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Genetic Etiology of Left-Sided Obstructive Heart Lesions: A Story in Development

Lauren E. Parker, BS; Andrew P. Landstrom, MD, PhD

ABSTRACT: Congenital heart disease is the most common congenital defect observed in newborns. Within the spectrum of congenital heart disease are left-sided obstructive lesions (LSOLs), which include hypoplastic left heart syndrome, aortic stenosis, bicuspid aortic valve, coarctation of the aorta, and interrupted aortic arch. These defects can arise in isolation or as a component of a defined syndrome; however, nonsyndromic defects are often observed in multiple family members and associated with high sibling recurrence risk. This clear evidence for a heritable basis has driven a lengthy search for disease-causing variants that has uncovered both rare and common variants in genes that, when perturbed in cardiac development, can result in LSOLs. Despite advancements in genetic sequencing platforms and broadening use of exome sequencing, the currently accepted LSOL-associated genes explain only 10% to 20% of patients. Further, the combinatorial effects of common and rare variants as a cause of LSOLs are emerging. In this review, we highlight the genes and variants associated with the different LSOLs and discuss the strengths and weaknesses of the present genetic associations. Furthermore, we discuss the research avenues needed to bridge the gaps in our current understanding of the genetic basis of nonsyndromic congenital heart disease.

Key Words: aortic stenosis ■ bicuspid aortic valve ■ coarctation of the aorta ■ congenital heart disease ■ hypoplastic left heart syndrome ■ interrupted aortic arch

Congenital heart disease (CHD) is a spectrum of structural malformations of the heart that impair efficient blood flow. It is the most common class of congenital abnormality, with a global prevalence of ≈1 in 100 live births and ≈1.35 million infants born with CHD each year.[1,2] Advances in diagnosis, surgical intervention, and perioperative management have significantly reduced mortality. Between 1987–1990 and 2002–2005, there was a 59% and 16% decrease in childhood and adult mortality respectively.[3] As a result of these advancements, the number of adults living with CHD now exceeds the number of children.[3] As more adults with CHD are able to live to have children of their own, understanding the genetics of CHD is critical to ensure early diagnosis and treatment of affected children.

BURDEN OF LEFT-SIDED OBSTRUCTIVE LESIONS

In a structurally normal heart, deoxygenated blood enters the right heart and travels through the pulmonary artery to the lungs. After gas exchange, oxygenated blood enters the left heart and is pumped through the aorta to the systemic circulation. Within the landscape of CHD, the class of left-sided obstructive lesions (LSOLs) includes structural and stenotic lesions that block left ventricular filling, output, and systemic blood flow. Among these are hypoplastic left heart syndrome (HLHS), aortic stenosis (AS), bicuspid aortic valve (BAV), coarctation of the aorta (CoA), and interrupted aortic arch (IAA). HLHS, a lethal univentricular defect, is characterized by an underdeveloped left ventricle and ascending aorta. The estimated population prevalence of HLHS is 2.60 (95% CI, 2.46–2.75) per 10 000 live births.[4] AS is a valvular defect that obstructs left ventricular output and prevalence per 1000 births is 0.46 (95% CI, 0.25–0.73).[5] AS is further subclassified as supravalvular, valvular, and subvalvular depending on the location of the stenotic lesion. In a classical tricuspid aortic valve, valvular AS can arise if the leaflets are dysplastic and narrow the aortic orifice but more commonly, congenital AS is due to cusp fusion.[6] BAV is the

Correspondence to: Andrew P. Landstrom, MD, PhD, Duke University Medical Center, Box 2652, Durham, NC 27710. E-mail: andrew.landstrom@duke.edu

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Nonstandard Abbreviations and Acronyms

AD	autosomal dominant
AS	aortic stenosis
AV	aortic valve
BAV	bicuspid aortic valve
CHD	congenital heart disease
CoA	coarctation of the aorta
DNV	de novo variant
ES	exome sequencing
GS	genome sequencing
HLHS	hypoplastic left heart syndrome
IAA	interrupted aortic arch
iPSC-CM	induced pluripotent stem cell derived cardiomyocytes
LOF	loss of function
LSOL	left-sided obstructive lesion
NS-CHD	non-syndromic CHD
S-CHD	syndromic CHD
SVAS	supravalvular aortic stenosis
TAA	thoracic aortic aneurysm

most common subtype of valvular AS and is the most prevalent form of CHD overall with a population prevalence of 1% to 2%.[7] BAV is frequently asymptomatic and undiagnosed until adulthood when AV stenosis, regurgitation, or other aortopathies develop. Though rare, cases of unicuspid and quadricuspid AVs have also been observed.[6,8] CoA is characterized by a narrowing of the aorta with an estimated prevalence of 5.57 (95% CI, 5.26–5.79) per 10 000 live births.[4] IAA is a rare defect with population prevalence 0.62 (95% CI, 0.55–0.70) per 10 000 live births and is characterized by the complete absence of part of the aortic arch.[4]

HERITABILITY OF LEFT-SIDED OBSTRUCTIVE LESIONS

Overall, the etiology of CHD is heterogenous and can roughly be divided into CHD in the setting of extracardiac abnormalities, so-called syndromic CHD (S-CHD), and nonsyndromic CHD (NS-CHD) where congenital abnormalities are isolated to the heart. Syndromic causes of LSOLs include a number of well-defined genetic syndromes resulting primarily from aneuploidies or variation in the typical diploid arrangement of portions of the chromosomes known as copy number variants (CNVs).[9] Down syndrome results from an extra copy of chromosome 21 and is associated with CHD in 40% to 50% of cases.[9] Turner syndrome is caused by the partial or total loss of the X chromosome in females and CHD occurs in 20% to 40% of these patients.[9] DiGeorge syndrome is caused by microdeletion at the 22q11.2 locus, resulting in the loss of over 40 genes.[9] Other LSOL-associated syndromes can be caused by single-gene variation, including Cantu syndrome (*ABCC9*), Kabuki syndrome (*KMT2D*), and Rubinstein-Taybi (*CBP* and *EP300*).[9]

NS-CHD has clear evidence for a heritable basis of disease and is the focus of this review. In contrast to S-CHD, NS-CHD typically have normal chromosomal number and arrangement without CNV. The incidence of CHD in a sibling of an affected child is 4% to 22% in comparison to the population prevalence of ≈1% among live births.[10,11] Nonsyndromic LSOLs are associated with a 20% incidence of CHD in first-degree relatives, with BAV being the most commonly detected lesion.[12,13] Given this high sibling recurrence risk, echocardiographic screening of first-degree relatives is recommended.[14-16] The risk of CHD varies by the proband's defect but is higher than the general population with prevalence in first-degree relatives of 19% for HLHS, 9% for CoA, and 1% for transposition of the great arteries.[16] Pedigree analysis of HLHS in 3 generations of 38 families found that over 55% of HLHS probands had more than 1 member affected and the recurrence risk of HLHS in siblings was 8%, which increased to 21% with an affected parent.[11] In the case of BAV, 9.4% of first- and second-degree relatives of a BAV proband were also affected.[17] Family studies demonstrate a heritable factor for CoA; however, ascertaining the genetics of CoA is difficult because only 16.2% of patients with CoA have isolated disease, whereas the majority have concomitant cardiac defects.[18,19] The high heritability of these lesions is strongly suggestive of an underlying genetic cause for LSOLs.[20]

Traditionally these inherited lesions are believed to be caused by the interaction of unidentified environmental and genetic factors.[21] However, the early presentation and familial clustering of CHD is reminiscent of early onset monogenic diseases such as severe autosomal recessive disorders and genetically more complex diseases such as Alzheimer disease, dyslipidemias, and autoimmune conditions.[22,23] In addition, a compound heterozygous inheritance pattern, a type of autosomal recessive inheritance where each copy of an allele harbors a different variant, has been identified in several LSOLs.[24,25] Finally, a number of cardiovascular diseases have been linked to autosomal dominant (AD), monogenic causes, including cardiomyopathies, channelopathies, and connective tissue diseases.[26] Forms of CHD associated with a high sibling recurrence risk and a multigenerational family history are consistent with an AD mode of inheritance with variable penetrance, such as in BAV,[27-29] and HLHS.[11] Sporadic cases of CHD may be because of either recessively inherited variants or de novo AD variants. Exome

sequencing (ES) of 1365 trios, 68 affected sibling pairs, and 458 singleton probands with S-CHD and NS-CHD revealed that de novo loss of function (LOF) variants were enriched in syndromic CHD, whereas inherited rare pathogenic variants were enriched in NS-CHD.[30] Incomplete penetrance of these rare inherited variants may explain the phenotypic spectrum observed in familial CHD and point toward a mono- or oligogenic cause.

TRANSCRIPTION FACTORS ARE IMPLICATED IN LEFT-SIDED OBSTRUCTIVE LESIONS

The heart is the first fetal organ to develop and arises from 2 cell lineages in the anterior lateral plate mesoderm. Gastrulation brings the cardiogenic mesodermal cells to form the cardiac crescent, and shortly after, the cells migrate to the midline to form a beating heart tube. Rightward looping occurs thereafter and positions the heart chambers, inflow, and outflow tracts, which are established by subsequent septal division. This process is governed by dynamic interaction between cell signaling cascades and transcription factors, namely NKX2-5, GATA, and NOTCH, among others.[31] Given the tight genetic control throughout cardiogenesis, any number of alterations in cardiogenic transcription factors or signal transduction could underlie NS-CHD phenotypes.

THE GENETIC LANDSCAPE OF LEFT-SIDED OBSTRUCTIVE LESIONS

The genetic causes of nonsyndromic LSOLs are heterogeneous with overlapping genetic substrate (Figure 1). Many of the genes implicated are associated with myocyte contractility (*MYH6*) or the transcriptional regulators of cardiac development (Figure 2). Identification of putative genetic loci have provided a foundation for understanding the genetics underlying heart development and CHD; however, there are limitations to these studies that are summarized next.

HYPOPLASTIC LEFT HEART SYNDROME

NOTCH1-Encoded Notch Receptor 1

NOTCH1 encodes the NOTCH1 receptor, 1 of 4 type 1 transmembrane receptors that interact with Jagged and Delta-like receptors on neighboring cells. When activated, the NOTCH receptors undergo a series of cleavage events that release the NOTCH intracellular domain, which interacts with transcriptional

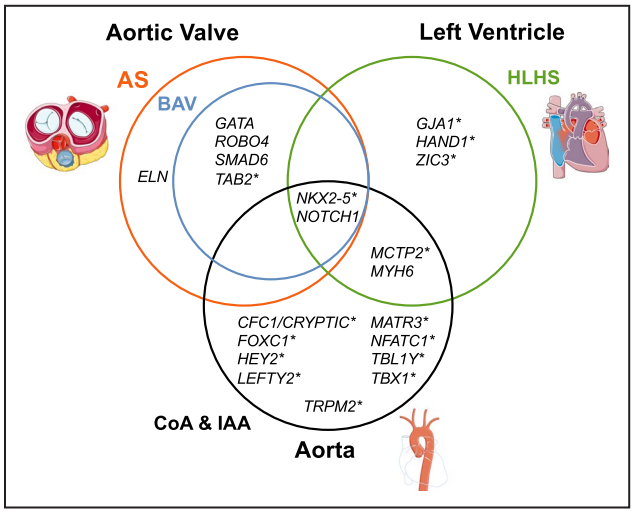

Figure 1. Venn diagram showing overlap of genes associated with LSOLs based on disease subtype.
Asterisk indicates genes without robust evidence of association. AS indicates aortic stenosis; BAV, bicuspid aortic valve; CoA, coarctation of the aorta; HLHS, hyperplastic left heart syndrome; IAA, interrupted aortic arch; and LSOL, left-sided obstructive lesion. This figure was created using images modified from Servier Medical Art Commons, licensed under a Creative Commons Attribution 3.0 Unported License (http://smart.servier.com).

repressor RBP-J to target genes in the HESR, CHF, and Hrt families. Despite the observation that *Notch1*$^{+/-}$ mice show no overt phenotype, several *NOTCH1* variants have been identified in patients with HLHS.[32] For instance, exome sequencing (ES) of 4 patients with HLHS identified 1 with a heterozygous protein-truncating variant C4662A, inherited through the patient's unaffected father.[33] Of note, the patient's paternal aunt was known to have tricuspid atresia, suggesting that this pathogenic variant may have variable expressivity. In another study, ES of 49 HLHS-affected families found likely pathogenic *NOTCH1* variants in 6% of HLHS probands and variants of unknown significance in 16% of the cohort.[34] Following this, a primary genetic association study in 1085 individuals with left ventricular outflow tract (LVOT) obstruction identified 2 rare intronic variants (g.chr9:139427582C>T and g.chr9:139435649C>T) with strong linkage disequilibrium, though the effects of these intronic variants remain unknown.[34] In addition to large genetic association studies that have implicated noncoding variation, family studies have identified variants in *NOTCH1* associated with likely autosomal recessive CHD. In 1 family, genome sequencing (GS) of an HLHS proband and 4 family members, 2 of whom had bicuspid aortic or pulmonary valves, identified compound heterozygous *NOTCH1* variants in the proband (P1964L and P1256L).[24] The P1256L variant was inherited from the

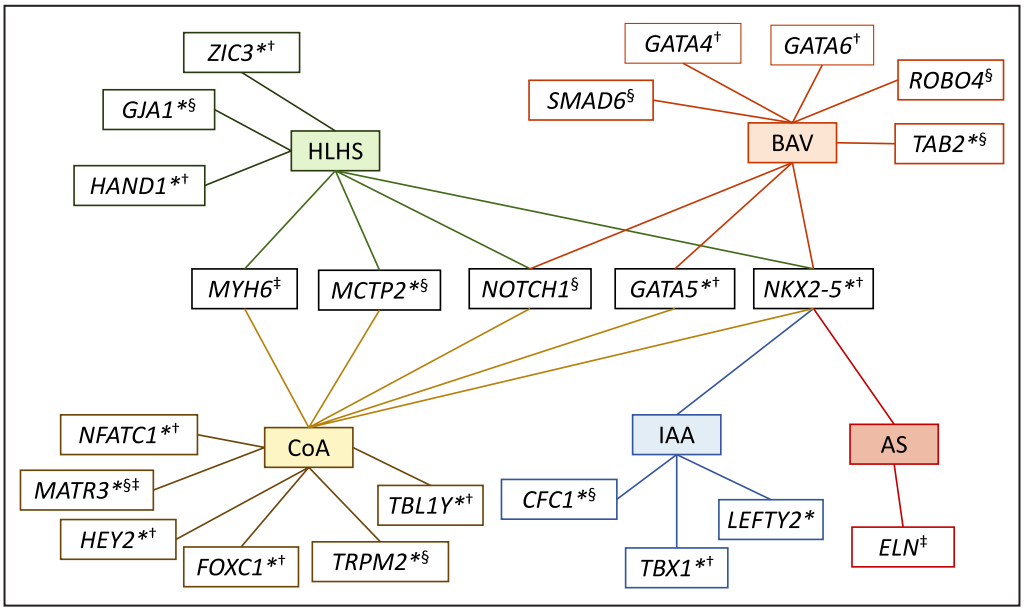

Figure 2. Genetic landscape of left-sided obstructive lesions.
Asterisk indicates genes without robust evidence of association. Genes code for †transcription factors, ‡structural or contractile proteins, §cell signaling components. AS indicates aortic stenosis; BAV, bicuspid aortic valve; CoA, coarctation of the aorta; HLHS, hyperplastic left heart syndrome; IAA, interrupted aortic arch; and LSOL, left-sided obstructive lesion.

patient's unaffected father, whereas the P1964L was inherited from the patient's mother who had BAV. The proband's induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) exhibited reduced Notch signaling, impaired myofilament organization, and altered nitrous oxide signaling while parent cells were unaffected. Independent HLHS-iPSC studies also observe impaired Notch signaling and differentiation, even in patient-derived cells without known *NOTCH1* variants.[35] *NOTCH1* variants associated with HLHS are repeatedly observed in unbiased genetic studies, though the current assessment by ClinVar is not currently supportive of a clear monogenic cause of disease based on the absence of many of these variants, and 1 is currently listed as benign. Animal and iPSC models have begun to delineate the role of *NOTCH1* in HLHS, but variants are still uncommon in the HLHS population and may represent a pathogenic substrate rather than a causative agent.

MYH6-Encoded Myosin 6

MYH6 encodes the minor ventricular myosin heavy chain isoform expressed during fetal development and the alpha-heavy chain subunit of cardiac myosin (MYH6).[36] *Myh6* ablation in mice is embryonic lethal and associated with gross cardiac defects, and recent identification of variants in HLHS patients suggests a role for *MYH6* in human CHD.[37] Unbiased genetic screens provide the strongest evidence for this association with HLHS, and suggest *MYH6* variants may predict poorer prognosis. GS in 5 patients with HLHS and reduced right ventricular ejection fraction post-Fontan operation identified 2 patients with rare inherited compound heterozygous variants (I704N and T1379M; D588A and E1207K) affecting the tail and head domains of MYH6.[38] Heterozygous family members were unaffected, and interestingly, *MYH6* variants were not identified in 21 patients with normal post-Fontan ejection fraction. Further, 1 group performed GS in a family with recurrent HLHS identified a rare inherited *MYH6*-R443P variant in both affected and unaffected individuals.[25] Case-control association testing in 190 unrelated patients with HLHS (who had previously undergone ES or GS) identified damaging variants in 20 patients (10.5%) versus 2.9% of controls. Of those with *MYH6* variants, 95% were heterozygous carriers.[25] Just as the previous study associated *MYH6* variants with reduced post-Fontan ejection fraction, transplant-free survival was significantly lower in *MHY6* variant carriers in the case-control cohort.[25,38] This suggests that beyond being a potential cause of HLHS, knowledge of *MYH6* variant status could inform the provider's choice between univentricular repair, biventricular repair, or transplant. Finally, a group derived iPSC-CMs from 16 patients with HLHS, 8 of whom were *MYH6* variant carriers. *MYH6* variant iPSC-CMs had 350% increased MYH7 expression in atrial and ventricular tissue, along with inefficient cardiomyocyte

differentiation and dysmorphic sarcomeres, all of which may represent compensatory responses to *MYH6* haploinsufficiency.[25] Taken together, *MYH6* variants in HLHS are well characterized by GS, ES, and iPSC models; however, ClinVar considers many of these variants of conflicting or uncertain significance. Given that independent studies have associated *MYH6* variants with a poorer post-surgery prognosis, it is possible that they are a disease-modifying factor rather than a disease-causing substrate.

GJA1-Encoded Gap Junction Alpha-1 Protein

GJA1 encodes the connexin43 (CX43) subunit of gap junction channels abundantly expressed in the heart. During development, these gap junctions are critical to facilitating cell-cell adhesion and communication via electrical and molecular signals.[39] *GJA1* knockout is embryonic lethal in mice and causes severe conotruncal defects and LVOT obstruction; however, the role in human LSOLs is debated.[40] *GJA1* variants were identified in an early study via denaturing gradient gel electrophoresis in 8 of 14 patients with HLHS post-transplant.[41] Each of these patients had 2 silent polymorphisms and missense variants R362Q and R376Q that, in vitro, abolish phosphorylation of the CX43 regulation domain.[41] Interestingly, this sequence is that of the *GJA1* pseudogene and the authors suggested that illicit recombination and loss of regulatory phosphorylation contributes to the HLHS phenotype. The *GJA1* pseudogene is implicated in nonsyndromic deafness and has been identified as a regulator of tumor growth; it has not been identified in HLHS probands in more recent studies and was likely an artifact from poor variant resolution by gel electrophoresis.[41-43] An independent group evaluated 300 patients with CHD for *GJA1* variants, including only 4 HLHS probands, and found no nonsynonymous variants in any patient with CHD.[44] Surprisingly, knock-in mice with variants blocking critical CK1 (S325A/S328Y/S330A) and PKC (S368A) phosphorylation sites shown in vitro to alter cell-cell communication exhibit no CHD.[44] Though animal models suggest an important role for *GJA1* in LVOT development, modern genetic screens, mouse models, and current ClinVar assessments do not support the original pseudogene hypothesis or *GJA1* variants as a genetic cause of HLHS.

NKX2-5-Encoded NK2 Homeobox 5

The *NKX2-5* homeobox transcription factor is expressed in the early first heart field, the second heart field pharyngeal mesoderm, and the pharyngeal endoderm.[45] *Nkx2-5*$^{-/-}$ mice develop a linear heart tube but fail to initiate looping, and *NKX2-5* haploinsufficiency in humans is associated with a spectrum of CHD.[46,47] The approach to *NKX2-5* variant identification in HLHS has been candidate-gene based because of established associations with ventricular septal defect, atrial septal defect, and tetralogy of Fallot but in patients with HLHS, *NKX2-5* variants are rarely observed. The first study describing an association between genetic variants in *NKX2-5* and HLHS identified a T178M variant in a patient with HLHS and a family history of atrial septal defect and atrioventricular conduction block.[48] Though a later study found a heterozygous R25C variant in 1 of 80 patients with HLHS, several groups have since sequenced *NKX2-5* in HLHS cohorts and repeatedly fail to identify any *NKX2-5* variant associated with sporadic or familial HLHS.[48-51] Despite this, iPSC-CMs from patients with HLHS exhibit downregulated *NKX2-5* expression and increased H3K27 methylation at the *NKX2-5* promoter relative to iPSC-CMs from patients with biventricular CHD.[52] Though mouse models illustrate the importance of *NKX2-5* in cardiogenesis, the few variants identified through targeted sequencing are conflicting in the ClinVar database, and current evidence does not support *NKX2-5* variants as a significant monogenic cause of HLHS.

HAND1-Encoded Heart and Neural Crest Derivatives-Expressed Protein 1

The *HAND1* gene encodes a basic helix-loop-helix (bHLH) transcription factor (HAND1) that is required for cardiac morphogenesis and regulates the balance between cardioblast proliferation and differentiation.[53,54] *HAND1* overexpression in mice leads to elongation of the LVOT whereas LOF causes abnormal looping and embryonic lethality.[54,55] The critical role and expression pattern of *HAND1* in left-heart development makes it a candidate gene in the etiology of HLHS; however, the existence and role of *HAND1* variants in the development of HLHS is controversial. Drawing on the outflow tract defects observed in mice, 1 group sequenced *HAND1* in formalin-fixed left ventricles and identified the *HAND1* frameshift variant A126fs (A126Pfs13X), in 24/31 patients with HLHS.[56] Formalin fixation can cause sequence artifacts due to cytosine deamination, crosslinking, and DNA fragmentation, and recent studies suggest that the A126fs variant was artefactual.[50,57,58] Direct *HAND1* sequencing in fresh frozen tissue from 24 HLHS hearts identified no *HAND1* variants.[50] This was corroborated by another group that failed to find *HAND1* variants in ventricular samples from 14 patients with HLHS.[33] Furthermore, recent ES studies also fail to find evidence of *HAND1* variants in HLHS, as do current ClinVar assessments.[30,59-61] Although

current understanding does not support any specific *HAND1* variant in the etiology of HLHS, it is possible that undiscovered alterations in *HAND1* regulatory elements may yet play an undiscovered role.

AORTIC STENOSIS

Supravalvular Aortic Stenosis

ELN-Encoded Elastin

The *ELN* gene, located at 7q11.23, encodes the elastin protein, a significant component of the extracellular matrix of the aorta. Large deletions of the *ELN* region cause Williams syndrome whereas smaller deletions affecting between 30 and 100 kb of the gene are associated with isolated supravalvular aortic stenosis (SVAS).[62-67] SVAS has an AD pattern of inheritance and >200 *ELN* variants, including microdeletions, intronic splice site, nonsense, and missense variants, are estimated to explain up to 35% of isolated SVAS.[66,68-74] $Eln^{-/-}$ mice died by P4.5 and recapitulate the aortic phenotype of SVAS whereas $Eln^{+/-}$ mice only recapitulate the hypertension, arterial stiffness, and compensatory overexpression of elastic lamellae and smooth muscle.[75,76] Overall, *ELN* haploinsufficiency is a well-established cause of inherited SVAS in a significant proportion of the patient population, but single gene variants underlying SVAS in the remaining patients have not been well described.

Subvalvar Aortic Stenosis

Although subvalvar, or subaortic, stenosis is typically believed to be an acquired condition because of its progressive nature and association with LVOT hypertrophy and scarring, there are a few case reports of familial occurrence.[77-80] Although a genetic predisposition has been observed in Newfoundland dogs, no recent genetic studies have identified subvalvar aortic stenosis associated genes in humans.[81]

BICUSPID AORTIC VALVE

ROBO4-Encoded Roundabout Homolog 4

ROBO4 is expressed in the endothelial and intimal cells of the aorta and regulates vascular permeability.[82] Zebrafish homozygous for a small deletion in exon 6 demonstrate perturbed outflow tract function, whereas mice with homozygous deletion of exons 1 to 3 demonstrate incompletely penetrant AV thickening, BAV, AS, regurgitation, and ascending aortic aneurysm.[82] These animal models were generated after discovery of *ROBO4* variants through ES in patients with BAV, and the consistent phenotypes of animal models support a causative role of *ROBO4* variants in a small percentage of BAV. Initially, ES in 5 affected individuals of a family with a history of BAV and ascending aortic aneurysm identified a heterozygous splice site variant at exon 13 (c.2056+1G>T). Further, ES in a mother with atrial septal defect/AS and her son with BAV/atrial septal defect/AS identified the rare heterozygous variant R64C.[82] After identification of these initial variants in family-based studies, candidate *ROBO4* sequencing in 441 probands with BAV/ascending aortic aneurysm identified 7 with heterozygous *ROBO4* variants (A95T, T232M, H411G, R568X, R64C, V247A, Y280S, G534Efs*49, N622H, A749L, and N510V) including an independent individual with the R64C variant.[82] Several of these variants are considered likely pathogenic in ClinVar; however, larger independent ES and functional studies are needed to more clearly define the role and prevalence of *ROBO4* variants in BAV.

GATA Factors

The GATA family of zinc-finger transcription factors includes GATA4, -5, and -6; each of which are required for cardiogenesis. *GATA5* variants are well characterized through candidate sequencing studies, and unlike *Gata4* and *Gata6*, *Gata5* knockout mice are not embryonic lethal but exhibit mild left ventricle hypertrophy and 25% frequency of BAV.[83] Targeted *GATA5* sequencing in 3 independent BAV cohorts identified several heterozygous variants, including L233P, S19Y, Y143H, G166S, Y16D, T252P, and a Q3R variant that appeared in 2 studies.[84-86] Although *GATA5* variants may be better characterized in the literature, a potential role for *GATA4* variants is supported by a genome-wide association study in a cohort of 466 patients with BAV that identified 2 novel variants, including an intergenic variant (rs6601627) identified in 8.3% of BAV cases relative to 4.2% in controls, as well as a near-significant S337G variant.[87] In addition, *GATA4* sequencing in a family with AD BAV found a heterozygous E147X variant that caused loss of transcriptional activity in vitro.[88] Finally, $Gata6^{+/-}$ mice exhibit BAV in 56% of males and 27% of females, consistent with the male predilection of human BAV.[89] *GATA6* sequencing in 152 patients with BAV identified a novel heterozygous E38X variant in 1 proband with a family history of BAV, and this variant caused a loss of transcriptional activity in vitro.[90] Current evidence suggests these rare variants play some causal role in between 0.4% and 8.3% of patients with BAV, but despite aforementioned genetic screens and promising animal models, neither *GATA4* nor *GATA6* variants appear in ClinVar, and only *GATA5*-Y16D is considered pathogenic.

NOTCH1-Encoded Notch Receptor 1

The Notch signaling cascade coordinates cell migration and differentiation in the conotruncal cushions

that give rise to the aortic and pulmonary valves.[91,92] A potential role for *NOTCH1* variants in BAV was first discovered through linkage analysis of a family with AV disease that revealed association with the 9q34-35 locus.[91] Direct sequencing of *NOTCH1* in this and another CHD-affected family revealed that the R1108X and H1505del nonsense variants segregated with CHD in the respective families.[91] *NOTCH1* variants have also been identified in sporadic BAV. One study found *NOTCH1* variants predicted to be pathogenic in silico, T596M and P1797H, in 2/48 patients with sporadic BAV.[93] Given that BAV is associated with an elevated risk of thoracic aortic aneurysm (TAA), *NOTCH1* was sequenced in 48 patients with BAV/TAA and 4 nonsynonymous *NOTCH1* variants in 5 probands (R1350L, P1377S, A1343V, and P1390T) were identified in both familial and sporadic cases.[94] Two of these variants, P1390T and A1343V, were absent from healthy controls and affect highly conserved residues. Whereas genetic variants in *NOTCH1* are emerging in their association with BAV and TAA, dysregulated Notch signaling in aortic endothelial cells may be present more broadly in BAV.[92] *NOTCH1* LOF variants would directly cause this dysregulation via haploinsufficiency, but the cause of attenuated Notch signaling in patients without *NOTCH1* variants warrants further investigation. Current ClinVar assessments consider the aforementioned variants benign or conflicting with the exception of R1107X, which is considered pathogenic. Further functional and animal studies are needed to better define the role not only of variants in *NOTCH1* but of other genes along the Notch signaling pathway.

SMAD6-Encoded Mothers Against Decapentaplegic Homolog 6

SMAD6 negatively regulates bone morphogenic proteins in response to elevated transforming growth factor-β signaling.[95] In mice, knockout of the *SMAD6* analog *Madh6* causes septation defects and cardiac valve hyperplasia, a finding that has driven *SMAD6* sequencing in patients with BAV.[95] In 1 study, targeted sequencing of unrelated probands found the P415L and C484F variants in patients with BAV/AS and BAV/CoA respectively, and both show inefficient bone morphogenic protein inhibition in vitro.[96] In another, pathogenic *SMAD6* variants were observed in 2.5% of a large BAV/TAA cohort, including 2 frameshift deletions (K242NfsX300 and Gly166VfsX23), 1 in-frame deletion (G26_S27del), 2 nonsense variants (Y279X and Y288X), and 6 missense variants clustered in the MH1 and MH2 functional domains (V239M, P257L, G271W, G406C, H408Q, and R443H).[97] The MH1 domain binds DNA and the MH2 domain interacts with transforming growth factor-β and bone morphogenic protein signaling cascades both of which are critical to SMAD6 function.[97] Finally, *SMAD6* resequencing in 473 patients with TAA, 65 of whom also had BAV, identified variants in 1.5% of the overall cohort and only in patients with concomitant BAV.[98] Of note, half of the *SMAD6* variant-positive patients with BAV/TAA had a family history of cardiovascular anomalies.[98] The P415L, C484F, and P257L variants are considered pathogenic in ClinVar but have not been validated by other independent studies at the present time. Other variants are either unlisted or of uncertain significance. In vivo interrogation of these variants, particularly those affecting the MH1 and MH2 domains, is still needed to fully understand the role of *SMAD6* in the development of BAV.

NKX2-5-Encoded NK2 Homeobox 5

As with HLHS, the search for *NKX2-5* variants in BAV has been driven by associations of variants with other forms of CHD and animal models that underscore the importance of NKX2-5 signaling in heart development. In the C57Bl/6 mouse strain, 11% of *Nkx2-5*$^{+/-}$ mice have BAV relative to the 1.4% in wild-type mice; however, this elevated frequency is not recapitulated in other strains and likely represents background genetic effect.[99] *NKX2-5* sequencing of 142 BAV probands and relatives identified the K192X variant in a family with AD BAV, which in vitro caused loss of transcriptional activity.[100] A second study, though limited by cohort size, failed to identify deleterious *NKX2*-5 variants in 19 patients with BAV.[101] ClinVar does not consider any *NKX2*-5 variant associated with BAV pathogenic and overall, the gene has modest association as a monogenic cause of BAV.

Other Candidate Genes

Recently identified variants have been proposed but their causal role warrants further study. A rare *MAT2A*-E344A variant has been associated with BAV/TAA.[102] *NRF2*F candidate sequencing identified an inherited heterozygous variant in the *NRF2F* transcription factor (C96X) in a family with nonsyndromic-BAV that caused complete loss of transcriptional activity in vitro.[103] Other variants identified in at least 2 patients through targeted sequencing include those in *AXIN1/2* (R841Q; A684V), *MCTP2* (T545M; L847F), *NFATC1* (P77L; V210M), and *TBX5* (S372L; V263M), each of which are conflicting, uncertain, or unlisted in ClinVar.[104] Further study of the functional consequences and heritability of each are needed to validate their role in the etiology of BAV.

INTERRUPTED AORTIC ARCH

TBX1-Encoded T-Box Transcription Factor 1

IAA is a rare form of CHD and can be divided into subtypes A, B, and C based on the location of interruption.

IAA type B interrupts the aorta between the left carotid and left subclavian arteries and is both the most common and genetically homogenous form of IAA and links to the 22q11 locus.[105] 22q11 deletion is most commonly associated with DiGeorge syndrome and is rare in isolated CHD; only 1 in a cohort of 628 patients with nonsyndromic conotruncal defects had 22q11 deletion.[106] No single gene is a definitive cause of the cardiovascular abnormalities associated with DiGeorge syndrome, though *TBX1* has been proposed as a likely candidate, as *TBX1* variants alone can recapitulate the cardiovascular and craniofacial defects, and Tbx1$^{+/-}$ mice develop abnormal aortic arch phenotypes.[107-111] Truly pathogenic *TBX1* variants in isolated IAA are uncommon; 1 study sequenced *TBX1* in 105 patients identified a 466-476dup10 duplication in a proband with IAA.[109] Further, another group failed to identify *TBX1* variants in 41 patients with conotruncal defects, and ClinVar does not consider *TBX1* variants pathogenic in IAA.[108]

CRKL-Encoded Crk-Like Protein

Like *TBX1*, *CRKL* is a candidate genetic susceptibility locus for the cardiac defects observed in children with DiGeorge syndrome. *CRKL* resides within the typical 3 Mb deletion that is characteristic of DiGeorge syndrome, and knockout in mice is embryonic lethal with severe outflow tract defects.[112,113] Interestingly, though *Crkl*$^{+/-}$ mice are typically normal, compound heterozygous *Crkl*$^{+/-}$*;Tbx1*$^{+/-}$ mice develop cardiac defects including IAA and other outflow tract defects, suggesting dose dependent interaction between *TBX1* and *CRKL* may underlie CHD in a subset of patients.[112] To date, there are no studies identifying *CRKL* genetic variants in patients with nonsyndromic LSOLs. Further investigation is needed to assess if *CRKL* is involved in cardiac defects apart from DiGeorge syndrome.

Other Identified Genes

Mouse studies have also identified genes in which LOF causes IAA and aortic arch defects. For example, *Foxc2* LOF causes the IAA phenotype in mice, *Pitx2* LOF causes a spectrum of conotruncal defects, and *Tgfβ1* LOF causes fourth pharyngeal arch artery hypoplasia.[111,114,115] Although promising animal models exist, the difficulty of assembling a large nonsyndromic cohort remains a barrier to defining the genetics of IAA.

COARCTATION OF THE AORTA

MYH6-Encoded Myosin 6

Genome-wide association study of 120 Icelanders with CoA, both with and without other CHD, was paired with GS data from 15 220 Icelanders, 39 of whom had CoA.[116] The genome-wide association study implicated the *MYH6*-encoding 14q11 locus, and GS identified the R721Y variant in 20% of the 39 chip-typed individuals with CoA.[116] This variant is rare outside of the Icelandic population and is not present in 6503 exomes from the NHBLI Exome Sequencing Project nor in ClinVar and appears only once in the 126 216 exomes and 15 136 genomes in the Genome Aggregation Database.[116] This variant also associates with BAV and other forms of CHD, but further functional studies and animal models, as well as further interrogation outside of the founder population of Iceland, are needed to establish the pathogenicity of this variant.[116]

HEY2-Encoded Hairy/Enhancer-of-Split Related With YRPW Motif Protein 2 and NOTCH Signaling

The zebrafish gridlock variant in *hey2* causes CoA and treatment with vascular endothelial growth factor pathway activators during angioblast cell fate determination and migration rescue the CoA phenotype.[117] *Hey2*$^{-/-}$ mice do not have CoA but do develop lethal postnatal cardiac hypertrophy; however, human *HEY2* variants have not been associated with CoA.[118] *HEY2* is a NOTCH1 target gene, and a large candidate-gene study of *NOTCH1* variants in families with CHD identified the E1262-G1301del and Y1843X variants in 2 probands with CoA and other cardiac defects.[119] The Y1843X variant was identified in 2 family members with AS and aortic valve insufficiency, whereas the E1262-G1301del was found in both affected and unaffected family members.[119] Neither variant is listed in ClinVar and an association of *NOTCH1* variants with CoA cannot be made because of the presence of other concomitant cardiac defects in variant positive individuals.

Other Candidate Loci

CNV analyses have identified a handful of CoA associated loci, including in *TRPM2*, FOXC1 binding sites, and along the X chromosome.[120,121] Beyond CNVs, a translocation in *MATR3* was identified in a proband with CoA and Noonan-like syndrome, but heterozygous *Matr3* ablation in mice causes diverse and incompletely penetrant cardiac defects.[122] *MCTP2* knockdown in *Xenopus* embryos causes outflow tract defects, and *MCTP2* duplications, deletions, and missense variants (A60T, G203D, and Y235C) have been found in patients with CoA both with and without other cardiac defects.[123] Finally, 1 study associated *TBL1Y* missense variants (D69H and R176W) with CoA, but cardiac defects associated with *TBL1Y* LOF have not been identified in animal models and have not been assessed in ClinVar.[120] As a whole, genetic studies

identifying single gene variants directly associated with CoA are not currently present.

EXOME AND GENOME-BASED STUDIES

Many inherited NS-CHD-associated genes were first identified through linkage analysis and targeted sequencing of large CHD-affected families. Initial findings led to candidate gene sequencing in larger CHD cohorts that typically return a handful of variants; however, it is rare for any gene to account for a significant proportion of affected individuals (Table 1). This low yield of known genetic variants is compounded by variable expression of disease. Many variants are shared among different LSOLs as well as other forms of CHD including tetralogy of Fallot and other right-sided or conotruncal defects. In one example, a paternally inherited *NOTCH1* variant was found in a patient with HLHS whose paternal aunt had a hypoplastic right ventricle.[33] Although the aunt was not available for sequencing to confirm presence of the putative variant, this report illustrates the variable expressivity that underlies the genetics of CHD.[33] The incidence of both right- and left-sided CHD in the same family also suggests these defects may also share common genetic and/or development etiology. In another example, the frequent association of BAV with CoA and AS invites the possibility that these LSOLs exist on a spectrum of disease severity.

It is likely that this variable expressivity is due to a myriad of factors, both intrinsic and extrinsic to the developing heart. In HLHS, for example, it is still unclear whether ventricular hypoplasia arises as the result of intrinsic defect in the left ventricular myocardium, whether it is secondary to hemodynamic changes caused by valvular defects or if both mechanisms are at play.[141] To this end, it is necessary to understand not only what genetic variants are associated with a particular lesion but also the cell population and tissue location that is perturbed developmentally. In addition, the developmental stage at the time of the extrinsic/environment stressor exposure also plays a key role in modifying the phenotypic expression of pathologic genetic variation.[141] For example, retinoic acid is involved in first and second heart-field development, myocardial proliferation, and coronary artery angiogenesis. Vitamin A deficiency, or toxicity, at any stage of cardiac development will lead to altered gene expression with downstream developmental consequences.[142]

Taken together, epigenetic and environmental influences play a role in CHD development and may further modify a pathologic genetic substrate. This complex interplay has been elegantly reviewed elsewhere.[143,144] Briefly, hypermethylation of cardiac transcription factors like *NKX2-5* and *HAND1* have been associated with tetralogy of Fallot, and monozygotic twins discordant for CHD have different DNA methylation at transcription factor binding sites.[145,146] Expression-altering histone modifications and miRNA dysregulation are also implicated in several defects.[143,147] These epigenetic changes may mediate the effects of environmental risk factors for CHD, including vitamin A exposure, thalidomide, maternal diabetes mellitus, and rubella infection, among others.[144,148] The precise mechanisms for these effects are poorly understood and represent the complex interaction between genetics, epigenetics, and the environment. Here, we discuss how an unbiased, systematic study of the heritable component of CHD through ES, GS, and transcriptomics is critical in defining the complex genetic mechanisms underlying CHD.

Overall Yield of NS-CHD Genes by ES Is Low

A recent multi-institutional study of the exomes of 2871 individuals with CHD, including 2645 trios, found significant association with several of the genes mentioned in this review, namely *NOTCH1*, *SMAD6*, *ELN*, and *GATA6*. In this large cohort, recessive genotypes contributed to 0.9% of CHD cases, de novo variants (DNVs) alone contributed to 3.1% of isolated CHD and inherited and DNVs together contributed to 10.1% of CHD.[149] Notably, this study included probands with neurodevelopmental defects and extracardiac abnormalities, and it is likely that cohorts stratified based on defect type and the presence of extracardiac syndromes would reduce the heterogeneity of returned candidate genes. In the case of laterality defects, ES of 323 unrelated probands identified 28 candidate variants in known heterotaxy related genes, nearly all of which were inherited from unaffected parents, potentially the result of parental mosaicism.[150] Gene-based aggregation analyses significantly associated *PXDNL* and *BMS1*, and in total, monogenic candidate variants were identified in 7.1% of the heterotaxy cohort.[150] In nonsyndromic-LSOLs specifically, a series of large ES studies have made strides in identifying putative pathogenic variants and many genes are shared between cohorts (*DNAH5*, *ACVR1*, *KMT2D*, *NOTCH1*, *POGZ*, *ROCK2*, *JARID2*).[30,59-61] Even in these large studies, the yield of variant-positive ES is low, ranging between 7.8% and 23.5%.[59-61]

Common and Noncoding Genetic Variation

There is evidence that common variants in genes expressed in cardiac development such as in *ERBB4*, *BMP4*, and *ISL1* may confer risk for LSOLs and might

Table 1. Genes Implicated in Nonsyndromic Left-Sided Obstructive Congenital Heart Lesions

Gene	Chr.	Protein	Mode of Inheritance	Genetic Yield	Online Mendelian Inheritance in Man	Reference
HLHS						
GJA1	6q22.31	Gap junction alpha-1 protein	AR	Rare	121014	41,44,124
HAND1	5q33.2	Heart and neural crest derivatives-expressed protein 1		Rare	241550	56,125-127
MCTP2	15q26.2	Multiple C2 and transmembrane domain-containing protein 2		Rare	616297	128
MYH6	14q11.2	Myosin-6	AR, AD, de novo, CH	≈11%	160710	25,38
NKX2-5	5q35.1	Homeobox protein Nkx-2.5	AD	Rare	600584	47,51,129
NOTCH1	9q34.3	Neurogenic locus notch homolog protein 1	AD	≈6% to 22%	190198	24,33,130,131
ZIC3	Xq26.3	Zinc finger protein ZIC3	XR	Unknown	300265	132
Aortic stenosis						
ELN	7q11.23	Elastin	AD	≈11% to 35%	185500	63,64,133
NKX2-5	5q35.1	Homeobox protein Nkx-2.5		Unknown	600584	47
Bicuspid aortic valve						
GATA4	8p23.1	Transcription factor GATA-4	AD	≈0.4% to 8%	600576	87,88
GATA5	20q13.33	Transcription factor GATA-5	AD	≈3% to 4%	611496	83-86
GATA6	18q11.2	Transcription factor GATA-6	AD	Rare	600001	90,134
NKX2.5	5q35.1	Homeobox protein Nkx-2.5	AD	Rare	600584	100
NOTCH1	9q34.3	Neurogenic locus notch homolog protein 1	AD	≈4% to 10%	190198	24,93,135
ROBO4	11q24.2	Roundabout homolog 4	AD	≈2%	607528	82
SMAD6	15q22.31	Mothers against decapentaplegic homolog 6		≈2% to 3%	602931	97,98
TAB2	6q25.1	TAK1-binding protein 2	AD	Rare	605101	136,137
Interrupted aortic arch						
CFC1	2q21.1	Cryptic protein		Unknown	605194	138
LEFTY2	1q42.12	Left-right determination factor 2		Unknown	601877	139
NKX2.5	5q35.1	Homeobox protein Nkx-2.5	AD	Unknown	600584	51
TBX1	22q11.21	T-box transcription factor TBX-1		Rare	602054	107,109-111
Coarctation of the aorta						
FOXC1	6p25/3	Forkhead box protein C1		Rare*	601090	121
GATA5	20q13.33	Transcription factor GATA-5		Unknown	611496	83
HEY2	6q22.31	Hairy/enhancer-of-split related with YRPW motif protein 2		Unknown	604674	117,118
MATR3	5q31.2	Matrin-3		Unknown	164015	122
MCTP2	15q26.2	Multiple C2 and transmembrane domain-containing protein 2	AD	Rare*	616297	128
MYH6	14q11.2	Myosin-6	AD, de novo	≈0% to 20%	160710	116
NFATC1	18q23	Nuclear factor of activated T-cells, cytoplasmic 1		Unknown	600489	104
NKX2.5	5q35.1	Homeobox protein Nkx-2.5	AD	Unknown	600584	47
NOTCH1	9q34.3	Neurogenic locus notch homolog protein 1	AD	Rare*	190198	119

(*Continued*)

Table 1. Continued

Gene	Chr.	Protein	Mode of Inheritance	Genetic Yield	Online Mendelian Inheritance in Man	Reference
SMAD6	15q22.31	Mothers against decapentaplegic homolog 6		Unknown	602931	98
TBL1Y	Yp11.2	F-box-like/WD repeat-containing protein TBL1Y		Rare*	400033	140
TRPM2	21q22.3	Transient receptor potential cation channel subfamily M member 2		Rare*	603749	120

Asterisk indicates a higher percentage was reported in a small study, but robust, independent evaluation is required for an association to be established. Unknown indicates the variant was associated with concomitant defects and a disease-specific yield could not be established. AD indicates autosomal dominant; AR, autosomal recessive; CH, compound heterozygous; and XR, X-linked recessive.

be otherwise missed in conventional searches for rare nonsynonymous variants.[151-153] This points toward the complex genetic architecture underlying CHD and a need to better understand how common, rare, inherited, and de novo variants interact to produce a CHD phenotype. ES analyses interrogating the differences between recessive and de novo CHD identified an enrichment of cilia-related gene variants in recessive CHD but an enrichment in chromatin-modifying genes in de novo CHD genotypes.[154] Similar findings were observed in an independent ES study that identified an excess of DNVs in genes involved in H3K4 and H3K27 methylation and H2BK120 ubiquitination.[155] These chromatin-modifying variants are also observed in patients with extracardiac and neurodevelopmental delays, suggesting these DNVs may play a greater role in syndromic CHD, a finding supported by previous ES analyses suggesting a higher burden of DNVs in S-CHD relative to NS-CHD.[30,155]

Genome Studies are Needed to Close the Gap in Our Understanding of NS-CHD Heritability

Although informative in identifying coding variants, ES alone fails to capture the full genetic landscape of inherited CHD, which is reflected in its overall yield of around 10%. GS has been a powerful tool in the accurate diagnosis of pediatric disease, particularly in critically ill neonates. A 2018 study performed rapid trio-based GS in critically ill neonates admitted to the neonatal intensive care unit had a diagnostic yield of 45% and was actionable within 2 to 5 days of sample collection.[156] Moreover, the Undiagnosed Diseases Network regularly leverages the power of ES and GS to identify a genetic cause of disease in over 40% of cases and identifies intronic and CNVs otherwise undetected by ES.[157,158] Given that LSOLs are associated with a spectrum of morbidity and mortality that ranges from HLHS, which is uniformly fatal if not corrected within the first few days of life, to BAV, which can remain undetected until adulthood when AS or aortic aneurysm develop and reveal the underlying congenital defect, GS could serve to significantly improve long-term quality of life through early intervention. GS will soon be widely used for all newborns, sick and ostensibly healthy. If the genetics underlying LSOL, or CHD in general, can be fully delineated, it opens the door for identifying at-risk infants by genetic testing.

Future Directions: Overcoming the Challenges of Genome Sequencing in LSOL

Implementation of GS is not without practical challenges, the most important of which is the need to distinguish truly pathogenic variants from background genetic noise. This is most challenging in noncoding areas of the genome that are not amenable to classic transgenic tools. Allele-specific expression analysis may provide a methodology for identifying candidate noncoding genetic variation through a combination of RNAseq and GS to identify variants predicted to cause changes in expression, including those in noncoding regions. This technique has identified pathogenic noncoding variation in several complex genetic diseases and similar methodologies have been applied to CHD.[159-161] One study linked RNAseq and ES in a cohort of 144 patients with surgically repaired CHD and observed significant expression differences pointing toward new candidate genes.[162] More recent studies have extended this strategy to combine GS and transcriptome sequencing in a cohort of 13 sudden cardiac death and sudden unexplained death in infancy victims with previously negative exome studies.[163] Of the 23 candidate variants identified in cardiac gene regulatory regions, the most significant was a *NEXN*-promoter variant associated with decreased NEXN expression and cardiac hypertrophy.[163] Finally, the largest GS in the CHD population sequenced 763 CHD trios with previously variant-negative ES

studies.[164] Transcriptomic profiling through effect neural network analysis and ATAC-seq identified an enrichment in damaging DNVs in noncoding cardiac regulatory regions in CHD trios relative to controls.[164] Overall, the group estimated that noncoding DNVs contribute to between 17% and 45% of CHD cases, underscoring the need to fully appreciate the role of the noncoding genome in the etiology of CHD.[164] Further stratification based on disease phenotype is needed to understand the precise underpinnings of each defect.

CONCLUSIONS

Inheritance studies suggest that LSOLs have a significant heritable component, and though progress has been made in identifying genes and variants associated with LSOLs, each fails to explain more than a small fraction of the patient population. A change in the approach to ascertaining the genetic underpinnings of CHD is needed. GS, paired with transcriptomics and allele-specific expression analysis, has the potential to detect variation in regulatory regions that cause haploinsufficiency. Streamlining this comprehensive, unbiased approach will pave the way for the development of better diagnostic algorithms and unlock potential therapeutic avenues.

RESOURCES

A number of genetics data repositories and resources are available to interested clinicians and researchers to aid in the study of LSOL genetics or interpretation of genetic variant pathogenicity (Table 2).[165-170] The Genome Aggregation Database provides information on sequence variation in the general population, whereas Online Mendelian Inheritance in Man serves as an encyclopedia of genetic disease in humans.[165,166] ClinVar relies on user submission of variants and phenotypes and interprets pathogenicity accordingly.[167]

Table 2. Resources for Studying Congenital Heart Disease Genetics

Resource	URL	Reference
ClinGen	https://clinicalgenome.org/	168
ClinVar	https://www.ncbi.nlm.nih.gov/clinvar/	167
Genome Aggregation Database	https://gnomad.broadinstitute.org/	165
Human Gene Mutation Database	http://www.hgmd.cf.ac.uk/ac/index.php	169
Online Mendelian Inheritance in Man	https://www.omim.org/about	166
Pediatric Cardiac Genomics Consortium	https://benchtobassinet.com/	170

Similarly, ClinGen is a data sharing portal to speed identification of clinically relevant variants.[168] The Human Gene Mutation Database accomplishes a similar aim by curating a list of published pathogenic genetic lesions.[169] The Pediatric Cardiac Genomics Consortium aims to identify and characterize CHD causing variants and makes limited data available to researchers on an application basis.[170] Finally, the most recent scientific statement from the American Heart Association on the genetic basis for congenital heart disease provides a comprehensive overview of genetics underlying syndromic and nonsyndromic heart lesions.[9] Given that many of these resources rely on user submission of data, there are inevitable information gaps. Further, our understanding of the pathogenicity of noncoding variation is lacking, and the recent advances in sequencing and analysis technology will help bridge this gap.

ARTICLE INFORMATION

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Affiliations

From the Division of Cardiology, Department of Pediatrics (L.E.P., A.P.L.) and Department of Cell Biology, Duke University School of Medicine, Durham, NC (A.P.L.).

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