Overlapping but distinct roles for NOTCH receptors in human cardiovascular disease

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Running title: Notch receptors in cardiovascular disease

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Abstract

The NOTCH signalling pathway is an essential pathway, involved in many cellular processes, including cell fate decision, cell proliferation, and cell death and important in the development of most organs. Mutations in genes encoding components of the NOTCH signalling pathway lead to a spectrum of congenital disorders. Over the past decades mutations in human NOTCH signalling genes have been identified in several diseases with cardiovascular involvement. *NOTCH1* mutations have been described in bicuspid aortic valve disease, left-sided congenital heart disease, and Adams-Oliver syndrome. *NOTCH2* mutations lead to the development of Alagille syndrome, while mutations in *NOTCH3* cause cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy. To date, mutations in *NOTCH4* have not been associated with cardiovascular disease. This review focuses on the mutations described in *NOTCH1*, *NOTCH2*, and *NOTCH3* and their associated cardiovascular phenotypes.

Key words

NOTCH Receptor

Mutation

Cardiovascular diseases

Alagille Syndrome

Adams-Oliver syndrome

Bicuspid Aortic Valve

CADASIL

Congenital heart defects

Introduction

The Notch signalling pathway is an essential pathway in human development and highly conserved throughout species.[1] It is involved in many cellular processes, including cell fate decision, cell proliferation, and cell death, and has an important function throughout the development of most organs. Therefore, one can imagine that mutations in genes encoding components of the NOTCH signalling pathway lead to a wide range of congenital disorders. Over the past decades, mutations in human NOTCH signalling genes have mainly been identified in different types of cardiovascular disease. This review elaborates on the different mutations described in *NOTCH1* (MIM#190198), *NOTCH2* (MIM#600275), and *NOTCH3* (MIM#600276) and the resulting development of cardiovascular disease in humans.

Canonical NOTCH signalling pathway

In humans, four different NOTCH receptors exist, comprising NOTCH1-4. These receptors interact with the canonical ligands, which belong to the Delta and Jagged (JAG) families in vertebrates.[1,2] JAG1 and JAG2 are part of the Jagged family of NOTCH ligands, while Delta-like protein (DLL)1, DLL3, and DLL4 belong to the Delta family of NOTCH ligands.[3] Both receptors and ligands are single pass transmembrane proteins and therefore signalling is restricted to neighbouring cells.

In the Golgi compartment, the precursor of the NOTCH receptor is cleaved at cleavage site S1 by FURIN, a proprotein convertase, after which the two parts form a heterodimer (Figure 1). Next, the receptor will translocate to the cell surface, where the N-terminus will be located extracellularly and the C-terminus will be located in the intracellular compartment. The N-terminus mainly consists of epidermal growth factor (EGF)-like repeats, which are involved in ligand binding. These EGF-like domains within the NOTCH receptors and its ligands have a specific structure and consist of approximately 40 amino acids and contain six cysteine residues, which will form three disulfide bridges. These disulfide bridges determine the secondary structure of the domain and as a consequence also of the protein. In addition to the EGF-like repeats, the N-terminus of the NOTCH

receptor consists of Lin12-NOTCH (LNR) repeats and a heterodimerization (HD) domain, both located close to the transmembrane domain (TMD). Together, the LNR repeats and the HD domain form the negative regulatory region. This region prevents activation of the signalling cascade in the absence of a ligand.

Binding of a ligand to the receptor initiates the signalling cascade (Figure 1) and this process causes mechanical forces to pull on the NOTCH receptor, leading to conformational changes,[4] which will expose the S2 cleavage site of the receptor, which is located in the HD domain. The exposure of this cleavage site allows its cleavage by a disintegrin and metalloproteinase domain (ADAM) 10 or Adam17,[5,6] which will release the extracellular part of the receptor. The cell expressing the ligand will ultimately internalize its ligand and the extracellular domain of the NOTCH receptor, a process termed *trans*-endocytosis.[7-9] The remainder of the NOTCH receptor is still attached to the cell surface and the intracellular part consists of a Recombining binding protein suppressor of hairless (RBPJ)-association module (RAM) domain, seven ankyrin (ANK) repeats[10] surrounded by three nuclear localisation signals (NLS), a transactivation domain (TAD), and a proline (P)-, glutamic acid (E)-, serine (S)-, threonine (T)-rich (PEST) motif. After cleavage by ADAM10/17, the membrane bound intracellular part of the receptor is cleaved by the γ-secretase complex at cleavage sites S3 and S4,[11-13] which are located just outside the TMD. This γ-secretase complex is composed of several proteins, including Presenilin, a transmembrane protease, Nicastrin, Presenilin enhancer 2 (PEN-2), and anterior pharynx-defective (APH-1).[1] After cleavage by the γ-secretase complex, the NOTCH intracellular domain (NICD) will be released and will translocate to the nucleus, based on the presence of an NLS. In the nucleus, the NICD will regulate transcription of the target genes, hairy and enhancer of split (HES) and HES-related with YRPW motif (HEY), which are evolutionarily conserved basic helix-loop-helix (bLHL) transcription factors.[14,15]

Before NICD enters the nucleus, RBPJ, which consists of an N-terminal, a β-trefoil and a C-terminal domain,[16] forms a corepressor complex by associating with several corepressor proteins and histone

deacetylase complexes (HDAC) to repress transcription of NOTCH target genes. Upon entering the nucleus, the NICD forms a ternary protein complex with RBPJ, by binding of the RAM domain of NOTCH to the β-trefoil domain of RBPJ and Mastermind-like protein (MAML) 1. This protein complex will bind to the DNA helix by the β-trefoil domain of RBPJ and will recruit coactivators of transcription, in order to replace the corepressor proteins.[17]

The NOTCH receptors and its ligands display an extensive expression pattern, both during foetal development and in adult life, and are involved in the development of the vast majority of organs.Even though all NOTCH receptors are expressed in the vascular system, NOTCH2 and NOTCH3 show a more pronounced expression compared to NOTCH1 and NOTCH4 (GTEx database), while NOTCH1 and NOTCH4 are highly expressed in endothelial cells.[18] Numerous processes have been identified that are involved in the refinement of NOTCH signalling, including posttranslational modifications of the receptor and ligands, such as glycosylation, hydroxylation, phosphorylation, and ubiquitination.[19] Additional finetuning is performed by interactions with coactivators and inhibitors at various levels of the pathway. The duration of the transcriptional event mediated by NICD is determined by MAML proteins, as they recruit cyclin dependent kinases (CDKs), which will phosphorylate the PEST domain of NICD, triggering the disassembly of the protein activator complex and ubiquitination of the NICD.[20] Ultimately, the NICD will undergo proteasomal degradation.

NOTCH1

Congenital heart disease

Bicuspid aortic valve (BAV) is the most common congenital heart defect, affecting 1-2% of the general population. BAV often goes unnoticed, but can lead to cardiovascular complications like calcific aortic valve disease (CAVD)[21], coarctation, stenosis, and valve dysfunction[22] and in at least 20% of BAV patients, the malformation is accompanied by the development of thoracic aortic aneurysms (TAA).[23] These complications are associated with significant mortality rates. Despite the fact that BAV is common, very few genes have been linked to this condition and the hitherto known genes only explain disease in a minority of patients.

In 2005, a first report was published describing the involvement of *NOTCH1* mutations in congenital heart disease (CHD).[21] Truncating *NOTCH1* mutations were identified in two families, members of whom presented with various aortic and cardiac anomalies, including BAV, CAVD, aortic stenosis, aortic insufficiency, TAA, Tetralogy of Fallot (TOF), ventricular septal defect (VSD), mitral atresia, hypoplastic left ventricle, and double-outlet right ventricle (Figure 2).[21] This initial report was followed by many others, describing an association between variations in *NOTCH1* and BAV, BAV/TAA, hypoplastic left heart syndrome (HLHS), aortic valve stenosis, and coarctation.[23-37] Most of the identified variants in these reports are missense variants, which do not replace or create critical cysteine or other conserved residues in the EGF-like domains. As the vast majority of these variants are also present in public databases (e.g. gnomAD[38]), sometimes at high allele frequencies, these are definitively not as convincing as the loss-of-function (LOF) mutations from the initial report. An overview of the published exonic and splicing *NOTCH1* variants, with a minor allele frequency (MAF) <0.05, associated with CHD is given in the supporting information (Table S1).

In 2016, a large scale screening of 428 probands with left-sided CHD (LS-CHD), confined to aortic valve stenosis, BAV, coarctation of the aorta, and HLHS, revealed the presence of 14 *NOTCH1* mutations, including splicing mutations, truncating mutations and a whole gene deletion (Figure 2, Table S1).[39] A specific frameshift mutation reported in this study (p.Ser2486Leufs*21) is located within the last exon and is therefore predicted to escape nonsense mediated decay (NMD) of the mutant mRNA transcript. Consequently, this mutation is hypothesized to lead to a dominant negative effect, which is different from to the other truncating mutations in this study, for which haploinsufficiency (HI) would be the most likely mechanism of disease. In addition, 18% of mutation carriers suffered from right-sided CHD (RS-CHD) or conotruncal heart disease, revealing that the observed CHD has both a left- and right-sided localisation.[39] Furthermore, in 10% of *NOTCH1* mutation carriers TAA was identified. Familial segregation was performed and 25% of mutation carriers were asymptomatic, indicating a significantly decreased penetrance.[39]

In contrast, a recent study in 441 BAV/TAA probands revealed a possible protective role for *NOTCH1* variants, as missense/splicing variants were observed more frequently amongst control populations compared to the BAV/TAA cohort (Table S1). However, the authors state that sample selection bias might have contributed to this observation, as *NOTCH1* variants appear to associate with early and severe valve calcification and seem to be enriched in families with highly penetrant BAV but far lower penetrance of TAA ..[23]

To study the role of this receptor, several Notch1 mouse models have been generated. Homozygous knock-out of Notch1 leads to embryonic lethality due to vascular defects, indicating an essential role for Notch signalling in early cardiovascular development.[40] Heterozygosity of Notch1 on a Nos3-null background, a model previously known for the development of BAV,[41] is characterized by high penetrance of BAV.[42] Endothelial-specific loss of Notch1 contributes to the development of BAV.[43,44] Endothelial Dll4 is essential for epithelial-mesenchymal transition (EMT), a process defined by the detachment of endocardial cells in the atrioventricular canal and outflow tract and their transition to mesenchyme cells of the endocardial cushions. Endocardial Jag1 on the other hand, is required for proper cushion formation at post EMT-stages.[45] Calcification studies of the aortic valves showed that immortalized Notch1$^{+/-}$ aortic valve interstitial cells resemble a myofibroblast-like phenotype, expressing higher amounts of mediators of dystrophic calcification.[46] Recent work has shown that a heterozygous loss of Notch1 (Notch1$^{+/-}$) leads to the development of TAA on a 129SV background, a phenomenon not observed on a mixed background (C59Bl6, 129SV, BTBR).[47]

This overview indicates that truncating mutations in *NOTCH1* cause a wide range of CHD, characterized by incomplete penetrance and variable expression. The causative potential of missense variants in NOTCH1 is less convincing and should be investigated in greater detail. It seems plausible that common pathophysiological mechanisms are underlying BAV and CHD, in which BAV is to be considered as the milder form of the more severe left ventricular outflow tract (LVOT) malformations.

Adams-Oliver syndrome

Adams-Oliver syndrome (AOS) is a rare developmental disorder with an estimated frequency of one affected individual per 225,000 live births.[48] AOS is characterized by the presence of both aplasia cutis congenita (ACC) of the scalp vertex and transverse terminal limb defects (TTLD). AOS patients also frequently present with cardiac, vascular and neurological symptoms. Approximately 20% of AOS patients have CHD, including valvular and ventricular abnormalities, atrial septal defect (ASD), and TOF. Vascular anomalies typically include cutis marmorata telangiectatica congenita (CMTC), which is present in approximately 20% of AOS patients. A clear variability in clinical expression can be observed. This is also illustrated by scalp defects that range from complete absence of skin with underlying skull defect to small patches of skin that lack hair, and limb defects that can range from severe amputation defects to small nails or short distal phalanges. Furthermore, recent studies have demonstrated incomplete penetrance in several AOS families.[49,50]

In total, mutations in six genes have been identified as a cause of AOS. The autosomal dominant (AD) form of AOS is caused by mutations in *ARHGAP31*, *RBPJ*, *NOTCH1*, or *DLL4*.[49-54] The autosomal recessive form is caused by recessive mutations in *DOCK6* and *EOGT*.[55-57] *ARHGAP31* and *DOCK6* encode regulatory proteins that specifically control the activity of the Rho GTPases RAC1 and CDC42, which are important for the maintenance of the actin cytoskeleton.[53] The remaining four genes, *RBPJ*, *NOTCH1*, *DLL4*, and *EOGT,* are components of the NOTCH pathway. Mutations in these NOTCH signalling genes provide a definitive diagnosis in the majority of molecularly solved AOS cases. As such, the NOTCH pathway plays a key role in AOS pathogenesis. Compared to the other established AOS genes, mutations in *DLL4, NOTCH1,* and *RBPJ* are more often associated with cardiovascular anomalies (unpublished results).[49] Epidermal growth factor (EGF) domain-specific O-linked N-acetylglucosamine transferase (EOGT) has been shown to act on NOTCH receptors in mammals.[58] Mutations in components of the NOTCH pathway are predicted to lead to dysregulated NOTCH signalling, likely through HI or LOF, as the induction of Notch target genes by several mutant NOTCH1

receptors has been shown to be diminished.[49] The exact mechanism underlying the clinical features observed in AOS remains unknown to date. However, it is hypothesized that the congenital anomalies in AOS are the consequence of an impaired circulation or vasculogenesis.[51,59,60]

Various mouse models of AOS genes have been studied over the years. The phenotype of Notch1, Dll4, or Rbpj (heterozygous) KO mice is mainly cardiac and vascular, including impaired trabeculation, defective cardiac looping, arteriovenous malformations, and hypoplastic endocardial cushions.[40,61,62] Remarkably, phenotypic features similar to ACC or TTLD have not been reported in these mouse models.

To date, *NOTCH1* is the most important contributor to the genetic basis of AD AOS/ACC/TTLD, providing a molecular diagnosis in 10% of all AOS cases. *NOTCH1* harbours deleterious variation across the major mutation categories, including large deletions, frameshift, nonsense, splice site, and missense mutations.[49,51] Truncating mutations are distributed across the length of the *NOTCH1* gene and are predicted to lead to NMD of the mutant mRNA transcript. The distribution of the truncating mutations does not differ from those observed in the BAV/CHD spectrum (Figure 2) and as such, the nature of the *NOTCH1* mutation does not explain the divergence of the associated phenotypes. Most likely, additional genetic modifiers, tissular second hits[63] or environmental factors determine the fate towards BAV/CHD or AOS. Missense mutations in *NOTCH1* most often, but not exclusively, remove or create cysteine residues, thereby giving rise to an odd number of cysteines and in turn lead to a disruption of the tertiary structure, as the conventional disulfide bonds structure will be interrupted. Furthermore, a clustering of missense NOTCH1 mutations in and around EGF-like domains 11-13 is observed,[49] a region crucial for binding of the ligand to the receptor. An overview of all published exonic and splicing *NOTCH1* variants with a MAF <0.05 in relation to AOS is given in the supporting information (Table S1).

NOTCH2

Alagille syndrome

Alagille syndrome (ALGS) is an AD multisystemic disorder characterized by the presence of bile duct paucity in combination with three out of five major criteria, including cholestatic liver disease, cardiac anomalies, ocular abnormalities, skeletal defects, and characteristic craniofacial features.[64] It was first described by Alagille et al. in 1987 and has an estimated prevalence of 1 in 70.000.[64] However, this prevalence is likely an underestimation, as it was based on the presence of neonatal liver disease and it was later discovered that a highly variable phenotype is present.[65] In the vast majority (94-96%) of patients, the phenotype is caused by *JAG1* mutations.[66-69] The second gene identified for ALGS is *NOTCH2*, explaining 1-2% of cases.[69,70] Recent studies have indicated that ALGS is accompanied by reduced penetrance and markedly variable expression. Importantly, familial segregation analyses revealed a substantial number of mutation carriers that did not fulfil all clinical diagnostic criteria of ALGS.[71] Therefore, the clinical diagnostic criteria might be too stringent and more emphasis should be placed on the molecular identification of pathogenic variants in this disease.

The vast majority of ALGS patients present with liver disease, including mild cholestasis, jaundice, and pruritis, and could progress to liver failure within the first three months of life.[72] Similarly, cardiac manifestations also vary widely between affected individuals and range from benign heart murmurs to major structural malformations. Approximately 94% of ALGS patients suffer from these cardiac manifestations.[73] Most commonly, the pulmonary system is affected, characterized by the presence of stenosis..[72] Other cardiac defects include TOF, VSD, ASD, aortic stenosis, and coarctation of the aorta.[72] Rarely, anomalous left coronary artery arising from the pulmonary artery is also observed and is associated with myocardial infarction and/or sudden death.[74] Ocular abnormalities often include posterior embyotoxon.[72] The most typical skeletal manifestation is butterfly vertebrae, a failure of fusion of the lateral halves of the vertebral body, which is present in the majority of ALGS patients.[72,75] Characteristic facial features include a prominent forehead, a pointed chin, deep-set

eyes, moderate hypertelorism, downslanting palpebral fissures, large ears, a prominent mandible, a depressed nasal bridge and a straight nose with bulbous tip.[76] The presence of these features will result in an upside down triangular shaped face. Other typical features include renal abnormalities, growth failure, delayed motor development, and neurovascular accidents, which are most often caused by aneurysms.[69,77]

The exact role of *JAG1* and *NOTCH2* in the development of ALGS remains unclear to date, but several hypotheses have been raised to explain the phenotypic features. One hypothesis states that ALGS is mainly a vasculopathy, as the vascular anomalies are widespread and NOTCH signalling has an important role in angiogenesis. Jag1 KO mice are embryonically lethal and show vascular defects.[78] In contrast, Jag1 heterozygous mice are viable, but do not show a cardiovascular phenotype. Combined HI of Jag1 and Notch2 does result in multisystem defects that resemble ALGS.[79] Despite the fact that gene-dosage sensitivity differs between mice and humans, the pulmonary artery stenosis and VSD observed in these mice suggest that the combination of Jag1 and Notch2 is necessary for the proper development of the cardiac outflow tract (OFT). A similar phenotype is observed upon KO of Psen1, a component of the γ-secretase complex, which is indicative of a common pathway.[80] Cardiac defects in ALGS often affect the cardiac OFT and the great vessels. These defects are recapitulated in a mouse model with Notch signalling specifically abrogated within the cardiac neural crest lineage. The latter is known to give rise to the vascular smooth muscle cells (VSMC) of the OFT and great vessels.[81] However, many of the other defects observed in ALGS, including liver, heart, skeleton, eye, face, and kidney defects, are caused by roles of Notch signalling in other cell types than VSMC. Kidney and eye defects, for example, are recapitulated in a homozygous, hypomorphic Notch2 mouse model.[82] This could indicate that tissular second hits may play a role in the phenotypic variability observed in ALGS patients. To date, over 400 mutations in *JAG1* have been reported to cause ALGS.[83] In up to 7% of ALGS patients, a large deletion of this gene was identified.[76] In the remainder of ALGS patients, truncating and missense mutations in *JAG1* are identified. No particular mutational hotspots have been identified within this gene. Only a small number of *NOTCH2* mutations have been published to

date, as only 1-2% of ALGS patients carries a mutation in this gene (Figure 3). These mutations involve missense mutations, frequently replacing a cysteine residue within an EGF-like domain, frameshift, splicing and nonsense mutations, suggesting LOF as the mechanism of action. Despite the limited number of identified *NOTCH2* mutation carriers, a few genotype-phenotype correlations can be observed. Compared to *JAG1* mutation carriers, *NOTCH2* probands less often seem to present with cardiac abnormalities, skeletal defects and characteristic facial features.[71] This could be explained by the fact that JAG1 is also able to bind the other NOTCH receptors, which could explain the wider phenotype in JAG1 mutation carriers.

NOTCH3

CADASIL syndrome

Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is a hereditary, progressive, systemic arterial vessel disease characterized by the presence of recurrent subcortical infarctions, migraine with aura, cognitive decline resulting in dementia, diffuse white matter lesions, and psychiatric disorders, including mood disturbances and apathy. It was first recognized as a separate disease entity in 1993.[84] To date, the minimum prevalence is calculated to be at least 4 in 100,000, but could be as high as 1 in 10,000.[85] It is recognized as the most common cause of inherited stroke and vascular cognitive impairment in adults.[86] CADASIL has an age of onset ranging from young to middle-aged adulthood. Large phenotypic variability can be observed both between and within families, without the presence of clear genotype-phenotype correlations.

CADASIL is caused by mutations in *NOTCH3*[87] and is pathologically defined by an accumulation of granular osmiophilic material (GOM) in VSMC, which show a progressive degeneration. GOM is located in the extracellular space, close to the surface of VSMC. The presence of GOM can be confirmed on a skin biopsy. On brain magnetic resonance imaging, extensive hyperintensities of the

white matter in the periventricular region, external capsule, and anterior part of the temporal lobes are observed in the vast majority of patients, even 10-15 years before clinical symptoms, like cognitive decline, arise.[88,89] The presence of subcortical infarcts and leukoencephalopathy are pathognomonic for the diagnosis of CADASIL.

The clinical diagnosis of CADASIL is made based upon the combined occurrence of distinctive brain MRI abnormalities, AD family history of stroke or dementia, and unexplained cerebral ischemic events and/or cognitive decline at a relatively young age. This clinical diagnosis can be confirmed by the identification of a pathogenic mutation in the *NOTCH3* gene. If no pathogenic mutation or a variant of uncertain significance (VUS) is observed, a skin biopsy is required to confirm the clinical diagnosis.[86] Skin biopsies present with typical vessel wall abnormalities, which include the presence of GOM and a positive NOTCH3 immunostaining. This immunostaining of the vessel wall for the extracellular domain of NOTCH3 is highly sensitive and specific for CADASIL.[90]

NOTCH3 is predominantly expressed in VSMC of the small arteries,[91,92] where it plays an important role in maturation and differentiation.[93] An odd number of cysteines will lead to misfolding of the EGF-like domain and in turn to increased multimerization of the NOTCH3 receptor. This multimerization is the consequence of sulfhydryl crosslinking.[94] Accumulation of the extracellular domain of NOTCH3 is seen predominantly in close proximity to VSMC in the vessel wall[91] and will lead to degeneration of the VSMC, due to a direct or indirect toxic effect.[95,96] Recently, it has been suggested that CADASIL-causing NOTCH3 mutations lead to disease through a neomorphic or gain-of-function (GOF) effect, rather than LOF, because most mutated NOTCH3 receptors are still able to activate downstream signalling.[97,98] It is hypothesized that the mutant protein will form novel protein-protein interactions, which will in turn lead to the disease phenotype. Even though arteriopathy in CADASIL is systemic, the small cerebral and leptomeningeal arteries are most severely affected.[86] Affected arteries show thickening of the arterial wall, which is accompanied by lumen stenosis, destruction of VSMC, and abundance of extracellular matrix proteins.[99] Consequently, an

impaired cerebrovascular reactivity and a decreased cerebral blood flow and metabolism develop and this will result in the ischemic events observed in this disease.[100] These events correlate with the cognitive decline, motor disability, cortical atrophy, and apoptosis of the neurons. Conversely, the pathogenesis of migraine with aura and mood disturbances remains largely unknown.

Several mouse models of CADASIL have been developed, which shed some light on the pathogenesis of this disease. A knock-in (KI) model of the p.Arg142Cys mutation, which causes disease in human, does not develop any disease phenotype,[101] while ectopic overexpression of mutant Notch3 results in the development of GOM deposits and recapitulates many of the phenotypic features of CADASIL in humans.[102] While hypomorphic mutations in humans do not cause CADASIL, Notch3-null mice do show arteriopathy and some disruption of VSMC in cerebral arteries.[103,104] Supporting the GOF hypothesis is the identification of several new binding partners of mutant Notch3, including tissue inhibitor metalloproteinase 3 (TIMP3) and vitronectin. These proteins seem to colocalize at sites where the extracellular domain of Notch3 aggregates. TIMP3, which inhibits degradation of the ECM by matrix metalloproteinases, has also been observed to be upregulated in vessels of CADASIL patients.[95] Increased inhibition may correspond with the thickening and fibrosis of the arteriole wall. As these studies were not able to rule out any of the possible disease causing mechanisms (i.e. GOF or LOF), additional studies are required to pinpoint the exact pathogenic mechanisms.

To date, over 200 distinct pathogenic CADASIL mutations have been described in *NOTCH3*.[105] These mutations lead to an odd number of cysteines in one of the 34 EGF-like domains of the extracellular region of the receptor. Several mutational hotspots in *NOTCH3* have been suggested for CADASIL. However, these are likely the result of founder mutations that segregate within specific populations, as these hotspots seem to vary between populations. It is debated whether non-cysteine variants also cause CADASIL. In several patients, variants for which the pathogenicity is unclear, have been described. These variants are mostly LOF or missense that do not involve a cysteine residue. They can only be considered pathogenic if all EGF-like domains have been screened for mutations and GOM

are visible on skin biopsy.[105] For variants that do not associate with GOM on a skin biopsy, further research is necessary to determine whether these variants are pathogenic. All rare, published non-cysteine NOTCH3 variations associated with CADASIL are depicted in Figure 4. A clustering can be identified within the first seven EGF-like domains. However, the significance of this clustering remains unclear.

Discussion

NOTCH signalling is important in the development of many organs. Over the past few decades, the NOTCH signalling pathway has been shown to be particularly important for cardiovascular development. Therefore, it is not surprising that mutations in genes encoding NOTCH signalling components lead to various types of cardiac and vascular disorders. Mutations in three out of the four NOTCH receptors are linked to cardiovascular disease in humans. NOTCH4 is the only NOTCH receptor for which an association with congenital cardiovascular anomalies has not yet been established.

NOTCH1 mutations are linked to a wide range of phenotypes: AOS, severe CHD and BAV/TAA. AOS patients, characterized by the presence of ACC and TTLD, show frequent involvement of cardiovascular disease. Similar cardiovascular malformations are observed in NOTCH1 mutation carriers without any ACC or TTLD involvement. However, since ACC and/or TTLD manifestations can be very subtle, *NOTCH1* patients with CHD require a closer look to rule out (subtle) involvement of AOS features.

By comparing the different mutations identified for (severe) LS-CHD, and AOS, one preliminary genotype-phenotype correlation emerged. EGF-like domains 11-13 have been identified as hotspot mutational region for AOS.[49] So far, no mutations within this region have been observed as a cause of BAV or LS-CHD only. Outside of this ligand binding region, there seems to be no difference in the type or location of AOS- or CHD-causing mutations.

NOTCH1 truncating variations have been found in both CHD and AOS. We believe that these obvious LOF mutations are the only convincing *NOTCH1* mutations in BAV/CHD, whereas the causality of the hitherto reported *NOTCH1* missense mutations in BAV/CHD patients may be questioned. Using patient derived induced pluripotent stem cells, some functional evidence was gathered for the causative potential of compound heterozygous *NOTCH1* missense mutations in a patient with HLHS.[34] It is hypothesized that rare monogenic causes of BAV/CHD exist, which is demonstrated by the fact that severe LOF mutations often segregate within families.[21,39] However, in view of the fact that the majority of reported missense *NOTCH1* variants have a rather high frequency in public databases, are not demonstrated unequivocally to be LOF mutations, and show marked decreased penetrance, BAV/CHD is most likely oligogenic in nature. The multifactorial nature could be the result of an interplay between multiple genetic variations, epigenetic factors, tissular second hits,[63] and environmental factors, such as hemodynamic shear stress, which is the consequence of altered blood flow over the abnormal valve. Altered expression levels of the genes of interest and their interaction partners could provide another source of phenotypic modification. An integrated approach is necessary to reveal the underlying mechanisms and to provide an individualized risk assessment.

Interestingly, in some *NOTCH1* families, both individuals with AOS only and CHD only have been described.[106] Moreover, identical mutations have been found in unrelated AOS and CHD cases as well,[39,49] further demonstrating that the same primary mutation can lead to different phenotypic outcomes. Both phenotypes may thus be part of a disease spectrum and not necessarily represent distinct phenotypes. The difference in phenotype may be the consequence of tissular second hits, modifier genes, (epi)genetic alterations and environmental factors. Even though no AOS-specific features were observed in the LS-CHD cohort, this does not rule out that both diseases are part of the same spectrum, as segregation analysis in AOS patients has revealed incomplete penetrance in addition to significant variability in clinical expression. For example, some individuals carrying a *NOTCH1* mutation do not present with TTLD. Furthermore, ACC can be present in various degrees and one could imagine that minor skin findings are easily missed in patients presenting with LS-CHD.

Further research is necessary to pinpoint the mechanisms discriminating *NOTCH1*-related AOS from isolated cardiovascular disease. Recent work has demonstrated that two ligands of the Notch receptor (Dll1 and Dll4) are able to activate distinct targets through the same Notch receptor. This discrimination is made based upon activation dynamics, whereby Dll1 induces a pulsed Notch activation and Dll4 induces a sustained activation.[107] Therefore, activation dynamics adds an extra layer of complexity to the Notch pathway and could be important in the development of distinct phenotypes.

When comparing mutations between the different NOTCH receptors, it is striking that the disease-causing mechanism in NOTCH1 and NOTCH2 is similar, while this differs from NOTCH3. CHD/AOS and Alagille syndrome are likely the result of a LOF mechanism. In contrast, GOF or a neomorphic function is the likely cause of disease for NOTCH3 mutations. Interestingly, cysteine-creating or replacing mutations in either NOTCH1 or NOTCH2 give rise to a LOF, while these types of mutations seem to give rise to GOF in NOTCH3. This difference could be due to different spatial and temporal expression of the receptors and the resulting different interaction partners at hand.

In conclusion, research during the recent decades has provided many insights into the development of cardiovascular disease caused by mutations in the NOTCH receptors. However, many aspects of the disease pathogenesis remain unexplained. Further research is necessary to explain how mutations within the same gene lead to different phenotypes and to gain more insight into disease pathogenesis, in order to develop more personalized preventive therapeutic applications.

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Figure legends

Figure 1: Canonical NOTCH signalling.
An overview of canonical NOTCH signalling. NOTCH ligands include JAG1, JAG2, DLL1, DLL3, and DLL4 and NOTCH receptors are represented by NOTCH1, NOTCH2, NOTCH3, and NOTCH4. HDAC, histone deacetylase complex; HES, hairy and enhancer of split; HEY, HES-related with YRPW motif; MAML1, Mastermind-like protein 1; NICD, NOTCH intracellular domain and RBPJ, Recombining binding protein suppressor of hairless.

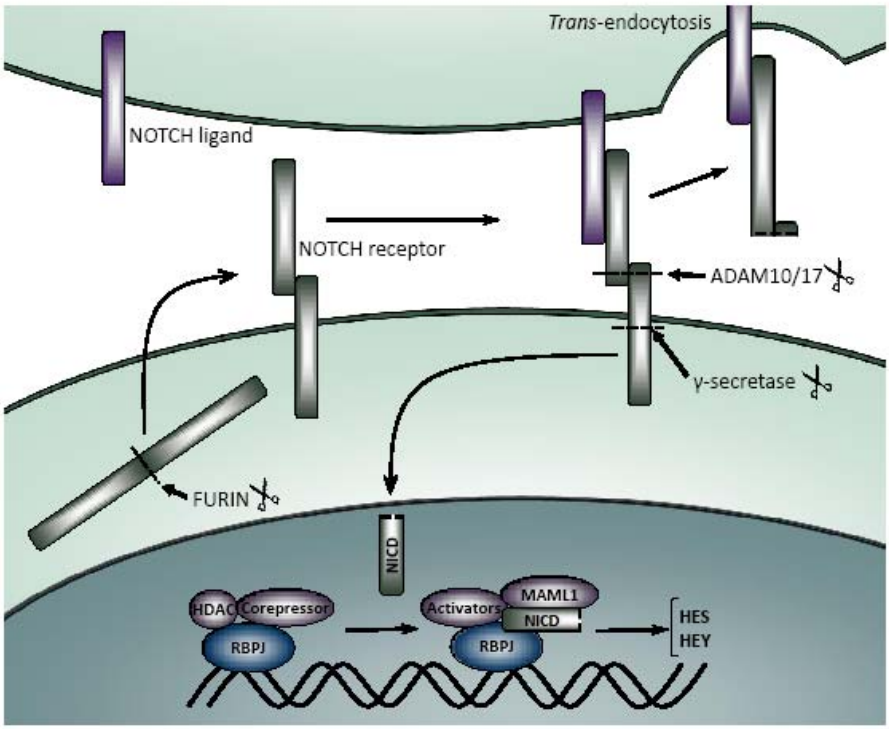

Figure 2: NOTCH1 mutations in LS-CHD, BAV, and AOS.
An overview of NOTCH1 LOF mutations in LS-CHD, BAV, and AOS. EGF 11-13 highlights the ligand-binding domain. ANK, ankyrin; AOS, Adams-Oliver syndrome; BAV, bicuspid aortic valve; cbEGF-like, calcium-binding Epidermal Growth Factor-like domain; ECD, Extracellular domain; EGF-like, Epidermal Growth Factor-like domain; ICD, Intracellular domain; LS-CHD, left-sided congenital heart disease; LNR, Lin-12/Notch repeat; RAM, RBP-Jkappa-associated module; TAD, transcriptional activation domain and PEST, proline (P), glutamic acid (E), serine (S), and threonine (T)-rich peptide sequence; TM, transmembrane domain.

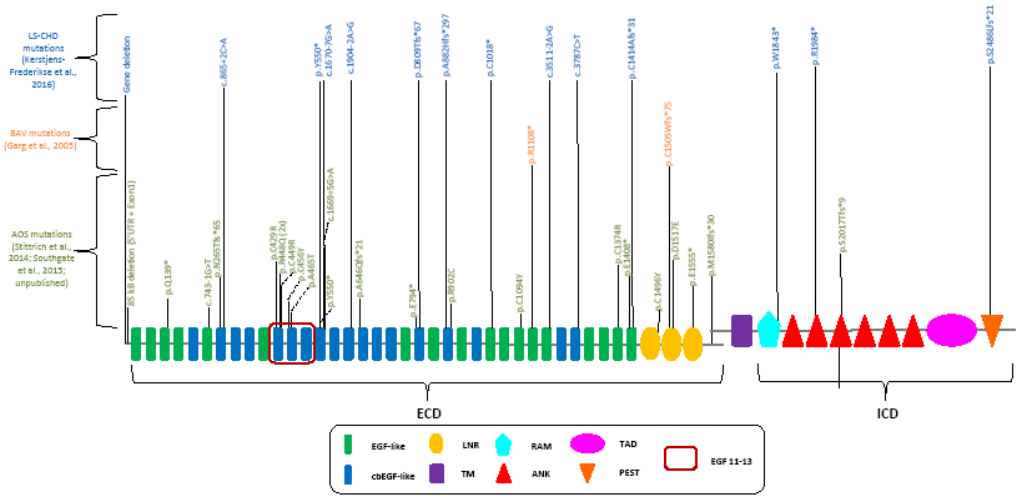

Figure 3: NOTCH2 mutations in Alagille syndrome.
An overview of all NOTCH2 mutations causing Alagille syndrome described in literature. ECD, Extracellular domain; ICD, Intracellular domain; EGF-like, Epidermal Growth Factor-like domain; cbEGF-like, calcium-binding Epidermal Growth Factor-like domain; LNR, Lin-12/Notch repeat; TM, transmembrane domain; RAM, RBP-Jkappa-associated module; ANK, ankyrin; TAD, transcriptional activation domain and PEST, proline (P), glutamic acid (E), serine (S), and threonine (T)-rich peptide sequence.

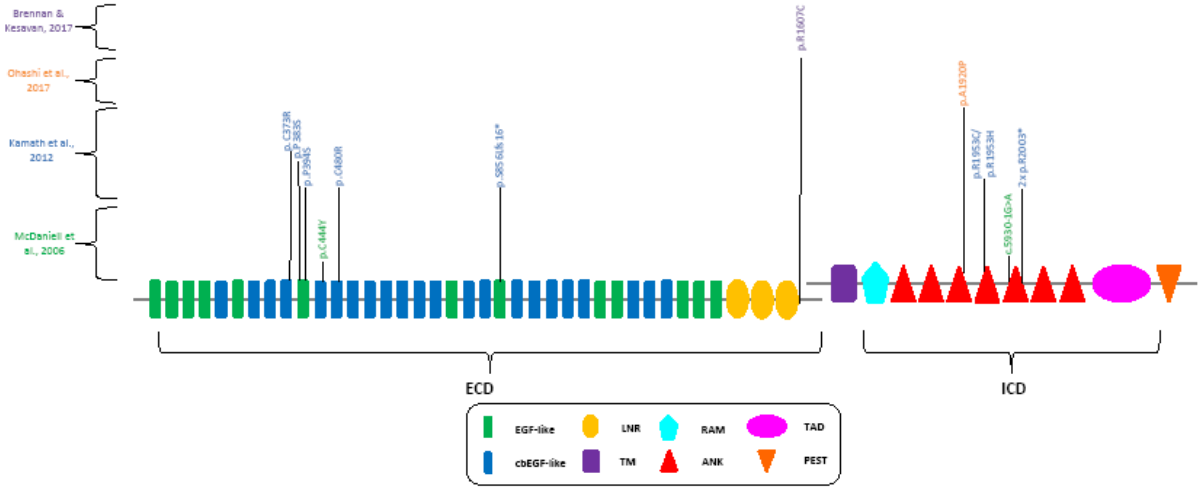

Figure 4: Cysteine sparing NOTCH3 variants in CADASIL syndrome.
Cysteine sparing NOTCH3 variants reported in literature. Variants absent from gnomAD are depicted in green. Variants with a minor allele frequency < 0.01 in gnomAD are depicted in black. Variants reported as polymorphisms by Rutten et al. (2004) are depicted in red. ANK, ankyrin; CADASIL, Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy; cbEGF-like, calcium-binding Epidermal Growth Factor-like domain; ECD, Extracellular domain; EGF-like, Epidermal Growth Factor-like domain; ICD, Intracellular domain; LNR, Lin-12/Notch repeat; PEST, proline (P), glutamic acid (E), serine (S), and threonine (T)-rich peptide sequence; RAM, RBP-Jkappa-associated module; TM, transmembrane domain, and ?, unknown.