P104, P115, P138, P146, P208; B: Nested proximal LCR A-B deletion in proband P39; C: Classical LCR A-D deletion and distal LCR E-H duplication in proband P09 and her mother; D: Distal LCR E-H duplication in family members of proband P09; E: Central LCR C-D deletion and LCR D-E duplication in proband P56 and in the proband's mother; F: Proximal LCR A-D duplication in probands P134 and P185; G: Central-distal LCR D-H duplication in proband P209; H: Distal LCR F-H duplication in proband P165; I: TOP3B probe deletion in proband P54; J: TOP3B probe duplication in probands P51 and P69 and family members; K: TOP3B and YWHAE probe duplication in proband P32 and her mother; L: YWHAE probe duplication in family members of proband P32

Based on the interpretation guidelines (Riggs et al., 2020), 11 CNVs were interpreted as pathogenic variant: 6 were typical deletions of LCR A-D (P38, P104, P115, P138, P146 and P208), 1 proximal nested deletion of LCR A-B (P39), 2 duplications of LCR A-D (P134 and P185), a combination of the proximal deletion of LCR A-D with duplication of LCR E-H (P09) and a duplication of LCR D-H (P209). Two further CNVs (one combination of a central deletion of LCR C-D with duplication of LCR D-E (P56) and one duplication of LCR F-H (P165)) were classified as VUS (Figure 4, Table 5). Four CNVs were also detected in the *TOP3B* gene (P54, P32, P51 & P69) (Figure 5I-5L). These four CNVs (three 268-kbp duplications and one 278-kbp deletion) in the *TOP3B* gene occurred due to one probe alteration in the MLPA reaction. These CNVs were subsequently confirmed with chromosomal microarray analysis (Figure 6).

Figure 6 CNVs confirmed by chromosomal microarray analysis



A: 278 kbp microdeletion in 22q11.22 in proband P54, arr[GRCh38] 22q11.22(21,946,279-22,224,671)x1. B: 268 kbp microduplication in 22q11.22 in proband P32, arr[hg19] 22q11.22(22,311,348-22,579,775)x3, *TOP3B* (#603582). C: 201 kbp microduplication in 17p13.3 in proband P32, arr[hg19] 17p13.3(1,070,538-1,271,913)x3, *ABR* (#600365), *TUSC5* (#612211), *YWHAE* (#605066)

Pathogenic results were highest in the TOF group of the CHD cohort (17% of all TOF patients), followed by the bicuspid aortic valve group (10%) (Figure 4, Table 4).

Table 4 Distribution of pathogenic and VUS 22q11 CNVs in the various CHD groups

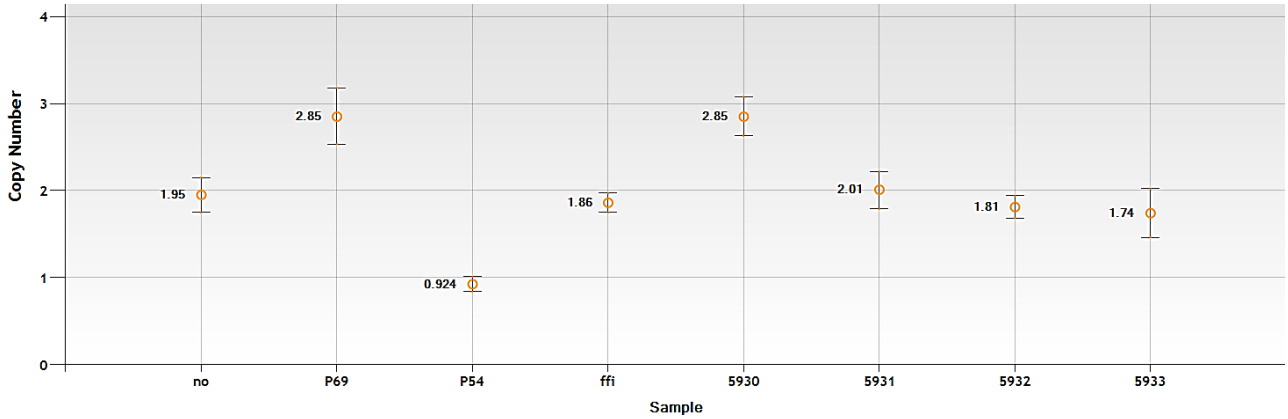
TYPE OF CHD	NUMBER OF PATHOGENIC CNVs OR VUS / NUMBER OF PATIENTS WITH CHD (%)	DEL.	DUPL.	DEL+DUPL
Fallot IV	**5 path / 30 (17%)**	**4**	0	**1**
Bicuspid aortic valve	2 path / 19 (10%)	**1**	**1**	0
AoS	2 path / 31 (6.5%)	0	**2**	0
CoA	1 VUS / 17 (6%)	0	0	**1**
VSD	1 path/ 36 (2.7%) 1 VUS/36 (2.7%)	**1** 0	0 **1**	0 0
TAPVD + VSD + PA	1path / 4 (25%) *	**1**	0	0
TOTAL	**11 path/212 (5.2%)** **2 VUS/212 (0.9%)**	**7** **0**	**3** **1**	**1** **1**

VUS: variant of unknown significance; CHD: congenital heart defect; CoA: coarctation of the aorta; AoS: congenital aorta stenosis; Fallot IV: tetralogy of Fallot; TAPVD: total anomalous pulmonary venous drainage; VSD: ventricular septal defect; PA: pulmonary atresia; DEL.:22q11microdeletion; DUPL.:22q11 microduplication. *This proportion is biased since this patient could also be classified in the VSD or PA group.

3.1.3 Determination of the frequency of TOP3B CNVs

TOP3B CNVs were identified in a relatively high proportion in our patient cohort. We recorded in overall four patients out of the 212 (19%): deletion in 1/212, 0.5% and duplication in 3/212, 1.4%. Considering this relatively high proportion in our patient cohort we decided to perform an independent analysis with ddPCR to determine the frequency of *TOP3B* CNVs in the healthy controls. *TOP3B* deletion was detected in one control sample (0.5%) and a duplication in four control samples (1.9%) (Figure 7). Altogether, CNVs were identified in 2.4 % of the controls (5/211). The difference in the CNV frequency between patients and controls was not significant (p=0.751). Thus, we ultimately classified *TOP3B* CNVs as rare benign variants, which are more frequent in the Hungarian population than in the global database (frequency in DECIPHER: 0.36%).

Figure 7 Droplet digital PCR results of *TOP3B* copy number variants in the patient and control samples.



no, ffi: Reference samples with normal 2 copies of *TOP3B*. P69: CHD-sample with *TOP3B* duplication. P54: CHD-sample with *TOP3B* deletion. 5930: Healthy control sample with *TOP3B* duplication. All other samples are healthy controls with normal *TOP3B* copy number.

3.1.4 Familial segregation

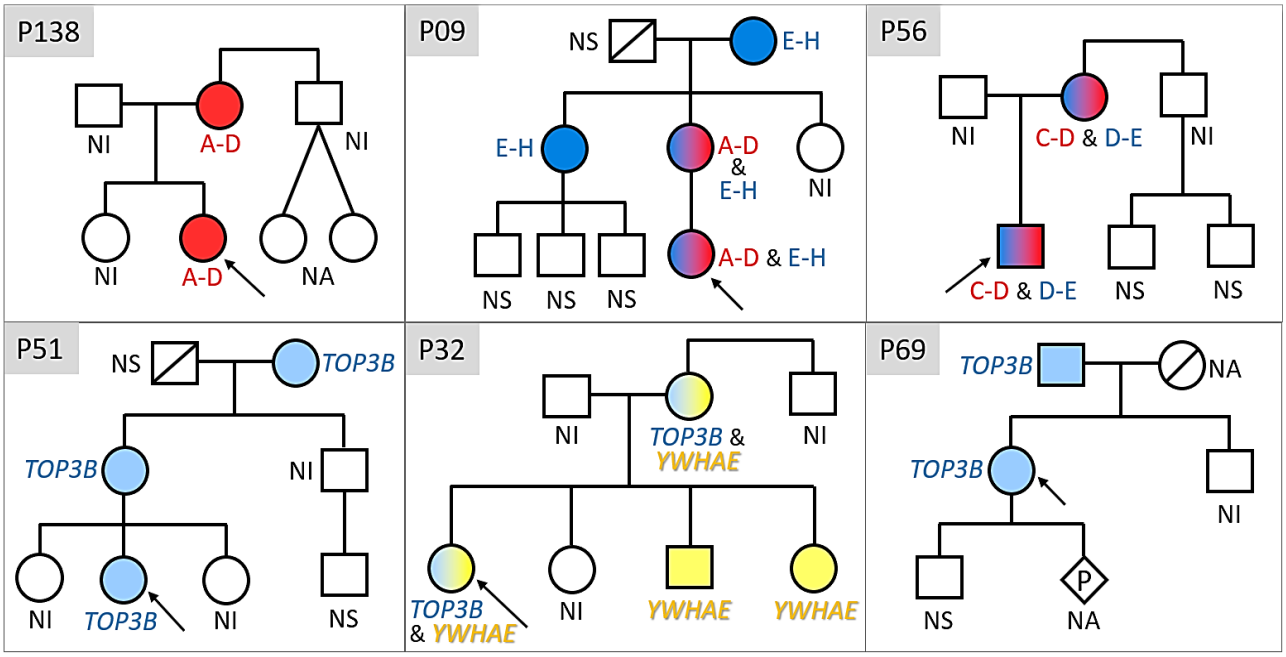
We were able to perform segregation analysis for 14 of the 17 positive CNVs cases. Six cases proved to be familial, two of these were found for patients with pathogenic CNVs, one for a patient with VUS and three for patients with benign *TOP3B* variants (Figure 8).

The segregation analysis detected familial occurrence for 18% (2/11) of the pathogenic CNVs and 50% (1/2) of VUS. In these three cases (P09, P56 and P138), the probands' mother carried the same chromosome imbalance. The phenotype of the mothers was of the same severity (P138) or milder (P09, P56) than the probands'. The suspicion of an underlying 22q11.2 CNV prior to the genetic testing was not raised at any of the affected family members.

For proband P09 and her mother, the typical ~2.5-3 Mb-large 22q11.2 microdeletion was combined with a distal ~1.5-2 Mb-large 22q11.2 microduplication. The segregation analyses of the family showed that the maternal grandmother and one sibling of the mother carried only the duplication with no cardiological symptom or developmental malformation, even though they had been diagnosed with mild anxiety disorder and depression. The deletion occurred most probably *de novo* in the mother and was transferred to the child.

The mother of proband P56 had only a bicuspid aortic valve (clinically diagnosed after the genetic diagnosis) and similar facial features as the proband without torticollis or severe scoliosis. The detailed clinical features of patients and their family members are shown in (Table 5).

Figure 8 Segregation analyses of the familial CNVs



Dark red: pathogenic microdeletion; Dark blue: pathogenic microduplication; Light blue and yellow: benign microduplication. NA: not analyzed; NI: no imbalance, NS: no symptom; P: pregnancy; Arrow indicates the proband. Proband in our study was defined as the first family member in whom CNV was detected.

A-D: the typical ~2.5-3 Mb microdeletion in region 22q11.21; C-D: ~0.5 Mb central microdeletion in region 22q11.21; D-E: ~1.2 Mb central microduplication in region 22q11.21q11.22; E-H: ~1.55-2 Mb distal microduplication in region 22q11.22q11.23; *TOP3B*: microduplication including *TOP3B* gene in region 22q11.2; *YWHAE*: microduplication including *YWHAE* gene in region 17p13.3.

Table 5 Clinical features of probands and parents with pathogenic and VUS MLPA results

Proband no./ Age*	CNV Extension (size)	Classification**	Type of CHD	Extracardiac manifestations	DD/ID	Facial features	Classical phenotype***	Hypocalcemia	Miscellaneous	Familiar inheritance or *de novo* mutation
P38/ Adulthood	**DEL LCR A-D** in 22q11.21 (~2.5-3 Mb)	Path	TOF	Recurrent bronchitis and otitis media, tonsillectomy, vesicoureteral reflux, renal cyst, velopharyngeal insufficiency, nasal speech, early teeth lost, small stature, kyphoscoliosis, block vertebrae, lower leg cramps, dyslexia, anxiety disorder, microcephaly, juvenile cataract, autoimmune hypothyroidism	DD/mild ID	Narrow face, micrognathia, low-set ears, narrow, small palpebral fissures, hypertelorism, hypoplastic alae nasi, pointed ear tips, thin lips	Yes	Yes	Obstipation, GOR, feeding difficulties in childhood, hypomagnesemia	*De novo*
P104/ Adulthood	**DEL LCR A-D** in 22q11.21 (~2.5-3 Mb)	Path	TOF	Arrythmia (radio frequent ablation), recurrent otitis media in childhood (Grommet tubes, adenotonsillectomy), inguinal hernia, scoliosis, mild kyphosis, thorax asymmetry, narrow shoulders, recurrent urinary infections, nephrolithiasis, nasal speech, learning difficulties.	DD/low normal IQ	Narrow, long face, narrow palpebral fissures, deep-set eyes, marked hypertelorism, large ear lobes, thin small lip, hypoplastic alae nasi, malar flattening	Yes	Yes	Lumbago, pulmonary embolism. Deceased postoperative in nosocomial infection before the genetic diagnosis	*De novo*
P115/ Adulthood	**DEL LCR A-D** in 22q11.21 (~2.5-3 Mb)	Path	VSD, PDA	Right main bronchus stenosis, congenital lacrimal duct stenosis, many recurrent upper and lower airway infections until puberty, severe scoliosis, hernia diaphragm + severe GOR (fundoplication), inguinal hernia (operated), palatoschisis, velopharyngeal insufficiency, thorax and hip deformity, learning difficulties, nasal speech, episodic hand tremor and foot paresthesia, small stature, microcephaly	DD/ low normal IQ	Narrow, long face low-set ears, narrow, small palpebral fissures, hypertelorism, hypoplastic alae nasi, pointed ear tips, thin small lips, malar flattening, mild facial asymmetry	Yes	-	Nasogastric tube feeding in infancy	*De novo*

P138/ Childhood	**DEL LCR A-D** in 22q11.21 (~2.5-3 Mb)	Path	Bicuspid aortic valve	Recurrent lower and upper airway infections and otitis media, nasal speech, hypermetropy, astigmia, no developmental delay, normal kindergarten and preschool	No/No	Long face, small mouth, straight nose, narrow eyelids, hypertelorism, pointed ear tip, fleshy ear lobes	Yes	Yes	Severe obesity (due to diet failure), secondary hypertonia	Maternally inherited: mother has similar outer appearance, umbilical hernia, vestibular neuronitis, impaired hearing, but no CHD
P146/ Adulthood	**DEL LCR A-D** in 22q11.21 (~2.5-3 Mb)	Path	TOF	Recurrent respiratory infection and otitis media, mastoiditis, cryptorchism (orchidopexy), severe scoliosis, thorax asymmetry (small right scapula), nasal speech, neuropsychiatric problems, learning difficulties, special school	Speech delay/mild ID	Narrow, long face, straight nose, low-set ears, narrow palpebral fissures	Yes	Yes	Brain MRI and abdominal ultrasound: normal	*De novo*
P208/ Childhood	**DEL LCR A-D** in 22q11.21 (~2.5-3 Mb)	Path	TPAVD, PA, VSD	Thymus aplasia, many respiratory infections in small childhood, prolonged Candidiasis, small stature	DD/mild ID	Narrow face, micrognathia, low-set ears, narrow, small palpebral fissures	Yes	Yes	Epidermal skin problems	*De novo*
P39/ Adulthood	**DEL LCR A-B** in 22q11.21 (~2.5-3 Mb)	Path	TOF	Recurrent respiratory infections, learning difficulties, special school, neuropsychiatric problems	DD/low normal IQ	Narrow face, low-set ears, narrow, small palpebral fissures	Yes	-	-	ND
P09/ Childhood	**DEL LCR A-D** in 22q11.21 (~2.5-3 Mb) + **DUPL LCR E-H** 22q11.22q11.23 (~1.55-2 Mb)	Path + VUS	TOF	Congenital laryngeal stenosis, thymus aplasia, respiratory infections (postoperative as well), pes calcaneovalgus	? / ?	Small mouth, pointed ear tips, hypoplastic alae nasi, low-set ears	Yes	No	Small for gestational age, transient nasogastric tube feeding	Maternally inherited. mother: TOF, severe scoliosis, nasal speech, classical DGS phenotype, low normal IQ, anxiety disorder
P56/ Adulthood	**DEL LCR C-D** in 22q11.21 (~0.5 Mb) + **DUPL LCR D-E** 22q11.21q11.22 (~1.2 Mb)	VUS + VUS	CoA	Torticollis, scoliosis, nasal speech, socially withdrawn, studies in higher education	No/No	Facial asymmetry, pointed ear lobes, small philtrum, low-set ears, triangular chin, retrognathia	No	No	-	Maternally inherited: mother has bicuspid aortic valve, similar facial features without asymmetry and torticollis
P134/ Adulthood	**DUPL LCR A-D** in 22q11.21 (~2.5-3 Mb)	Path	AoS	None	No/No	No	No	No	Atopy, asthma	*De novo*

P185/ Adulthood	**DUPL LCR A-D** in 22q11.21 (~2.5-3 Mb)	Path	AoS	None	No/No	No	No	No	-	ND
P209/ Adulthood	**DUPL LCR D-H** 22q11.21q11.23 (~3.1-3.5 Mb)	Path	Bicuspid aortic valve	Horseshoe kidney, pyeloureteral stenosis, Ewing-sarcoma in childhood, frequent tonsillitis (tonsillectomy), primary amenorrhea, special school	Speech delay/mild ID	Hypertelorism, divergent strabismus, prominent long mandible, uvula elongata	No	No	Obesity. Twin sibling died of pulmonary atresia after birth	No CNV in father, mother not tested
P165/ Adulthood	**DUPL LCR F-H** in 22q11.23 (~1-1.2 Mb)	VUS	VSD	Myopia, bilateral inguinal hernia, truncal obesity, learning difficulties	No/No	Micrognathia	No	No	Preterm birth, normal catch-up development, bronchial asthma	*De novo*

CHD: congenital heart disease; CNV: copy number variation; LCR: low copy repeat region; TOF: Tetralogy of Fallot; AoS: congenital stenosis of the aorta; PA: pulmonal atresia; VSD: ventricular septal defect; TPAVD: Total anomalous pulmonary venous return; CHD: congenital heart disease; CoA: coarctation of the aorta; AVSD: atrioventricular septal defect. ND: not done; DD: developmental delay; ID: intellectual disability, GOR: gastro-esophageal reflux; Path: pathological; VUS: variant of uncertain significance. * Denotes the life period when the genetic diagnosis was set up. ** Denotes the classification of CNVs based on the joint consensus of ACMG and ClinGen Guidelines (Riggs et al, 2020). ***Classical phenotype of 22q11.2 deletion syndrome, previously described as DiGeorge syndrome.

In addition to the *TOP3B* microduplication, proband P32 also had a 201bp microduplication on chromosome 17p13.3, which included the *YWHAE* gene (Figure 6 and 8). The analysis revealed both chromosomal imbalances were inherited from an asymptomatic parent. Two asymptomatic siblings also carried the *YWHAE* microduplication but without the *TOP3B* CNV. Therefore, the *YWHAE* CNV was interpreted as a rare benign variant.
In the other *TOP3B* microduplications (P51 and P69), a healthy parent also carried the variant (Figure 8). In the patient with *TOP3B* deletion (P54, Figure 8), segregation analysis was not performed; the proband had two healthy children. The individuals with *TOP3B* CNVs were excluded from the genotype-phenotype comparison based on these results, the high frequency of *TOP3B* CNV in the controls and the fact that these patients exhibited no other malformations or comorbidities in addition to CHD.

3.1.5 Genotype-phenotype comparison

The probands' age at the genetic diagnosis with pathogenic or VUS 22q11.2 CNVs ranged from 2 months to 52 years (median age: 21 years). Three patients out of 13 were diagnosed in childhood, one child in the first year of life. The two oldest patients and the affected family members were born before the molecular diagnostic era. No correlation could be observed between the severity of the phenotype and the age at the diagnosis.
The prevalence of the common clinical features in the different CNVs is comparable to previously reported prevalence data in the literature (Table 6). Patients presented with more marked phenotypic features for 22q11.2 microdeletions than those with microduplications in the same region. Besides the CHDs, the typical microdeletions of LCR A-D with or without accompanying CNVs resulted in the classical phenotype of 22q11.2 deletion syndrome.
The co-occurring duplication in proband P09 and her mother did not modify their phenotype significantly as compared to other LCR A-D microdeletion phenotypes.
In proband P56 and his mother with the combination of central deletion and distal duplication, the phenotype differed completely from that of 22q11.2 deletion syndrome (except the CHD) and the impact of the duplication could not be determined precisely (Table 5).
Among patients with deletions, Fallot-tetralogy was the most occurring CHD. For patients with duplications: congenital aorta stenosis, coarctation of the aorta and bicuspid aortic valve were the most common CHD types (Table 4). These three entities may be described on the spectrum for one disease.

CHDs were overrepresented in our 22q11.2 CNV patients, and their affected family members, compared to the data in the literature (94% *vs* 74%), which could be the result of the patient enrolment criteria.

Neuropsychiatric disorders were underrepresented among our patients (19% vs 60%). However, other characteristics including facial features, velopharyngeal insufficiency, immunodeficiency, hypocalcemia, skeletal anomalies, developmental delay and learning difficulties had a distribution in our cohort similar to that reported in the literature (Table 6).

Table 6 Prevalence of clinical features in the 22q11.2 region (pathogenic CNVs and VUS)

Symptoms	**Classical LCR A-D deletion**	**All other deletions***	**Only Duplications**	**CNVs in this study**	**Prevalence in literature****
CHD	8/9 (89%)	3/3 (100%)	4/4 (100%)	**15/16 (94%)**	64-74%
Facial dysmorphia Classical facial features in 22q11.2 deletion	9/9 (100%) 9/9 (100%)	3/3 (100%) 1/3 (33%)	2/4 (50%) 0/4	**14/16 (87.5%)** **10/16 (62.5%)**	46-88% ND
Velopharyngeal insufficiency	7/9 (78%)	1/3 (33%)	0/4	**8/16 (50%)**	55-69%
Immunodeficiency, recurrent infections	8/9 (89%)	1/3 (33%)	1/4 (25%)	**10/16 (62.5%)**	50-77%
Skeletal anomalies	6/9 (67%)	1/3 (33%)	0/4	**7/16 (44%)**	15-50%
Other anomalies***	7/9 (78%)	0/3	2/4 (50%)	**9/16 (56%)**	67-81%
Developmental delay (motor ± speech) in childhood	6/9 (67%)	1/3 (33%)	1/4 (25%)	**8/16 (50%)**	70-90%
Intellectual disability Learning difficulties	4/9 (44%) 6/9 (67%)	0/3 1/3 (33%)	1/4 (25%) 1/4 (25%)	**5/16 (31%)** **8/16 (50%)**	28-31% 66-93%
Neuropsychiatric problems	3/9 (33%)	0/3	0/4	**3/16 (19%)**	60-73%
Hypocalcemia	5/9 (55.5%)	0/3	0/4	**5/16 (31%)**	17-60%

CHD: congenital heart defect; **ID:** intellectual disability; *In this category **LCR A-B nested deletion and LCR C-D deletion combined with LCR D-E duplication are included;** ** Data for 22q11.2 deletions described by (Burnside, 2015; Campbell et al., 2018; McDonald-McGinn et al., 1993; Niarchou et al., 2019) are presented; *** Including clinically diagnosed thymus aplasia, respiratory, gastrointestinal and/or urogenital abnormalities; ND: not determined.

Velopharyngeal insufficiency, nasal speech could not be assessed in the two youngest probands. Intellectual disability, learning difficulties were only assessed in individuals in

school age or older. Psychiatric disorders were taken into account only if they were clinically diagnosed.

The presence of immunodeficiency was deduced from the recurrence of respiratory and ear infections occurring mostly in childhood. Based on regular laboratory check-ups, the average absolute lymphocyte count was in the lower normal range (2.13 G/L, normal range: 1.5-3.2 G/L); whereas the average relative lymphocyte count was below normal (23.8%, normal range: 27-34%). Flow-cytometry and serum immunoglobulin levels were not measured regularly. Likewise, the immune status and infections of 22q11.2 CNV patients were not strictly controlled before the genetic diagnosis. Proband P104 died of a fulminant postoperative infection before genetic diagnosis (Table 5).

Hypocalcemia (average serum calcium level: 1.84 mmol/l, normal range: 2.2-2.55 mmol/l) was often present in patients with the typical 22q11.2 microdeletions with or without clinical symptoms. However, hypocalcemia was not considered relevant for therapy before genetic diagnosis. Severe hypomagnesemia was also detected in one 22q11.2 microdeletion patient. The thrombocyte count was in the normal range with an average of 156x10⁹/l. Thyroid and parathyroid hormone levels and vitamin D levels were not measured in these patients before genetic diagnosis. These laboratory abnormalities, could not be consistently identified for patients with CNVs other than the typical microdeletion.

3.1.6 *TBX1* gene sequencing

All CHD patient samples were analysed for *TBX1* variants by Sanger sequencing. There was no pathogenic variant detected in the *TBX1* gene, although three missense variants were found in exon 9: c.1189A>C; p. Asn397His with a 21% minor allele frequency (MAF), c.1049G>A; p.Gly350Asp with 0.48% MAF and c.1341_1342insCCGCACGCGCAT; p.Ala450_His453dup with 0.24% MAF (Table 7).

The frequency of p.Asn397His variant was as well 21% for the controls. The two less frequent variants were also detected in one of the proband's healthy parents and were recorded in the Hungarian or the global database with very low frequencies (Table 7).

Of the ten probands and mothers with proximal 22q11.2 microdeletions, encompassing the *TBX1* gene, two (20%) carried the common p.Asn397His variant in a hemizygous form (P09 and P138). Proband P09 exhibited a severe phenotype, while proband P138 presented only milder symptoms. The two rare variants were not detected in any of the microdeletion patients.

Based on the allele frequencies, ACMG criteria and segregation analyses, all three variants were ultimately classified as benign.

Table 7 Comparison of MAF of TBX1 variant in the CHD and control cohort

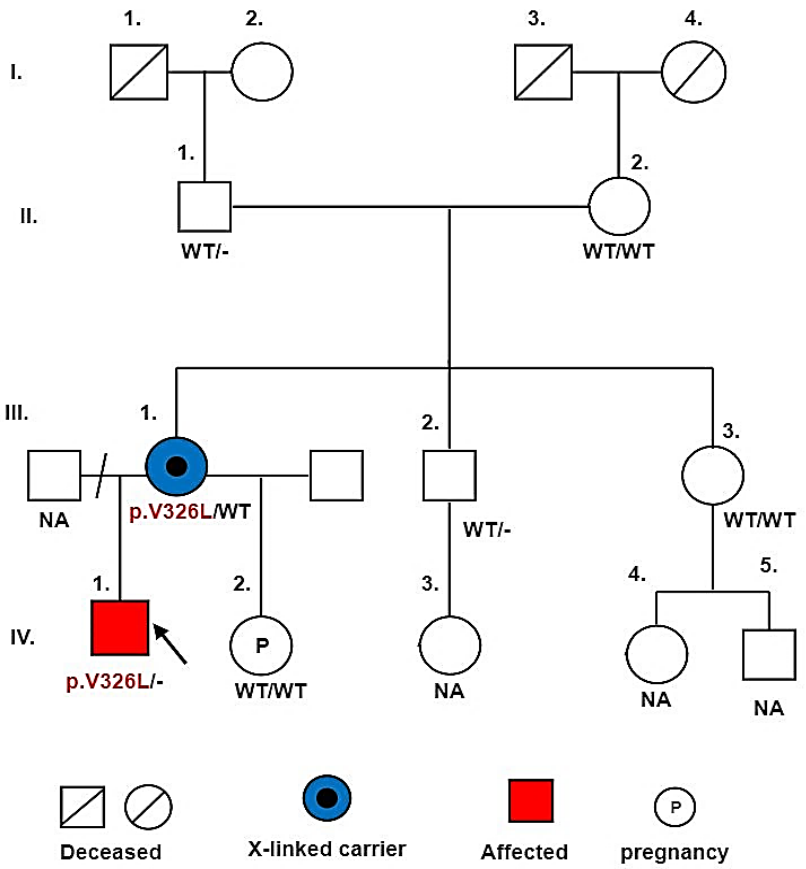
Variant	**MAF in CHD (%)**	**MAF in controls (%)**	***p***	**MAF in GnomAD (%)**	***p***	***In silico* predic-tion***
c.1189A>C; p.Asn397His (rs72646967)	21	21	*0.809*	23.19	*0.3583*	benign
c.1049G>A;p.Gly350Asp (rs781731042)	0.48	0.95	*0.686*	0.0402	***0.0138***	benign
c.1341_1342insCCGCACGCGCAT p.Ala450_His453dup (rs1341195668)	0.24	0	*0.498*	0.00325	***0.0267***	VUS

In *TBX1* locus, CHD cohort contained overall 418 alleles, control cohort 422 alleles. MAF: minor allele frequency; CHD: congenital heart defect, GnomAD. *TBX1* transcript number: NM_080647. *In silico variant prediction was performed with VarSome, based on the ACMG criteria.

3.2 *PAK3* mutation analysis

During the genetic assessment of a 14-year-old male patient with intellectual disability, a novel variant; NM_001128167.2:c.976G>C;p.(Val326Leu) (ClinVar submission number: SCV000927119; LOVD accession number: #0000578234, DB-ID: PAK3_000063) was identified with exome analysis in exon 10 of the *PAK3* gene. The variant was present in the proband in a hemizygous form and his unaffected mother in a heterozygous form but not in any other healthy family members tested (Figure 9 and 10) or in the control population databases: (141,456 whole exome/genome sequences contained in GnomAD, in +500 exome sequences of the in-house database of qGenomics or in 151 exome sequences of Hungarian patients recruited in other projects).

Figure 9 **Pedigree of the patient presenting with intellectual disability and the novel variant identified**



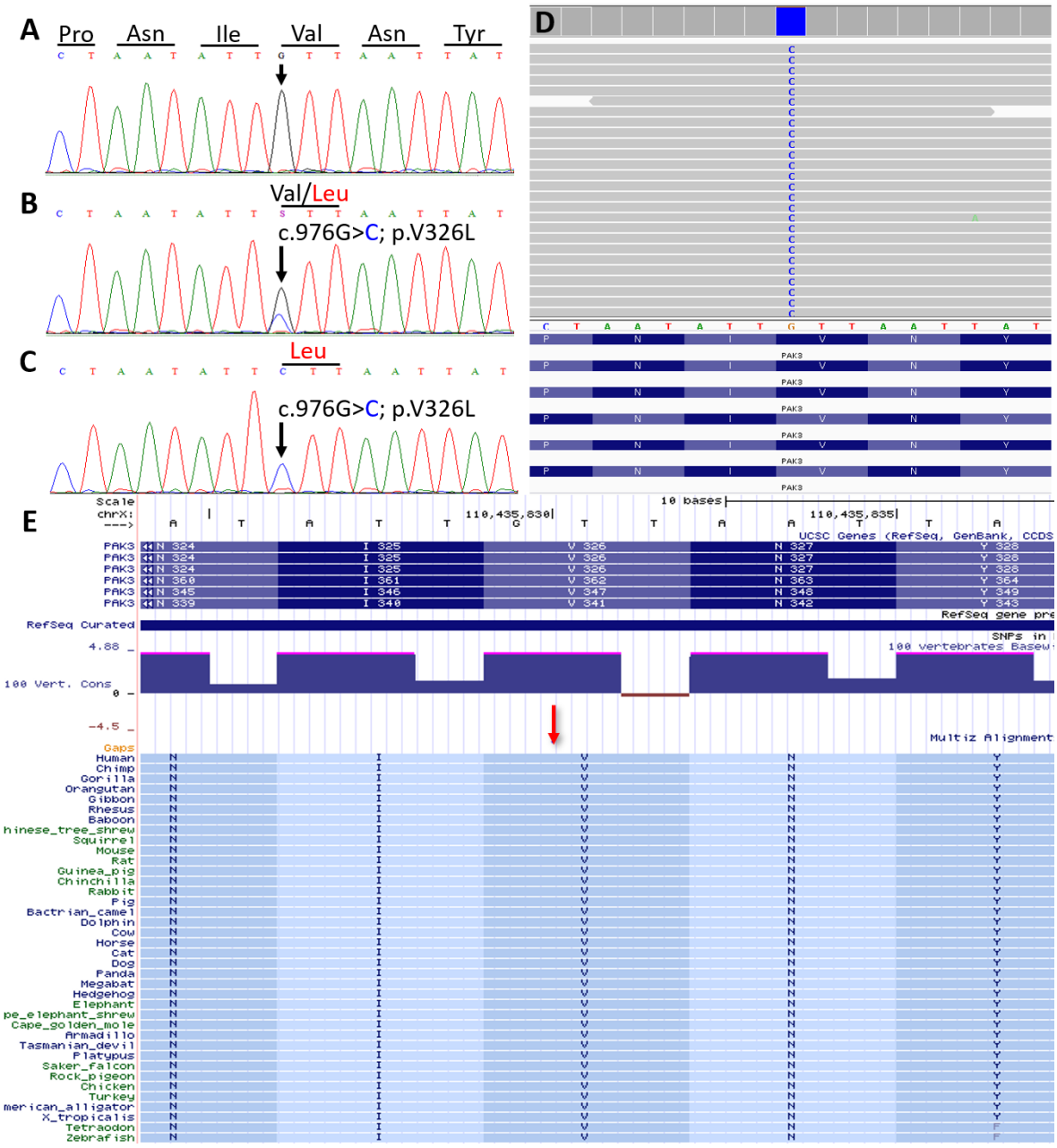
The mother of the proband was pregnant with a female, non-carrier foetus, as confirmed by karyotyping and targeted mutation analysis. Proband is indicated with an arrow. NA: not assessed. WT: Wild type.

The Val326Leu variant was predicted to be likely damaging by the *in silico* predictions. The Val326Leu variant is located in the highly conserved protein kinase domain of the *PAK3* gene (Figure 10E).

The *in silico* modelling (Figure 11) suggested that the wild-type residue Val326 is located on the surface of the ATP-binding recess of the kinase domain of *PAK3* in close vicinity to the ATP molecule (at a distance of 4.0 angstrom); however, it does not bind to ATP. The amino acid change to Leu326 resulted in a shortening of the distance between the ATP molecule and residue 326 (3.6 angstrom), a change in the surface area of the ATP-binding recess and the formation of a new hydrogen bond between residues Leu326 and Leu403, thus supporting its impact on protein structure and function.

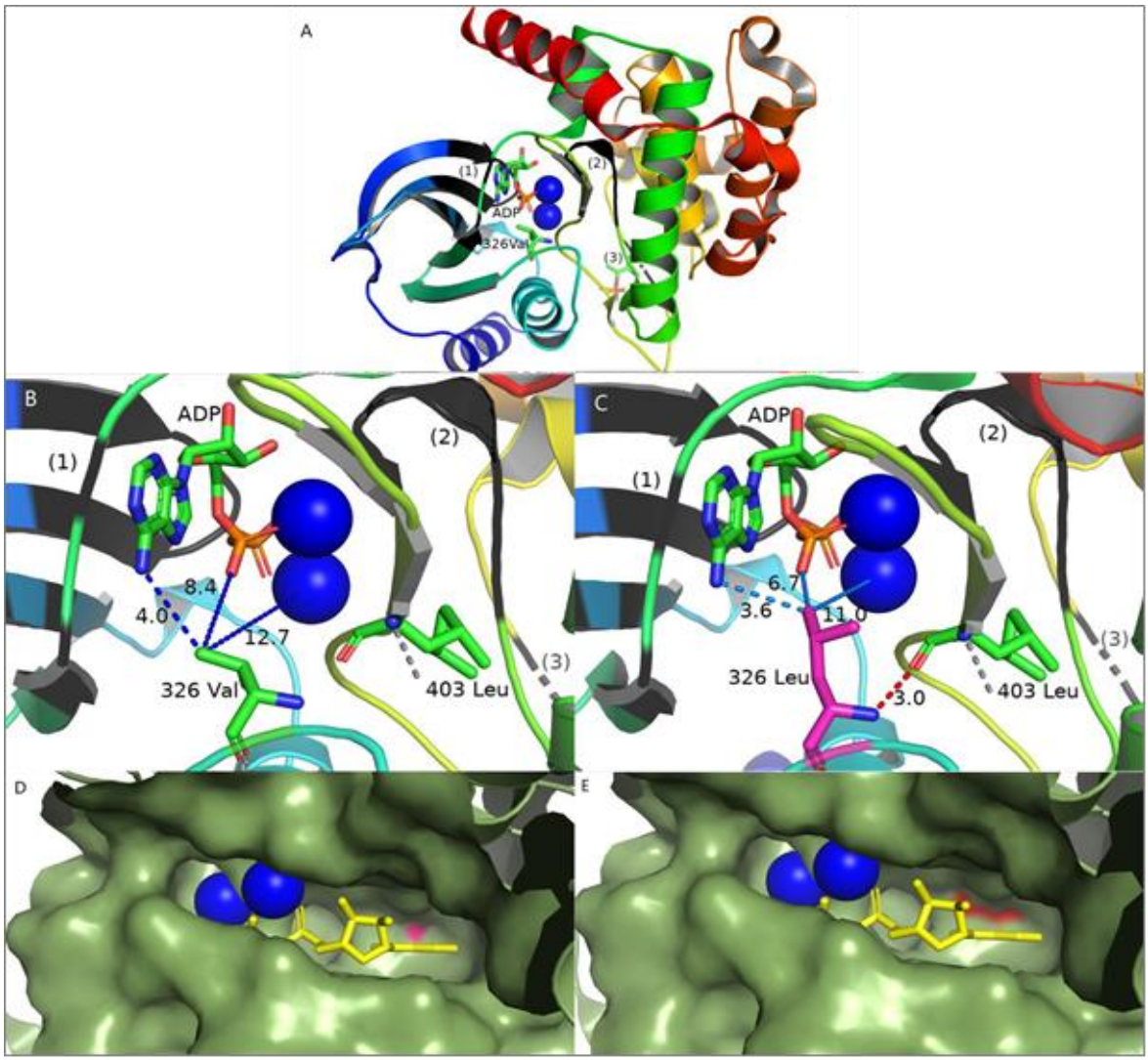
The variant occurred *de novo* in the proband's mother. Besides, based on the ACMG criteria and the detailed clinical comparison with previously described patients (Table 8: supplementary material), the results supported the ethiopathogenicity of the novel Val326Leu*PAK3*-variant.

Figure 10 Analysis of clinical exome sequencing and bidirectional Sanger sequencing



Electropherogram of the **(A)** wild-type sequence, **(B)** heterozygous female carrier (mother) and **(C)** hemizygous proband. **(D)** The screen shot from the Integrative Genomic Viewer shows part of the (51/51) reads supporting the c.976G>C variant in the proband. **(E)** Screen shot from the UCSC Genome Browser represents the high conservation of the amino acid residue in position 326 and surrounding genomic context. Arrows indicate the nucleotide change.

Figure 11 ***In silico*** **modelling of the PAK3 kinase domain of wild-type and mutant proteins**



Magnesium-ions are represented as blue spheres, adenosine-5'-diphosphate (ADP) molecule and amino acid residues of interest as sticks. Stick color indicates carbon (green), nitrogen (blue), oxygen(red) and phosphorus (orange) atoms. Important positions are indicated based on the RCSB Protein Data Bank description, residues are numbered according to the sequence of the NCBI reference transcript (number NM_001128167.2).

(A) Cartoon representation of the three-dimensional model of the kinase domain of the wild-type PAK3 protein, which consists of a smaller N-terminal part, composed mostly of β-sheets (in the left lower part), and of a larger C-terminal part, composed mostly by α-helices (in the right upper part). Black loops and residues indicate: (1) the ATP-binding site, residues 274–282, 295, 297, 342–343 and 345, (2) the magnesium-binding catalytic site, residues 387–394, and (3) the activation site withthe phosphorylated Thr^{421} residue.

(B) Enlargement of the PAK3 kinase domain, showing the wild-type Val^{326} residue in green and **(C)**the mutant Leu^{326} residue in magenta. Grey dotted lines represent the hydrogen bonds between residues, blue dotted lines the distance in angstrom between ADP and Val^{326} residue and red dotted line the new hydrogen bond between Leu^{326} and Leu^{403} with a 3-angstrom distance.

(D) Surface modelling of the ATP-binding site of the wild-type PAK3 protein and **(E)** of the mutantPAK3 protein. Yellow sticks indicate ADP molecule. Magenta coloration indicates the contribution of the wild-type Val^{326} residue and red coloration the contribution of the mutant Leu^{326} to the overallsurface of the ADP-harboring area.

4 DISCUSSION

4.1 Copy number variants in the CHD cohort

Our study was the first systemic, large-scale genetic screening study conducted in Hungarian CHD patients. All patients with cardiological verified CHDs were enrolled in the study without further selection. Although the enrolled patients were cardiologically diagnosed with non-syndromic CHDs prior to this study, 13 were found to be syndromic after the genetic screening. We observed a higher median age (21 years) and a similar or wider age range (0.17-52years) at the genetic diagnosis in our cohort as compared to previously described cohorts (median age: 17.3 years, range: 0.1-59.4 years in Canadian patients: median age: 2.9 years, range: 0-17.6 years in US patients) (Palmer et al., 2018). This difference may partly be explained by the fact, that the 22q11.2 duplication patients with more variable phenotypes were also included in the present study, whereas Palmer and colleagues analyzed only the 22q11.2 deletions.

The frequency of CHDs was representative and corresponded to the frequency described in large epidemiological studies (van der Linde et al., 2011). All types of CNVs in the 22q11.2 chromosomal region were present in 8% of the CHD cohort, while pathogenic CNVs in 5.2%, VUS in 0.9% and benign CNVs in 1.9%.

Our patients presented pathogenic 22q11.2 CNVs more often compared to other CHD cohorts, such as 1.27% in Brazilian, 2.8% in Cameroonian and 2.9% in Chinese population (Huber et al., 2014; Z. Li et al., 2019; Wonkam et al., 2017). However, this difference may also be explained by the fact that most of these studies focused on the detection of 22q11.2 deletion but not on duplications.

For tetralogy of Fallot, the proportion of pathogenic CNVs was significantly higher, 17% in our cohort, which corresponds with literature on the basis that 22q11.2 CNVs are common in conotruncal heart defects (McDonald-McGinn et al., 1993). 22q11.2 deletion can be detected in approximately 20% of all conotruncal heart defects (Wozniak et al., 2010), within this category its prevalence can be as high as ~50% in interrupted aortic arch type B, ~35% in truncus arteriosus or 10-25% in tetralogy of Fallot (Goldmuntz, 2020). All these suggest an absolute indication for 22q11.2 CNV analysis in these CHD groups, especially when co-occurring with at least one extracardiac manifestation or dysmorphic traits (Wozniak et al., 2010). The congenital bicuspid aortic valve is common (0.5–2%) and, without the complication of stenosis, regurgitation or dissection, highly considered as a benign congenital heart defect

(Y. Li et al., 2017). Therefore, based on our result, it should not be ignored during genetic testing.

The most common pathogenic CNV was the typical microdeletion of the LCR A-D region on chromosome 22q11.2 (64% of all pathogenic variants), which corresponds with the literature (Du et al., 2020). Towards the LCR F-H region, the frequency of deletions decreased. This was reflected in our results as well, since nested and central deletions were rare and distal deletions were not detected in our cohort. Proximal and distal duplication, likewise, two combined CNVs were also identified: LCR A-D deletion combined with LCR E-H duplication and a rare LCR C-D deletion combined with LCR D-E duplication. Although most patients with the typical LCR A-D deletion showed the majority of the characteristic features of 22q11.2 deletion syndrome (velopharyngeal insufficiency, skeletal malformation, gastrointestinal and nephrological anomalies, hypocalcemia, frequent infections due to immunodeficiency and common facial features), these symptoms were present less frequently (Table 6) with the non-typical deletions and duplications, as expected (Burnside, 2015; Du et al., 2020). In addition to the presence of CHDs, no typical common characteristics were detected for these patients. These could be due to the low number of patients with single CNVs in our cohort or to the even wider phenotypic spectrum of these CNVs, for instance, in the case of 22q11.2 duplications (A. Yu et al., 2019). Therefore, several individuals with only mild symptoms or no detectable malformations or dysmorphy may remain undetected.

Hypocalcemia, hypomagnesemia, lymphocytopenia, thrombocytopenia and abnormalities of the thyroid, parathyroid hormones or vitamin D levels may remain undiscovered in 22q11deletion patients, most especially in the absence of genetic diagnosis. Nevertheless, these conditions may significantly contribute to co-morbidities, such as increased susceptibility to infections, bleeding diathesis and heightened prevalence of autoimmune disorders, and thus, should be considered for treatment (Goldmuntz, 2020; Lambert et al., 2018; Legitimo et al., 2020).

Neuropsychiatric disorders (attention deficit hyperactivity disorder, autism spectrum disorder, schizophrenia, anxiety symptoms and sleep disturbances) are frequent in patients with 22q11.2 CNVs (Brzustowicz and Bassett, 2012; Moulding et al., 2020). Complex presentation of three or more psychiatric traits may occur in 73% of patients with 22q11.2 CNVs (Chawner et al., 2019; Niarchou et al., 2014 and 2019). Though these were markedly underrepresented (19%) in our patient cohort (Table 6), and this is most probably due to the lack of awareness and screening rather than to their absence. This result further emphasizes the importance of multidisciplinary management of patients with 22q11.2 CNVs.

The large phenotypic variability of 22q11.2 microdeletions has recently been the focus of much research but is still not yet fully understood. Haploinsufficiency of the coding genes, including *TBX1, DGCR8, CRKL* among others, alone does not seem to account for the highly variable phenotypes and incomplete penetrance of the affected individuals (Du et al., 2020). Some recent studies have investigated the role of possible genetic and epigenetic factors which contribute to the diversity of phenotypes associated with 22q11.2 deletions (Bertini et al., 2017; Brzustowicz & Bassett, 2012; Du et al., 2020).

Breakpoint analysis of the LCR A-D region showed that small variations in the deletion size within this region have no significant role in phenotypic variability (Bertini et al., 2017). Pathogenetic sequential variations in the remaining single copy of the genes (with an emphasis on *TBX1* gene) encompassed in the deletion region, were not yet revealed by previous investigation (Brzustowicz & Bassett, 2012). This was further supported by our *TBX1* sequencing results since no pathogenic *TBX1* variant was detected in our patients with 22q11.2 CNVs or the overall CHD-cohort. Thus, pathogenic *TBX1* mutations may be causal most probably only in a small fraction of CHD patients and 22q11.2 CNV patients, if at all. It was also hypothesized that sequential variations elsewhere in the genome (for example, *de novo* mutations in histone-modifying genes) may collectively contribute to this diversity (Zaidi et al., 2013).

Bertini and colleagues have investigated additional rare and common CNVs in typical 22q11.2 patients (2017). According to their results, these additional CNVs often contain miRNA genes or mitochondrial genes, which may interact with 22q11.2 deletion and lead to metabolic and energetic problems rather than a decreased dosage of morphogenetic genes. The *DGCR8* gene is located within the typical 22q11.2 region and plays a crucial role in miRNA biosynthesis and, in combination with other CNV-miRNAs, may orchestrate highly variable phenotypic outcomes (Bertini et al., 2017). Previously, these additional CNVs, miRNAs have been investigated exclusively for 22q11.2 microdeletion patients, but not for duplication patients. Studying these duplication patients may further refine diversity.

The family segregation study proved to be beneficial in cases with pathogenic CNVs, since in 18% further affected family members were identified. The number of familial cases was higher in our cohort than the previously described 6-10% (Goldmuntz, 2020; McDonald-McGinn et al., 1993). The affected family members of our cohort exhibited similar or milder symptoms than the probands. This phenomenon has already been observed (Goldmuntz, 2020; Wozniak et al., 2010).

22q11.2 is considered one of the most unstable regions of the human genome, due to the low-copy repeat regions on chromosome 22. This instability predisposes the region to deletions and duplications through non-allelic homologous recombination events. Hence, the presence of a parental CVN may trigger the development of another CNV in the same or nearby chromosomal region in the offspring, as seen in family P09 and as described by Capra and colleagues (Capra et al., 2013).

In conclusion, based on the present results and those described in the literature (Goldmuntz, 2020; Z. Li et al., 2019; Wozniak et al., 2010),we suggest the implementation of the genetic screening of CNVs in the postnatal management of CHD patients, regardless of the type of CHDs. For this purpose, MLPA is a cost-effective, fast and specific method suitable for the screening of a large number of samples. Early diagnosis allows better patient care through regular laboratory check-ups, cardiological, orthopedic, endocrinological, immunological, neurodevelopmental, psychiatric examinations and follow-ups. Additionally, informed decisions can be made to enable positive family planning.

4.2 Intellectual disability and *PAK3* gene

Concerning non-syndromic X-linked intellectual disability, this study and to the best of our knowledge, this family is the first Hungarian and the tenth family reported worldwide with *PAK3*-associated non-syndromic XLID. Previously, one nonsense, one splice site and seven missense mutations have been reported for the *PAK3* gene. Eight of the ten mutations are located in the kinase domain of the protein, which could have probably deactivated its enzymatic function. The site of the Leu326 mutation in the kinase domain and the presence of an extra hydrogen bond formation suggests that it may influence the ATP-binding capacity and could affect the structure of the protein. The function of PAK3-protein and its regulation is highly complex. Loss-of-function mutations in the gene are thought to result in decreased neural plasticity and cognitive impairment without major structural brain abnormalities. However, brain developmental abnormalities have been reported in some patients carrying variants of the *PAK3* gene, which may be a consequence of *PAK3* protein involvement in other signalling pathways (Magini et al., 2014).

We also provide a thorough, comprehensive clinical review of *PAK3*-patients described in the literature to date (Table 8: supplementary material), which helped us deduce the typical phenotypic features in *PAK3*-XLID: microcephaly, mild-to-moderate intellectual disability in

males, large ears, low frontal hairlines, elongated face, muscle hypotonia in infancy, drooling, seizures, aggression, anxiety and autistic behaviour. Besides, this is the first reported patient who also has occult spina bifida and mild thoracolumbar deformity, however, these findings are common in the general population and thus, may also be unrelated features.

Copy number variations in the *PAK3*-containing chromosome (Xq23) have also been reported in syndromic female patients with moderate-to-severe intellectual disability (Hoischen et al., 2009; Jin et al., 2015). However, these phenotypes are distinct from *PAK3*-XLID due to the haploinsufficiency of other genes involved.

Besides the genetic importance of the diagnosis of *PAK3*-associated XLID, it may also have therapeutic consequences, as presented in a previous report (Horvath et al., 2018). They reported their patient with epilepsy, cerebral laceration as a result of early-onset, intractable, self-injurious behaviour due to decreased levels of dopamine and serotonin metabolites in the cerebrospinal fluid. Low-dose replacement therapy drastically improved and stabilized his condition. It was hypothesized that PAK3 dysfunction may lead to diminished dendritic spines and consequentially diminished postsynaptic dopamine receptors or may impair the phosphorylation of the tyrosine hydroxylase, ultimately leading to decreased catecholamine synthesis (Daubner et al., 2011; Horvath et al., 2018).

Thus, in case of behavioural or psychiatric deterioration, determination of the neurotransmitter levels and if necessary, supplementation may be considered. However, further studies are needed for final recommendations.

In conclusion, this examination provided in-depth knowledge of the genetic and phenotypic background of the novel missense variant of *PAK3*-XLID. It also further expanded the *PAK3* mutation spectrum which may be of help to others in terms of genetic diagnosis by emphasising the common typical *PAK3*-associated features.

5 SUMMARY

Rare diseases are thought to affect a smaller number of people, but together they become a major health concern. Due to advancements in molecular techniques, early diagnosis is essential to the genetic cause of these diseases. This may improve medical care and the survival of affected individuals. Copy number variations or pathogenic single gene mutations are the cause of most of these disorders. Two main rare disease categories were in the focus of our research: congenital heart defects and non-syndromic intellectual disability.

Genetic factors, including copy number variations (CNVs), play an important role in the development of CHDs. The most common CNVs are found on chromosome 22q11.2. The genomic instability of this region, caused by the eight low copy repeats (LCR A-H), may result in several recurrent and/or rare microdeletions and duplications, including the most common, ~3Mb large LCR A-D deletion (classical 22q.11.2 deletion syndrome, known as DiGeorge syndrome). Many patients with 22q11.2 CNVs, especially in the adult population may remain undiagnosed due to the high phenotypical variability of these CNVs.

Therefore, we aimed to perform a systemic molecular genetic screening for 22q11.2 CNVs in the paediatric and adult patients of the Southern-Hungarian CHD Registry, regardless of the type of their CHDs.

All the enrolled participants were cardiologically diagnosed with non-syndromic CHDs. A combination of multiplex ligation-dependent probe amplification, chromosomal microarray analysis and droplet digital PCR methods were used to comprehensively assess the detected 22q11.2 CNVs in overall 212 CHD-patients. Additionally, capillary sequencing was performed to detect variants in the *TBX1* gene, a cardinal gene located in 22q11.2.

Pathogenic CNVs were detected in 5.2% (11/212), VUS in 0.9% and benign CNVs in 1.8% of the overall CHD cohort. In patients with tetralogy of Fallot the rate of pathogenic CNVs was 17% (5/30). Sixty-four percent of all CNVs were typical proximal deletions (LCR A-D). However, nested (LCR A-B) and central deletions (LCR C-D), proximal (LCR A-D) and distal duplications (LCR D-E, LCR D-H, LCR E-H, LCR F-H) and rare combinations of deletions and duplications were also identified.

Segregation analysis detected familial occurrence in 18% (2/11) of the pathogenic variants. Based on in-depth clinical information, a detailed phenotype–genotype comparison was performed. No pathogenic variant was identified in the *TBX1* gene, which may question the ethiopathogenetic role of TBX1 mutations alone in the development of CHDs and DiGeorge syndrome.

Our findings confirmed the previously described large phenotypic diversity in the 22q11.2 CNVs. MLPA proved to be a highly efficient and cost-effective genetic screening method for our CHD-cohort. Our results highlight the necessity for large-scale genetic screening of CHD-patients and the importance of early genetic diagnosis in their adequate clinical management.

In the frame of genetic examinations in X-linked non-syndromic intellectual disabilities we identified a novel mutation in *PAK3* gene in a 14-year-old boy.

PAK3 is a p21-activated serine/threonine kinase, an essential downstream effector in the Rho-GTPase signaling. It has been reported to play an important role in dendritic spine morphogenesis, synaptic network dynamics and neuronal plasticity. *PAK3* gene mutations have been described to cause non-syndromic X-linked intellectual disability with neuropsychiatric disorders and dysmorphic but not distinctive features in the affected individuals. Our patient presented with cognitive impairment, autistic features, temper tantrums, episodic aggression, prior episodes of convulsions, spina bifida occulta, mildly dysmorphic facial features and microcephaly without structural brain abnormalities. Exome sequencing identified a novel hemizygous missense variant in the kinase domain of *PAK3* gene (c.976G>C; p.V326L) which is interspecies highly conserved. *In silico* variant predictions, *in silico* functional modeling and segregation analysis in the family supported the ethiopathogenicity of the variant. Our detailed clinical findings together with the data from the few reported families allowed further insight in the phenotype of the disease, to expand the mutation spectrum of *PAK3* gene and support the importance of PAK3 in neural synaptic function.

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SUPPLEMENTARY MATERIAL

Table 8: Comparison of the clinical presentation of *PAK3*- XLID male patients and carrier women in the present and previous studies

	Allen et al. 1998	**Bienvenu et al. 2000**	**Gedeon et al. 2003**	**Peippo et al. 2007**	**Rejeb et al. 2008**	**Magini et al. 2014**	**Hertecant et al. 2017**	**Muthusamy et al. 2017**	**Horvath et al. 2018**	***Present study***
***PAK3* mutations**	c.1255C>T; p.R419X nonsense	c.199C>T; p.R67C missense	c.1049C>A; p.A365E missense	c.1337G>A; p.W446S missense	c.276+4A>G p.G92VfsX35 splice site	c.1167G>T; p.K389N missense	c.1279T>C; p.Y427H missense	c.880G>A; p.V294M missense	c.1579A>G; p.S527G missense	c.G976C p.V326L missense
	rs121434611	rs121434612	rs121434613	rs121434614	CS084886	CM146392	–	–	rs200474454	–
Number of patients tested	4	6	19 (13 presented)	5	4 (2 presented)	2	2	3	1	1
Number of female carriers tested	4 (unaffected)	ND	14 (unaffected)	4 (3 affected)	4 (unaffected)	3 (affected only with mild ichthyosis)	0	2 (unaffected)	2 (unaffected)	1 (unaffected)
Geographical origin	USA	France	Australia	Finland	Tunisia	Italy	United Arab Emirates	India	Canada	Hungary
Facial features	ND	ND	**Long ears** (12) Prominent nose (in 3 elderly) **Low forehead** (2) Thin upper lip (7)	**Large ears** (5 M, 0 F) **Low forehead** (3 M) Thin upper lip (5 M) **Drooling** and open mouth (1 M)	**Large ears** **Low forehead** Upslanting palpebral fissures Short nose Thick upper lip **Drooling** Large teeth	**Large ear** Ptosis, squint High palate Pectus excavatum Camptosyndactyly of hands	No obvious dysmorphic feature	Elongated face Synophrys **Long,** low set **ears** Short neck	Facial asymmetry Elongated mid-face Full lips Long jaw	**Large ears** Downslanting palpebral fissures, Prominent nose **Low forehead** Flat occiput **Drooling**
Microcephaly	**Present** (1)	ND	ND	**Present** (2 M, 0 F)	**Present** (1)	**Present** (2)	Macroce.-phaly	**Present**	ND	**Present**
Stature	ND	Normal (6)	Normal (3)	Normal (5 M, 4 F)	Normal (2)	Normal (2)	Normal	ND	ND	Normal
Intellectual disability	Present	Moderate-severe	Present (13) IQ: 65-80	Borderline-mild (F) **Mild**-moderate (M)	**Mild** (IQ:54) – moderate	Present (2)	Present	Moderate	**Mild**	**Mild-moderate**
Gross motor development	ND	ND	Delayed (5)	Delayed (5 M) Normal (F)	Delayed (2)	Delayed (2)	Delayed	Delayed	Delayed	Mildly delayed
Fine motor development	ND	ND	Delayed (3)	Moderate (M) Normal–mild (F)	Delayed (2)	Severely delayed (2)	Delayed	Delayed	Delayed	Moderately delayed
Language development	ND	ND	Delayed (5)	Delayed (M)	Delayed (2)	Severely delayed (2)	Delayed	Delayed	Delayed	Mildly delayed

Language skills Verbal expression and comprehension Reading and writing	ND	ND	ND	Moderate–severe (M) Normal-mild (F)	Moderate (2)	ND	ND	ND	Delayed	Moderately delayed
Visual skills	ND	ND	ND	Severe (M) Normal-mild (F)	Mild (2)	ND	ND	ND	ND	Moderately delayed
Socialization	ND	ND	Laborer jobs (9)	Sheltered job (3 M)	Sheltered job	ND	ND	ND	Lives in group home	Mildly delayed
Behavior and neuro-psychological profile	ND	ND	Non-categorized learning difficulties (12) **Aggression** (1) Schizophrenia (2) Myoclonic **epilepsy** (1)	Paranoid psychosis (1 M) **Epilepsy** (1 M) **Aggression** (4 M) Inattention (1 M, 1 F) Learning difficulties (3 F)	**Aggressive**, clastic episodes Hyperactivity Agitation **Epilepsy** in infancy	**Epilepsy** in infancy	**Autistic** characteris-tics Temper tantrums Avoiding social interaction	Attention deficit hyperactivity disorder (2) **Aggression** (1)	Irritable, poor sleep Self-injury (head rubbing, hitting) **Autism** Attention deficit **Epilepsy**	**Aggressive** episodes Agitation **Autistic characteristics** **Seizures** in early childhood
Brain imaging	Small brain, otherwise normal on MRI	ND	ND	Non-progressive hydrocephalus on CT (1 M) CT normal (2 M)	ND	Cerebellar hypoplasia (2) Corpus callosum agenesis/ hypoplasia (2) Lateral ventriculomegaly (1)	Normal MRI	ND	Ventriculomegaly Thin corpus callosum White matter cavitations (due to contusions)	Normal MRI
EEG	ND	ND	ND	Posterior slow wave (4 M/1 F)	Normal (2)	ND	ND	ND	Abnormal (variable)	Normal
Other clinical features	ND	ND	Obesity in 3 elderly	Stooping posture (2 M) Scoliosis (1 M) Childhood **hypotonia** (3 M, 1 F)	**Hypotonia** in infancy (2)	Ichthyosis (2) Early childhood **hypotonia** (2)	Mild axial **hypotonia**	Hypogonadism (1)	Marfanoid habitus Kyphosis Syndactyly Calcaneovalgus deformity **Hypotonia** in infancy	Mild kypho-scoliosis Pectus carinatum Calcaneovalgus deformity Wide-spaced nipples Spina bifida occulta

ND: not described; M: male; F: female; (number): number of patients examined and found positive for the described features. In case of no numbers, all affected male patients exhibited the feature. Common features of patients are written in bold.

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ORIGINAL RESEARCH
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Systemic Screening for 22q11.2 Copy Number Variations in Hungarian Pediatric and Adult Patients with Congenital Heart Diseases Identified Rare Pathogenic Patterns in the Region

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Congenital heart defects (CHD) are the most common developmental abnormalities,affecting approximately 0.9% of livebirths. Genetic factors, including copy number variations (CNVs), play an important role in their development. The most common CNVs are found on chromosome 22q11.2. The genomic instability of this region, caused by the eight low copy repeats (LCR A-H), may result in several recurrent and/or rare microdeletions and duplications, including the most common, ∼3 Mb large LCRA-D deletion (classical 22q.11.2 deletion syndrome). We aimed to screen 22q11.2 CNVs in a large Hungarian pediatric and adult CHD cohort, regardless of the typeof their CHDs. All the enrolled participants were cardiologically diagnosed with non-syndromic CHDs. A combination of multiplex ligation-dependent probe amplification (MLPA), chromosomal microarray analysis and droplet digital PCR methods were used to comprehensively assess the detected 22q11.2 CNVs in 212 CHD-patients. Additionally, capillary sequencing was performed to detect variants in the *TBX1* gene, a cardinal gene located in 22q11.2. Pathogenic CNVs were detected in 5.2% (11/212), VUS in 0.9% and benign CNVs in 1.8% of the overall CHD cohort. In patients with tetralogy of Fallot the rate of pathogenic CNVs was 17% (5/30). Fifty- four percent of all CNVs were typical proximal deletions (LCR A-D). However, nested(LCR A-B) and central deletions (LCR C-D), proximal (LCR A-D) and distal duplications (LCR D-E, LCR D-H, LCR E-H, LCR F-H) and rare combinations of deletions and duplications were also identified. Segregation analysis detected familial occurrence in 18% (2/11) of the pathogenic variants. Based on in-depth clinical information, a detailed phenotype–genotype comparison was performed. No pathogenic variant wasidentified in the *TBX1* gene. Our findings confirmed the previously described large

phenotypic diversity in the 22q11.2 CNVs. MLPA proved to be a highly efficient genetic screening method for our CHD-cohort. Our results highlight the necessity for large-scale genetic screening of CHD-patients and the importance of early genetic diagnosis in their clinical management.

Keywords: 22q11.2 deletion syndrome, *TBX1* gene, multiplex ligation-dependent probe amplification, copy number variations, droplet digital PCR, syndromic and non-syndromic congenital heart defects, chromosomal microarray analysis

INTRODUCTION

Congenital heart defects (CHDs) are the most common congenital developmental defects and affect approximately 0.9%of livebirths (van der Linde et al., 2011). Thirty to forty percent of CHDs are syndrome-associated and are causedby copy number variants (CNVs) or a mutation in a singlegene. The most common human CNVs affect chromosomal region 22q11.2 (Fahed et al., 2013; Digilio and Marino, 2016). Proximal microdeletions of 1.5–3 Mb in this chromosomal region typically include the sequence between low copy repeat regions A and D (LCR A-D, LCR A-B) and may lead to the classical phenotype of 22q11.2 deletion syndrome, also knownas DiGeorge syndrome. Central (LCR B-D, LCR C-D) or distal deletions (LCR C-H) may cause other, variable phenotypes (Burnside, 2015; Kruszka et al., 2017). Duplications have also been identified in this chromosomal region and are associated with even more significant phenotypic variability than deletions (Wentzel et al., 2008).

DiGeorge syndrome (also known as velocardiofacial syndrome and conotruncal anomaly face syndrome) is mostly characterized by CHD, thymus hypoplasia, immunodeficiency and skeletal, gastrointestinal and urogenital defects as well as by developmental delay, learning difficulties, susceptibility to neuropsychiatric disorders and, in some cases, by mild to moderate intellectual disability. 22q11.2 CNVs have reducedpenetrance and incomplete expression and may be detected in asymptomatic or mildly affected individuals; approximately 7–10% of the cases are familial (McDonald-McGinn et al., 1993-2020; Campbell et al., 2018).

The *T-box transcription factor 1 (TBX1)* gene is located withinthe proximal 22q11.2 region, encodes a transcription factor that plays an important role in early embryonic development and is hypothesized to contribute to 22q11.2 deletion phenotype as wellas to non-syndromic CHDs. (Griffin et al., 2010; Heike et al., 2010; Guo et al., 2011).

Clinical diagnosis may be challenging and significantly delayed due to the large phenotypic spectrum resulting from 22q11.2 CNVs (from asymptomatic appearance to multiple defects) (van Engelen et al., 2010). Previous studies have drawn attention to the importance of routine screening for 22q11.2CNVs in patients with congenital heart defects, especially with conotruncal anomalies (Wozniak et al., 2010; Huber et al., 2014;Goldmuntz, 2020).

Based on the low number of patients referred for 22q11.2 CNV analysis at our genetic department over the last decade, we hypothesized that some patients with 22q11.2 CNVs—especially in the adult population—may have remained undiagnosed. The aim of our study was therefore to test for 22q11.2 CNVs and *TBX1* gene variants for the pediatric and adult patients of the Southern-Hungarian CHD Registry, cardiologically diagnosed with non-syndromic CHDs, and to carry out genotype–phenotype comparison in positive cases based on in- depth clinical data.

MATERIALS AND METHODS

Overall, 212 unrelated patients (110 females, 112 males; mean age: 26.9 years; age range: 2 weeks to 74 years) previously cardiologically diagnosed with non-syndromic congenital heart defects were enrolled in the study at the University of Szeged between 2016 and 2019. The distribution of the patients with the different CHD types are presented in **Table 1**.

The DNA of 211 Hungarian individuals with no CHDs (confirmed with cardiological examination), and with no family history of CHD (144 females, 67 males, mean age: 37 years, age range: 8–73 years) was used as controls for the comparative analyses.

In positive cases, genetic testing was offered to all first-degree family members.

All investigations were performed according to the Helsinki Declaration 2008 and approved by the National Medical Research Council (No CHD-01/2016—IF-6299-8/2016) and the Local Ethical Committee of the University of Szeged (No 105/2016-SZTE). Participants/legal guardians/parents gave their informed consent to the study.

Sample Preparation and Multiplex Ligation-Dependent Probe Amplification (MLPA)

DNA was extracted from peripheral blood with the QIAamp DNA Blood Mini Kit (QIAGEN, Gödöllõ, Hungary).

To detect CNVs in the 22q11.2 locus, all patient sampleswere processed using the P250-B2 DiGeorge SALSA MLPA Probemix (IVD, MRC-Holland, Amsterdam) according to the manufacturer's instructions. The MLPA probe mix contained48 probes, 29 of which are located in the 22q11.2 region (24in the LCR A to H region and 5 in the Cat-Eye syndromeregion) and 19 in regions 4q35, 8p23, and 9q34 (Kleefstra syndrome), 10p14 (DiGeorge syndrome 2) and 17p13 and 22q13(Phelan-McDermid syndrome), deletions in the latter may result in phenotypical similarity to DGS. Amplicon fragment length analysis was performed on an ABI 3500 Genetic Analyzer

TABLE 1 | The distribution of different types of congenital heart defects among patients (*N* = 212).

Type of congenital heart defect	Number of patients (%)
Ventricular septal defect	**36 (16.9%)**
VSD alone	25
VSD + ASD + PDA	5
VSD + ASD	2
VSD + PDA	2
VSD + PS	2
Atrial septal defect	**35 (16.5 %)**
ASD alone	27
ASD + PDA	3
ASD + VSD	3
ASD + PS	2
Congenital aorta stenosis	**31 (14.6%)**
AoS alone	17
AoS + bicuspid aortic valve	13
AoS + ASD	1
Fallot IV	**30 (14.1%)**
TGA	**21 (9.9%)**
Bicuspid aortic valve	**19 (8.9%)**
Coarctation of the aorta	**17 (8%)**
CoA alone	10
CoA + bicuspid aortic valve	3
CoA + VSD + PDA	4
Atrioventricular septal defect	5 (2.4%)
Anomalous pulmonary venous drainage	4 (2%)
TAPVD alone	2
TAPVD + PA + VSD	1
PPAVR	1
Pulmonary stenosis (congenital)	4 (2%)
Univentricular heart	3 (1.4%)
Hypoplastic left heart syndrome	2 (0.9%)
Pulmonary atresia	2 (0.9%)
Truncus arteriosus communis	1 (0.5%)
Double outlet right ventricle	1 (0.5%)
Ebstein anomaly	1 (0.5%)

AoS, congenital aorta stenosis; ASD, atrial septal defect; CoA, coarctation of theaorta; Fallot IV, Tetralogy of Fallot; PA, pulmonary atresia; PDA, patent ductus arteriosus; PPAVR, partial pulmonary anomalous venous return; PS, pulmonary stenosis; TGA, transposition of the great arteries; TAPVD, total anomalous pulmonary venous drainage; VSD, ventricular septal defect. The most frequent CHD groups in the cohort are indicated in bold.

(Thermo Fisher Scientific, Waltham, MA) and analyzed by Coffalyser.net software (MRC-Holland, Amsterdam).

Validation of Positive Cases: FISH, Chromosomal Microarray Analysis, ddPCR

MLPA was repeated for all samples in which CNVs were found. Deletions and duplications were confirmed with an independent method, including FISH (Vysis DiGeorge Region LSI N25 SO/ARSA SGn Probes, Abbott Molecular Inc., Des Plaines, IL, United States, and SureFISH 22q11.21 CRKL, Agilent Technologies, Cedar Creek, TX, United States), a supplementaryMLPA kit (P372-SALSA MLPA Microdeletions 6, MRC-Holland, Amsterdam, Netherlands) or chromosomal microarray analysis (CMA, Affymetrix, CytoScan 750 K, Thermo Fisher Scientific, Waltham, MA, United States). CMA was performed as describedby Nagy et al. (2019). In cases where one probe was deleted,the probe region was sequenced with bidirectional capillary sequencing to exclude MLPA-interfering SNPs in the sample DNA. These validation methods confirmed all positive MLPA results (i.e., no false positives).

A droplet digital PCR (ddPCR) method was designed forthe confirmation of recurrent single-probe CNVs in the *TOP3B* gene from CHD patient samples. This method was also used to determine the frequency of *TOP3B* CNVs in the control cohort aswell. The analysis was performed on the QX100 Droplet Digital PCR system (Bio-Rad Laboratories, Hercules, CA, United States), according to the manufacturer's instruction. Primers and probes were designed for *TOP3B* exon 7 and for the *PRDM15* gene as reference region on chromosome 21 (**Supplementary Material**).*TOP3B* CNVs found in the controls with ddPCR were confirmedwith MLPA.

Sequencing of the TBX1 Gene

Bidirectional capillary sequencing of *TBX1* coding regions was performed for all patient samples with an ABI 3500 Genetic Analyzer. The primers used are listed in **Supplementary Material**. The non-synonymous variants (all located in exon 9 of *TBX1* gene) were tested in the control cohortas well.

CNV and Variant Interpretation

Identified CNVs and single nucleotide variants (SNVs) were classified according to the standards and guidelines of the American College of Medical Genetics (Richards et al., 2015; Riggs et al., 2020). The following websites and databases were used for CNV interpretation: Database of Chromosomal Imbalance, Phenotype of Humans using Ensemble Resources (DECIPHER, Firth et al., 2009), Database of Genomic Variation(DGV, MacDonald et al., 2013), PubMed and GeneReviews(McDonald-McGinn et al., 1993-2020). For SNV interpretation, VarSome (Kopanos et al., 2011), ClinVar (Landrum et al., 2020),and Genome Aggregation Database (GnomAD, Karczewski et al.,2020) databases were used.

Statistical Analysis

GraphPad Prism (GraphPad Software, San Diego, California, United States), version 4.00 for Windows, was used for statistical analysis. The frequency of *TOP3B* CNVs and *TBX1* variantsin the patient cohort was compared with the frequency in the control cohort and also with the frequency in the global dataset of GnomAD using the Fisher exact test and χ^2-test. $P < 0.05$ wasconsidered to be statistically significant.

RESULTS

Distribution of CHD Types in Patients

In the CHD cohort, the four most common CHD typeswere ventricular septal defect (VSD), atrial septal defect (ASD), congenital aorta stenosis (AoS) and tetralogy of Fallot

(TOF) (**Table 1**). In 81% of the patients, only one cardiac entity was diagnosed; whereas, in 19% of the cases, twoor more CHDs occurred together. The distribution of the different CHDs among the South-Hungarian Registry patients corresponded well with the frequency described in the literature (van der Linde et al., 2011).

Distribution of Positive MLPA Results and Classification of the Detected CNVs

Overall, 17 cases of 212 patients (8%) diagnosed prior with non-syndromic CHD were yielded positive MLPA result, and after evaluation 11 of these copy number changes (5.2%) were interpreted as pathogenic variant, two as variant of unknown significance (VUS, 0.9%) and four as benign (1.8%) (**Figure 1** and **Supplementary Material**). The most frequent CNVs of the positive MLPA results were microdeletions (8/17); however, microduplications (7/17) and a combination of deletions and duplications (2/17) were also observed.

Among pathogenic CNVs 7 microdeletions, 2 duplications and 1 combination of a deletion and a duplication was detected, while among the VUS one duplication and one combined CNV and among the benign variants one deletion and three duplications.

Pathogenic results were observed most frequently in the TOF group: in 17% of all TOF patients, followed by the group of bicuspid aortic valve with 10% (**Figure 1** and **Table 2**).

Based on the interpretation guidelines (Riggs et al., 2020), 11CNVs were interpreted as pathogenic:

6 typical deletions of LCR A-D, one proximal nested deletionof LCR A-B, two duplications of LCR A-D, one combinationof the proximal deletion of LCR A-D with a duplication ofLCR E-H and one duplication of LCR D-H. Two further CNVs (one combination of a central deletion of LCR C-D with duplication of LCR D-E and one duplication of LCR F-H) were classified as VUS (**Figure 1** and **Table 3**). Four CNVs (three268 kbp duplications and one 278 kbp deletion) were detectedin the *TOP3B* gene and resulted from one probe alteration in the MLPA reaction. These CNVs were confirmed with chromosomal microarray analysis (**Supplementary Material**). Considering the relatively high proportion of *TOP3B* CNVs in our patient cohort (overall 4/212, 1.9%: deletion in 1/212, 0.5% and duplication in 3/212, 1.4%), we decided to perform an independent analysis with ddPCR to determine the frequencyof *TOP3B* CNVs in the healthy controls. The *TOP3B* deletion was detected in one control sample (0.5%) and a duplicationin four control samples (1.9%); i.e., CNVs were identified in 2.4% of the controls (5/211). The difference in the CNV

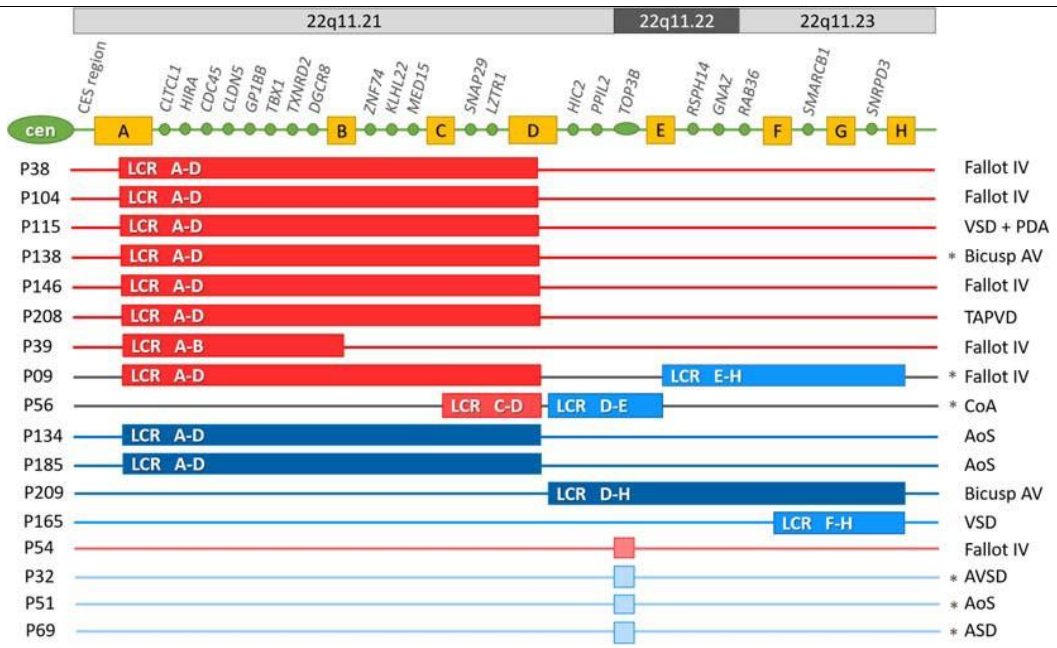

FIGURE 1 | Pathogenic variants, variants of uncertain significance and benign 22q11.2 copy number variations in the CHD cohort. Cen, centromere; CES, Cat eyesyndrome region; A-H yellow boxes: low copy repeat regions in locus 22q11.2. Genes indicated between the LCR regions, are the ones that have corresponding probes in the P250-B2 DiGeorge SALSA MLPA Probemix; LCR: low copy repeat region. Dark red: pathogenic microdeletion; Dark blue: pathogenic microduplication; Middle red: microdeletion of uncertain significance; Middle blue: microduplication of uncertain significance; Light red: benign microdeletion; Light blue: benign microduplication. LCR A-D: the typical ∼2.5–3 Mb microdeletion in region 22q11.21; LCR A-B: ∼1.5 Mb proximal microdeletion in region 22q11.21;LCR C-D: ∼0.5 Mb central microdeletion in region 22q11.21; LCR D-E: ∼1.2 Mb central microduplication in region central-distal microduplication in region 22q11.21q23; LCR E-H: ∼1.55–2 Mb distal microduplication in region 22q11.22q11.23; LCR F-H: ∼1–1.2 Mb microduplication in region 22q11.23; Asterix denotes familial CNVs. AoS, congenital aorta stenosis; AVSD, atrioventricular septal defect; bicuspid AV: bicuspid aorticvalve; CoA, coarctation of the aorta; Fallot IV, tetralogy of Fallot; PDA, patent ductus arteriosus; TAPVD, total anomalous pulmonary venous drainage; VSD, ventricular septal defect.

TABLE 2 | The distribution of the pathogenic and VUS 22q11 CNVs in thedifferent CHD groups.

Type of CHD	Number of pathogenic CNVs or VUS/Number of patients with CHD (%)	Del	Dupl	Del + Dupl
Fallot IV	**5 path/30 (17%)**	**4**	0	**1**
Bicuspid aortic valve	2 path/19 (10%)	**1**	**1**	0
AoS	2 path/31 (6.5%)	0	**2**	0
CoA	1 VUS/17 (6%)	0	0	**1**
VSD	1 path/36 (2.7%)	**1**		0
1 VUS/36 (2.7%)			**1**	
TAPVD + VSD + PA	1 path/4 (25%)*	**1**	0	0
Total	**11 path/212 (5.2%)**	**7**	**3**	**1**
	2 VUS/212 (0.9%)	**0**	**1**	**1**

*VUS, variant of uncertain significance; CHD, congenital heart defect; CoA,coarctation of the aorta; AoS, congenital aorta stenosis Fallot IV, tetralogy of Fallot; TAPVD, total anomalous pulmonary venous drainage; VSD, ventricular septal defect; PA, pulmonary atresia; DEL, 22q11 microdeletion; DUPL, 22q11 microduplication. *This proportion is biased, since this patient could be also classified in VSD or PA group.*

frequency between patients and controls was not significant (p = 0.751). Thus, we ultimately classified *TOP3B* CNVsas rare benign variants, which are more frequent in the Hungarian population than in the global database (frequency in DECIPHER: 0.36%).

Familial Segregation

It was possible to perform segregation analysis for 14 of the17 positive cases. Six cases proved to be familial (**Figure 1**),two of these were for patients with pathogenic CNVs, one fora patient with VUS and three for patients with benign *TOP3B* variants. In addition to the *TOP3B* microduplication, proband P32 also had a 201-bp microduplication on chromosome 17p13.3, which included the *YWHAE* gene (**Supplementary Material**). Both chromosome imbalances were inherited from an asymptomatic parent. Two asymptomatic siblings also carried the *YWHAE* microduplication but without the *TOP3B* CNV.Therefore, the *YWHAE* CNV was interpreted as a rare benign variant. In case of the other *TOP3B* microduplications (P51and P69) one healthy parent carried also the variant. Inthe patient with the *TOP3B* deletion, segregation analysis was not performed, the proband had two healthy children. The individuals with *TOP3B* CNVs were excluded from the genotype–phenotype comparison based on these results, the highfrequency of *TOP3B* CNV in controls and the fact that these patients displayed no other malformations or comorbidities in addition to CHD.

The segregation analysis detected familial occurrence for 18%(2/11) of the pathogenic CNVs and 50% (1/2) of VUS. In these three familial cases (P138, P09 and P56), the proband's mother carried the same chromosome imbalance. The phenotypes ofthe mothers were the same severity (P138) or milder (P09, P56) than the probands'. The suspicion of an underlying 22q11.2CNV prior to the genetic testing was not raised at any of the affected family members.

For proband P09 and for the proband's mother, the typical ∼2.5–3 Mb 22q11.2 microdeletion was combined with a distal 1.5–2 Mb 22q11.2 microduplication. The segregation analysis of the family showed that the maternal grandmother andone sibling of the mother carried only the duplication with no cardiological symptom or developmental malformation, although they had been diagnosed with mild anxiety disorder and depression. The deletion occurred most probably *de novo* in the mother and was transferred to the child.

The mother of proband P56 had only bicuspid aortic valve (clinically diagnosed only after the genetic diagnosis) and similar facial features as the proband without torticollis or severe scoliosis. The clinical features of patients and family members are shown in **Table 3**.

Genotype-Phenotype Comparison

The probands' age at the genetic diagnosis with pathogenic or VUS 22q11.2 CNVs ranged from 2 months to 52 years (median age: 21 years). Three patients out of 13 were diagnosed in childhood, one child in the first year of life. The two oldest patients and the affected family members were born before the molecular diagnostic era. No correlation could be observed between the severity of the phenotype and the ageat the diagnosis.

The prevalence of common clinical features for different CNVs is comparable to previously reported prevalence data in the literature (**Table 4**). Patients presented with more marked phenotypic features for 22q11.2 microdeletions than with microduplications in the same region. In addition tothe CHDs, the typical microdeletions of LCR A-D—withor without accompanying CNVs—resulted in the classical phenotype of 22q11.2 deletion syndrome. The co-occurring duplication in proband P09 and mother has not modified their phenotype significantly compared to other LCR A-D microdeletion phenotypes. In proband P56 and his mother with the combination of central deletion and distal duplication, the phenotype differed completely from that of 22q11.2 deletion syndrome (except the CHD) and the impact of the duplication could not be determined precisely (**Table 3**).

Among patients with deletions, Fallot tetralogy was the most common CHD. Among patients with duplications, congenital aorta stenosis, coarctation of the aorta and bicuspid aortic valve were the most common CHD types (**Table 2**). These three entities may be considered on the spectrum for one disease.

CHDs were overrepresented in our 22q11.2 CNV patients andtheir affected family members compared to data in the literature (94% vs. 74%), which may be the result of the patient enrollment criteria. Neuropsychiatric disorders were underrepresented among our patients (19% vs. 60%). Other characteristics (facial features, velopharyngeal insufficiency, immunodeficiency, hypocalcemia, skeletal anomalies, developmental delay, and learning difficulties) had a distribution in our cohort similarto that described in the literature (**Table 4**). The presenceof immunodeficiency was deduced from the recurrence of respiratory and ear infections occurring mostly in childhood.Based on regular laboratory check-ups, the average absolute

TABLE 3 | Clinical features of probands and parents with pathogenic and VUS MLPA results.

Proband no./Age*	CNV Extension (size)	Classification**	Type of CHD	Extracardiac manifestations	DD/ID	Facial features	Classical phenotype***	Hypocalcemia	Miscellaneous or *de novo* mutation	Familial inheritance
P38/ Adulthood	DEL LCR A-D in 22q11.21 (~2.5-3 Mb)	Path	TOF	Recurrent bronchitis and otitis media, tonsillectomy, vesicoureteral reflux, renal cyst, velopharyngeal insufficiency, nasal speech, early teeth lost, small stature, kyphoscoliosis, block vertebrae, lower leg cramps, dyslexia, anxiety disorder, microcephaly, juvenile cataract, autoimmune hypothyroidism	DD/mild ID	Narrow face, micrognathia, low-set ears, narrow, small palpebral fissures, hypertelorism, hypoplastic alae nasi, pointed ear tips, thin lips	Yes	Yes	Obstipation, GOR, feeding difficulties in childhood, hypomagnesemia	*De novo*
P104/ Adulthood	DEL LCR A-D in 22q11.21 (~2.5–3 Mb)	Path	TOF	Arrythmia (radio frequent ablation), recurrent otitis media in childhood (Grommet tubes, adenotonsillectomy), inguinal hernia, scoliosis, mild kyphosis, thorax asymmetry, narrow shoulders, recurrent urinary infections, nephrolithiasis, nasal speech, learning difficulties.	DD/low normal IQ	Narrow, long face, narrow palpebral fissures, deep-set eyes, marked hypertelorism, large ear lobes, thin small lip, hypoplastic alae nasi, malar flattening	Yes	Yes	Lumbago, pulmonary embolism. Deceased postoperative in nosocomial infection before the genetic diagnosis	*De novo*
P115/ Adulthood	DEL LCR A-D in 22q11.21 (~2.5–3 Mb)	Path	VSD, PDA	Right main bronchus stenosis, congenital lacrimal duct stenosis, many recurrent upper and lower airway infections until puberty, severe scoliosis, hernia diaphragm + severe GOR (fundoplication), inguinal hernia (operated), palatoschisis, velopharyngeal insufficiency, thorax andhip deformity, learning difficulties, nasal speech, episodic hand tremor and footparesthesia, small stature, microcephaly	DD/low normal IQ	Narrow, long face low-set ears, narrow, small palpebral fissures, hypertelorism, hypoplastic alae nasi, pointed ear tips, thin small lips, malar flattening, mild facial asymmetry	Yes	–	Nasogastric tube feeding in infancy	*De novo*
P138/ Childhood	DEL LCR A-D in 22q11.21 (~2.5–3 Mb)	Path	Bicuspid aortic valve	Recurrent lower and upper airway infections and otitis media, nasal speech, hypermetropy, astigmia, no developmental delay, normal kindergarten, and preschool	No/No	Long face, small mouth, straight nose, narrow eyelids, hypertelorism, pointed ear tip, fleshy ear lobes	Yes	Yes	Severe obesity (dueto diet failure), secondary hypertonia	Maternally inherited: mother has similar outer appearance, umbilical hernia, vestibular neuronitis, impaired hearing, butno CHD
P146/ Adulthood	DEL LCR A-D in 22q11.21 (~2.5–3 Mb)	Path	TOF	Recurrent respiratory infection and otitis media, mastoiditis, cryptorchism (orchidopexy), severe scoliosis, thorax asymmetry (small right scapula), nasal speech, neuropsychiatric problems, learning difficulties, special school	Speech delay/mild ID	Narrow, long face, straight nose, low-set ears, narrow palpebral fissures	Yes	Yes	Brain MRI and abdominal ultrasound: normal	*De novo*

TABLE 3 | Continued

Proband no./Age*	CNV Extension (size)	Classification**	Type of CHD	Extracardiac manifestations	DD/ID	Facial features	Classical phenotype***	Hypocalcemia	Miscellaneous or *de novo* mutation	Familial inheritance
P39/ Adulthood	DEL LCR A-B in 22q11.21 (~2.5–3 Mb)	Path	TOF	Recurrent respiratory infections, learning difficulties, special school, neuropsychiatric problems	DD/low normal IQ	Narrow face, low-set ears, narrow, small palpebral fissures	Yes	–	–	ND
P09/ Childhood	DEL LCR A-D in 22q11.21 (~2.5–3 Mb) + DUPL LCR E-H 22q11.22q11.23 (~1.55–2 Mb)	Path + VUS	TOF	Congenital laryngeal stenosis, thymus aplasia, respiratory infections (postoperative as well), pes calcaneovalgus	?/?	Small mouth, pointed ear tips, hypoplastic alae nasi, low-set ears	Yes	No	Small for gestational age, transient nasogastric tube feeding	Maternally inherited. mother: TOF, severe scoliosis, nasal speech, classical DGS phenotype, low normal IQ, anxiety disorder
P56/ Adulthood	DEL LCR C-D in 22q11.21 (~0.5 Mb) + DUPL LCR D-E 22q11.21q11.22 (~1.2 Mb)	VUS + VUS	CoA	Torticollis, scoliosis, nasal speech, socially withdrawn, studies in higher education	No/No	Facial asymmetry, pointed ear lobes, small philtrum, low-set ears, triangular chin, retrognathia	No	No	–	Maternally inherited: mother has bicuspid aortic valve, similar facial features without asymmetry and torticollis
P134/ Adulthood	DUPL LCR A-D in 22q11.21 (~2.5–3 Mb)	Path	AoS	None	No/No	No	No	No	Atopy, asthma	*De novo*
P185/ Adulthood	DUPL LCR A-D in 22q11.21 (~2.5–3 Mb)	Path	AoS	None	No/No	No	No	No	–	ND
P209/ Adulthood	DUPL LCR D-H 22q11.21q11.23 (~3.1–3.5 Mb)	Path	Bicuspid aortic valve	Horseshoe kidney, pyeloureteral stenosis, Ewing-sarcoma in childhood, frequent tonsillitis (tonsillectomy), primary amenorrhea, special school	Speech delay/mild ID	Hypertelorism, divergent strabismus, prominent long mandible, uvula elongata	No	No	Obesity. Twin sibling died of pulmonary atresia after birth	No CNV in father, mother not tested
P165/ Adulthood	DUPL LCR F-H in 22q11.23 (~1–1.2 Mb)	VUS	VSD	Myopia, bilateral inguinal hernia, truncal obesity, learning difficulties	No/No	Micrognathia	No	No	Preterm birth, normal catch-up development, bronchial asthma	*De novo*

*CHD, congenital heart disease; CNV, copy number variation; LCR, low copy repeat region; TOF, Tetralogy of Fallot; AoS, congenital stenosis of the aorta; PA, pulmonal atresia; VSD, ventricular septal defect; TPAVD, Total anomalous pulmonary venous return; CHD, congenital heart disease; CoA, coarctation of the aorta; AVSD, atrioventricular septal defect. ND, not done; DD, developmental delay; ID, intellectual disability, GOR, gastro-esophageal reflux; Path, pathological; VUS, variant of uncertain significance.*Denotes the life period when the genetic diagnosis was set up. **Denotes the classification of CNVs based on the joint consensus of ACMG and ClinGen Guidelines (Riggs et al., 2020). ***Classical phenotype of22q11.2 deletion syndrome, previously described as DiGeorge syndrome*

TABLE 4 | The prevalence of the common clinical features in all probands and family members carrying pathogenic CNVs and VUS in the 22q11.2 region compared tothe prevalence in the literature.

Symptoms	Classical LCR A-D deletion	All other deletions*	All duplications alone	All CNVs of this study	Prevalence in the literature**
CHD	8/9 (89%)	3/3 (100%)	4/4 (100%)	15/16 (94%)	64–74%
Facial dysmorphia	9/9 (100%)	3/3 (100%)	2/4 (50%)	14/16 (87.5%)	46–88%
Classical facial features in 22q11.2 deletion	9/9 (100%)	1/3 (33%)	0/4	10/16 (62.5%)	ND
Velopharyngeal insufficiency	7/9 (78%)	1/3 (33%)	0/4	8/16 (50%)	55–69%
Immunodeficiency, recurrent infections	8/9 (89%)	1/3 (33%)	1/4 (25%)	10/16 (62.5%)	50–77%
Skeletal anomalies	6/9 (67%)	1/3 (33%)	0/4	7/16 (44%)	15–50%
Other anomalies***	7/9 (78%)	0/3	2/4 (50%)	9/16 (56%)	67–81%
Developmental delay (motor ± speech) in childhood	6/9 (67%)	1/3 (33%)	1/4 (25%)	8/16 (50%)	70–90%
Intellectual disability	4/9 (44%)	0/3	1/4 (25%)	5/16 (31%)	28–31%
Learning difficulties	6/9 (67%)	1/3 (33%)	1/4 (25%)	8/16 (50%)	66–93%
Neuropsychiatric problems	3/9 (33%)	0/3	0/4	3/16 (19%)	60–73%
Hypocalcemia	5/9 (55.5%)	0/3	0/4	5/16 (31%)	17–60%

CHD, congenital heart defect; ID, intellectual disability.

In this category LCR A-B nested deletion and LCR C-D deletion combined with LCR D-E duplication are included; ** Data for 22q11.2 deletions described by McDonald- McGinn et al. (1993-2020), Burnside (2015); Campbell et al. (2018), and Niarchou et al. (2019) are presented; * Including clinically diagnosed thymus aplasia, respiratory, gastrointestinal and/or urogenital abnormalities; ND: not determined. Velopharyngeal insufficiency, nasal speech could not be assessed in the two youngest probands.Intellectual disability, learning difficulties could be only assessed in individuals in school age or older. Psychiatric disorders are taken into account only if they were clinically diagnosed.*

lymphocyte count was in the lower normal range (2.13 G/L, normal range: 1.5–3.2 G/L); whereas the average relative lymphocyte count was below normal (23.8%, normal range: 27–34%). Flow-cytometry and serum immunoglobulin levels were not measured regularly. The immune status and infections of 22q11.2 CNV patients were not strictly controlled before the genetic diagnosis. Before genetic diagnosis, proband P104 died of a fulminant postoperative infection (**Table 3**).

Hypocalcemia (average serum calcium level: 1.84 mmol/l, normal range: 2.2–2.55 mmol/l) was often present in patients with the typical 22q11.2 microdeletions—with or without clinical symptoms. However, hypocalcemia was not considered relevant for therapy before genetic diagnosis. Severe hypomagnesemia was also detected in one 22q11.2 microdeletion patient. The thrombocyte count was in the low normal range with an average of 156 10^9/l.Thyroid and parathyroid hormone levels and vitamin D levels were not measured in these patients before genetic diagnosis. These laboratory abnormalities could not be consistently identified for patients with CNVs other than the typical microdeletion.

Results of the *TBX1* Gene Sequencing

No apparently pathogenic variant was detected in the *TBX1*gene. For CHD patients, three missense variants were foundin exon 9: c.1189A>A; p.Asn397His with a 21% minor allele frequency (MAF), c.1049G>A; p.Gly350Asp with 0.48% MAF and c.1341_1342insCCGCACGCGCAT; p.Ala450_His453dup with 0.24% MAF (**Table 5**). The frequency of the p.Asn397His variant was also 21% for the controls. The two less frequent variants were also detected in one of the proband's healthyparents and are listed in the Hungarian or the global database with very low frequencies (**Table 5**). Of the 10 probands and mothers with proximal 22q11.2 microdeletions encompassing the *TBX1* gene, two (20%) carried the common p.Asn397His variantin hemizygous form (P09 and P138). Proband P09 exhibited a severe phenotype; whereas proband P138 presented only milder symptoms. The two rare variants were not detected in any of the microdeletion patients. Based on the allele frequencies, ACMG criteria and segregation analyses, all three variants were ultimately classified as benign.

Discussion

This was the first systemic, large-scale genetic screening study of Hungarian CHD patients. All patients with cardiologically verified CHDs were enrolled in the study without further selection. Although the enrolled patients were cardiologically diagnosed with non-syndromic CHDs prior to this study, 13 were found to be syndromic after the genetic screening.

We observed a higher median age (21 years) and a similar or wider age range (0.17–52 years) at the genetic diagnosis in our cohort as compared to previously described cohorts (median age:

17.3 years, range: 0.1–59.4 years in Canadian patients; median age: 2.9 years, range: 0–17.6 years in American patients) (Palmeret al., 2018). This difference may partly be explained by thefact, that the 22q11.2 duplication patients with more variable phenotypes were also included in the present study, whereas only 22q11.2 deletions were analyzed by Palmer and colleagues.

The frequency of CHDs was representative and correspondedto the frequency described in large epidemiological studies (van der Linde et al., 2011).

All types of CNVs in the 22q11.2 chromosomal region were present in 8% of the CHD cohort, while pathogenic CNVs in 5.2%, VUS in 0.9% and benign CNVs in 1.8%. Our patients

TABLE 5 | Minor allele frequencies of *TBX1* variants in the CHD cohort and control cohort compared to the allele frequency in the global database.

Variant	MAF in CHD	MAF in controls	*p*	MAF in GnomAD	*p*	*In silico* prediction*
c.1189A>A; p.Asn397His(rs72646967)	21%	21%	*0.809*	23.19%	*0.3583*	Benign
c.1049G>A; p.Gly350Asp(rs781731042)	0.48%	0.95%	*0.686*	0.0402%	*0.0138*	Benign
c.1341_1342insCCGCACGCGCAT; p.Ala450_His453dup (rs1341195668)	0.24%	0%	*0.498*	0.00325%	*0.0267*	VUS

In TBX1 locus CHD cohort contained overall 418 alleles, control cohort 422 alleles.MAF, minor allele frequency; CHD, congenital heart defect.
*GnomAD, Genome Aggregation Database (Karczewski et al., 2020). TBX1 transcript number: NM_080647. *In silico variant prediction was performed with VarSome,based on the ACMG criteria (Kopanos et al., 2011; Richards et al., 2015).*

presented pathogenic 22q11.2 CNVs more often comparedto other CHD cohorts, such as 1.27% in Brazilian, 2.8% in Cameroonian and 2.9% in Chinese population (Huber et al.,2014; Wonkam et al., 2017; Li et al., 2019). However, thisdifference may be also explained by the fact that most of these studies focused on the detection of 22q11.2 deletion but noton duplications.

For tetralogy of Fallot, the proportion of pathogenic CNVs was significantly higher, 17% in our cohort, which is in agreement with the fact that 22q11.2 CNVs are common in conotruncal heart defects (McDonald-McGinn et al., 1993-2020). 22q11.2 deletion can be detected in approximately 20% of all conotruncal heart defects (Wozniak et al., 2010), within this category its prevalence can be as high as 50% in interrupted aortic arch type B, 35% in truncus arteriosus or 10–25% in tetralogy of Fallot (Goldmuntz, 2020). All these suggest an absolute indicationfor 22q11.2 CNV analysis in these CHD groups, especially whenco-occurring with at least one extracardiac manifestation or dysmorphic traits (Wozniak et al., 2010).

Congenital bicuspid aortic valve is common (0.5–2%) and, without complication of stenosis, regurgitation or dissection,considered a largely benign congenital heart defect (Li et al., 2017). However, based on our results, it should not be ignoredin genetic testing.

The most common (64%) pathogenic CNV among our patients was the typical microdeletion of the LCR A-D region onchromosome 22q11.2, which is in agreement with the literature (Du et al., 2020). The frequency of deletions decreased toward the LCR F-H region, which was reflected in our results as well, since nested and central deletions were rare, and distal deletions were not detected. Proximal and distal duplication as well as two combined CNVs were also identified. Although most patients with the typical LCR A-D deletion showed the majority of the characteristic features of 22q11.2 deletion syndrome (velopharyngeal insufficiency, skeletal malformation, gastrointestinal and nephrological anomalies, hypocalcemia, frequent infections due to immunodeficiency and common facialfeatures), these symptoms were present less frequently (**Table 4**) with the non-typical deletions and the duplications, as expected (Burnside, 2015; Du et al., 2020). In addition to the presence of CHDs, no typical common characteristics could be found for these patients. This may be due to the low number of patients with single CNVs in our cohort or to the even wider phenotypic spectrum of these CNVs, e.g., in the case of 22q11.2 duplications (Yu et al., 2019). Therefore, several individuals with only mild symptoms or no detectable malformation or dysmorphia may remain undetected.

Hypocalcemia, hypomagnesemia, lymphocytopenia, thrombocytopenia, and abnormalities of the thyroid, parathyroid hormone or vitamin D levels may remain undiscovered in 22q11.2 deletion patients, especially without genetic diagnosis. However, these conditions may significantly contribute to co-morbidities, such as increased susceptibility to infections,bleeding diathesis and heightened prevalence of autoimmunedisorders, and, thus, should be considered for treatment (Lambert et al., 2018; Goldmuntz, 2020; Legitimo et al., 2020).

Neuropsychiatric disorders (attention deficit hyperactivity disorder, autism spectrum disorder, schizophrenia, anxiety symptoms and sleep disturbances) are frequent in patients with 22q11.2 CNVs (Brzustowicz and Bassett, 2012; Moulding et al., 2020). Complex presentation of three or more psychiatric traits may occur in 73% of patients with 22q11.2 CNVs (Niarchou et al., 2014; Chawner et al., 2019; Niarchou et al., 2019). However, these were markedly underrepresented (19%) in our patient cohort(**Table 4**), and this is most probably due to the lack of awarenessand screening rather than to their absence. This result further emphasizes the importance of multidisciplinary management of patients with 22q11.2 CNVs.

The large phenotypic variability of 22q11.2 microdeletions has recently been the focus of much research but is stillnot yet fully understood. The haploinsufficiency of the coding genes, including *TBX1, DGCR8, CRKL* among others, alonedoes not seem to account for the highly variable phenotypes and incomplete penetrance of affected individuals (Du et al., 2020). Some recent studies have investigated the role of possiblegenetic and epigenetic factors which contribute to the diversity of phenotypes associated with 22q11.2 deletions (Brzustowicz and Bassett, 2012; Bertini et al., 2017; Du et al., 2020). Breakpoint analysis of the LCR A-D region showed that small variations in the deletion size within this region have no significant role on phenotypic variability (Bertini et al., 2017). Pathogenic sequential variations in the remaining single copy of the genes (with an emphasis on *TBX1* gene) encompassed in the deleted region, were not yet revealed by previous investigation (Brzustowiczand Bassett, 2012). And this was further supported by our *TBX1* sequencing results, since no pathogenic *TBX1* variant

was detected in our patients with 22q11.2 CNVs or in the overall CHD-cohort. Thus, pathogenic *TBX1* mutations may be causal most probably only in a small fraction of CHD patients and 22q11.2 CNV patients, if at all. It was also hypothesized that sequential variations elsewhere in the genome (for example, *de novo* mutations in histone modifying genes) may collectively contribute to this diversity (Zaidi et al., 2013). Bertini and colleagues have investigated additional rare and common CNVs in typical 22q11.2 patients (2017). Accordingto their results, these additional CNVs often contain miRNA genes or mitochondrial genes, which may interact with 22q11.2 deletion and lead to metabolic and energetic problems ratherthan a decreased dosage of morphogenetic genes. The *DGCR8* gene is located within the typical 22q11.2 region and playsa crucial role in miRNA biosynthesis and, in combination with other CNV-miRNAs, may orchestrate highly variable phenotypic outcomes (Bertini et al., 2017). Previously, these additional CNVs, miRNAs have been investigated exclusively for 22q11.2 microdeletion patients, but not for duplication patients. Studying these duplication patients may further refine the diversity.

The family segregation study proved to be beneficial in cases with pathogenic CNVs, since in 18% further affected family members were identified. The number of familial cases washigher in our cohort than the previously described 6–10% (McDonald-McGinn et al., 1993-2020; Goldmuntz, 2020). The affected family members in our cohort exhibited similar or milder symptoms than the probands. This phenomenon has already been observed (Wozniak et al., 2010; Goldmuntz, 2020).

22q11.2 is considered one of the most unstable regions of the human genome, due to the low-copy repeat regions on chromosome 22. This instability predisposes the regionto deletions and duplications through non-allelic homologous recombination events. Hence, the presence of a parental CNV may trigger the development of another CNV in the same or nearby chromosomal region in the offspring, as seen in the familyP09 and as described by Capra et al. (2013).

In conclusion, based on the present results and on those described in the literature (Wozniak et al., 2010; Li et al., 2019; Goldmuntz, 2020), we suggest the implementation of the genetic screening of CNVs in the postnatal management of CHD patients, regardless of the type of CHDs. For this purpose, MLPA is a cost-effective, fast and specific method suitable for the screening of a large number of samples. Patients and families benefit greatly from early diagnosis, through the regular cardiological, orthopedic, endocrinological, immunological, neurodevelopmental, and psychiatric follow-ups, the more aggressive infection control and the possibility of positive family planning.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are includedin the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the National Medical Research Council (No. CHD-01/2016—IF-6299-8/2016) and by the Local Ethical Committee of the University of Szeged (No. 105/2016-SZTE). Written informed consent to participate in this study was provided by theparticipants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

DN and MS: conceptualization, review, editing, and supervision. DN, GZ, MO, AK, KH, GR, MK, AU and ON: methodology, investigation, and validation. DN and MO: data curation. GZ and DN: writing and original draft preparation. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at:https://www.frontiersin.org/articles/10.3389/fgene.2021.635480/full#supplementary-material

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Further delineation of the phenotype of *PAK3*-associated x-linked intellectual disability: Identification of a novel missense mutation and review of literature

1. Introduction

X-linked intellectual disability (XLID) accounts for approximately 5–16% of males with intellectual disability. It is estimated that at least 200 genes are implicated in XLID, and the approximately 170 XLID entities are clinically classified as syndromic or non-syndromic (Stevenson et al., 2009; Lubs et al., 2012).

The p21-activated kinase 3 (*PAK3*) gene was the fourth to be associated with non-syndromic XLID, type 30 (OMIM: #300558) (Allen et al., 1998). PAK3 is a serine/threonine kinase and its sequence is highly conserved between species. The kinase acts as a downstream effector of Rac1 and Cdc42 Rho-GTPases and has important roles in actin cytoskeletal reorganization, dendritic spine morphology, density, stability and dynamics and also in synaptic currents (Kreis et al. 2007; Dubos et al. 2012; Thévenot et al., 2011).

Since 1998, nine different *PAK3* mutations have been identified in 46 affected individuals from nine families of different ethnicity. Here, we report the first case of a Hungarian patient with intellectual disability associated with a novel *PAK3* mutation and review the cases previously described in the literature.

2. Clinical report

The proband presented at genetic counselling at the age of 14 years with intellectual disability, autistic characteristics and behavioral problems.

He was born at term by spontaneous delivery following a normal pregnancy, with normal birth weight and length as a first child of Caucasian non-consanguineous parents. Autistic characteristics and delayed psychomotor development were first noted at the age of 3 years (Brunet-Lézine test: gross motor skills: 65; fine motor skills: 61; language skills: 58; sociability: 52; overall developmental quotient of 59). Special training was initiated. He started to speak and maintain eye contact at 4 years and let his mouth hang open with constant drooling until the age of 4.5 years. He was toilet-trained by the age of 5.5 years, but accidental soiling still happens.

He had three generalized tonic seizure episodes with fever in early childhood and experienced short absence-like episodes and unusual grimacing in the 1.5 year previous to examination. Baseline and sleep-deprived EEGs were repeatedly normal and brain MRI detected no abnormality. Temper tantrums and occasional aggressive behavior has been reported, but no sleep disturbance. At present, he receives risperidone treatment and attends special school.

On examination, he was cooperative, his body weight (43 kg, 10–25 percentile) and height (158 cm, 25 percentile) were normal. Microcephaly (Supplementary Table 1), mild thoracic kyphosis, dorsolumbar scoliosis, ankle valgus, pectus carinatum, wide-spaced nipples and spina bifida occulta with a sacral dimple were noted. His facial features included large ears, prominent but not bulbous nose, low forehead, downslanting palpebral fissures, thin upper lip and high-arched palate (Fig. 1A). His sexual maturation and testicular size were normal. Neurological examination revealed small muscle bulk in the limb-girdle muscles with normal tone and strength, mild postural and intentional tremor, symmetric brisk reflexes without spasticity and no gait disturbance.

Neuropsychological assessment showed mild-to-moderate intellectual disability with moderate impairment of visuo-spatial, reading, writing, comprehension and counting skills and severe attention deficit, mood imbalance, anxiety and autistic traits (Woodcock-Johnson and Snijders-Oomen nonverbal intelligence tests: age equivalent of 5;2 and 5;3, respectively).

Quantitative and qualitative blood count, serum electrolytes, lactate, carbamide, uric acid, creatinine, creatine kinase and liver enzyme levels, inflammatory and autoinflammatory parameters, serum amino acid and acyl-carnitine profile, serum and urine dopamine and serotonin levels showed no marked discrepancy. Audiology detected mild sensorineural hearing loss, however, the examination was inconclusive due to lack of cooperation. On nephrological examination, underactive bladder function was detected. Abdominal ultrasound, echocardiography, ECG and ophthalmology showed no abnormality. Karyotyping on G-banded chromosomes using standard procedures detected no major aberration and testing for Fragile-X syndrome showed no triplet repeat expansion in *FMR1*.

No relatives had intellectual disability or dysmorphic facial features (Fig. 1B).

3. Methods

Genomic DNA was isolated from peripheral blood samples from the proband and his relatives using the Promega Maxwell® RSC Blood DNA Kit. Clinical exome analysis was carried out on the whole exome sequence obtained using Illumina NextSeq500 sequencer after library preparation with Roche KAPA HyperPrep library kit and SeqCap EZ MedExome capture kit.

Mean average depth of on-target coverage in the sequenced exome was 69X (target bases at 10x coverage: 96%; at 20x coverage: 93%; at 30x coverage: 86%). Reads were aligned to the human reference genome (GRCh37) using BWA (v.0.7.12). Among 120,469 variants, deleterious ones were prioritized on the basis of the functional re-levance of genes, inheritance models and minor allele frequency (MAF) in the general population (gnomAD and in-house databases). As a resultof the filtering, a novel variant in the *PAK3* gene was identified as the most probable pathogenic variant. The variant was submitted to a combination of 14 variant prediction tools and was confirmed by bi- directional Sanger sequencing (Supplementary Table 1).

PyMOL Molecular Graphics System (version 2.0 Schrödinger, LLC)

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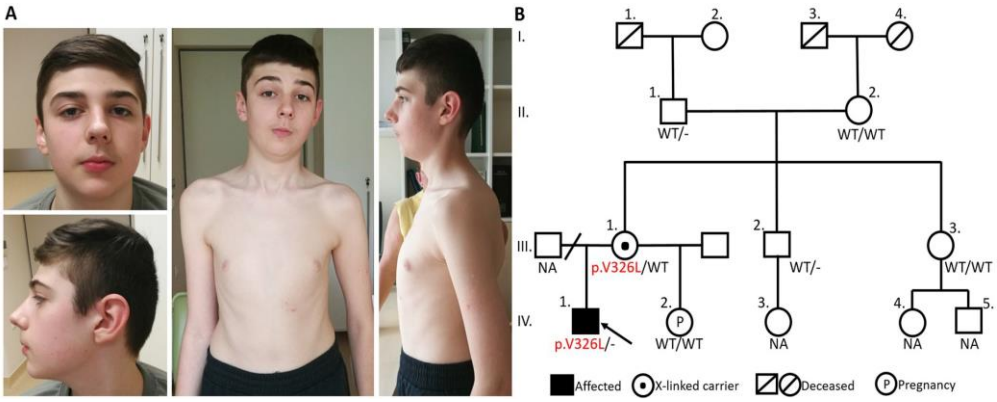

Fig. 1. Pictures and pedigree of the patient.
(A) The images of the proband were captured at the age of 14 years (first column) and 14.5 years (second and third column). **(B)** The mother of the proband was pregnant with a female, non-carrier fetus, as confirmed by karyotyping and targeted mutation analysis. Proband is indicated with an arrow. NA: not assessed.

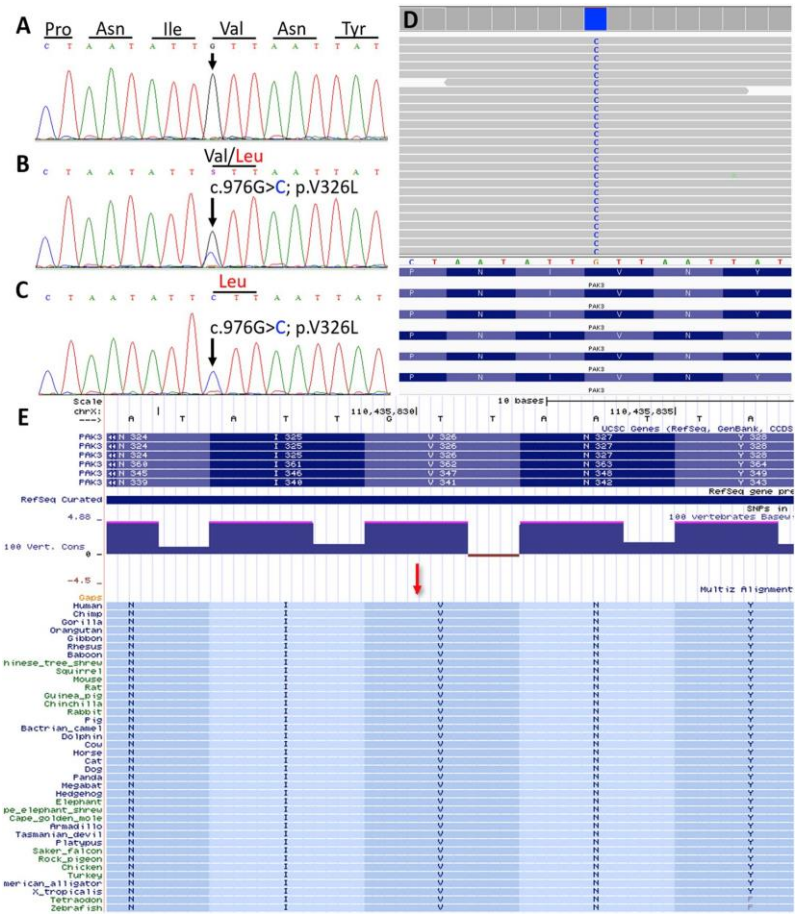

Fig. 2. Analysis of the novel Val326Leu *PAK3* variant by clinical exome sequencing and bidirectional Sanger sequencing.
Electropherogram of the **(A)** wild-type sequence, **(B)** heterozygous female carrier (mother) and **(C)** hemizygous proband. **(D)** The screen shot from the Integrative Genomic Viewer shows part of the (51/51) reads supporting the c.976G > C variant in the proband. **(E)** Screen shot from the UCSC Genome Browser represents thehigh conservation of the amino acid residue in position 326 and surrounding genomic context. Arrows indicate the nucleotide change.

Table 1
Comparison of the clinical presentation of male patients and carrier women in the present and previous studies.

	Allen et al. (1998)	Bienvenu et al. (2000)	Gedeon et al. (2003)	Peippo et al., 2007	Rejeb et al. (2008)	Magini et al. (2014)	Hertecant et al. (2017)	Muthusamy et al. (2017)	Horvath et al. (2018)	*Present study*
PAK3 mutations	c.1255C > T; p.R419X nonsense	c.199C > T; p.R67C missense	c.1049C > A; p.A365E missense	c.1337G > A; p.W446S missense	c.276+4A > G p.G92VfsX35 splice site	c.1167G > T; p.K389N missense	c.1279T > C; p.Y427H missense	c.880G > A; p.V294M missense	c.1579A > G; p.S527G missense	c.G976C p.V326L missense
	rs121434611	rs121434612	rs121434613	rs121434614	CS084886	CM146392	–	–	rs200474454	–
Number of patients tested	4	6	19(13 presented)	5	4(2 presented)	2	2	3	1	1
Number of female carriers tested	4(unaffected)	ND	14(unaffected)	4(3 affected)	4(unaffected)	3(affected only with mild ichthyosis)	0	2(unaffected)	2(unaffected)	1(unaffected)
Geographical origin	USA	France	Australia	Finland	Tunisia	Italy	United Arab Emirates	India	Canada	Hungary
Facial features	ND	ND	**Long ears** (12) Prominent nose (in 3 elderly) **Low forehead** (2) Thin upper lip (7)	**Large ears** (5 M, 0 F) **Low forehead** (3 M) Thin upper lip (5 M) **Drooling** and open mouth (1 M)	**Large ears** **Low forehead** Upslanting palpebral fissures Short nose Thick upper lip **Drooling** Large teeth	**Large ear** Ptosis, squint High palate Pectus excavatum Camptosyndactyly of hands	No obvious dysmorphic feature	Elongated face Synophrys **Long,** low set **ears** Short neck	Facial asymmetry Elongated mid-face Full lips Long jaw	**Large ears** Downslanting palpebral fissures, Prominent nose **Low forehead** Flat occiput **Drooling**
Microcephaly	**Present** (1)	ND	ND	**Present**(2 M, 0 F)	**Present** (1)	**Present** (2)	Macroce.-phaly	**Present**	ND	**Present**
Stature	ND	Normal (6)	Normal (3)	Normal (5 M, 4 F)	Normal (2)	Normal (2)	Normal	ND	ND	Normal
Intellectual disability	Present	Moderate-severe	Present (13) IQ: 65-80	Borderline-mild (F) **Mild**-moderate (M)	**Mild** (IQ:54) – moderate	Present (2)	Present	Moderate	**Mild**	**Mild-moderate**
Gross motor development	ND	ND	Delayed (5)	Delayed (5 M) Normal (F)	Delayed (2)	Delayed (2)	Delayed	Delayed	Delayed	Mildly delayed
Fine motor development	ND	ND	Delayed (3)	Moderate (M) Normal–mild (F)	Delayed (2)	Severely delayed (2)	Delayed	Delayed	Delayed	Moderately delayed
Language development	ND	ND	Delayed (5)	Delayed (M)	Delayed (2)	Severely delayed (2)	Delayed	Delayed	Delayed	Mildly delayed
Language skills **Verbal expression and comprehension** **Reading and writing**	ND	ND	ND	Moderate–severe (M) Normal-mild (F)	Moderate (2)	ND	ND	ND	Delayed	Moderately delayed
Visual skills	ND	ND	ND	Severe (M) Normal-mild (F)	Mild (2)	ND	ND	ND	ND	Moderately delayed
Socialization	ND	ND	Laborer jobs (9)	Sheltered job (3 M)	Sheltered job	ND	ND	ND	Lives in group home	Mildly delayed
Behavior and neuro-psychological profile	ND	ND	Non-categorized learning difficulties (12) **Aggression** (1)			Schizophrenia			(2)	Myoc

Table 1 (*continued*)

	Allen et al. (1998)	Bienvenu et al. (2000)	Gedeon et al. (2003)	Peippo et al., 2007	Rejeb et al. (2008)	Magini et al. (2014)	Hertecant et al. (2017)	Muthusamy et al. (2017)	Horvath et al. (2018)	*Present study*
EEG	ND	ND	ND	Posterior slow wave (4 M/1 F)	Normal (2)	ND	ND	ND	Abnormal (variable)	Normal
Other clinical features	ND	ND	Obesity in 3 elderly	Stooping posture (2 M) Scoliosis (1 M) Childhood **hypotonia** (3 M, 1 F)	**Hypotonia** in infancy (2)	Ichthyosis (2) Early childhood **hypotonia** (2)	Mild axial **hypotonia**	Hypogonadism (1)	Marfanoid habitus Kyphosis Syndactyly Calcaneovalgus deformity **Hypotonia** in infancy	Mild kyphoscoliosis Pectus carinatum Calcaneovalgus deformity Wide-spaced nipples Spina bifida occulta

ND: not described; M: male; F: female; (number): number of patients examined and found positive for the described features. In case of no numbers, all affected male patients exhibited the feature. Common features of patients are written in bold.

was used to evaluate *in silico* the changes in the mutant PAK3 protein structure. The wild-type three-dimensional protein structure has been obtained from RCSB Protein Data Bank (ID: 6fd3) and submitted to PyMOL's Wizard/Mutagenesis on protein application to create and visualize the specific mutant PAK3 protein.

Additional testing included maternity testing on the sample from the proband, maternity-paternity testing on the samples from the proband's mother and maternal grandparents (Promega PowerPlex® ESX 17 System) and X-chromosome inactivation assay (Supplementary Table 1) (Kiedrowski et al., 2011).

The results were assessed and classified according to the ACMG guideline (Supplementary Table 1) (Richards et al., 2015).

1. Results

One novel variant – NM_001128167.2:c.976G > C;p.(Val326Leu) (ClinVar submission number: SCV000927119; LOVD accession number: #0000578234, DB-ID: PAK3_000063) – has been detected in exon 10 of *PAK3* gene, which is associated with X-linked non-syndromic intellectual disability. The variant was present in the proband in a hemizygous form and in unaffected mother in a heterozygous form but not in any other healthy family members tested (Figs. 1B, Fig. 2A–D) or in the control databases (141,456 whole exome/genome sequences contained in gnomAD, in +500 exome sequences of the in-house database of qGenomics or in 151 exome sequences of Hungarian patients recruited in other projects).

The Val326Leu variant was predicted to be probably damaging by PANTHER and PolyPhen2 and damaging by the other 12 prediction tools. The Val326Leu variant is located in the highly conserved protein kinase domain of the *PAK3* gene (Fig. 2E).

The *in silico* modelling suggested that the wild-type residue Val^{326} is located on the surface of the ATP-binding recess of the kinase domain of PAK3 in close vicinity to the ATP molecule (at a distance of 4.0 Å); however, it does not bind to ATP. The amino acid change to Leu^{326} resulted in a shortening of the distance between the ATP molecule and residue 326 (3.6 Å), a change in the surface area of the ATP-binding recess and the formation of a new hydrogen bond between residues Leu^{326} and Leu^{403} (Supplementary Fig. 1), thus supporting its impact on protein structure and function.

Maternity and paternity testing revealed no discrepancy and, therefore, confirmed the *de novo* origin of the variant in the proband's mother.

Based on the ACMG criteria (Supplementary Table 1) and a detailed clinical comparison with previously described patients (Table 1), the results supported the ethiopathogenicity of the novel Val326Leu *PAK3*-variant.

2. Discussion

To the best of our knowledge, this family is the first Hungarian and the tenth family reported worldwide with *PAK3*-associated non-syndromic XLID. Until now, one nonsense, one splice site and seven missense mutations have been reported for the *PAK3* gene. Eight of ten mutations are located in the kinase domain of the protein, presumably disabling its enzymatic function. The location of the Leu^{326} mutation in the kinase domain and the additional hydrogen bond formation suggests that it may influence the ATP-binding capacity and also the structure of the protein.

PAK3 function and regulation is complex. When activated by GTP-bound Rho GTPases (Cdc42 and Rac1), PAK3 kinase phosphorylates other signaling molecules in neurons. The PAK3 function is important for the fine-wiring of the synaptic network in the brain. Therefore, loss-of function mutations in the *PAK3* gene are believed to lead to decreased neural plasticity and cognitive impairment without major structural brain abnormalities, also referred to as synaptopathies (Horvath et al., 2018). However, brain developmental abnormalities

have been reported in some patients carrying variants of the *PAK3* gene, which may be a result of PAK3 protein involvement in other signaling pathways (Magini et al., 2014).

In the current paper, we provide a thorough, comprehensive clinical review of *PAK3*-patients described in the literature to date (Table 1), which allowed us to deduce the typical phenotypic features in *PAK3*-XLID: microcephaly, mild-to-moderate intellectual disability in males, large ears, low frontal hairlines, elongated face, muscle hypotonia in infancy, drooling, seizures, aggression, anxiety and autistic behavior. In addition, this is the first reported patient who also has occult spina bifida and mild thoracolumbar deformity, however these findings are common in the general population and thus, may also be unrelated features.

Copy number variations in the *PAK3*-containing chromosomal region (Xq23) have also been reported in syndromic female patients with moderate-to-severe intellectual disability (Hoischen et al., 2009; Jin et al., 2015). However, these phenotypes are distinct from *PAK3*-XLID due to the haploinsufficiency of other genes involved.

Beside the genetic importance of the diagnosis of *PAK3*-associated XLID, it may also have therapeutic consequence, as presented in a previous report (Horvath et al., 2018). Their patient had epilepsy, cerebral laceration as a result of early-onset, intractable, self-injurious behavior due to decreased levels of dopamine and serotonin metabolites in the cerebrospinal fluid. Low-dose replacement therapy drastically improved and stabilized his condition. It was hypothesized that PAK3 dysfunction may lead to diminished dendritic spines and consequentially diminished postsynaptic dopamine receptors or may impair the phosphorylation of the tyrosine hydroxylase, ultimately leading to decreased catecholamine synthesis (Horvath et al., 2018; Daubner et al., 2011). Thus, in case of behavioral or psychiatric deterioration, determination of the neurotransmitter levels and if necessary, supplementation may be considered. However further studies are needed for final recommendations.

In conclusion, our paper provides further insight into the genetic and phenotypic background of *PAK3*-XLID, expands the *PAK3* mutation spectrum, and may help others with the genetic diagnosis by highlighting the common typical *PAK3*-associated features.

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Consent for participation and publication

Written informed consent was obtained from the proband and family members for clinical and genetic testing using a consent form approved by the Ethics Review Committee, Faculty of Medicine, University of Szeged. The study was conducted according to the Principles of the Helsinki Declaration. Written informed consent for publication of the patient's clinical details and images was obtained from the proband's parent.

Declaration of competing interest

The authors have no conflict of interest to report.

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Appendix A. Supplementary data

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