



# Genetics of congenital heart disease: a narrative review of recent advances and clinical implications

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**Abstract:** Congenital heart disease (CHD) is the most common human birth defect and remains a leading cause of mortality in childhood. Although advances in clinical management have improved the survival of children with CHD, adult survivors commonly experience cardiac and non-cardiac comorbidities, which affect quality of life and prognosis. Therefore, the elucidation of genetic etiologies of CHD not only has important clinical implications for genetic counseling of patients and families but may also impact clinical outcomes by identifying at-risk patients. Recent advancements in genetic technologies, including massively parallel sequencing, have allowed for the discovery of new genetic etiologies for CHD. Although variant prioritization and interpretation of pathogenicity remain challenges in the field of CHD genomics, advances in single-cell genomics and functional genomics using cellular and animal models of CHD have the potential to provide novel insights into the underlying mechanisms of CHD and its associated morbidities. In this review, we provide an updated summary of the established genetic contributors to CHD and discuss recent advances in our understanding of the genetic architecture of CHD along with current challenges with the interpretation of genetic variation. Furthermore, we highlight the clinical implications of genetic findings to predict and potentially improve clinical outcomes in patients with CHD.

**Keywords:** Congenital heart disease (CHD); genetics; genetic testing; clinical outcomes

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## Introduction

Congenital heart disease (CHD) is the most common birth defect, affecting nearly 1% of all live births (1). CHD encompasses a wide spectrum of defects from simple malformations with a favorable prognosis to more complex and severe lesions that require multiple catheter-based or

surgical interventions with uncertain long-term outcomes. Although CHD remains a leading cause of morbidity and mortality in childhood, the population of adults with CHD is dramatically expanding. Now, more than 90% of children with CHD survive into adulthood due to significant advances in disease recognition and improved medical and

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surgical management across the lifespan (2-5). Therefore, understanding the genomic architecture of CHD is increasingly clinically important (6). While there have been significant advances in the elucidation of the genetic etiologies for other forms of inherited cardiac disease such as cardiomyopathy and arrhythmias, it has only been with the increased understanding of the molecular pathways regulating cardiovascular development over the past couple of decades that the genetic basis of CHD has become more defined (7-10). However, the detailed genetic architecture of CHD and how disruption of these underlying regulatory mechanisms result in the spectrum of CHD phenotypes is actively being investigated.

While numerous genes have been discovered to be implicated in the pathogenesis of syndromic CHD, the identification of the genetic contributors of non-syndromic CHD is more challenging due to genetic heterogeneity, incomplete segregation and potentially oligogenic or polygenic origins. The initial discoveries of disease-causing genes were primarily restricted to milder forms of CHD in non-syndromic and syndromic cases by using linkage analysis to study large families with autosomal dominant disease or by targeted sequencing of candidate genes in affected populations (7,8,10). Remarkable advances in genetic sequencing technologies, such as massively parallel or next-generation sequencing (NGS) have enabled the discovery of rare variants in new candidate genes that are likely contributing to non-syndromic CHD (11-15). Although *in vivo* and *in vitro* genetic models have allowed for assessment of the potential functional deficits of specific variants on gene function, these sequencing studies still have practical challenges in establishing pathogenicity of identified variants (16-18). Recent advances in powerful new technologies known as single-cell RNA sequencing (scRNA-seq) have facilitated the discovery of the role of individual cells during cardiac development and pathogenic mechanisms by which small subset of cells affected by genetic mutations lead to cardiac malformations (19).

In this review, we aimed to summarize the well-established genetic contributors to CHD and also discuss the recent advances in our understanding of the genetic architecture of CHD along with the challenges associated with the interpretation of newly discovered genetic variants in individuals with CHD. In addition, we sought to highlight the clinical implications of these genetic findings, which have the potential to predict and improve clinical outcomes in patients with CHD.

This narrative review was compiled through study,

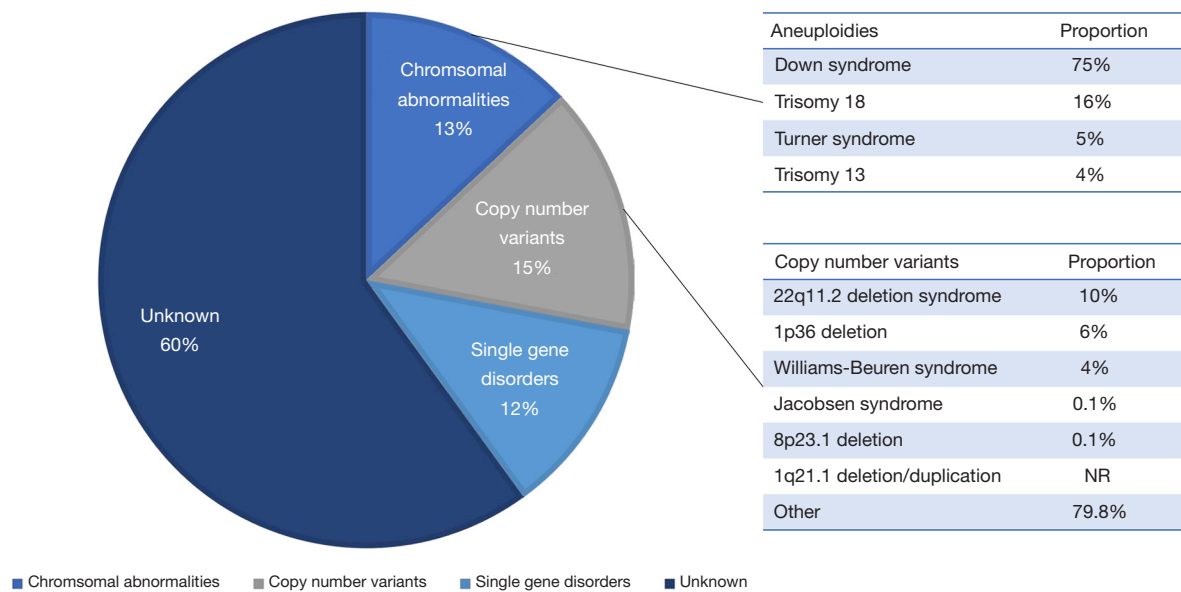
analysis, and discussion of previously published literature. PubMed was searched without time limitations and language restrictions, including articles related to the etiology and genetic contributors of CHD, NGS studies in large CHD cohorts, challenges with interpretation of NGS findings, functional genomics of CHD, and clinical implications of genetic testing and genetic prediction of clinical outcomes in patients with CHD. Search terms included congenital heart defects in combination with genetics, etiology, pathogenesis, mutations/genetic variation, environmental factors, NGS, exome sequencing, whole genome sequencing (WGS), variant prioritization, scRNA-seq, functional genomics, genetic animal models, human induced pluripotent stem cells (iPSCs), noncoding variants, or genetic testing and a combination of congenital heart disease, genetics and clinical outcomes. We present the following article in accordance with the Narrative Review reporting checklist (available at <https://dx.doi.org/10.21037/tp-21-297>).

### Established etiologic contributors to CHD

The etiology of CHD is multifactorial as both genetic and environmental factors have been implicated in its etiology (20). Specific genetic causes can be detected in an estimated 40% of CHD cases (*Figure 1*). Genetic causes of CHD are extremely heterogeneous, including chromosomal anomalies or aneuploidies (estimated 13%, range from 9% to 18%) (21), copy number variants (CNVs) (estimated 10–15%: range from 3% to 25% in syndromic CHD and 3% to 10% in non-syndromic CHD) (22-24), and single gene disorders (12%) (13,25-27). The genetic basis of CHD can be divided into syndromic CHD and non-syndromic CHD, where congenital abnormalities are isolated to the heart.

### Genetic abnormalities associated with syndromic CHD

Numerous commonly observed syndromes have been found to be caused by chromosomal aneuploidies and CNVs as well as pathogenic variation of single genes (28). Common syndromes associated with CHD are summarized in *Table 1*. Chromosomal aneuploidies include the trisomies (13, 18 and 21) and monosomies such as Turner syndrome, that are detectable by karyotyping (29-34). CNVs are large deletions or duplications of DNA and pathogenic CNVs that are associated with syndromic CHD. These include 22q11.2 deletion syndrome (DiGeorge syndrome)



**Figure 1** Established genetic causes of congenital heart disease. Chromosomal abnormalities, copy number variation and single gene variants are associated with ~40% of congenital heart disease cases but the majority (60%) of congenital heart disease remains unknown. All percentages are approximate based on recent publications (13,21-27). NR, not reported.

(35-41), 1p36 deletion syndrome (42-44), 7q11.23 deletion (Williams-Beuren syndrome) (45,46), terminal deletions of 11q (Jacobsen syndrome) (47-50), 1q21.1 deletion/duplication (69-72), and 8p23.1 deletion syndrome (73,74), which can be detected by fluorescent *in situ* hybridization and/or chromosomal microarray (CMA). Syndromes caused by single gene variants have additionally been found to be genetically heterogeneous, including mutations in transcription factors and chromatin modifiers that are important for normal cardiac development. Single gene etiologies, inherited in a Mendelian manner, were initially detected by classic linkage analyses and targeted sequencing of candidate genes in large, multigenerational kindreds where multiple family members were affected with CHD associated with syndromes.

### Monogenic causes of non-syndromic CHD

Pathogenic variants that result in non-syndromic CHD can be broadly divided into transcription factors, cell signaling molecules and cardiac structural proteins (17,25). Monogenic causes of non-syndromic CHD with sufficient evidence are summarized in *Table 2* and briefly described below. The expression and function of these factors are critical for cardiac progenitor lineages and the

spatiotemporal regulation and formation of the complex three-dimensional heart structure.

### Transcription factors

Transcription factors involved in cardiac development have been identified by genetic studies with multiple animal model systems (152,153). NKX, GATA and T-box family members constitute the core regulatory network that is responsible for normal cardiogenesis and are causative genes in CHD (154).

Mutations in the homeobox transcription factor *NKX2-5* were first reported as the cause of non-syndromic CHD by studying four kindreds with autosomal dominant disease (98). The common phenotype associated with *NKX2-5* mutations is atrial septal defect (ASD) along with atrioventricular conduction abnormalities (99). *NKX2-5* mutations have since been reported in a wide spectrum of CHD, including ventricular septal defect (VSD), tetralogy of Fallot (TOF), subvalvar aortic stenosis (AS), pulmonary atresia and hypoplastic left heart syndrome (HLHS), as well as atrioventricular conduction abnormalities, leading to complete heart block and sudden cardiac death (100-103). Previous studies demonstrated that mutations in the homeodomain of *NKX2-5* are a cause of ASD, while mutations

**Table 1** Common syndromes associated with congenital heart disease (selected)

Syndrome	Gene	Loci	Cardiac defect	Clinical findings	References
Chromosomal aneuploidies					
Down syndrome	Unknown	Trisomy 21	AVSD, ASD, VSD, PDA, TOF	CHD (40–50%), short stature, cognitive deficits, atlantoaxial instability, immune system dysfunction, hypotonia, hypothyroidism	(29-31)
Turner syndrome	Unknown	45, X (monosomy X)	CoA, BAV, dilated Ao, AS, HLHS	CHD (30%), short stature (partially growth hormone responsive), cognitive deficits, ADHD, lymphedema, webbed neck, primary amenorrhea	(32-34)
CNVs					
22q11.2 deletion syndrome (DiGeorge syndrome)	<i>TBX1</i>	22q11.2 deletion	Conotruncal defects (TOF, PTA), VSD, IAA, ASD	CHD (74–85%), cleft palate, bifid uvula, velopharyngeal insufficiency, microcephaly, hypocalcemia/hypoparathyroidism, thymic hypoplasia/immune deficit, psychiatric disorder, learning disability	(35-41)
1p36 deletion syndrome	Unknown	1p36 deletion	PDA, VSD, ASD, BAV, Ebstein's anomaly, LVNC	CHD (70%), Growth deficiency, intellectual disability, microcephaly, deep-set eyes, low-set ears, hearing loss, hypotonia, seizures, central nervous system defects, genital anomalies	(42-44)
Williams-Beuren syndrome	<i>ELN</i>	7q11.23	SVAS, PPS, VSD, ASD	CHD (80%), dysmorphic facies, thick lips, strabismus, stellate iris pattern, intellectual disability, infantile hypercalcemia	(45,46)
Jacobsen syndrome	<i>ETS1</i> <i>FLI1</i>	11q terminal deletion	HLHS, AS, VSD, CoA, Shone's complex	CHD (56%), growth retardation, developmental delay, thrombocytopenia, platelet dysfunction, widely spaced eyes, strabismus, broad nasal bridge, thin upper lip, prominent forehead, intellectual disability	(47-50)
Single-gene variation					
Alagille syndrome	<i>JAG1</i> <i>NOTCH2</i>	20p12.2 1p12-p11	PPS, TOF, PA	CHD (>90%), bile duct paucity, posterior embryotoxon, butterfly vertebrae, renal defects	(51-53)
Char syndrome	<i>TFAP2B</i>	6p12.3	PDA, VSD	CHD (58%), wide-set eyes, down-slanting palpebral fissures, thick lips, hand anomalies	(54)
CHARGE syndrome	<i>CHD7</i>	8q12	TOF, PDA, DORV, AVSD, VSD	CHD (75–85%), coloboma, choanal atresia, genital hypoplasia, ear anomalies, hearing loss, developmental delay, growth retardation, intellectual disability	(55,56)
Costello syndrome	<i>HRAS</i>	11p15.5	PS, ASD, VSD, HCM, arrhythmias	CHD (44–52%), short stature, feeding problems, broad facies, bitemporal narrowing, redundant skin, intellectual disability	(57)
Ellis-van Creveld syndrome	<i>EVC</i> <i>EVC2</i>	4p16.2 4p16.2	Common atrium	CHD (60%), skeletal dysplasia, short limbs, polydactyly, short ribs, dysplastic nails, respiratory insufficiency	(58,59)
Holt-Oram syndrome	<i>TBX5</i>	12q24.1	VSD, ASD, AVSD, conduction defects	CHD (50%), absent, hypoplastic, or triphalangeal thumbs, phocomelia, defects of radius, limb defects more prominent on left	(60,61)

**Table 1** (continued)

Table 1 (continued)

Syndrome	Gene	Loci	Cardiac defect	Clinical findings	References
Kabuki syndrome	<i>KMT2D</i>	12q13	CoA, BAV, VSD, TOF, TGA, HLHS	CHD (50%), growth deficiency, wide palpebral fissures, large protuberant ears, fetal finger pads, intellectual disability, clinodactyly	(62-64)
	<i>KDM6A</i>	Xp11.3			
Noonan syndrome	<i>PTPN11</i>	12q24.13	Dysplastic PVS, ASD, TOF, AVSD, HCM, VSD, PDA	CHD (75%), short stature, hypertelorism, down-slanting palpebral fissures, ptosis, low posterior hairline, pectus deformity, bleeding disorder, chylothorax, cryptorchidism	(65-68)
	<i>SOS1</i>	2p22.1			
	<i>RAF1</i>	3p25.2			
	<i>KRAS</i>	12p12.1			
	<i>NRAS</i>	1p13.2			
	<i>RIT1</i>	1q22			
	<i>SHOC2</i>	10q25.2			
	<i>SOS2</i>	14q21.3			
	<i>BRAF</i>	7q34			

ADHD, attention deficit/hyperactivity disorder; AS, aortic stenosis; ASD, atrial septal defect; AVSD, atrioventricular septal defect; BAV, bicuspid aortic valve; CHARGE, coloboma, heart defects, choanal atresia, retarded growth and development, genital anomalies, and ear anomalies; CHD, congenital heart disease; CNVs, copy number variants; CoA, coarctation of the aorta; dilated Ao, dilated ascending aorta; DORV, double-outlet right ventricle; HCM, hypertrophic cardiomyopathy; HLHS, hypoplastic left heart syndrome; IAA, interruption of aortic arch; LVNC, left ventricular noncompaction cardiomyopathy; PA, pulmonary atresia; PDA, patent ductus arteriosus; PPS, peripheral pulmonary stenosis; PS, pulmonary stenosis; PTA, persistent truncus arteriosus; PVS, pulmonary valve stenosis; SVAS, supraaortic stenosis; TGA, transposition of great arteries; TOF, tetralogy of Fallot; VSD, ventricular septal defect.

outside the homeodomain may result in TOF (155). In addition, mice harboring *NKX2-5* mutations have been reported to recapitulate cardiac phenotypes found in humans (156-158). Identification of *NKX2-5* mutations is clinically beneficial in terms of detecting patients with the increased risk of progressive conduction system disease, sudden cardiac death or asymptomatic ASD.

Mutations in the GATA family members *GATA4*, *GATA5*, and *GATA6*, characterized by zinc finger domains and transcriptional activation domains, have been identified in patients with various types of CHD. Heterozygous mutations in *GATA4* were first discovered in familial cardiac septal defects (76). *GATA4* mutations have been associated with ASD, VSD, atrioventricular septal defect (AVSD), pulmonary stenosis (PS), and TOF (77-80). These findings have been supported by the reports of similar cardiac phenotypes in mice haploinsufficient for *GATA4* or those harboring disease-causing *GATA4* mutations (159-161). Additionally, rare sequence variants in *GATA5* have been reported in affected individuals with CHD, including bicuspid aortic valve (BAV), VSD, TOF and double outlet right ventricle (DORV) (82,162,163). Genetic deletion of *GATA5* and endothelial cell-specific deletion of *GATA5*

using *Tie2-Cre* in mice led to BAV (164). Furthermore, *GATA6* mutations were first reported in patients with persistent truncus arteriosus (PTA) (85) and have been implicated in a variety of CHD, including TOF, DORV, transposition of the great arteries (TGA), ASD, VSD and PS (86,165). However, murine models display BAV, including mice haploinsufficient for *GATA6* and those with second heart field specific deletion of *GATA6* using *Isl1-Cre* (166). Interestingly, *GATA6* mutations were also found to be an important cause of pancreatic abnormalities (hypoplasia and agenesis) and associated type 1 diabetes mellitus (87,165). *GATA6* was recently shown to function as a pioneer factor in cardiac development, regulating transcriptional activation of critical genes associated with the development of the heart as well as endodermal lineages, pancreas and diaphragm (167). These findings illuminated the molecular mechanisms for diverse developmental defects such as cardiac outflow tract defects, pancreas and diaphragm dysgenesis in patients with distinct *GATA6* variants.

The T-box family consists of important transcription factors in cardiac development. *TBX5* and *TBX1* are implicated in the etiology of Holt-Oram syndrome and

**Table 2** Genes associated with non-syndromic congenital heart disease (selected)

Gene	Cardiovascular malformation	OMIM	References
Transcription factors			
<i>CITED2</i>	ASD, VSD	602937	(75)
<i>GATA4</i>	ASD, VSD, AVSD, PS, TOF	600576	(76-81)
<i>GATA5</i>	ASD, VSD, DORV, TOF, BAV	611496	(82-84)
<i>GATA6</i>	PTA, TOF	601656	(85-89)
<i>HAND1</i>	AVSD, DORV, HLHS, ASD, VSD	602406	(90,91)
<i>HAND2</i>	TOF, LVNC, VSD	602407	(92-94)
<i>JARID2</i>	Left-sided lesions	601594	(95)
<i>MED13L</i>	TGA	608771	(96)
<i>NR2F2</i>	AVSD, AS, CoA, VSD, HLHS, TOF	107773	(97)
<i>NKX2-5</i>	ASD, atrioventricular conduction delay, TOF, HLHS, VSD	600584	(98-103)
<i>NKX2-6</i>	PTA	611770	(104,105)
<i>TBX1</i>	DORV, TOF, IAA, PTA, VSD	602054	(106)
<i>TBX5</i>	AVSD, TOF, BAV, CoA, ASD, VSD	601620	(107)
<i>TBX20</i>	ASD, VSD, MS, DCM	606061	(108-110)
<i>MEF2C</i>	DORV	600602	(111)
<i>NFATC1</i>	TA, AVSD	600489	(112,113)
<i>ZFPM2/FOG2</i>	TOF, DORV	603693	(114-116)
Cell signaling and adhesion proteins			
<i>ACVR1/ALK2</i>	AVSD	102576	(117)
<i>CFC1</i>	TGA, DORV	605194	(118)
<i>CRELD1</i>	ASD, AVSD	607170	(119-122)
<i>FOXH1</i>	TOF, TGA, VSD	603621	(123)
<i>GDF1</i>	ASD, DORV, TGA, TOF	602880	(124)
<i>GJA1</i>	HLHS, VSD, PA	121014	(125-127)
<i>HEY2</i>	AVSD	604674	(128)
<i>JAG1</i>	TOF, PS	601920	(129-131)
<i>NODAL</i>	TGA, DORV, TOF, VSD	601265	(123,132)
<i>NOTCH1</i>	BAV, AS, HLHS, TOF, PS, ASD, VSD, CoA, DORV	190198	(12,133,134)
<i>PDGFRA</i>	TAPVR	173490	(135)
<i>SMAD6</i>	BAV, CoA, AS	602931	(136)
<i>TAB2</i>	BAV, AS, TOF	605101	(137)
<i>VEGFA</i>	TOF, PDA, AS, BAV, CoA, IAA, VSD	192240	(138,139)

Table 2 (continued)



Table 2 (continued)

Gene	Cardiovascular malformation	OMIM	References
Structural proteins			
<i>ACTC1</i>	ASD, HCM, DCM, LVNC	102540	(140)
<i>DCHS1</i>	MVP	603057	(141)
<i>ELN</i>	SVAS	130160	(142-145)
<i>MYH6</i>	ASD, HCM, DCM	160710	(146-148)
<i>MYH7</i>	Ebstein's anomaly, LVNC, HCM, DCM	160760	(149,150)
<i>MYH11</i>	PDA, TAA	160745	(11,151)

AS, aortic stenosis; ASD, atrial septal defect; AVSD, atrioventricular septal defect; BAV, bicuspid aortic valve; CoA, coarctation of the aorta; DCM, dilated cardiomyopathy; DORV, double-outlet right ventricle; HCM, hypertrophic cardiomyopathy; HLHS, hypoplastic left heart syndrome; IAA, interruption of aortic arch; LVNC, left ventricular noncompaction cardiomyopathy; MS, mitral valve stenosis; MVP, mitral valve prolapse; PA, pulmonary atresia; PDA, patent ductus arteriosus; PPS, peripheral pulmonary stenosis; PS, pulmonary stenosis; PTA, persistent truncus arteriosus; SVAS, supraaortic stenosis; TA, tricuspid atresia; TAA thoracic aortic aneurysm; TAPVR total anomalous pulmonary venous return; TGA, transposition of great arteries; TOF, tetralogy of Fallot; VSD, ventricular septal defect.

22q11.2 deletion syndrome, respectively (168,169). In addition to the link to syndromic CHD, mutations in *TBX5* and *TBX1* have been identified in non-syndromic CHD such as TOF and cardiac septal defects (106,107,170). Another member of the family, *TBX20*, has subsequently been implicated in non-syndromic CHD, including cardiac septal defects, mitral valve stenosis, dilated cardiomyopathy and TOF (108-110).

### Cell signaling and adhesion molecules

Notch signaling is important for cellular differentiation regulating the development of cardiac valves and chambers, and is associated with syndromic as well as non-syndromic CHD (51,129,171). Variants in *NOTCH1* were the first reported genetic cause of aortic valve disease (133) and then have been described in not only left-sided CHD such as BAV, AS, coarctation of the aorta (CoA), and HLHS, but also TOF and other right-sided CHD (15,134,172).

Another important cell signaling pathway in cardiovascular development is the Nodal signaling that regulates left-right patterning. *NODAL* mutations have been reported in patients with heterotaxy as well as non-syndromic CHD including TGA, conotruncal heart defects and VSD (123,132). Mutations in several downstream targets of *NODAL* (*GDF1*, *CFC1* and *FOXH1*) were also identified in CHD cohorts (118,124,173,174).

### Structural proteins

Cardiac sarcomere and extracellular matrix proteins are

crucial for the structure and function of cardiac muscle. Mutations in structural cardiac proteins are common causes of cardiomyopathy; however, some of these genes have also been associated with non-syndromic CHD. *MYH6* ( $\alpha$ -myosin heavy chain 6) mutations have been described in familial ASD along with hypertrophic or dilated forms of cardiomyopathy (146,175). Mutations in *MYH7* ( $\beta$ -myosin heavy chain) have been associated with Ebstein's anomaly and left ventricular noncompaction (LVNC) (149,150). Similarly, mutations in *ACTC1* ( $\alpha$ -cardiac actin), another sarcomere protein gene, have been identified in familial ASD and cardiomyopathy (140,176). *MYH11* (myosin heavy chain 11) mutations have been implicated in familial thoracic aortic aneurysm with patent ductus arteriosus (PDA) (11,151). *ELN* (elastin) haploinsufficiency causes syndromic CHD in Williams-Beuren syndrome (45,142), whereas mutations in *ELN* have been reported in non-syndromic supraaortic AS and PS (143,144).

### Environmental contributors to CHD

Environmental causes are implicated in 2–10% of CHD cases, and include maternal illnesses such as diabetes mellitus, obesity and phenylketonuria, maternal infection such as rubella and influenza, nutritional deficiencies such as folic acid, vitamin A and vitamin D, and teratogens such as thalidomide, alcohol, smoking and drugs (177-181). Although a significant proportion of CHD cases are likely to have some environmental etiologic contribution, it has been

difficult to quantify the specific role these environmental contributors play in disease development. The underlying mechanisms by which environmental factors disrupt molecular pathways during cardiac development to cause CHD remain unknown. Moreover, CHD has been shown to be caused by gene-environment interactions in mice, where haploinsufficiency of *Notch1* in developing embryos together with maternal hyperglycemic or hypoxic exposure resulted in increased incidence of CHD (182-184).

### Recent advances in the understanding of the genetic architecture of CHD

#### Summary of NGS in large CHD cohorts

Over the past decade, remarkable advancements in NGS technologies have allowed for the identification of novel genetic etiologies for CHD and better understanding of the complex genetic architecture of non-syndromic CHD. Recent multi-institutional studies describing the results of exome sequencing of 2,871 CHD probands (among which were 2,645 trios) conducted by the Pediatric Cardiac Genomics Consortium (PCGC) have uncovered damaging rare transmitted variants and *de novo* variants (DNVs) in 8% of patients with sporadic CHD including 28% of syndromic and 3% of non-syndromic CHD (14,185). These DNVs were frequent in genes associated with proteins that function in chromatin modification, transcriptional regulation and RNA processing. A recent gene-burden analysis of 2,391 CHD trios revealed that cilia-related genes are enriched for rare, damaging recessive variants but comparatively less enriched for damaging DNVs (186). In contrast, chromatin-modifying genes were highly enriched for damaging DNVs. Similar findings were observed in another exome sequencing study that confirmed an excess of DNVs in chromatin-modifying genes involved in H3K4 methylation, H3K27 methylation, and H2BK120 ubiquitination (25). These DNVs in chromatin-modification genes were also associated with neurodevelopmental delays and extra-cardiac anomalies, indicating a potential role of these DNVs in syndromic forms of CHD. Furthermore, a large international study using exome sequencing of 1,891 probands has revealed a distinct genetic architecture for syndromic versus non-syndromic CHD, with unique enrichment of loss-of-function DNVs in syndromic CHD and incompletely penetrant inherited protein-truncating variants in non-syndromic CHD, respectively (13). Incomplete penetrance of these rare variants may contribute

to the phenotypic heterogeneity in familial CHD and could also contribute to oligogenic causes of CHD (187,188).

With the advances in NGS technologies and large patient cohort studies, new genes and genetic variants were implicated in the pathogenesis of CHD as well as identifying a subset of patients with pathogenic variants identified in known CHD genes as described in *Table 2*. Variants in the gene, *FLT4*, which encodes vascular endothelial growth factor receptor 3 (VEGFR3), were identified in several large cohort exome sequencing studies. Jin *et al.* (14) found 2.3% of TOF patients to have LOF *FLT4* variants and Page *et al.* (15) reported that deleterious *FLT4* variants were identified in 2.4% of TOF cases. Majority of *FLT4* variants were predicted LOF variants. A recent study of WGS in 231 individuals with CHD, most with TOF, demonstrated a significant truncating variant burden for *FLT4* (189). Not surprisingly, these studies also identified pathogenic variants in *NOTCH1* in patients with TOF. Additional studies are necessary to understand the mechanistic roles of *FLT4* variants in the etiology of TOF, which may influence the subtypes of TOF and clinical outcomes (190). In addition to *FLT4*, other genes in the vascular endothelial growth factor (VEGF) pathway have been associated with TOF. LOF variants in *KDR*, encoding the vascular endothelial growth factor receptor 2 (VEGFR2), were identified in TOF cohorts (138) and more recently, exome sequencing in a familial case of TOF and large-scale genetic studies revealed rare variants in *KDR* in a family with TOF (191). These studies proposed novel mechanisms that dysregulated VEGF signaling contributes to the pathogenesis of TOF.

Recessive genotypes in *MYH6* were identified ~11% of patients with Shone complex, and left ventricular dysfunction was demonstrated in 4 of 7 probands with *MYH6* mutations in the recent large exome sequencing study by PCGC (14). A study of WGS in 5 patients with HLHS and reduced right ventricular ejection fraction revealed recessive compound heterozygous *MYH6* variants in 2 patients (192). Furthermore, in a case-control study in 190 unrelated patients with HLHS, an increased burden of damaging *MYH6* variants (10.5%) was noted compared with controls (2.9%) (193). A recent WGS study in 197 probands with HLHS identified rare, damaging *MYH6* variants in 10% of cohorts (194). Interestingly, these studies suggest that *MYH6* variants are associated with poor clinical outcomes in HLHS, as will be discussed further in the section titled genetic prediction of clinical outcomes.

RBFOX2, an RNA-binding protein that regulates alternative splicing, has also been identified as a new



candidate CHD gene (195). *RBFOX2* has a variety of biological activity contributing to neuronal maturation and axon assembly as well as cardiac remodeling and cardiac decompensation in pressure overload-induced heart failure (196,197). Damaging DNVs in *RBFOX2* have been found in patients with HLHS in the large patient cohort studies (14,27). In addition, Verma *et al.* (198) uncovered a new role of *RBFOX2* in the pathogenesis of HLHS, demonstrating that *RBFOX2* was functionally impaired in HLHS patients leading to transcriptomic changes in the right ventricle of HLHS patients.

*KMT2D* encodes the histone-lysine N-methyltransferase 2D enzyme, responsible for H3K4 methylation, and regulates the genes involved in early embryonic development (199). Pathogenic variants in *KMT2D* are a well-known genetic cause of Kabuki syndrome (200). Recently, significant enrichment of damaging DNVs in *KMT2D* have been observed in CHD cases (14,27), and clustering of missense *KMT2D* variants have been found to cause a novel phenotype distinct from Kabuki syndrome, that includes CHD (201).

### **Challenges with interpretation of NGS results**

Although NGS technologies in CHD cohorts have contributed to identifying variants associated with CHD risk, NGS techniques have some challenges that have limited translation to the clinical setting. First, there are difficulties in variant prioritization and interpretation. Second, establishing the pathogenicity of identified variants is still challenging even with advances in *in vitro* and *in vivo* genetic modeling of CHD. Third, with the increased availability and use of WGS, the role of noncoding variation in the genetic architecture of CHD is not clear.

### **Variant prioritization**

In spite of the tremendous advances in genetic sequencing technologies, the identification of pathogenic variants in patients with non-syndromic CHD has been challenging, even in familial cases of CHD. Previously, we and others have successfully used exome sequencing and a variant prioritization pipeline to identify novel pathogenic variants in familial CHD using known CHD-causing gene lists. Blue *et al.* (202) reported that a targeted NGS gene panel of 57 candidate genes in a cohort of CHD families identified pathogenic variants in 31% of families. Consequently, they have successfully performed exome sequencing along with bioinformatics pipelines and filtering strategies to

identify candidate variants in familial non-syndromic CHD (203). In our work, we identified a genetic etiology in 3/9 (33%) of familial cases of CHD using similar approaches (11). In these studies, variants were filtered using bioinformatics pipelines and prioritized according to *in silico* prediction programs, predicted mode of inheritance, minor allele frequencies, and presence in databases such as dbSNP (Single Nucleotide Polymorphism Database), the National Heart, Lung and Blood Institute Exome Sequencing Projects (ESP) (<https://evs.gs.washington.edu/EVS/>), 1000 Genomes (<https://www.internationalgenome.org/>), the Exome Aggregation Consortium (ExAC) and the Genome Aggregation Database (gnomAD) (<https://gnomad.broadinstitute.org/>). Variants were then classified based on the American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology (AMP) guidelines (204). Since the release of these guidelines, several online tools and repositories have been developed for classification and interpretation of genetic variants, including the National Institutes of Health-funded Clinical Genome Resource (ClinGen) (<https://clinicalgenome.org/working-groups/sequence-variant-interpretation/>), Varsome (<https://varsome.com/>), Franklin (<https://franklin.genoox.com/clinical-db/home>) and Clinvar (<https://www.ncbi.nlm.nih.gov/clinvar/>). Recently, Szot *et al.* (205) reported the utility of their dual approaches for analyzing exome sequencing data to identify likely pathogenic variants. They achieved overall high diagnostic rate in families with sporadic and familial CHD by interrogating high confidence CHD-causing genes as well as an unbiased screen in which the exome sequencing data were analyzed comprehensively for additional variants not identified through the CHD gene list. In addition, a recent study has reported the effectiveness of pathway enrichment analyses of DNVs in exomes of CHD patients to explore novel CHD risk genes and validate potentially damaging variants (206).

With the advent of scRNA-seq technology, the profiling and analysis of single-cell transcriptomes is now possible with unprecedented resolution and throughput. The resolution of scRNA-seq datasets coupled with machine learning approaches has led to numerous findings in CHD. scRNA-seq has been utilized to generate cell atlases of cardiac cell types at various stage of cardiac development by profiling the anatomical locations of the embryonic heart (207,208). Additionally, scRNA-seq has been used to elucidate the mechanisms regulating the emergence and segregation of the early cardiac lineage

during heart development (209,210). A network-based computational method for scRNA-seq analysis has revealed the mechanisms by which relatively small populations of cells are affected during cardiogenesis and how regulatory defects in discrete cell subsets can lead to morphologic developmental defects (19). Single-cell genomics will help to understand the pathogenic mechanisms of CHD at the single-cell level and provide novel insights into the genetic architecture of CHD. These approaches can refine variant prioritization pipeline by filtering novel candidate genes from large patient cohorts along with validation of cardiac expression from scRNA-seq datasets to expand our ability to identify pathogenic variants in CHD. The incorporation of these additional filtering criteria will result in continued improvement of the pathogenic variant detection algorithm and increasingly identify the genetic underpinnings of CHD.

### Functional genomics of CHD

Although the ability to identify potential genetic variants contributing to CHD is improving, the determination of variant pathogenicity remains challenging. Accordingly, genetic model systems are required to characterize potentially pathogenic variants and elucidate pathogenic mechanisms (18). Numerous *in vivo* and *in vitro* models are available, including zebrafish, fruit fly, frog, chick and large mammals, and each model has strengths and weaknesses. Murine models have been widely used to study cardiovascular development because they share a high degree of sequence conservation to humans and recapitulate human cardiac development. However, genotype-phenotype relationships can be different between humans and mice. Furthermore, using the murine model system is not amenable for the analysis of large number of recently identified genetic variants. Therefore, additional genetic models that allow for a higher throughput analysis are increasingly necessary.

Human iPSCs provide a unique *in vitro* platform to study genetic mechanisms of non-syndromic CHD by single-gene defects (211). Human iPSCs can be differentiated into a variety of cell types including cardiomyocytes (iPSC-CMs), endothelial cells (iPSC-ECs), vascular smooth muscle cells (iPSC-SMCs) and cardiac fibroblasts (iPSC-CFs). In addition, patient-specific iPSCs can be tailored to the unique individual genetics of patients and are crucial in investigating the complex genetic mechanisms of CHD by incorporating with advanced NGS technologies as well as transgenic animal models. These significant advantages have

made it possible to expand the use of patient-derived iPSCs to study a variety of CHD, including supravalvular AS (212), and BAV and calcific aortic valve disease (213), cardiac septal defects (214,215), HLHS (216-219), pulmonary atresia with intact ventricular septum (220), and LVNC (187,221). A recent examination of transgenic murine hearts and patient-derived iPSC-CMs study revealed the oligogenic inheritance of LVNC with the evidence for a *NKX2-5* variant as a genetic modifier (187). Another recent study reported genome-wide transcriptome profiles of iPSC-CMs that were generated from patients with single ventricle heart disease and non-syndromic TOF (222). These studies provide growing evidence for the effectiveness of using iPSCs to model CHD and open the door to identifying how a modifier gene or multiple genes interact to cause CHD. Furthermore, the single-cell omics approaches in cardiovascular precision medicine combined with iPSC platforms, epigenomics and proteomics will contribute to the development of patient-specific therapeutics for CHD.

### Noncoding genetic variation

WGS captures both coding and noncoding regions of the genome. Although the overall yield of exome sequencing in patients with CHD is low, WGS allows for the discoveries of a large number of CNVs and single nucleotide variations (SNVs) in regulatory and noncoding regions of the entire human genome. However, establishing a link between noncoding genetic variation and CHD is still challenging because classic transgenic mouse methods are not as applicable to noncoding regions. Allele-specific expression analysis can identify candidate noncoding genetic variants by combining RNA-seq and WGS in complex genetic diseases including CHD (195,223). The largest WGS in CHD demonstrated an enrichment in damaging DNVs in noncoding regions in CHD trios compared to controls and estimated that noncoding DNVs are associated with 17–45% of CHD cases (224). These studies highlight the potential of WGS to elucidate the role of noncoding variants contributing to the pathogenesis of CHD. Future studies are needed to establish the transcriptional and post-transcriptional regulatory effects of noncoding variants on cardiac development.

## Clinical implications of recent genetic advances

### Genetic testing

Over the past 20 years, the advanced genetic testing

methodologies (e.g., CMA and exome sequencing) are increasingly being incorporated into the genetic evaluation of patients with CHD, and the results of this testing has important clinical implications. The clinical benefits of genetic testing for patients with CHD include establishing a genetic diagnosis, anticipatory management of CHD and associated extra-cardiac conditions, and clinical screening of at-risk family members (225). In addition, genetic testing can provide the information about the genetic causes and the recurrence risk of CHD to support reproductive decisions and to guide perinatal management. While CMA and single-gene testing for specific syndromes are currently utilized as standard genetic testing for patients with CHD and extra-cardiac abnormalities, diagnostic use of NGS technology is evolving, allowing the interrogation of large datasets of genetic variants. The overall diagnostic yield of genetic testing varies from the low single digits to close to 40%, which depends on the tests available, the anatomical lesions, and the presence of extra-cardiac or other relevant clinical features (13,27). Despite the potential utility, variant interpretation is challenging as genotype-phenotype correlations remain elusive and phenotypic heterogeneity or incomplete penetrance is present. Communicating complex genetic findings to potentially unsuspecting patients is also challenging. Given the potential psychosocial impact of genetic testing in asymptomatic, phenotype-negative individuals, it is therefore critical that genetic testing should be offered within the context of appropriate genetic counseling so that families are given the opportunity to discuss insurance and other risks well in advance (6).

### *Genetic prediction of clinical outcomes*

Despite advances in our understanding of genetics underlying CHD, prediction of clinical outcomes by using genetic findings remains challenging. Initial successful examples of linking common genetic variants for clinical care were limited to areas of prediction of disease risk, disease classification, and drug response (226). Apolipoprotein E genotypes were associated with adverse neurodevelopmental outcomes after cardiac surgery in patients with CHD (227,228). Additionally, in patients with single ventricle, adrenergic receptor genotypes were associated with poor postoperative outcomes, and renin-angiotensin-aldosterone receptor genotypes were linked to failure of ventricular remodeling after surgery, impaired renal function and somatic growth (229).

Discovery of an increasing number of genetic

contributors to CHD, large-scale data of clinical phenotypes and the growing knowledge about the biological and physiological impact of genetic variants are providing evidence to help understand clinical implications of genetic findings to predict clinical outcomes in CHD patients. Underlying genetic variants are increasingly recognized to affect clinical outcomes such as long-term/event-free survival, growth, neurodevelopmental performance, and ventricular function (230). Additionally, a significant increase in genetic burden of novel and rare variants in genes implicated in CHD and neurodevelopmental disabilities (NDD) was identified to be associated with the development of NDD in CHD patients (231). Therefore, identification of genetic variants associated with CHD and neurodevelopmental abnormalities could be useful for early learning intervention strategies.

Furthermore, specific genetic variation influences clinical outcomes in some categories of CHD patients. Pathogenic variants in cilia genes may predict postoperative and respiratory outcomes (25). Patients with single ventricle heart defects segregated by the presence of potentially pathogenic CNVs had worse outcomes as well as worse linear growth and neurodevelopmental performance at 14 months of age than those without CNVs (22). Pathogenic CNVs have been associated with increased risk of death or transplant in non-syndromic CHD (23). A 22q11.2 deletion in CHD patients also affects their surgical complication rate and survival. Patients with TOF and 22q11.2 deletion syndrome were found to have a longer stay in the intensive care unit and a worse quality of life (232). A recent study of exome sequencing in a large cohort of 2,517 patients with CHD demonstrated that 11.7% of patients carried clinically significant DNVs and patients with DNVs were more likely to have extra-cardiac anomalies (233). This study also found that DNVs were associated with lower transplant-free survival and worse postoperative respiratory outcomes such as longer times on the ventilator in patients who underwent open-heart surgery. Interestingly, the magnitude of the association between DNVs and clinical outcomes was shown to be different for patients with versus without extra-cardiac anomalies. In patients with extra-cardiac anomalies, the association of DNVs with worse outcomes was modest without statistical significance. In contrast, DNVs were strongly associated with adverse outcomes among patients without extra-cardiac anomalies. These important findings suggest a benefit for genetic testing even in patients without extra-cardiac anomalies who are not suspected to have genetic abnormalities in routine clinical practice.

Although only a few HLHS candidate genes have been validated by robust genetic and functional studies, several genetic factors have been reported to have an impact on clinical outcomes in patients with HLHS. Patients with HLHS harboring pathogenic CNVs were reported to be associated with significantly decreased survival compared to those with normal CMA or variants of undetermined significance (VUS) (234). In addition, HLHS with risk of poor outcomes has been linked to *MYH6* variants. Patients harboring *MYH6* variants had abnormal myocardial physiology and reduced right ventricular ejection fraction (192). Transplant-free survival was significantly lower in HLHS patients with damaging *MYH6* variants compared with HLHS patients without *MYH6* variants (193). Most recently, compound heterozygosity for rare damaging variants in *MYH6* or *MYBPC3*, encoding myosin binding protein C3, was found to be a risk factor for myocardial dysfunction in patients with HLHS (194). These findings could help identify patients at risk for poor outcomes and develop precision medicine approaches tailored to the genetic information of each patient in the future.

### Future directions

The increased understanding of genetics of CHD is providing new insights into the etiology of CHD as well as the impact of genetic variants on clinical outcomes in patients with CHD. More accurate detection and interpretation of pathologic genetic variants in CHD patients will enable clinicians to identify extra-cardiac manifestations, predict post-operative course and long-term outcomes, leading to the improvements of clinical outcomes. Further refinement of the clinical variant interpretation framework such as ACMG/AMP guidelines will construct a more accurate, consistent and transparent approach to variant classification. In addition, larger studies in well-phenotyped CHD cohorts, including important long-term clinical outcomes, will be required to determine further genetic factors contributing to the pathogenesis of CHD and its associated morbidities.

The ultimate goals are to develop therapies to slow the progression or prevent the occurrence of CHD. Current advances in sequencing technologies and functional genomic models of CHD will allow for the integration of genome editing, cardiac bioengineering and cardiac organoid models. The maturation of these technologies will open the door for new regenerative and preventive therapeutic approaches to treat the core disease mechanisms

in CHD patients in the future.

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