Chapter 4

Cardiovascular malformations caused by *NOTCH1* mutations do not keep left: data on 427 LVOTO probands and their families

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ABSTRACT

Background: Left ventricular outflow tract obstruction (LVOTO) includes bicuspid aortic valve, congenital aortic valve stenosis, coarctation of the aorta and hypoplastic left heart syndrome and is highly heritable. Mutations in *NOTCH1* are associated with LVOTO, but no reliable estimations of mutation frequency or phenotypic variability have been published.

We aimed to determine the prevalence and phenotypic spectrum of *NOTCH1* mutations in congenital heart defects (CHDs).

Methods and Results: *NOTCH1* was screened for mutations in 427 non-syndromic LVOTO probands, referred for genetic counseling. Familial LVOTO was defined as LVOTO in a first, second or third degree relative, or CHD in a first degree relative. When a *NOTCH1* mutation was detected, the proband's relatives were also screened for the mutation.

In 147/427 patients (34%) LVOTO was familial. Thirteen mutations (4 RNA splicing mutations, 8 truncating mutations and one whole gene deletion) were detected in 427 probands (3%). Most mutations were found in familial disease (9/147 (7%)) and less in sporadic disease (3/280 (1%)). In total, 55 (obligate) mutation carriers were identified in the families of the 13 probands with mutations. Their phenotypes included not only LVOTO, but also conotruncal malformations and thoracic aortic aneurysms (TAA). Nine mutation carriers (9/55) had a normal cardiac ultrasound (16%).

Conclusion: Pathogenic mutations in *NOTCH1* occur in 7% of familial LVOTO and in 1% of sporadic LVOTO. The phenotypic spectrum includes LVOTO, conotruncal CHDs and TAA. The high penetrance of mutations (84%) suggests that testing *NOTCH1* for an early diagnosis in familial LVOTO/CHD is warranted.

INTRODUCTION

Left ventricular outflow tract obstruction (LVOTO) is a highly heritable congenital heart defect, including bicuspid aortic valve (BAV), aortic valve stenosis (AVS), coarctation of the aorta (COA) and hypoplastic left heart syndrome (HLHS).[1, 2] HLHS is a severe disease, with limited options for surgical corrections, that may lead to death at a very young age.[3] BAV, however, may be asymptomatic and undetected in infancy, but has a risk for serious complications and sudden cardiac death later in life.[4, 5]

LVOTO is reported in more than 200 different syndromes and in many of these the associated gene or chromosomal region is known, for example in Andersen syndrome (KCNJ2 gene), Turner syndrome (monosomy X), Marfan(-like) syndrome (*FBN1, TGFBR1, TGFBR2* and *TGFB2* genes), Kabuki make-up syndrome (*MLL2*), Williams syndrome (microdeletion 7q11.2, *ELN* gene), Rieger syndrome (*PITX2, FOXC1*), and Smith-Magenis syndrome (microdeletion 7p11.2, *RAI1* gene).[6-9]

In non-syndromic LVOTO only a few genes are known to be associated with the disease (*NOTCH1*, *GJA1, NKX2.5, GATA5, SMAD6*) and the number of patients with mutations in these genes is low.[10-15] *NOTCH1* is the only gene reported with truncating mutations segregating with LVOTO. 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.[16-18] 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 neighbouring cells. After ligand binding, the receptor is cleaved and an intracellular domain enters the nucleus, where it interacts with DNA-binding proteins. Downstream targets of NOTCH1 are amongst others Hes (hairy-enhancer-of split) and Hrt (Hes related) families of genes.[10, 17, 19, 20]

Germline truncating mutations in *NOTCH1* were first reported segregating in two families with mainly aortic valve disease and one patient with Fallot's tetralogy.[10] One of these mutations was also reported in a patient with a stenotic tricuspid aortic valve.[21] In 5 series of LVOTO patients screened for *NOTCH1* mutations only two new truncating mutations and one RNA splicing mutation and several possibly pathogenic missense variants were reported.[11, 22-25] As these series are small and the number of mutations reported limited, the role of *NOTCH1* in LVOTO is still unclear.

In this study the prevalence, phenotypic spectrum and pedigrees of patients with truncating or RNA splicing *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 LVOTO, referred for genetic counseling to one of three participating university hospitals in the Netherlands between January 1st 2006 and January 1st 2014 were included in the

study. Intrauterine deaths and terminations of pregnancy were also included. All patients had a detailed cardiac evaluation by a (pediatric) cardiologist, including ECG and cardiac ultrasound/ Doppler imaging. MRI was performed if the aortic arch could not be visualized by ultrasound. Cardiac diagnoses included were BAV, AVS, aortic valve insufficiency (AVI), COA (with or without BAV), HLHS or other left sided cardiac diseases, including sub-valvular or supra-valvular aortic stenosis, hypoplastic aortic arch, interruption of the aorta 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 stenotic, normally functioning, 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. Cardiac ultrasounds were offered to a proband's parents, siblings and children, as described previously.[2] Cases were considered familial if a first, second or third degree relative also had LVOTO or pulmonary valve disease, or a first degree relative had a congenital heart defect other than LVOTO or pulmonary valve disease.

Sequence analysis of the *NOTCH1* gene

Mutation analysis of the coding exons and flanking intronic sequences of the *NOTCH1* gene 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 (40ng/µl) genomic DNA. The samples were PCR amplified on a Perkin-Elmer (ABI) Geneamp 9700 using the following program: an initial denaturation at 94°C for 1 minute, 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. The remaining PCR products were purified with **ExoSAP-IT** (Amersham Pharmacia Biotech, Biscataway, NY,USA) and subjected to direct sequencing on an ABI 3730 automated sequencer, using the specific primers.

Array-comparative genomic hybridization (aCGH) 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 V.9.1 software. This analysis was not included in the genetic work-up by all clinical geneticists. Data were available in 180 patients and these were checked 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 to be pathogenic. The Alamut (vs 2.3) missense prediction and splicing prediction modules from Interactive Biosoftware were used to predict pathogenicity (www.interactive-biosoftware.com). 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 (MAF Data from NHLBI exome sequencing project (ESP) on European Americans (http://evs.gs.washington.edu/EVS/)).

cDNA analysis of c.3787 C>T;p.Arg1263Cys a potential RNA splicing mutation

All missense variants and synonymous DNA variants were analyzed *in silico* for their effect on RNA splicing. One variant, c.3787 C>T, p.Arg1263Cys, was predicted to introduce a new splice donor site and was therefore tested. Total RNA was extracted from peripheral blood, using the RNABee procedure (Cinna Biotecx, Friendswood, Texas, USA) and cDNA was obtained using the GoScript Reverse Transcription System (Promega).

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.3787 C>T mutation in exon 23. 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, USA) for the statistical analyses. A p-value of < 0.05 was considered to be statistically significant. Interquartile range (IQR) was used to show statistical dispersion.

RESULTS

Patients

A cohort of 427 patients (286 male, 141 female) with non-syndromic LVOTO, including 31 intra uterine deaths/terminated pregnancies, was included. The median age was 10 years (IQR 29,9). In 147 of 427 probands (34%) the heart defect was familial. Cardiac phenotypes are described in table 1.

Table 1. Diagnoses and family history in 427 probands

Diagnosis	Male (n)	Female (n)	Familial (n)	Total
BAV/AVS/AVI	153	51	93	204
COA (+/- BAV)	80	55	34	135
HLHS	49	26	15	75
Other	4	9	5	13
Total	**286 (67%)**	**141 (33%)**	**147 (34%)**	**427**

AVI= congenital aortic valve insufficiency; AVS= congenital aortic valve stenosis; AV= bicuspid aortic valve; COA= coarctation of the aorta; HLHS= hypoplastic left heart syndrome; other= other left-sided heart defects: interruption of the aorta (3), sub-valvular aortic stenosis (3), supra-valvular aortic stenosis (1), hypoplastic aortic arch without HLHS (4), congenital mitral valve insufficiency (1), congenital mitral valve stenosis (1).

Truncating and RNA splicing mutations

In total we detected 13 novel pathogenic mutations in 427 index patients (probands) (3%): eight truncating (nonsense or frameshift) mutations, four RNA splicing mutations and one whole gene deletion. (figure 1) One RNA splicing mutation (c.3787 C>T, p.Arg1263Cys) within exon 23 was predicted to introduce a new splice donor site and was therefore tested by cDNA analysis. Sequence data 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, p.Glu1262_Gly1301del.

The truncating and RNA splicing mutations were found in probands with BAV (n=4), AVS (n=5), COA (n=2), HLHS (n=2). Three mutations were detected in 280 non-familial cases (1%): two were truncating mutations (in HLHS and BAV) and there was one whole gene deletion (in HLHS, detected by aCGH). One of these mutations was present in a non-affected parent, the other two were *de novo*. Ten mutations were detected in 147 probands with familial LVOTO (7%), six were truncating and four were RNA splicing mutations.

Phenotypes in families with truncating or RNA splicing mutations

Relatives of the 13 index patients who had truncating and RNA splicing mutations were subsequently tested and in these families we identified 42 new mutation carriers, eight of which were obligate carriers. Nine other relatives had a congenital heart defects but DNA was not available for testing

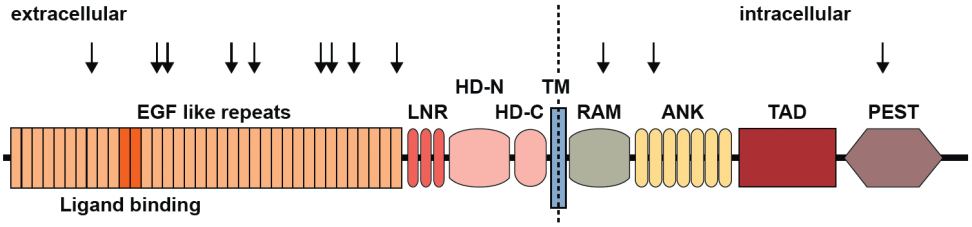

Figure 1. NOTCH1 protein, position of truncating and RNA-splicing mutations
↓Truncating and RNA-splicing mutations. EGF=epidermal growth factor, LNR=Lin/Notch repeats, HD=heterodimerization domain, TM=transmembrane domain, RAM=RBPjk-association module, ANK=ankyrin repeats, TAD=transcriptional activation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

Mutation		Domain	Effect
cDNA change	**protein change**		
del 9q34.3 (137,4-140,2)		whole gene	deletion
in 5 c.865+2C>A	p.?	EGF-like 8 Ca-binding	RNA splicing
ex 10 c.1650C>G	p.(Tyr550*)	EGF-like 14	truncating
in 11 c.1904-2A>G	p.?	EGF-like15	RNA splicing
ex 15 c.2425delG	p.(Asp809Thrfs*67)	EGF-like 21 Ca-binding	truncating
ex 17 c.2643delC	p.(Ala882Hisfs*297)	EGF-like 23 Ca-binding	truncating
ex 19 c.3054C>A	p.(Cys1018*)	EGF-like 29	truncating
in 21 c.3511-2A>G	p.?	EGF-like 30	RNA splicing
ex 23 c.3787C>T	p.(Glu1262_Gly1301del)	EGF-like 32 Ca-binding	RNA splicing
ex 25 c.4240delT	p.(Cys1414Alafs*31)	EGF-like 36	truncating
ex 30 c.5529G>A	p.(Trp1843*)	RAM	truncating
ex 32c.5950C>T	p.Arg1984*	ANK	truncating
ex 34 c.7455dupC	p.Ser2486Leufs*21	PEST	truncating

Pedigrees are presented in figure 2 and the phenotypes of the probands and family members are summarized in table 2.

Bicuspid aortic valve was present in 16/55 mutation carriers (probands included) (29%), AVS without BAV was present in 7/55 mutation carriers (13%), AVI without BAV in 4/55 (7%), COA in 2/55 (4%), HLHS in 2/55 (4%). Six mutation carriers (11%) presented with a conotruncal heart defect: pulmonary atresia or pulmonary valve stenosis with or without VSD in two, Fallot's tetralogy in two, truncus arteriosus in one, persistent ductus arteriosus in one. Sudden cardiac death without a previous cardiac diagnosis (at ages 37, 39 and 53 years) was reported in 3 obligate mutation carriers. One deceased obligate carrier was reported by the family to have a "valve insufficiency", unfortunately medical records were not available. Two mutation carriers with a bicuspid aortic valve also had pulmonary valve stenosis, one had associated VSD. Four mutation carriers had thoracic

Table 2. Cardiac phenotypes of families with splice site and truncating *NOTCH1* mutations

Fam	nr	Age*	Change cDNA	Change AA	LVOTO	Conotruncal	Other
A	II.1	44	c.3511-2A>G	p.?	AVS, AVR age 38	PVS	
	III.1	15	c.3511-2A>G	p.?	BAV, mild AVS		
	III.2	14	c.3511-2A>G	p.?	BAV, no AVS/AVI		
B	I.2	54	c.865+2C>A	p.?	US normal		
	II.1	39	c.865+2C>A	p.?	BAV, no AVS/AVI		TAA 43mm
	II.2	30	c.865+2C>A	p.?	BAV, AVS, AVR age 33		
	II.3	26	c.865+2C>A	p.?	AVS, tricuspid, asymmtric AV		
	III.1	16	c.865+2C>A	p.?	BAV, no AVS/AVI		
	III.3	4	c.865+2C>A	p.?	BAV, no AVS/AVI		
	III.5	0,5 †	c.865+2C>A	p.?		PA, VSD,rDA	
	III.6	4	c.865+2C>A	p.?	Tricuspid, asymmetric AV		
C	I.1	69	c.5950C>T	p.(Arg1984*)	AVS (AVR age 60)		TAA 48 mm
	II.2	33†	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,, intact VS	HRV, TA ASDII, ODB
E	II.1	46	c.5529G>A	p.(Trp1843*)	Aortic sclerosis		
	II.2	39	c.5529G>A	p.(Trp1843*)	AVI		

	III.1	0,2†	not tested			PA	VSD
	III.2	25	c.5529G>A	p.(Trp1843*)	BAV, AVS, COA		MS, MI
F	II.1	56	c.4240delT	p.(Cys1414Alafs*31)	AVS (AVR age 56)		
	II.2	60	not tested		AVS (AVR age 58)		
	III.1	0.8†	not tested			TOF, PA	
G	I.1	66	not tested		BAV, AVS, (AVR age 42)		TAA 50 mm
	II.1	30	c.7455dupC	p.(Ser2486Leufs*21)	BAV	PVS, severe	TAA 36 mm sinus
	III.1	1,2	c.7455dupC	p.(Ser2486Leufs*21)		TA	
H	I.1	70†	obl. carrier		AVS,MVS (AVR +MVR age 50)		AF,VF
	I.3	55†	obl. carrier		AVS (no AVR)		VF
	II.1	34	c.3787C>T#	p.(Glu1262_Gly1301del)	US normal		VT
	II.2	38	c.3787C>T#	p.(Glu1262_Gly1301del)	US normal		
	II.3	37†	obl.carrier	p.(Glu1262_Gly1301del)	u		
	II.4	41	c.3787C>T#	p.(Glu1262_Gly1301del)	US normal		
	II.5	27	c.3787C>T#	p.(Glu1262_Gly1301del)	BAV, COA		LVNC
	III.1	16	c.3787C>T#	p.(Glu1262_Gly1301del)	US normal		
	III.2	23	c.3787C>T#	p.(Glu1262_Gly1301del)		PDA	LV↓
I	I.1	80	not tested		"AVS calcified valve"		
	II.2	40	c.2425delG	p.(Asp809Thrfs*67)	US normal		
	II.3	4	c.2425delG	p.(Asp809Thrfs*67)	BAV, mild AVS, MVS		
J	I.1	66	c.1904-2A>G	p.?	US normal		
	II.1	45	c.1904-2A>G	p.?	BAV, mild AVS		
	II.2	41	c.1904-2A>G	p.?		TOF rDA	
K	*de novo*	0†	del 9q34.3 (137,4-140,2)		HLHS		

Fam	nr	Age*	Change cDNA	Change AA	LVOTO	Conotruncal	Other
L	*de novo*	TOP	c.3054C>A	p.(Cys1018*)	HLHS		
M	II.3	67†	obl. carrier		Thickened aortic valves?		AF, LV↓
	II.5	53†	obl. carrier		u		SCD
	II.7	43†	obl. Carrier		u		"Valve insufficiency"
	II.8	60†	obl. carrier		"AVR"		
	II.9	39†	obl. carrier		u		SCD
	III.10	57	c.1650C>G	p.(Tyr550*)	US normal		
	III.11	54	c.1650C>G	p.(Tyr550*)	US normal		
	III.14	47	c.1650C>G	p.(Tyr550*)	BAV, aortic sclerosis, AVI		
	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 gr I, MVR		CAD
	III.29	33	c.1650C>G	p.(Tyr550*)	AVI gr I-II		
	III.30	45	c.1650C>G	p.(Tyr550*)	BAV		TAA39 mm
	III.32	48	c.1650C>G	p.(Tyr550*)	BAV, AVI gr I-II		
	IV.8	0,2†	not tested		AVS		TAPVR
	IV.12	10	c.1650C>G	p.(Tyr550*)			VSD (patch), OFO
	IV.15	23	c.1650C>G	p.(Tyr550*)	BAV, AVI gr II		
	IV.16	21	c.1650C>G	p.(Tyr550*)	BAV		VSD (spont. closed)
	V.1	1	c.1650C>G	p.(Tyr550*)		TOF	
	V.2	5	not tested		AVS		
	V.3	1	not tested				ASD II, VSD, OFO

Legend table 2.

*Age in years at last contact with hospital, † age at death; # splice effect: deletion last 120 bp
AF= Atrial Fibrillation; AVI=aortic valve insufficiency; ASD=atrial septal defect; AV=Aortic Valve; AVS=congenital Aortic Valve Stenosis; AVR= Aortic Valve Replacement; BAV= bicuspid aortic valve; CAD=coronary artery disease; HLHS=hypoplastic left heart syndrome; LV ↓=impaired LV function; LVNC=Left Ventricular Noncompaction Cardiomyopathy; LVOTO= congenital Left Ventricular Outflow Tract Obstruction; MI= Mitral Valve Insufficiency; MVR=mitral valve replacement; MVS=Mitral Valve Stenosis; obl.=obligate; OFO=open foramen ovale; PA=pulmonary atresia; Patnr= number of patient in the pedigree; PDA=Persistent Ductus Arteriosus; PVS= congenital Pulmonary Valve Stenosis; rDA= right Descending Aorta; SCD= Sudden Cardiac death; spont.=spontaneously; TA=truncus arteriosus; TAA=thoracic aortic aneurysm; TAPVR=total anomalous pulmonary venous return; TOF= Tetralogy of Fallot; TOP=termination of pregnancy; u=unknown; US= cardiac Ultrasound; VF= in hospital Ventricular Fibrillation; VS=ventricular septum; VSD= Ventricular Septal Defect; VT= registered Ventricular Tachycardias treated with ablation therapy.

aortic aneurysm (TAA), this was associated with BAV in three and with AVS in one. Four of the nine relatives who were not tested had AVS, one of these had associated total anomalous pulmonary venous return (TAPVR), two had BAV and one of these had associated TAA. Pulmonary atresia and/ or Fallot's tetralogy was present in two untested relatives and VSD and ASDII in one. In 9/55 mutation carriers (16%) cardiac ultrasound was normal, so apparently the mutation was non-penetrant in these individuals. Non-penetrance occurred in 3 patients with maternally inherited *NOTCH1* mutations and in 4 patients with paternally inherited mutations and no DNA was available from the parents of 2 mutation carriers with a normal ultrasound (family B I.2 and J I.1).

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 patients (35/427, (8%)). These variants are listed in table 3, 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 cardiac ultrasound 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 and DNA were available). 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 13 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.

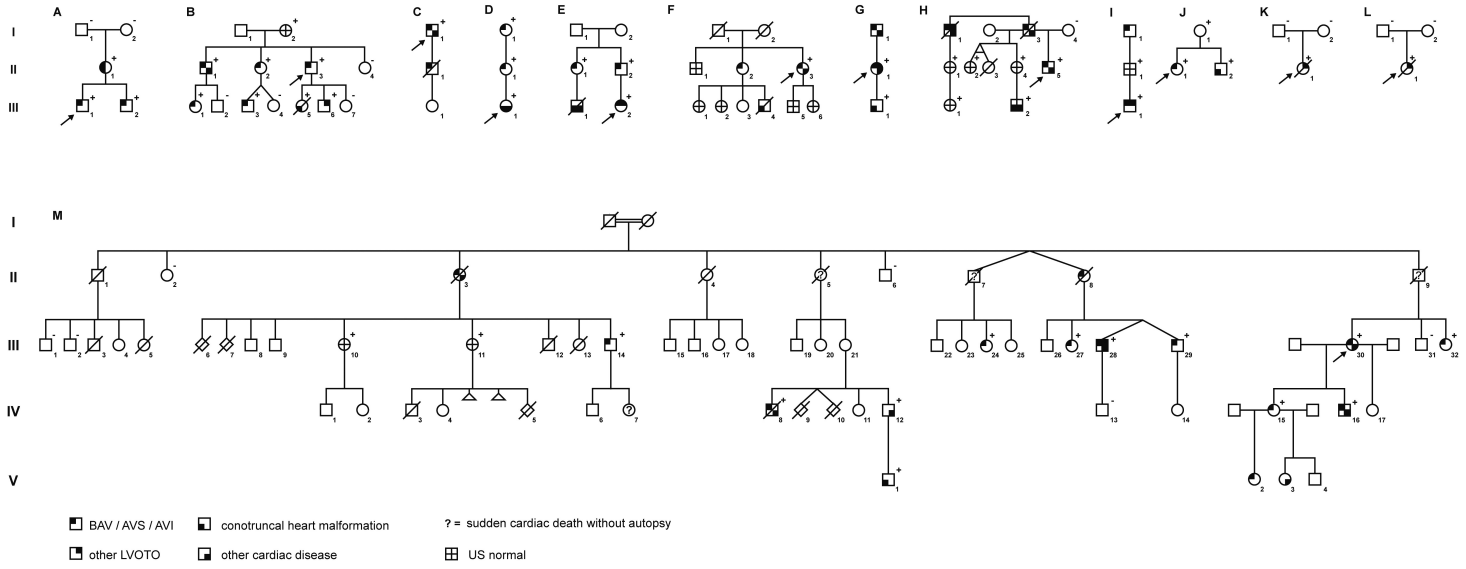

Figure 2. Pedigrees of patients with truncating and RNA splicing mutations in *NOTCH1*

Thirteen families of probands with mutations. The mutations in family K and L were *de novo*. See table 2 for description of the phenotypes.

Table 3. Non-synonymous variants (24 non-synonymous variants in 35 patients)

Pos cDNA	Protein	Origin	Phenotype	Sift	Poly	aGVGD	MT	Segregation	Controls	Concl
136T>G	Cys46Gly	mat	COA	1	1	C0	1	no US	nr	VOUS
701G>A	Arg234His	nt	AVS	1	1	C0	1	unknown	0/7612	VOUS
839A>G	Asn280Ser	mat	COA	1	1	C45	1	thickened AV	1/8302	P
1030T>A	Cys344Ser	mat	HLHS	1	1	C65	1	norm US#	nr	P
1801G>A	Glu601Lys	mat	AVS	1	0	C0	1	norm US	nr	B
2003C>T	Pro668Leu	pat	BAV+Aodilat	1	1	C0	1	norm US	nr	VOUS
2080G>A	Glu694Lys	mat	BAV	0	0	C0	0	norm US	8/8488	B
2542G>A	Glu848Lys	pat	COA	1	1	C0	1	Aodilat	32/8360	VOUS
		nt	HLHS					unknown		
		pat	HLHS					norm US		
		nt	AVS					unknown		
		nt*	BAV					unknown		
2636G>A	Arg879Gln	mat	HLHS	0	0	C0	1	norm US	1/8340	B
2734C>T	Arg912Trp	pat	COA	1	0,5	C15	1	BAV	27.8344	VOUS
		mat	COA					norm US		
		nt	COA					unknown		
		nt	BAV					unknown		
		pat	AVS					no US		
3271G>A	Gly1091Ser	mat	BAV	1	1	C0	1	norm US	nr	VOUS
		mat	HLHS					norm US		
3328G>A	Val1110Ile	mat	COA	0	0	C0	0	norm US	nr	B
3859C>T	Arg1287Cys	nt	HLHS	1	0,5	C25	1	unknown	nr	VOUS

Pos cDNA	Protein	Origin	Phenotype	Sift	Poly	aGVGD	MT	Segregation	Controls	Concl
4028C>T	Ala1343Val	nt	HAoA	1	0,5	C0	1	unknown	1/7492	VOUS
4031C>T	Thr1344Met	nt	BAV	1	0,5	C0	1	unknown	4/7508	B
		mat	HLHS					norm US		
4049G>T	Arg1350Leu	pat	HLHS	1	0,5	C25	0	norm US	4/7262	B
		pat	COA					norm US		
		nt	AoI+VSD					unknown		
4382A>G	Lys1461Arg	mat	BAV	1	0	C25	1	pat BAV$	nr	B
4971C>G	Ser1657Arg	nt*	BAV	0	0	C0	0	unknown	nr	B
5006T>G	Met1669Arg	nt	BAV	0	0	C0	1	unknown	nr	B
5011G>A	Val1671Ile	nt	BAV+Ao diss	1	0	C45	1	sib BAV$	2/6752	B
6938G>A	Arg2313Gln	mat	HLHS	1	1	C35	1	norm US	1/8380	VOUS
7397C>T	Thr2466Met	pat	HLHS	1	0,5	C0	0	norm US	1/8414	B
7432G>A	Ala2478Thr	pat	HLHS	0	0	C0	0	norm US	nr	B
7606G>A	Val2536Ile	nt	HLHS	0	0	C0	0	unknown	3/8314	B

Prediction: SIFT: tolerated=0, deleterious=1; Polyphen (Poly): benign=0, possibly damaging=0.5, probably damaging=1; align GVGD (aGVGD): (C0, C15, C25, C35, C45, C55, C65) with C65 most likely to interfere with function and C0 least likely. Mutation Taster (MT): benign=0, disease causing =1.
Ao= aorta, AoI= interruption of the aorta, AV= aortic valve, B= probably benign, BAV= bicuspid aortic valve, COA= coarctation of the aorta, concl= conclusion, dilat= dilatation, diss= dissection, HAoA= hypoplastic aortic arch, HLHS= hypoplastic left heart syndrome, mat= maternal: mutation present in mother, no US= not affected, but no US performed, norm US= normal cardiac ultrasound, nt= variant not tested in parents, nr= variant not reported in NHLBI exome sequencing project; pat= paternal: mutation present in father, P = probably pathogenic, Poly= Polyphen, Pos.= position, *two variants present in the same patient, VOUS= variant of unknown significance. # mother has affected relatives, $ Not a carrier of familial *NOTCH1* variation.

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 LVOTO diagnosis subgroup

	Number of patients with					
Diagnosis	**truncating or RNA splicing mutations**		**non-synonymous mutations**		**Total**	
	total	*familial*	*total*	*familial*	*total*	*familial*
BAV/AVS/AVI	9 (4%)	8 (9%)	13 (6%)	4 (4%)	204	93
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	0	13	5
Total	**13**	**9**	**35**	**5**	**427**	**147**
%	**(3%)**	**(7%)**	**(8%)**	**(3%)**		**(34%)**

AVI= congenital aortic valve insufficiency; AVS= congenital aortic valve stenosis; BAV= bicuspid aortic valve; COA= coarctation of the aorta; HLHS= hypoplastic left heart syndrome; other= other left-sided heart defects: interruption of the aorta (3), sub-valvular aortic stenosis (3), supra-valvular aortic stenosis (1), hypoplastic aortic arch without HLHS (4), congenital mitral valve insufficiency (1), congenital mitral valve stenosis (1).

DISCUSSION

We present the results on *NOTCH1* mutation analysis in 427 probands with non-syndromic congenital LVOTO. We detected 13 pathogenic (truncating or RNA splicing) *NOTCH1* mutations in 427 patients (3%). The mutation rate was seven times as high in familial compared to non-familial LVOTO patients. Mutations were present in probands with BAV, AVS, COA as well as HLHS. In non-familial LVOTO we detected three mutations (1%), two of which were *de novo*. In 13 families of probands with truncating and RNA-splicing mutations, we show that the spectrum of disease does not only involve left-sided heart defects, but also conotruncal defects including pulmonary atresia, Fallot's tetralogy and truncus arteriosus, and other anomalies, such as anomalous pulmonary venous return, ASD, VSD. Non-penetrance in these families was determined in nine *NOTCH1* mutation carriers (16%) and their mutations were inherited from the mother or from the father in equal frequencies.

Apart from these truncating and RNA splicing mutations, which are considered definitely pathogenic, we detected 24 non-synonymous variants (11 novel, 13 reported in the ESP (http://evs.gs.washington.edu/EVS/)) in 35 patients (8%).

The frequency of truncating and RNA splicing mutations in LVOTO patients is higher in our study than in previous studies. Five studies on *NOTCH1* have been published in LVOTO cohorts and one in stenotic tricuspid aortic valves.[11, 21-25] In total, the five studies in LVOTO patients included 273 probands, and reported 3/273 pathogenic (truncating or RNA splicing) mutations (1%): two from a cohort of 53 HLHS patients[24] and one from a cohort of 11 familial BAV patients.[25] In the other three

studies only non-synonymous variants of unknown significance were reported.[11,22,23] Our higher frequency of mutations compared to other studies may be due to our sequencing protocols (in one study only 4 exons were screened),[23] or to their low numbers of patients or to patient/study population characteristics. Our study population was a selection of LVOTO patients referred for genetic counseling and this was reflected in a relatively high percentage of familial cases (34%) compared with other studies.[11, 22, 24] In addition, our population was relatively young, with a median age of 10 years, while in three of the other studies mainly adult patients were included.[22, 23, 25] 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 truncating and RNA splicing *NOTCH1* mutations in our study included a wide variety of congenital heart defects and were different from other studies: in the initial report on *NOTCH1* mutations in two families, LVOTO was present in most patients and Fallot's tetralogy and double outlet right ventricle were each present in one.[10] Since that initial report, LVOTO has been the focus of most studies on *NOTCH1*. However, copy number variations including the *NOTCH1* and *JAG1* regions were reported in non-syndromic Fallot's tetralogy.[26] In addition, Fallot's tetralogy and pulmonary valve disease in Alagille syndrome are caused by mutations in *JAG1* and *NOTCH2*, indicating that NOTCH signaling is involved in a broader spectrum of conotruncal heart diseases. Activation of NOTCH1 represses differentiation of embryonic stem cells into cardiomyocytes and stimulates endocardial epithelial-to mesenchymal-transition (EMT), which is an important step in the process of cardiac valve formation.[19, 27] NOTCH signaling is also involved in cardiovascular development through fate determination of neural crest derived cells.[28, 29] The occurrence of not only left sided valvular defects, but 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 regulating neural crest derived cells and EMT.[18, 30]

Data on the penetrance of heart defects in *NOTCH1* mutation carriers have not been published, but non-penetrance has been reported in studies on LVOTO.[31] The reduced penetrance we found, and the variability we observed in the severity of the heart defects within families with pathogenic mutations, indicate that the inheritance is not simply monogenic, but more complex, and that the phenotype also depends on unknown modifiers.

It has been suggested in the literature that the penetrance of a congenital heart defect depends on the gender of the transmitting parent, with a higher risk for offspring of mothers with a congenital heart defect. This has been based on weak evidence from family studies and it has been hypothesized to be due to imprinting effects.[32] We found that *NOTCH1* mutations were paternally versus maternally transmitted in 4 versus 3 non-affected mutation carriers, but our study population is far too small to confirm or reject this hypothesis.

In 8% of our patients we found non-synonymous variants (MAF < 0,01), compared to 5,5% in the 5 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 two of these variants, present in 6/91 patients, to be pathogenic.[11] We found most non-synonymous variants in HLHS (16%) compared to lower frequencies in COA and in BAV/AVS/AVI (6%). This difference was significant but not reported in an earlier study also focusing on all LVOTO subgroups.[11] Why these variants are more frequent in the most severe LVOTO group is not clear, but they may more often act together with other variants in an oligogenic or complex disease model. The *NOTCH1* gene is a large gene, containing 34 exons, and shows many variants in coding and non-coding regions. These variants may not cause, but may well contribute to disease development in a complex model, as reported for variants in other genes involved in congenital heart defects.[33] Currently, these variants are not very helpful for genetic counseling in individual families and the pathogenicity of these variants can only be estimated on the basis of software prediction programs and the observed segregation in the family. Sequencing data from large cohorts and functional studies are needed to conclude on the contribution of these variants to congenital heart defects.

This study has some limitations, since we have only included patients with LVOTO who were referred for genetic counseling, we did not perform any functional studies, we did not look into synonymous and intronic variants extensively and we did not screen for mutations in other genes that have been incidentally reported to be associated with LVOTO. However, this is the largest study on *NOTCH1* in LVOTO so far, showing that *NOTCH1* mutations do play an important role in congenital heart defects.

However, although *NOTCH1* may play an important role, we detected *NOTCH1* mutations in only 7% of the familial cases of this cohort, indicating that there must be other genes involved in the patients without *NOTCH1* mutations. Massive parallel sequencing will hopefully reveal more about the etiology of LVOTO and other congenital heart defects in the near future.[34]

We conclude that disease causing *NOTCH1* mutations were detected in 7% of familial non-syndromic LVOTO and in 1% of sporadic LVOTO. In addition, we show that the penetrance of heart defects in mutation carriers is high (84%) and the expression of *NOTCH1* mutations is variable between and within families, in severity as well as in the location of the malformation. Mutations in *NOTCH1* are the major disease-causing factors in some families, but there must be additional factors involved which modify the phenotype.

We recommend *NOTCH1* mutation screening in patients with LVOTO or pulmonary valve disease and other conotruncal anomalies, especially in familial cases, because this will help to detect relatives at risk for complications of a previously unknown congenital heart defect, for instance BAV with TAA. It will also identify relatives at risk for affected offspring. Future research is needed to explain the phenotypic variability in *NOTCH1* mutations and to find other factors involved in familial and sporadic LVOTO.

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