Rare damaging variants in the sex differences of congenital heart disease: an exome sequencing study

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Summary

Background Congenital heart disease (CHD) exhibits a marked male predominance in birth prevalence, yet the genetic mechanisms underlying this sex disparity remain poorly understood. This study investigates the contribution of rare damaging variants on autosomes and the X chromosome to sex differences in foetal CHD.

Methods Parents of foetuses with CHD were recruited for the study. Rare damaging variants were identified by analysing whole-exome sequencing data from foetus-parental trios, and their contributions to sex differences were estimated through case–control studies. Functional enrichment analysis was conducted to assess functional differences in genetic variants between sexes.

Findings 820 foetal probands with CHD were recruited, including 487 males and 333 females. We identified a significant enrichment of X-linked rare damaging variants, primarily driven by maternally inherited hemizygous variants (MIHVs) in male probands (OR = 1·84, $P < 0·05$), which accounted for 7·2% of male cases in our cohort. These variants were not found to be enriched in female probands. Additionally, X-linked rare damaging de novo variants (DNVs) were not enriched in either male or female probands (female probands: 1·8% versus female controls: 0·7%, $P = 0·6789$; no DNVs observed in males). Gene-level variant burden analysis revealed three X-linked CHD candidate genes: *DCX*, *CACNA1F*, and *MAP3K15*. Autosomal rare variants did not show significant differences in variant burdens between sexes. Notably, male probands showed specific functional enrichments in cilia-related pathways for autosomal recessive variants, as well as in chromatin remodelling and nervous system development pathways for autosomal DNVs.

Interpretation Male and female foetal CHD have significantly different genetic landscapes. The enrichment of X-linked rare damaging MIHVs in males provides a genetic explanation for the higher prevalence of CHD in males. This finding highlights the importance of incorporating sex-stratified approaches in clinical diagnostics and research.

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Keywords: Congenital heart disease; Sex differences; Whole exome sequencing; Pediatric; Prenatal

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Research in context

Evidence before this study

Previous studies have shown that males and females exhibit intrinsic differences in the prevalence, severity, treatment outcome, and survival related to congenital heart disease (CHD), suggesting that sex can serve as a risk stratification factor among patients with CHD. Recently, efforts to define personalized prevention, disease identification, prognosis, and individualized therapeutic strategies have highlighted the necessity of considering sex differences. However, the underlying mechanisms, including genetic factors responsible for these sex differences, remain poorly understood. We searched PubMed for studies related to whole exome sequencing in CHD published in English on or before Dec 31, 2023. We utilized the MeSH terms "Exome Sequencing" and "Heart Defects, Congenital" to identify relevant studies. Notably, none of these studies has evaluated the impact of rare damaging variants on sex differences in CHD. There is a need for further knowledge and experience to incorporate sex factors into clinical practice effectively.

Added value of this study

We performed whole exome sequencing in a cohort of foetal CHD to assess the contribution of rare damaging variants to the sex differences in CHD.

Our analysis revealed a significant enrichment of X-linked rare damaging variants in male probands (7·2%), driven exclusively by maternally inherited hemizygous variants. In contrast, no statistically significant enrichment of X-linked variants was observed in female probands. In addition, we identified novel X-linked CHD candidate genes (*DCX, CACNA1F, MAP3K15*) with solid statistical evidence. Autosomal de novo variants (DNVs) and recessive variants (RVs) did not show significant sex-based burden differences. However, male probands showed enrichment in cilia-related genes for RVs and genes associated with chromatin remodelling and nervous system development for DNVs. This male-specific enrichment may underlie the male predominance of heterotaxy syndrome and neurodevelopmental deficits in CHD patients.

Implications of all the available evidence

Male and female CHD have a significantly different genetic landscape. Sex differences in burden and function of rare damaging variants provide a novel insight into the underlying genetic mechanism for the sexual dimorphism of CHD. Our findings underline the need to consider sex as a stratifying factor in clinical treatment and basic research. Future studies should investigate how these genetic sex differences can be used for better risk assessment, early intervention, and targeted treatment of CHD.

Introduction

Congenital heart disease (CHD) is the most common and often severe type of birth defect, accounting for about 1% of all liveborn infants.[1] It accounts for approximately 40% of deaths among children with birth defects worldwide.[2] With advances in medical and surgical management, the survival of children with CHD has dramatically improved. Nevertheless, there are still considerable individual differences in clinical manifestations and prognosis.[3,4] Increasingly, research is focussing on understanding the differences between individual patients, including sex differences and the underlying mechanisms, including genetic factors that predict or influence clinical outcomes.[4–7]

There are notable sex differences in the prevalence of CHD at birth.[8] Overall, there is a significant male predominance in the total CHD birth prevalence.[9–16] A meta-analysis of nearly 77 million births in China found that the birth prevalence of total CHD was significantly higher—by 1·2 times—among male births compared to female births.[11] Similarly, an analysis of 18 registries from 24 European countries, involving over 100,000 samples, also indicated a male excess in CHD cases.[12] However, the relationship between sex and specific subtypes of CHD varies across different studies.[9,10,15,16] This inconsistency may be influenced by racial differences and variations in CHD categories.[8,10] Generally, it is recognized that male predominance is associated with critical CHD, while female predominance tends to be associated with milder forms of CHD.[8,12]

Beyond the differences in prevalence, sex disparities are also evident in the outcomes and comorbidities associated with CHD. Neurodevelopmental deficits (NDD) are the most common comorbidities that severely reduce the quality of life and limit the educational and employment opportunities of patients with CHD.[17,18] Recent clinical investigations have demonstrated that male patients with CHD are more susceptible to neurodevelopmental and cognitive functional deficits, leading to poorer neurodevelopmental outcomes compared to females.[18–20] Clinical studies indicate a notable sex disparity within RASopathies, a common form of syndromic CHD, with male patients exhibiting a higher predisposition to neurodevelopmental issues.[21] Notably, a study analysing CHD mortality in the US from 1999 to 2017 identified a higher mortality rate attributable to CHD in males than in females.[2]

Although there are significant differences in prevalence, prognosis, and outcomes of CHD between sexes, little is known about the underlying mechanisms of these differences. Understanding the sex differences and the underlying mechanisms is crucial for improving risk stratification, early intervention, and targeted treatment of patients with CHD. Clearly, genetic factors are important contributors to differences between patients.[22] Large-scale whole exome sequencing (WES) in CHD has shown that rare damaging de novo variants (DNVs) and recessive variants (RVs) contribute significantly to CHD.[23] Specifically, sequencing of CHD trios has identified an overrepresentation of damaging DNVs, particularly in genes highly expressed in the developing heart and brain, as well as in genes involved in cardiac morphogenesis and chromatin modification.[24]

Despite these findings, few systematic studies have investigated the genomic differences between male and female patients with CHD. It remains unclear whether the contribution of rare damaging variants is related to the observed sex differences in CHD. To understand the genetic reasons behind the sex differences in CHD, we performed WES in a large foetal CHD cohort without known chromosomal abnormalities or pathogenic copy number variations (CNVs) to evaluate the impact of rare damaging variants of both autosomal and X chromosome (ChrX) regarding sex differences in CHD.

Methods

Ethics

This is a retrospective study approved by the institutional review board of the Medical Ethics Committee of Beijing Anzhen Hospital (NO2019030), and all parents provided written informed consent to participate.

Patient subjects

Between June 5, 2015, and March 22, 2022, parents of foetuses with CHD who opted for genetic testing across our centres were retrospectively screened for eligibility for inclusion in this study. All families included in this study were of Han Chinese descent. Cases of CHD were classified according to the guidelines of the American Heart Association.[25] Foetus-parental trio samples were collected for genetic sequencing. Copy number variation sequencing (CNV-seq) was performed to identify aneuploidies and CNVs. foetal sex was initially recorded based on parental report and subsequently confirmed or determined bioinformatically through analysis of sex chromosome read counts and heterozygosity patterns derived from the foetal CNV-seq and WES data. Consideration of sex was integral to the study design, as the primary aim was to investigate the genetic basis of observed sex differences in CHD prevalence and associated features. Foetuses with known chromosomal abnormalities or pathogenic CNVs identified by CNV-seq were excluded from the study to focus on the role of rare damaging single-gene variants.

Controls

Controls consisted of 365 in-house families who underwent trio WES at our hospital for suspected monogenic diseases. All controls in this study were of Han Chinese descent. Diseases involved mainly included the urinary, nervous, skeletal, endocrine, and metabolic system. The mean age in the control group was 12 years.

Whole exome sequencing

WES was performed in the setting of a purely research-based protocol, using methods as described previously.[26] Briefly, genomic DNA was extracted from the umbilical cord and parental blood using a Qiagen DNA Blood Midi/Mini kit (Qiagen GmbH, Hilden, Germany). DNA libraries were prepared using an Agilent liquid capture system (Agilent SureSelect Human All Exon V6; Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's protocol. The DNA library was sequenced on Illumina HiSeq 4000 or Illumina NovaSeq (Illumina, Inc., San Diego, CA, USA) for paired-end 150 bp reads according to the manufacturer's protocol, producing an average of 10 million pair-end reads per sample. Low-quality sequencing reads were filtered using a quality score of ≥20 by fastp.[27] Duplicated reads caused by PCR were marked by samblaster.[28] The clean reads were aligned to the human reference genome (hg38) by the Burrows-Wheeler Aligner[29] with default settings. The aligned reads were sorted by sambamba[30] according to the physical coordinate of the reference genome and stored in cram format. The alignment summaries were calculated by samtools.[31] Per-sample variants were detected by the HaplotypeCaller module of the Genome Analysis Tool Kit.[32] Per-trio variants were combined by the CombineGVCFs module from per-sample variants in a trio. The qualities of variants were recalibrated by the VQSR module. High-quality variants of a trio were identified based on the following quality control criteria: (1) map quality >55, (2) variant quality >30, (3) each individual with a threshold of genotype quality >30, (4) each individual with a threshold of read depth >10, (5) all individuals meet criteria 3 and 4.

De novo SNVs and INDELs were identified by ForestDNM[33] at thresholds of probability >0·6 for SNV and >0·9 for INDEL, respectively. De novo variants (DNVs) that were presented in more than one trio have been excluded.

Variant annotation and filtering

After variant detection, the Variant Effect Predictor (VEP)[34] was used for variant annotation. The variant frequency was determined in the gnomAD database[35] and the ChinaMAP database[36] to remove common

variants (minor allele frequency ≥0·1% in any of the global, East Asian populations, or Chinese population). In particular, variants on the male ChrX were excluded if the variant had a hemizygous record in the gnomAD database male population. Because males possess only one ChrX, a single maternally inherited damaging variant results in a hemizygous state, functionally similar to the manifestation of autosomal recessive conditions. Throughout this manuscript, these variants are specifically termed maternally inherited hemizygous variants (MIHVs). To avoid ambiguity, any subsequent reference herein to RVs specifically in the context of the male ChrX will be referred to as MIHVs.

To prioritize variants with potential functional consequences, we selected the canonical transcript for annotation when a variant was annotated to multiple transcripts. The canonical transcript, as annotated by VEP,[34] represents the most representative or principal transcript for each gene. Then, variants located in exonic regions and canonical splice sites (intronic GT donor and AG acceptor sequences) were selected due to their high likelihood of impacting gene function. Finally, we included potentially disease-causative variants, defined as loss-of-function (LoF) variants (frameshift, nonsense, start loss and canonical splice-site variants), damaging missense variants (D-mis), and non-frameshift insertion/deletion variants. The pathogenicity of missense variants was assessed using the Combined Annotation Dependent Depletion (CADD) score prediction.[37] Specifically, missense variants with a CADD score of 25·3 or higher were classified as D-mis and included in further analysis. This threshold of 25·3 aligns with the recommendations for using CADD scores in variant interpretation according to the ACMG guidelines.[38] In our study, these potentially disease-causative variants were further classified as rare damaging variants if their MAF was less than 0·1% in both the gnomAD and ChinaMAP databases.

Gene-level enrichment analysis of damaging recessive variants

We implemented a one-tailed Poisson distribution to quantify the enrichment of rare damaging RVs in a specific gene, including compound heterozygous and homozygous variants. The cumulative allele frequency (CAF) of damaging inherited variants in a gene was calculated by the formula:

$$CAF = 1 - \prod_{i=1}^{n}(1 - AF_i) \quad (1)$$

where AF_i refers to the allele frequency of the i th variant in the gnomAD database, and n refers to the number of variants in a gene.

If the probability of a gene having a damaging variant in one copy was CAF, the probability of having damaging variants in both copies should be CAF^2, then the expected number (λ) of RVs was calculated by:

$$\lambda = CAF^2 \times N \quad (2)$$

where N refers to the number of trios.

The expected number (λ) of MIHVs in males was estimated as:

$$\lambda = CAF \times N \quad (3)$$

where N refers to the number of trios.

The P-value of the enrichment of observed number (m) of RVs versus expected was calculated by the exact Poisson test:

$$P(X \geq m) = 1 - \sum_{k=0}^{m-1} \frac{\lambda^k}{k!} e^{-\lambda} \quad (4)$$

Gene-level enrichment analysis of de novo variants

The one-tailed Poisson distribution was also utilized to quantify the enrichment of damaging DNVs in a specific gene. The expected number of DNV was calculated by:

$$\lambda = \mathrm{R} \times \sum_{i=1}^{n} L_i = \mathrm{R} \times \sum_{i=1}^{n} \sum_{j=1}^{l} G_{ij} \quad (5)$$

$G \in (0, 1)$

where n refers to the number of trios. L refers to the effective length of a transcript with a recorded length of l. G_{ij} refers the high-quality genotypes at the position j of the i th trio. The number 1 indicates there is a high-quality genotype and 0 indicates there is not. R refers to the average rate of DNV estimated by Samocha et al.[39] Specifically, the rate of damaging missense was estimated by a weight of ω from:

$$R_{damaging\ missense} = \omega \times R_{missense} = \frac{N_{gnomAD_{damaging\ missense}}}{N_{gnomAD_{rare\ missense}}} R_{missense} \quad (6)$$

The exact Poisson test was then used to test for enrichment of observed DNVs versus expected as follows:

$$P(X \geq m) = 1 - \sum_{k=0}^{m-1} \frac{\lambda^k}{k!} e^{-\lambda} \quad (7)$$

Sex difference analysis

Enrichment was calculated by the ratio of proportion of damaging variants in cases compared to controls. The proportion test was used to test for the significance of

enrichment. The significance of odds ratio (OR) of a given type of variants (damaging, LoF, and D-mis) to synonymous variants was calculated by Fisher's exact test. The difference in ORs between males and females was estimated by Wald test:

$$\delta = log\ OR_{male} - log\ OR_{female} \tag{8}$$

$$se(\delta) = \sqrt{se^2_{log\ OR_{male}} + se^2_{log\ OR_{female}}} \tag{9}$$

$$se(\log OR) = \sqrt{\frac{1}{n_1}+\frac{1}{n_2}+\frac{1}{n_3}+\frac{1}{n_4}} \tag{10}$$

where n_1, n_2, n_3, n_4 refer to the number of damaging variants and synonymous variants in case probands and unaffected probands, respectively.

$$Z = \delta / se(\delta) \tag{11}$$

$$P = 1 - \phi(Z) \tag{12}$$

where ϕ refers to the cumulative distribution function of standard normal distribution.

Considering potential stratified factors (e.g. subtypes and extra-cardiac abnormality), we adjusted the OR of Fisher's exact test via a fixed-effect model (formula 13-15) and evaluated the heterogeneity among stratified factors by Cochran's Q test (formula 16):

$$\log(OR_{combined}) = \frac{\sum \omega_i \log(OR_i)}{\sum \omega_i} \tag{13}$$

where OR_i refers to the OR of the group i.

$$se(\log(OR_{combined})) = \sqrt{1/\sum \omega_i} \tag{14}$$

$$\omega_i = \frac{1}{se(\log(OR_i))^2} \tag{15}$$

where ω_i refers to the inverse variance weight, $se(\log(OR_i))$ was denoted by formula 10.

$$Q = \sum_{i=1}^{n} \omega_i (\log(OR_i) - \log(OR_{combined}))^2 \sim \chi^2_{n-1} \tag{16}$$

where n refers to the number of groups of stratified factors.

We estimated the contribution of RVs/DNVs by the narrow sense heritability:

$$h^2 = \frac{\sigma^2_G}{\sigma^2_P} = \frac{var(X\beta)}{\sigma^2_Y} = \frac{2f(1-f)\beta^2}{\sigma^2_Y} \tag{17}$$

where f refers to the frequency of RVs/DNVs in male or female participants. β refers to the effect estimated by linear regression model:

$$Y = \beta X + \varepsilon \tag{18}$$

where σ^2_P and σ^2_Y refer to the variance of disease status.

For the binary trait in our study, the heritability of liability scale (h^2_l) is adjusted from the observed case–control scale (h^2_o).[40]

$$h^2_l = h^2_o \frac{K(1-K)}{\phi(\phi^{-1}[K])} \frac{K(1-K)}{P(1-P)} \tag{19}$$

where K refers to the incidence of CHD in population that was estimated from 0.008 to 0.012.[1] We employed $K = 0{\cdot}01$ in our study. P refers to the proportion of affected probands in our study. ϕ and ϕ refer to the cumulative distribution function and density distribution function of standard normal distribution respectively.

The difference in proportion of RVs/DNVs between affected and unaffected probands is calculated by:

$$diff = \frac{x_{case}}{N_{case}} - \frac{x_{control}}{N_{control}} \tag{20}$$

where x_{case} and $x_{control}$ refer to the number of affected probands with damaging RVs/DNVs, unaffected probands with damaging RVs/DNVs, respectively. N_{case} and $N_{control}$ refer to the total number of affected probands and unaffected probands, respectively.

$$se(diff) = \sqrt{\hat{p}(1-\hat{p})\left(\frac{1}{N_{case}}+\frac{1}{N_{control}}\right)} \tag{21}$$

where p-hat was calculated by:

$$\hat{p} = \frac{x_{case} + x_{control}}{N_{case} + N_{control}} \tag{22}$$

P-value of the different proportion is calculated by:

$$Z = diff / se(diff) \tag{23}$$

$$P = 1 - \phi(Z) \tag{24}$$

The significance of sex-differed proportion is calculated by:

$$Z = \frac{diff_{male} - diff_{female}}{\sqrt{se^2_{(diff_{male})} + se^2_{(diff_{female})}}} \quad (25)$$

$$P = 1 - \phi(Z) \quad (26)$$

Gene ontology enrichment analysis

Functional enrichment analysis was performed using the PANTHER online tool (version 18, https://pantherdb.org). A false discovery rate of 0·05 was used as the cutoff.

Statistical analysis

Comparisons of basic proportions were performed using the chi-square test or Fisher's exact test, as appropriate. The gene-level burden of X-linked RV was estimated by formulae 1-4. The gene-level burden of DNV was estimated by formulae 5-7. The chrX-wide significance threshold for Bonferroni correction was set to $P < 6{\cdot}19 \times 10^{-5} = 0{\cdot}05/808$. The exome-wide significance threshold for Bonferroni correction was set to $P < 5{\cdot}28 \times 10^{-6} = 0{\cdot}05/19{,}399$. The quantile function of Poisson distribution was used to estimate the power of burden analysis for our sample size and the other five sample sizes ranging from 500 to 10,000 (Supplementary Figure S1). The enrichment of X-linked RVs was estimated by two-sample z-test. The OR of X-linked RVs to synonymous variants was estimated by Fisher's exact test. The heterogeneity of OR of subgroups was tested by formulae 13-16. The sex-differed OR was estimated by formulae 8-12. The explained variance of X-linked RV/DNVs was estimated by formulae 17-19. The explained proportion of X-linked RV/DNVs was estimated by formulae 20-24. The sex-differed proportion of X-linked RV/DNVs was estimated by formulae 25-26. All analyses were performed using the R-program unless otherwise specified.

Role of funders

Funders had no role in study design, data collection, data analyses, interpretation, and writing of the report.

Results

Clinical and sequencing features

Between June 5, 2015, and March 22, 2022, a total of 1005 pregnant women identified with a foetus diagnosed with CHD chose to terminate their pregnancy and underwent genetic testing. CNV-seq and WES were performed sequentially on specimens from these foetuses and their parents. The initial CNV-seq analysis identified chromosomal abnormalities in 185 foetuses, accounting for 18·4% of the total cases. Consequently, these cases were excluded from the subsequent WES analysis as we continued to investigate potential genetic etiologies in the remaining cases. Ultimately, a total of 820 foetal probands with CHD and their parents were included in this retrospective study. The sample size from 820 trios provides adequate power for performing burden analysis at both the gene and chromosome levels (Supplementary Figure S1). The clinical characteristics of probands are shown in Supplementary Table S1. The cohort consisted of 487 males and 333 females, with a significantly higher proportion of males (59·4% ± 1·7%) than females (40·6% ± 1·7%) ($diff_{male\text{-}female}$ = 18·8%; ratio of male to female = 1·4; two-sample z-test, $P = 1{\cdot}13 \times 10^{-8}$). There was no significant difference in the male-to-female ratio of each subtype of CHD compared with the whole cohort. There was also no significant difference in the proportion of extra-cardiac malformations between the sexes. The controls include 365 in-house non-CHD trios. All patients and controls in this study were of Han Chinese descent. There was no significant difference in the male-to-female ratio between the cases and controls (Fisher's exact test, $P = 0{\cdot}22$). Meanwhile, no statistically significant differences in the main sequencing metrics were observed between probands with CHD and their unaffected parents and control trios (Supplementary Figure S2 and Supplementary Table S2).

Analysis of recessive, de novo, and hemizygous variants on chromosome X

Rare damaging maternally inherited hemizygous variants on chromosome X are enriched in male probands but not females

The haploid nature of the ChrX in males makes them more susceptible to X-linked damaging variants. Therefore, we initially focused on the role of X-linked rare damaging variants in contributing to sex differences in CHD. Since males and females possess different copies of X-linked genes, we cannot directly compare the burden of the X-linked variants between male and female probands. Instead, we compared male and female probands with same-sex controls to determine whether there was an enrichment of X-linked variants. Additionally, we directly compared the burden of rare damaging variants between male and female probands for autosomal variants.

We first investigated the contribution of X-linked rare MIHVs in males, by comparing the male probands to male controls. We found a significant enrichment of X-linked rare damaging MIHVs in male probands, with an enrichment ratio of 2 (Two-sample z-test, $P = 0{\cdot}0021$**; Table 1). To estimate the effect of all rare damaging MIHVs on CHD risk, we compared the OR of rare damaging MIHVs to rare synonymous variants in both male probands and male controls. Our analysis revealed that rare damaging MIHVs contribute to CHD

	Sex	Number of variants		Enrichment (Ratio)	P-value of enrichment	OR	P-value of OR	P-value of sex different OR
		Probands	Controls					
Damaging	Male	80	19	2	0·0021**	1·84 [1·06~3·32]	0·0143*	0·0168*
	Female	60	17	1·42	0·1027	0·77 [0·41~1·5]	0·8451	
LoF	Male	26	4	3·08	0·0198*	2.83 [0·96~11·36]	0·0311*	0·1357
	Female	22	4	2·21	0·0933	1·2 [0·39~4·94]	0·4956	
D-mis	Male	54	15	1·71	0·0347*	1·57 [0·84~3·09]	0·0877	0·0261*
	Female	38	13	1·18	0·355	0·64 [0·31~1·38]	0·928	
Syn	Male	362	158	–	–	–	–	–
	Female	316	69	–	–	–	–	

CHD, Congenital heart disease; D-mis, Missense variants with a CADD prediction score ≥25·3. Damaging, D-Mis + LoF; Syn, Synonymous variants. *refers to $P < 0·05$, **refers to $P < 0·01$, ns refers to $P > 0·05$.

Table 1: **The burden of rare X-linked maternally inherited damaging variants in males and females CHD.**

risk in male probands, with an OR of 1·84 (Fisher's exact test, $P = 0·0143$*). Then, we explored the role of different types of variants and discovered that both LoF and D-mis were significantly enriched in male probands. Notably, LoF variants showed a more substantial contribution, with an enrichment ratio of 3·08 (Two-sample z-test, $P = 0·0198$*) and an OR of 2·83 (Fisher's exact test, $P = 0·0311$*; Table 1).

To confirm our findings, we replicated the enrichment of rare damaging MIHVs in male patients by comparing the male probands to their unaffected fathers, which yielded an OR of 1·49 (Fisher's exact test, $P = 0·0375$*; Supplementary Table S3). Moreover, considering the potential confounding of CHD subtype and extra-cardiac abnormalities, we adjusted the OR by a fixed-effect model, which yielded a combined OR of 1·89–1·91 for the damaging variant in males (Supplementary Table S4). There is no heterogeneity among CHD subtypes, as well as extra-cardiac abnormality (Cochran's Q test, $P > 0·1$). These results indicate that our statistical evidence is robust, regardless of the source of controls and/or confounding.

We propose that if all genotypes were to exhibit complete penetrance, female probands would not be affected by heterozygous variants transmitted from their healthy mothers. Supporting this hypothesis, we found that rare damaging maternally transmitted heterozygous variants were not significantly enriched in female probands compared to female controls (Table 1).

X-linked rare damaging de novo variants are not enriched in either male or female probands

No X-linked rare damaging DNVs were identified in male probands or male controls. In females, X-linked DNVs were observed in 1·8% (6/333) of probands and 0·7% (1/134) of controls; however, this difference was not statistically significant (Fisher's exact test, $P = 0·6789$). These findings suggest that X-linked rare damaging DNVs do not significantly impact the observed sex differences within this cohort.

Quantifying the contribution of X-linked rare damaging variants to CHD

Next, we quantified the proportion of cases attributed to X-linked rare damaging variants, which include MIHVs and DNVs, in male and female probands. Our analysis revealed that X-linked rare damaging variants accounted for 7·2% ± 2·7% (Wald test, $P < 0·05$*) of male probands, as shown in Fig. 1 and Table 2. In contrast, the contribution of X-linked rare damaging variants was not statistically significant in females (1·1% ± 1·2%), suggesting limited relevance to the sex disparity in CHD. The simple calculated difference in proportion between sexes was 6·1% ± 2·9% (Wald test, $P = 0·018$*). Additionally, the expanded variance analysis, namely heritability in the narrow sense, indicated that X-linked rare damaging variants contributed a higher heritability in males (1·29%) compared to females (0·21%). Notably, the rare damaging variants identified in male probands were exclusively MIHVs, with no DNVs observed. In contrast, the rare damaging variants identified in female probands were exclusively DNVs, with no X-linked RVs identified. These data suggest that male and female probands have significantly different landscapes of X-linked rare damaging variants and that MIHVs in males, rather than DNVs, are responsible for the male bias observed in our cohort of foetal CHD.

Gene-level variant burden analysis identified X-linked CHD genes

To search for novel X-linked CHD genes, we compared the observed and expected distribution of all rare damaging MIHVs in each gene on ChrX in male probands. There were four genes (*NONO, DCX, CACNA1F,* and *MAP3K15*) which significantly departed from expectation, reaching a threshold of ChrX-wide significance (Bonferroni correction, $P < 6·19 \times 10^{-5}$) (Fig. 2, Supplementary Tables S5 and S6). *NONO* and *DCX* also reach exome-wide significance (Bonferroni correction, $P < 2·58 \times 10^{-6}$). In addition, we also observed three genes (*AFF2, DRP2,* and *FRMPD3*) with

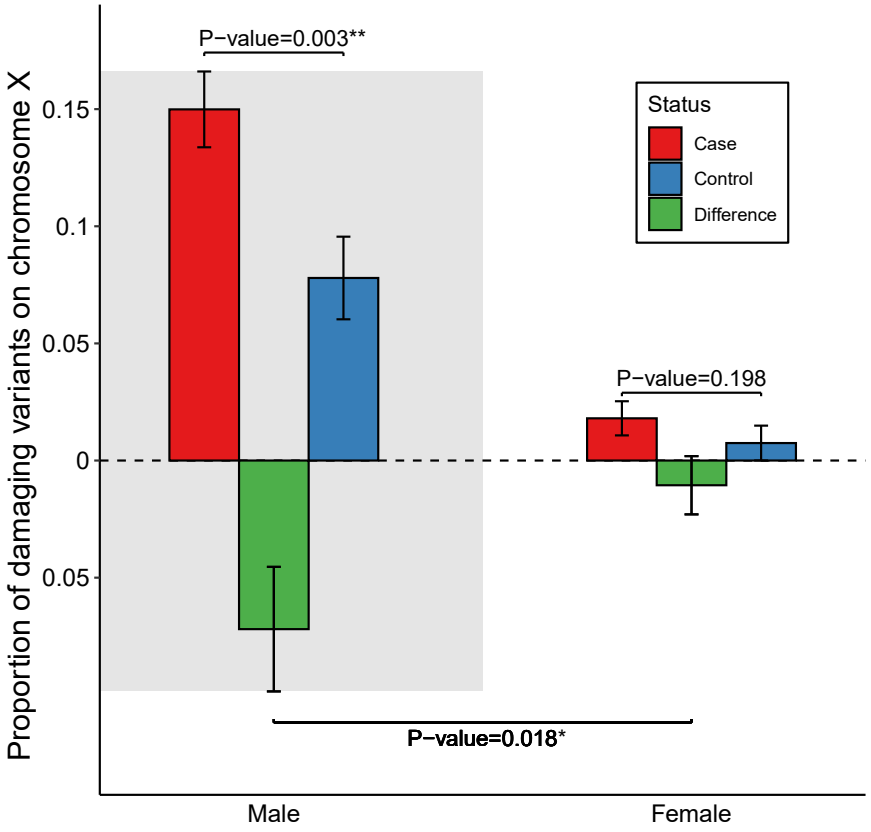

***Fig. 1:* Sex differences in rare X-linked damaging variants.** Rare X-linked damaging variants, which include maternally inherited hemizygous variants and de novo variants in males and recessive variants and de novo variants in females, explained a significant proportion (Wald test, $P = 0{\cdot}003^{**}$) for male probands and a small proportion (Wald test, $P = 0{\cdot}198$) for female probands (upper panel). The explained proportions differed significantly (Wald test, $P = 0{\cdot}018^{*}$) by sex (lower panel).

suggestive levels of association. In contrast, we did not identify any significant enrichment in male controls.

Because hemizygous LoF variants in males have the effect of homozygous knockouts, we paid particular attention to these variants. In total, 26 LoF variants were identified in 24 genes in the male probands (Supplementary Table S6). Of the 24 genes, *NONO* is a known CHD gene,[41] another six genes (*CACNA1F, ELF4, SERPINA7, TBC1D8B, USP26,* and *XPNPEP2*) are associated with non-CHD diseases, and the remaining 17 genes have not been associated with Mendelian disease in the OMIM database.[42] No LoF variant of these 24 genes was found in male controls.

Analysis of autosomal recessive variants

We identified 76 rare damaging RVs in all CHD cases (Supplementary Table S7). There are four genes (*PLD1, IQGAP3, WDFY3,* and *ANK1*) with two rare damaging

Sex	Group	X-linked RVs and DNVs		Proportion	Differed proportion	Sex-differed proportion	Explained variance
		with	without				
Male	Case	73	414	15% [±1·6%]	7·2% [±2·7%]**	6·1% [±2·9%]*	1·29%
Male	Control	18	213	7·8% [±1·8%]			
Female	Case	6	327	1·8% [±0·7%]	1·1% [±1·2%]ns		0·20%
Female	Control	1	133	0·7% [±0·7%]			

CHD, Congenital heart disease; DNVs, de novo variants; RVs, recessive variants for females and maternally inherited hemizygous variants for males. *refers to $P < 0{\cdot}05$, **refers to $P < 0{\cdot}01$, ns refers to $P > 0{\cdot}05$.

***Table 2:* The genetic contribution of X-linked RVs and DNVs to CHD.**

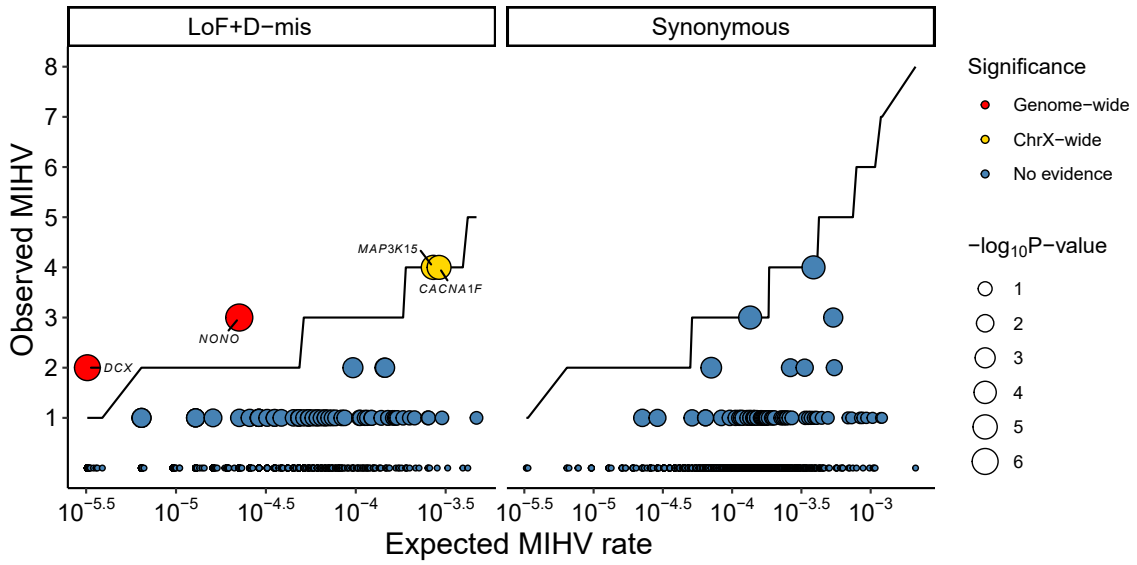

Fig. 2: **The burden of maternally inherited hemizygous variants on X chromosome in male probands.** The X-axis refers to the expected rate of RVs for each gene. The Y-axis refers to the number of observed MIHVs for each gene in 487 male probands. The black polyline indicates the exome-wide significant threshold of 0·05/19,399. Gene symbols of genes with significant enrichment of DNVs surpassing the exome-wide (red) or ChrX-wide (yellow) threshold are shown. DNV, de novo variant; RVs, recessive variants; MIHV, maternally inherited hemizygous variant.

RVs (Supplementary Table S7). However, no significant enrichments were observed at the gene level (Supplementary Figure S3), indicating that no single autosomal gene showed a disproportionately high burden of RVs. Interestingly, there is evidence suggesting that RVs in PLD1 could lead to cardiac valvular dysplasia, and the cardiac features observed in the two patients with PLD1 RVs were consistent with previous reports,[43] suggesting a potential role for *PLD1* in this specific cardiac phenotype. The other three genes have not yet been associated with CHD, highlighting potential candidates for further investigation.

In addition, rare damaging RVs in three genes (*ENKUR, NME7,* and *ENPP1*) comprised double LoF variants (Supplementary Table S7). *ENKUR*, a ciliary transient receptor potential channel-interacting protein crucial for patterning the left–right axis in vertebrates,[44] has been associated with a familial case featuring situs inversus and dextrocardia. The similar laterality defects in our case suggest that the RV in *ENKUR* is a causative factor for human laterality defects. *NME7*, a known member of the cilium, exhibited situs inversus as a prominent feature in the Nme7 knock-out mice model and patients with RVs in *NME7*.[45]

The cardiac phenotype of our case with *ENPP1* compound heterozygous LoF variants was characterized by generalized aortic calcification, consistent with the typical feature of generalized arterial calcification of infancy caused by *ENPP1* deficiency, further supporting the established link between *ENPP1* deficiency and this specific cardiac manifestation.

Differences of autosomal RVs between male and female probands

We observed 39 rare damaging autosomal RVs in male and 33 in female probands. The overall burden of autosomal RVs did not significantly differ between the sexes (Chi-square test, $P = 0{\cdot}33$), suggesting that these variants may not be a primary driver of the male predominance observed in CHD.

Gene ontology (GO) analysis of the rare damaging RVs (Supplementary Table S7) identified significant enrichment for cilium and sarcomere pathways in male probands (Supplementary Table S8). The involvement of cilium and sarcomere pathways in cardiac development and CHD has been well-documented.[46,47] Additionally, we observed an enrichment of RVs in the biological pathway of calmodulin binding. In contrast, no significant GO enrichment was observed in female probands. These findings suggest that while the overall burden of autosomal RVs is similar between sexes, the distinct biological pathways affected may lead to different functional consequences, potentially contributing to the observed sex-specific aspects of disease phenotypes.

Analysis of autosomal de novo variants

The number of observed DNVs closely fit the Poisson distribution (Supplementary Figure S2), consistent with previous reports.[23] In total, there were 319 rare damaging DNV in 296 genes, including 198 D-mis and 121 LoF variants (Supplementary Table S9). There were 14 genes with >1 rare damaging DNV, of which three

genes, *KMT2D, CHD7,* and *MYRF,* had more rare damaging DNV than expected (Supplementary Figure S4 and Supplementary Table S10). *KMT2D* and *CHD7* are the well-known and most common CHD genes. There were 3 *MYRF* DNVs, all of which were LoFs. The cardiac phenotype of all three probands was a left-sided lesion, and no extracardiac abnormalities were observed. Among 93 probands with left-sided lesions in our cohort, 3·2% had de novo *MYRF* LoF variants (Exact Poisson test, $P = 6{\cdot}4 \times 10^{-11}$). Of the remaining 11 genes, *MEGF8* is a recently discovered CHD gene.[48] The association between the other ten genes and CHD needs to be further verified and studied.

Differences of autosomal DNVs between male and female probands

Rare damaging DNVs occurred in 219 CHD cases, including 180 males and 139 females (Supplementary Table S9). The number of rare damaging DNVs did not significantly differ between male and female probands (Two-sample z-test, $P = 0{\cdot}16$), suggesting that autosomal DNVs play little role in the sex difference of CHD incidence.

GO analysis of the DNVs revealed enrichment of genes involved in previously identified biological pathways in CHD pathogenesis,[47] including chromatin remodelling, Wnt signalling, and heart development in male probands (Supplementary Table S11). We also observed several GO enrichments in male probands, such as nervous system development and male sex differentiation. Interestingly, none of these biological pathways enriched in males, with the exception of heart development, showed similar enrichment in female probands. In contrast, the most notable feature in female probands was the enrichment of pathways related to heart development, including ventricular development, myocardial development, cardiomyocyte proliferation, and trabecular development (Supplementary Table S11). This male-specific enrichment of autosomal DNVs in chromatin remodelling and nervous system pathways offers a potential explanation for the higher incidence of NDDs observed in male patients with CHD, suggesting a sex-specific impact of autosomal DNVs on disease manifestation.

Discussion

Our study evaluated the sex differences in rare damaging variants on autosomes and ChrX by performing WES in a Chinese cohort of foetuses with CHD. Our analysis revealed that the enrichment of X-linked MIHVs in males significantly contributes to the male predominance of CHD cases. We identified three potential X-linked CHD genes through gene-level variant burden analysis in males. Additionally, our enrichment analysis revealed distinct functional characteristics of rare damaging variants in male and female patients, which may help explain the differences in CHD subtypes and associated comorbidities between the sexes. These findings highlight the importance of researching sex differences, particularly in the context of sex-specific healthcare. Our findings also provide a genetic perspective on sex differences in the prevalence of CHD, emphasizing the need to consider sex as a stratifying factor in future clinical and basic research.

Males and females exhibit intrinsic differences in the anatomy, physiology, and function of the heart, as well as in prevalence, severity, treatment outcome, and survival from cardiovascular disease.[7,49,50] In cases of CHD, the male predominance in CHD prevalence and severity has long been recognized,[11,13] yet its underlying mechanisms remain elusive.[7] Differences in the canonical composition of sex chromosomes may contribute to male "susceptibility" or female "resilience" in human diseases.[51,52] Our study provides critical genetic evidence that the haploid state of the X chromosome in males amplifies the impact of damaging X-linked MIHVs, offering a genetic explanation for this sex disparity. Specifically, we observed significant enrichment of X-linked MIHVs in male probands (OR = 1·84, Fisher's exact test, $P < 0{\cdot}05$), which accounted for 7·2% of male CHD cases in our cohort. In contrast, no such enrichment was detected in females. This male-specific vulnerability aligns with the unique genetic architecture of the X chromosome: hemizygosity in males exposes recessive alleles to phenotypic effects without compensation from a second allele, a protective mechanism inherent to female diploidy.

Contrary to the "female protective effect" observed in neurodevelopmental disorders like autism, where females require a higher variant burden for phenotypic manifestation,[53,54] our data revealed no evidence of increased variant burden in female patients with CHD. Instead, the male predominance in CHD appears rooted in the intrinsic genetic susceptibility conferred by X-chromosome hemizygosity. These findings underscore the necessity of sex-stratified genetic analyses in CHD diagnostics, particularly for male patients where X-linked variants may be underrecognized.

Recent research at transcriptome and proteome levels revealed that a set of genes on ChrX regulate cardiac protein expression through a dose-sensitive mechanism, which appears to be at least partly responsible for male-female sex differences in adult mouse hearts.[55] They also identified some sex-biased proteins associated with CHD types showing sex differences, suggesting that sex chromosome mechanisms significantly influence heart development and the sex differences in CHD.

Three novel X-linked CHD genes with strong statistical evidence were identified. For *CACNA1F*, three of the four rare maternally inherited damaging variants in male probands were missense. *CACNA1F* was intolerant to the missense variant in gnomAD (Z score of

missense = 2·6). Interestingly, all three variants occurred at the N-terminal of *CACNA1F*, and the cardiac phenotype of the three patients was highly consistent, all of which were atrioventricular septal defects. Among 54 male probands with atrioventricular septal defect in our cohort, 5·6% had *CACNA1F* missense variants. The *CACNA1F* gene encodes calcium signalling protein, namely the α1 subunit of the Cav1.4 voltage-gated calcium channel.[56] A recent study identified abnormal calcium signalling as a pathophysiological mechanism in human CHD.[57] These pieces of evidence strongly support a pathogenic role for the *CACNA1F variants in CHD*. Future studies need to establish the molecular mechanism of the pathology underlying *CACNA1F*-related cardiac phenotype.

Children with CHD are at increased risk of developing NDDs, such as developmental disorders, disabilities, or developmental delays.[58] These NDDs can limit their ultimate educational achievement, employability, lifetime earnings, insurance, and quality of life.[58] Recent studies have found a significant male bias in the prevalence of NDDs among individuals with CHD.[19,20]

Previous studies have demonstrated that DNVs in chromatin modifiers are a major contributor to CHD, especially those associated with NDDs.[23,24] Consistent with the higher risk of NDDs in male patients, our research found that males but not females exhibited enrichments of DNVs in processes related to nervous system development and chromatin remodelling. These findings highlight a sex difference in DNVs associated with chromatin modifiers and nervous system development, which could help explain the greater incidence of NDDs in male patients with CHD. Future studies should validate the male bias for neurodevelopmental and chromatin remodelling-related DNVs in larger cohorts.

Cilia-associated damaging RVs were enriched and accounted for the highest proportion of RVs (6/39) in male probands, confirming the importance of the ciliary pathway in CHD pathogenesis. Enrichment of damaging cilia-related RVs was particularly marked in male but not female probands with laterality defects. This finding may partially explain the higher incidence of heterotaxy syndrome in males compared to females.

Genes related to sex/reproductive system development were enriched in DNVs of male probands but not in females (Supplementary Table S11). After reanalysing the DNVs reported by Jin et al,[23] we found similar enrichment of genes related to male reproductive system development in DNVs in their cohort. However, due to the lack of sex information, we could not further understand the sex distribution of this enrichment. CHD is the most important structural malformation associated with urinary system malformation.[59] Recent studies suggest that CHD and genitourinary abnormalities have overlapping genetic causes.[60] Interestingly, in this study, several male probands with DNVs affecting genes involved in male reproductive development did not have genitourinary malformations, indicating variability in the clinical presentation of these genes.

Our study design, which focused on exome sequencing in a large foetal CHD cohort, has several notable strengths. By specifically excluding cases with known chromosomal abnormalities or pathogenic CNVs, we were able to more precisely investigate the contribution of rare damaging single-gene variants, including both RVs and DNVs, to the observed sex differences in CHD. This targeted approach reduced etiological heterogeneity within our study population, potentially increasing the statistical power to detect sex-differential effects of these specific types of genetic variants, particularly those on the X chromosome. Furthermore, this subgroup of patients, without chromosomal abnormalities or pathogenic CNVs, constitutes the majority of CHD cases, making our findings relevant to a significant portion of the CHD population.

However, this deliberate exclusion also presents certain weaknesses. By focussing on cases without chromosomal abnormalities or pathogenic CNVs, our findings may not fully reflect the sex differences present in the entire spectrum of CHD. It is recognized that chromosomal abnormalities and large CNVs are significant contributors to CHD, and the sex ratios and underlying mechanisms in these cases may differ considerably from those driven by single-gene variants. Therefore, our conclusions regarding the genetic basis of sex differences in CHD are primarily applicable to the subgroup of patients without these genetic etiologies. Future research should aim to investigate sex differences in CHD across the complete spectrum of underlying causes, including chromosomal abnormalities and CNVs, to provide a more comprehensive understanding of this complex aspect of the disease.

The study has several limitations. First, the sample size (820 trios) provided limited statistical power to explore subtype-specific or comorbidity-adjusted analyses (Supplementary Figure S1). Only three genes reached genome-wide significance for autosomal DNVs, and two genes for X-linked MIHVs in 820 trios, collectively explaining 0·24–0·85% of cases. Larger cohorts are essential to validate these candidate genes and identify additional contributors, particularly for low-frequency variants, such as MIHVs with population frequencies $<10^{-4}$. Second, selection bias may skew our cohort toward severe phenotypes, as our center serves as a national CHD referral hub, and families with severe foetal anomalies are more likely to opt for genetic testing. Third, the age mismatch between foetal cases and postnatal controls introduces potential confounding. Although germline variants are fixed at conception, developmental and environmental differences between foetal and postnatal stages could modulate gene expression or phenotype penetrance. Thus, our findings should be interpreted cautiously until replicated in age-

matched cohorts. Finally, while our results highlight sex-specific genetic architectures, functional validation in cardiac development models and independent cohorts is critical to confirm causality.

In conclusion, our study shows that male and female CHD have a significantly different genetic landscape. Sex differences in burden and function of rare damaging variants provide insight into the underlying genetic mechanism for the sexual dimorphism of CHD. Future studies should investigate how these genetic sex differences can improve risk assessment, early intervention, and targeted treatment of CHD.

Contributors

Yihua He proposed and designed the study. Feng Lan, Jianbin Wang, and Jianguo Zhang assisted with the designing of the study. Hankui Liu and Hairui Sun analysed the whole exome data. Hairui Sun, Xiaoyan Hao, Xiaoyan Gu, and Siyao Zhang interpreted and assessed variants. Hairui Sun, Hankui Liu, Xiaoyan Gu, and Xiaoyan Hao produced the figures and did the statistical analyses. Core members of the clinical review panel (Yihua He, Hongjia Zhang, Xiaoyan Gu, Jiancheng Han, Ye Zhang) and local center representatives (Hongmei Xia, Xueqin Ji, and Yan Xu) worked on the clinical information. Tingting Liu, Xian Yang, Jiaqi Fan, Yuxuan Guan, and Jiaoyang Xie collected data. Hairui Sun, Xiaoyan Hao, Hankui Liu, Siyao Zhang, Xiaoyan Gu and Yihua He have accessed and verified the underlying data presented in the study. Xiaoyan Hao, Hairui Sun, Hankui Liu, Xiaoyan Gu, and Siyao Zhang wrote the first draft, and all authors critically reviewed iterations of the manuscript and approved the final draft for submission.

Data sharing statement

Due to the requirements of Chinese government departments for the protection of genetic resources, all raw sequencing data will not be available to others. The bioinformatic pipeline and necessary statistical code were released on GitHub (https://github.com/liuhankui/CHD).

Declaration of interests

All authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ebiom.2025.105736.

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