Cardiovascular malformations caused by *NOTCH1* mutations do not keep left: data on 428 probands with left-sided congenital heart disease and their families

Wilhelmina S. Kerstjens-Frederikse*, Ingrid M.B.H. van de Laar*, Yvonne J. Vos, Judith M.A. Verhagen, Rolf M.F. Berger, Klaske D. Lichtenbelt, Jolien S. Klein Wassink-Ruiter, Paul A. van der Zwaag, Gideon J. du Marchie Sarvaas, Klasien A. Bergman, Catia M. Bilardo, Jolien W. Roos-Hesselink, Johan H.P. Janssen, Ingrid M.E. Frohn-Mulder, Karin Y. van Spaendonck-Zwarts, Joost P. van Melle, Robert M.W. Hofstra*, Marja W. Wessels*

**These authors contributed equally*

Genetics in Medicine 2016;18(9):914-23

Abstract

Purpose

We aimed to determine the prevalence and phenotypic spectrum of *NOTCH1* mutations in left-sided congenital heart disease (LS-CHD). LS-CHD includes aortic valve stenosis, bicuspid aortic valve, coarctation of the aorta and hypoplastic left heart syndrome.

Methods

NOTCH1 was screened for mutations in 428 non-syndromic LS-CHD probands and family histories were obtained in all. When a mutation was detected, relatives were also tested.

Results

In 148/428 patients (35%) LS-CHD was familial. Fourteen mutations (5 RNA splicing mutations, 8 truncating mutations, one whole gene deletion) were detected (3%), 11 in familial disease (11/148 (7%)) and three in sporadic disease (3/280 (1%)). Fourty-nine additional mutation carriers were identified in the 14 families, 12 were asymptomatic (12/49 (25%)). Most had LS-CHD, but 9 (also) had right-sided congenital heart disease (RS-CHD) or conotruncal heart disease (CTD) (9/49 (18%)). Thoracic aortic aneurysms (TAA) occurred in 6 mutation carriers (probands included 6/63, (10%)).

Conclusion

Pathogenic mutations in *NOTCH1* occur in 7% of familial LS-CHD and in 1% of sporadic LS-CHD. The penetrance is high, a cardiovascular malformation was found in 75% of *NOTCH1* mutation carriers. The phenotypic spectrum includes LS-CHD, RS-CHD, CTD and TAA. Testing *NOTCH1* for an early diagnosis in LS-CHD/RS-CHD/CTD/TAA is warranted.

Introduction

Left-sided congenital heart disease (LS-CHD), represents a group of highly heritable congenital heart defects, including bicuspid aortic valve (BAV), aortic valve stenosis (AVS), coarctation of the aorta (COA) and hypoplastic left heart syndrome (HLHS). LS-CHD includes the previously used term left ventricular outflow tract obstruction (LVOTO) [1-5]. BAV may be asymptomatic and undetected in infancy, but has a risk for serious complications and sudden cardiac death later in life [6, 7]. HLHS is a severe disease, which needs extensive surgical corrections and which may lead to death at a young age [8, 9]. LS-CHD often presents as a non-syndromic condition, but is also reported in more than 200 different syndromes and in many of these the associated gene or chromosomal region is known, for example in Jacobsen syndrome (11q23 deletion), Turner syndrome (monosomy X), Kabuki make-up syndrome (*MLL2, KDM6A*) and Rieger syndrome (*PITX2, FOXC1*) [10-13]. In non-syndromic LS-CHD only a few genes are known to be associated with the disease (*NOTCH1*, *GJA1, NKX2-5, GATA5, SMAD6, MYH6*) and the number of patients with mutations in these genes is low [14-21]. *NOTCH1* is the only gene reported with truncating mutations segregating with LS-CHD, most often BAV and/or early onset calcific AVS.

The *NOTCH1* gene codes for the transmembrane receptor protein NOTCH1, which is part of the NOTCH signaling pathway. NOTCH signaling is evolutionary conserved and plays an important role in embryonic development, by influencing cell fate decisions [22]. NOTCH signaling mediates short-range intercellular communication: the transmembrane receptors NOTCH1-4 interact with ligands (Delta like (DLL) 1, 3, 4, Jagged (JAG) 1, 2) from neighboring cells. After ligand binding, the receptor is cleaved and an intracellular domain enters the nucleus, where it interacts with DNA-binding proteins [23]. Downstream targets of NOTCH1 are amongst others Hes (hairy-enhancer-of split) and Hrt (Hes related) families of genes [24, 25].

Activation of NOTCH1 represses differentiation of embryonic stem cells into cardiomyocytes and stimulates endocardial epithelial-to mesenchymal-transition (EMT), which is important in the process of cardiac valve formation [26, 27]. In addition, *NOTCH1* haploinsufficiency leads to calcification of the aortic valve by dysregulating downstream transcription of genes involved in osteogenesis, inflammation, and oxidative stress [28, 29]. Germline truncating mutations in *NOTCH1* were first reported segregating in two families with mainly aortic valve disease and two patients with conotruncal heart defects (CTDs) (Fallot's tetralogy and double outlet right ventricle) [14]. One of these mutations was also reported in a patient with a stenotic tricuspid aortic valve [30]. In 5 series of LS-CHD patients screened for *NOTCH1* mutations only two new truncating mutations and one RNA splicing mutation and several possibly pathogenic missense variants were reported [15, 31-34]. As these series are small and the number of mutations reported limited, the role of *NOTCH1* in LS-CHD is still unclear.

In this study the phenotypic spectrum and pedigrees of patients with clearly pathogenic *NOTCH1* mutations are presented, as well as data on non-synonymous variants. This information will help clinicians to make decisions upon DNA-testing and in counseling patients on risk profiles for their relatives and their offspring.

Methods

Patients

Patients with LS-CHD referred for genetic counseling to one of three participating university hospitals in the Netherlands between 1 January 2006 and 1 January 2014 were included in the study. This is a selected population, which can be expected to have a higher prevalence of familial disease than described earlier in LS-CHD (20-35%, depending on the definition of familial disease) [4]. Intrauterine deaths and terminations of pregnancy were also included. All patients had a detailed cardiac evaluation by a (pediatric) cardiologist, including ECG and cardiac echo/Doppler imaging. MRI was performed if the aortic arch could not be visualized by echo. Cardiac diagnoses included were BAV, AVS, aortic valve insufficiency (AVI), COA (with or without BAV), HLHS or other left sided cardiac diseases, including subvalvular or supravalvular aortic stenosis, hypoplastic aortic arch, interrupted aortic arch (type B) and mitral valve anomalies. In patients with combined lesions the primary diagnosis was defined as the most relevant anomaly, so if a BAV and a COA were present, the diagnosis was COA. All normally functioning, stenotic or insufficient bicuspid aortic valves were labelled BAV. HLHS was defined as underdevelopment of the left ventricle and ascending aorta together with anomalies of the mitral and/or aortic valve. A complete physical examination was performed and a detailed family history was taken by a clinical geneticist. Patients with major extracardiac malformations or known syndromes were excluded. Familial LS-CHD was defined as LS-CHD in the proband and LS-CHD or any CHD in a first degree relative, or LS-CHD in the proband and LS-CHD or right-sided CHD (RS-CHD)/CTD in a second or third degree relative. The RS-CHD/CTD group included pulmonary valve stenosis, pulmonary atresia, Fallot's tetralogy, and truncus arteriosus. Cardiac echos were offered to first degree relatives as described previously [4].

Sequence analysis of the *NOTCH1* gene

Mutation analysis of the coding exons and flanking intronic sequences of the *NOTCH1* gene (NM_0176173) was carried out using flanking intronic primers (primer sequences available upon request). PCR was performed in a total volume of 15 µl containing 10 µl AmpliTag Gold ®Fast PCR Master Mix (Applied Biosystems), 1.5 pmol/µl of each primer (Eurogentec, Serian, Belgium) and 2 µl (40 ng/µl) genomic DNA. To the PCR mix of exon 1 10% DMSO was added. The samples were PCR amplified on a Perkin-Elmer (ABI) Geneamp 9700 (see Detailed Methods,

Supplemental Information). The PCR products were purified with ExoSAP-IT (Amersham Pharmacia Biotech, Piscataway, NJ) and subjected to direct sequencing on a PRISM 3730XL DNA analyzer (Applied Biosystems, Bleiswijk, the Netherlands) using the specific primers.

Array-comparative genomic hybridization analysis

To detect deletions, array-comparative genomic hybridization (aCGH) analysis was performed using the 180K oligo array from Agilent (custom design ID: 23363; Agilent Technologies Inc., Santa Clara, CA, USA). A mix of 40 healthy male or 40 female DNA samples was used as a reference (sex-matched). Procedures were performed according to the manufacturer's protocol. Data were extracted using Feature Extraction software version 9.1. This analysis was not included in the genetic work-up by all clinical geneticists. Data were available in 180 patients and these were analysed for deletions in the chromosome 9q34 region, which contains the *NOTCH1* gene.

Classification of mutations

Mutations that cause premature truncation (nonsense and frameshift mutations) or a complete deletion of the protein were classified pathogenic. Moreover, mutations within 2 base pairs upstream or downstream from the exon were considered to affect RNA splicing and therefore also assigned as pathogenic. The Alamut (version 2.3) missense and splicing prediction modules from Interactive Biosoftware were used to predict pathogenicity. The missense prediction module includes Align GVGD, SIFT, PolyPhen-2 and MutationTaster and the splicing module includes SpliceSiteFinder, MaxEntScan, NNSplice, GeneSplicer and Human Splicing Finder. Variants with a minor allele frequency (MAF) > 0.01 were considered to be polymorphisms and were not registered. Allele frequency data were derived from NHLBI exome sequencing project on European Americans (http://evs.gs.washington.edu/EVS/) and the Exome Aggregation Consortium on non-Finnish Europeans (http://exac.broadinstitute.org).

cDNA analysis of potential RNA splicing mutations

All missense variants, synonymous DNA variants and intronic variants further than 2 base pairs upstream or downstream from the exon were analyzed *in silico* for their effect on RNA splicing. Two variants, c.3787 C>T, p.(Arg1263Cys) and c.1670-7G>A were predicted to introduce a new splice donor site and were therefore tested. Total RNA was extracted from peripheral blood, using the RNABee procedure (Cinna Biotecx, Friendswood, TX) and complementary DNA (cDNA) was obtained using the GoScript Reverse Transcription System (Promega, Leiden, the Netherlands). Primers amplifying a product from the exon 21-exon 22 transition site to exon 24 (f) TGCAAGTGCGTGGCCGGCTACCA and (r) CCATTCTTGCAGGGCTTGCCTTT were used to characterize the cDNA sequence around the c.3787C>T mutation in exon 23. Primers amplifying a product from the exon 9-exon 10 transition site to exon 12, GGGCTTCACTGGGCATCTG (forward) and GGCACACTCGTAGCCATCG (reverse) were used to

characterize the cDNA sequence around the mutation in intron 10, c.1670-7G>A. The PCR products were loaded on to a 3% agarose gel, purified with ExoSAP-IT, and subjected to direct sequencing to confirm the presence of an aberrant transcript.

Statistical analysis

We used SPSS for Windows (version 20; Chicago, IL) for the statistical analyses. A *p*-value <0.05 was considered statistically significant. Interquartile range was used to show statistical dispersion.

Results

Patients

A cohort of 428 probands (286 male, 142 female) with non-syndromic LS-CHD, including 31 intrauterine deaths/terminated pregnancies, was included. The median age (terminated pregnancies excluded) was 10 years (interquartile range, 2-30 years). In 148 of 428 probands (35%) the heart defect was familial. Cardiac phenotypes are described in **Table 1**.

Table 1. Diagnoses and family history in 428 probands

Diagnosis	Male	Female	Familial	Total
BAV/AVS/AVI	153	52	94	205
COA (±BAV)	80	55	34	135
HLHS	49	26	15	75
Other[a]	4	9	5	13
Total	**286 (67%)**	**142 (33%)**	**148 (35%)**	**428 (100)**

Data are *n* or *n* (%). AVI, aortic valve insufficiency; AVS, aortic valve stenosis; BAV, bicuspid aortic valve; COA, coarctation of the aorta; HLHS, hypoplastic left heart syndrome. [a] Other left-sided heart defects: interrupted aortic arch (n=3), subvalvular aortic stenosis (n=3), supravalvular aortic stenosis (n=1), hypoplastic aortic arch without HLHS (n=4), congenital mitral valve insufficiency (n=1), and congenital mitral valve stenosis (n=1).

Truncating and RNA splicing mutations and whole gene deletions

In total, we detected 14 novel clearly pathogenic mutations in 428 probands (3%): 8 truncating (nonsense or frameshift) mutations, 5 RNA splicing mutations, and 1 whole gene deletion. (**Figure 1** and **Table 2**) Two variants (c.3787C>T, p.Arg1263Cys and c.1670-7G>A) were predicted to introduce a new splice donor site and were therefore tested by cDNA analysis. c.3787 C>T, p.Arg1263Cys: cDNA sequencing confirmed that a new splice donor site was introduced in exon 23, causing a deletion of 120 bp at the 3'site of the exon, resulting in p.Glu1262_Gly1301del. c.1670-7G>A: cDNA sequencing confirmed that a new splice acceptor site was introduced five nucleotides downstream from the original splice acceptor site (r.1670-5_1670-1ins) causing a frameshift p.Gly557fs.The 14 clearly pathogenic mutations were found in probands with BAV (n=7, families A, D, G I; J, M and N as shown in **Table 3**), AVS

(n=3, families B, C and F), COA with BAV (n=2, families E and H), HLHS (n=2, families K, and L). Eleven mutations were detected in 148 probands with familial LS-CHD (7%); six were truncating and five were RNA splicing mutations. Three mutations were detected in 280 non-familial cases (1%) (families I, K and L): two were truncating mutations (one *de novo* in a patient with HLHS and one inherited from a non-affected parent in a patient with BAV) and one was a *de novo* whole-gene deletion, in a patient with HLHS (detected by aCGH).

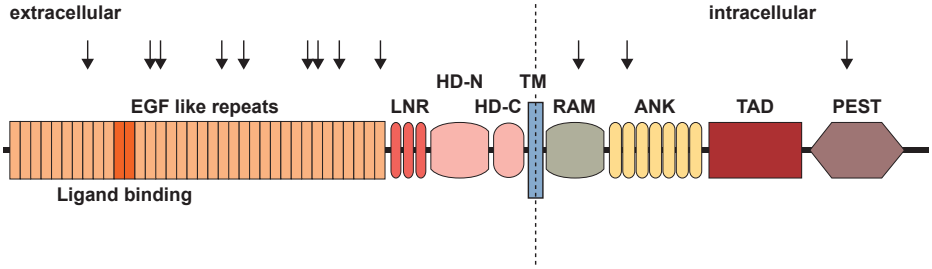

Figure 1. NOTCH1 protein, position of truncating and RNA splicing mutations. ↓Truncating and RNA-splicing mutations. ANK, ankyrin repeats; EGF, epidermal growth factor; HD, heterodimerization domain; LNR, Lin/Notch repeats; PEST, PEST domain; RAM, RBPjk-association module; TAD, transcriptional activation domain; TM, transmembrane domain.

Table 2. Pathogenic mutations in *NOTCH1*

cDNA change	Protein change	Domain	Effect
del 9q34.3 (137.4-140.2)		Whole gene	Deletion
c.865+2C>A	p.?	EGF-like 8 Ca-binding	RNA splicing
c.1650C>G	p.(Tyr550*)	EGF-like 14	Truncating
c.1670-7G>A	p.Gly557fs	HD-domain	RNA splicing
c.1904-2A>G	p.?	EGF-like15	RNA splicing
c.2425delG	p.(Asp809Thrfs*67)	EGF-like 21 Ca-binding	Truncating
c.2643delC	p.(Ala882Hisfs*297)	EGF-like 23 Ca-binding	Truncating
c.3054C>A	p.(Cys1018*)	EGF-like 29	Truncating
c.3511-2A>G	p.?	EGF-like 30	RNA splicing
c.3787C>T	p.Glu1262_Gly1301del	EGF-like 32 Ca-binding	RNA splicing
c.4240delT	p.(Cys1414Alafs*31)	EGF-like 36	Truncating
c.5529G>A	p.(Trp1843*)	RAM	Truncating
c.5950C>T	p.(Arg1984*)	ANK	Truncating
c.7455dupC	p.(Ser2486Leufs*21)	PEST	Truncating

Phenotypes in families with clearly pathogenic mutations

Relatives of the 14 probands who had pathogenic mutations were subsequently tested; in these families we identified 49 new mutation carriers, 8 of whom were obligate carriers. Thirteen other relatives had a congenital heart defect, but DNA was not available for testing.

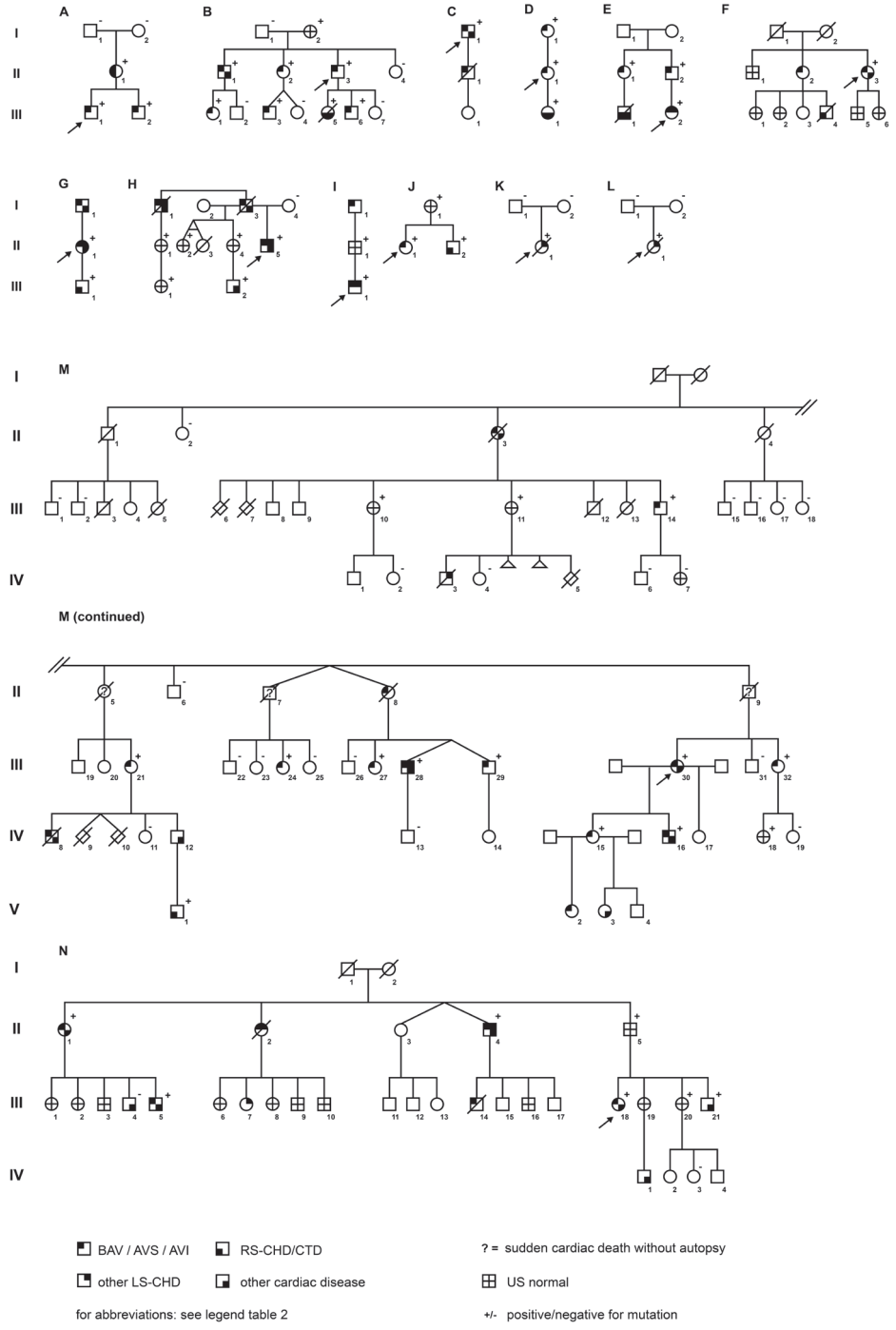

Figure 2. Pedigrees of patients from fourteen families with truncating and RNA splicing mutations in *NOTCH1*. The mutations in families K and L were *de novo*. See Table 3 for description of the phenotypes.

Table 3. Cardiac phenotypes of families with clearly pathogenic *NOTCH1* mutations (truncating mutations, splice-site mutations, whole-gene deletions)

Family	No. in pedigree[a]	Age (years)[b]	Nucleotide change	Protein change	LS-CHD	RS-CHD/ CTD	Other
A	II.1	44	c.3511-2A>G	p.?	AVS, AVR 38 years	PVS	-
	III.1	15	c.3511-2A>G	p.?	BAV, mild AVS	-	-
	III.2	14	c.3511-2A>G	p.?	BAV	-	-
B	I.2	54	c.865+2C>A	p.?	-	-	-
	II.1	39	c.865+2C>A	p.?	BAV	-	TAA 43mm
	II.2	30	c.865+2C>A	p.?	BAV, AVS, AVR 33 years	-	-
	II.3	26	c.865+2C>A	p.?	AVS, asymmTAV	-	-
	III.1	16	c.865+2C>A	p.?	BAV	-	-
	III.3	4	c.865+2C>A	p.?	BAV	-	-
	III.5	0.5[c]	c.865+2C>A	p.?	-	PA, rDA	VSD
	III.6	4	c.865+2C>A	p.?	asymmTAV	-	-
C	I.1	69	c.5950C>T	p.(Arg1984*)	AVS, AVR 60 years	-	TAA 48 mm
	II.1	33[c]	Not tested	-	BAV, AVS	-	SCD
D	I.1	68	c.2643delC	p.(Ala882Hisfs*297)	AVS	-	-
	II.1	37	c.2643delC	p.(Ala882Hisfs*297)	BAV , AVS	-	-
	III.1	16	c.2643delC	p.(Ala882Hisfs*297)	-	PA-IVS , TA	HRV, ASDII, PDA
E	II.1	46	c.5529G>A	p.(Trp1843*)	Aortic sclerosis	-	-
	II.2	39	c.5529G>A	p.(Trp1843*)	AVI	-	-
	III.1	0,2[c]	Not tested	-	-	PA	VSD
	III.2	25	c.5529G>A	p.(Trp1843*)	COA, BAV, AVS, MVS, MI	-	-
F	II.2	60	Not tested	-	AVS, AVR 58 years	-	-
	II.3	56	c.4240delT	p.(Cys1414Ala fs*31)	AVS, AVR 56 years	-	TAA 42 mm
	III.4	0.8[c]	Not tested	-	-	TOF, PA	-
G	I.1	66	Not tested	-	BAV, AVS, AVR 42 years	-	TAA 50 mm
	II.1	30	c.7455dupC	p.(Ser2486Leufs*21)	BAV	Severe PVS	Aortic root 36 mm
	III.1	1,2	c.7455dupC	p.(Ser2486Leufs*21)	-	TA	-
H	I.1	70[c]	Obl. carrier	-	AVS, MVS, AVR + MVR 50 years	-	AF, VF
	I.3	55[c]	Obl. carrier	-	AVS (no AVR)	-	VF
	II.1	34	c.3787C>T[d]	p.(Glu1262_ Gly1301del)	Normal echo	-	VT
	II.2	38	c.3787C>T[d]	p.(Glu1262_ Gly1301del)	Normal echo	-	-
	II.3	37[c]	Not tested	p.(Glu1262_ Gly1301del)	Unknown	-	SCD
	II.5	27	c.3787C>T[d]	p.(Glu1262_ Gly1301del)	BAV, COA	-	LVNC
	III.1	16	c.3787C>T[d]	p.(Glu1262_ Gly1301del)	Normal echo	-	-

Table 3. Cardiac phenotypes of families with clearly pathogenic *NOTCH1* mutations (truncating mutations, splice-site mutations, whole-gene deletions) (*continued*)

Family	No. in pedigree[a]	Age (years)[b]	Nucleotide change	Protein change	LS-CHD	RS-CHD/ CTD	Other
	III.2	23	c.3787C>T[d]	p.(Glu1262_ Gly1301del)	-	-	PDA[e], LV ↓
I	I.1	80	Not tested	-	AVS	-	-
	II.1	40	c.2425delG	p.(Asp809Thrfs*67)	Normal echo	-	-
	III.1	4	c.2425delG	p.(Asp809Thrfs*67)	BAV, mild AVS, MVS	-	-
J	I.1	66	c.1904-2A>G	p.?	Normal echo	-	-
	II.1	45	c.1904-2A>G	p.?	BAV, mild AVS	-	-
	II.2	41	c.1904-2A>G	p.?	-	TOF rDA	-
K	II.1	0[c]	del 9q34.3 (137.4-140.2)	-	HLHS	-	-
L	II.1	TOP	c.3054C>A	p.(Cys1018*)	HLHS	-	-
M	II.3	67[c]	Obl. carrier	-	Thickened aortic valves	-	AF, LV↓
	II.5	53[c]	Obl. carrier	-	Unknown	-	SCD
	II.7	43[c]	Obl. carrier	-	Unknown	-	-
	II.8	60[c]	Obl. carrier	-	AVR 39 years	-	-
	II.9	39[c]	Obl. carrier	-	Unknown		SCD
	III.10	57	c.1650C>G	p.(Tyr550*)	Normal echo	-	Aortic root 39 mm
	III.11	54	c.1650C>G	p.(Tyr550*)	Normal echo	-	-
	III.14	47	c.1650C>G	p.(Tyr550*)	BAV, aortic sclerosis, AVI	-	-
	III.21	53	c.1650C>G	p.(Tyr550*)	AVS	-	-
	III.24	47	c.1650C>G	p.(Tyr550*)	BAV	-	-
	III.27	44	c.1650C>G	p.(Tyr550*)	AVI	-	-
	III.28	34	c.1650C>G	p.(Tyr550*)	AVI, MVR	-	CAD
	III.29	33	c.1650C>G	p.(Tyr550*)	AVI	-	-
	III.30	45	c.1650C>G	p.(Tyr550*)	BAV	-	TAA 43 mm
	III.32	48	c.1650C>G	p.(Tyr550*)	BAV, AVI	-	-
	IV.3	0.3[c]	Not tested	-	MVS	-	-
	IV.8	0.2[c]	Not tested	-	AVS	-	TAPVR
	IV.12	10	Obl. carrier	-	-		VSD (patch)
	IV.15	23	c.1650C>G	p.(Tyr550*)	BAV, AVI	-	
	IV.16	21	c.1650C>G	p.(Tyr550*)	BAV	-	VSD
	IV.18	28	c.1650C>G	p.(Tyr550*)	Normal echo	-	-
	V.1	1	c.1650C>G	p.(Tyr550*)	-	TOF	-
	V.2	5	Not tested	-	AVS	-	-
	V.3	1	Not tested	-	-	-	ASD II, VSD, PFO
N	II.1	79	c.1670-7G>A	p.Gly557fs	BAV, AVS, AVR 42 years	-	CABG 64 years

Table 3. Cardiac phenotypes of families with clearly pathogenic *NOTCH1* mutations (truncating mutations, splice-site mutations, whole-gene deletions) (*continued*)

Family	No. in pedigree[a]	Age (years)[b]	Nucleotide change	Protein change	LS-CHD	RS-CHD/ CTD	Other
	II.2	60[c]	Not tested	-	AVI, MVS, AVR + MVR 37 years	-	-
	III.4	52	Negative	-	-	-	HCM
	III.5	58	c.1670-7G>A	p.Gly557fs	BAV, AVI, AVS, AVR 53 years	-	PCI 47 years, TAA 45 mm
	III.7	53	Not tested	-	Mild MI	-	-
	III.14	9[c]	Not tested	-	AVS	-	-
	III.18	41	c.1670-7G>A	p.Gly557fs	BAV, AVS, MVS, AVR 34 years, MVR 9 years	-	OHCA 36 years due to VF
	III.20	45	c.1670-7G>A	p.Gly557fs	Normal echo	-	-
	III.21	45	c.1670-7G>A	p.Gly557fs	-	PVS	VSD
	IV.1	23	Not tested	-	-	-	VSD

AF, atrial fibrillation; ASD, atrial septal defect; asymmTAV, asymmetric tricuspid aortic valve; AVI, aortic valve insufficiency; AVR, aortic valve replacement; AVS, aortic valve stenosis; BAV, bicuspid aortic valve; CABG, coronary artery bypass graft; CAD, coronary artery disease; cDNA, complementary DNA; COA, coarctation of the aorta; CTD, conotruncal heart disease; HCM, hypertrophic cardiomyopathy; HLHS, hypoplastic left heart syndrome; HRV, hypoplastic right ventricle; IVS, intact ventricular septum; LS-CHD, left-sided congenital heart disease; LV ↓, impaired left ventricular function; LVNC, left ventricular noncompaction; MI, mitral valve insufficiency; MVR, mitral valve replacement; MVS, mitral valve stenosis; obl., obligate; OHCA, out of hospital cardiac arrest; PA, pulmonary atresia; PCI, percutaneous coronary intervention; PDA, persistent ductus arteriosus; PFO, patent foramen ovale; PVS, pulmonary valve stenosis; rDA, right descending aorta; RS-CHD, right-sided congenital heart disease; SCD, sudden cardiac death; TA, truncus arteriosus; TAA, thoracic aortic aneurysm; TAPVR, total anomalous pulmonary venous return; TOF, tetralogy of Fallot; TOP, termination of pregnancy; VF, in hospital ventricular fibrillation; VSD, ventricular septal defect; VT, registered ventricular tachycardias treated with ablation therapy. [a] Probands are underlined. [b] Age at last contact with hospital. [c] Age at death. [d] Splice effect: deletion last 120 bp of exon 23. [e] PDA detected at age 22 years, no pulmonary hypertension, not operated.

Pedigrees are presented in **Figure 2** and the phenotypes are summarized in **Table 3**. BAV was present in 19/63 mutation carriers (probands included) (30%), AVS without BAV was present in 10/63 mutation carriers (16%), AVI without BAV in 5/63 (8%), COA in 2/63 (3%), HLHS in 2/63 (3%). Nine mutation carriers (14%) presented with a RS-CHD/CTD, in three of those this was together with a LS-CHD. Sudden death without a previous cardiac diagnosis (at ages 37, 39 and 53 years) was reported in 2 obligate mutation carriers and one twin sister of a mutation carrier. One deceased obligate carrier was reported by the family to have a "valve insufficiency", but medical records were not available. Six mutation carriers had thoracic aortic aneurysm (TAA) larger than 40 mm; this was associated with BAV in four and with AVS without documented BAV in two. Seven of the 13 affected relatives who were not tested had AVS; two of these had documented BAV (one with associated TAA), and one AVS patient had associated total anomalous pulmonary venous return (TAPVR). Mitral valve disease was present in three untested relatives, pulmonary valve atresia with Fallot's tetralogy or ventricular septap defect (VSD) in two, VSD in one and VSD and atrial septal defect II in one. Twelve of the 49 mutation

carriers (25%) were asymptomatic (confirmed by echocardiography in all), so apparently the mutation was non-penetrant in these individuals. The age range of these relatives was 16-75 years; the younger ones might still develop calcification of the aortic valve and/or TAA in the future. Nonpenetrance occurred in four patients with maternally inherited *NOTCH1* mutations and in four patients with paternally inherited mutations and no DNA was available from the parents of four mutation carriers with a normal echocardiogram (family B I.2,I II.1, J I.1 and N II.5). In patient II.1 from family H, sustained ventricular tachycardia was registered in the hospital and treated with ablation. In her daughter (III.1), who complained of palpitations, no arrhythmia was registered on Holter registration.

Non-synonymous variants

Apart from these pathogenic mutations, 24 non-synonymous variants (11 novel) were detected in 35 of the 428 patients (8%). These variants are listed in **Supplemental Table 1**, with data of the phenotype, outcome of the prediction programs, number of controls tested and segregation of the variants in the families. In one patient with BAV and TAA, two non-synonymous variants were present. The parents were available for testing in 22 patients. We found 18 carrier parents were not affected, and this was confirmed with echocardiography in 16 of them. In the family with variant p.(Cys344Ser), however, the unaffected carrier mother had two close relatives who were reported by the family to have had a congenital heart defect but were deceased (no medical files or In two families, the variant was not present in an affected relative, suggesting that these variants (p.(Lys1461Arg) and p.(Met1669Arg)) are not the main disease-causing factors. Based on the segregation observed, prediction programs and data from controls, we consider 12 variants to be probably benign and two variants to be probably pathogenic (p.(Cys344Ser) and p.(Asn280Ser)). The data were inconclusive in 9 non-synonymous variants. In **Table 4** the numbers of truncating, splice site and non-synonymous mutations are summarized per heart defect diagnosis group.

Table 4. Summary of all mutations and variants in *NOTCH1* per left-sided congenital heart disease diagnosis subgroup

	Patients with truncating or RNA splicing mutations		Patients with non-synonymous mutations		Total tested	
Diagnosis	**Total**	**Familial**	**Total**	**Familial**	**Total**	**Familial**
BAV/AVS/AVI	10 (5%)	9 (10%)	13 (6%)	4 (4%)	205	94
COA (± BAV)	2 (1%)	1 (3%)	8 (6%)	1 (3%)	135	34
HLHS	2 (3%)	0	12(16%)	0	75	15
Other	0	0	2 (15%)	0	13	5
Total %	**14 (3%)**	**10 (7%)**	**35 (8%)**	**5 (3%)**	**428**	**148 (35%)**

AVI, aortic valve insufficiency; AVS, aortic valve stenosis; BAV, bicuspid aortic valve; COA, coarctation of the aorta; HLHS, hypoplastic left heart syndrome. [a] Other left-sided heart defects: interrupted aortic arch (n=3), subvalvular aortic stenosis (n=3), supravalvular aortic stenosis (n=1), hypoplastic aortic arch without HLHS (n=4), congenital mitral valve insufficiency (n=1), and congenital mitral valve stenosis (n=1).

Discussion

We present the results on *NOTCH1* mutation analysis in 428 probands with non-syndromic congenital LS-CHD. We detected 14 clearly pathogenic *NOTCH1* mutations (truncating, RNA splicing or whole gene deletion) in 428 patients (3%). The mutation rate was seven times as high in familial compared to non-familial LS-CHD patients. Mutations were present in probands with BAV, AVS, COA, as well as HLHS. In non-familial LS-CHD we detected three mutations (1%), two of which were *de novo*. *NOTCH1* mutations were highly penetrant: 75% of all mutation carriers (probands excluded) showed a cardiovascular abnormality at examination. Non-penetrance in these families was determined in 12 *NOTCH1* mutation carriers (25%), and their mutations were inherited from the mother just as frequently as from the father. The frequency of clearly pathogenic mutations in this cohort of patients with LS-CHD is higher than in previous studies. In five previous studies on *NOTCH1* in LS-CHD cohorts, a total of 273 probands was included, and only three clearly pathogenic mutations (truncating or RNA splicing) were reported (1%) [15, 31-34] two from a cohort of 53 patients with HLHS [33] and one from a cohort of 11 patients of familial BAV [34]. In three other studies, only non-synonymous variants of unknown significance were reported [15, 31, 32]. The higher frequency of *NOTCH1* mutations in our population may be due to our sequencing protocols (in one study only four exons were screened) [32], or to patient/study population characteristics. Our study population was a selection of LS-CHD patients referred for genetic counseling, and this was reflected in the relatively high percentage of familial cases (35%) compared with other studies [15, 31, 33]. In addition, our population was relatively young, with a median age of 10 years, whereas three of the other studies included mainly adult patients [31, 32, 34]. Congenital heart defects detected in childhood are generally more severe, and this may indicate that *NOTCH1* mutations are more often found in severe disease.

The phenotypes of the probands and relatives with pathogenic *NOTCH1* mutations in our study included a wide variety of congenital heart defects; in the majority of families with pathogenic mutations, we show that the spectrum of disease involves not only left-sided heart defects but also right-sided heart defects affecting the pulmonary valve, conotruncal disease including pulmonary atresia with intact ventricular septum, Fallot's tetralogy and truncus arteriosus, and other CHDs, such as anomalous pulmonary venous return, ASD, and VSD. The occurrence of a wider range of defects affecting the conotruncus of the heart in patients with *NOTCH1* mutations is in agreement with the reported role of NOTCH signaling in determining the fate of neural crest-derived cells [35, 36]. Copy number variations including the *NOTCH1* and *JAG1* regions were reported in non-syndromic Fallot's tetralogy [37]. In addition, Fallot's tetralogy and pulmonary valve disease in Alagille syndrome are caused by mutations in *JAG1* and *NOTCH2*, supporting our findings that NOTCH signaling is not only involved in aortic valve development, but also in pulmonary valve development, suggesting a general

role in the development of semilunar valves. The finding of left ventricular noncompaction in one patient might be associated with the *NOTCH1* mutation because *MIB1* mutations, affecting the NOTCH signaling pathway, were reported in left ventricular noncompaction [38]. The variability we observed in the severity of the heart defects within families with pathogenic mutations and the reduced penetrance indicate that the inheritance is not simply monogenic but more complex, and that the phenotype also depends on unknown modifiers. Truncating *NOTCH1* mutations were also recently reported in Adams Oliver syndrome, a genetically heterogeneous syndrome with aplasia cutis and heart malformations in some patients [39, 4]. The cardiac malformations reported in *NOTCH1* mutation carriers among these patients are in the LS-CHD and RS-CHD/CTD spectrum, comparable with the findings in our families. Of note is that no features of AOS were present in our families.

Apart from these clearly pathogenic mutations, which are considered definitely disease causing, we detected 24 non-synonymous variants; 6 were novel and 18 were reported in the ExAC database (http://exac.broadinstitute.org) in 35 patients (8%), compared with 5.5% in the five previous studies (corrected for polymorphisms c.3836G>A, p.(Arg1279His) (MAF 0.0231), c.4129C>T, p.(Pro1377Ser) (MAF 0.0234), c. 6853G>A, p.(Val2285Ile) (MAF 0.0162) which were excluded in our study). One report included functional studies suggesting that two of these variants, present in 6/91 patients, are pathogenic [15]. The *NOTCH1* gene is large, containing 34 exons, and shows many variants in coding and non-coding regions. These variants may not be causative alone but may well contribute to disease development in a complex model, as reported for variants in other genes involved in congenital heart defects [41]. This may be supported by our finding that the allele frequency of five variants, p.(Glu848Lys), p.(Arg912Trp), p.(Gly1091Ser), p.(Thr1344Met), and p.(Arg1350Leu), detected in more than one family is higher than the allele frequency reported in the Exome Sequencing Project and ExAC databases (**Supplemental Table 1**). We found most non-synonymous variants in HLHS (16%) and lower frequencies in COA and in BAV/AVS/aortic valve insufficiency (6%). This difference was significant but not reported in an earlier study that also focused on all LS-CHD subgroups [15]. Why these variants are more frequent in the most severe LS-CHD group is not clear, but this may indicate that there is a contribution of (some) of these *NOTCH1* variants acting in a complex disease model. The pathogenicity of these variants can only be estimated on the basis of software prediction programs and the observed segregation in the family. Therefore, sequencing data from other large cohorts and functional studies are needed to determine the contribution of these variants to congenital heart defects before they can be used in genetic counseling of individual families.

This study has some limitaitons. Since we only included patients with LS-CHD who were referred for genetic counseling, functional studies were not carried out, synonymous and intronic variants were not analyzed extensively, and mutation analysis of other genes (inciden-

tally) associated with LS-CHD was not performed. However, this is to our knowledge the largest study to date on *NOTCH1* in LS-CHD, showing that *NOTCH1* mutations do play an important role in both LS-CHD and RS-CHD/CTD. Although we detected *NOTCH1* mutations in 7% of the familial cases of this cohort, a large number of familial LS-CHD remained unsolved, indicating the involvement of other genes associated with a phenotype similar to that presented in the *NOTCH1*-related families. Massive parallel sequencing will hopefully reveal more about the etiology of LS-CHD and other congenital heart defects in the near future [42].

In conclusion, disease-causing *NOTCH1* mutations were detected in 7% of familial non-syndromic LS-CHD and in 1% of sporadic LS-CHD. In addition, we show that the penetrance of heart defects in mutation carriers is high (75%), and the expression of *NOTCH1* mutations is variable between and within families, in severity as well as in the cardiac phenotype. Mutations in *NOTCH1* are the major disease-causing factors in some families, but additional factors might be involved that modify the phenotype.

We recommend *NOTCH1* mutation screening in patients with LS-CHD, pulmonary valve disease, or conotruncal anomalies, especially in familial cases. The finding of a pathogenic *NOTCH1* mutation not only helps to counsel the patients at risk for complications such as early calcification of the aortic valve and TAA, it also enables the identification of relatives at risk for complications of a previously unknown congenital heart defect, for instance, BAV with TAA. In addition, it identifies relatives at risk for having affected offspring. Future research is needed to explain the phenotypic variability in *NOTCH1* mutations and to reveal other factors involved in familial and sporadic LS-CHD.

Acknowledgements

We thank all patients for their participation in this study. We thank Jackie Senior for editing the manuscript, Tom de Vries Lentsch for preparing the artwork and Lennart F. Johansson, Krista K. van Dijk-Bos, Esther J. de Jong, Krista A. Kooi and Ludolf G. Boven for sequencing *NOTCH1* and performing the RNA splicing studies.

References

1. Cripe L, Andelfinger G, Martin LJ, Shooner K, Benson DW. Bicuspid aortic valve is heritable. *J Am Coll Cardiol*. 2004;44:138-143.
2. McBride KL, Pignatelli R, Lewin M, Ho T, Fernbach S, Menesses A, Lam W, Leal SM, Kaplan N, Schliekelman P, Towbin JA, Belmont JW. Inheritance analysis of congenital left ventricular outflow tract obstruction malformations: Segregation, multiplex relative risk, and heritability. *Am J Med Genet A*. 2005;134:180-186.
3. Hinton RB,Jr., Martin LJ, Tabangin ME, Mazwi ML, Cripe LH, Benson DW. Hypoplastic left heart syndrome is heritable. *J Am Coll Cardiol*. 2007;50:1590-1595.
4. Kerstjens-Frederikse WS, du Marchie Sarvaas GJ, Ruiter JS, Van Den Akker PC, Temmerman AM, Van Melle JP, Hofstra RM, Berger RM. Left ventricular outflow tract obstructions: Should cardiac screening be offered to first-degree relatives? *Heart*. 2011;97:1228-1232.
5. Hitz MP, Lemieux-Perreault LP, Marshall C, Feroz-Zada Y, Davies R, Yang SW, Lionel AC, D'Amours G, Lemyre E, Cullum R, Bigras JL, Thibeault M, Chetaille P, Montpetit A, Khairy P, Overduin B, Klaassen S, Hoodless P, Nemer M, Stewart AF, Boerkoel C, Scherer SW, Richter A, Dube MP, Andelfinger G. Rare copy number variants contribute to congenital left-sided heart disease. *PLoS Genet*. 2012;8:e1002903.
6. Michelena HI, Khanna AD, Mahoney D, Margaryan E, Topilsky Y, Suri RM, Eidem B, Edwards WD, Sundt TM,3rd, Enriquez-Sarano M. Incidence of aortic complications in patients with bicuspid aortic valves. *JAMA*. 2011;306:1104-1112.
7. Siu SC, Silversides CK. Bicuspid aortic valve disease. *J Am Coll Cardiol*. 2010;55:2789-2800.
8. Hirsch JC, Copeland G, Donohue JE, Kirby RS, Grigorescu V, Gurney JG. Population-based analysis of survival for hypoplastic left heart syndrome. *J Pediatr*. 2011;159:57-63.
9. Shuhaiber J, Morgan B, Gottliebson W. Survival outcomes following norwood procedure for hypoplastic left heart. *Pediatr Cardiol*. 2015;36:57-63.
10. Mattina T, Perrotta CS, Grossfeld P. Jacobsen syndrome. *Orphanet J Rare Dis*. 2009;4:9.
11. Pierpont ME, Basson CT, Benson DW,Jr., Gelb BD, Giglia TM, Goldmuntz E, McGee G, Sable CA, Srivastava D, Webb CL. Genetic basis for congenital heart defects: Current knowledge: A scientific statement from the american heart association congenital cardiac defects committee, council on cardiovascular disease in the young: Endorsed by the american academy of pediatrics. *Circulation*. 2007;115:3015-3038.
12. Schrander-Stumpel CT, Spruyt L, Curfs LM, Defloor T, Schrander JJ. Kabuki syndrome: Clinical data in 20 patients, literature review, and further guidelines for preventive management. *Am J Med Genet A*. 2005;132A:234-243.
13. Gripp KW, Hopkins E, Jenny K, Thacker D, Salvin J. Cardiac anomalies in axenfeld-rieger syndrome due to a novel FOXC1 mutation. *Am J Med Genet A*. 2013;161A:114-119.
14. Garg V, Muth AN, Ransom JF, Schluterman MK, Barnes R, King IN, Grossfeld PD, Srivastava D. Mutations in NOTCH1 cause aortic valve disease. *Nature*. 2005;437:270-274.
15. McBride KL, Riley MF, Zender GA, Fitzgerald-Butt SM, Towbin JA, Belmont JW, Cole SE. NOTCH1 mutations in individuals with left ventricular outflow tract malformations reduce ligand-induced signaling. *Hum Mol Genet*. 2008;17:2886-2893.
16. Elliott DA, Kirk EP, Yeoh T, Chandar S, McKenzie F, Taylor P, Grossfeld P, Fatkin D, Jones O, Hayes P, Feneley M, Harvey RP. Cardiac homeobox gene NKX2-5 mutations and congenital heart disease: Associations with atrial septal defect and hypoplastic left heart syndrome. *J Am Coll Cardiol*. 2003;41:2072-2076.
17. Padang R, Bagnall RD, Richmond DR, Bannon PG, Semsarian C. Rare non-synonymous variations in the transcriptional activation domains of GATA5 in bicuspid aortic valve disease. *J Mol Cell Cardiol*. 2012;53:277-281.
18. Shi LM, Tao JW, Qiu XB, Wang J, Yuan F, Xu L, Liu H, Li RG, Xu YJ, Wang Q, Zheng HZ, Li X, Wang XZ, Zhang M, Qu XK, Yang YQ. GATA5 loss-of-function mutations associated with congenital bicuspid aortic valve. *Int J Mol Med*. 2014;33:1219-1226.

19. Dasgupta C, Martinez AM, Zuppan CW, Shah MM, Bailey LL, Fletcher WH. Identification of connexin43 (alpha1) gap junction gene mutations in patients with hypoplastic left heart syndrome by denaturing gradient gel electrophoresis (DGGE). *Mutat Res*. 2001;479:173-186.
20. Tan HL, Glen E, Topf A, Hall D, O'Sullivan JJ, Sneddon L, Wren C, Avery P, Lewis RJ, ten Dijke P, Arthur HM, Goodship JA, Keavney BD. Nonsynonymous variants in the SMAD6 gene predispose to congenital cardiovascular malformation. *Hum Mutat*. 2012;33:720-727.
21. Theis JL, Zimmermann MT, Evans JM, Eckloff BW, Wieben ED, Qureshi MY, O'Leary PW, Olson TM. Recessive MYH6 mutations in hypoplastic left heart with reduced ejection fraction. *Circ Cardiovasc Genet*. 2015.
22. Artavanis-Tsakonas S, Rand MD, Lake RJ. Notch signaling: Cell fate control and signal integration in development. *Science*. 1999;284:770-776.
23. Bray SJ. Notch signalling: A simple pathway becomes complex. *Nat Rev Mol Cell Biol*. 2006;7:678-689.
24. Hori K, Sen A, Artavanis-Tsakonas S. Notch signaling at a glance. *J Cell Sci*. 2013;126:2135-2140.
25. Bray S, Bernard F. Notch targets and their regulation. *Curr Top Dev Biol*. 2010;92:253-275.
26. Timmerman LA, Grego-Bessa J, Raya A, Bertran E, Perez-Pomares JM, Diez J, Aranda S, Palomo S, McCormick F, Izpisua-Belmonte JC, de la Pompa JL. Notch promotes epithelial-mesenchymal transition during cardiac development and oncogenic transformation. *Genes Dev*. 2004;18:99-115.
27. Luna-Zurita L, Prados B, Grego-Bessa J, Luxan G, del Monte G, Benguria A, Adams RH, Perez-Pomares JM, de la Pompa JL. Integration of a notch-dependent mesenchymal gene program and Bmp2-driven cell invasiveness regulates murine cardiac valve formation. *J Clin Invest*. 2010;120:3493-3507.
28. Theodoris CV, Li M, White MP, Liu L, He D, Pollard KS, Bruneau BG, Srivastava D. Human disease modeling reveals integrated transcriptional and epigenetic mechanisms of NOTCH1 haploinsufficiency. *Cell*. 2015; 160:1072-1086.
29. Acharya A, Hans CP, Koenig SN, Nichols HA, Galindo CL, Garner HR, Merrill WH, Hinton RB, Garg V. Inhibitory role of Notch1 in calcific aortic valve disease. *PLoS One*. 2011;6:e27743.
30. Ducharme V, Guauque-Olarte S, Gaudreault N, Pibarot P, Mathieu P, Bosse Y. NOTCH1 genetic variants in patients with tricuspid calcific aortic valve stenosis. *J Heart Valve Dis*. 2013;22:142-149.
31. Mohamed SA, Aherrahrou Z, Liptau H, Erasmi AW, Hagemann C, Wrobel S, Borzym K, Schunkert H, Sievers HH, Erdmann J. Novel missense mutations (p.T596M and p.P1797H) in NOTCH1 in patients with bicuspid aortic valve. *Biochem Biophys Res Commun*. 2006;345:1460-1465.
32. McKellar SH, Tester DJ, Yagubyan M, Majumdar R, Ackerman MJ, Sundt TM,III. Novel NOTCH1 mutations in patients with bicuspid aortic valve disease and thoracic aortic aneurysms. *J Thorac Cardiovasc Surg*. 2007; 134:290-296.
33. Iascone M, Ciccone R, Galletti L, Marchetti D, Seddio F, Lincesso A, Pezzoli L, Vetro A, Barachetti D, Boni L, Federici D, Soto A, Comas J, Ferrazzi P, Zuffardi O. Identification of de novo mutations and rare variants in hypoplastic left heart syndrome. *Clin Genet*. 2012;81:542-554.
34. Foffa I, Ait Ali L, Panesi P, Mariani M, Festa P, Botto N, Vecoli C, Andreassi MG. Sequencing of NOTCH1, GATA5, TGFBR1 and TGFBR2 genes in familial cases of bicuspid aortic valve. *BMC Med Genet*. 2013;14:44.
35. Wakamatsu Y, Maynard TM, Weston JA. Fate determination of neural crest cells by NOTCH-mediated lateral inhibition and asymmetrical cell division during gangliogenesis. *Development*. 2000;127:2811-2821.
36. High FA, Zhang M, Proweller A, Tu L, Parmacek MS, Pear WS, Epstein JA. An essential role for notch in neural crest during cardiovascular development and smooth muscle differentiation. *J Clin Invest*. 2007; 117:353-363.
37. Greenway SC, Pereira AC, Lin JC, DePalma SR, Israel SJ, Mesquita SM, Ergul E, Conta JH, Korn JM, McCarroll SA, Gorham JM, Gabriel S, Altshuler DM, Quintanilla-Dieck ML, Artunduaga MA, Eavey RD, Plenge RM, Shadick NA, Weinblatt ME, De Jager PL, Hafler DA, Breitbart RE, Seidman JG, Seidman CE. De novo copy number variants identify new genes and loci in isolated sporadic tetralogy of fallot. *Nat Genet*. 2009;41: 931-935.
38. Luxan G, Casanova JC, Martinez-Poveda B, Prados B, D'Amato G, MacGrogan D, Gonzalez-Rajal A, Dobarro D, Torroja C, Martinez F, Izquierdo-Garcia JL, Fernandez-Friera L, Sabater-Molina M, Kong YY, Pizarro G, Ibanez B, Medrano C, Garcia-Pavia P, Gimeno JR, Monserrat L, Jimenez-Borreguero LJ, de la Pompa JL.

Mutations in the NOTCH pathway regulator MIB1 cause left ventricular noncompaction cardiomyopathy. *Nat Med*. 2013;19:193-201.

39. Stittrich AB, Lehman A, Bodian DL, Ashworth J, Zong Z, Li H, Lam P, Khromykh A, Iyer RK, Vockley JG, Baveja R, Silva ES, Dixon J, Leon EL, Solomon BD, Glusman G, Niederhuber JE, Roach JC, Patel MS. Mutations in NOTCH1 cause adams-oliver syndrome. *Am J Hum Genet*. 2014;95:275-284.
40. Southgate L, Sukalo M, Karountzos AS, Taylor EJ, Collinson CS, Ruddy D, Snape KM, Dallapiccola B, Tolmie JL, Joss S, Brancati F, Digilio MC, Graul-Neumann LM, Salviati L, Coerdt W, Jacquemin E, Wuyts W, Zenker M, Machado RD, Trembath RC. Haploinsufficiency of the NOTCH1 receptor as a cause of adams-oliver syndrome with variable cardiac anomalies. *Circ Cardiovasc Genet*. 2015.
41. Wessels MW, Willems PJ. Genetic factors in non-syndromic congenital heart malformations. *Clin Genet*. 2010;78:103-123.
42. Pediatric Cardiac Genomics Consortium. The congenital heart disease genetic network study: Rationale, design, and early results. *Circ Res*. 2013;112:698-706.

Supplemental Information

Detailed Methods

Samples were PCR amplified using the following program: an initial denaturation at 94°C for 10 minutes, followed by 5 cycles of denaturation at 94°C for 5 seconds, annealing starting at 65°C for 30 seconds with a step-down of 1°C every cycle, and elongation at 72°C for 1 minute, followed by 20 cycles of denaturation at 94°C for 5 seconds, annealing at 60°C for 30 seconds, and elongation at 72°C for 1 minute, followed by 15 cycles of denaturation at 94°C for 5 seconds, annealing at 55°C for 30 seconds, and elongation at 72°C for 1 minute with a final step at 72°C for 5 minutes, after which the samples were cooled down to 20°C. 5 µl of the PCR products were loaded with 5 µl loading buffer and run on a 2% agarose gel with a FastRuler Low Range DNA Ladder (Fermentas) for comparison.

Supplemental Table 1. Non-synonymous *NOTCH1* variants detected in our cohort

cDNA change	Protein change	Phenotype(s)	Origin	Segregation	ExAC MAF	Conservation	Classification
c.136T>G	p.(Cys46Gly)	COA	Maternal	Unaffected parent	Absent	++	Uncertain significance
c.701G>A	p.(Arg234His)	AVS	Unknown	Unknown	3/3188	+	Uncertain significance
C.839A>G	p.(Asn280Ser)	COA	Maternal	Thickened aortic valve	9/63734	++	Likely pathogenic
c.1030T>A	p.(Cys344Ser)	HLHS	Maternal	Unaffected parent	Absent	++	Likely pathogenic
c.1801G>A	p.(Glu601Lys)	AVS	Maternal	Unaffected parent	Absent	+	Likely benign
c.2003C>T	p.(Pro668Leu)	BAV, TAA	Paternal	Unaffected parent	3/65372	++	Uncertain significance
c.2080G>A	p.(Glu694Lys)	BAV	Maternal	Unaffected parent	54/59512	-	Likely benign
c.2542G>A	p.(Glu848Lys)	COA	Paternal	TAA	238/64828	++	Uncertain significance
		HLHS	Unknown	Unknown			
		HLHS	Paternal	Unaffected parent			
		AVS	Unknown	Unknown			
		BAV, TAA	Unknown*	Unknown			
c.2636G>A	p.(Arg879Gln)	HLHS	Maternal	Unaffected parent	9/63598	+	Likely benign
c.2734C>T	p.(Arg912Trp)	COA	Paternal	BAV	143/51644	-	Uncertain significance
		COA	Maternal	Unaffected parent			
		COA	Unknown	Unknown			
		BAV	Unknown	Unknown			
		AVS	Paternal	Unknown			
c.3271G>A	p.(Gly1091Ser)	BAV	Maternal	Unaffected parent	4/65478	++	Uncertain significance

Supplemental Table 1. Non-synonymous *NOTCH1* variants detected in our cohort (*continued*)

cDNA change	Protein change	Phenotype(s)	Origin	Segregation	ExAC MAF	Conservation	Classification
		HLHS	Maternal	Unaffected parent			
c.3328G>A	p.(Val1110Ile)	COA	Maternal	Unaffected parent	1/63248	-	Likely benign
c.3859C>T	p.(Arg1287Cys)	HLHS	Unknown	Unknown	1/42208	+	Uncertain significance
c.4028C>T	p.(Ala1343Val)	HAoA	Unknown	Unknown	110/40574	+	Likely benign
c.4031C>T	p.(Thr1344Met)	BAV	Unknown	Unknown	56/42400	++	Uncertain significance
		HLHS	Maternal	Unaffected parent			
c.4049G>T	p.(Arg1350Leu)	HLHS	Paternal	Unaffected parent	54/48172	-	Uncertain significance
		COA	Paternal	Unaffected parent			
		IAA, VSD	Unknown	Unknown			
c.4382A>G	p.(Lys1461Arg)	BAV	Maternal	Father BAV	Absent	++	Likely benign
c.4971C>G	p.(Ser1657Arg)	BAV, TAA	Unknown*	Unknown	2/3122	-	Likely benign
c.5006T>G	p.(Met1669Arg)	BAV	Unknown	Sibling BAV	Absent	+	Likely benign
c.5011G>A	p.(Val1671Ile)	BAV, AAD	Unknown	Unknown	2/1896	-	Likely benign
c.6938G>A	p.(Arg2313Gln)	HLHS	Maternal	Unaffected parent	15/64068	++	Uncertain significance
c.7397C>T	p.(Thr2466Met)	HLHS	Paternal	Unaffected parent	3/45352	+	Likely benign
c.7432G>A	p.(Ala2478Thr)	HLHS	Paternal	Unaffected parent	Absent	-	Likely benign
c.7606G>A	p.(Val2536Ile)	HLHS	Unknown	Unknown	25/54918	-	Likely benign

AAD, acute aortic dissection; AVS, aortic valve stenosis; BAV, bicuspid aortic valve; COA, coarctation of the aorta; ExAC MAF, minor allele frequency in Exome Aggregation Consortium, non-Finnish European population; HAoA, hypoplastic aortic arch; HLHS, hypoplastic left heart syndrome; IAA, interrupted aortic arch; TAA, thoracic aortic aneurysm; VSD, ventricular septal defect; * two variants present in the same patient.