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Analysis of Copy Number and Sequence Variants Linked to Cardiac Development in Children with Syndromic Congenital Heart Defects

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Abstract

Congenital heart defects (CHDs) are the most common congenital anomalies, with identifiable genetic etiologies in approximately 5–30% of affected infants, depending on the clinical presentation and comorbidities. This study included 216 children with CHD, predominantly syndromic, to explore the role of genetic variants in their morphological phenotypes. Chromosomal microarray (CMA) and whole-exome sequencing (WES) were performed, revealing clinically significant copy number variations (csCNVs) in 59 (27.3%) patients. The most frequent were 22q11.21 (8/59; 13.6%) and 7q11.23 (5/59; 8.5%) deletions. WES was conducted in 28.0% of cases, achieving a detection rate of 29.5%, primarily identifying variants related to Noonan syndrome. Genetic diagnoses were confirmed in 33.3% of patients, with clinically significant CNVs and SNV/INDELs found exclusively in those with syndromic CHD, leading to a 36.5% diagnosis rate in those patients. The identified variants most frequently affected genes encoding transcription factors (40.4%), followed by genes involved in the RAS signaling pathway and structural proteins (17.0%), and chromatin remodeling proteins (12.8%).

Keywords: congenital heart defects (CHD); syndromic CHD; chromosomal microarray (CMA); whole exome sequencing (WES); genetic variation

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1. Introduction

Congenital heart defects (CHD) are structural malformations of the heart and great vessels, with a wide spectrum of clinical manifestations. CHD is the most common congenital anomaly and the leading cause of death in the childhood period [1,2]. These defects can occur in sporadic, familial, or syndromic forms. The causes of CHD are multifactorial, involving a combination of genetic and environmental factors [3]. Genetic causes can be identified in approximately 20–30% of affected children [3], encompassing aneuploidies, copy number variations (CNVs), and single-nucleotide variants (SNVs). Microarray

analysis is a first-tier diagnostic tool for the copy number variations (CNVs) [4]. Its diagnostic yield ranges from approximately 5% to 20% in patients with developmental delay/intellectual disability (DD/ID), autism spectrum disorder, and multiple congenital anomalies. In children with the syndromic form of CHD, the diagnostic yield of microarray analysis has been reported to be as high as 25%, while in the sporadic form, it ranges from 3 to 10% [5]. Whole-exome sequencing (WES) is a powerful tool for the detection of causative single-nucleotide variants (SNVs) and small insertions/deletions (INDELs). The diagnostic yield of trio-based WES in children with syndromic and familial forms of CHD ranges from 25% to 46%, and 2–10% in sporadic form, according to De Bacher et al. [5].

CHDs comprise a broad spectrum of cardiac abnormalities, from single defects, such as atrial septal defect (ASD) or ventricular septal defect (VSD), which are typically acyanotic, to more complex malformations, such as tetralogy of Fallot (ToF), which are usually cyanotic [6]. During embryogenesis, cardiogenic mesodermal cells arise bilaterally from the first heart field (FHF) and the second heart field (SHF), forming the cardiac crescent [7]. Cells derived from the FHF give rise to the primitive beating heart tube, which subsequently develops into the atria and the left ventricle. In contrast, SHF cells contribute to the formation of the right ventricle, the outflow tract, and parts of both atria [7–9]. All these processes that include cell proliferation, differentiation, migration and structural formation are achieved through precisely regulated expression of genes that code for transcription factors (*TBX1*, *TBX5*, *GATA4*), chromatin regulating proteins (*KMT2D*, *NSD1*), signaling proteins (*PTPN11*, *BRAF*, *SOS1*), structural proteins (ELN, *FBN1*), growth factors (*VEGF*, *ACVR1*), and others. The morphological type of CHD is associated with the timing of developmental disruption during cardiogenesis and is closely linked to genes that play critical roles in normal cardiac development [6].

Additionally, genes that regulate the cell cycle, such as those encoding growth factor receptors and tumor suppressors, as well as other critical regulatory proteins, play significant roles in normal cardiac morphogenesis. These essential genes are highly sensitive to alterations in gene dosage, which may result from CNVs. In contrast, SNVs and INDELS may preserve physiological gene dosage while impairing gene function [5]. Such genetic alterations can lead to developmental abnormalities and contribute to the pathogenesis of CHD [10].

Therefore, the present study investigated the diagnostic utility of genomic analyses, including molecular karyotyping and whole-exome sequencing (WES), in children with CHD. Additionally, we analyzed the contribution of the detected gene variants to the morphological phenotypes of congenital heart defects.

2. Materials and Methods

2.1. Participants

Between January 2018 and December 2025, 2650 children with congenital anomalies and neurodevelopmental disorders underwent chromosomal microarray (CMA) at the Institute of Human Genetics, Faculty of Medicine, University of Belgrade, as a part of diagnostic testing. Patients were referred from various clinics across Serbia. From this cohort, we selected 216 children with isolated or syndromic CHD, collected detailed phenotypic information and results of exome sequencing (ES). Clinical data were obtained from patients' medical records and a structured internal questionnaire specifically designed for comprehensive phenotypic characterization. Phenotypic assessment was performed by a multidisciplinary team comprising clinical geneticists, pediatric cardiologists and neurologists.

CHD in newborns was primarily diagnosed by fetal or neonatal echocardiography, including Doppler and three-dimensional imaging. For older children, a comprehensive cardiological evaluation was conducted, and the morphological classification of heart defects is determined using transthoracic echocardiography, Doppler and three-dimensional imaging.

Types of CHD are specified according to the morphological classification of congenital heart defects [11]. Septal defects are characterized by abnormal communications between cardiac chambers, typically resulting in left-to-right shunting and, less commonly, right-to-left shunting. This category includes ASD, VSD, and atrioventricular septal defect (AVSD). Conotruncal defects are characterized by abnormalities of the great arteries and are often associated with cyanosis. This group included ToF, dextro-transposition of the great arteries (TGA), double outlet right ventricle (DORV), and truncus arteriosus communis (TAC). Valve anomalies included aortic stenosis (AS), pulmonary stenosis (PS), and Ebstein anomaly. Obstructive lesions were classified into two categories: left ventricular outflow tract obstructions (LVO) and right ventricular outflow tract obstructions (RVO). LVO conditions included coarctation of the aorta (CoA), hypoplastic left heart syndrome (HLHS), aortic stenosis (AS), and interrupted aortic arch (IAA). RVO conditions included pulmonary stenosis (PS) and pulmonary atresia with ventricular septal defect (PAVSD). The classification also covered other complex congenital heart defects (OCHD) and patent ductus arteriosus (PDA).

2.2. Molecular Karyotyping

After DNA was extracted from 3–5 mL of peripheral blood using the standard salting-out method [12], aCGH was conducted for all children included in our study. We used Agilent microarray oligonucleotide slides, specifically the SurePrint G3 Human CGH Microarray 8 × 60K and the SNP + CGH Microarray 4 × 180K (Agilent Technologies, Santa Clara, CA, USA), according to the manufacturer's protocol. Genomic positions were referenced based on the human genome sequence GRCh37/hg19. All identified CNVs were analyzed and classified according to the latest guidelines from the American College of Medical Genetics and Genomics (ACMG) [13]. Pathogenic and likely pathogenic variants are regarded as clinically significant (csCNV).

2.3. Whole Exome Sequencing (WES)

WES results for children without csCNVs detected by CMA were collected by reviewing the Heliant database and the University Children's Hospital, Belgrade internal database. WES was commercially performed by Centogene (Rostock, Germany) and the Institute of Molecular Genetics and Genetic Engineering (IMGGI), Belgrade, Serbia, upon request of the Genetic Counselling Department at the University Children's Hospital. Full exome capture and sequencing were carried out using an Illumina platform.

Variants were extracted from clinical reports provided by Centogene and IMGGI, with classifications taken directly from the external laboratory reports. WES was performed only for the proband. Additionally, for all positive findings, segregation analysis was conducted using Sanger sequencing at the same laboratories to determine the inheritance of the variants.

2.4. Association of Genes with CHD and Its Dosage Sensitivity

All csCNVs encompassing multiple genes were analyzed to identify candidate genes involved in the CHD development. A thorough literature review (PubMed) and database search (DECIPHER, ClinVar) aimed at establishing a connection or providing experimental evidence for the role of one or more genes in the embryonic heart development supported this analysis. Dosage sensitivity of the genes was established by prediction parameters (pLi,

pHaplo, pTriplo) in DECIPHER and ClinGen databases. For the deletions, we considered genes with a known association with CHD or embryonic heart development, as well as those with high haploinsufficiency scores (pLi and/or pHaplo between 0.8 and 1, and/or a ClinGen haploinsufficiency score of 2 or 3) as potentially causative. Conversely, for cases of copy number gain, we considered genes with high triplosensitivity scores (pTriplo between 0.8 and 1, and/or a ClinGen dosage curation score of 2 or 3).

Statistical analysis was performed using SPSS software (version 20.0; SPSS Inc., Chicago, IL, USA). Statistical significance of the association between CHD morphological types and presence or absence of csCNVs was analyzed using Chi-square and Fisher's exact tests. p values lower than 0.05 were considered statistically significant.

Informed consent was obtained from the legal guardians of all patients. The study protocol was approved by the Ethics Committee of the Faculty of Medicine at the University of Belgrade (Approval No. 1322/VII-4), and the use of next-generation sequencing (NGS) data was authorized by the Ethics Committee of the University Children's Hospital, Tirsova, Belgrade (Approval No. 017:16/47).

3. Results

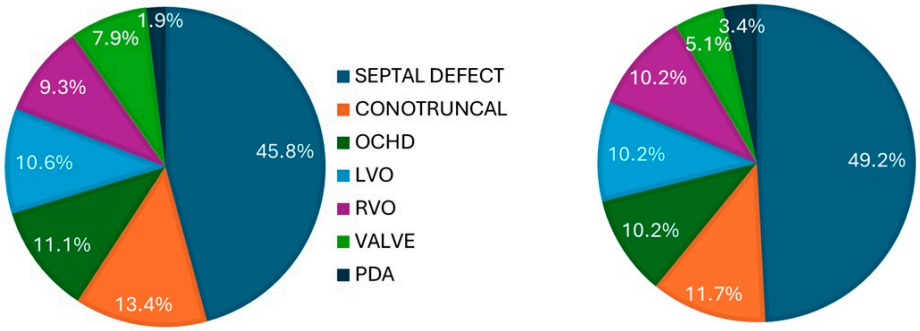
3.1. Patients' Demographic and Clinical Data

Among the 216 children examined, there was an equal distribution of boys and girls. Phenotypic analysis indicated that 91.2% of the children had a syndromic form of CHD. A neurocognitive assessment identified DD/ID in 108 (50%) cases, but could not be performed in 60 newborns and infants (27.8%). The gender, age, and clinical data for the children are presented in Table 1.

Table 1. Characteristics of children with CHD.

Children with Congenital Heart Defects		**N (%)**
Gender	Boys	108 (50.0)
	Girls	108 (50.0)
Age	Mean (+SD)	3.19 (5.18)
	Median (min–max)	0.9 (0.1–18)
DD/ID	Unknown	60 (27.8)
	Yes	108 (50.0)
	No	48 (22.2)
Facial dysmorphia		150 (69.4)
Head and CNS anomalies		52 (24.1)
Urogenital tract anomalies		29 (13.4)
Skeletal and joint system anomalies		21 (9.8)
Autism spectrum disorders		9 (4.2)
Epilepsy		14 (6.5)
Isolated CHD		19 (8.8)

Using a morphological classification of CHD, we assessed the prevalence of specific types of heart defects within the study population. Septal defects were the most prevalent, occurring in 45.8% of the children, and distribution of all morphological types is presented in Figure 1a.



(**a**) (**b**)

Figure 1. (**a**) Distribution of morphological types of congenital heart defects; (**b**) Distribution of csCNV by morphological type of CHD.

3.2. Chromosomal Microarray Analysis Results

Clinically significant CNVs were identified in 27.3% of the infants, and they were found only in children with the syndromic form of CHD. Deletions accounted for 50.6%, duplications for 33.8%, and 15.6% of the children had two or more concurrent variants (either deletions and/or duplications). The size of these CNVs ranged from 0.33 to 64.28 Mb. Notably, 49.2% of all detected csCNVs were identified in children with septal defects. The csCNV distribution across morphological types of CHD is shown in Figure 1b. The detection rates of csCNVs across different morphological types of CHD were as follows: 29.3% (29/99) in septal defects, 30.0% (6/20) in RVO, 26.1% (6/23) in LVO, 24.1% (7/29) in conotruncal defects, 17.6% (3/17) in valve anomalies, 50.0% (2/4) in PDA, and 25.0% (6/24) in other complex heart defects (OCHD). No statistically significant differences were observed in the distribution of morphological CHD types between children with and those without detected csCNVs ($p = 0.812$). These results should be interpreted cautiously, given the small subgroup sizes.

Consistent with expectations, the most frequent csCNVs were deletions in the 22q11.2 (13.6%; 8/59) and 7q11.23 (8.5%; 5/59) regions. Two patients were diagnosed with Noonan syndrome: one had a duplication in the 2p22.1 region, and the other had a deletion in the 11p15.2 region. This diagnosis was based on clinical findings and confirmed dosage sensitivity of the genes within the CNVs.

Overall, we diagnosed common syndromes linked to CHD in 30.5% of the cases (18 out of 59 patients), as shown in Table 2. The remaining 41 patients in our cohort were individual cases, as presented in Table 3.

Table 2. Common syndromes associated with CHD diagnosed in more than one patient by CMA.

Syndrome	Region	CNV Type	Size (Mb)	Gene (OMIM)	N (%)	CHD Type (N)
DiGeorge	22q11.2	Deletion	2.50	*TBX1* (602054)	8 (13.6)	ToF (4), AVSD (2), VSD (2) PAToA (2), OCHD (1)
22q11.2 duplication	22q11.2	Duplication	2.80		3 (5.1)	
Williams-Beuren	7q11.23	Deletion	1.40	*ELN* (130169)	5 (8.5)	* PS (3), * AS (2)
Noonan	2p22.1	Duplication	2.73	*SOS1* (182530)	2 (3.4)	VSD (1)
	11p15.2	Deletion	4.20	*RRAS2* (600098)		PS (1)

Legend: *supravalvular; AS: aortic stenosis, pulmonary stenosis; AVSD—atrioventricular septal defect; OCHD—Other complex heart defect; PAToA—pulmonary atresia with D-transposition of aorta and atrial septal defect; VSD—ventricular septal defect.

Table 3. Clinically significant CNVs observed in individual cases within our cohort of children with CHD.

	CMA Findings (GRCh37)	Size Mb	Syndromes (OMIM)	Gene(s) (OMIM)	Phenotype
1	1p31.3(61782914_62322790)x1	0.54	NA	NA	OCHD, corpus callosum agenesia, congenital inguinal hernia
2	1p36.13-p36.11(18900374_23966858)x1	5.1	1p36 microdeletion (#607872)	*CDC42* (16952) *ECE1* (600423)	ASD, facial dysmorphia, hypotonia, clubfoot, right sided hydronephrosis
3	1q21.1(145413388_145747269)x1	0.33	Thrombocytopenia Absent Radius-TAR (#274000)	*RBM8A* (605313)	AVSD, TAR
4	1q23.2-q23.3(160465291_165429037)x1	4.9	NA	*PBX1* (176310)	ASD, facial dysmorphia, ectopic kidney, glaucoma, cerebral palsy
5	2q11.1-q11.2(96779631_98021592)x1	1.2	NA	NA	PDA, preterm birth, microcephaly, craniosynostosis, metatarsus varus
6	2q22.2-q22.3(143986161_146890297)x1	2.9	Mowat–Wilson (#235730)	*ZEB2* (605802)	CoA, aortic bicuspid valve, facial dysmorphia, microcephaly, corpus callosum agenesia
7	2q23.3-q24.1(151373825_158622730)x1	7.3	NA	*ACVR1* (102576)	PDA, VSD, facial dysmorphia, microcephaly
8	3q22.1-q29(133562250_197840339)x3	64.3	Noonan type 11 (#618499)	*MRAS* (608435)	OCHD, facial dysmorphia, cleft soft palate, IUGR
9	3q28-q29 (191861311_197840339)x3 5p15.33-p15.31 (151737_7144623)x1	6.0 7.0	3q29 microduplication (#611936) Cri du chat (#123450)	*PAK2* (605022)	AV block I, PS, PFO, facial dysmorphia, hypospadias, hypopituitarism, hip dysplasia, scoliosis, myopia
10	4p16.3-p16.1(71552_7760991)x1 17q25.3(76890486_81029941)x3	7.7 4.1	Wolf-Hirschhorn (#194190)	*MSX1* (142983)	ASD, PS, facial dysmorphia, complete cleft palate, microcephaly, hypotonia
11	4q21.21(81561965_82010110)x3	0.45	NA	NA	AVSD, facial dysmorphia, rhizomelia, hypotonia
12	5q21.3-q23.1(105623245_116004172)x1	10.4	Familial Adenomatous Polyposis (#175100)	*APC* (175100)	ToF, facial dysmorphia, imperforate anus
13	7p15.3-p14.3(20993642_30739239)x1	9.7	NA	*HOXA1* (142955)	ASD, facial dysmorphia, IUGR, kidney hypoplasia, skeletal anomalies
14	7q33-q36.3(133749674_158909738)x3	25.2	NA	*KMT2C* (606833)	PDA, facial dysmorphia, cleft palate, brain anomalies
15	8p23.3-p23.1(221611_9261350)x1 8q21.2-q24.3(86842195_146280020)x3	9.0 59.4	Cornelia de Lange Syndrome 4 (#614701)	*RAD21* (606462)	AVSD, PDA, ptosis, Pierre Robin sequence
16	8p23.3-p22(524066_17541888)x3 9p24.3-p24.2(271257_42776209)x1	17.0 4.0	8p23.1 microduplication	*GATA4* (600576)	OCHD, omphalocele, scoliosis, arachnodactyly

Table 3. *Cont.*

	CMA Findings (GRCh37)	Size Mb	Syndromes (OMIM)	Gene(s) (OMIM)	Phenotype
17	8q23.3-q24.23(113589865_136427632)x4	22.8	Cornelia de Lange Syndrome 4 (#614701)	*RAD21* (606462)	VSD, facial dysmorphia, cryptorchidism
18	8q22.2-q22.23(100973253_103335730)x1	2.4	NA	*SPAG1* (603395)	AVSD, facial dysmorphia, complete palate cleft
19	9p23-p22.3(12772471_14680180)x1	1.9	NA	*MPDZ* (603785)	AVSD, craniofacial dysmorphia, macrocephaly
20	9q31.1-q31.3(106828041_112710753)x1	5.9	9q31.1-q31.3 microdeletion (#618619)	*ZNF462* (617371)	VSD, facial dysmorphia, bilateral VUR
21	12p13.3-p11(511504_34189943)x4	35 p arm	Pallister- Killian (#601803)	NA	VSD, bicuspid aortic valve, facial dysmorphia, acromelia, brain anomalies, anal atresia, cryptorchidism
22	12p13.3-p11(511504_34189943)x4	35 p arm	Pallister- Killian (#601803)	NA	PDA, tricuspid valve insufficiency, DD/ID
23	14q32.3(105717621_106327993)x1	0.60	NA	*PACS2* (610423)	ASD, hypertelorism, micrognathia
24	15q11.2-q13.1(23699701_28525460)x1	4.8	Angelman type II (#105830)	NA	mitral valve dysplasia, DD/ID, facial dysmorphia, strabismus
25	15q13.2-q13.3(31014508_32510863)x1	1.5	15q13.3 microdeletion (#612001)	*KLF13* (605328)	MVP, left ventricular hypertrophy facial dysmorphia
26	15q21.1(48905243_49084691)x1	0.29	Marfan (#154700)	*FBN1* (134797)	MVP, voluminous left ventricle, Marfan-like phenotype
27	15q26.2-q26.3(94447479_102383473)x1	7.9	NA	*NR2F2* (107773)	AVSD, facial dysmorphia, short stature, VUR bilateral
28	16p11.2(29673954_30198600)x1	0.52	16p11.2 proximal microdeletion (#611913)	*TBX6* (602427)	ToF, polycystic kidney disease
29	16q13.11(15048751_16249607)x1	1.2	NA	*MYH11* (160745)	PDA, preterm birth, craniofacial dysmorphia, bilateral inguinal hernias, hyperbilirubinemia
30	16q11.2-q12.1(46564557_49053314)x3 16q12.2-q22.2(55361181_71354431)x3	2.5 16.0	NA	NA	ASD, micrognathia, short neck, torticollis, umbilical hernia, pes varus
31	16q11.2-q22.2(46564557_71127772)x3	24.6	NA	NA	OCHD, facial dysmorphia, hypotonia
32	16q24.2-q24.3(88653937_89429735)x3	0.78	NA	*ANKRD11* (611192)	PDA, PFO, mitral valve anomaly, hydrops fetalis,
33	17q12(34817422_36168104)x1	1.4	17q12 microdeletion (#614527)	NA	VSD, facial dysmorphia, polycystic kidney disease
34	17p13.3-p13.2(51885_3882130)x1	3.8	Miller-Dieker (#247200)	*DPH1* (60352) *NXN* (612895)	VSD, IUGR, hypotrophy, brain anomalies, toe anomalies
35	17q21.31(43717703_44159862)x1	0.44	Koolen-De Vries (#610443)	*KANSL1* (612452)	ASD, facial dysmorphia, kidney agenesis, unilateral cleft lip

Table 3. *Cont.*

	CMA Findings (GRCh37)	Size Mb	Syndromes (OMIM)	Gene(s) (OMIM)	Phenotype
36	5q35.2-q35.3(176033642_177013961)x3[0.412] 18p11.32-p11.21(142096_14748636)x1[0.412] 18q21.2-q23(49545872_77901872)x1[0.412]	0.98 14.6 28.4	NA Chromosome 18 ring	*NSD1* (606681) *NFACT1* (600488)	OCHD, facial dysmorphia, microcephaly, cleft lip and palate
37	18q21.33-q23(59653070_78621175)x1	17.2	18q microdeletion (#601808)	*NFACT1* (600488)	mitral and tricuspid valve dysplasia, facial dysmorphia
38	18q23(75814123_78014123)x1	2.2	18q microdeletion (#601808)	*NFACT1* (600488)	ASD, facial dysmorphia, microphthalmia, hypertrichosis, microcephaly, brain atrophy
39	21q22.(46411778_48067924)x1	1.7	NA	*COL6A1* (120220)	ASD, VSD, right aortic arch, pulmonary artery atresia, facial dysmorphia, NEC
40	22q11.1-q11.21(17096855_18953065)x3	1.9	Cat-eye (#115470)	NA	TAPVR, facial dysmorphia, congenital hypothyroidism
41	22q11.23(23739437_24988455)x3	1.2	NA	*SMARCB1* (601607)	VSD, facial dysmorphia, epilepsy, dolichocephaly

Legend: ASD—atrial septal defect; AVSD—atrioventricular septal defect; CoA—coarctation of aorta; DD/ID—developmental delay/intellectual disabilities; IUGR—intrauterine growth retardation; MVP—mitral valve prolapses; NEC—necrotizing enterocolitis; OCHD—Other complex heart defect; PDA—patent ductus arteriosus; PFO—patent foramen ovale; PS—pulmonary stenosis; TAPVR—total anomalous pulmonary venous return; ToF—Tetralogy of Fallot; VSD—ventricular septal defect; VUR—vesicoureteral reflux.

In our analysis of the gene content within CNVs from 9 of those patients, we were unable to identify a specific gene linked to CHD development. In an additional group of 9 patients, we identified pathogenic and likely pathogenic CNVs that included genes associated with CHD, such as *MRAS*, *KMT2C*, *RAD21*, *GATA4*, and *NSD1*. However, these variations were either too large (primarily duplications), encompassing many genes, or too complex, involving two or more chromosomes, making it impractical to pinpoint a single gene responsible for the heart defects.

Finally, among the patients shown in Table 3, we identified a candidate gene for CHD in 16 of them. In addition to the 18 previously described in Table 2 in which the causative gene was already known or suspected, this accounted for 57.6% (34/59) of patients who had a genetic diagnosis by CMA.

3.3. WES Analysis Results

For the remaining cohort, (157 patients with negative or non-diagnostic CMA findings), we collected WES results. At that time, only 28.0% (44 out of 157) of these children had WES results. The detection rate of clinically significant variants in this group was 29.5% (13/44), with the following distribution according to CHD morphology: 30.8% (4/13) in septal defects, 23.1% (3/13) in valve anomalies, 15.4% (2/13) in OCHD, and 7.6% (1/13) in conotruncal defects, LVO, RVO, and PDA. No statistically significant differences were observed in the distribution of morphological CHD types between children with and without clinically significant SNV or INDEL variants ($p = 0.315$).

Table 4 presents all clinically relevant SNVs and INDELs in genes potentially associated with CHD. Almost all identified variants were inherited in an autosomal dominant pattern and occurred de novo. Notably, two boys had X-linked variants; one had recessive variants in the *FGD1* gene, and the other had dominant variants in the *AMER1* gene. In Patient 4, who has Coffin–Siris syndrome type 1, and Patient 9, who has Aarskog–Scott syndrome,

de novo variants of uncertain significance (VUS) were detected. However, based on a thorough clinical evaluation and phenotypic correlation, these variants were deemed possibly pathogenic.

Table 4. Clinically significant SNV/INDEL detected in genes responsible for CHD.

	Syndrome	Gene (OMIM)	Transcript	SNV/INDEL	AAC	Zyg	Class	CHD Type
1 2	Noonan type 1	*PTPN11* (176876)	NM_002834.5 NM_002834.5	c.767A > G c.228G > T	p.Gln256Arg p.Glu76Asp	Het Het	P P	PS PS
3	Noonan type 7	*BRAF* (164757)	NM_004333.6	c.1785T > G	p.Phe595Leu	Het	P	PS
4	MIM 616977 MIM 619522	*HIVEP2* (143054) *ZMYM2* (602221)	NM_006734.4 NM_197968.4	c.3566T > C c.2320C > T	p.Leu1189Ter p.Gln774Ter	Het Het	P LP	PDA
5	Sotos	*NSD1* (606681)	NM_022455.5	c.6206_6209del TTTG	p.Val2069fs	Het	P	VSD
6	Alagille type 1	*JAG1* (601920)	NM_000214.3	c.2113 + 1G > A	splice site variant	Het	LP	PAVSD PDA
7	Kabuki type 1	*KMT2D* (602113)	NM_003482.4	c.12598C > T	p.Gln4200Ter	Het	P	ASD
8	Stankiewicz-Isidor	*PSMD12* (604450)	NM_002816.5	c.47_56del	p.Met12Thrfs Ter16	Het	P	CCA
9	MIM 612621	*SYNGAP1* (603384)	NM_006772.3	c.3361del	p.Ser1121Ala fs*9	Het	LP	ASD
10	MIM 300373	*AMER1* (300647)	NM_152424.4	c.1275C > A	p.Tyr425Ter	Hem	P	VSD
11	MIM 618672	*CNOT3* (604910)	NM_014516.4	c.1438dupG	p.Ala480fs	Het	LP	IAA
			Possibly clinically significant VUS					
12	Coffin-Siris 1	*ARID1B* (614556)	NM_001374828.1	c.1520C > T	p.Pro507Leu	Het	VUS PP#	HA
13	Aarskog-Scott	*FGD1* (300546)	NM_004463.3	c.2046G > T	p.Gln682His	Hem	VUS PP#	OCHD

Legend: AAC—amino acid change; ASD—atrial septal defect; CCA—Coronary cameral fistulas; HA—hemitruncus arteriosus; IAA—interrupted aortic arch; LP—likely pathogenic MIM300373—Osteopathia striata with cranial sclerosis; MIM618672—Intellectual developmental disorder with speech delay, autism, and dysmorphic facies; MIM616977—Intellectual developmental disorder, autosomal dominant 43; MIM619522 Neurodevelopmental-craniofacial syndrome with variable renal and cardiac abnormalities; MIM 612621—Intellectual developmental disorder, autosomal dominant 5; OCHD—Other complex heart defect; PAVSD—pulmonary atresia with ventricular septal defect; P—pathogenic; PS—pulmonary stenosis; PDA—patent ductus arteriosus;—VUS PP#—variant of uncertain significance, possible pathogenic; VSD—ventricular septal defect; Zyg—Zygosity.

3.4. Analysis of the Diagnostic Yield and Candidate Genes for CHD

In this study, a genetic diagnosis was established, either by CMA or WES, for 72 out of 216 cases, representing 33.3% of the total. Specifically, 59 were confirmed CMA-positive. Of 157 CMA-negative patients, 44 underwent WES, and 13 had positive findings. For syndromic CHD, this percentage is higher- 36.5% (72/197), while no genetic cause was found in patients with isolated heart defects (0/19). We analyzed all genes with sequence variants identified by WES (see Table 4) as well as those considered critical for CHD within the csCNVs (Tables 2 and 3). Overall, we identified a causative or potentially causative gene in 65.3% (47 of 72) of cases. We subsequently classified these genes into six distinct functional groups (Table 5).

Variants in genes encoding transcription factors (TF) represent the most frequent cause of CHD (40.4%; 19/47). Among children with TF variants, 42.1% had septal defects and 21.0% had conotruncal defects. RAS signaling variants primarily caused valve anomalies and outflow tract obstructions. Other gene variants were mostly associated with septal defects.

Table 5. CHD type according to candidate genes and their specific function.

Function	Genes	Type CHD (n)	Patients n (%)
Transcription Factors	*TBX1*, *PBX1*, *CNOT3*, *ZEB2*, *HIVEP2*, *ZMYM2*, *NR2F2*, *MSX1*, *NFATC1*, *ZNF462*	ToF (4), PAToA (2), ASD (2), AVSD (3), VSD (3), CoA (1), IAA (1), PDA (1), OCHD (1), PS (1)	19 (40.4)
RAS signaling pathway	*RRAS2*, *PTPN11*, *BRAF*, *SOS1*, *FGD1*, *CDC42*, *SYNGAP1*	PS (4), ASD (2), VSD (1), OCHD (1)	8 (17.0)
Structural proteins	*ELN*, *FBN1*, *MYH11*, *COL6A1*	PS * (3), AS * (2), MVP (1), PDA (1), OCHD (1)	8 (17.0)
Chromatin regulating	*ARID1B*, *SMARCB1* *ANKRD11*, *NSD1*, *KMT2D*, *KANSL1*	HA (1), PDA (1), ASD (2), VSD (2)	6 (12.8)
GFR and tumor suppressors	*ACVR1*, *AMER1*, *APC*	VSD (2), ToF (1)	3 (6.4)
Ungrouped protein	*PACS2*, *JAG1*, *PSMD12*,	CCA (1), ASD (1), PAVSD (1)	3 (6.4)

Legend: PS—pulmonary stenosis; ASD—atrial septal defect; AS-aortic stenosis; AVSD—atrioventricular septal defect; CCA—Coronary cameral fistulas; CoA—coarctation of the aorta; GFR—growth factor receptors; IAA—interrupted aortic arch; MVP—Mitral valve prolapse; OCHD—Other complex heart defect; PAToA—pulmonary atresia with D-transposition of aorta and atrial septal defect; PAVSD—pulmonary atresia with ventricular septal defect; PDA—patent ductus arteriosus; ToF—Tetralogy of Fallot; VSD—ventricular septal defect; * supravalvular stenosis.

4. Discussion

We analyzed children with CHD who predominantly exhibited a complex phenotype, and less than 10% of our cases were non-syndromic. As expected, none of the children with isolated heart defects had a clinically significant CNV or sequence variants. Conversely, among children with DD/ID, dysmorphic features, or other major congenital anomalies, the diagnostic yield of CMA and WES was substantial. The highest diagnostic yield of CMA—up to 28%—occurs in cases associated with extracardiac malformations or DD/ID, which aligns with our study [14]. The absence of csCNVs in patients with non-syndromic CHD in our cohort may be due, in part, to the small number of such patients and the strict phenotypic categorization used. For example, a 22q11.2 deletion, characteristic of DiGeorge syndrome, was identified in two infants who did not yet exhibit additional phenotypic traits. Nonetheless, they were classified as syndromic because other typical symptoms are expected to evolve. Besides, early diagnosis had predictive value, allowing for more cautious planning of the operative intervention for the heart defect, immunological, developmental, and other early interventions that were anticipated.

WES diagnostic yield was slightly higher than that of CMA (29.5% vs. 27.3%). However, only 44 patients had WES results available at the time of this retrospective analysis. It should be emphasized that over a quarter of these children were under the age of one, and, in our country, CMA is typically the first-tier genetic test for children with congenital anomalies. Published data consistently show that the diagnostic yield is higher in syndromic forms of CHD than in sporadic cases, with reported rates ranging from 39.0% to 43.0% across different populations [15–17]. We established genetic diagnosis in 33.3% patients, although this result should be interpreted with caution since WES analysis was not performed for all individuals.

Septal defects were the most prevalent type of CHD in our cohort, followed by conotruncal anomalies. The Pediatric Cardiac Genomics Consortium reports higher rates of septal defects (53%) and conotruncal anomalies (36%) than our study [6]. These discrepancies may be due to differences in classification, as valvular anomalies and other complex heart defects were not analyzed as separate subgroups in the Consortium reports. Nevertheless, septal defects remained the most common type of CHD across all populations, with VSD being the most frequent subtype [18]. Also, the largest number of children with csCNV had a septal defect (49.2%) as well as patients with pathogenic sequence variants (30.8%), which corresponds to literature data [19]. However, no statistically significant differences were observed in the distribution of morphological CHD types between children with detected csCNVs and those without a molecular diagnosis.

Further analysis of both CNV and sequence variants revealed likely causative gene(s) for CHD in 65.3% of children. The observed variants affected different groups of genes with specific functions. The most common were among genes encoding transcription factors, which aligns with their crucial role in early embryonic and cardiac development. Among those genes, the most common were variants in *TBX1*, with a well-known association with cardiac defects in DiGeorge or 22q11.2 duplication syndrome [20]. ToF is a typical heart defect associated with *TBX1* [1,11,21], which was also found in our 4 out of the 11 patients with variants in this gene.

Similarly, the *PBX1* gene is a critical regulator of heart morphogenesis. Disruption of its function or dosage-sensitive interactions with *PBX2* and *PBX3* can lead to severe cardiac defects, including ToF, PDA, and septal defects, often as part of CAKUTHED syndrome (Congenital Anomalies of the Kidney and Urinary Tract, Hearing loss, Ear abnormalities, and Developmental delay) [22]. Patient 4 in Table 3 presented with developmental delay, facial dysmorphia, renal ectopia, congenital glaucoma, cerebral palsy, and ASD. Interestingly, *PBX1* interacts with another TF gene important for embryonic development—*ZNF462*, which is deleted in our patient with VSD, facial dysmorphia, and bilateral vesicoureteral reflux. Pathogenic variants of *ZNF462*, primarily loss-of-function, are associated with Weiss-Kruszka syndrome, which is characterized by distinctive craniofacial dysmorphism, developmental delay, and, less frequently, CHD or growth hormone deficiency. *ZNF462* prevents the heterodimerization of *PBX1* and its binding to DNA, suggesting that its deletion may be crucial for CHD expression [23]. Variants in the *CNOT3* gene are also associated with DD/ID and cardiac defects [24], and our patient with a variant in this gene has developmental delay and an interrupted aortic arch. This gene is part of the CCR4-NOT transcription complex, which regulates gene expression and is involved in processes such as mRNA degradation, miRNA-mediated repression, and general transcription regulation [25]. The CCR4-NOT complex is crucial for heart development, as it promotes cardiomyocyte proliferation by regulating the degradation of cell cycle inhibitor mRNAs.

A patient with Mowat–Wilson syndrome had CoA, along with other typical features. Critical gene in this microdeletion syndrome is *ZEB2*, which encodes a zinc-finger transcription factor that binds E-box sequences to regulate organ development, nervous system maturation, and immune function. About 60% of patients have CHD [1,26]. Another patient with PDA had variants in two different genes: *HIVEP2* and *ZMYM2*. First encodes a zinc-finger transcription factor that is primarily active in the brain, regulating genes essential for neuronal growth, maturation, and synaptic function. While no published studies have linked variants in *HIVEP2* to cardiac defects, *ZMYM2* functions as a nuclear transcriptional corepressor and chromatin regulator. Variants in this gene have been associated with a rare neurodevelopmental and craniofacial syndrome that can also include renal and cardiac abnormalities [27]. The *NR2F2* gene encodes a ligand-inducible transcription factor that is crucial for embryonic development, angiogenesis, and cell fate determina-

tion. Pathogenic variants in the *NR2F2* gene are linked to various complex cardiovascular anomalies, including AVSD, which our patient with 15q26.2-q26.3 deletion has [1,28]. The *MSX1* gene encodes a transcription factor vital for cardiac development, particularly in the formation of the outflow tract and atrioventricular cushions. The combined loss of function of both *MSX1* and *MSX2* in animal models leads to severe cardiac defects, including DORV, septal defects, and ventricular hypoplasia [29]. Based on this, in our patient with Wolf-Hirschhorn syndrome, who had PS and ASD, this gene was considered a candidate for the CHD phenotype. Finally, the *NFATC1* gene encodes a transcription factor crucial for immune response, bone remodeling, and embryonic development. Variants in *NFATC1* are linked to CHD, particularly septal and valve anomalies [30]. Three patients in our cohort with an 18q23 deletion encompassing *NFATC1* were identified, but two of them had large deletions including dozens of morbid genes; it was only counted as a critical gene for CHD in the patient with the smallest deletion of 2,2 Mb, which did not contain any other monogenic dosage sensitive gene. This patient presented with an ASD.

The second most common were variants affecting genes within the RAS signaling pathway. Five patients had Noonan syndrome, and four of them presented with pulmonary stenosis. Two had variants in the *PTPN11* gene, while individual cases had variants in *BRAF*, *RRAS2*, and *SOS1*. Variants in *PTPN11* were associated with valvular pulmonary stenosis (LVO), and the *SOS1* variant was linked to septal defects, particularly VSD [1,26,28,31]. One patient has a 1p36 microdeletion affecting the *CDC42* and *ECE1* genes. *ECE1* is important for the development of neural crest-derived cells, especially in cardiac and craniofacial structures [32]. Variants in *ECE1* are associated with conditions like Hirschsprung disease and cardiac defects, including ASD. Our patient, besides ASD, also had hypotonia, clubfoot, and right-sided hydronephrosis. Additionally, a patient with a variant in the *FGD1* gene—classified as VUS, possibly pathogenic, based on clinical phenotype—was diagnosed with OCHD.

We also identified gene variants affecting structural proteins, specifically in the *ELN*, *FBN1*, *MYH11*, and *COL6A1* genes. Five patients were diagnosed with Williams-Beuren syndrome due to deletions in the *ELN* gene, and all had typical heart defects [28,33]: two patients had supravalvular AS, and three had PS. One patient with mitral valve prolapse had a deletion of the first exon of the *FBN1* gene which is well-known for its role in the structural integrity of connective tissues. Mutations in this gene lead to Marfan syndrome, with deletions accounting for less than 10% [31,34]. The *MYH11* gene encodes smooth muscle myosin heavy chain 11, a crucial protein involved in muscle contraction in blood vessels. Variants in *MYH11* are associated with thoracic aortic aneurysms and isolated PDA [1,26,28]. Our patient with a deletion in the 16q13.11 region, which includes the *MYH11* gene, has PDA. Ultimately, the *COL6A1* gene provides instructions for producing the alpha-1 chain of type VI collagen, an essential structural component of microfibrils within the extracellular matrix. *COL6A1* pathogenic variants primarily cause collagen VI-related myopathies, mainly affecting skeletal muscle rather than the heart [35]. While *COL6A1* variants have been investigated as potential genetic modifiers or contributors to CHD in individuals with Down syndrome, specific cases, such as Patient 39 in Table 3, have presented with right aortic arch, pulmonary artery atresia, ASD, and VSD.

Chromatin-regulating proteins play major roles in gene transcription and DNA replication, repair, and recombination. They regulate chromatin conformation by modulating DNA-histone interaction and binding of functional DNA-regulating protein complexes [36].

The *ARID1B* and *SMARCB1* genes encode distinct subunits of the SWI/SNF chromatin remodeling complex, which regulates gene expression by modifying chromatin structure and DNA accessibility using energy from ATP hydrolysis [1,37]. Dominant variants in *ARID1B* and *SMARCB1* are primarily associated with Coffin–Siris syndrome type 1 and type 3, respectively. CHDs are frequently reported in Coffin–Siris syndrome and include AVSD, PDA, and ToF [38]. In our cohort, the patient carrying a VUS, possibly pathogenic, in the *ARID1B* gene, presented with hemitruncus arteriosus, while the patient with a *SMARCB1* duplication was diagnosed with a VSD, craniofacial dysmorphia, and epilepsy. Another chromatin regulator, *ANKRD11*, interacts with histone deacetylases in cardiac neural crest cells, and pathogenic variants in this gene are associated with outflow tract septation defects, persistent truncus arteriosus, and ventricular dysfunction [26]. Haploinsufficiency of *ANKRD11* is known to cause KBG syndrome (MIM148050), where CHD are reported in approximately 10–26% of cases, most commonly septal defects. In our cohort, one patient with 16q24.2-q24.3 duplication that interrupts *ANKRD11* gene presented with a mitral valve anomaly, patent foramen ovale, and PDA. The *KMT2D* and *NSD1* genes encode distinct histone methyltransferases that are crucial for gene expression during development and cellular growth [26,39]. Variants in *KMT2D* are linked to Kabuki syndrome, where CHDs occur in up to 70% of patients, commonly involving LVO issues and septal defects. In our cohort, one patient with Kabuki syndrome type 1 had an ASD. Variants in *NSD1* cause Sotos syndrome, with about 60% of patients experiencing CHDs, including septal defects in 44.4% of cases [40,41]. A patient in our cohort with Sotos syndrome was diagnosed with a VSD. The *KANSL1* gene encodes an essential component of the NSL histone acetyltransferase complex. Deletions involving *KANSL1* are associated with Koolen–de Vries syndrome, which leads to CHD in 25–50% of cases, AVSD, ventricular anomalies, and PDA [42]. In our cohort, a patient with a KANSL1 deletion was diagnosed with PDA and other characteristics of the syndrome.

The *ACVR1* gene encodes a bone morphogenetic protein type I receptor within the TGFβ receptor superfamily. It is involved in a wide variety of biological processes, including the development of the heart, bone, cartilage, brain, and reproductive system. Loss-of-function variants in *ACVR1* are linked to CHD, particularly VSD and valve malformations [43]. For example, our patient with deletion of 2q23.3-q24 encompassing this gene presented with VSD, PDA, facial dysmorphia, and microcephaly.

The *AMER1* gene functions as a tumor suppressor and a key negative regulator of the Wnt signaling pathway. Variants in *AMER1* are associated with osteopathia striata with cranial sclerosis, a rare X-linked disorder that may include CHD. Li Chang et al. described a three-generation family with CHD caused by variants in the *AMER1* and *KCNE1* genes [44]. Our patient presented with a VSD. Another tumor suppressor important for cardiac development is APC, which negatively regulates the Wnt/β-catenin signaling pathway. Loss of *APC* function leads to abnormal accumulation of β-catenin, which can cause severe cardiac defects, such as VSD and persistent truncus arteriosus, by inducing apoptosis in cardiac neural crest cells during embryonic development [45]. We detected a 5q21.3-q23.1 deletion including *APC* in a patient presented with ToF, facial dysmorphia, and imperforate anus.

Finally, we identified CHD-related genes that code for ungrouped proteins, such as *PACS2*, *JAG1*, and *PSMD12*. The *PACS2* gene encodes a protein that regulates membrane trafficking between the endoplasmic reticulum, mitochondria, Golgi apparatus, and lysosomes [46]. Approximately 21% of patients with *PACS2* variants experience CHD, often associated with the VACTERL association (vertebral defects, anal atresia, cardiac defects, tracheoesophageal fistula, renal anomalies, and limb abnormalities) [47]. Our patient had DD/ID, facial dysmorphism, and ASD.

The *JAG1* gene encodes Jagged-1, a key ligand in the Notch signaling pathway that mediates cell-to-cell communication. Variants in *JAG1* lead to Alagille syndrome, which affects over 95% of patients with cardiac defects, such as pulmonary stenosis, VSD, overriding aorta, and right ventricular hypertrophy [1,26,28]. Our patient exhibited pulmonary atresia with VSD. The *PSMD12* gene encodes a non-ATPase regulatory subunit of the 26S proteasome, which is essential for proteasome assembly, scaffolding, and protein degradation. SNV or deletions of *PSMD12* cause Stankiewicz–Isidor syndrome, a neurodevelopmental disorder associated with variable CHD. Experimental models of cardiac defects further support the role of *PSMD12* variants in CHD [48]. Our patient with a pathogenic variant in this gene presented with OCHD.

In this paper, we present genetic findings in a cohort of children with syndromic CHD, along with an extensive description of candidate genes. The systematic functional classification of candidate genes is strength of this work, albeit our results are predominantly confirmatory. This study also has several important limitations. Its retrospective nature resulted in missing some data regarding the patients' phenotype, since most of the patients were neonates and infants at the time of genetic analysis. Only a small proportion of patients in our cohort underwent WES diagnostics, limiting the ability to identify additional genetic variants that may contribute to the CHD. Some parents declined further genetic testing after negative CMA results, and some patients passed away before completion of additional procedures. Furthermore, WES (whole exome sequencing) was more frequently requested for patients with complex phenotypes, which may lead to an overestimation of the reported detection rate. Additionally, although targeted segregation analysis was performed for patients with positive WES results, trio analysis was not conducted beforehand, which could limit the overall diagnostic yield.

5. Conclusions

In our study, we identified a genetic diagnosis in 36.5% of patients with syndromic CHD, while no genetic cause was found in patients with isolated heart defects. Morphological type of CHD did not influence the diagnostic yield of CNVs or sequence variants. In 40.4% of cases, confirmed or candidate CHD genes encode transcription factors. The most common phenotype observed in patients with these gene variants was septal defects. The second most prevalent group consisted of variants in genes associated with the RAS signaling pathway and those encoding structural proteins, primarily linked to obstructions in the valves and outflow tracts.

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Abbreviations

The following abbreviations are used in this manuscript:

CHD	congenital heart defect
CNV	copy number variation
SNV	single-nucleotide variants
DD/ID	developmental delay/intellectual disability
WES	whole-exome sequencing
INDELs	insertions/deletions
ASD	atrial septal defect
VSD	ventricular septal defect
ToF	tetralogy of Fallot
CMA	chromosomal microarray
ES	exome sequencing
AVSD	atrioventricular septal defect
TGA	dextro-transposition of the great arteries
DORV	double outlet right ventricle
TAC	truncus arteriosus communis
AS	aortic stenosis
PS	pulmonary stenosis
LVO	left ventricular outflow tract obstructions
RVO	right ventricular outflow tract obstructions
HLHS	hypoplastic left heart syndrome
CoA	coarctation of the aorta
PAVSD	pulmonary atresia with ventricular septal defect
OCHD	other complex congenital heart defects
PDA	patent ductus arteriosus
PAToA	pulmonary atresia with D-transposition of aorta and atrial septal defect
IUGR	intrauterine growth retardation
MVP	mitral valve prolapses
NEC	necrotizing enterocolitis
PFO	patent foramen ovale
TAPVR	total anomalous pulmonary venous return
VUR	vesicoureteral reflux
AAC	amino acid change
CCA	coronary cameral fistulas
IAA	interrupted aortic arch

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