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Subtype-Specific Genetic Spectrum of Fetal Congenital Heart Disease: A Retrospective Prenatal Cohort Study

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Abstract

Objectives: To assess the diagnostic yield of copy number variation sequencing (CNV-seq) and trio-based whole exome sequencing (trio-WES) across fetal congenital heart disease (CHD) subtypes and compare genetic findings between isolated and non-isolated cases. **Methods**: This retrospective study included 543 fetuses with CHD diagnosed by prenatal ultrasound who underwent invasive prenatal testing between January 2022 and September 2025. All cases underwent CNV-seq; karyotype analysis was performed in 294 cases, and 168 CNV-seq–negative cases subsequently received trio-WES. **Results**: In this retrospective cohort of 543 CHD fetuses, clinically significant chromosomal abnormalities were detected in 61 cases (11.23%), including 36 numerical chromosomal abnormalities and 25 pathogenic CNVs. A total of 168 fetuses with negative CNV-seq results received trio-WES testing. Pathogenic/likely pathogenic SNV-indels were identified in 15 of these 168 cases (8.93%). Chromosomal diagnostic yields were highest in endocardial cushion defects (41.67%), followed by single ventricle (27.27%) and left-sided lesions (21.62%). Higher molecular diagnostic rates were observed in fetuses with left-sided lesions (27.78%), tumors (16.67%) and right-sided lesions (9.52%). Notably, all abnormalities identified in endocardial cushion defects were chromosomal in origin, predominantly aneuploidies. The positive rate of chromosomal anomalies was higher in non-isolated CHD (20.33%, 25/123) compared with isolated CHD (8.57%, 36/420, $p < 0.01$). **Conclusions**: CNV-seq followed by trio-WES improves the diagnostic yield in fetal CHD. The marked variation across CHD subtypes supports systematic genetic evaluation, particularly in subtypes with a higher likelihood of genetic etiology.

Keywords: congenital heart disease; prenatal diagnosis; copy number variation sequencing; trio-exome sequencing; genetic spectrum

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1. Introduction

Congenital heart defects are the most common type of birth defects and one of the leading causes of infant mortality associated with congenital anomalies, affecting approximately

8–12 per 1000 live births [1]. This likely underestimates the true incidence of congenital heart disease because prenatal fetal deaths are not taken into account. The etiology of CHD is complex and involves multiple factors, including genetic, environmental, and maternal factors, such as maternal infections, maternal diseases and medication exposure during pregnancy [2,3]. Advances in cardiac surgery have significantly improved the survival of patients with CHD by correcting or alleviating structural cardiac defects. However, many patients remain at risk of long-term complications [4], including heart failure and arrhythmia, and CHD with underlying genetic etiologies is often associated with poorer clinical outcomes [5,6]. Therefore, prenatal detection of CHD and identification of the underlying genetic etiology is essential for prenatal counseling, pregnancy management, neonatal care, and surgical planning [7].

With the development of molecular diagnostics, copy number variation sequencing (CNV-seq) and whole exome sequencing (WES) have been increasingly applied in the genetic evaluation of CHD. In the prenatal setting, chromosomal microarray analysis (CMA) is the standard genetic test in many developed countries because pathogenic copy number variations can substantially affect development, quality of life, and life expectancy in children with CHD [8,9]. Previous studies have shown that CMA identifies genetic abnormalities in approximately 10–20% of fetuses with CHD [10–13], mainly chromosomal aneuploidies and pathogenic copy number variations, whereas ES detects single-nucleotide variants and small insertions/deletions and can further improve diagnostic yield after negative chromosomal testing [14]. However, most studies still classify CHD simply as "simple" or "complex," which may not adequately capture phenotypic heterogeneity or subtype-specific genetic risk [15]. Some studies incorporated postnatal phenotypic data [16], which may overestimate the diagnostic yield of ES. Others relied on targeted gene panels [17], included biased sample sources [18], or had small sample sizes [19]. Moreover, most studies focused on ES or CMA separately, with limited assessment of the combined use of multiple genomic technologies. This study aims to evaluate the diagnostic yield of CNV-seq and trio-WES in different types of fetuses with CHD using a refined phenotypic classification framework. We further assessed the incremental diagnostic value of trio-WES following CNV-seq and compared genetic detection rates across CHD subtypes, with the goal of improving the accuracy and clinical utility of genetic testing in prenatal CHD.

2. Materials and Methods

2.1. Subjects and Sampling

In this single-center retrospective study, we collected all prenatal cases diagnosed with CHD by ultrasound and undergoing genetic testing at the Department of Prenatal Diagnosis, Beijing Obstetrics and Gynecology Hospital, Capital Medical University, between January 2022 and September 2025. CHD cases included structural cardiac defects and vascular anomalies. The inclusion criteria were as follows: (1) fetuses diagnosed with CHD by prenatal ultrasonographic examination at our hospital; (2) availability of complete clinical data and clearly documented prenatal ultrasound findings; (3) completion of genetic testing with valid and interpretable results; and (4) written informed consent obtained from the parents, who had been fully informed about the study and voluntarily agreed to participate. The exclusion criteria included: (1) incomplete clinical or prenatal ultrasound information; (2) unavailable or invalid genetic testing results; and (3) duplicate records involving repeated sampling or testing of the same fetus. Ultimately, 543 fetuses with CHD were included in the study. The samples collected for genetic testing in this study included amniotic fluid, chorionic villi, and products of conception.

This retrospective study was approved by the Ethics Committee of Beijing Obstetrics and Gynecology Hospital, Capital Medical University (No. 2023-KY-009-01). The CNV-seq

and trio-WES projects had previously received ethical approval in August 2021, prior to the collection of clinical genetic testing data beginning in January 2022. Written informed consent for prenatal genetic testing was obtained from all participants. The study was conducted in accordance with the principles of the Declaration of Helsinki (2013 revision).

Fetal cardiac diagnoses were performed or reviewed by at least two fetal cardiology specialists. In cases of disagreement, ultrasound images were jointly reviewed and discussed to reach a consensus, with a senior fetal cardiology specialist making the final determination if disagreements persisted. Pregnancy outcomes were collected through hospital medical records and telephone follow-up, with follow-up continuing until the end of pregnancy to document live birth or termination of pregnancy. All samples in the cohort underwent CNV-seq, and karyotype analysis was performed in 294 cases. CNV-seq was performed in all 543 fetuses with CHD, while karyotyping was performed in 294 cases based on clinical indications and parental preferences. Among the 482 fetuses with negative CNV-seq results, 168 underwent additional trio-ES. The selection of cases for karyotyping and trio-ES was based on prenatal ultrasound findings, suspected genetic etiology, family history, and parental preferences. Trio-ES was not performed systematically in all CNV-seq-negative cases. Trio-WES analysis was conducted using complete parent–fetus trios, and comprehensive clinical and genetic information was collected for most cases. Only pathogenic or likely pathogenic (P/LP) variants identified by CNV-seq or trio-WES were included in the final analysis. Both P/LP variants and VUS were evaluated, but only P/LP variants were included in the calculation of diagnostic yield. VUS were reported separately and were not considered definitive molecular diagnoses. Secondary findings unrelated to the prenatal phenotype were not systematically investigated. Clinically significant chromosomal findings were defined as pathogenic or likely pathogenic aneuploidies and CNVs. Mosaic and low-penetrance variants were assessed according to their pathogenicity classification and available clinical and phenotypic evidence.

CHD phenotypes were classified according to the method proposed by Stephens et al. [15], which categorizes CHD into six main groups: left-sided lesions, right-sided lesions, conotruncal defects, endocardial defects, looping/arterial malposition defects and other CHD types. Cases were further classified as isolated or non-isolated CHD. Isolated CHD was defined as CHD without major extracardiac structural anomalies or fetal growth restriction (FGR). FGR was defined as an estimated fetal weight or abdominal circumference below the 10th percentile for gestational age. Fetuses with CHD accompanied only by soft markers—including echogenic intracardiac focus, echogenic bowel, choroid plexus cyst, single umbilical artery, urinary tract dilation, short humerus and/or femur, increased nuchal fold thickness, absent or hypoplastic nasal bone, or mild ventriculomegaly—were also classified as isolated CHD. Cardiac phenotypes and associated extracardiac structural abnormalities were assessed based on prenatal ultrasonographic examinations, including fetal echocardiography and systematic evaluation of other fetal organ systems. The prenatal ultrasound findings served as the primary basis for phenotypic characterization and classification in this prenatal cohort.

2.2. Chromosomal Karyotyping

Chromosomal karyotype analysis was performed on amniotic fluid and chorionic villus samples using standard cytogenetic procedures, with sample-specific processing and cell culture protocols. Following informed consent, amniocentesis was performed to obtain 10 mL of amniotic fluid. Amniocytes were isolated by centrifugation and cultured in 4 mL of medium at 37 °C with 5% CO_2 for 5–7 days. Colchicine (70 µL) was added 40 min prior to harvest to arrest cells at metaphase. Standard procedures, including hypotonic treatment,

fixation, and G-banding, were performed. A minimum of 30 metaphases were analyzed routinely. For suspected mosaicism, 50–100 metaphases were examined.

Approximately 3–4 chorionic villi were selected and washed with saline containing penicillin and streptomycin to remove maternal blood contamination. After microscopic confirmation of chorionic villus tissue, the samples were finely minced using sterile scissors. The tissue fragments were then digested with 0.25% trypsin–EDTA for 3–5 min, and digestion was terminated by adding 1 mL of fetal bovine serum. Following centrifugation for 10 min, the supernatant was discarded, and the resulting cell pellet was resuspended in 20 mL of culture medium. Subsequent cell culture, chromosome harvesting, G-banding, and karyotype analysis were performed according to the standard cytogenetic protocol.

2.3. Copy Number Variation Sequencing

Genomic DNA was extracted from tissue, amniotic fluid, and other clinical samples using silica-coated superparamagnetic nanobeads under chaotropic salt conditions. Genomic DNA was extracted using a DNA extraction kit, and 50 ng of genomic DNA was used for library preparation according to standard next-generation sequencing (NGS) protocols. Briefly, sequencing libraries were constructed through end repair, A-tailing, adapter ligation, and PCR amplification. Library quality and concentration were assessed using an Agilent 2100 Bioanalyzer and quantitative PCR (qPCR). Sequencing was performed on the BGISEQ-500 platform to detect chromosomal aneuploidies and copy number gains or losses ≥ 100 kb. Maternal cell contamination was assessed using short tandem repeat (STR) genotyping. Sequencing data were analyzed using the Population-Scale Copy Number Variation (PSCC) algorithm, and reads were aligned to the human reference genome GRCh37/hg19. Copy number gains and losses ≥ 100 kb with a confidence score ≥ 90% were further annotated and interpreted using public databases, including the Database of Genomic Variants (DGV), DECIPHER, Online Mendelian Inheritance in Man (OMIM), and ClinGen. CNVs were classified according to the American College of Medical Genetics and Genomics (ACMG) guidelines (2020) into five categories: pathogenic, likely pathogenic, variants of uncertain significance (VUS), likely benign, and benign. A mosaicism reporting threshold of 20% was applied.

2.4. Trio Whole-Exome Sequencing (Trio-WES) and Bioinformatic Analysis

Genomic DNA was isolated from fetal samples and parental peripheral blood using the Nucleic Acid Extraction and Purification Kit (AmCare Genomics Laboratory, Guangzhou, China). Following enzymatic fragmentation, sequencing libraries were prepared using the Gene Sequencing Library Kit (AmCare, Guangzhou, China), and exonic regions were enriched using the NEXome XP Panel v1.0 (AmCare, Guangzhou, China). Sequencing was performed on the AmCareSeq-2000 platform (AmCare, Guangzhou, China) with 150-bp paired-end reads, achieving an average depth of 100× and at least 20× coverage for more than 98% of target regions.

Raw sequencing data were processed using fastp (v0.20.2) to remove adapter sequences and low-quality bases ($Q < 20$). Clean reads were aligned to the GRCh37/hg19 reference genome using BWA-MEM (v0.7.15), and single-nucleotide variants (SNVs) and small insertions/deletions (indels) were identified using GATK HaplotypeCaller (v4.3.0.0). Variants with a variant allele frequency (VAF) below 15% were excluded. Variant annotation and filtering were performed using ANNOVAR (https://annovar.openbioinformatics.org/en/latest/, accessed on 15 March 2023) and an in-house bioinformatics pipeline (AmCare). Population frequencies and clinical relevance were evaluated using gnomAD, ClinVar, OMIM, and an internal database of 100,000 Han Chinese individuals (AmCare). Variant effects were predicted using SIFT, PolyPhen-2, MutationTaster, and FATHMM, while poten-

tial splicing alterations were assessed using SpliceAI, MaxEntScan, and Human Splicing Finder (v3.1).

Candidate variants were evaluated in relation to prenatal phenotypes and inheritance patterns, including segregation within the trio. Selected variants were confirmed by Sanger sequencing using an ABI 3730xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Variant pathogenicity was classified according to the 2015 ACMG/AMP guidelines, with evidence strength calibrated using the Bayesian framework endorsed by the ClinGen Sequence Variant Interpretation Working Group. Only pathogenic and likely pathogenic variants were considered molecular diagnostic findings, whereas variants of uncertain significance were evaluated separately and excluded from diagnostic yield calculations.

2.5. Statistical Analysis

Statistical analyses were performed using IBM SPSS Statistics version 27.0 (IBM Corp., Armonk, NY, USA). Continuous variables were presented as medians and ranges, and categorical variables as frequencies and percentages. Group comparisons were performed using Pearson's chi-square test or Fisher's exact test, as appropriate. Fisher's exact test was applied when expected cell frequencies were <5. Diagnostic yields were reported with 95% confidence intervals (CIs) calculated using the exact binomial method. All tests were two-sided, with $p < 0.05$ considered statistically significant.

3. Results

Between January 2022 and September 2025, a total of 543 fetuses diagnosed with CHD underwent invasive prenatal diagnosis at our center. The median maternal age was 32 years (range: 20–44 years), and the median gestational age at invasive sampling was 24 weeks (range: 12–38 weeks) (Table 1). All cases were analyzed by CNV-seq; karyotype analysis was performed in 294 cases, and 168 cases further received trio-WES (Figure 1). Genetic diagnoses were established in 80 cases, including 61 cases with chromosomal abnormalities and 15 cases with sequence variants. The types of CHD and the detection rates of pathogenic or likely pathogenic findings for fetuses with CHD in different subgroups are summarized in Tables 2 and 3. Comparisons between isolated and non-isolated CHD are presented in Table 4, and descriptions of the 15 cases with positive trio-WES results are shown in Table 5.

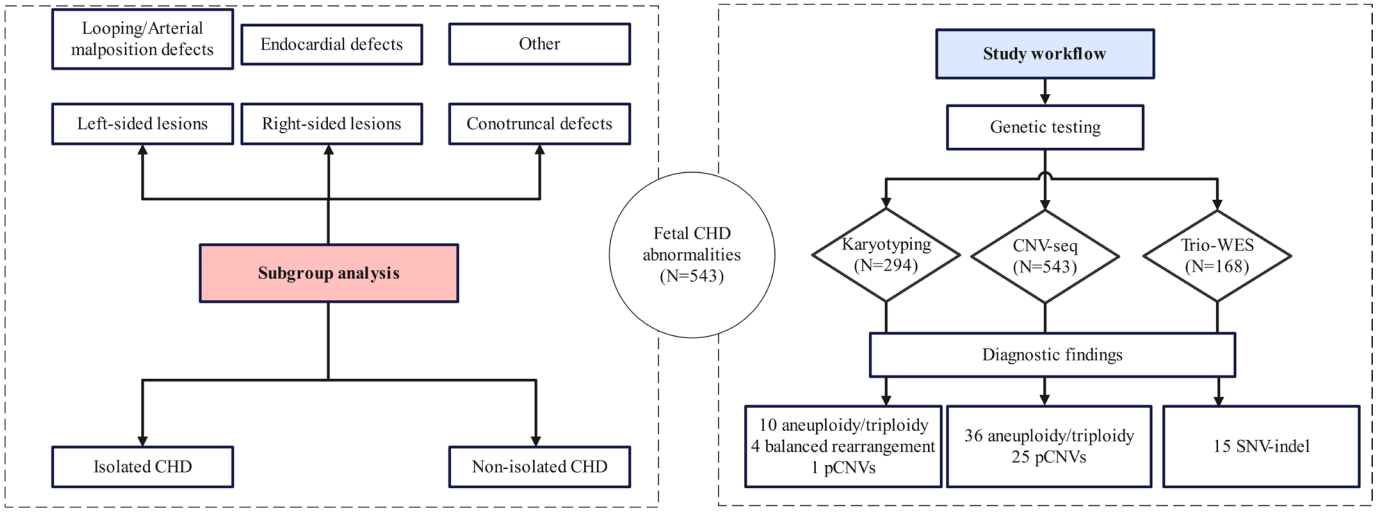


Figure 1. Flowchart of the study design and genetic testing workflow for prenatal congenital heart disease.

Table 1. Maternal and fetal baseline characteristics of the study cohort.

	Total (N = 543)
Maternal age	24 (20–44)
Gestational weeks	32 (12–38)
The types of CHD	
VSD	206 (37.94%)
Conotruncal defects	175 (32.23%)
Right-sided lesions	45 (8.29%)
Left-sided lesions	37 (6.81%)
PLSVC	25 (4.60%)
Hex	12 (2.21%)
Endocardial defects	12 (2.21%)
SV	11 (2.03%)
Looping/arterial malposition defects	11 (2.03%)
Tumor	7 (1.29%)
TAPVC	2 (0.37%)
Isolated CHD	
Yes	420 (77.35%)
No	123 (22.65%)

CHD, congenital heart disease. VSD, ventricular septal defect. PLSVC, persistent left superior vena cava. Hex, heterotaxia syndrome. SV, single ventricle. TAPVC, total anomalous pulmonary vein connection.

3.1. Genetic Diagnostic Yield Across CHD Categories

CHD cases were categorized into six major groups. Ventricular septal defect (VSD) was the most common subtype, accounting for 37.94% (206/543) of cases, followed by conotruncal defects (32.23%, 175/543) and right-sided lesions (8.29%, 45/543) (Table 1). The highest chromosomal diagnostic yields were observed in endocardial defects (41.67%), single ventricle (SV) (27.27%) and left-sided lesions (21.62%) (Table 3). In contrast, relatively low detection rates were identified in tumor, total anomalous pulmonary venous connection (TAPVC), and looping/arterial malposition defects (Table 3). Among the eight fetuses with left-sided lesions in whom chromosomal abnormalities were detected, seven were accompanied by extracardiac anomalies. Notably, all three fetuses diagnosed with mitral valve stenosis (MVS) were found to harbor a positive rate of SNVs. In endocardial defects, all detected genetic abnormalities were chromosomal, with aneuploidy accounting for the majority (33.3%). The detection rate of pathogenic copy number variants in this group was 8.33% (1/12). Given that atrioventricular septal defect (AVSD) represents the predominant phenotype within endocardial defects, this finding is consistent with the well-established association between this phenotype and chromosomal abnormalities. In addition, fetuses with left-sided lesions (27.78%, 95%CI: 9.7–53.5%), tumor (16.67%, 95%CI: 0.4–64.1%), and right-sided lesions (9.52%, 95%CI: 1.17–30.38%) were revealed to have a relatively higher prevalence of molecular diagnosis.

Table 2. Clinical and genetic characteristics of fetuses with CHD and chromosomal abnormalities.

Chromosome Abnormalities	CHD Causative	Case Number	Isolated Group	Non-Isolated Group	CHD Types	Inheritance	Outcome
Trisomy 18	Yes	13	5	8			13 Termination
Trisomy 21	Yes	10	10	0			10 Termination
45, X	Yes	4	0	4			4 Termination
Trisomy 13	Yes	2	0	2			2 Termination
Trisomy 15	Yes	1	1	0			1 Termination
47, XXX	No	1	1	0			1 Termination
Mosaic trisomy 20	No	1	1	0			1 Delivery
Mosaic 47, XXX	No	1	1	0			1 Delivery
69, XXX	Yes	1	0	1			1 Termination
Mosaic trisomy 7	No	1	0	1			1 Lost follow up
Mosaic 47, XXY	No	1	1	0			1 Delivery
22q11.2 deletion	Yes	7	4	3	2 PA-VSD/1 VSD/1 PLSVC/1 Coa-IVS/1 Tetralogy of Fallot/1 Double aortic arch/vascular ring	6 Unknown/1 De novo	4 Termination/1 Delivery/2 Lost follow up
7q11.23 deletion	Yes	3	2	1	1 Coa-IVS/1 AS/1 Double aortic arch/vascular ring	3 De novo	2 Termination/1 Unknown
15q11.2 deletion	No	2	2	0	2 VSD	1 Unknown/1 Parental	2 Delivery
8p23.1 deletion	Yes	1	1	0	1 AVSD	1 Unknown	1 Termination
9p24.3p13.1 duplication	No	1	1	0	1 VSD	1 Maternal	1 Termination
16p12.2 deletion	Yes	1	1	0	1 Right aortic arch	1 Unknown	1 Delivery

Table 2. *Cont.*

Chromosome Abnormalities	CHD Causative	Case Number	Isolated Group	Non-Isolated Group	CHD Types	Inheritance	Outcome
16p11.2 deletion	Yes	1	1	0	1 Tetralogy of Fallot	1 De novo	1 Termination
16p11.2 duplication	No	1	1	0	1 VSD	1 Parental	1 Delivery
7q34q36.3 deletion	Yes	1	0	1	1 HRHS	1 Unknown	1 Termination
2q37.1q37.3 deletion with 5q33.3q35.5 duplication	Yes	1	0	1	1 Coa-IVS	1 De novo	1 Termination
5q32q35.3 duplication	Yes	1	0	1	1 MVS	1 De novo	1 Termination
15q26.1q26.3 deletion	Yes	1	0	1	1 VSD	1 De novo	1 Termination
4p16.3p15.32 deletion	Yes	1	0	1	1 Double aortic arch/vascular ring	1 De novo	1 Termination
6p25.3p25.2 deletion	Yes	1	0	1	1 Truncus arteriosus	1 De novo	1 Termination
8q12.1q12.2 deletion	Yes	1	1	0	1 Right aortic arch	1 Unknown	1 Termination
11q23.3q25 deletion with 3q26.33q29 duplication	Yes	1	0	1	1 Truncus arteriosus	1 De novo	1 Termination

CHD, congenital heart disease. PA-VSD, pulmonary atresia with ventricular septal defect. VSD, ventricular septal defect. PLSVC, persistent left superior vena cava. Coa-IVS, coarctation of the aorta with intact ventricular septum. AS, aortic stenosis. AVSD, atrioventricular septal defects. HRHS, hypoplastic right heart syndrome. MVS, mitral valve stenosis.

Table 3. Genetic diagnostic yield across CHD subtypes in the total, isolated, and non-isolated cohorts.

Types	Total					Non-Isolated CHD					Isolated CHD				
	n	Chromosomal Diagnosis (*n* (%))	Chromosomal Abnormalities		SNV-Indel (*n*/*N*)	*n*	Chromosomal Diagnosis (*n* (%))	Chromosomal Abnormalities		SNV-Indel (*n*/*N*)	*n*	Chromosomal Diagnosis (*n* (%))	Chromosomal Abnormalities		SNV-Indel (*n*/*N*)
			NCA	CNV				NCA	CNV				NCA	CNV	
Conotruncal	175	17(9.71%)	6	11	3/43	35	7(20%)	3	4	2/16	140	10(7.14%)	3	7	1/27
DORV	11	2(18.18%)	2	0	1/5	6	2(33.3%)	2	0	1/4	5	0	0	0	0/1

Table 3. *Cont.*

Types	Total					Non-Isolated CHD					Isolated CHD				
	n	Chromosomal Diagnosis (*n* (%))	Chromosomal Abnormalities		SNV-Indel (*n*/*N*)	*n*	Chromosomal Diagnosis (*n* (%))	Chromosomal Abnormalities		SNV-Indel (*n*/*N*)	*n*	Chromosomal Diagnosis (*n* (%))	Chromosomal Abnormalities		SNV-Indel (*n*/*N*)
			NCA	CNV				NCA	CNV				NCA	CNV	
IAA, type B	2	0	0	0	0/1	0	0	0	0	0/0	2	0	0	0	0/1
Tetralogy of Fallot	41	4(9.76%)	2	2	0/14	8	1(12.5%)	0	1	0/5	33	3(9.09%)	2	1	0/9
PA-VSD	6	2(33.33%)	0	2	0/2	1	1(100%)	0	1	0/0	5	1(20%)	0	1	0/2
Right aortic arch	54	3(5.56%)	1	2	1/13	9	0	0	0	1/3	45	3(6.67%)	1	2	0/10
Truncus arteriosus	7	3(42.86%)	1	2	1/1	3	2(66.67%)	1	1	0/0	4	1(25%)	0	1	1/1
Double aortic arch/vascular ring	54	3(5.56%)	0	3	0/7	8	1(12.5%)	0	1	0/4	46	2(4.35%)	0	2	0/3
Left-sided lesions	37	8(21.62%)	3	5	5/18	13	7(53.85%)	3	4	2/5	24	1(4.17%)	0	1	3/13
Coa-IVS	18	4(22.22%)	0	4	2/9	5	3(60%)	0	3	0/2	13	1(7.69%)	0	1	2/7
AS	5	0	0	0	2/4	2	0	0	0	2/2	3	0	0	0	0/2
HLHS	7	1(14.29%)	1	0	1/3	2	1(50%)	1	0	0/0	5	1(20%)	0	0	1/3
IAA, type A	1	0	0	0	0/0	0	0	0	0	0/0	1	0	0	0	0/0
Supravalvar aortic stenosis	3	0	0	0	0/2	1	0	0	0	0/1	2	0	0	0	0/1
MVS	3	3(100%)	2	1	0/0	3	3(100%)	2	1	0/0	0	0	0	0	0/0
Right-sided lesions	45	2(4.44%)	1	1	2/21	5	0	0	0	0/3	40	2(5%)	1	1	2/18

Table 3. *Cont.*

Types	Total					Non-Isolated CHD					Isolated CHD				
	n	Chromosomal Diagnosis (*n* (%))	Chromosomal Abnormalities		SNV-Indel (*n*/*N*)	*n*	Chromosomal Diagnosis (*n* (%))	Chromosomal Abnormalities		SNV-Indel (*n*/*N*)	*n*	Chromosomal Diagnosis (*n* (%))	Chromosomal Abnormalities		SNV-Indel (*n*/*N*)
			NCA	CNV				NCA	CNV				NCA	CNV	
Ebstein's	2	0	0	0	0/1	0	0	0	0	0/0	2	0	0	0	0/1
PA/PS-IVS	5	0	0	0	0/0	0	0	0	0	0/0	5	0	0	0	0/0
Pulmonary valve stenosis	26	0	0	0	2/16	4	0	0	0	0/2	22	0	0	0	2/14
HRHS	5	2(40%)	1	1	0/0	0	0	0	0	0/0	5	2(40%)	1	1	0/0
TVA	7	0	0	0	0/4	1	0	0	0	0/1	6	0	0	0	0/3
endocardial defects	12	5(41.67%)	4	1	0/4	2	1(50%)	1	0	0/0	10	4(40%)	3	1	0/4
ASD	1	0	0	0	0/1	0	0	0	0	0/0	1	0	0	0	0/1
AVSD	11	5(45.45%)	4	1	0/3	2	1(50%)	1	0	0/0	9	4(44.44%)	3	1	0/3
Looping defects	11	0	0	0	0/5	0	0	0	0	0/0	11	0	0	0	0/5
DTGA with VSD	3	0	0	0	0/2	0	0	0	0	0/0	3	0	0	0	0/2
DTGA without VSD	8	0	0	0	0/3	0	0	0	0	0/0	8	0	0	0	0/3
Other	263	29(11.03%)	22	7	5/77	68	10(14.71%)	9	1	2/24	195	19(9.74%)	13	6	3/53
VSD	206	24(11.65%)	18	6	4/53	54	8(14.81%)	7	1	2/18	152	16(10.53%)	11	5	2/35
PLSVC	25	1(4%)	0	1	0/8	5	0	0	0	0/3	20	1(5%)	0	1	0/5
SV	11	3(27.27%)	3	0	0/5	5	2(40%)	2	0	0/2	6	1(16.67%)	1	0	0/3

Table 3. *Cont.*

Types	Total					Non-Isolated CHD					Isolated CHD				
	n	Chromosomal Diagnosis (*n* (%))	Chromosomal Abnormalities		SNV-Indel (*n*/*N*)	*n*	Chromosomal Diagnosis (*n* (%))	Chromosomal Abnormalities		SNV-Indel (*n*/*N*)	*n*	Chromosomal Diagnosis (*n* (%))	Chromosomal Abnormalities		SNV-Indel (*n*/*N*)
			NCA	CNV				NCA	CNV				NCA	CNV	
Hex	12	1(8.33%)	1	0	0/3	3	0	0	0	0/0	9	1(11.11%)	1	0	0/3
TAPVC	2	0	0	0	0/2	1	0	0	0	0/1	1	0	0	0	0/1
Tumor	7	0	0	0	1/6	0	0	0	0	0/0	7	0	0	0	1/6
Total	543	61(11.23%)	36	25	15/168	123	25(20.33%)	16	9	6/48	420	36(8.57%)	20	16	9/120

CHD, congenital heart disease. NCA, numerical chromosomal abnormality. CNV, copy number variation. SNV-indel, single-nucleotide variants and small insertions/deletions. DORV, double outlet right ventricle. IAA, interrupted aortic arch. PA-VSD, pulmonary atresia with ventricular septal defect. Coa-IVS, coarctation of the aorta with intact ventricular septum. AS, aortic stenosis. HLHS, hypoplastic left heart syndrome. MVS, mitral valve stenosis. PA/PS-IVS, pulmonary atresia/pulmonary stenosis with intact ventricular septum. HRHS, hypoplastic right heart syndrome. TVA, tricuspid valve atresia. ASD, atrial septal defect. AVSD, atrioventricular septal defect. DTGA, d-transposition of great arteries. VSD, ventricular septal defect. PLSVC, persistent left superior vena cava. SV, single ventricle. Hex, heterotaxia syndrome. TAPVC, total anomalous pulmonary vein connection.

Table 4. Comparison of chromosomal abnormalities and SNV/indel findings in different groups.

CHD	*n*	Chromosomal Abnormalities			SNV-Indel
		NCA	CNV	Total	
Non-isolated CHD	123	16 (13.00%)	9 (7.32%)	25 (20.33%)	6 (12.50%)
Isolated CHD	420	20 (4.76%)	16 (3.81%)	36 (8.57%)	9 (7.50%)
p-value		<0.01	0.10	<0.01	0.370

3.2. Prenatal Genetic Diagnosis in Isolated and Non-Isolated CHD

The prevalence of isolated and non-isolated CHD and the corresponding genetic detection rates across different groups are summarized in Tables 3, 4 and A1. Specifically, chromosomal abnormalities were more frequently detected in non-isolated CHD compared with isolated CHD (20.33% vs. 8.57%, $p < 0.01$). Aneuploidy (13.00% vs. 4.76%, $p < 0.01$) was significantly more common in non-isolated cases, while the frequencies of CNVs (7.32% vs. 3.81%, $p = 0.10$) and sequence variants (12.50% vs. 7.50%, $p = 0.370$) showed no significant differences between the two groups. Among the 123 fetuses with non-isolated CHD, 95 had a single additional anomaly, and 28 had multiple additional anomalies. Chromosomal abnormalities were detected more frequently in fetuses with multiple additional anomalies than in those with a single additional anomaly (42.86%, 12/28 vs. 13.68%, 13/95; $p < 0.01$). Cystic hygroma was associated with the highest detection rate of chromosomal abnormalities (85.71%, 6/7). Detailed results are presented in Appendix A, Table A1.

3.3. Diagnostic Performance of Chromosomal Abnormalities in CHD

Karyotype analysis was performed in 294 cases, among which 10 cases showed aneuploidy or triploidy, 4 cases had balanced rearrangements, and 1 case presented with pathogenic CNVs (Figure 1). CNV-seq identified clinically significant abnormalities in 61 of 543 fetuses with CHD, yielding an overall diagnostic rate of 11.23% (Table 3). Numerical chromosomal abnormalities accounted for 6.63% of all CHD cases. Among the 61 cases with clinically significant chromosomal abnormalities, 36 (59.02%) were chromosomal number abnormalities, including one case of triploidy (69, XXX) and 35 cases of aneuploidy. The most frequently observed aneuploidies were trisomy 18, trisomy 21, and Turner syndrome. The remaining 25 cases (40.98%) harbored pathologic CNVs, with DiGeorge syndrome and Williams–Beuren syndrome representing the most common recurrent CNV syndromes (Table 2).

3.4. Incremental Diagnostic Yield of Trio-WES in Fetuses with CHD

Trio-WES was performed in 168 fetuses with CHD following negative CNV-seq results. All cases underwent proband–parent trio analysis, including 120 isolated CHD cases and 48 non-isolated CHD cases with extracardiac structural anomalies (Table 3). Among fetuses with CHD and negative CNV-seq results, trio-WES identified pathogenic/likely pathogenic (P/LP) sequence variants in 8.93% (15/168) of cases, thereby providing an additional diagnostic yield beyond CNV-seq (Table 3). The CHD types and the detection rates of P/LP variations and variants of uncertain significance (VUS) with CHD are summarized in Tables 3, 5 and A2. Among the 168 fetuses undergoing trio-WES, the detection rates of P/LP and VUS were 8.93% (15/168) and 4.17% (7/168).

Table 5. Clinical and molecular findings in fetuses with CHD and pathogenic or likely pathogenic variants identified by trio-WES.

Patient ID/Gender	CHD Types	ECM	Gene	Disease (MIM#ID)	CHD Related	Mode	Refseq	Variants	Inherited from	Reported?	Class
P1	Coa-IVS	NA	RPL5	Diamond-Blackfan anemia 6 (MIM#612561)	Yes	AD	NM_000969.5	c.172_173delAG	De novo	Yes	P
P2	Truncus arteriosus	NA	ZMYM2	Neurodevelopmental-craniofacial syndrome with variable renal and cardiac abnormalities (MIM#6119522)	Yes	AD	NM_197968.4	c.3279_3286del	De novo	No	P
P3	VSD	NA	RAF1	Cardiomyopathy, dilated, 1NN#LEOPARD syndrome 2#Noonan syndrome 5 (MIM#615916#611554#611553)	Yes	AD	NM_002880.4	c.770C>T	De novo	No	P
P4	VSD	Intestinal obstruction	CHD7	CHARGE syndrome#Hypogonadotropic hypogonadism 5 with or without anosmia (MIM#214800#612370)	Yes	AD	NM_017780.4	c.7957C>T	De novo	Yes	P
P5	HLHS	NA	MYRF	Cardiac-urogenital syndrome#Encephalitis/encephalopathy, mild, with reversible myelin vacuolization (MIM#618280#618113)	Yes	AD	NM_001127392.3	c.782dupC	De novo	Yes	P
P6	VSD	NA	SMAD2	Congenital heart defects, multiple types, 8, with or without heterotaxy#Loeys-Dietz syndrome 6 (MIM#619657#619656)	Yes	AD	NM_005901.6	Ex3-5del	De novo	No	P
P7	DORV	Dandy-Walker syndrome	PKP2	Arrhythmogenic right ventricular dysplasia 9 (MIM#609040)	Yes	AD	NM_001005242.3	c.715delG	Paternal	No	LP

Table 5. *Cont.*

Patient ID/Gender	CHD Types	ECM	Gene	Disease (MIM#ID)	CHD Related	Mode	Refseq	Variants	Inherited from	Reported?	Class
P8	AS	FGR	FLNA	FG syndrome 2#Cardiac valvular dysplasia, X-linked (MIM#300321#314400)	Yes	XL	NM_001110556.2	c.6632_6633dup	Maternal	No	LP
P9	Tumor	NA	TSC2	Focal cortical dysplasia, type II, somatic#Lymphangioleiomyomatosis, somatic#Tuberous sclerosis-2 (MIM#607341#606690#613254)	Yes	AD	NM_000548.5	c.4287_4291del	De novo	No	P
P10	Coa-IVS	NA	FOXP1	Intellectual developmental disorder with language impairment with or without autistic features (MIM#613670)	No	AD	NM_001349338.3	c.1241delT	De novo	Yes	P
P11	VSD	Open spina bifida with myelomeningocele, hydrocephalus, Double-foot eversion.	CUL3	Neurodevelopmental disorder with or without autism or seizures#Pseudohypoaldosteronism, type IIE (MIM#619239#614496)	Yes	AD	NM_003590.5	c.1549_1552del	De novo	Yes	P
P12	Right aortic arch	Urinary tract dilatation, Bronchopulmonary Sequestration	SUZ12	Imagawa-Matsumoto syndrome (MIM#618786)	No	AD	NM_015355.4	c.1291C>T	De novo	No	P

Table 5. *Cont.*

Patient ID/Gender	CHD Types	ECM	Gene	Disease (MIM#ID)	CHD Related	Mode	Refseq	Variants	Inherited from	Reported?	Class
P13	AS	Micrognathia	EFTUD2	Mandibulofacial dysostosis, Guion-Almeida type (MIM#610536)	Yes	AD	NM_004247.4	c.698dupA	De novo	No	P
P14	Pulmonary valve stenosis	NA	RPL11	Diamond-Blackfan anemia 7 (MIM#612562)	Yes	AD	NM_000975.5	c.268dupC	De novo	No	P
P15	Pulmonary valve stenosis	NA	PTPN11	PTPN11-related Noonan syndrome (MIM#163950)	Yes	AD	NM_002834.5	c.417G>C	De novo	Yes	P

Coa-IVS, coarctation of the aorta with intact ventricular septum. VSD, ventricular septal defect. HLHS, hypoplastic left heart syndrome. DORV, double outlet right ventricle. AS, aortic stenosis. AD, autosomal dominant. XL, X-linked. P, Pathogenic. LP, Likely pathogenic. FGR, fetal growth restriction.

Among the 15 fetuses with a molecular diagnosis of P/LP variants, 13 variants were de novo, whereas two were inherited, including one paternally inherited and one maternally inherited variant. Among all CHD subtypes, left-sided lesions had the highest diagnostic yield by trio-WES (27.78%, 5/18, 95% CI: 9.7–53.5%). Overall, trio-WES identified P/LP variants in 7.50% (9/120, 95%CI: 3.49–13.76%) of isolated CHD cases and 12.50% (6/48, 95%CI: 4.73–25.25%) of non-isolated CHD cases (Table 3).

4. Discussion

Genetic etiology is an important determinant of the phenotypic presentation and prognosis of fetal CHD. Accurate genetic testing is therefore essential for etiologic diagnosis and for informing prenatal counseling and clinical management [20]. In this study, we applied a combined CNV-seq and trio-WES to evaluate the genetic basis of different CHD subtypes. CNV-seq identified chromosomal abnormalities in 11.23% of cases, and trio-WES provided an additional diagnostic yield of 8.93% (15/168). We further characterized the distribution of genetic abnormalities across CHD subtypes and compared the genetic findings between isolated and non-isolated CHD. Together, these results support the clinical utility of an integrated genomic approach in the prenatal evaluation of fetal CHD.

Previous studies have commonly adopted the classification framework proposed by Botto et al. [21], which provided an important basis for etiologic studies of CHD by integrating cardiac phenotype, cardiac complexity and extracardiac anomalies. However, this framework predates more recent advances in the understanding of the genetic heterogeneity and hierarchical clinical complexity of specific CHD subtypes, and offers limited practical guidance for the hierarchical classification of complex phenotypes. Most importantly, the National Birth Defects Prevention Study on which it was based excluded cases with known etiologies, including chromosomal or genetic syndromes, which may reduce its applicability in prenatal genetic studies. By contrast, the more recent C-CLOC system proposed by Stephens et al. [15] provides a clearer mutually exclusive hierarchical approach based on core lesions, common co-occurring defects and expected clinical complexity. We therefore primarily adapted the hierarchical logic of the C-CLOC system, with modifications based on prenatal ultrasound phenotypes and subgroup sample size.

The detection rate of P/LP variants by CNV-seq in our cohort was 11.23%, comparable to the 11.5% reported by Sukenik-Halevy et al. in a prenatal cohort [22]. However, several more recent studies have reported higher CNV detection rates in fetal CHD. In our cohort, the overall detection rate of aneuploidy was 6.63%, whereas Yi et al. [18] reported an overall prenatal CNV detection rate of 24.37%, including 10.80% attributable to aneuploidy. This discrepancy may partly reflect differences in cohort composition, as that study appeared to preferentially include CHD cases undergoing termination of pregnancy. Differences in CHD subtype distribution may also have contributed, as CTD, left ventricular outflow tract obstruction, and right ventricular outflow tract obstruction were the most frequent categories in the study by Yi et al. [18], whereas VSD, CTD, and right-sided lesions predominated in our cohort. Given that VSD generally has a lower genetic diagnostic yield, variation in subtype composition is likely to influence the overall CNV detection rate. Collectively, these findings suggest that differences in reported CNV yields across studies may be related to case selection, subtype distribution, testing strategies, and potential selection bias.

In the present study, the CNV-based genetic diagnostic yield was significantly higher in non-isolated CHD than in isolated CHD (20.33% vs. 8.57%, $p < 0.01$), consistent with previous observations that non-isolated CHD carries a greater burden of genetic abnormalities. Nevertheless, the rates of genetic defects reported in earlier prenatal studies, particularly aneuploidy, were generally higher than those observed in our cohort [23]. One possible explanation is the widespread use of noninvasive prenatal testing in China, which may

have led to the early detection and termination of some aneuploid fetuses before invasive diagnostic evaluation. Although chromosomal abnormalities remained significantly more frequent in non-isolated than in isolated CHD, the magnitude of this difference was smaller than that reported previously. This may partly reflect the limited sensitivity of prenatal ultrasound for certain extracardiac anomalies, resulting in partial misclassification of some non-isolated cases as isolated CHD. By contrast, no significant difference in trio-WES yield was observed between the two groups, which may be related to limited sample size and differences in sequencing strategies.

Beyond routine CNV-seq, trio-WES provided an additional diagnostic yield of 8.93%, further supporting the value of incorporating trio-WES into the prenatal genetic evaluation of fetal CHD. Previous studies have reported variable incremental yields of ES, generally ranging from 5% to 15.9% [17,23,24]. Such variability is likely attributable to differences in phenotypic composition, sequencing strategies, and sample selection across cohorts. Non-isolated CHD has consistently been associated with a higher detection rate of monogenic variants; however, in the prenatal setting, extracardiac anomalies may be incompletely characterized by ultrasound, and subtle syndromic features are often difficult to recognize in utero [25], thereby affecting phenotypic stratification and overall diagnostic yield. Methodological differences may also contribute. Previous studies have used either ES or targeted gene panels, and these approaches are not directly comparable. For example, Hu et al. [17] reported a prenatal diagnostic yield of 15.9% (7/44) using a 77-gene targeted panel in fetuses with CHD; however, panel-based testing may be influenced by cohort selection and predefined gene content. In addition, some studies included postnatally phenotyped cases, in which more complete clinical information facilitates genotype–phenotype correlation and may increase the likelihood of establishing a molecular diagnosis [26,27]. These factors should be considered when comparing the incremental value of ES across studies.

Subtype-specific analysis showed that endocardial defects (41.67%) and left-sided lesions (21.62%) had relatively high genetic diagnostic yields in our cohort. In endocardial defects, both aneuploidy and pathogenic CNVs were frequently identified, with trisomy 21 representing the most common abnormality [6]. We also identified one AVSD case with a microdeletion involving GATA4, a key cardiac transcription factor involved in cardiomyocyte differentiation and cardiac development, further supporting its role in CHD pathogenesis. Previous studies have likewise reported relatively frequent detection of GATA-related variants by ES [18]; however, this may be influenced by cohort composition and sequencing strategy.

Of the 15 cases with P/LP variants detected by ES, 33.3% (5/15) had left-sided lesions, with the implicated genes including RPL5, MYRF, FLNA, FOXP1, and EFTUD2. This finding further highlights the marked genetic heterogeneity of CHD, which involves multiple disease-causing genes and diverse variant types. By comparison, Sun et al. [28] reported KMT2D variants in 7 of 13 P/LP cases in a prenatal left-sided lesions cohort, corresponding to a detection rate of 10.6%, including fetuses with HLHS, aortic valve atresia, mitral atresia, and coarctation of the aorta. The higher yield reported in that study may reflect differences in case selection, as only fetuses with severe CHD undergoing termination of pregnancy were included.

We also identified two cases of Diamond–Blackfan anemia associated with RPL5/RPL11 variants. Experimental studies have shown that RPL5 and RPL11 interact with MDM2 under ribosomal stress, thereby stabilizing p53 through disruption of the MDM2–p53 regulatory pathway. Consistent with this mechanism, previous studies have reported that, compared with individuals without RPL5 or RPL11 variants, carriers of RPL5 and RPL11 variants have an approximately 5-fold and 6-fold increased risk of cardiac malformations, respectively [29]. Together, these findings underscore the importance of considering both

subtype-specific and gene-specific patterns when interpreting the genetic architecture of fetal CHD.

Our study was based on a relatively large, consecutively recruited single-center prenatal CHD cohort, allowing a less biased evaluation of the diagnostic performance of CNV-seq and ES. In addition, we applied a comprehensive CHD classification framework and performed all ES analyses in parent–fetus trios, which improved phenotypic stratification and variant interpretation. Several limitations should be acknowledged. First, the limited sensitivity of prenatal ultrasonography may have led to misclassification between isolated and non-isolated CHD, while the lack of systematic postnatal verification prevented confirmation of prenatal findings. Second, the non-uniform application of genetic testing, influenced by clinical indications and parental preferences, may have introduced selection bias. As only 168 CNV-seq-negative fetuses underwent trio-WES, its incremental diagnostic yield (8.93%) may not be generalizable to the entire CHD cohort, and the overall genetic burden may have been underestimated. Third, small subgroup sizes and incomplete consideration of potential confounders, including maternal age, consanguinity, and ethnicity, may have affected the interpretation of our findings. Finally, the clinical uncertainty associated with variants of uncertain significance (VUS) remains a challenge. Larger studies with comprehensive clinical data and systematic postnatal follow-up are warranted.

5. Conclusions

In conclusion, integrated prenatal genetic testing with CNV-seq and trio-WES improves the diagnostic yield in fetal CHD. Our study further demonstrates that genetic abnormalities are unevenly distributed across CHD subtypes and are more frequent in non-isolated CHD, underscoring the value of detailed phenotypic stratification. These results support the use of a stepwise genomic testing strategy in fetal CHD and provide a basis for improving prenatal diagnosis, genetic counseling, and clinical decision-making.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Medical Ethics Committee of Beijing Obstetrics and Gynecology Hospital, Capital Medical University (No. 2023-KY-009-01; 23 February 2023).

Informed Consent Statement: Written informed consent was obtained from all participants for participation in this study and for the publication of anonymized data.

Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request. Due to patient privacy and ethical restrictions, the data are not publicly available.

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Abbreviations

The following abbreviations are used in this manuscript:

CHD	Congenital heart disease
CNV-seq	Copy number variation sequencing
trio-WES	Trio-based whole exome sequencing
ES	Exome sequencing
CMA	Chromosomal microarray analysis
P/LP	Pathogenic or likely pathogenic
FGR	Fetal growth restriction
SV	Single ventricle
TAPVC	Total anomalous pulmonary venous connection
MVS	Mitral valve stenosis
AVSD	Atrioventricular septal defects
NCA	Numerical chromosomal abnormality
SNV-indel	Single-nucleotide variants and small insertions/deletions
DORV	Double outlet right ventricle
IAA	Interrupted aortic arch
PA-VSD	Pulmonary atresia with ventricular septal defect
Coa-IVS	Coarctation of the aorta with intact ventricular septum
AS	Aortic stenosis
HLHS	Hypoplastic left heart syndrome
PA/PS-IVS	Pulmonary atresia/pulmonary stenosis with intact ventricular septum
HRHS	Hypoplastic right heart syndrome
TVA	Tricuspid valve atresia
ASD	Atrial septal defect
DTGA	D-transposition of great arteries
VSD	Ventricular septal defect
PLSVC	Persistent left superior vena cava
Hex	Heterotaxia syndrome

Appendix A

Table A1. Detection rates of chromosomal abnormalities in fetuses with non-congenital heart disease.

CHD with Additional Symptom	Case Number	Chromosomal Abnormalities			Positive Rate of SNVs
		Total	NCA	CNV	
CHD with a single anomaly	95	13	7	6	4/48
Urinary tract system	23	0	0	0	0/5
Central nervous system	11	0	0	0	1/6
Cystic hygroma	7	6	4	2	0/0
Skeletal system	6	0	0	0	0/3
Abdominal wall	4	3	3	0	0/1
Face	4	0	0	0	1/1
Gastrointestinal system	4	0	0	0	1/3
Respiratory system	3	1	0	1	0/1
FGR	33	3	0	3	1/17
CHD with multiple additional anomalies	28	12	9	3	2/11

Table A2. Variants of unknown significance (VOUS) ($n = 10$) identified using exome sequencing in 7 fetuses with congenital heart defects and without chromosomal abnormality or pathogenic copy number variation.

Patient	Gene	Variant	NM Number	Zygosity	Inheritance	CHD	Additional Anomalies
1	PBX1	c.700C>T (p.R234W)	NM_002585.4	Het	AD	Pulmonary valve stenosis	microgastria; tracheal stenosis; midline liver; pulmonary hypoplasia; thoracic hypoplasia; diaphragmatic eventration.

Table A2. *Cont.*

Patient	Gene	Variant	NM Number	Zygosity	Inheritance	CHD	Additional Anomalies
2	DNAH5	c.9781A>G (p.K3261E)	NM_001369.3	CH	AR	Hex	
		c.6437C>G (p.S2146C)	NM_001369.3				
3	CHD5	c.5200G>A (p.D1734N)	NM_015557.3	Het	AD	VSD	FGR; Blake's pouch cyst
4	SCAF4	c.1291A>G (p.N431D)	NM_020706.2	Het	AD	SV	right-sided stomach; midline liver; common arterial trunk
5	KIDINS220	c.3197G>A (p.R1066H)	NM_020738.4	CH	AR	VSD	
		c.1024G>C (p.A342P)	NM_020738.4				
6	MYH7	c.4520-2A>C	NM_000257.4	CH	AR	TVA	
		c.1729T>C (p.F577L)	NM_000257.4				
7	EP300	c.4136A>C (p.Q1379P)	NM_001429.4	Het	AD	TVA	FGR

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