Genetics of congenital heart disease

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Genetics of congenital heart disease

Erfelijkheid (genetica) van aangeboren hartziekten

(met een samenvatting in het Nederlands)

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Contents

CHAPTER 1

Introduction and outline of this thesis

Introduction: genetics of congenital heart defects

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Introduction

The first reference in history to the presence of congenital heart defects comes from a Babylonian tablet which dates back to around 4000 BC. The description mentions: "When a woman gives birth to an infant that has the heart open and has no skin, the country will suffer from calamities", which might refer to ectopia cordis [1]. Leonardo da Vinci then was the first to describe a congenital heart defect (atrial septal defect) in humans in his Quaderni de Anatomia [1] (Figure 1).

As a group congenital heart defects are the most common birth defect and occur in approximately 4-13 per 1000 live births (excluding bicuspid aortic valves and minor self resolving ventricle septal defects) [2]. This is certainly an underestimation of the total incidence of congenital heart disease, as the incidence of congenital heart disease is much higher in fetuses that die prenatally [3]. Since many life born children with congenital heart defects nowadays survive into adulthood, congenital heart defects are more frequently seen in adults which require specialized care (reproductive issues, heart failure, arrhytmias etc).

Figure 1.

Figure 1. *Leonardo da Vinci, drawing of atrial septal defect in his Quaderni de Anatomia II published in 1513. The inscription read right to left: "I have found that a, left auricle, to b, right auricle, a perforating channel from a to b, which I not here to see whether this occurs in other auricles of other hearts (Rashkind WJ: Historical Perspective of Pediatric Cardiology. Pediatric Cardiology 1979; 63-71).*

Reproductive loss is common and a high proportion of early fetal loss is associated with chromosomal defects (20-70%), which often cause congenital heart defects [4-6]. The most common congenital heart defects at birth are ventricular septal defect (26-50.5%), atrial septal defect (3.4-14.3%), pulmonary stenosis (2.4-13.5%), tetralogy of Fallot (2-10.4%) and coarctation of aorta (2-9.8%) [2, 7].

Since a long time, congenital heart defects have been considered multifactorial in origin. Special effort has been put into identifying modifiable risk factors for risk reduction. Identified modifiable risk factors are for example maternal diabetes (strict glucose control during pregnancy), maternal infections, and therapeutic drug use such as anticonvulsants. Several reports suggest that maternal multivitamin use, including folic acid, is associated with a reduction of congenital cardiovascular defects (for review see Jenkins et al. Circulation 2007) [8-13]. As a result, the American Academy of Pediatrics has made specific recommendations for prospective parents to reduce the risk for having a child with a congenital heart defect [12]. They recommend to take a multivitamin with folic acid daily, optimize preconception and prenatal care with specific attention to detection and management of maternal illness (diabetes, rubella vaccination etc.), discuss any medication use, avoidance of contact with people with febrile illnesses and avoidance of exposure to organic solvents [12]. As maternal smoking and maternal alcohol use have been found to substantially increase the relative risk for an infant with a congenital heart defect, soon-to-be mothers should be advised to refrain from these adverse habits [14-17].

Although environmental factors do play a role in the pathogenesis, the prevalence of heart defects among family members of patients with isolated congenital heart disease is higher than expected (2-16% vs 0.4-0.8%). Heritability estimates (percentage of phenotype explained by inherited factors) suggest that a genetic component is very likely to contribute [18-22]. Sanchez-Cascos et al. 1978, demonstrated that 50-95% of 1148 isolated cases of congenital heart disease could be explained by heritable factors [22]. The recurrence risk for a sibling or offspring of an affected patient to have a congenital heart defect varies between specific cardiac defects, but the overall recurrence risk for non-mendelian inherited congenital heart defects lies between 3-15% (Table 1) [23, 24]. Surprisingly, for still unknown reasons, congenital heart defects occur more frequently in offspring when the mother is affected than when the father is affected.

Table 1.

Recurrence risks of congenital heart disease in offspring			
Type of Heart disease	**Total risk**	**Mother affected**	**Father affected**
Acyanotic congenital heart disease			
• Atrial septal defect	3-5%	4.5-6%	1.5-3.5%
• Ventricular septal defect	4-8%	6-9.5%	2-3.6%
• Atrio-ventricular septal defect	10-15%	7.5-15%	1-7%
• Patent ductus arteriosus	3-4%	4%	2%
• Pulmonary Stenosis	4%	5.3-6.5%	2-3.5%
• Left ventricular obstruction	11-15%	10-11%	3%
• Coarctation of aorta	6%	4-6.3%	2.5-3%
Cyanotic congenital heart disease			
• Tetralogy of Fallot	2.2-3.1%	2-2.5%	1.5-2.2%
• Transposition of great vessel	0.5%		
Mendelian disorders			
• Holt-Oram syndrome	50%	50%	50%
• Noonan syndrome	50%	50%	50%
• Marfan syndrome	50%	50%	50%

For clinicians it is important to identify whether a congenital heart defect has an underlying genetic component. Congenital heart disease is frequently associated with syndromes, which can be recognized by other dysmorphic features. In certain syndromes associated with congenital heart defects, other organ systems can be involved as well. An example is the 22q11.2 deletion syndrome (DiGeorge's syndrome/ velo-cardio-facial syndrome). Children with 22q11.2 deletion syndrome most often have intellectual disabilities and dysmorphic characteristics are usually minor (may be very difficult to detect especially in infancy). Frequently outflow tract defects (conotruncal heart defects) such as tetralogy of Fallot are observed in these children [25-28]. Often, these children are immune compromised because of thymus hypoplasia [25], therefore requiring irradiated erythrocytes after cardiac surgery in order to prevent graft versus host reaction. The

Table 2. Gene mutation and phenotype

Gene	Chromosomal location	Cardiac defect
ALK2	2q23-q24	Primum type ASD, MVP [30, 31]
BMPR2	2q33	AVSD, ASD, PDA, PAPVR + PAH [32]
CFC1	2q21.1	Heterotaxia, TGA, DORV, common AV canal, AA hypoplasia, pulmonary artresia, DIRV [33, 34]
CITED2	6q23.3	TOF, VSD, ASD, anomalous pulmonary venous return, RVOT obstruction [35]
CRELD1	22p13	AVSD, cleft mitral valve, ASD type I, heterotaxy [36, 37]
ELN	7q11.2	Supravalvular AoS [38]
FOG2	8q23	TOF [39]
GATA 4	8p23.1-p22	ASD, AVSD, pulmonary valve thickening, insufficiency of cardiac valves [40, 41]
JAG1	20p12	TOF, VSD with aortic dextroposition, PPS [42]
KRAS	12p12.1	ASD, VSD, valvular PS, HCM, HOCM, MVP, TVP, LVH [43]
MYH6	14q12	Secundum ASD [44]
NKX2.5	5q34	ASD, VSD, TOF, AoS, VH Pulmonary atresia, Mitral valve anomalies, conduction disturbances [45, 46]
NKX2.6	8p21	TA [47]
NOTCH1	9q34.3	Bicuspid aortic valve, mitral valve stenosis, TOF, VSD [48, 49]
PROSIT240	12q24	TGA [50]
TBX1	22q11.2	Interrupted aortic arch, TA, other aortic arch anomalies [51]
TBX5	12q24.1	ASD, AVSD [52]
ZIC3	Xq26.2	TGA, DORV, ASD, AVSD [53]

AA: aortic arch, AoS: aortic stenosis, ASD: atrial septal defect, AV: atrioventricular, AVSD: atrioventricular septal defect, DIRV: double inlet right ventricule, DORV: double outlet right ventricle, HCM: hypertrophic cardiomyopathy, HOCM: hypertrophic obstructive cardiomyopathy, MVP: mitral valve prolapse, PAH: pulmonary artery hypertension, PAPVR: partial anomalous venous return, PDA: patent ductus arteriosus PPS: peripheral pulmonic stenosis, PS: pulmonary stenosis, RVOT: right ventricle outflow tract, TA: truncus arteriosus, TGA: transposition of great arteries, TOF: tetralogy of Fallot, TVP: tricuspid valve prolapse, VSD: ventricular septal defect, VH: ventricular hypertrophy

presence of an underlying syndrome, such as a 22q11.2 deletion syndrome, may influence prognosis. Children with a microdeletion 22q11.2 and congenital heart defects are at relatively high risk for mortality and morbidity, as determined by both the severity of the cardiac lesions and the extracardiac anomalies associated with the microdeletion [29]. Moreover, establishing a syndrome diagnosis can change follow-up protocols. For example,

special attention should be paid to the follow up patients with Turner syndrome. These patients have a relatively high risk of aortic aneurysm, coarctation of the aorta, aortic valve disease and coronary artery disease and should be monitored regularly. Additionally, identifying a syndrome or isolated genetic defect will have implications for reproductive risk (genetic counseling) and for family members (screening for genetic defects and/or cardiac anomalies). In summary, diagnosing a syndrome as the underlying cause of a heart defect can have implications for treatment and prognosis of the patient and the change on recurrence.

Congenital heart defects and monogenic disease

In isolated congenital heart disease a monogenic cause is rarely found. Only few families demonstrate clear Mendelian inheritance patterns. In these families an autosomal dominant pattern or, rarely, autosomal recessive inheritance patterns are found. In families with an autosomal dominant inherited congenital heart defect, genetic research has been able to identify several mutations in genes important for the occurrence of congenital heart defects such as atrial septal defects and bicuspid aortic valves (further discussed below) [40, 41, 45, 48, 49]. Penetrance in these families seems to be incomplete and these single gene mutations demonstrate a high phenotypic variability (pleiotropy) (Table 2). Conversely, mutations in different genes may also cause identical phenotypes, demonstrating the genetic heterogeneity of cardiac defects (Table 2). A possible explanation for this phenomenon comes from animal studies [54]. Cardiac development is controlled by multiple genetic pathways. Even in relatively small steps of development, for example in endocardial cushion formation important to cardiac valve development, numerous genetic pathways are involved. Disturbances in each single element of a such a pathway, such as ligands, receptors and transcription factors, could theoretically cause similar cardiac defects.

As the population frequency of recessive genetic mutations associated with congenital heart defects is small, recessive disease occurs rarely and therefore is difficult to identify. Only in regions were consanguinity is common, recessive disease has been found more frequently. Persistent Ductus Arteriosus (PDA) is such an example. PDA occurs. generally sporadically, but in an Iranian population PDA was mapped to a recessive locus at 12q24 [55].

Figure 2.

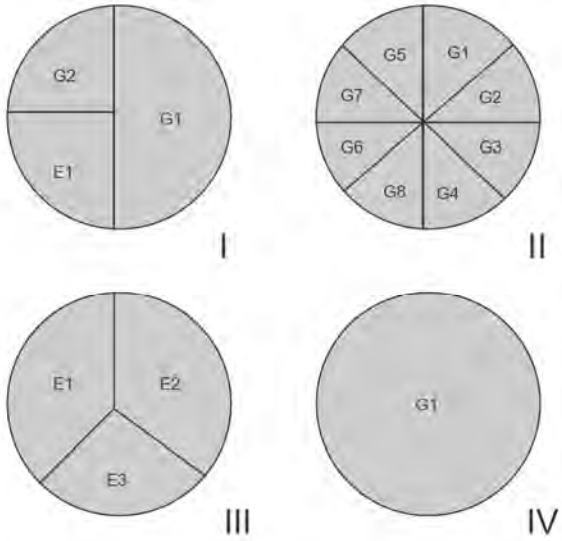

Figure 2. Sufficient cause model. *Constellation of components that combined has a sufficient cause to develop a congenital heart defect. G: genetic variation, E: environmental factor or exposure. Different combinations can lead to a sufficient cause. For example in the first tart a combination of 2 genetic variants and 1 environmental factor (e.g. maternal diabetes or no folic acid intake) are sufficient to cause the development of a heart defect while in the second tart different genetic variations altogether is sufficient to cause congenital heart disease. Also a combination of only environmental factors could lead to congenital heart disease as depicted in tart III. Of course for disease causing mutations only one component is sufficient to cause the defect as depicted in tart IV.*

Complex role of genes in congenital heart defects

Most of the isolated congenital heart defects do not show a typical Mendelian inheritance pattern, but as mentioned earlier a genetic component is very likely to contribute. This can be explained by applying the polygenic threshold theory for discontinuous traits. Consider a very large number of genes that are associated with heart development. Variation in all these genes may have relatively small deleterious or protective effects on the risk of congenital heart disease. All these effects added up create a certain individual disease susceptibility. This susceptibility follows a normal (Gaussian) distribution in the population. Embryos whose susceptibility exceeds a critical threshold will develop a congenital heart defect; those, whose susceptibility is below the threshold, will not. Affected people have inherited an unfortunate combination of risk-susceptibility genes. Their relatives who share may of these unfavorable genetic variants with them will therefore, as a group, also have an above average susceptibility but will, usually, not exceed the treshold. In line with such a multifactorial model in a recent study Roesler et al.

reported 35 different genetic variations or single nucleotide polymorphism (SNP's) in three genes of from the Nodal pathway in 375 unrelated individuals with left-right asymmetry cardiac defects [56]. They detected only a few pathogenic mutations, but they did find functionally relevant common SNP's more frequently than expected. In addition, in several patients they found more than one functionally relevant SNP. These common SNP's could theoretically act as unfavorable variants adding to the susceptibility for congenital cardiac defects. Therefore, in most patients, cumulative impairment of a genetic pathway by several minor variants with a small effect seems more likely to explain the congenital heart defect than a single gene mutation [56].

A multifactorial genesis congenital heart defects is also in line with the epidemiological principles of the sufficient cause model described by Dr. Kenneth Rothman [57]. A sufficient cause is a complete causal mechanism or a minimal set of conditions (components) and events, that together are sufficient for the outcome, in this case a congenital heart defect (Figure 2). Each component (for example a certain genetic variant) itself is unable to cause the defect but when acting together with other component causes (e.g. other genetic variants or environmental factors) will result in the development of a congenital heart defect [57]. There are several combinations of different components possible to cause a congenital heart defect and thus several sufficient causes.

Genetic variation may in addition alter the response to our environment. If the genetic susceptibility is already high, only small additional environmental factors may induce disease while in persons with low genetic susceptibility it may not. This is demonstrated in infants carrying the Reduced Folate Carrier (*RFC1* gene) polymorphism. A study of Shaw et al. investigated whether an interaction existed between this SNP and maternal use of vitamin supplement containing folic acid on risk of conotruncal heart defects (and orofacial clefts). They found that infants who carried the *RFC1* G80 polymorphism where at increased risk (1.6- 2.3 fold) of developing conotruncal heart defects. When taking into account maternal vitamin use (including folic acid), a substantially higher risk was observed for infants carrying the *RFC1* G80 polymorphism, whose mothers were nonusers of vitamin, versus infants both with and without the *RFC1* G80 polymorphism, whose mother did use vitamins. Moreover, infants with the *RFC1* A80 polymorphism seemed not at increased risk for conotruncal heart defects, even when their

mother did not use vitamins (protective effect). This example nicely demonstrates how genetic variation may interact with environmental factors [58].

Isolated congenital heart defects

As described above, only in a minority of isolated congenital heart defects autosomal dominant inheritance patterns have been identified. Below, a description is given of some of the isolated congenital heart defects in which specific genes have been implicated in a Mendelian fashion.

Bicuspid aortic valves

The prevalence in the general population of a bicuspid aortic valve (BAV) is estimated to be 0.5-1.3% [59-61]. Bicuspid aortic valves progress more rapidly into regurgitation or stenosis of the valve [62]. This results in a higher occurrence of aortic valve replacement, especially at a younger age [63]. Patients with a bicuspid aortic valve are also more prone for endocarditis and ascending aorta dilatation which may ultimately result in aortic dissection or rupture (aortic dissection occurs 9 times more often with BAV than with tricuspid aortic valves) [64-66]. Although, infective endocarditis is more observed in patients with a bicuspid aortic valve the last task force criteria from the American Heart Association (2007) have changed their recommendations for endocarditis prophylaxis [67]. They shifted their recommendation from all antibiotic prophylaxis for all patients with an increased lifetime risk for developing infective endocarditis to only patients with an underlying cardiac condition that increases the risk of an adverse outcome from infective endocarditis, which does not include bicuspid aortic valves [67]. The European guidelines (2004) still recommend antibiotic prophylaxis for patients with a bicuspid aortic valve, but it's suspected the European guidelines will adopt the American guidelines, when they're revised [68]. Patients with BAV are more likely to die because of cardiac death, aortic syndromes and sudden unexplained death, even after aortic valve replacement (compared with patients with a tricuspid aortic valve and aortic vale replacement) [69]. Therefore, early identification of patients with a bicuspid aortic valve is warranted.

Pedigree analysis suggests two types of inheritance patterns: autosomal dominant inheritance with reduced penetrance and a non-mendelian pattern of inheritance. In 36.7% of families with a bicuspid aortic valve patient, at least one additional family member can

be identified with such a condition [70]. In a later report heritability of a bicuspid aortic valve was estimated to be 0.89 (89 % of the bicuspid aortic valve cases is explained by inherited factors) [71]. Family-based genome-wide linkage analysis linked several loci with BAV such as a locus on chromosome 18 (LOD score 3.8: this means the logarithm of the odds ratio in this case indicating the odds of ~6300:1 that the disease gene in this family is linked to this locus). Other interesting loci are on chromosome 9q34-35 and on chromosome 15q15-21 and 13q33-qter (suggestive linkage) [48, 72]. Thus, the origin of a bicuspid aortic valve displays genetic heterogeneity. Until now two studies have found mutations in the *NOTCH1* gene (chromosome 9q34) in either sporadic patients or familial bicuspid aortic valve disease [48, 49]. NOTCH1 encodes a transmembrane receptor and is important for cellular differentiation, proliferation and apoptotic programs during embryogenesis and could therefore influence organ formation and morphogenesis [73]. In approximately 4% of patients with a bicuspid aortic valve, mutations in *NOTCH1* are implicated [49]. A bicuspid aortic valve can also occur in a family as part of a cluster of left ventricular outflow tract abnormalities, including hypoplastic left ventricle, aortic stenosis, coarctation of the aorta, mitral valve stenosis, supra valvular mitral valve stenosis and a hypoplastic aortic arch (the shone complex) [74]. As in most other familial isolated congenital heart defects, the phenotypic variability within families is considerably high.

Atrial septal defect (type secundum)

Secundum type atrial septal defect (ASD II) is a common congenital heart defect (3.4-14.3% of all CHD) and frequently occurs sporadic [2, 75]. However, some families have been identified with an autosomal dominant inheritance pattern of ASD II both with and without conduction abnormalities [76-79]. Schott et al. was the first to link ASD II with atrioventricular (AV) conduction delay with mutations in *NKX2.5* (Chromosome 5q34) [45]. NKX2.5 is a cardiac homeobox protein which plays a critical role in cardiac-specific gene expression and is essential for cardiac differentiation [80]. In four families described in the literature, all carriers of the *NKX2.5* mutations had AV conduction delay and 27 of 33 had an ASD. Other structural heart malformations identified in carriers included ventricular septal defects, tetralogy of Fallot, pulmonary atresia and subvalvular aortic stenosis (Table 2). Garg et al. and Okubo et al. identified loss of function mutations in *GATA4* (chromosome 8p23.1-p22) -a zinc-finger transcriptional factor that controls cardiac specific gene expression and modulates cardiogenesis- segregating in 3 families with ASD II

without conduction abnormalities [40, 41, 81]. Although, in these kindreds ASD II was the most common cardiac malformation, also atrioventricular septal defects, ventricular septal defects, pulmonic stenosis and persistent ductus arteriosus were observed (Table 2). Though the mutations in *GATA4* explained the congenital heart defects observed in these families, only in a small percentage (0.5-3%) of ASD patients GATA4 mutations are detected [30, 82].

Additionally, mutations in *TBX5* have been found to be associated with ASD. *TBX5* is located on chromosome 12q21.3-q22 and has an essential role in cardiac specification and morphogenesis [83]. Mutations in *TBX5* were first found responsible for the Holt-Oram syndrome, a developmental disease affecting limbs and heart (described below) [84]. Examination of heart tissue from 68 formalin fixed hearts of patients with complex heart disease (including ASD) identified 2 recurrent mutations in *TBX5* in the hearts of 16 unrelated patients with ASD [52]. It should be taken into account that these mutations were found in heart tissue and not in peripheral blood lymphocytes. This likely implies that these mutations are somatic mutations and not germ line mutations. Somatic mutations are acquired in a somatic cell, e.g. a cardiac progenitor cell, and passed on to the progeny of this mutated cell, resulting for example in mutations only in the heart and not in other organs/ tissues. Somatic mutations are frequently caused by environmental factors such as radiation, UV light and chemical agents, but can also occur spontaneously when aging. Germ line mutations on the other hand, are inherited genetic alterations that occur in the germ cells and can be passed on to offspring. Somatic mutations have also been described in *NKX2.5* in patients with ASD II [85].

Concluding: ASD II is a complex genetic heterogeneous disease (several genes are associated with one defect). Although most often the defect is isolated, a genetic component should be considered. If either a first degree relative or two or more second degree relatives are also known with a congenital heart defect, referral for further genetic analysis and counseling may be appropriate. Both a complex inheritance pattern and autosomal dominant inheritance pattern have been described. In addition, ASD II might be part of a syndrome. If suspicion of a syndromal or chromosomal disorder should arise, based on developmental or unexplained growth delay, or the presence of dysmorphic features, referral for syndrome diagnostics is indicated. In any case a thorough family history for heart defects should be obtained.

Atrioventricular septal defect

Atrioventricular septal defects (AVSD) originate from an abnormal or inadequate fusion of the superior and inferior endocardial cushion with the atrial and muscular portion of the ventricular septum and can be divided in complete AVSD and partial AVSD. Of all live born children with a congenital heart defect, approximately 2.0-8.6% have an AVSD [2, 86]. Digilio et al. investigated the prevalence of congenital heart defects among relatives of patients with non-syndromic AVSD and found in circa 12% of pedigrees one or more relatives with concordant or discordant congenital heart disease [87]. Congenital heart disease occurred in 1.9% of the 206 parents of an AVSD proband, in 3.6% of the 111 siblings and in 0.8% of the 644 second degree relatives [87]. Up to now, two genetic loci have been associated with isolated AVSD. First, analysis of a large pedigree consisting of 14 affected individuals with AVSD, demonstrated linkage with a locus on chromosome 1 (1p31-21), now called AVSD1. The causative gene, however, has not yet been identified. A second locus was identified in a population of patients with 3p25 deletion syndrome, a syndrome characterized by mental retardation, growth retardation, microcephaly, ptosis and micrognathia. In a third of patients with 3p25 deletion syndrome congenital heart defects, mainly AVSD, are found [88]. Analysis of the critical region for congenital heart defects in this chromosomal syndrome led to the discovery of the second AVSD locus (AVSD2), located on chromosome 3p25.3, with *CRELD1* as the responsible gene (Cysteine-Rich protein with EGF-like domain 1) [88-91]. Mutations in *CRELD1* were subsequently identified in patients with isolated AVSD as well (Table 2). Additionally, *GATA4* mutations have been described in patients with AVSD [40, 41].

Most often AVSD is associated with chromosomal defects. The most frequently associated chromosomal defect (with both complete and partial AVSD) is trisomy 21 (Down's syndrome). In up to 76% of patients with AVSD, a chromosomal aberration of chromosome 21 is found (see below) [92]. Other syndromes associated with AVSD are for instance Ivemark syndrome, Ellis van Creveld syndrome and the CHARGE syndrome (Table 3) [93-96].

Congenital heart defects associated with syndromes

Frequently, congenital heart disease is associated with dysmorphic features, associated congenital malformations, mental retardation or unexplained growth deficits, thus suggesting a syndrome diagnosis. Syndromes can be caused by both chromosomal aberrations as well as single gene mutations. Below a description of some of the most well

known syndromes associated with congenital heart defects is given. Table 3 gives an overview of the most common syndromes associated with congenital heart disease.

Down syndrome/ Trisomy 21

Down syndrome is the most frequent form of mental retardation. It is caused by a microscopically demonstrable chromosomal 21 aberration (trisomy 21) and occurs in one of 800-1000 live births [97-99]. This syndrome is characterized by well-defined and distinctive phenotypic features including characteristic facies, minor dysmorphisms of the limbs, hypotonia and growth retardation [100]. Frequently, Down syndrome is associated with other specific congenital malformations such as Hirschprung's disease and duodenal abnormalities [100]. The incidence of congenital heart defects in Down syndrome is about 40-60%, most frequently atrioventricular septal defects (AVSD) and ventricular septal defects (VSD) [92, 101].

The risk for having a child with Down syndrome increases with maternal age [97, 98, 102, 103]. Besides, it is indicated that certain maternal genetic polymorphisms of genes involved in the folate/ homocysteine metabolism or the combination of these polymorphisms can be associated with higher incidence of offspring with trisomy 21, although results from different reports have been conflicting and/or inconclusive [104-109]. Additionally it has been hypothesized and suggested in some reports that factors in the maternal grandmother such as age and altered folate/homocysteine metabolism can be implicated in chromosome 21 nondisjunction risk as well [110-114]. In 95% of Down syndrome patients three free copies of chromosome 21 are identified, in most instances caused by an error in maternal meiosis I. In most of the remaining patients, one copy of chromosome 21 is translocated to another acrocentric chromosome (robertsonian translocation) [98]. Robertsonian translocations may be the cause of familial occurrence of Down syndrome. Although chromosome analysis is often not absolutely necessary to establish a diagnosis of Down syndrome, it should be performed to determine whether there may be an increased risk for future offspring.

Though, it can be assumed that increased dosage of specific chromosome 21 genes plays a role, thus far no chromosome 21 genes responsible for AVSD have been

unambiguously identified. Importantly only a percentage of Down syndrome patients exhibit a congenital heart defect. This suggests that factors other than overexpression of genes on chromosome 21 are needed for cardiac defects to occur. These may be environmental factors or genetic variation at other loci. Specific environmental risk factors have been implicated with the occurrence of Down syndrome with the co-occurrence of a congenital heart defect such as maternal diabetes (OR20.6) and smoking, but these findings have not been confirmed in other studies [92, 145, 146]. The presence of specific gene variants could, in addition to trisomy 21, further increase susceptibility for cardiac defects. Mutations in *CRELD1* have been found in Down syndrome patients with AVSD [88, 129, 147].

Turner (Ullrich-Turner) syndrome

Turner syndrome appears in 32-50 per 100.000 females and is cytogenically characterized by the absence of one of both X-chromosomes (45,X) or a structurally abnormal X-chromosome, where part has been deleted. Clinical manifestations include short stature, specific dysmorphic features (that are not always present), primary amenorrhea, absence of secondary sex characteristics and cardiovascular malformations [148-151]. Additionally, there is an increased incidence of hypothyroidism, other congenital malformations (such as malformations of the urinary tract), deafness and fractures (osteoporotic fractures in adulthood, non-osteoporotic fractures in childhood) [152, 153].
Congenital cardiac defects occur in about 22-55% of affected individuals. Most frequently, coarctation of the aorta and bicuspid aortic valves, but also anomalous pulmonary venous drainage and other types of aortic valve disease can be found [154-156]. Not completely unexpected, the presence of cardiac lesions seems to be dependent on the karyotype. In a report of Prandstraller et al severe congenital heart defects and multiple lesions were found only in patients with a 45,X karyotype. Patients with an X-ring pattern had a higher prevalence of bicuspid aortic valves (BAV) while patients with deletions of the X-chromosome did not demonstrate any cardiac defects [155]. Specific phenotypic features may hint on the presence of congenital heart defects, such as neck webbing; 50% of patients with webbing of the neck were found to have congenital heart defects, versus 23% of the patients without neck webbing [156]. Additionally, a specific ECG pattern can be found in Turner patients. More frequently a left posterior fascicular Block and accelerated AV conduction is observed and the QTc is significantly longer (average 423 mm vs 397 ms) [157].

Table 3.

Syndrome	Chromosome	Gene	Congenital Heart Defect	Other features
Adams-Oliver syndrome	unknown	unknown	Pulmonary vein stenosis, ToF, VSD, DORV, pulmonary HT [116]	aplasia cutis congenital, vascular defects
Allagile syndrome	20p12, 1p13-p11	*JAG1, NOTCH2* [119, 120]	Pulmonic valvular stenosis, peripheral arterial stenosis	Cholestasis, skeletal abnormalities, ocular abnormalities and characteristic facies
Bindewald syndrome	Unknown; AR	Unknown	ToF, DORV with subaortic VSD and PS [117]	minor facial anomalies, failure to thrive, mental retardation
Cardio-facio-cutaneous syndrome (CFC)	7q34, 12p12.1, 15q21, 7q32, 2p22-p21	*BRAF, KRAS, MEK1, MEK2, SOS1* [121-123]	PS, ASD, hypertrophic cardiomyopathy	Similar features as NS, but more severe mental retardation + ectodermal abnormalities, friable hair, absent eyebrows
Costello Syndrome	11p15.5, 7q34, 15q21	*HRAS, BRAF, MEK1* [124-126]	Hypertrophic cardiomyopathy, VSD, PS, PDA, MVP, atrial arrhytimias [118]	Characteristic facies, short stature, distinctive hand posture and appearance, feeding difficulties, developmental disabilities
DiGeorge (VCF/ Takao/ 22q11 deletion/ CATCH22)	Microdeletion 22q11, 18q21.33, 10p13 [130, 131]	*TBX1* [127, 128]	VSD, ToF, pulmonary atresia with VSD, interrupted AA, TA, DORV, double AA, ASD	Neonatal hypocalcaemia (hypoplasia parathyroid gland) T-cell deficiency (hypoplasia thymus), low set ears, cleft palate, short stature, mental retardation, typical facies
Down's syndrome/ Trisomy 21 syndrome	Trisomy 21	AVSD associated in some cases with *CRELD1* mutations (3p25.3) [129]	AVSD, VSD, ASD	Neonatal hypocalcaemia (hypoplasia parathyroid gland) T-cell deficiency (hypoplasia thymus), low set ears, cleft palate, short stature, mental retardation, typical facies
Ellis van Creveld syndrome	4p16	*EVC1, EVC2* [96]	Common atrium	Dwarfism, polydactyly [115]

Syndrome	Chromosome	Gene	Congenital Heart Defect	Other features
Ivemark syndrome	Unknown	Unknown	Dextrocardia, ASD, VSD, PS, endocardial cushion defects, conotruncal defects	Asplenia, malposition + maldevelopment of abdominal organs, abnormal lobation of lungs or polysplenia and abnormalities of visceral lateralization
Leopard syndrome	12q24.1, 3p25	*PTPN11, RAF1* [135, 136]	PS, conduction abnormalities, left sided obstructive cardiomyopathy	Multiple lentigines, ocular hypertelorism, abn.genitalia, growth retardation, sensorineural deafness
Noonan Syndrome (NS)	12q24.1, 7q34, 12p12.1, 2p22-p21, 3p25, 15q21	*PTPN11*(~50%,), *BRAF, KRAS, SOS1, RAF1, MEK1* [43, 136-141]	PS, PDA	Short stature, facial features, high arched palate, webbed and/or short neck, curly hair, coagulation defects
Opitz/GBBB Syndrome	Xp22	*MID1* [142]	ASD, VSD, ToF, HLV, PS	Hypertelorism, telecanthus, cleft lip/ palate, genitourinary anomalies, ear abnormality
Thoraco-abdominal syndrome	Xq25-q26.1 [143]	Unknown	TGA, PDA [132]	Diaphragmatic and ventral hernia, hypoplasia of lungs, hydrocephalus, anencephaly, cleft lip, renal agenesis, hypospadias
Turner's syndrome	45, X	Unknown	Coarctation of the aorta, BAV	Growth retardation, skeletal defects, high arched palate, webbed neck, lymphoedema, ovarian failure, infertility, renal anomalies
Williams-Beuren syndrome	Deletion 7q11.3 (ELN locus) [144]	Unknown	Supravalvular AoS, multiple pheripheral pulmonary artery stenosis, MVP, BAV [133, 134]	Elfin face, mental and statural deficiency, dental malformation, infantile hypercalcemia

AA: Aortic Arch, AoS: Aortic Stenosis, AR: autosomal recessive, ASD: Atrial septal defects, AV: atrioventricular, BAV: bicuspid aortic valve, DORV: Double Outlet Right Ventricle, HLV: Hypoplastic left ventricle, HT: Hypertension, NS: Noonan syndrome, MVP: Mitral valve prolapse PDA: Persistent Ductus Arteriosus, PS: Pulmonic Stenosis, TA: Truncus Arteriosus,TGA: Transposition of Great Arteries, ToF: Tetralogy of Fallot

While Turner syndrome is a relatively common syndrome, there is a considerable delay in diagnosis of the syndrome. Although, many are ascertained at an earlier age because of short stature, it is not uncommon for these patients to come to medical attention because of primary amenorrhea (around 15 years of age) [150]. Women with Turner syndrome have an increased cardiovascular risk as a result of an increased incidence of diabetes (both insulin dependent and non-insulin dependent), hypertension and decreased estrogen levels [152, 154]. They need life-long estrogen replacement therapy starting from puberty onwards. Among Turner females ischemic heart disease and stroke are more frequently observed than in the general population [152].

All cause mortality is increased considerably in patients with Turner syndrome (standardized mortality ratio of 3) [150, 158]. Mortality risks are generally greatest in women with 45,X monosomy and in patients diagnosed young [158]. Mortality is specifically increased for circulatory diseases such as aortic aneurysm, aortic dilatation and dissection, aortic valve disease, hypertensive and ischemic heart disease and cerebrovascular disease. Therefore, the aorta and aortic valve (aortic diameter, as well as the presence of aortic valve disease) should be monitored regularly. Furthermore, primary and secondary preventive strategies should be developed for further decreasing cardiovascular risk, such as myocardial infarction and strokes, in this patient population. Next to circulatory diseases, respiratory disease mortality (mainly pneumonia) and mortality from endocrine and associated disease (especially diabetes mellitus type 2) are raised [150, 158].

Noonan syndrome

In 1962 dr. JA Noonan presented a new syndrome in 9 children with pulmonary stenosis. The syndrome was characterized by small stature, ptosis, hypertelorism, mild mental retardation and, in some instances, undescended testes and skeletal malformations [159]. Later she described the typical facies, cardiac features and clinical phenotype as resembling Turner syndrome (described above), but with normal chromosomes and occurring in both males and females [160]. Subsequently, more clinical details were identified in individuals with Noonan syndrome, including low set posteriorly rotated ears with a thick helix, deafness and pectus carinatum and excavatum, and blood clotting anomalies (Figure: picture of Noonan patient) [161]. Phenotypic craniofacial appearance changes with age. Subtle features must be searched for in parents of affected children [162]. Congenital

Figure 3.

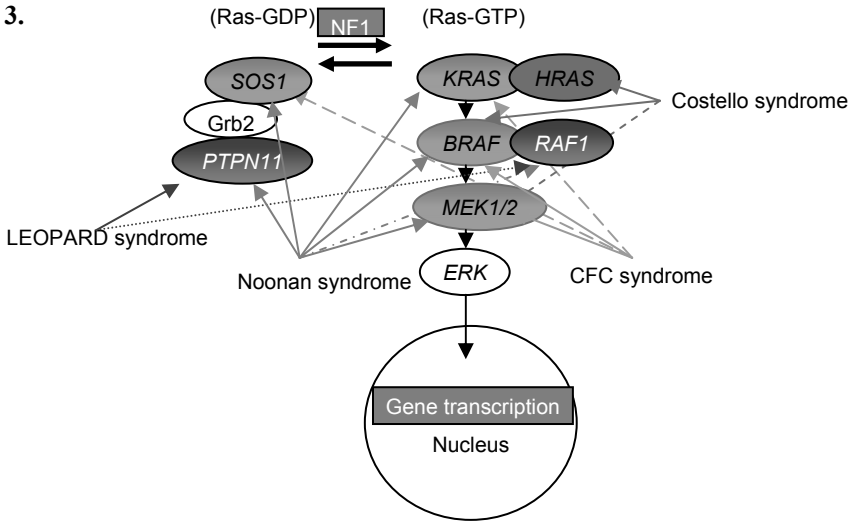

Figure 3. MAPK- pathway and associated syndromes. *Mutations in genes of the MAPK pathway have been found in individuals with Noonan syndrome, Cardiofaciocutaneous (CFC) syndrome, LEOPARD syndrome and Costello's syndrome. These syndromes phenotypically overlap and as the same genes are implicated, these syndromes probably belong to the same disease entity. PTPN11 and RAF1 can be mutated in Noonan syndrome and in LEOPARD syndrome and are shown in both grey and blue. Mutations have been found in SOS1 and KRAS in patients with both Noonan syndrome and CFC syndrome and are shown in both grey and green, whilst BRAF and MEK mutations have been found in the latter two syndromes plus Costello syndrome (encircled in red). Mutations in HRAS have only been found in patients with Costello syndrome depicted in red here. For figure in colour: Supplement colour figures page 197.*

cardiac defects are found in two-third of patients. Common anomalies are pulmonary valvular abnormalities in 50-62% (both dysplastic and stenotic valves), hypertrophic cardiomyopathy in 10-25% (especially anterior septal hypertrophy) and atrial septal defect in 8-10% [161, 163-165]. This listing is not exhaustive and other congenital heart defects have been described in individuals with Noonan syndrome (Table 3) [165]. Although exact birth prevalence of Noonan syndrome is unknown, it has been suggested to be between 1 in 1000 to 1 in 2500 live births [161].

The syndrome is inherited in an autosomal dominant fashion and has been initially linked to the *PTPN11* gene (chromosome 12q24.1) [137, 166-168]. Approximately 33-50% of Noonan patients have activating missense mutations in *PTPN11*, but also mutations in other genes of the MAPK pathway (e.g. *SOS1*, *KRAS, MEK1, MEK2, RAF1,HRAS, BRAF*) have been found in affected individuals (Table 3, Figure 3)[138, 139, 169, 170]. Some of these genes have also been implicated in Cardiofaciocutaneous (CFC) syndrome, Costello's syndrome

and LEOPARD syndrome [121, 124, 135, 171]. However, distinct distinct genotype-phenotype correlations exist (RAF1 associates with hypertrophic cardiomyopathy, PTPN11/ SOS1 dysplastic pulmonic valves etc). This, taken together with the fact that these syndromes phenotypically overlap and can occasionally be found within one family indicates that the old syndromal nomenclature is not always adequate and stressed the need for molecular diagnosis.

DiGeorge syndrome/ Velocardiofacial syndrome/ 22q11 deletion

DiGeorge was the first who reported in 1965 congenital absence of the thymus and parathyroid glands in 4 children [172], resulting in hypocalcaemia and defective cellular immunity. Subsequently congenital heart defects and a characteristic facial appearance were found to be associated with this finding (Figure: picture DiGeorge patient) [173, 174]. Additional features found in DiGeorge syndrome are velopharyngeal dysfunction, or cleft palate, discrete dysmorphisms, developmental and behavioral problems and psychiatric disorders in adulthood (mainly psychosis and schizophrenia) [28, 175-177]. Congenital heart defects are detected in circa 80% of DiGeorge patients and consist primarily of outflow tract anomalies like interrupted aortic arch (IAA), truncus arteriosus (TA) and tetralogy of Fallot (TOF)(Table 3) [174, 177, 178].

The incidence of this syndrome is estimated to be at least 1 in 4000-6000 live births, but this might be an underestimation as many cases with mild features may remain undiagnosed [178-180]. Familial cases of DiGeorge have been described demonstrating an autosomal dominant inheritance pattern [181-184], but usually DiGeorge syndrome occurs sporadic and results from a de novo 22q11.2 (micro)deletion [130, 131, 182, 183, 185, 186]. Although the penetrance of a 22q11 deletion is nearly 100%, the severity of the disorders is variable [28, 187]. Importantly, 22q11.2 deletions supposedly are found in 6-11% of patients diagnosed with isolated tetralogy of Fallot (TOF) as well [188, 189]. Especially when TOF is associated with pulmonary atresia the chance of finding a 22q11.2 deletion is very high. Mutations in *TBX1*, located within the minimal deleted region, have been found responsible for the major features of this syndrome, but are a rare cause of this syndrome (Table 3) [127, 128]. Other genes within the deletion definitely contribute to the phenotype, especially to the mental retardation and psychiatric symptoms. In rare cases of isolated aortic arch anomalies and truncus arteriosus mutations in TBX1 have also been found [51]. In a minority of patients with

a DiGeorge syndrome phenotype other genetic chromosome defects have been associated with the defect such as deletions at 18q21.33 and 10p13 [131].

Various diagnostic terms have been assigned to the constellation of features of DiGeorge syndrome including Velo-cardio-facial syndrome (VCF), 22q11.2 deletion syndrome, Takao syndrome and CATCH22. All of these terms are now acknowledged to represent variant manifestations of the same entity, as all of these syndromes are caused by the same 22q11.2 microdeletion and demonstrate an extensive overlap of phenotypes [178, 190].

For the clinician it is important to be aware of the possibility of this syndrome in patients with conotruncal cardiac malformations. Mortality and morbidity after corrective surgery for congenital heart defects is higher in these patients than in those with isolated congenital heart defects. In addition, irradiated blood products and blood sero-negative for CMV should be given when blood transfusion is indicated (because of immuno incompetence due to thymus hypoplasia and T-cell dysfunction). Furthermore, because of parathyroid hypoplasia there is always a chance of hypocalcemia, even in patients who have never experienced episodes of hypocalcemia before [191]. As dysmorphic features can be very subtle, especially in neonates newly identified with a heart defect, it is recommended to screen newborns with TOF, TA and IAA for the presence of a 22q11.2 deletion.

Holt-Oram

The Holt-Oram syndrome, also called heart-hand syndrome, was first described in 1960 [192]. It is an inherited disorder causing anomalies of the upper limb (mainly the thumbs) and heart with an incidence of approximately 1 per 100.000 births [192, 193]. Affected individuals exhibit skeletal abnormalities ranging from subclinical radiographic findings (abnormal carpal bones are almost always present) to more obvious radial defects (flattened thenar, fingerlike triphalangeal thumbs or reduction defects) [192, 194-196]. Heart defects occur in circa 76-95% of patients and are most often atrial septal defects (secundum type), ventricular septal defects, conduction abnormalities and supraventricular arrhythmias [194, 195, 197]. But, as for most syndromes, other heart defects are sometimes observed as well (Table 3). The syndrome is transmitted as an autosomal dominant trait that is highly penetrant, although the clinical manifestations vary significantly, even within families [192, 194, 197].

Sporadic cases of Holt-Oram occur frequently as well, presumably due to *de novo* mutations [195, 196, 198]. This syndrome maps to a gene named *TBX5*, a transcription factor important for cardiac tissue specification and formation, located on chromosome 12q24.1 [84, 198-200]. Mutations in *TBX5* (loss of function of the transcription factor *TBX5*) are found in circa 35-74% of affected individuals, depending on (strict) clinical criteria used for diagnosis [201, 202]. In a small minority of Holt-Oram cases mutations in *SALL4* have been demonstrated and also deletions on chromosome 14 (14q23.3q31.1) and on chromosome 6 have been found [203-205]. Therefore, the Holt-Oram syndrome is genetically heterogeneous.

CHARGE syndrome

The combination of choanal atresia and coloboma with other congenital defects, specific for the CHARGE syndrome, was first described by Hall and Hittner in 1979 and has a birth prevalence of circa 1 in 8500 live births [206-209]. The CHARGE acronym stands for Coloboma of the eye, Heart defects, Atresia of the choanae (congenital abnormality of the anterior base skull, characterized by blockage of one or both of the posterior nasal cavities), Retardation of growth and development, Genital hypoplasia and Ear abnormalities [208, 210]. Although this acronym helped identification of affected individuals, diagnostic uncertainties occurred in patients with only some CHARGE features, warranting more specific diagnostic criteria which were developed by Blake and further specified by Verloes (Table 4) [211, 212]. Especially absence or abnormal anatomy of the semicircular canals on imaging of the os petrosum seem to be a very sensitive and specific diagnostic marker for this syndrome. In approximately 83-85% of affected individuals congenital heart defects can be observed (of any type), ranging from persistent ductus arteriosus, ventricle septal defects, atrial septal defects and conotruncal abnormalities [95, 208, 209]. CHARGE appears usually sporadic but some familial cases have been described as well. In circa 65-73% of CHARGE patients, mutations or deletions of the chromodomain helicase DNA-binding 7 gene (CHD7) can be detected, mostly *de novo* mutations [209, 213, 214]. When the diagnostic criteria according to Blake and Verloes are strictly applied this percentage is even higher [213]. Therefore CHD7 seems to be the major gene involved in the development of this syndrome. However, microdeletions in 22q11 have been detected in some patients classified as CHARGE syndrome as well [215, 216]. The CHD7 mutations are autosomal dominant. The mostly sporadic nature of CHARGE syndrome results from the fact that severely affected patients rarely reproduce.

Williams (Williams-Beuren) syndrome

Williams syndrome is a developmental disorder affecting circa 1 in 7500, caused by a heterozygous deletion of circa 1.5-2 Mb of chromosome 7q11.23 including the Elastin (ELN) locus in 90-94% [144, 217-219]. Usually, it occurs sporadically, but some families with an autosomal dominant inheritance pattern have been described as well [144, 220]. This syndrome is characterized by a characteristic facies (described as an elfin face in the older literature), developmental delay of variable severity, a friendly and very sociable personality, infantile hypercalcaemia and congenital heart defects (in circa 80%) (figure: picture of patient with Williams syndrome) [220-223]. Supravalvular aortic stenosis (SVAS) and peripheral pulmonary artery stenosis are most frequently detected heart defects in Williams syndrome. SVAS has been specifically related to ELN hemizygosity [144, 224]. Smaller deletions encompassing this gene cause SVAS in absence of other Williams syndrome features. Besides SVAS other congenital heart defects have been described in patients with Williams syndrome, such as atrial and ventricular septal defects, bicuspid aortic valve, pulmonary valve stenosis, coarctation of the aorta and mitral valve prolapse [133, 223, 225, 226].

Severity of SVAS can change over time. In one report, with a mean follow-up of 12.9 years in 59 patients with Williams syndrome and SVAS, it became evident that pressure gradients of less than 20 mmHg in infancy generally remained unchanged during the first 2 decades of life. Pressure gradients exceeding 20 mmHg, increased from an average of 36 to 53 mmHg in 13 patients. Additionally, after corrective surgery for SVAS, frequently restenosis occurred [227]. Conversely, in an Asian population progression of SVAS was uncommon and even regression was documented in round 30% of patients [226]. For reasons unknown, a higher prevalence and on average, increased severity of SVAS (and other heart defect(s) has been observed in male patients with Williams syndrome as compared to females. As age at diagnosis of Williams syndrome will probably strongly correlate with the age at which a heart defect is detected, this could explain why male Williams patients are diagnosed, on average, at a younger age than female Williams patients [223].

Besides congenital heart defects, in circa 17-55% of both young and older patients with Williams syndrome hypertension can be diagnosed [220, 222, 228, 229]. Increased aortic stiffness and decreased arterial compliance have been described in William syndrome patients, which could contribute to the high prevalence of hypertension in these patients [230]. Also renal artery stenosis and abdominal aortic narrowing, as underlying cause of hypertension, have been described in patients with Williams syndrome [220, 231].

Summary and future research projects

Isolated congenital heart defects are most frequently sporadic. Although sporadic, a genetic component is very likely to contribute to the occurrence of these defects [22].
A Mendelian inheritance pattern is only rarely observed and therefore, up to now, molecular genetic diagnostic tests, apart from 22q11.2 deletion testing in newborns and small children, only occasionally play a role of importance. However, the clinician should be aware that congenital heart defects are frequently associated with syndromes and in these syndromic cases a genetic diagnosis is important. Syndromes associated with congenital heart defects may have very subtle dysmorphic features, and can easily be missed (table 4). Therefore, clinicians should be trained to be able to recognize at least the most common syndromes. Features that should raise suspicion of a syndrome are associated birth defects, learning disabilities or mental retardation and short stature. Besides, the presence of family members with a syndrome diagnosis or congenital heart defect may indicate the possible occurrence of a syndrome in your patient. As phenotypical variability has been proven to be high, even the presence of another type of congenital heart defect (with or without dysmorphic features) in one first degree relative or two second or third degree relatives or request of a family member, warrants consultation from a clinical geneticist. Therefore, special attention should be given to a thorough family history.

Finally, specific types of congenital heart defects may also indicate the possible presence of a genetic defect. In patients with Tetralogy of Fallot for example, frequently a 22q11 deletion is found and as explained above this has consequences for treatment, reproductive risk and might have implications for other family members as well (Table 4) [188, 189]. For family members of patients with a bicuspid aortic valve, the presence of such a bicuspid aortic valve or other obstructive left ventricular outflow tract anomaly should be suspected [71, 74] (Table 4). Additionally, prenatal evaluation should be offered to prospective parents when one parent has a congenital heart defect or when they previously had a child with a congenital heart defect. In specific cases where there is strong evidence for a genetic contribution to cardiac defects within the families, also prenatal evaluation can be useful (Table 4). Prenatal evaluation is primarily needed to medically plan and prepare the delivery of a child with a congenital heart defect, such as delivery in a hospital with specialized neonatal care and prostaglandin administration to maintain an open ductus for cyanotic congenital heart defects depending on ductal flow.

Table 4.

Recommendations for Clinical Care in patients with Congenital Heart Defects
1) Obtain a thorough family history (pedigree analysis)
2) Consider physical examination with a special emphasis on cardiac murmurs in first degree relatives, especially for defects known to cluster within families (bicuspid aortic valve and other left ventricular outflow tract obstructions)
3) Consider consultation of clinical geneticist if:
- a congenital heart defect present in one first degree or two second/ third degree relatives
- family history of other congenital defects or syndromes (e.g.laterality defects, mental retardation, choanal atresia, cleft lip/palate)
- suspicion of a syndrome (e.g additional birth defects, learning disabilities/mental retardation, short stature and dysmorphic features)
- certain cardiac defects (tetralogy of Fallot/ truncus arteriosus/interupted aortic arch type B (22q11del))
4) Offer prenatal evaluation during pregnancy if:
- congenital heart defect is present in parent
- congenital heart defect is present in one or more offspring(s)
- evidence for strong genetic predisposition for cardiac defects based on family history

Future research in isolated congenital heart defects will focus on unraveling the intricate role of genes in the development of congenital heart defects. We expect through both candidate gene sequencing screens and genome wide association studies, (GWAs) more and more genes will be discovered to be involved in human congenital heart defects. The advantage of GWAs is that it is hypothesis free. Using Single Nucleotide Polymorphism (SNP)-arrays, the whole genome is scanned for regions associated with the trait of interest. It's now used to detect genetic factors contributing to common complex diseases such as myocardial infarction. One important assumption is that the genetic factor one is looking for is a common variant, to be able to reliably detect an association if present. In congenital heart disease the genetic variants associated are supposedly rare. Variants with relatively large affected will be self limiting as a result of selection and therefore will not be consistently linked to a specific haplotype. To detect an association with a rare variant (with a small effect), very large numbers of patients and controls are needed. When interesting variants are picked up, a replication cohort should be sought for to confirm the findings [232]. As the traits of interest are also relatively rare, the number of

affected persons is limited. Only through large world wide collaborations, this type of research will be within reach in the near future.

Another hot topic at the moment is whole genome sequencing. A competition has been set up named “the 1000 dollar genome”. Researchers all over the world are now trying to determine an individuals whole genome sequence for only 1000 dollars [233]. Therefore, in the near future whole genome sequencing will not only be achievable but also come within reach for advanced clinical diagnostic test. Whole genome sequencing will generate a lot of new information and interpreting these results will be difficult. Tools for the interpretation of such huge amounts of data in the context of congenital heart defects are not readily available. The more complex and heterogeneous the disorder, the more difficult this will be. While determining which genetic variations are associated with the development of congenital heart defects, also interactions between genes (gene-gene interaction) and between genes and environmental factors (gene-environment interaction) should be further investigated. In summary, in the coming decade a lot of new information will be generated and this will probably change the understanding of the pathogenesis of congenital heart defects tremendously.

As the genetic knowledge of the development of congenital heart defects progresses, collaboration between the clinician involved in the treatment of patients with congenital heart defects and the department of clinical genetics is warranted for both interpretation of new findings and genetic counseling.

Outline of this thesis

In chapter 2 an overview is given of genes and genetic pathways associated with congenital heart defects with a special emphasis on congenital atrioventricular valve and septal defects. In Chapter 3 and 4, genetic variation in the *ALK2* receptor is described in patients with primum type atrial septal defects with or without trisomy 21. In both chapters the *ALK2* receptor is functionally evaluated using *in vitro* and *in vivo* assays and family studies. Chapter 5 appraises whether GATA4 duplications can cause congenital heart defects in a family with a (132,9 kb) small duplication of 8p23.1 including only the *GATA4* gene. Furthermore, in chapter 6 the discovery of a new candidate gene by a translocation breakpoint analysis, disrupting a gene of unknown function, SPOCK3, is described. Additionally, in chapter 7 (a+b) the design and clinical characteristics of the study "genes and gene function in bicuspid aortic valves" are presented. This study has been started for the identification of genetic mutations or variations associated with the development of a bicuspid aortic valve. Tissue samples are collected as well to study the morphological consequence and down-stream targets of the genes involved. Finally, a summary of this thesis, outlined against the current scientific knowledge, is given in chapter 9.

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CHAPTER 2

Genes in congenital heart disease: atrioventricular valve formation

Genes in congenital heart disease: atrioventricular valve formation

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Abstract

Through the use of animal studies, many candidate genes (mainly encoding transcriptional factors and receptors) have been implicated in the development of congenital heart disease. Thus far, only a minority of these genes have been shown to carry mutations associated with congenital disease in humans, e.g. *GATA 4, TBX-5, NOTCH1* and *NKX2-5*. Mutations in these genes can cause a variety of cardiac defects even within the same family. Conversely, similar phenotypes are observed for different gene mutations suggesting a common pathway.

Multiple genes and genetic pathways have been related to atrioventricular valve formation, although most of these genes have not yet been demonstrated as causative in human atrioventricular valve defects. Key pathways include the epidermal growth factor receptor pathway and related interacting pathways, most importantly the pathway of UDP-glucose dehydrogenase, resulting ultimately in activation of Ras. Other examples of interacting pathways include that of Nodal/Cited2/Pitx2, Wnt, Notch and ECE. Further studies are needed to investigate the pathways which are crucial for atrioventricular valve formation in humans. Understanding the underlying molecular process of abnormal atrioventricular valve formation in patients with congenital heart disease may provide important insight, in the etiology and possibly into preventive or treatment regimes. **(Basic Res Cardiol 2008; 103: 216-27)**.

Keywords: Heart defects, congenital, valves, genes, genetic pathways

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Introduction: genes in congenital heart disease

Congenital heart disease is the most common form of birth defect. One-quarter of these congenital heart disease patients is associated with dysmorphic features, thus suggesting a syndromic diagnosis (such as trisomy 21, Noonan's syndrome and Holt-Oram syndrome) [1-3]. In many of these cases both chromosomal as well as gene mutations have been described as causative for their defect. However, in isolated congenital heart disease a monogenic cause is rarely found. Since the prevalence amongst family members of patients with isolated congenital heart disease is higher than based solely on probability (2-16% vs 0.4-0.8%), a genetic component is likely to contribute [4-7].

Atrial septal defects (ASD) are, to date, the most commonly investigated type of isolated congenital heart defects. Although a high frequency of index patients present with an ASD, within families the phenotype is extremely diverse. This phenotype variability is observed despite the similarity of mutations found in the index patients. Phenotypic variation within families includes tetralogy of fallot (TOF), transposition of the great arteries (TGA), ventricular septal defects (VSD) and pulmonary artery outflow obstruction. Another frequently investigated type of defect is the atrioventricular septal defect (AVSD) which is often associated with trisomy 21 (or other syndromic diagnosis) [3], but also found in isolated cases of congenital heart disease [8]. Surprisingly, after linkage analyses no genes on chromosome 21 could be related to the occurrence of AVSD. Recently few studies have linked mutations in *CRELD1* to AVSD in both trisomy 21 as well as non-syndromic patients [9-11].

Although many genes (mainly encoding transcriptional factors) have been found to be important for cardiac development in animal studies, thus far only a minority of mutations in these genes have been related to congenital heart disease in humans, e.g. *GATA 4, TBX-5, NOTCH1* and *NKX2.5* (see Table 1) [12-15]. *GATA 4*, for example, encodes a transcription factor at chromosome 8p23.1. Garg et al. and Okubo et al. found mutations in the *GATA4* gene associated with mostly ASD in an autosomal dominant inheritance pattern. A missense mutation, affecting the transcriptional activity of *GATA4*, was found to segregate with the heart defects observed [12, 16]. In addition a frameshift mutation, resulting in a predicted premature stop codon inducing early truncation of the protein has been

described [16]. Congenital heart defects associated with this second mutation included atrioventricular septal defects and pulmonary valve thickening or insufficiency of cardiac valves. *GATA4* has been shown to interact with *TBX5*, and *NKX2-5* (described below), both of which act as co-activators for *GATA4* activity [12, 17]. Strikingly, similar cardiac defects have been observed in patients with *TBX5, GATA4* and *NKX2-5* mutations. This is consistent with the observation that these proteins form a complex to regulate a specific group of genes necessary for cardiac septation.

Another example is *NKX2.5*. The *NKX2-5* gene encodes the homeobox transcription factor *NKX2-5*. Analyses of the Tinman gene in Drosophila, which is the orthologue of the human *NKX2-5 gene*, has shown its essential role in cardiac development. In humans, Schott et al. found mutations (10 missense nucleotide substitutions and 1 in-frame deletion) in this gene in four families with congenital heart disease and atrioventricular block [15]. The congenital heart defects found in patients with these *NKX2-5* mutations were secundum ASD, VSD, TOF, subvalvular aortic stenosis, ventricular hypertrophy, pulmonary atresia and redundant mitral valve leaflets with fenestrations. Similar outcomes were observed in a study of König et al.[18].

To date, a number of genes have been linked to congenital heart disease, but only very few have focused on potential mutations associated with atrioventricular valve defects in humans. Animal models and *in vitro* studies have been performed to unravel the intricate process of atrioventricular valve formation and might provide important insight for further study in humans. This review will give an overview of embryogenesis, the genetic pathways associated with atrioventricular valve formation thus far, and the effect of potential disturbances in the genetic routes on phenotype.

Atrioventricular valve formation

During embryonic development the atrioventricular valves start appearing around gestational day 22 (E9.5 in mice), immediately following cardiac looping [19]. At this time the primitive heart is a single heart tube when cardiac cushions start to develop dividing the heart tube into a right and left canal. Cardiac cushion formation is an intricate process characterized by epithelial- mesenchymal transdifferentiation (EMT). During EMT, subsets of endothelial cells overlying the future valve site are specified to delaminate, differentiate

Table 1. Gene Mutation and phenotype

Gene	Chromosomal	Cardiac defect	References
ALK2	2q23-q24	Primum type ASD, MVP	Joziasse, Circulation 2007 [59]
BMPR2	2q33	AVSD, ASD, PDA, PAPVR + PAH	Roberts, Eur Respir J 2004 [110]
CFC1/Cryptic	2q21.1	Heterotaxia, TGA, DORV, common AV canal, AA hypoplasia, pulmonary artresia, DIRV	Bamford, Nat Genet, Goldmuntz Am J Hum Genet 2002 [111,112]
Cited2	6q23.3	TOF, VSD, ASD, anomalous pulmonary venous return, RVOT obstruction	Sperling, Hum mutat 2005 [98]
CRELD1	22p13	AVSD, cleft mitral valve, ASD type I, heterotaxy	Robinson, Am J Hum Genet 2003 [9]
Elastin	7q11.2	Supravalvular AoS	Metcalfe, Eur J Hum Genet 2000 [113]
FOG2	8q23	TOF	Pizzuti, Hum mutat 2003 [75]
GATA4	8p23.1-p22	ASD, AVSD, pulmonary valve thickening, insufficiency of cardiac valves	Garg, Nature 2003 [12] Okubo, J Med Genet 2004 [16]
JAG1	20p12	TOF, VSD with aortic dextroposition, PPS	Eldadah, Hum Mol Genet 2001 [91]
KRAS	12p12.1	ASD, VSD, valvular PS, HCM, HOCM, MVP, TVP, LVH	Schubbert Nat Genet 2006 [114]
MYH6	14q12	Secundum ASD	Ching, Nat Genet 2005 [115]
NKx2.5	5q34	ASD, VSD, TOF, AoS, VH Pulmonary atresia, Mitral valve anomalies, conduction disturbances	Schott, Science 1998 [15] König, Clin Res Cardiol 2006 [18]
NKx2.6	8p21	TA	Heathcote, Hum Mol genet 2005 [116]
NOTCH1	9q34.3	Bicuspid aortic valve, mitral valve stenosis, TOF, VSD	Garg, Nature 2005 [13]; Mohamed, Biochem Biophys Res Commun 2006 [90]
PROSIT240	12q24	TGA	Muncke, Circulation 2003 [117]
TBX1	22q11.2	Interrupted aortic arch, TA, other aortic arch anomalies	Gong, J Med Genet 2001 [118]
TBX5	12q24.1	ASD, AVSD	Reamon-Buettner,Hum Mutat 2004 [14]
Zic3	Xq26.2	TGA, DORV, ASD, AVSD	Ware, Am J Hum Genet 2003 [119]

AA: aortic arch, AoS: aortic stenosis, ASD: atrial septal defect, AV: atrioventricular, AVSD: atrioventricular septal defect, DIRV: double inlet right ventricule, DORV: double outlet right ventricle, HCM: hypertrophic cardiomyopathy, HOCM: hypertrophic obstructive cardiomyopathy, MVP: mitral valve prolapse, PAH: pulmonary artery hypertension, PAPVR: partial anomalous venous return, PDA: patent ductus arteriosus PPS: peripheral pulmonic stenosis, PS: pulmonary stenosis, RVOT: right ventricle outflow tract, TA: truncus arteriosus, TGA: transposition of great arteries, TOF: tetralogy of Fallot, TVP: tricuspid valve prolapse, VSD: ventricular septal defect, VH: ventricular hypertrophy

and migrate into the cardiac jelly (extracellular matrix, in-between the endocardium and myocardium, for review see Eisenberg and Markwald [19]. By day 40 (E12.5), EMT abates and the cushions begin to thin out and form mature valvular structures [20]. For an extensive review on heart development and atrioventricular valve formation, see Moorman et al. 2003 [21-23].

EMT is induced and maintained by several important signaling proteins, receptors and transcription factors. Thus far a number of pathways have been described, which are essential for endothelial cell signaling and differentiation. A major route essential for this process includes the epidermal growth factor receptor pathways and interacting pathways, notably of UDP-glucose dehydrogenase, resulting ultimately in activation of Ras. Other examples of interacting pathways are of Nodal/Cited2/Pitx2, Wnt, Notch and endothelin converting enzyme (ECE). These are described below, however, the way these factors interact is still not fully understood.

Extracellular matrix proteins

UDP-glucose dehydrogenase (UDGH) is an enzyme required for the conversion of UDP-glucose to UDP-gluronic acid, which is used in the biosynthesis of hyaluronan (HA), glycosaminoglycans (GACs), heparin sulfate and chondroitin. Zebrafish heterozygous in Jekyll, which encoded for UDGH fail to develop atrioventricular valves [24, 25]. It is important to underline the fact that in this case a phenotype is observed in the heterozygous state since mostly a phenotype is only established when the gene is completely absent. Generally, homozygous mutations cause early lethality, resulting in early termination of pregnancy, which might be the reason for the lack of observed human mutations in congenital heart disease. Another gene which causes cardiac defects in the heterozygous state is FGF8 (of the extracellular signaling fibroblast growth factor family). $FGF8^{+/-}$ mice have severe cardiac defects (TGA, PTA, ASD, VSD, hypoplastic aortic arch, hypoplastic left ventricle and singular atrioventricular valve [26]. HA is synthesized by hyaluronic acid synthase (Has). Humans possess three Has genes, *Has1,-2,* and *Has3.* Only Has2 has been identified as pivotal in HA synthesis during cardiac development, especially in atrioventricular cushion formation. Mutations in this gene are lethal at E9.5 and null mice display pericardial edema, disordered vessel growth and an absence of cardiac jelly, with these latter phenotypes likely responsible for the lack of observable endocardial cushions [27]. In addition, several studies

Figure 1.

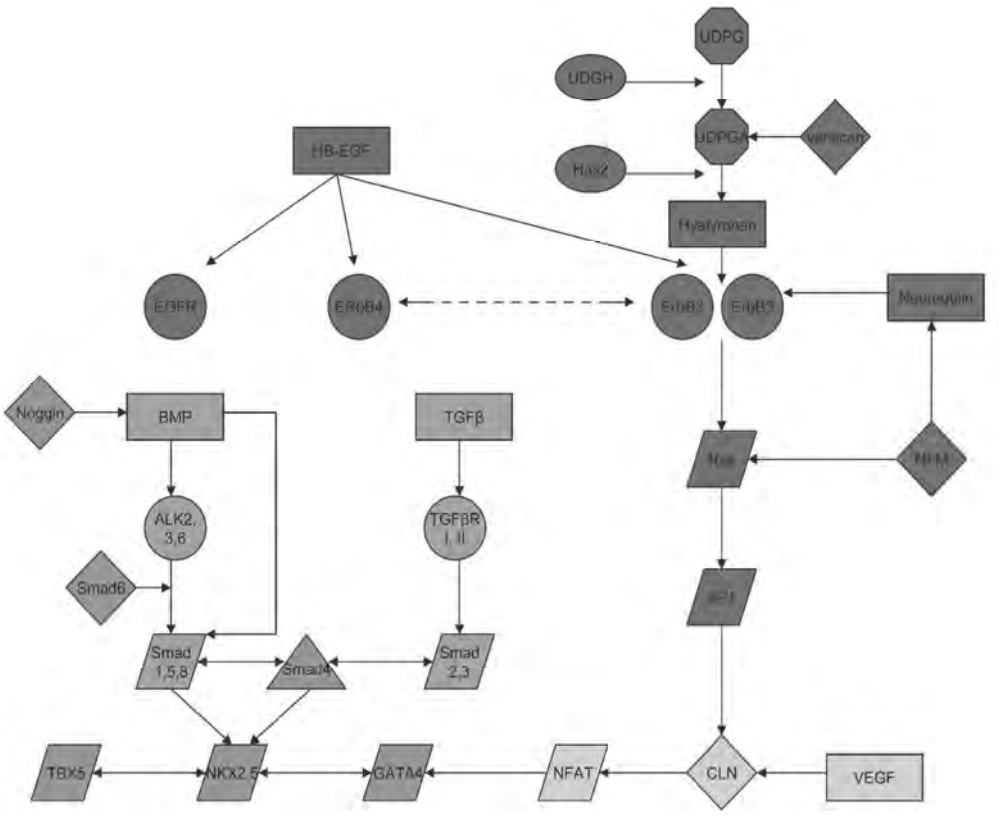

Figure 1. Signaling pathways in atrioventricular valve formation. *In blue, transforming growth factor β (TGFβ) and Bone Morphogenetic Protein (BMP) signaling, through Smad proteins. In pink: ErbB and Ras Signaling. In yellow: Vascular endothelial growth factor (VEGF) - nuclear factor of activated T-cells pathway (NFAT). In green: shared (common) genetic signaling proteins/ Transcription factors. Rectangles: Ligands. Circles: receptors. Parallelogram: transcription factors. Triangle: carrier protein. Diamonds: miscellaneous. Octagon: component of other protein. Dashed arrow: indirect effect. Minus sign: inhibitory effect. HB-EGF: heparin binding- Endothelial Growth Factor; EGFR: Endothelial Growth Factor Receptor; BMP: Bone Morphogenetic protein, ALK: Activine receptor-like Kinase BMPR: Bone Morphogenetic Protein Receptor; TGFβR: Transforming Growth Factor β Receptor; UDPG: UDP-Glucose; UDPGA: UDP-Glucuronic Acid, UDGH: UDP-Glucose dehydrogenase; NFM: Neurofibromin; AP1: Activator Protein 1; CLN: calcineurin. Commonly used alternative names are: ALK2/ActR-I, ALK3/BMPR-IA, ALK5/TβR-I, and ALK6/BMPR-IB. For figure in colour: Supplement colour figures page 198.*

have reported HA is required for EMT and proliferation in the endocardial cushions and can activate RAS and RAC1 [27, 28]. Versican, which is a HA-binding proteoglycan and encoded by the Cspg2 gene, proved to be necessary for atrioventricular valve formation besides HA, since versican-deficient mice have defects comparable with Has2 null mice [29]. These results imply that extracellular matrix products have an essential function in

atrioventricular valve formation, presumably the result of their fundamental role in endocardial cushion formation [27, 28]. Interestingly, HA can activate the ErbB2/ErbB3 (epidermal growth factor) receptor indirectly by heterodimerization, which in turn activates the Ras pathway. ErbB2-deficient mice have severe cardiac defects, although it is currently unclear whether this cardiac phenotype is mediated by HA or by HB-EGF [27, 30]. The Ras pathway is important in the atrioventricular cushion formation signaling cascade and can directly initiate EMT as described further below.

Another important gene in atrioventricular valve formation is the *NF1* gene. The *NF1* gene is a tumor suppressor gene, which encodes neurofibromin. Neurofibromin contains a GAP domain, which, in wild type mice, accelerates intrinsic activity of Ras-GTPase which results in an active Ras-GTP to be converted into an inactive Ras-GDP. Mutation in this gene gives rise to Von Recklinghauses or Type 1 neurofibromatosis. In addition to neurofibromatosis, NF1 -/- mice have an overabundance of spongy tissue in the endocardial cushions of the outflow tract and the atrioventricular valve region and cardiac endothelial cells demonstrate an abnormal EMT. Cardiac defects in endothelial conditionally deleted NF1 -/- mice mimic the cardiac defects discovered in NF1 null mice, suggesting these defects originate from the endothelial cells. Thus cannot be contributed to altered neurofibromin function in the cardiac neural crest cells as earlier suggested since, no cardiac defects where found in neural crest cells conditionally deleted NF1 mice [31]. Additionally, NF1-/- mice have altered expression of several genes: Msx2, ErbB3 and Del1 are down regulated whilst neuregulin is upregulated. A consequence of this downregulation of the ErbB3 receptor, the NF1 gene has indirectly an effect on the Ras pathway as well (See Figure 1) [20]. The major downstream effector pathway of Ras in atrioventricular valve formation is probably the activation of MAPK (mitogen activated protein kinase or also named ERK) that arises through c-Raf and MEK1 activation. This ultimately results in NFAT activation (possibly through calcineurin activation) [32].

NFAT is a transcription factor which is heavily phosphorylated in resting cells. NFAT requires dephosphorylation for translocation into the nucleus. Calcineurin dephosphorylates NFAT when activated by Ca^{2+}, thus triggering NFAT nuclear accumulation and activation of NFAT target genes [33]. Contiguous to calcineurin, VEGF (vascular endothelial growth factor) stimulates NFATc1 nuclear translocation, possibly via

KDR/VEGF-R2, by a calcineurin-dependent mechanism [34]. Both VEGF and NFAT1 are important for cardiac valve formation. NFAT1 knock-out mice lack semilunar valves, have a hypertrophic left ventricle and the atrioventricular valves are underdeveloped whereas overexpression of VEGF leads to inhibition of EMT [33, 35-37]. Furthermore, NFAT influences transcription synergistically with several families of transcription factors, including the GATA zinc finger proteins and EGR, the helix-turn-helix domain proteins Oct, HNF3, and IRF-4, the MADS-box proteins MEF2 and nuclear receptor PPAR-γ [38] (reviewed in Hogan, P.G. et al) [39].

Growth factors

I. Epidermal growth factor signaling

Heparin-binding epidermal growth factor (HB-EGF) is a ligand which is synthesized as a type I transmembrane protein (proHB-EGF). HB-EGF is required for a number of physiological and pathological processes such as cardiac hypertrophy and wound healing. HB-EGF activates multiple receptors, either directly (epidermal growth factor receptor [EGFR/ ErbB1/HER1] and ErbB4/HER4) or indirectly, by heterodimerization (ErbB2 and ErbB3). HB-EGF knock out mice develop severe heart failure caused by diminished cardiac function, enlarged ventricular chambers and enlarged cardiac valves. Interestingly, mice lacking epidermal growth factor receptors (EGFR) and cardiac specific deletion of ErbB2 have similar cardiac defects. These results imply that HB-EGF-mediated signaling by ErB2 and EGFR is essential for heart development [40-42]. In addition, other EGF receptors (ErbB3 and ErbB4) have proven to be crucial for cardiac morphogenesis since mice deficient in ErbB3 demonstrate heart valve malformation and ErbB4 null mice die early *in utero* due to abnormal ventricular trabecularization (See Figure 1) [30, 43].

II. Transforming growth factor β signaling

TGFβ binding initiates the formation of a complex between the type I and type II transforming growth factor β receptors (TGFβR). Upon complex formation the type II TGFβR phosphorylates type I TGFβR propagating the signaling events into the cytoplasm (for review, see Shi& Massague,) [44]. This is followed by the activation of Smad Transcription factors required for EMT. Also TGBβ can induce non-Smad dependent pathway, often referred to as noncanonical TGFβ signaling. The most important other

pathway is probably that of the MAPK or ERK, important for EMT (described above) and activation of this pathway can increase TGFβ1 expression (for review see Derynck et al) [45].

Around gestational day E7.25 TGFβ2 is expressed in the cardiogenic plate of the precardiac mesoderm and gives signals to the endothelial cells to start EMT [46, 47]. These endothelial cells commence intruding the extracellular matrix and start to proliferate and differentiate (around E9.5). Until recently it was believed that myocardial signaling, together with TGFβ has a pivotal role in initiating EMT. This has been demonstrated in chick explant studies where endothelial cells failed to initiate EMT in the absence of either myocardial cells or TGFβ2 supplementation [48]. In addition, antisense inhibition of TGFβ3 in cell culture reduced EMT by 80% [49], although this was not observed with inhibition of TGFβ2. However, this could, to date, not be confirmed *in vivo* with mouse genetic studies using TGFβ (1-3) null mice. TGFβ3 null mice have only a mild cardiac phenotype with minor differences in the position and curvature of aortic arches and ventricular wall thickness [47, 50]. No cardiovascular defects have been reported for TGFβ1 null mice although TGFβ1 is expressed extensively throughout the developing heart [47, 51]. TGFβ2 knockout mice, on the other hand, have extensive cardiac defects, including VSD, double outlet right ventricles and the mitral and tricuspid valve are attached to the left ventricular wall [50]. Recently, conditional myocardial and endocardial deletion of TGFβR-II in mice, which is an essential receptor for TGFβ signaling, did not show an essential role for TGFβ in EMT. However, mutant mice did show a reduced growth rate of inferior cushion mesenchyme while that of the superior cushion was normal. This is likely due to reduced expression of Cyclin D1 in the inferior cushion, whereas expression in the superior mesenchymal cushion is normal [52]. Also in explanted chick atrioventricular cushion tissue, a blocking antibody to the TGFβ receptor III (TGFβR-III) significantly reduced the number of mesenchymal cells formed in the explants in response to induction by cushion myocardium. Again, null mutant mice only showed mild delay in the fusion of the endocardial cushion tissue with the muscular interventricular septal and no apparent other atrioventricular valve defects [53, 54].

III. Bone morphogenic proteins (BMP)

BMPs are members of the TFGβ superfamily and ligand of the BMP receptors (ALK2, ALK3, ALK6, BMPR-II). ALK2 and ALK3 appear to be indispensable for cardiac morphogenesis as conditional deletion of ALK2 and/or ALK3 results in severe defects in

atrioventricular valve, -canal and septum formation [55-57], the latter of which also results in depressed expression of TGFβ2 [58]. Moreover, mutations in *ALK2* have been recently found to segregate with primum type ASD in humans [59]. Conditional deletion of BMP2, -4 and -6/7 in mice, the purported ligands of ALK2 and ALK3, give similar heart defects [60-63].

Binding of BMPs to their receptor (such as ALK2, 3 and 6) activates Smad transcription factors. Smad proteins can be divided in 3 different functional classes; the receptor-activated R-Smads (Smad 1,-5,-8), the comediator Co-Smad (Smad 4) and the inhibitory I-Smads (Smad 6,-7) (reviewed in Euler-Taimor 2006) [64]. When Smad 1, Smad 5 and Smad 8 are activated, Smad 4 coseggregates with Smad 1, 5 or 8 and the complexe translocates to the nucleus where transcription is initiated. Smad1 and Smad5 null mice have severe cardiac defects as well as other developmental defects (primordial germ cell specification, ruffled visceral yolk sac, allantois and Yolk sac vascularisation defects), while $Smad1^{+/-}$ or $Smad5^{+/-}$ mice have no cardiac defects at all [65, 66].
Remarkably $Smad1^{+/-}$ $Smad5^{+/-}$ double heterozygous mice have defects that encompass the entire range of disturbances described for Smad1- and Smad5-deficient embryos, suggesting that Smad1 and Smad5 function cooperatively to direct BMP dependent gene expression. This could explain the low observed penetrance within families with a congenital heart defect when affected embryos (with a phenotype) are generally homozygous which most of the time leads to early miscarriage. Heterozygous mutation carriers might only develop heart defects in a certain genetic background, or genetic variation.

In contrast with Smad 1 and Smad 5, Smad 8 is confined to the visceral endoderm and does not have any effect on cardiac (valve) morphogenesis [67]. Smad 6, on the other hand, is an inhibitory protein which binds type I BMP receptors (ALK2,-3 and -6) and prevents the binding and phosphorylation of Smad 1 and Smad 5 [68]. Mice deficient in Smad 6 are viable but have large, thickened atrioventricular valves due to ectopic activation Smad 1 and 5 (See Figure 1) [69].

Owing to its essential function as the only carrier of Smad proteins into the nucleus, it is hardly surprising that Smad 4 null mice have severe gastrulation defects. These knock-out mice die early in embryogenesis preceding cardiogenesis. Consequently,

the specific function of Smad 4 in atrioventricular valve formation has not been established [70]. The Smad 4 complex is transported to the nucleus where it binds to promoter sites. Together with NKX2-5 and GATA 4, Smad 4 forms a complex which synergistically facilitates transcription of target genes. Although NKX2-5 or GATA 4 are capable of acting independently, the formation of this complex increases the binding affinity to DNA and thus enhances transcription (51-192 fold) [17, 71-73]. FOG-2 on the other hand acts as a repressor of GATA 4 function and has not been found to be able to function in a GATA 4 independent manner. Since GATA 4 is a positive regulator of EMT, mice deficient of FOG-2 demonstrate an increased EMT in the endocardial cushions of both the outflow tract as well as the AV canal and die early in infancy of heart failure due to various cardiac malformations (including a common atrioventricular valve) [74]. Also sporadic cases of TOF have been found to be associated with mutations in the FOG-2 gene [75].

Other extracellular Signaling Molecules

a. Endothelin cascade (vasoconstrictor peptides)

Endothelins are composed of three structurally related isoforms; ET-1, -2 and -3 which act on two subtypes of G protein-coupled heptahelical receptors, the ET A and ET B receptor. Endothelin converting enzyme-1 and -2 (ECE-1, -2) are membrane-bound metalloproteases that can cleave the inactive endothelin-1 (big ET-1) to form the active ET-1. Interruptions in the endothelin cascade cause severe developmental defects. In particular ET-1, ETA and ECE-1 null mutations give rise to cardiac developmental defects, comprising an interrupted or tubular hypoplastic aortic arch, double outlet right ventricle (DORV), ventricular septal defects (VSD), transposition of the great arteries (TGA), persistent truncus arteriosus (PTA) and localizing defects of the aortic branches [76-79]. Conversely, ECE-2-deficient mice show no developmental defects at all, although ECE-1 -/-, ECE-2 -/- double knock-out mice have more severe cardiac defects than ECE-1 null mice, including the total absence of atrioventricular valves [79].

b. Wingless-type (Wnt) signaling

Wnt signaling has proven to be indispensable for cardiac development and signaling important during other embryonic patterning. The two distinct routes of Wnt signaling, the canonical Wnt/β-Catenin pathway and the non-canonical Wnt pathway, have

distinct functions in the developing heart. In the canonical pathway and the absence of Wnt, β-Catenin is phosphorylated by a multiprotein complex which includes the tumour suppressor gene product, Adenomatous Polyposis Coli (APC), Axin and GSK-3β [80]. Once phosphorylated, β-catenin is ubiquitinated and degraded. In the presence of Wnt, the receptor (of the Frizzled family) is activated and the phosphorylation/degradation complex is not recruited. β-catenin accumulates in the cytoplasm and is translocated to the nucleus where it regulates gene expression through interaction with transcription factors (TCF/LEF) (reviewed in Brade 2006) [81]. Canonical Wnt signaling has been associated with cardiac valve formation and its main role is thought to be in EMT, as overexpression of β-catenin (due to a mutated APC protein) in zebrafish gave rise to markedly expanded endocardial cushions and an excessive endocardial layer fused with the atrioventricular outflow tract (all endocardial cells seemed to have undergone EMT) [82]. Conversely, truncation of Wnt9A (inhibiting Wnt signaling) in chick embryos displays

Figure 2.

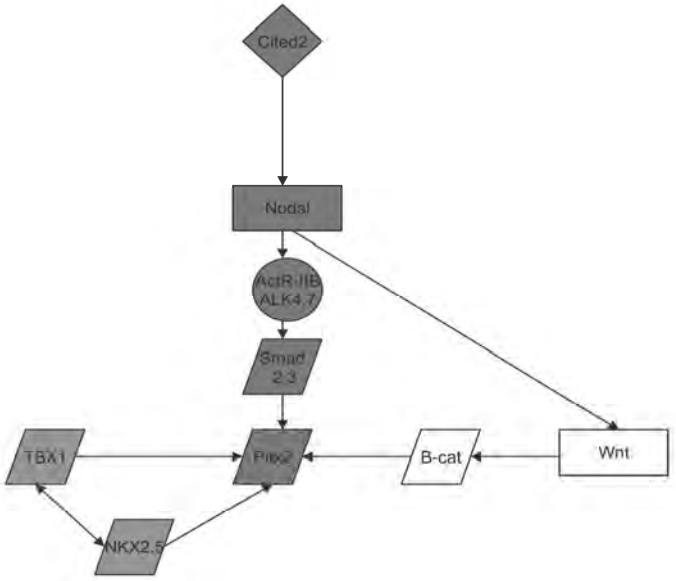

Figure 2. Nodal interactions and Wnt pathway*. In orange: Cited2/Pitx2 pathway. In green: shared (common) genetic signaling proteins/ Transcription factors. In white/grey: Wnt pathway. Rectangles: Ligands. Circles: receptors. Parallelogram: transcription factors. Diamantes: miscellaneous. B-Cat: β-catenin. Commonly used names: ALK4/ActR-IB. For figure in colour: Supplement colour figures page 197.*

hypocellular endocardial cushions, delayed cardiac looping and caused severe cushion hypoplasia. Notably, in the same study overexpression of Wnt9 not only resulted in an expansion of endocardial cushions, but also an increase in the number of mesenchymal cells compared with controls and a decrease in apoptotic activity [83]. These results suggest that Wnt is involved in both EMT and proliferative activity. Moreover, conditional Wnt1 deletion of β-catenin in the neural crest reveals a marked decrease in Pitx2 expression [84], which is, as described above, also important for cardiac valve development. Furthermore, TGFβ acts contemporaneously with β-catenin in inducing cardiac valve formation [85].

The non-canonical Wnt pathway functions independently of β-catenin and has not been shown to be as important in cardiac valve development. Non-canonical Wnt signaling is necessary for cadherin-mediated cell adhesion in neonatal cardiac myocytes and a proper development of the proximal outflow tract. It has been suggested that non-canonical Wnt also contributes to the inhibition of β-catenin signaling (See Figure 2) [81, 86].

Receptors

Notch signaling

The Notch genes encode transmembrane receptors containing an extracellular domain (variable number of EGF-like repeats) and an (nuclear) intracellular domain (NIC). When a Notch specific ligand binds to the Notch receptor, proteolytic processing of the receptor starts and consequently NIC is translocated to the nucleus, where it binds to the transcription factor RBPJK [87].

Notch1b is, like all the other genes described above, involved in atrioventricular valve formation. Notch is expressed throughout the embryonic endocardium and loss of Notch1b expression in zebrafish inhibits the transition of AV endocardial cells from squamous to cubical and EMT is dramatically reduced [88]. Moreover Notch and RBPJK mutants show reduced expression of TGFβ2 and downregulation of receptors downstream of TGFβ2 (TBRI-III) [88]. In the reciprocal setting, chemical induction of transgenic overexpression of Notch in zebrafish results in embryos with enlarged atrioventricular valves and hypertrophic endocardial cushions [89]. *NOTCH1* mutations have also been found in patients with bicuspid aortic valves and subsequently aortic valve calcification [13, 90]. Jagged1 (*JAG1*), a ligand of Notch is related to the Alagile syndrome, a dominant multisystem

syndrome characterized by cardiac, skeletal, ophthalmologic manifestations, hepatic bile duct paucity and cholestasis. Thus far its role in isolated cases of congenital heart disease has not yet been fully elucidated, although in one family with tetralogy of fallot, mutations in this gene have been found [91, 92]. When looking at the primary target genes of Notch (Hey1,2 and HeyL), loss of Hey2 in mice, causes congenital heart defects, including atrioventricular valve defects. Double knockout embryo's of Hey1/Hey2 die early in midgestation due to severe vascular problems, while Hey1$^{-/-}$ mice are healthy without any pathological findings, this could therefore be explained by a partial redundancy of Hey1 and Hey2. Also HeyL$^{-/-}$are grossly normal and fertile, but double knockout mice Hey1/HeyL have mostly ventricular septal defects and dysplastic atrioventricular valves (demonstrating functional redundancy between the two), due to impaired EMT [93].

Transcription regulation

CITED2, Pitx2 pathway

Cited2 is a transcriptional mediator of *TFAP2* (encodes a member of the AP-2 family of transcription factors), which is related to Char syndrome, characterized by patent ductus arteriosus, facial dysmorphism and abnormalities of the fifth finger [94, 95]. Cited2 controls left-right patterning and heart development through regulation of Nodal and Pitx2. Cited2 null mice have severe heart defects (besides serious defects in the nervous system, adrenal system and fibroblast proliferation) including ASD, VSD, DORV, TGA, PTA and in some Cited2 null mice, endocardial cushion formation is reduced [96, 97]. In addition, isolated cases of congenital heart disease have been associated with mutations in the *CITED2* gene, causing TOF, VSD, ASD, abnormal pulmonary venous return to the right atria, and right ventricular outflow tract obstruction [98]. Nodal, a member of the TGFβ superfamily, acts directly downstream of Cited2 and is consequently involved in left-right patterning. In addition to activation by Cited2, BMP2 signaling also plays a substantial role in activating Nodal [99-101]. Nodal itself signals through a TGFβ receptor (ACVRII) phosphorylating Smad 2 and 3 (see also the pathway of TGFβ receptor which is described in detail above) and consequently activating Pitx2 expression [96, 102].

Pitx2, a transcription factor, is downstream of nodal signaling and classically described for its role in left-right patterning. Mutations in the *PITX2* gene are associated

with Rieger syndrome, a syndrome distinguished by cardiac and eye defects, missing or misplaced teeth and umbilical abnormalities [103]. Pitx2 null mice show, also manifest these distinctive defects including defects in eye and tooth development, hypoplasia of the right ventricle, enlarged left atrium, the mitral and tricuspid valve fail to develop resulting in a common atrioventricular valve, ASD and VSD [104, 105]. At the molecular level, Pitx2 binds with TBX1 or Nkx2.5 and synergistically promotes DNA transcription. Remarkably, Pitx2 +/-, TBX1 +/- double heterozygous mice demonstrate a phenotype not found in either TBX1 homo- or heterozygous mice or Pitx2 knock out mice i.e. DORV, VSD, ASD, atrioventricular valve defects, pulmonary trunk stenosis and an abnormal drainage of the pulmonary vein. This, therefore, suggests that nodal is necessary for initiation of Pitx2 expression, which is then maintained by the transcription factors NKx2.5 and TBX1 (See Figure 2) [106].

Summary and conclusion

As this review shows, many genes have already been found essential for normal atrioventricular valve formation in animal or *in vitro* studies. What stands out is: atrioventricular valve formation is regulated by different genetic pathways. Disturbances in each subset of a particular pathway, ligand, receptor, transcription factor or extracellular matrix proteins for example, can all result in similar cardiac defects. For example when looking at the pathway of BMP, conditional deletion of BMP (ligand), ALK2, 3 or 6 (receptor) or Smad transcriptional factors result in similar cardiac defects. In addition, there seems to be a hierarchical pattern as disturbances high up in the genetic route result in altered consequences for all genes or gene products further downstream. In our example, conditional deletion of BMP results in altered expression of Smad transcriptional factor, an effect also observed when conditional deleting ALK2 or 3. Also, all genetic pathways seem to be connected with other pathways and again similar phenotypes are observed among different pathways.

Altogether, this might explain the comparability of (cardiac) phenotypes, with different gene mutations as is demonstrated in human congenital heart disease. Also it could explain why, within a certain gene mutation, the cardiac phenotypes can be quite diverse, as not one but multiple genes are involved in the cardiac embryogenesis of the

atrioventricular valves. Future study should endeavor, in a large population of patients with atrioventricular valve defects, the existence of gene mutations in those genes found important in animal models or *in vitro* studies. Although one gene mutation can cause a certain phenotype in some families with a dominant inheritance pattern, in many cases such an inheritance pattern is not found but merely a higher occurrence rate of congenital heart defects in the family, suggesting low penetrance. Also somatic mutations rather than germline mutations might play a role in the development of congenital heart defects, as demonstrated by Reamon-Buettner and coworkers, where mutations in *NKX2.5* where identified in (human) affected cardiac tissue, while unaffected cardiac tissue didn't possess any mutations [107]. With normal diagnostic DNA sequencing methods, lymphocytic DNA is used and therefore somatic mutations in a cardiac line would be left undetected. Many genes are implicated in atrioventricular valve formation, but in many cases a phenotype is only established in the homozygous state (in animal models) and generally these mutations cause early lethality, resulting in early termination of pregnancy. There are a few exceptions as for example heterozygous zebrafish for Jekyll, which encodes for *UDGH*, fail to develop atrioventricular valves. Heterozygous mutation carriers might only develop heart defects in a certain genetic background, or genetic variation. This is demonstrated in a model with $Smad1^{+/-}$ $Smad5^{+/-}$ double heterozygous mice, where these mice have defects that encompass the entire range of disturbances described for Smad1- and Smad5-deficient embryos, while $Smad1^{+/-}$ or $Smad5^{+/-}$ mice have no cardiac defects at all. Another explanation would be that of a specific environmental background is needed for heterozygous mutation carriers to develop cardiac defects. In addition, gene-expression might be altered by environmental factors as well [108, 109]. Thus, disturbances in a genetic pathway might be the result of environmental differences, as well as gene mutations or the combination of the two. Future study should focus on the potential importance of both mentioned factors. For example in patients with congenital heart disease the prevalence of multiple gene mutations can be assessed.

However, it is important to consider that genes important in a model (animal or *in vitro* studies) might point to relevant genes for human cardiac development, but since it is a model, could also be unrepresentative for mechanisms essential for human heart development and therefore result in the absence of mutations in these genes in humans.

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CHAPTER 3

A dominant-negative *ALK2* allele associates with congenital heart defects

A dominant-negative *ALK2* allele associates with congenital heart defects

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Background - Serious congenital heart defects (CHDs) occur due to improper atrioventicular septum (AVS) development during embryogenesis. Despite extensive knowledge of the genetic control of AVS development, few genetic lesions have been identified that are responsible for AVS-associated CHDs.

Methods and Results - We sequenced 32 genes known to be important in AVS development in patients with AVS defects (AVSD) and identified 11 novel coding single nucleotide polymorphisms (cSNPs) that are predicted to impair protein function. We focused on variants identified in the bone morphogenetic protein (BMP) receptor, *ALK2,* and subjected two identified variants to functional analysis. The cSNPs, R307L and L343P, are heterozygous missense substitutions and were each identified in single individuals. The L343P allele had impaired functional activity as measured by *in vitro* kinase and BMP-specific transcriptional response assays and dominant-interfering activity *in vivo. In vivo* analysis of zebrafish embryos injected with ALK2 L343P RNA revealed improper atrioventricular canal formation.

Conclusion - These data identify the dominant-negative allele, ALK2 L343P, in a patient with AVSD. **(Circulation 2009; 119: 3062-69)**

Keywords: Congenital heart defects, candidate screen, BMP signalling, ALK2

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Introduction

The primitive heart tube of vertebrates consists of an inner layer of endothelial cells, the endocardium, and an outer, muscular layer of myocardial cells. After formation of the heart tube, endothelial cells delaminate from the endocardium and migrate into an extracellular matrix, termed "cardiac jelly", which resides between the endocardium and myocardium. These invading endothelial cells undergo an endothelial-to-mesenchymal transition (for detailed review see [1]) and give rise to swellings known as endocardial cushions (EC). ECs contribute to the valves and septa of the heart and disruptions in their formation result in valvular and septal defects [2]. A number of signalling pathways, including vascular endothelial growth factor (VEGF) signalling, Notch, Wnt/β-catenin, BMP/Transforming growth factor β (TGFβ) signalling have been implicated in AVS development either *in vitro* or *in vivo* [1].

This extensive knowledge of the genetic control of AVS development has yet to be translated into a broader clinical knowledge of the genetic determinants of CHD. This is largely due to the complex pathogenesis of CHD and the scarcity of large families with multiple affected individuals suitable for conventional genetic analyses. More recently, candidate screening approaches have been utilized to circumvent this limitation. Such approaches, when coupled with kindred linkage and/or detailed functional analyses, can identify novel, causative mutations in genes previously suspected to function in AVS development [3-5].

In an effort to identify genetic lesions causative of CHD, we used a candidate approach and sequenced the coding regions of 32 candidate genes in DNA from patients with defects associated with improper EC development. We focussed on the functional characterisation of a number of cSNPs in the *ALK2* gene. ALK2 is a bone morphogenetic protein (BMP) receptor and roles for BMP signalling in EC development have been well documented [6]. Compound mutants of BMP ligands (including BMP6/BMP7 and BMP5/BMP7) have been shown to result in defective EC development in mouse [7-10]. Moreover, the genetic ablation of ALK2 in the endocardium of developing mouse embryos, results in hypoplastic ECs [11]. This role for ALK2 in EC development is also conserved evolutionarily whereby an absence of ALK2 in the ALK2/8 zebrafish mutant, *lost-a-fin*,

results in reduced EC development (manuscript in preparation). Despite the well characterised role of ALK2 in AVS development, examples of aberrant BMP signalling in patients with CHDs have yet to be reported. Here we report the identification of 11 genetic lesions in 10 different genes predicted to affect protein function. Furthermore, we report a detailed characterisation of two genetic lesions in the BMP receptor gene, *ALK2*.

Materials and Methods

SNP/mutation discovery

cSNPs in selected genes was determined by dideoxy resequencing of PCR-amplified exonic fragments. PCR primers (sequences available upon request) for amplification were designed using an in-house developed management system (http://www.limstill.niob.knaw.nl), which was also used for the semi-automated identification and classification of mutations [12].

Clinical evaluation, sample collection and genotyping.

DNA material from the Netherlands national congenital heart disease registry, CONCOR, was obtained as previously described [13]. DNA material from patients with non-cardiac-related diseases (Fragile X [n=170], male infertility [n = 90] and Rett Syndrome [n = 90]) was used for controls. Controls were sourced from the Dutch population which comprises 80% of individuals of European ancestry (according to the Central Bureau of Statistics, The Netherlands, for 2008). The two probands harbouring the ALK2 variants (R307L and L343P) were of European ancestry and the remaining 10 mutations were identified in patients from the Dutch population. Following identification of variant alleles, patients and kindred were examined and samples collected in accordance with the Local Research Ethics Committee of the University Medical Center Utrecht. Clinical evaluation was performed by history and physical examination and positive carriers, by transthoracic echocardiographic examination. Genotyping was performed on DNA from peripheral blood, oral epithelium, hair follicles or tissue biopsy by PCR amplification of exon 6, followed by sequencing using Big Dye Terminator chemistry.

Cell lines and transfections.

Bovine aortic endothelial cells [14] were used for luciferase assays and cos7 cells were used for construct expression and kinase assay experiments. All transfections were performed using Lipofectamine (Invitrogen) according to manufacturer's instructions.

Constructs, luciferase assay and kinase activity assays.

Expression constructs for full-length human ALK2 have been previously described [15] and mutant variants were generated by site-directed mutagenesis using a QuikChange kit (Stratagene). Inserts were fully sequenced and recloned into the original parental vector or the pCS2+ vector (for mRNA synthesis). Luciferase assay was performed as previously described [16] in combination with a short hairpin RNA (shRNA) targeting bovine ALK2 construct. The shRNA targeting construct was made by cloning complementary oligonucleotides into the pSuper vector (5'–gatccccGATGAGAAGTCGTGGTTTAttcaagagaTAAACCACGACTTCTCATCttttggaaa –3') to knockdown endogenous bovine ALK2 but not vector-based, human ALK2. Kinase assay was performed previously described [17] using γ-ATP (Perkin Elmer).

Protein isolation and western blot analysis

Protein for construct expression analysis was isolated from transfected cells by direct lysis in laemmli buffer. For embryos lysis, lysates were collected as previously described [18]. Western blotting was performed as previously described [18] using an HA antibody (12CA5; Roche) at 1:1000 and rabbit anti-phospho-Smad1,5,8 (Cell Signaling) at a concentration of 1:1000.

Fish lines, mRNA synthesis, injections, in situ hybridisation (ISH) and immunofluorescence.

Wild type and *Tg(Tie2:EGFP)*^{s849} [19] fish were kept under standard conditions [20]. pCS2+ ALK2 constructs were linearised with NotI enzyme and capped mRNA prepared with the Message Machine kit (Ambion). mRNA was diluted in nuclease-free water and 1 nl per embryo injected at the 1-2 cell stage. ISH was carried out as previously described [20]. Embryos were cleared in methanol and mounted in benzylbenzoaat/benzylalcohol (2:1) before pictures were taken. Immunohistochemistry was performed as previously described

[21]. Mouse anti-Tropomyosin (Sigma) and mouse anti-GFP (Santa Cruz) were applied at 1:200.

Statistical analysis

Results are expressed as mean ± SEM. Data were transformed to normality with equal variance by rank transformation. Statistical significance was determined by two-way analysis of variance using the program SigmaStat version 3.0 (Jandel Scientific, San Rafael, CA). The overall significance of the ANOVA for each of the two factors was $p < 0.001$. All pair-wise comparisons were made using the Holm-Sidak method.

Results

Identification of cSNPs in patients with CHD

In an effort to identify SNPs that may be causative of CHD, the coding regions of 32 candidate genes (selected on the basis of relevant phenotypes from model organisms) were analyzed in DNA from 190 patients with AVSDs. 7 genes showed no variation in their coding sequences, while 418 variants were identified in the other 25 genes, including 199 out of 200 known SNPs that were present in dbSNP at that time, indicating high sensitivity for SNP detection. A total of 86 SNPs were identified resulting in non-synonymous substitutions or cSNPs of which 43 cSNPs were unique to the patient group and did not occur in any of the control DNA samples derived from a Dutch population with non-cardiac diseases (see Materials and Methods for details; Supplementary Table 1). The potential functional effect of all cSNPs was analyzed using the SIFT [22] and PolyPhen [23] programs (Supplementary Table 1), showing that 18 cSNPs are predicted to affect protein function by both algorithms. Interestingly, only 7 were found in the class of cSNPs not unique to the patient group while 11 cSNPs that are predicted to affect protein function were found in the patient specific class of cSNPs and may thus contribute to the disease phenotype in these patients (Table I).

We focussed on the functional characterisation of a number of cSNPs in the *ALK2* gene. *ALK2* was found to contain three cSNPs: Ala15Gly (A15G) (located in the predicted signal peptide), Arg307Leu (R307L) and Leu343Pro (L343P) (these latter two located in the kinase domain) (Figure 1a-d). Of the 350 control individuals screened, 9 possessed the

Table 1. List of patient specific cSNPs predicted to be damaging to protein function.

No.	Gene and resultant substitution	Number of patients	Sift prediction [18]	Polyphen prediction [19]
1	ALK2 L343P	1	affects, 0.00	Probably damaging
2	ALK3 R443C	1	affects, 0.0	Possibly damaging
3	APC N1217T	1	affects, 0.01 *	Possibly damaging
4	ECE2 Y417C	1	affects, 0.01	Probably damaging
5	EGFR D1152H	1	affects, 0.03	Possibly damaging
6	EGFR P848L	2	affects, 0.00	Probably damaging
7	ERBB3 P1283R	1	affects 0.00 *	Probably damaging
8	FOXP1 P604L	1	affects, 0.04	Possibly damaging
9	GATA4 R285C	1	affects, 0.00	Probably damaging
10	ADAM19 P694S	1	affects, 0.00	Probably damaging
11	UGDH R141C	2	affects, 0.02	Probably damaging

* Low confidence for this prediction, due to low sequence number for comparison.

A15G variant (allele frequency 1.3%), indicating that this cSNP is common to the general population. No controls (n=350) were found to possess the R307L or L343P variant. Only L343P was predicted to affect protein function.

Analysis of the crystal structure of TGFβRI, which is highly homologous to the cytoplasmic domain of the ALK2 receptor, suggests that R307 is solvent exposed whereas L343 is part of a β-sheet that shields the nucleotide binding site. Interestingly, the side chain of L343 is in van-der-Waals interaction distance to the base of the nucleotide. The L343P substitution would interrupt this interaction and is likely to disrupt the β-sheet, thereby destabilising the overall structure of the kinase domain (Figure 1e).

Pedigree analysis of patients carrying cSNPS in ALK2

The proband carrying the R307L allele (Supplementary Figure 1a, III:2) was diagnosed soon after birth with a complex congenital defect which included a common atrium, a cleft of the anterior mitral valve (MV) leaflet, interruption of the inferior caval vein (Figure 1f) and a partial abdominal situs inversus. The proband experienced a miscarriage in early pregnancy (IV:1). At physical examination no evidence for other

Figure 1.

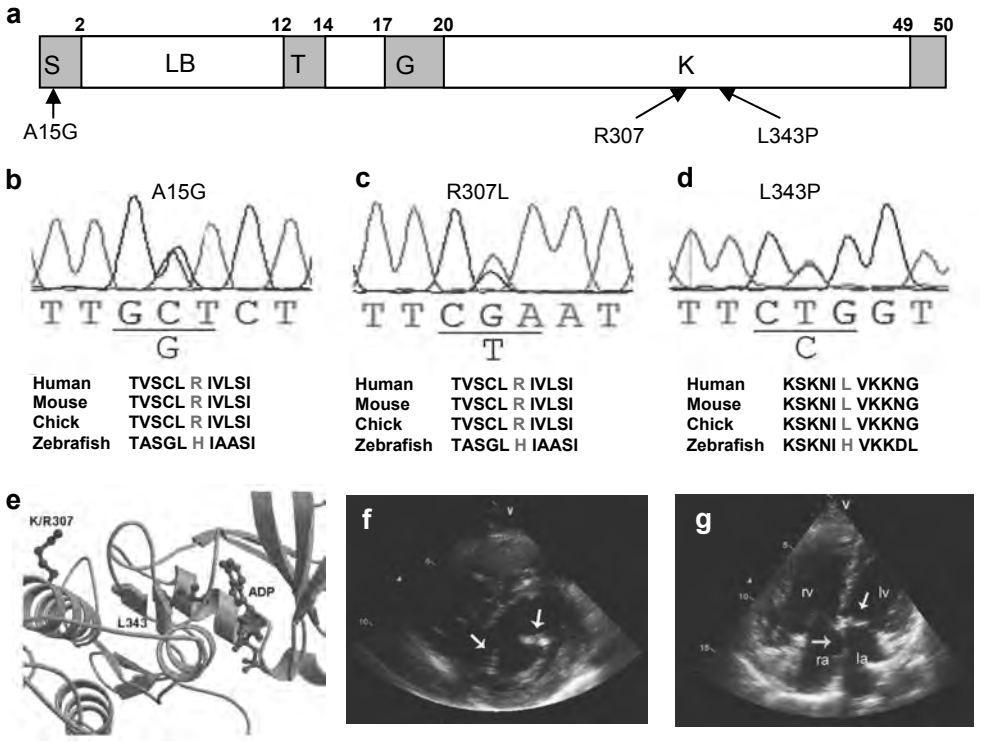


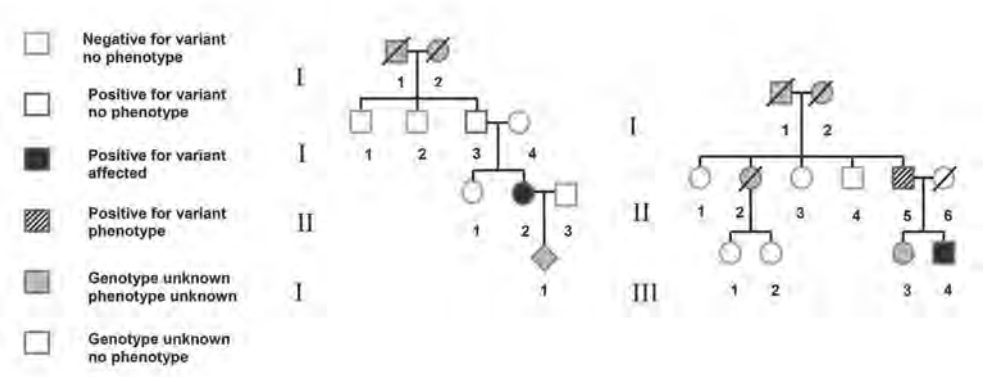
Figure 1. Identification of ALK2 variations in patients with CHDs. ***a***. *Schematic representation of the ALK2 protein and structural domains: signal peptide (SP), ligand binding domain (LBD), transmembrane domain (TM), GS domain (GS) and kinase domain (KD).* ***b-d***. *Sequencing chromatograms showing heterozygous cSNPs A15G (**b**) R307L (**c**) and L343P (**d**) in genomic DNA from affected individuals with accompanying protein alignments below (aligned by ClustalW). The protein alignment shows the poorly conserved nature of the A15 and R307 residues and strong conservation for the L343 residue.* ***e***. *TGFβRI (pdb entry 1PY5) as a model for ALK2, showing R307 and L343 in red, ADP in blue and the β-sheet, where L343 resides in yellow.* ***f,g***. *Echocardiograms of probands carrying variations R307L (**f**; individual III:2) and L343P (**g**; individual III:4).* ***f***. *Echocardiogram of R307L proband (after surgery, four-chamber view). Corrected common atrium, cleft of anterior MV and interruption of inferior caval vein, showing a thickened anterior MV leaflet (white arrow), thickened portion of atrial septum (yellow arrow) and an enlarged right chamber due to pulmonary hypertension. RV: right ventricle, LV: left ventricle, RA: right atrium, LA: Left atrium.* ***g.*** *Echocardiogram of L343P proband (short axis parasternal), showing a cleft MV (arrows), located in the middle of the anterior MV leaflet. Doppler examination showed mild MV regurgitation. For figure in colour: Supplement colour figures page 199.*

congenital malformations or other dysmorphic features were found. Pedigree analysis revealed that the proband's father (II:3) carried the R307L allele however ultrasound and examination revealed no CHD in this individual. No other living relatives were positive for the variant or had cardiac complaints.

The proband carrying the L343P variant (Supplementary Figure 1b, III:4) was born with a primum type atrial septal defect (ASD I) with a cleft anterior MV leaflet and a small left-right shunt (Figure 1g). The MV showed mild regurgitation and no other cardiac defects were found. There was no evidence for other congenital malformations or other dysmorphic features and his blood cell count was within normal parameters. His father (II:5) had a cardiac murmur since he was 14 years of age and DNA sequencing demonstrated he was a carrier of the L343P allele. There was no evidence for mosaicism in the father in DNA derived from skin fibroblasts, hair roots, blood and oral epithelium. Additionally, equal amounts of the wt and L343P allele were found to be expressed in skin fibroblast, as determined by sequencing of cloned RT-PCR products (data not shown). Ultrasound examination showed calcification of the annulus of the posterior MV leaflet and prolapse of both leaflets. No other relatives carried the mutation or had cardiac complaints.

ALK2 L343P has reduced transcriptional and kinase activity in vitro

To determine whether the cSNPs affect ALK2 function, the various alleles were tested for their capacity to induce BMP-specific transcriptional reporter activity [16] (Figure 2a). There was no significant difference in the activity of either the A15G or R307L alleles



Supplementary Figure 1. *Family pedigrees of patients (identified by asterisk) with identified mutations.* ***a.*** *The family carrying the R307L variant exhibited no obvious inheritance pattern.* ***b.*** *The L343P pedigree was uninformative for inheritance of the mutation with CHD. The phenotype of II:5 could not be ruled out as degenerative and thus could not unambiguously be classified with a CHD. Carriers of non-carriers of variants are depicted with a red or green outlines, respectively, and individuals with or without CHDs are shaded black or white, respectively. The ambiguous phenotype is represented by hatching. Grey colouring indicates the genotype or phenotype was unable to be determined. The diamond indicates a miscarriage and deceased individuals are annotated by a diagonal strike-through.* *For figure in colour: Supplement colour figures page 203.*

Figure 2.

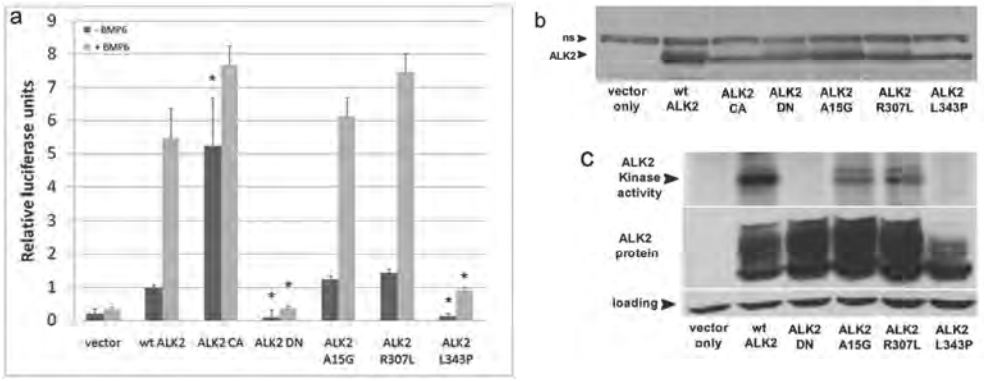


Figure 2. The ALK2 L343P allele has reduced activity in vitro. *Representative luciferase assay (**a**) on bovine aortic endothelial cells transfected with a BMP-responsive element fused to luciferase, renilla (as transfection control), ALK2 constructs and bovine ALK2 RNAi construct (to knockdown endogenous bovine, but not vector-based, human ALK2). ALK2 activity was measured as relative luciferase units (RLU), without (dark grey) and with (light grey) induction by BMP6 protein. Significantly higher RLU was observed for ALK2 CA without induction whereas significantly lower RLU was observed for ALK2 DN with and without BMP6 induction. No significant differences were observed for ALK2 A15G or ALK2 R307L without or with induction compared with wtALK2. Significantly lower RLU was observed for ALK2 L343P both without and with induction compared with wtALK2. No statistically significant interaction was detected between BMP6 treatments and the various ALK2 alleles (as determined by two-way ANOVA; $p = 0.140$). RLU were measured as luciferase activity/renilla activity and expressed relative to unstimulated wtALK2 activity. RLU are depicted as mean ± SEM, where n = 3-8 experiments (3 replicates per experiment). Data were transformed to normality with equal variance by rank transformation. Statistical significance was determined by two-way ANOVA. All pair-wise comparisons were made using the Holm-Sidak method and alleles significantly different from wtALK2 are indicated by * $p < 0.05$. **b**. Construct expression was approximately equal for all constructs when transfected into cos7 cells, as determined by western blot for the HA-tag harboured by each ALK2 construct. **c**. Kinase activity assay showing kinase activity present for wtALK2, ALK2 A15G and ALK2 R307L but no detectable activity for alleles ALK2 DN or ALK2 L343P (autoradiogram). Western blotting for HA-tagged protein shows that the protein was expressed for each construct approximately equally (middle blot) and the total loading was approximately equal for all samples (lower blot).*

compared with wtALK2 using this assay, indicating that *in vitro* receptor activity is not altered by these substitutions. Strikingly, however, expressing the L343P variant resulted in significantly lower luciferase activity in both the uninduced (without BMP) and induced (with BMP) state when compared with wtALK2 ($p < 0.05$). Using a kinase assay, kinase activity was observed in wtALK2, ALK2 A15G and ALK2 R307L (albeit slightly reduced in these latter two alleles) however no measurable kinase activity was detected for the ALK2 L343P allele, comparable with that of the classical dominant-negative form of the receptor [24] (ALK2 DN; Figure 2c). These data demonstrate that the L343P cSNP reduces ALK2 signalling *in vitro* and disrupts the kinase activity of the receptor.

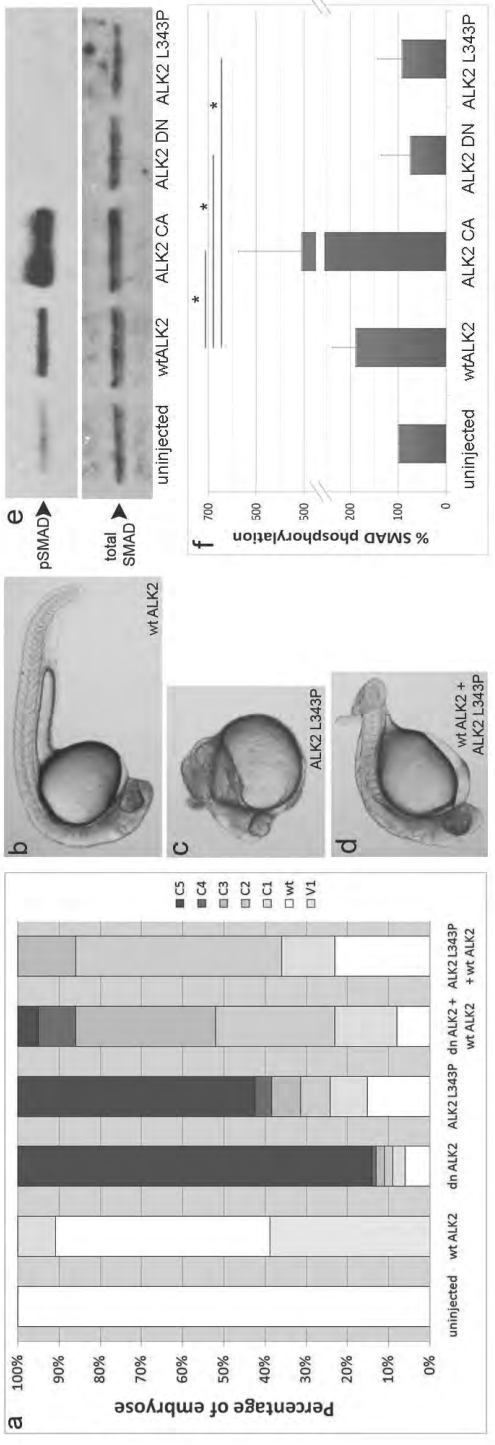


Figure 3. ALK2 L343P acts in a dominant-negative fashion in vivo. *Zebrafish embryos uninjected and injected with variant RNA at the single cell stage.* ***a****. Graphical depiction of the DV phenotype of embryos uninjected or injected with wtALK2, ALK2 DN alone or coinjected with wt ALK2, ALK2 L343P alone or coinjected with wtALK2 at the single cell stage and scored at 28 hpf.* ***b-d****. Images of* ***b****. wtALK2,* ***c****. ALK2 L343P and* ***d****. wtALK2 and ALK2 L343P injected embryos at 28 hpf.* ***a,d****. wtALK2 RNA resulted in a wild type phenotype in the majority of embryos (51% of 292 embryos) whereas the ALK2 DN RNA resulted in the majority of embryos with severe C5 dorsalisation (86% of 149 embryos). ALK2 L343P RNA also severely dorsalised embryos to C5 (c; 57% in 150 embryos) although not as potently as ALK2 DN RNA. Coinjection of wtALK2 with ALK2 L343P RNA partially rescued the dorsalisation phenotype resulting in a more intermediate C2 phenotype in the majority of embryos (****d****; 50% in 70 embryos). A similar affect was observed after coinjection of wtALK2 with ALK2 DN RNA.* ***e,f****. pSMAD levels in wt embryos injected with ALK2 variant RNA.* ***e****. Representative western blots of pSMAD (top) and total SMAD (bottom) proteins.* ***f****. Graphical representation of 4 independent experiments showing significantly lower pSMAD signaling in embryos injected with ALK2 L343P RNA. Bars are mean ± SEM and represent the percentage of SMAD phosphorylation, relative to uninjected embryos. The percentage of pSMAD was determined as a ratio of optical density of the pSMAD band compared with the total SMAD band. Statistical significance was determined by t test, where * $p < 0.05$. For figure in colour: Supplement colour figures page 200.*

ALK2 L343P has dominant-negative activity in vivo

To investigate the *in vivo* functionality of the L343P allele, ALK2 RNA was injected into zebrafish embryos at the single cell stage (Figure 3 and Supplementary Table 2). BMP signalling plays a pivotal role in dorsoventral (DV) patterning of the zebrafish embryo, whereby abrogation of signalling results in more dorsal tissue (dorsalisation) and constitutive activation results in more ventral tissue (ventralisation) of the embryo [25]. Injection of human wtALK2 RNA into wt zebrafish embryos caused mild ventralisation (V1 in 38% of embryos; Figure 3a; classification according to [26, 27]) and this effect increased upon injection of higher concentrations (V1 and V2 in 21 and 26% of embryos, respectively; Supplementary Table 2). Mild ventralisation was observed in A15G and R307L variant injections, comparable with wtALK2. In contrast, injection of L343P RNA into wt embryos resulted in severe dorsalisation (C5 in 57% of embryos; Figure 3a,c), comparable with that of ALK2 DN, suggesting a dominant-interfering affect of this cSNP. This effect on DV patterning was confirmed by *in situ* hybridisation (ISH) using a number of DV markers (Supplementary Figure 2). To determine the extent of interference by the L343P allele on wtALK2 signalling, equimolar amounts of wt and L343P RNA were injected into zebrafish embryos. Embryos exhibited an intermediate dorsalising effect (C2 in 50% of embryos; Figure 3b,d), confirming the dominant nature of the L343P allele.

To investigate downstream signalling, western blot analysis for phosphorylated SMAD 1,5,8 (pSmad1) proteins, the downstream target of ALK2, was performed on RNA-injected embryos. pSmad1 levels were higher in wtALK2-injected embryos and higher still in embryos injected with a constitutively active form of ALK2 [28] whereas injection of ALK2 DN resulted in significantly lower pSmad1 levels, compared with wtALK2 injected embryos (Figure 3e,f; $p < 0.05$). Consistent with the dorsalising effect of ALK2 L343P RNA, L343P injection resulted in significantly lower levels of pSmad1 (Figure 3e,f; $p < 0.05$). Together these observations demonstrate the dominant-interfering effect of the L343P substitution on ALK2 function *in vivo*.

Overexpression of ALK2 L343P in zebrafish embryos disrupts AVC formation

Finally, we investigated heart morphology of embryos injected with ALK2 L343P RNA (Figure 4). ISH analysis for *cmlc2*, a cardiac myosin marker, showed the overall morphology of the heart tube was disrupted in ALK2 L343P injected embryos compared

Supplementary table 2. Phenotype of zebrafish at 28 hpf after injection at the one-cell stage with variations ALK2 construct

				Phenotype (percentage)									
RNA	Concentration	Genotype	Number	V4	V3	V2	V1	wt	C1	C2	C3	C4	C5
-	-	wildtype	441	0	0	0	0	100	0	0	0	0	0
wt ALK2	10 ng/uL	wildtype	292	0	0	1	38	51	9	2	0	0	0
wt ALK2	60 ng/uL	wildtype	76	0	0	26	21	44	9	0	0	0	0
dn ALK2	10 ng/uL	wildtype	149	0	0	0	0	6	3	2	2	1	86
ca ALK2	10 ng/uL	wildtype	160	99	0	0	1	0	0	0	0	0	0
ALK2 A15G	10 ng/uL	wildtype	174	0	0	0	24	71	3	1	1	0	0
ALK2 R307L	10 ng/uL	wildtype	142	0	0	4	21	68	5	1	1	0	0
ALK2 L343P	10 ng/uL	wildtype	150	0	0	0	0	15	9	7	7	4	57
wt + dn ALK2	20 ng/uL*	wildtype	92	0	0	0	0	8	15	29	34	9	5
wt + L343P ALK2	20 ng/uL*	wildtype	70	0	0	0	0	23	13	50	14	0	0

* 10 ng/uL of each RNA
Phenotypic classification based on [23, 24]
All constructs are based of human sequence

with uninjected and wtALK2 injected embryos, similar to that observed in the *laf/alk2/8* mutant [29]. *tbx2b*, *anf/nppa* and *has2*, markers of the AVC myocardium, the atrial and ventricular chambers and the endocardial cushions, respectively, revealed a loss of AVC identity. *tbx2b* expression was lost or reduced in ALK2 L343P injected embryos compared with uninjected and wtALK2 injected embryos. *anf* expression, which is characteristically absent in the AVC, was continuous throughout the heart tube in ALK2 L343P injected embryos compared with controls and *has2* expression was lost in ALK2 L343P injected compared with control embryos, reminiscent of mouse mutants with reduced BMP signalling which also exhibit a loss of *tbx2b* and *has2* expression [9]. Loss of these markers was not due to a disruption in cardiac looping as these markers were patterned normally in a non-looping mutant (HU119). Finally, we visualised the endocardial cells of the AVC cushions by immunofluorescent staining of uninjected, wtALK2 and ALK2 L343P RNA injected embryos. Using the transgenic line *Tg(Tie2:EGFP)*, which has enriched expression of GFP in ECs, the myocardium was counterstained using anti-Tropomyosin antibody. We observed reduced expression of GFP and a loss of endocardial cushions in ALK2 L343P RNA injected embryos. These data demonstrate that reduced ALK2 signalling in the zebrafish, via injection of L343P RNA, disrupts normal heart morphology and formation of the AVC.

Discussion

CHDs occur in more than 1 out of every 100 live births and yet only a limited number of determinants responsible for this set of diseases have been uncovered [30]. The number of causative mutations described to date has been restricted to a subset of genes, including *GATA4* [31], *NKX2.5* [32] and *TBX20* [3], which are essential for normal cardiac development. Most of the identified lesions, however, have been identified by conventional genetic approaches utilizing large families with multiple affected individuals. The incidence of such large families with an inheritance pattern of CHDs, however, is rare. The rarity of such families has been attributed to the multi-factorial nature of CHD and/or low penetrance of the causative lesion. In recent years a number of genetic lesions have been described using candidate approaches and this has yielded a vast number of putative mutations. However the inherently biased approach of this method requires that further validation of these mutations is performed, either by pedigree analyses and/or functional characterization of the gene products.

Figure 4. The AVC and ECs are improperly formed in embryos injected with ALK2 L343P RNA*. ISH staining for cardiac myosin (cmlc2), the AVC myocardium (tbx2b), the atrial and ventricular chambers (anf) and the ECs (has2) and immunofluorescent staining for ECs. Cmlc2 staining showed a disruption in the overall morphology of the heart tube in ALK2 L343P injected embryos (33/62 embryos) compared with uninjected (9/67 embryos) and wtALK2 injected embryos (10/69 embryos). A loss of AVC identity was apparent in embryos injected with ALK2 L343P RNA compared with uninjected and wtALK2 RNA injected controls. tbx2b expression was lost or reduced in ALK2 L343P RNA injected embryos (40/81 embryos; arrow head) compared with uninjected (9/62 embryos) and wtALK2 injected embryos (13/57 embryos). anf expression was continuous throughout the heart tube of ALK2 L343P injected embryos (22/32 embryos) compared with uninjected (13/39 embryos) and wtALK2 injected embryos (6/42 embryos). has2 expression was also lost in ALK2 L343P injected (23/124 embryos) compared with uninjected (0/50 embryos) and wtALK2 injected embryos (0/120 embryos)(red arrowhead: OFT, yellow arrowhead: AVC). Disruption of these markers is not due to defective cardiac looping as these markers were patterned normally in a non-looping mutant (HU119). AVC (white arrowhead) and OFT (green arrowhead) cushions were visualised using the transgenic line, Tg(Tie2:EGFP), counterstaining the myocardium with anti-Tropomyosin antibody. Reduced expression of GFP and a loss of EC structure were observed in ALK2 L343P RNA injected embryos. For figure in colour: Supplement colour figures page 201.*

We have identified 11 novel cSNPs predicted to be damaging to protein function in patients with AVSD. These putative mutations were identified in 10 genes shown through animal and cell culture experimentation to be important in EC formation and, to our knowledge, represent the largest collection of cSNPs for this disease. Surprisingly, of the 11 novel cSNPs predicted to be damaging to protein function we only identified 1 patient with a cSNP in GATA4 and none in NKX2-5 or TBX20. Our systematic analysis of a large group of patients with defects associated with improper EC development demonstrates that mutations in GATA4, NKX2-5 or TBX20 have a very small contribution to such CHDs in the Netherlands.

We have identified 43 cSNPs that are unique to the group of 190 patients. Amongst this collection of cSNPs we identified two variants in the BMP receptor gene, *ALK2*. This equates to 1% of the CHD patients screened (2/190). Pedigree analysis showed a lack of segregation of the R307L allele with a CHD. Furthermore, no functional differences were observed between the wt and R307L alleles in either the BMP-specific transcriptional response assays or by phenotypic evaluation of RNA-injected embryos. These findings do not preclude this allele from contributing to the R307L proband phenotype however this is difficult to determine with the current data. For the L343P variant, it was not possible to establish a pattern of inheritance for the variant with a cardiac phenotype. Although the atypical site of the mitral valve prolapse in the proband's father and the historical nature of the cardiac murmur from childhood favors a congenital origin for the defect, a degenerative cause for this phenotype could not be ruled out. The ambiguous nature of the heart defect was uninformative and, whilst not contradicting an inheritance pattern for this allele, could not support co-segregation either.

Structural modelling of the kinase domain of the ALK2 receptor in combination with kinase assay suggested that the L343P variation interferes with kinase activity of the ALK2 receptor and transcriptional assay results demonstrate a loss of BMP-responsive receptor activity. Interestingly, a dominant-negative affect was observed in RNA-injected embryos suggesting that the L343P variant is capable of participating in the receptor complex and interfering with BMP signalling. Whether this variant acts at the level of ligand binding, thus acting as a ligand sink, or by occupying binding sites of co-receptors was not addressed here. What is clear from the coinjection experiment of wt and L343P

RNA, however, is the L343P allele is capable of interfering with wt ALK2 signalling when present in equimolar amounts. Furthermore, the downstream effector of ALK2 signalling, pSMAD1, was activated at lower levels in L343P RNA-injected embryos. Finally, we show that the AVC in embryos injected with L343P RNA is disrupted. The overall morphology of the heart in L343P-RNA injected embryos was disturbed as characterized by a loss of cardiac looping. Importantly, however, a mutant with cardiac looping defects exhibited restricted expression patterns for markers of the AVC, cardiac cushions and cardiac chambers, similar to wt control embryos, demonstrating that normal cardiac looping is not required for patterning of these markers. This loss of AVC identity is, therefore, attributed to a specific disruption in BMP signalling.

To our knowledge, this is the first specific report linking variations in ALK2 with CHDs. A database study examining the chromosomal regions most frequently altered in patients with CHDs failed to identify patients with disruptions in the region harbouring the *ALK2* gene (region 2q24.1) [33]. A current investigation of the database, Decipher (DatabasE of Chromosomal Imbalance and Phenotype in Humans using Ensembl Resources), reveals six patients in possession of a deletion inclusive of the *ALK2* gene. Although there is only limited clinical information for these patients, none are listed as possessing a CHD. In contrast, examination of the Ecaruca database (European Cytogeneticists Association Register of Unbalanced Chromosome Aberrations) identifies more than 10 patients with deletions that map to this genomic region and possess CHDs manifesting as ASDs or ventricular septal defects (VSDs). Whilst these diseases are consistent with improper EC formation, it is difficult to determine whether or not these defects are the direct result of ALK2 hemizygosity or the surrounding genes that are also absent due to the deletion. Given the lack of phenotype in ALK2 heterozygous null mice [34, 35], however, it is unlikely that a loss of one copy of *ALK2* would be sufficient to be causative.

The requirement for ALK2 in EC formation has been well documented. Chick AV cushion explants fail to undergo EMT when exposed to ALK2 neutralizing antibodies [36]. Furthermore, endothelial-specific deletion of ALK2 in mice results in hypoplastic EC from a loss of EMT [11]. It is, therefore, not surprising that individuals carrying a dominant-negative allele of ALK2, such as the L343P variant, also possess EC-associated defects. What is surprising, however, is the viability of such individuals. ALK2-deficient mice die

during gastrulation [34, 35], demonstrating the necessity for ALK2-mediated signalling during early stages of embryonic development. That these individuals survive through gastrulation to develop an EC-associated cardiac defect suggests that a minimal amount of ALK2-mediated signalling still occurs in these individuals. It is also possible that, although equally expressed transcriptionally, the wt protein is more highly represented by virtue of a less stable L343P protein. Alternatively, other type I BMP receptors, such as ALK3, may be playing a compensatory role. Further studies will be necessary to tease out the details of how these individuals escape the devastating affect this allele is expected to exert during gastrulation.

Conclusion

To summarise, using a candidate sequencing approach we have screened the coding sequences of 33 genes known to be involved in AVS development in a DNA database derived from a host of AVSD patients. We identified 11 putative mutations that are predicted to affect protein function and thus potentially contribute to CHD in the screened patients. In this study we focus on the functional characterisation of the cSNPs identified in the BMP receptor, *ALK2*. We identified two novel heterozygous missense mutations in *ALK2,* one of which, the L343P variant, showed reduced signalling capacity *in vitro* and dominant-negative activity in *in vivo* assays. The dominant-negative activity of ALK2 L343P interferes with normal formation of the AVC and the development of ECs in developing zebrafish embryos. Finally, the *ALK2* L343P allele was demonstrated in a father and son, who both have a structural cardiac defect. However, whereas the son displayed an ASD1 with cleft MV, the father had an anomaly that could not be unambiguously classified as a CHD and was clinically non-penetrant, consistent with the notion that CHDs are usually complex diseases. Taken together our data suggest that mutations in ALK2 may be causative for AVSD and report a significant resource in the identification of 11 cSNPs potentially causative of AVSD.

Acknowledgements

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Disclosures

None.

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CHAPTER 4

ALK2 mutation in Down syndrome patient with congenital heart defect

ALK2 mutation in Down syndrome patient with congenital heart defect

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Abstract

Down syndrome, resulting from an additional copy of chromosome 21, is frequently associated with congenital heart defects. Though apparently increased dosage of chromosome 21 sequences are part of the etiology of cardiac defects, only a proportion of Down syndrome patients exhibit a congenital heart defect (birth prevalence 40-60%). Through a large candidate gene sequencing screen in patients with atrial ventricular septal defect, three heterozygous missense substitutions were identified in three different genes in a patient with DS and a primum type atrial septum defect. Structural modeling of the cytoplasmic domain of the ALK2 receptor suggests that H286 is in close proximity to the nucleotide binding site of the kinase domain. We investigated whether this p.His286Asp substitution altered ALK2 function by using both *in vitro* as well as *in vivo* assays.

The p.His286Asp variant demonstrated impaired functional activity as measured by BMP-specific transcriptional response assays. Furthermore, mild dominant-interfering activity was observed *in vivo* compared with wild type ALK2 as determined by RNA injection into zebrafish embryos. These data indicate that in the context of a Down syndrome background, ALK2 mediated reduction of BMP signalling may contribute to congenital heart defects. **(submitted)**

Keywords: Down Syndrome, Congenital Heart defects, gene mutation

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Introduction

Down syndrome (DS) is the most frequent cause of mental retardation, most often caused by trisomy 21 (in approximately 95% of cases). DS occurs in about one in 800-1000 live births [1-3]. The syndrome is characterized by well-defined and distinctive phenotypic features including characteristic facies, minor limb anomalies, hypotonia and growth retardation [4]. Whilst all individuals with DS manifest these phenotypes, the degree of the phenotype varies between DS individuals [5]. In conjunction with these phenotypic traits, DS individuals suffer a higher incidence of several conditions, including congenital heart defects (CHDs) [5].

The (birth) prevalence of congenital heart defects in DS is circa 40-60%, most frequently atrioventricular septal defects (AVSD) and ventricle septal defects (VSD) [6-8]. Efforts have been made to determine the critical chromosomal region for specific phenotypic features of DS by deletion mapping and characterization of patients with partial trisomy 21 [4, 9, 10]. One potential candidate gene for these trisomy 21-related congenital heart defects is collagen Type IV however the evidence for the involvement of collagen Type IV has, to date, been restricted to correlative expression pattern analysis [11, 12].

Whilst this susceptibility and variability of the DS phenotype is widely accepted, the factors contributing to this variability have not been established. It is postulated that variations in gene dosage of chromosome 21, environmental factors and genetic modifications not linked to chromosome 21 account for this variability and it is likely that contributions from each of these elements are involved. Genetic imbalance caused by the presence of an extra copy of chromosome 21 will seriously disrupt one or more developmental pathways. In addition the presence of non-chromosome 21 loci variation may predispose for a heart defect. To date, *CRELD1* (3p25.1) is the only non-chromosome 21 gene that has been reported to associate with DS-related CHDs [13-15].

We have previously reported mutations in *ALK2*, a type I receptor for bone morphogenetic proteins (BMPs), in patients with endocardial cushion-associated CHDs. One of these variants identified has a dominant-interfering effect on BMP signaling and is associated with primum type atrial septal defect (ASD type I) [16]. Here we report an additional *ALK2* variant that was identified in a patient with trisomy 21 and a primum type

atrial septal defect (ASD type I). In addition to the *ALK2* variant, two other variations were detected in another type I BMP receptor gene, *ALK3*, and in the epidermal growth factor receptor family, *ErbB3*. Using both that *in vitro* and *in vivo* assays, we show that the *ALK2* variant has reduced BMP-inductive capacity and has a mild dominant-interfering effect on BMP signaling. This effect on BMP signaling from the *ALK2* variation in conjunction with the additional variations identified in this patient is likely to contribute to the manifestation of a CHD in this individual.

Material and Methods

This study was prospectively reviewed and approved by the Local Research Ethics Committee of the University Medical Center Utrecht and the Academic Medical Center, Amsterdam, The Netherlands.

Genetic material and clinical evaluation

The patient described in this study was identified in a large-scale candidate gene sequencing screen as previously described [16]. In total 9 candidate genes were sequenced in this patient (*ALK2*, *ALK3*, *EGFR*, *GATA4*, *HAS2*, *UGDH*, *ERBB2*, *ERBB3*, *CRELD1*). Patient material was acquired from the CONCOR (CONgenital CORvitia) database [17]. DNA material was obtained from the patient and first degree relatives by buccal swaps. Family members were clinically evaluated and individuals testing positive for the *ALK2*, *ERBB3* and *ALK3* variant were examined by echocardiography. DNA material from patients with non-cardiac-related diseases (Fragile X [n=170], male infertility [n=90] and Rett Syndrome [n=90]) was used for controls. Controls were sourced from the Dutch population which comprises 80% of individuals of European ancestry (according to the Central Bureau of Statistics, The Netherlands, for 2008).

Detection of variants

PCR primers (sequence available upon request) for amplification were designed using an in-house developed management system (http://www.limstill.niob.knaw.nl), which was also used for the semi-automated identification and classification of mutations [18]. Selected genes were analysed by dideoxy sequencing of PCR-amplified exonic fragments.

Figure 1.

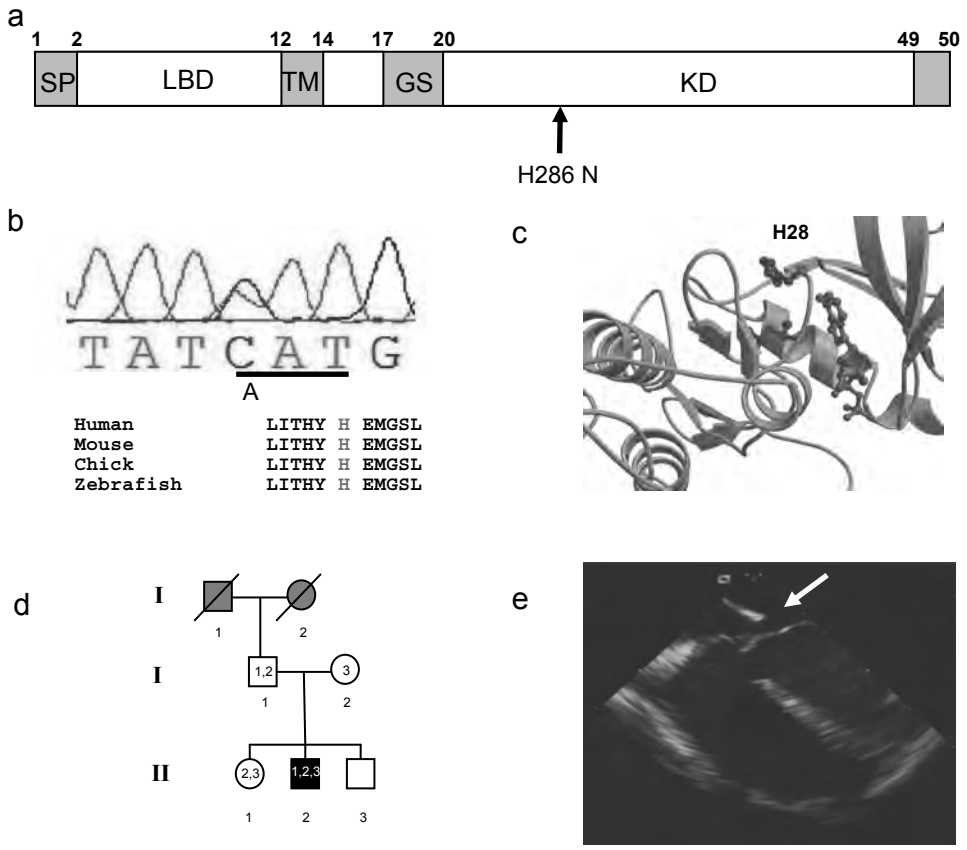


Figure 1. Identification of ALK2 variant in DS. ***a***. *Schematic representation of the ALK2 protein and structural domains: signal peptide (SP), ligand binding domain (LBD), transmembrane domain (TM), GS domain (GS) and kinase domain (KD). Arrows indicate approximate locations of p.His286Asp in ALK2.* ***b***. *Sequencing chromatogram showing the p.His286Asp variant in genomic DNA from the proband with DS and a primum type atrial septal defect with accompanying protein alignment below (aligned by ClustalW). The protein alignment shows strong conservation between species of the p.His286Asp residue.* ***c***. *TGFβRI (pdb entry 1PY5) as a model for ALK2, showing H268 in red, ADP in blue and the β-sheet in yellow.* ***d***. *Family pedigree of proband (III:2). Only the father, without DS, was in possession of the ALK2 variant and did not have a congenital heart defect at cardiac echocardiography. Carriers of variants are depicted with a number in the squares or circle; 1: ALK2 p.His286Asp variant, 2: ALK3 p.Glu414Lys and 3: ERBB3 p.Thr1169Il and individuals with or without CHDs are shaded black or white, respective. Grey colouration indicates that the genotype or phenotype could not be determined. Deceased individuals are annotated by a diagonal strike-through.* ***e***. *Echocardiogram of proband carrying the p.His286Asp variant (III:2) before surgery, demonstrating a large primum type atrial septal defect (arrow) and overriding AV valve. For figure in colour: Supplement colour figures page 202.*

Cell lines and transfections.

Bovine aortic endothelial cells [19] were used for luciferase assays and cos7 cells were used for construct expression. All transfections were performed using Lipofectamine (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions.

Constructs, luciferase assay and kinase activity assays.

Expression constructs for full-length human ALK2 have been cloned into the pcDNA3 vector as previously described [20] and mutant variants were generated by site-directed mutagenesis using a QuikChange kit (Stratagene, La Jolla, CA, USA). Inserts were fully sequenced and recloned into the original parental vector or the pCS2+ vector (for mRNA synthesis) to ensure against dditional mutations. Luciferase assay was performed as previously described [21]. This included a injection of vector only and in combination with a short hairpin RNA (shRNA), to knockdown endogenous bovine ALK2 but not vector-based human ALK2, as described previously [16].

Fish lines, mRNA synthesis and injections

Wild type fish were kept under standard conditions as previously described [22]. pCS2+ ALK2 constructs were linearised with NotI enzyme and capped mRNA was prepared with the Message Machine kit (Ambion, Austin, TX, USA). mRNA was diluted to appropriate concentration in nuclease-free water and 1 nl [22] per embryo injected at the 1-2 cell stage. Embryos were cleared in methanol and mounted in benzylbenzoaat/ benzylalcohol (2:1) prior to imaging.

Statistical analysis

Results are expressed as mean ± SEM. Statistical significance was determined by one-way analysis of variance, followed by a Bonferroni *t* test using the program SigmaStat version 2.03 (Jandel Scientific, San Rafael, CA).

Results

We recently performed a screen for the detection of cSNPs (coding single nucleotide polymorphisms) in patients with congenital heart defects arising from improper endocardial

Figure 2.

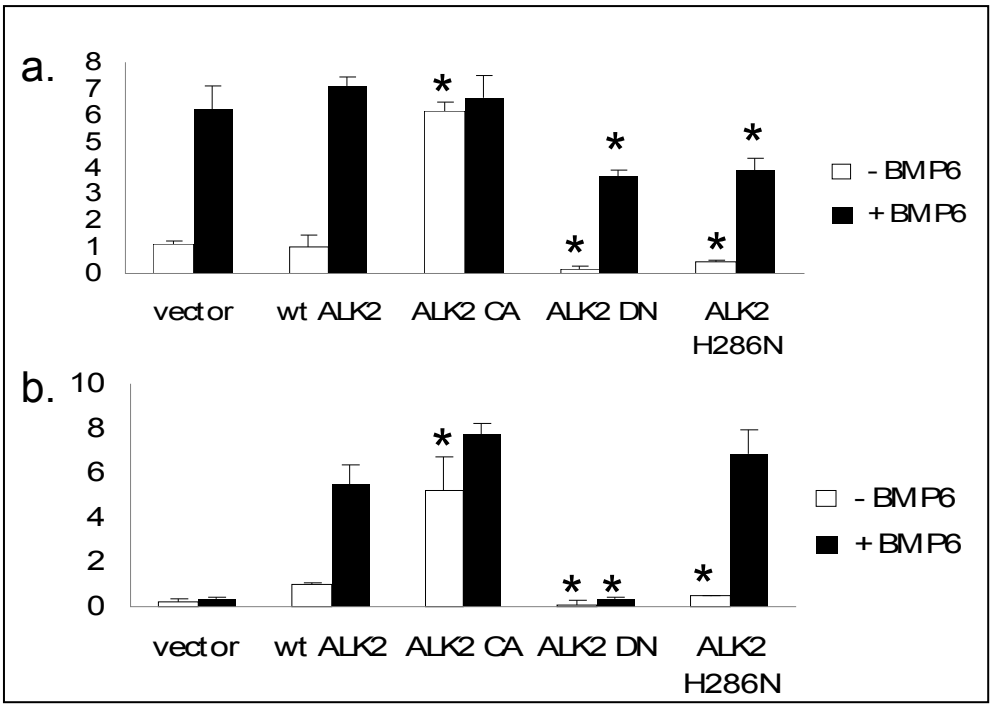
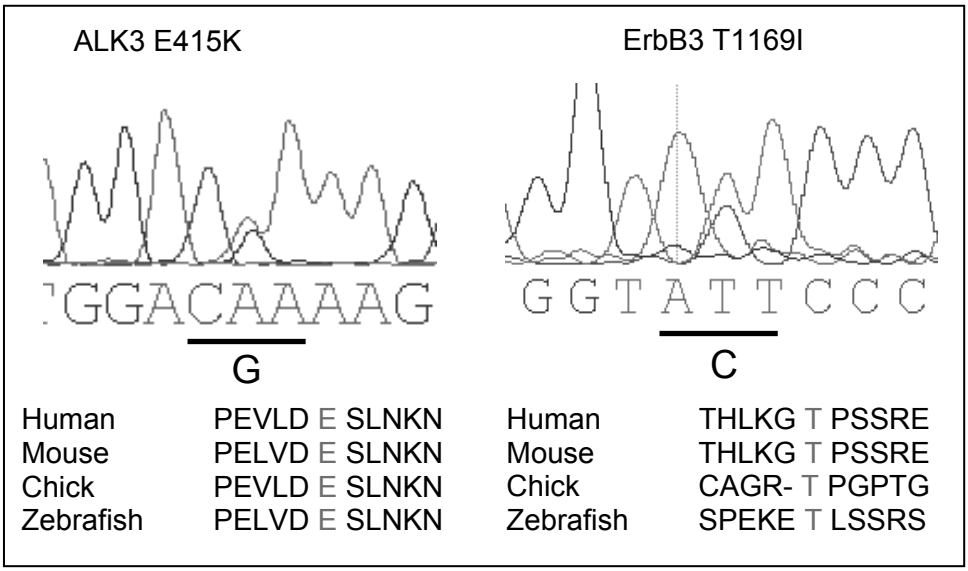


Figure 2. *Representative luciferase assay on bovine aortic endothelial (BAE) cells transiently transfected with a BMP-responsive element fused to luciferase, renilla (to control the transfection) and ALK2 constructs. ALK2 activity was measured as relative luciferase units (RLU), without (blue) and with (red) induction by BMP6 protein.* ***(a)*** *Significantly higher RLU was observed for ALK2 CA without induction whereas significantly lower RLU was observed for ALK2 DN with and without BMP6 induction. For the p.His286Asp variant both without and with induction with BMP6 significantly lower RLW was observed.* ***(b)*** *Luciferase assay on BAE as described above including injection of bovine ALK2 RNAi construct, to knockdown endogenous bovine ALK2 but not vector-based, human ALK2. A similar pattern for ALK2 CA, ALK2 DN and wt ALK2 was observed as with injection of vector only. However, transfection with the p.His286Asp variant demonstrated significantly lower RLU without BMP6 induction whilst induction with BMP6 did not demonstrate a significant difference between wt ALK2 and the p.His286Asp variant. RLU were measured as luciferase activity/renilla activity and expressed relative to unstimulated wt ALK2 activity. RLU are depicted as mean ± SEM, where n = 5 (3 replicates per experiment). Statistical significance, compared with wt ALK2, was determined by t test, where * p <0.05.*

cushion formation [16]. During this screen we identified a patient with Down syndrome in possession of compound cSNPs in *ALK2* resulted in a His286Asp substitution (*ALK2* p.His286Asp) (Figure 1b); the *ALK3* cSNP caused a Glu414Lys substitution (*ALK3* p.Glu414Lys); whilst the cSNP in *ERBB3* resulted in a Thr1169Ile substitution (*ERBB3* p.Thr1169Ile) (Supplement figure 1). These cSNPs were analyzed with the prediction

Supplement figure 1.



Supplement figure 1. *Sequencing chromatogram with alignment below (aligned by ClustalW) showing* ***a.*** *the ALK3 p.Glu414Lys. Protein alignment of the ALK3 p.Glu414Lys demonstrates strong conservation between species.* ***b.*** *Sequencing chromatogram of the ERBB3 p.Thr1169Ile variant. Conservation alignment did not show strong conservation of the ERBB3 p.Thr1169Ile region. For figure in colour: Supplement colour figures page 204.*

models PolyPhen [23, 24] and SIFT [25] (Table 1). Only *ALK2* p.His286Asp was predicted to be damaging protein function in both models and was not detected in 350 controls of the Dutch population. The coding regions of a total of 9 genes were sequenced in this individual, including *CRELD1*, and no other cSNPs were identified.

This patient with DS was diagnosed with a primum type atrial septal defect with a cleft mitral valve and the coronary sinus draining into the left atrium (Figure 1e). Sequencing of DNA from peripheral blood lymphocytes comfirmed the 3cSNPs in this individual (figure 1d). To determine heritability and segregation of the cSNPs, we screened all first degree relatives of the proband for the presence of each substitution (Figure 1d). The father of the proband was found to possess both the *ALK2* p.His286Asp and the *ALK3* p.Glu414Lys variants whereas the mother was a carrier of the *ERBB3* p.Thr1169Ile variant. Of the probands two sibling, only the sister carried the *ALK3* p.Glu414Lys and the *ERBB3*

Figure 3.

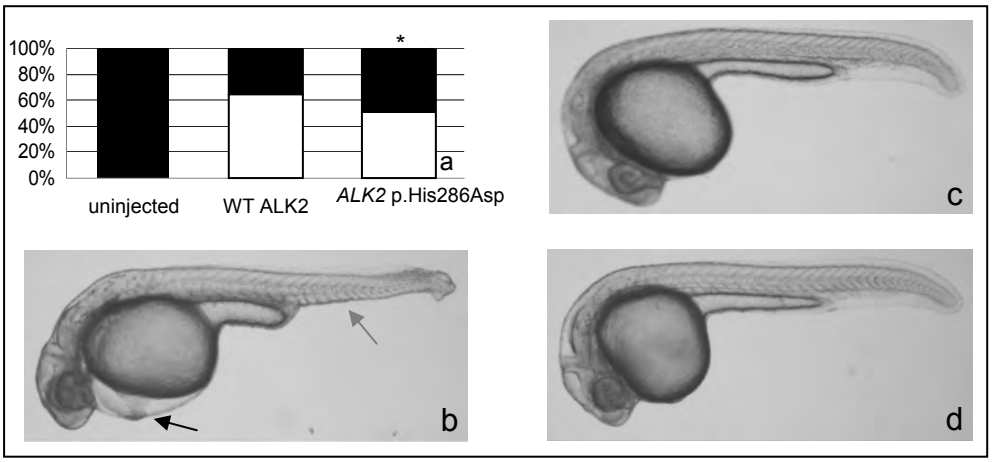


Figure 3. Rescue experiments in laf mutants. *Rescue experiments in the zebrafish ALK2/8 mutant, lost-a-fin (laf).* ***a.*** *Injection of wt ALK2 or p.His286Asp RNA at the single cell stage in laf/Alk2 mutants. Wt ALK2 RNA rescues phenotype in 65% whilst injection of p.His286Asp rescues phenotype in 51%. This is significantly less effective compared to wt RNA injection. Statistical significance was determined by Student t test, where * $p < 0.05$.* ***b.*** *laf embryo identifiable by its C1 dorsalisation phenotype; with cardiac edema (black arrow), absent ventral fin (blue arrow).* ***c.*** *rescued laf zebrafish sibling after injection with ALK2 p.His286Asp demonstrating wt phenotype* ***d.*** *wt zebrafish. For figure in colour: Supplement colour figures page 203.*

p.Thr1169Ile variants whereas the brother was negative for the three cSNPs. None of these relatives were found to have a congenital heart defect on cardiac echocardiogram.

We utilised our previous experience in functionally characterising ALK2 variants to investigate the *ALK2* p.His286Asp variant. Structural modelling of the cytoplasmic domain of the *ALK2* receptor demonstrated that H286 resides within the ATP binding pocket of the kinase domain, indicating that a missense substitution at this residue may impair receptor activity (Figure 1a,c). To test this hypothesis, *ALK2* p.His286Asp was examined for its capacity to induce BMP-specific transcriptional reporter activity [21]. In this reporter assay the AL2 p.His286Asp variant showed significantly lower luciferase activity with and without BMP6 induction compared with wtALK2 ($p < 0.05$) (Figure 2a). Endogenous ALK2 expression was knocked down by an RNAi construct as previously described [16]. Transfection with the ALK2 p.His286Asp variant demonstrated significantly lower levels of luciferase activity in the uninduced state ($p< 0.05$), but a difference from wtALK2 was not observed upon stimulation with BMP6 (Figure 2b). These results indicate that the ALK2 p.His286Asp receptor is capable of propagating a signal when stimulated

with a BMP ligand but this activity is impaired when the ALK2 p.His286Asp variant is in the presence of the wtALK2 form of the receptor.

To investigate the effect of the ALK2 p.His286Asp variant on receptor function *in vivo,* synthetic wtALK2 and ALK2 p.His286Asp mRNAs was injected into zebrafish embryos at the single cell stage. Injection of the ALK2 p.His286Asp variant resulted in mild dorsalisation of the zebrafish (C1 in 17%), which demonstrates a mild dominant-interfering effect of the ALK2 p.His286Asp variant (Figure 3a). As a further measure of the reduction of ALK2 signaling for the ALK2 p.His286Asp variant, rescue experiments were performed in the zebrafish ALK2/8 mutant, *lost-a-fin* (*laf*), which is identifiable for its C1 dorsalisation phenotype (Figure 3c). Whilst both wtALK2 and variant ALK2 mRNA were able to rescue the C1 dorsalization phenotype in *laf/alk2* embryos, the ALK2 p.His286Asp did so less effectively than wt (51% versus 65%, respectively; $p < 0.05$; Figure 3a).

Discussion

We recently reported a dominant-negative *ALK2* allele in a patient with a congenital heart defect [16]. In this study we report a patient with Down syndrome and a primum type atrial septal defect who harbours three cSNPs in genes associated with endocardial cushion development. The cSNPs result in missense subsitutions in the type I BMP receptors, ALK2 and ALK3 resulting in the genetic variants p.His286Asp and p.Glu414Lys, respectively, and in the epidermal growth factor receptor family gene, ERRB3, resulting in the p.Thr1169Ile variant.

We show that, whilst subtle, the *ALK2* p.His286Asp variation does alter BMP signalling activity compared with the wtALK2 allele. Additionally, a genetic variant in ALK3 was identified. How these compound substitutions in two distinct type I BMP receptors affects overall BMP signalling was not tested here but is expected to affect BMP signalling to a greater extent when present in a compound manner, since ALK2 and ALK3 can form a heterodimer complex [26]. Furthermore, it is difficult to predict what adverse effects may arise when occurring in concert with the non-conservative *ERBB3* p.Thr1169Ile substitution. What can be determined from the family data is that none of these variants are sufficient to cause a congenital heart defect when present in isolation in a non-down syndromic background.

The three variants were detected in the parents of the probands, demonstrating that these variants were not acquired *de novo*. The father of the proband was in possession of the *ALK2* p.His286Asp and *ALK3* p.Glu414Lys variants and the sister of the proband was in possession of the *ALK3* p.Glu414Lys and the *ERBB3* p.Thr1169Ile variants. Neither of these individuals presented with a congenital heart defect, demonstrating that the possession of either of these two combinations of cSNPs is either not sufficient to cause the heart defect or the variants are not completely penetrant.

Our data confirm that *ALK2* is a susceptibility gene for primum type atrial septal defects, in combination with variants at other loci, or in combination with trisomy 21. This case also demonstrates that it is feasible to identify additional predisposing genetic factors for heart defects in DS patients, by large scale candidate gene screens, thus underlining the true polygenic nature of these defects. Whilst not particularly useful from a clinical view point, it suggests that, also in cases of isolated heart defects, a large number of candidate genes will have to be screened to arrive at meaningful risk estimates based on molecular information.

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CHAPTER 5

A Duplication Including *GATA4* Does Not Co-Segregate With Congenital Heart Defects

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To the Editor

Chromosomal aberrations of chromosome band 8p23 have been frequently associated with congenital heart defects [1-4]. Some of the heart defects in patients with deletions can be attributed to loss of one *GATA4* allele (OMIM *600576), although deletion mapping suggests that at least one other candidate for heart defects may be located in this region [5]. In addition, loss of function mutations of *GATA4* have been described by Garg et al. [2003][6] and Okubo et al. [2004][7] in three families with atrial septal defects (ASD), with autosomal dominant inheritance, indicating the essential role of *GATA4* in cardiac development.

Duplications in this region have also been reported in patients with heart defects (Tetralogy of Fallot and pulmonary stenosis) [8, 9]. Interestingly, Barber et al. [2005][8] showed that a *de novo* duplication of *GATA4* was associated with a congenital heart defect (pulmonary stenosis), while an apparently similar duplication not including *GATA4* was not. This suggests that *GATA4* is a dosage sensitive gene that, when present in an additional copy, may also cause congenital heart disease.

Copy number changes (CNC's) are common and they cluster in the genome in a non-random fashion [10]. Many CNC's have been described in 8p23.1 immediately proximal and distal of *GATA4* [8, 9, 11, 12]. Although most of these do not disrupt or encompass *GATA4*, recently a large 8p23.1-p22 duplication containing *GATA4* (~extending 1.2 Mb telomeric of *GATA4* and ~3 Mb centromeric) was found among ~1,200 randomly selected North-Americans. The population frequency was estimated to be 0.6% (n=7) and the duplication was found in individuals of Caucasian, African and East Asian ethnicity [13]. Since no information on the cardiac status of the duplication carriers is available, this does not settle

the debate whether or not *GATA4* duplications cause abnormal cardiac development. However, it does show that *GATA4* duplications do occur in the general population, albeit at a low frequency. In this report we present a family with a duplication of *GATA4* in three generations not segregating with congenital heart defects.

The index patient (III:1) (*Figure 1*) was evaluated because of mild developmental delay, a congenital heart defect, bilateral inguinal hernias, unilateral cryptorchidism, asymmetric growth of the lower legs with unilateral club foot, without the presence of significant facial dysmorphisms. No specific syndrome was diagnosed after evaluation by senior staff members of the department of Clinical Genetics. He was born after an uncomplicated pregnancy of non-consanguineous parents, but he had a difficult start with asphyxia post-partum and an Apgar score of 3/5/7. His birth weight was 2900 grams, birth length was 49 cm and OFC was 35 cm and therefore appropriate for gestational age. He had a secundum type atrial septal defect (ASD) and a haemodynamically important perimembranous ventricular septal defect (VSD) and dysplastic pulmonary and aortic valves. During surgery for the ASD and VSD at age eleven, redundant tissue of the pulmonary valve was excised as well. The mother of the index patient (II:7) (see Figure 1) was of apparently normal intelligence, without a cardiac defect, but she was unable to raise her child due to psychosocial problems. The father was not available for study. He

Figure 1.

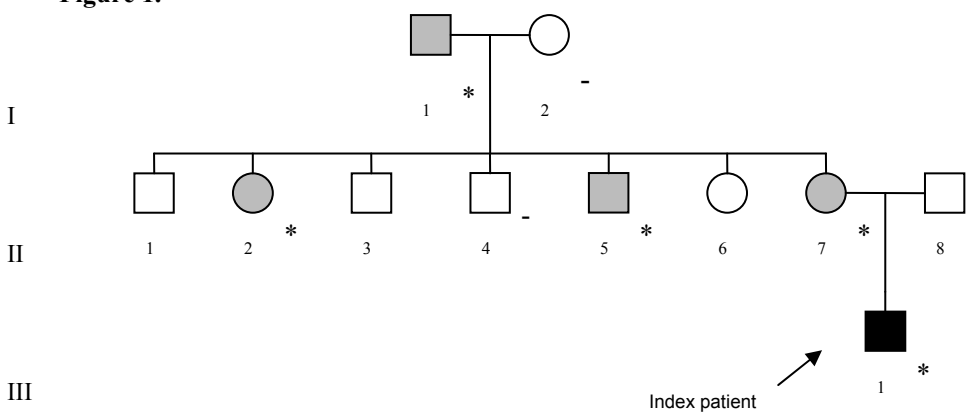

Figure 1. *Three generation pedigree of family with a GATA4 duplication.* presence of GATA4 duplication, - absence of GATA4 duplication, ■ presence of congenital heart defect, □ absence of congenital heart defect, ■ absence of congenital heart defect at echocardiogram*

Table 1. Information of supernumerary ring chromosomes identified in index patient.

Chrom	Start SNP	Position	Last SNP	Position	BAC probes	Genes in region
11	rs12224499	50,950,526	rs515667	55,009,954	RP11-135H8 and RP11-217G11	Olfactory receptor genes (OR4A5, OR4C46, OR4A16, OR4A15)*, TRIM48*
12	rs11053149	34,220,859	rs11053295	34,480,677	no duplicated BAC probes	None
X	rs7888435	61,920,847	rs5964445	64,727,677	no duplicated BAC probes	FAM123B, ASB12, MTMR8*, HCA127*, ZC3H12B & LAS1L

Nucleotide positions are according to the March 2006 human reference sequence (NCBI build 36.1). Genes are marked with an asterisk (*) are located within known CNC's (Database of Genomic Variants) [12]

presumably had no heart defect, but he may have had intellectual impairments. Cytogenetic analysis in peripheral blood lymphocytes revealed one to three distinct, supernumerary small ring chromosomes in the proband that were not seen in the mother. Fluorescence in situ hybridization (FISH) analysis with centromere-specific DNA-probes showed that these supernumerary ring chromosomes consisted of pericentromeric material of chromosomes 11, 12 and X (*Table 1*). BACarray-based, Comparative Genomic Hybridization (CGH) analysis [14], using the array described by [15] and SNP array-analysis (Infinium HumanHap300 Genotyping BeadChip (Illumina Inc., San Diego, CA, USA)) were performed to further characterize the genetic content of the small ring chromosomes. These analyses also revealed an additional copy of material in 8p23.1, that was narrowed down to a 132.9 kb interval, including rs1178941 (nucleotide position 11,523,629) to rs809204 (nucleotide position 11,656,572) (*Figure 2 and 3*). FISH with the RP11-235I5 BAC-probe (nucleotide position 11,538,840-11,721,811) on metaphase chromosomes, suggested that the duplication was located in band 8p23.1 in both the index patient and his mother with a different signal intensity between both homologous chromosomes (data not shown). Therefore, the additional copy seems to have arisen from a direct or inverted duplication in 8p23.1. The duplicated region contains only one gene, *GATA4* (nucleotide positions 11,599,162-11,654,918). All nucleotide positions are according to the March 2006 human reference sequence (NCBI build 36.1 and dbSNP 129). This duplication starts 76,433 bp upstream of exon 1 of *GATA4* and therefore contains its putative promoter (*Figure 2*) [16, 17]. DNA-sequence analysis of *GATA4* (coding regions including intron-exon boundaries) in both the index patient and his mother did not reveal any point-mutations. FISH using the

Figure 2.

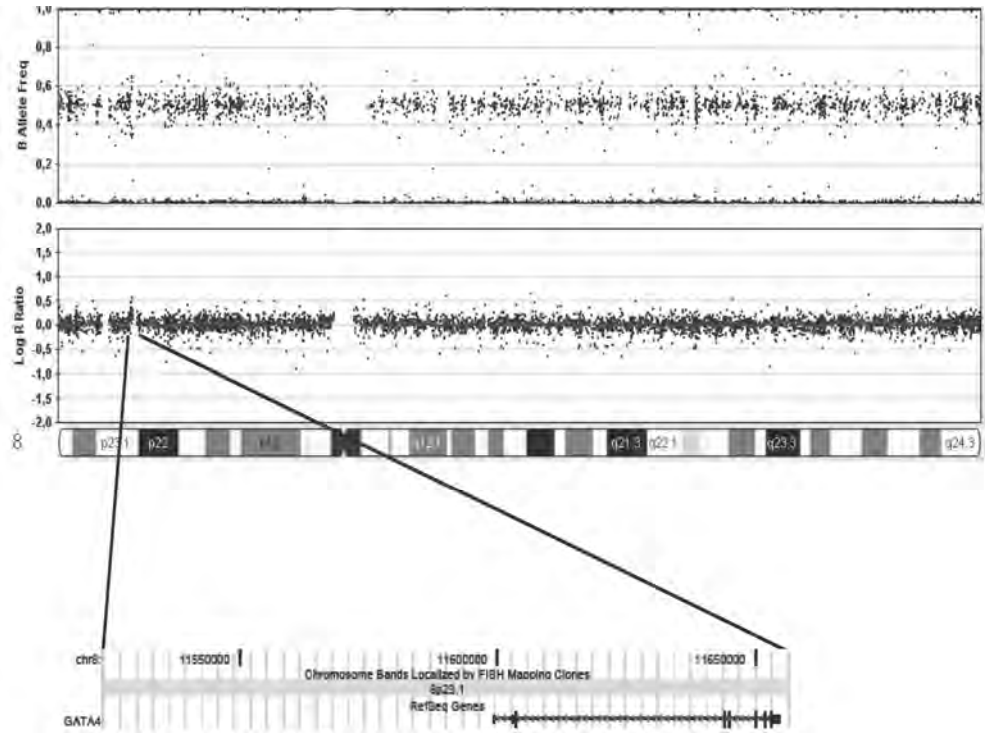

Figure 2. *A: SNP- array analysis of chromosome 8 demonstrating a small duplication of the region 8p23.1 ranging from the most proximal duplicated SNP rs1178941 to the most distally duplicated SNP rs809204 (for further details, see text). B: genes affected by duplication (based on the March 2006 human reference sequence (NCBI Build 36.1)) viewed with the UCSC genome browser.*

LSI-ELN probe was negative for the Williams-Beuren syndrome microdeletion and the sequence of NKX2.5 was normal.

To further evaluate the effect of the *GATA4* duplication on cardiac development, first degree and second degree relatives were approached for participation in this study. Medical records and history of family members were examined for congenital heart malformations. Those relatives willing to participate were evaluated for the *GATA4* duplication using the multiplex ligation probe amplification (MLPA) test (SALSA MLPA P.023B kit, MRC-Holland, Amsterdam, The Netherlands), after informed consent had been obtained. Family members carrying the *GATA4* duplication were further clinically evaluated, including echocardiographic (transthoracic) examination. In the mother of the index patient, the

Figure 3.

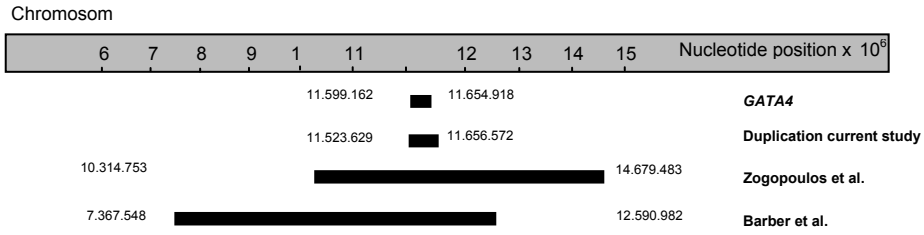

Figure 3. *Reported duplications containing GATA4. Nucleotide positions are represented as number x 10^6 and are based on the March 2006 human reference sequence (NCBI Build 36.1).*

presence of the duplication was confirmed with array-CGH. Of the five additional relatives that consented to participate, three had an extra copy of *GATA4* as determined by MLPA (*Figure 1*). In none of the four family members carrying the *GATA4* duplication was any cardiac defect detected, either clinically or with echocardiography. (Vysis-Abbott) was negative for the Williams-Beuren syndrome microdeletion and the sequence of NKX2.5 was normal.

Therefore, in this family a chromosomal duplication containing *GATA4* and its promoter appears not to segregate with a congenital heart defect. Our finding that sequence analysis of *GATA4* in the proband and his mother did not reveal any point mutations, suggests that they have three functional copies of *GATA4*. During adult life, *GATA4* is only expressed in the heart, gut epithelium and gonads [18]. As a result, we were unable to assess whether the *GATA4* duplication indeed resulted in increased *GATA4* expression.

Although it was not possible to obtain material for studying *GATA4* expression, it is reasonable to assume that the duplicated *GATA4* gene is expressed. This assumption is based on the fact that the duplication contains the entire *GATA4* gene, including 76 kb of DNA-sequences upstream from the putative transcription initiation site. In the mouse, 1.3 kb of upstream DNA sequence is sufficient to drive reporter gene expression of *Gata4* in cardiac myocytes that are induced to differentiate *in vitro*, indicating that DNA sequences essential for transcription in cardiac cells are located close to the transcription start site [17]. Furthermore, it has been shown that 5 kb of DNA upstream of the transcription start site of the rat *Gata4* gene is sufficient for reporter gene activity in several *Gata4*-expressing

organs in transgenic mice, including the heart, intestine, pancreas and testis [16]. In the zebrafish, 14.8 kb of upstream DNA sequence is sufficient to drive reporter gene expression in both chambers and valves of the heart [19]. Given that sequences and positions of transcription factor binding sites in the *GATA4* promoter are conserved between mouse, rat and man [17], we conclude that it is very likely, although not conclusively demonstrated, that the duplicated *GATA4* gene is fully functional.

Although the proband has a cardiac defect in the spectrum of cardiac defects previously found in patients with *GATA4* mutations, [6, 7, 20] none of four family members with a *GATA4* duplication had cardiac complaints or any observable congenital cardiac defect. This is in contrast to the findings of Barber et al. [2005], who described a *de novo* duplication of the 8p23.1 region containing *GATA4* in a girl with pulmonary stenosis and Tetralogy of Fallot, whereas a cytogenetically similar duplication in the same region, not including *GATA4*, was not associated with a heart defect [8]. The authors suggested that *GATA4* is dosage sensitive, with duplications conveying an increased risk for congenital heart defects. However, it should be noted that the *GATA4* duplication associated with a heart defect described by Barber et al. was larger than the one reported here (*Figure 3*). Therefore, in the studies of Barber et al. [2005] duplicated genes other than *GATA4* may have caused the pulmonic stenosis and Tetralogy of Fallot.

Since not all family members participated in our study, the number of observations (n=7) is relatively small. Therefore, the absence of observed heart defects could be merely due to reduced penetrance of heart defects associated with *GATA4* duplications, when compared to loss of function mutations. As well, spontaneously resolving congenital heart defects (some cases of VSD and persistent ductus arteriosus) could have been missed as a result of the adult age of our study participants. None of the other potential *GATA4* carriers in this family had any cardiac complaints or a history of congenital heart disease. They all survived into adulthood, thus decreasing the likelihood of any significant cardiac defect being present.

In order to find an alternative explanation for the combination of severe heart defect in our index patient we interrogated the supernumerary ring chromosomes. The supernumerary ring chromosome X that was found in the index patient encompasses the

FAM123B gene (*Table 1*). *FAM123B* negatively regulates *WNT* signaling by promoting β-catenin expression. Perturbations of *WNT*-signaling have been associated with congenital heart defects in animal models (reviewed by [21]). Therefore an additional copy of this gene may have contributed to the observed cardiac defect in the proband, rather than the *GATA4* duplication. Also, the combined duplication of *FAM123B* and *GATA4* could have led to the development of the cardiac defects. The only other duplicated gene that was considered a possible candidate is *ZC3H12B*. Little is known about this gene other than that this codes for a zinc-finger protein and is expressed in the fetal heart in mice [22]. Although no other likely candidate genes were found in the small supernumerary ring chromosomes, they may still have contributed to the heart defect by disturbing the complex processes involved in cardiac development.

In conclusion, elevated levels of *GATA4* protein, due to a *GATA4* gene duplication are not sufficient to cause congenital heart disease. This is in keeping with the recent finding that duplications containing *GATA4* occur in the general population at low frequency (*Figure 3*) [13].

Acknowledgments

We kindly acknowledge the cooperation of the index patient and his family members.

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CHAPTER 6

SPOCK3 is a candidate gene for Transposition of the Great Arteries in humans

SPOCK3 is a candidate gene for Transposition of the Great Arteries in humans

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Abstract

Transposition of the Great Arteries (TGA) is one of the most common cyanotic congenital heart defects. Although, usually sporadic, heritability estimates were circa 10%. To date, several genes have been implicated in cardiac laterality defects and TGA, but mutations in these genes have been found only in a small proportion of TGA patients. In this report we describe a case of a TGA patient with a de novo balanced translocation, disrupting SPOCK3 (4q32.3), encoding a member of a Ca(2+)-binding proteoglycan protein family. Proteoglycans are components of the extracellular matrix. Postnatal, SPOCK3 is primarily expressed in brain tissue, but in situ hybridization in murine fetus revealed a cardiac specific expression, with high expression of SPOCK3 in the ascending aorta and aortic valves. Therefore, SPOCK3 is an excellent candidate gene to explain the phenotype observed in the proband. **(submitted)**

Keywords: SPOCK3, Transposition of the Great Arteries, cardiac laterality defects, gene

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Introduction

Transposition of the great arteries (TGA) is one of the most common cyanotic congenital heart defects (5.4%) and is characterized by atrioventricular concordant and a ventriculo-arterial discordant connection [1]. Whether the pathogenesis of TGA has a genetic contribution has been debated in literature, due to low recurrence rates (0.27-1.8%) of TGA in families [2, 3]. A report of Digilio et al. on the other hand, estimated that 10% of all TGA cases can be explained by genetic factors [3].

Thus far, few genes have been associated with laterality defects and transposition of the great arteries, such as *Left„ PROSIT240*, *GDF*, *SESN1* (*PA26*) and *CFC1*, but only in a small proportion of all TGA patients [1, 4-9]. Therefore these genes cannot explain the high incidence of TGA. We've identified a novel gene, Sparc/Osteonectin, CWCV and Kazal-like domains Proteoglycan 3 (SPOCK3, 4q32.3), which is disrupted in an individual with TGA with an apparently balanced chromosomal translocation. His family history was negative for heart defects, apart from a daughter who died shortly after birth, who inherited an unbalanced translocation. SPOCK3 encodes a member of the Ca(2+)-binding proteoglycan protein family, a component of the extracellular matrix [10]. Previously, the extracellular matrix protein, perlecan (heparin-sulfate proteoglycan) has been associated with the development of TGA in mice. Mice deficient of perlican demonstrate TGA without ventricular septal defect in 73%. The remaining embryos showed a normal ventriculoarterial concordant connection, although the aortic root was slightly dextropositioned when compared with wildtype embryos [11]. Therefore extracellular matrix proteins, such as SPOCK3 are excellent candidate genes for TGA.

Postnatal, the expression pattern of SPOCK3 in mice demonstrates tissue specificity of SPOCK3 in the brain. No significant expression of SPOCK3 is found in the heart. Therefore, expression patterns of SPOCK3 will be assessed during cardiogenesis using in situ hybridization in mice. Additionally, in 97 patients with isolated TGA and an equal amount of controls, *SPOCK3* was sequenced.

Methods

Case report

The couple in this report was ascertained during their third pregnancy because of multiple congenital abnormalities at ultrasound. Two previous pregnancies ended in early miscarriage. At amniocentesis an unbalanced translocation between chromosomes 2 and 4 (46,XX,der (4),t(2;4)(p2?5;q3?3)pat) was identified. The father who was carrier of the balanced translocation, suffered from a transposition of the great arteries and pulmonic stenosis, which had been surgically corrected soon after birth. Both paternal grandparents had normal karyotypes. Family history was negative for heart defects. Ultrasound examination of the fetus revealed multiple abnormalities including a small left ventricle and a hypoplastic aortic arch. She was born after 40 5/7 week by vacuum extraction and weighted 3320 grams, length 47.5 cm, and Apgar score 2/5/7/9. Physical examination showed multiple dysmorphic features (especially in the head and neck region and complete inability to flex the 5th digits of both hands, which has been found earlier in 4qter deletions with similar breakpoints [12, 13]. Echocardiography demonstrated a small left ventricle, a narrow left ventricular outflow tract, a very wide persistent ductus arteriosus, a small aorta (smaller than pulmonic arteries) and a large ventricular septal defect. Due to respiratory failure she died 2 days later on the neonatal intensive care. Autopsy was denied.

Genetic analysis

Karyotyping and Fluorescence in situ hybridization (FISH) were performed according to standard protocols. FISH was performed, using the 963-K6, CEP-4, 820G20, paint 4 and paint 2 probes, in the proband and both her parents, to unambiguously demonstrate the translocation, which was difficult to detect in G-banded chromosomes. In order to fine map the breakpoints, making use of the fact that the proband had an unbalanced karyotype, DNA of her and her father was subjected to Single Nucleotide Polymorphism (SNP) array analysis, using the Infinium HumanHap300 Genotyping BeadChip (Illumina Inc., San Diego, CA, USA) according to the manufacturer's protocol. All nucleotide positions in this report are according to the March 2006 human reference sequence (NCBI build 36.1).

Mutation screening

DNA samples of the CONCOR cohort [14] of patients with TGA were requested. Polymerase chain reaction (PCR) was used to amplify coding regions of SPOCK3, including intron-exon boundaries, followed by deoxy- sequencing. Data analysis was performed by software (LIMSTILL) developed at the Hubrecht Institute [15]. DNA material from patients with non-cardiac-related diseases (Fragile X, male infertility and Rett Syndrome was used for controls. Controls were sourced from the Dutch population which comprises 80% of individuals of European ancestry (according to the Central Bureau of Statistics, The Netherlands, for 2008).

Results

Karyotyping and FISH analysis of peripheral blood lymphocytes of father demonstrated a de novo balanced translocation of chromosome 2 and 4 (46,XY,t(2;4)(p2?5,q3?3)). Cytogenetic analysis of the mother and both parents of the father revealed no abnormalities. Cultured amniocytes demonstrated an unbalanced translocation in the fetus resulting in a partial trisomy of chromosome 2 (2p2?5-2pter) and a partial monosomy of chromosome 4 (4q3?3-4qter).

The father showed no DNA copy number changes for any of the SNPs located in the breakpoint regions. The proband had a breakpoint in chromosome band 2p25.1 between SNP rs2013746 (retained) at nucleotide position 7,328,173 and SNP rs768447 (duplicated) at nucleotide position 7,323,816. In chromosome band 4q32.3 the breakpoint was situated between rs897514 (retained) at nucleotide position 168,013,448 and SNP rs7660401 (deleted) at nucleotide position 168,017,490 (Figure 1). All nucleotide positions are according to the March 2006 human reference sequence (NCBI build 36.1). Further analysis of the breakpoint regions revealed no disruption of a gene on chromosome 2; the breakpoint was located in a relative “gene desert”. On chromosome 4, the breakpoint disrupted a gene of unknown function, Sparc Osteonectin, CWCV and Kazal-like domains Proteoglycan 3 (*SPOCK3*) (Figure 1). *SPOCK3*, consisting of 7 exons, encodes for a calcium binding proteoglycan, which is a component of the extracellular matrix (OMIM 607989). The breakpoint of the translocation was situated in exon 5, making it unlikely that a functional protein is produced from the disrupted allele. Additionally, alignment of SPOCK3 revealed that this gene is evolutionary conserved between species, giving an extra indication that disruption of this gene might not be tolerated.

Figure 1.

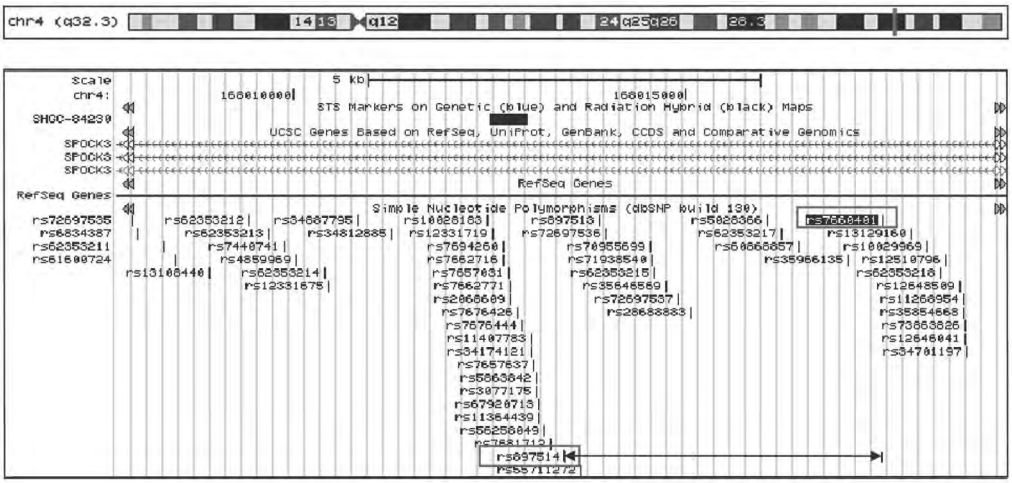

Figure 1. *SNPs positioned around the translocation breakpoint situated in SPOCK3, viewed with the UCSC genome browser. SNP- array analysis of the translocation breakpoint of chromosome 4 located the breakpoint between SNP rs897514 (retained) at nucleotide position 168,013,448 and SNP rs7660401 (deleted) at nucleotide position 168,017,490, both shown in a box.All nucleotide positions are according to the March 2006 human reference sequence (NCBI build 36.1).*

Mutation analysis of the *SPOCK3* gene in 97 unrelated individuals with Transposition of the Great Arteries identified no pathogenic mutations or patient population specific genetic variation.

Discussion

In this report we have demonstrated the presence of a de novo balanced translocation (t(2;4)(p25.1,q32.3)) disrupting *SPOCK3* in a patient with transposition of the great arteries. If a candidate region for a disease is identified by a balanced translocation, without any doubt, the gene disrupted is an immediate candidate since the prior chance of a gene being disrupted by a balanced translocation is small (approximately 3-6%) [16]. Therefore, *SPOCK3* is an excellent candidate gene for TGA, especially since also the daughter of the proband (although with an unbalanced karyotype) had severe cardiac defects, particularly of the cardiac outflow tract. In addition patients with distal deletions of the long arm of chromosome 4 including SPOCK3 frequently have cardiac defects, whereas deletions not including SPOCK3 do not as frequently [12].

SPOCK3 is a component of the extracellular matrix and previously perlecan (an extracellular matrix protein) was associated with TGA in mice. Mice deficient of perlecan demonstrate TGA without ventricular septal defect in 73%. The remaining embryos showed a normal ventriculoarterial concordant connection, although the aortic root was slightly dextropositioned when compared with wildtype embryos [11]. Additionally, in a mouse model of TGA, generated by the maternal administration of a single dose of all-trans retinoic acid (89% of fetuses demonstrate TGA), it has been suggested that certain components of the extracellular matrix might be involved in the pathogenesis of TGA [17]. Therefore, extracellular matrix proteins, such as SPOCK3 seem excellent candidate genes for TGA.

Although we failed to detect mutations in the coding sequence or intron-exon boundaries of *SPOCK3* in 97 patients with TGA, we cannot completely rule out any effect of this gene. *SPOCK3* may still be the major determinant of the TGA in our index case, but it should be concluded that it is not a major player in TGA morbidity (prevalence at least smaller than 1%).

Genetic factors are likely to contribute to the etiology of TGA, but a monogenic defect in humans is only found in a small proportion of patients [4-9]. Genetic variation in several genes with a small deleterious effect is likely to increase disease susceptibility as well. In line with this theory, in a recent study Roesler et al reported 35 different genetic variations or single nucleotide polymorphisms (SNP's) in three genes of from the Nodal pathway in 375 unrelated individuals with left-right asymmetry cardiac defects [18]. They detected only a few pathogenic mutations, but they did find functionally relevant common SNP's more frequently than expected. In addition, in several patients they found more than one functionally relevant SNP, which could theoretically act as unfavorable variants adding to the susceptibility for congenital cardiac defects in these patients. Therefore, in most patients, cumulative impairment of a genetic pathway by several minor variants with a small effect seems more likely to explain congenital heart defects than a single gene mutation. Thus, although no pathogenic mutations have been identified in SPOCK3 in 97 patients SPOCK3 may still contribute to TGA occurrence. Further studies, including in situ hybridization of SPOCK3 in mice embryos, should further establish the function of SPOCK3 in cardiogenesis.

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CHAPTER 7a

Gene and Gene function in bicuspid aortic valves: study design and preliminary results

Gene and Gene function in bicuspid aortic valves: study design

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Abstract

Background The prevalence of a bicuspid aortic valve (BAV) in the general population is circa 0.5-1.3% and it has been estimated that circa 50-95% of all BAV cases can be explained by genetic factors. Several genetic loci have been implicated with the occurrence of BAV. Two studies have identified mutations in the NOTHC1 gene in either related and unrelated patients with BAV, albeit that NOTCH1 mutations are only detected in a small proportion of patients (circa 4%). Therefore, other genes must be implicated in BAV as well. We hypothesize in this study that two specific genetic pathways are involved in the development of BAV, which are genes implicated in Epithelial to Mesenchymal Transformation (EMT) and genes of the secondary heart field.

Methods Patients planned for aortic valve replacement are approached for study. At surgery the morphology of the aortic valves is assessed and DNA is obtained for DNA isolation. The valve is snap frozen and stored in the biobank of the University Medical Center Utrecht. Polymerase chain reaction (PCR) will be used to amplify candidate genes of interest, followed by sequencing reactions to screen for mutation within the coding region of such genes. Then the nature and effect of any mutations identified will be characterized by functional analyses. Upon identification of a mutation, the morphological consequence and down-stream targets of the gene function can be analyzed in fixed tissue by histological and immunohistochemical methods.

For a substudy MRI and echocardiographic images are evaluated for diagnosis of the valve morphology as well.

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Introduction

The prevalence in the general population of a bicuspid aortic valve (BAV) is estimated to be 0.5-1.3% [1-3]. There are two categories of bicuspid aortic valves; a true bicuspid aortic valve in circa 24% (2 valve leaflets) and a bicuspid aortic valve with a tricuspid origin but with a fused commisure in 76% (3 valve leaflets but with a raphe) [4]. Bicuspid aortic valves progress more rapidly into regurgitation or stenosis of the valve [5]. This results in a higher occurrence of aortic valve replacement, especially at a younger age [6]. Patients with a bicuspid aortic valve are also more prone for endocarditis and ascending aorta dilatation which may ultimately result in aortic dissection or rupture (aortic dissection occurs 9 times more often with BAV than with tricuspid aortic valves) [7-9]. Patients with BAV are more likely to die because of cardiac death, aortic syndromes and sudden unexplained death, even after aortic valve replacement (compared with patients with a tricuspid aortic valve and aortic vale replacement) [10]. Therefore, early identification of patients with a bicuspid aortic valve is warranted.

Family studies suggest two types of inheritance patterns of BAV: autosomal dominant with reduced penetrance in few families and a non mendelian pattern in most. In a third of families with a bicuspid aortic valve patient, at least one additional family member can be identified with such a condition [11]. Even more so, genetic factors contribute in ~90% of all BAV patients to their occurrence [12]. Family-based genome-wide linkage analysis linked several loci with BAV such as a locus on chromosome 18 (Log odds (LOD) score 3.8, indicating odds of ~6300:1 that the disease gene in this family is linked to this locus). Other interesting loci are on chromosome 9q34-35 and on chromosome 15q15-21 and 13q33-qter (suggestive linkage) [13, 14]. Thus, the origin of a bicuspid aortic valve seems genetically heterogeneous. Until now two studies have found mutations in the *NOTCH1* gene (chromosome 9q34) in either related or unrelated patients with a bicuspid aortic valve [13, 15]. In approximately 4% of patients with a bicuspid aortic valve, mutations in *NOTCH1* are involved [15]. NOTCH1 encodes a transmembrane receptor and is important for cellular differentiation, proliferation and apoptotic programs during embryogenesis and could therefore influence organ formation and morphogenesis [16]. In animal models, NOTCH1 signaling has been found important for epithelial mesenchymal transformation (EMT) which is essential for endocardial cushion formation (cardiac valves) [17]. Other genes for

EMT have been found involved in cardiac valve formation as well, such as genes of the TGFβ pathway and the BMP pathway [18-20]. Also, genes form the secondary heart field might be implicated in aortic valve development as these genes are important for truncus arteriosus formation and primordial embryogenesis.

We hypothesize that both genes involved in EMT and genes of the secondary are important for correct aortic valve formation. Genes of the secondary heart field are most likely involved in the pathogenesis of a true BAV, seeing that early in embryogenesis tricuspid aortic valve primordial can be found, whereas a BAV with a tricuspid origin is expected to result from altered EMT, which occurs later on in embryogenesis.

The purpose of this study is to identify mutations or variations in genes important for EMT and the secondary heart field, involved in the pathogenesis of a bicuspid aortic valve using a large scale candidate gene sequencing screen approach. Because of a high association of a BAV with aortic dilatation a sub-study will focus on identifying genes important for a BAV with aortic dilatation as well. Additionally the nature of effect of the identified genetic variation or gene mutations will be assessed using both *in vivo* and *in vitro* functional assays and family studies.

Study design and Methods

Study design

This study is designed as a multicenter observational cross-sectional study. The participating centers are the UMC Utrecht and St. Antonius Hospital Nieuwegein. The study has been reviewed and approved by the ethical boards of both hospitals. The methodology and privacy regulations are in accordance with Dutch and European privacy Protection laws.

Research population

All patients planned for aortic valve replacement between 18 years and 80 years of age are eligible for participation in this study. Patients with endocarditis and prior aortic valve replacement are excluded from participation. Patients with a genetic diagnosis such as

Marfan's disease, Down syndrome, Turner syndrome and Noonan syndrome are excluded from participation as well.

Collecting data and tissue preparation

Informed consent is obtained from patients with aortic stenosis and who are scheduled for aortic valve replacement. During valve surgery 10 ml of blood will be obtained for genomic DNA extraction and the aortic valve is photographed *in vivo* and excised. In case of a bicuspid aortic valve the surgeon will document the raphal position, if present, on a standardized form. The presence of annular dilatation and distension or other pathology of the ascending aorta will be documented. After excision the aortic valve leaflets are collected on ice by dedicated staff and immediately taken to the department of pathology. Then the aortic valve leaflets will be inspected, measured and photographed by the pathologist. Each of the aortic valve leaflets will be subsequently bisected. One half of the valve leaflet will be snap frozen in liquid nitrogen and stored at -80 ˚C for further analyses. The remaining half will be immersion fixed in formalin overnight and embedded in paraffin. Haematoxylin and eosing (H&E) as well as Elastica van Giesson (EvG) stainings will be performed on 3 micron slides for routine histopathological purposes. Both frozen and formalin fixed and paraffin embedded tissue will be stored in the Biobank of the UMC Utrecht.

Echocardiography and MRI (for substudy)

An MRI of the aorta and aortic valves is made for identification of either a bicuspid or tricuspid aortic valve and for identification of possible aortic root dilatation and coarctation of the aorta before surgery. First of all the aorta will be examined in the transverse imaging planes. Then, imaging plane perpendicular to the long axis will be used to assess the diameters of the aortic root, ascending aortic, aortic arch and the descending aorta accurately. The morphology of the aortic valve is evaluated in the specific imaging planes perpendicularly or longitudinally oriented through the aortic valve. All images will be evaluated by a radiologist experienced in assessing cardiac MRIs. Echocardiographic examination images from the referring hospitals are reviewed by at least 2 investigators (ICJ and MJC). The aortic valve is examined in the left parasternal long axis view (a bicuspid aortic valve shows a doming configuration when it opens during systole), M-mode is used to determine whether the valve closes centrally and the short axis view is used for

determining further valve morphology ("fish-mouth" opening in true bicuspid aortic valve, or somewhat altered with a fused commissure). If the aortic valve is too calcified to determine the valve morphology the valve is defined to be "undetermined". The aorta is measured at several positions in the parasternal long axis view. Measured are the, annulus, sinus valsalvae (aortic root) sinotubular junction and the ascending aorta measured 4 cm from the annulus in diastole. The aortic root is also evaluated at the M-mode recording.

DNA samples

Genomic DNA is extracted from peripheral blood obtained directly from the patient at surgery. Polymerase chain reaction (PCR) will be used to amplify candidate genes of interest, followed by sequencing reactions to screen for mutation within the coding region of these genes (exon and exon-intron boundaries). Candidate genes are selected based on a large literature search with an emphasis on genes important for valve formation (especially those important for EMT or those genes from the secondary heart field). The screening will be conducted in collaboration with the group of Jeroen Bakkers at the Hubrecht Institute. This will involve utilizing the established pipeline for large-scale mutational discovery. The pipeline includes a bioinformatics platform for efficient design, management and interpretation of experimental results and high-throughput sequencing facility with a capacity of sequencing more than 30.000 amplicons (up to 15 x 106 bp) per week. For quality control all genetic variations have to be found in duplo. If not this variant will be resequenced and if not consistent, this patient will be excluded from further analysis. Data will be checked for errors (such as sequencing errors).

Functional characterization

Luciferase-reporter assays will be used as an initial screening tool for all putative mutations found. Luciferase-reporter assays have proven effective for quantitating the induction of receptor-signaling pathways by using a responsive promoter element for the given pathway of interest coupled to luciferase [21]. Wild-type and mutant components of the signaling pathway are then expressed in cultured cells. Endogenous equivalent pathway members will also be knocked down using RNAi expression vectors, in instances where the endogenous protein expression masks the effects of the introduced proteins of interest. The activity of each putative mutant of a given signaling pathway member can then be compared with that of the wild-type protein by measuring luciferase activity. This assay can

be applied to numerous components of any signaling pathway from exogenously applied ligands to receptors and intracellular components. All can be introduced into cell culture and assayed for induction of luciferase activity [21]. For testing functionality of specific components of signaling pathways, specialized assays will be employed. These assays include binding assays to test ligand binding, kinase assays to test receptor kinase activity and electromobility shift assays (EMSA) to test the DNA binding capacity of transcription factors. These particular assays are all currently established by the Bakkers group and will be employed when deemed appropriate. All functional experiments will be repeated at least three times and if not consistent another approach will be used to evaluate functionality of identified genetic variations. If not possible, this specific genetic variation will not be further evaluated.

Morphological analysis

Upon identification of a mutation, the morphological consequence and down-stream targets of the gene function can be analyzed in snap frozen or formalin fixed tissue by histological and immunohistochemical methods.

Endpoints

The endpoints of this study will be the identification of genetic variation in patients with a bicuspid aortic valve -differentiating between BAVs with a true bicuspid origin- and with a trisucpid origin and in patients with a bicuspid aortic valve plus aortic dilation, in patients with severe aortic stenosis who are accepted for aortic valve replacement surgery. Of all identified genetic variation the nature of effect of the variation will be tested using functional assays. Depending on the functional assay, specific endpoints will be sought for (for example difference in light emission (URLs) for the luciferase assays). For a substudy, differences between MRI and echocardiography versus the golden standard assessment at surgery for the diagnosis of a bicuspid aortic valve will be determined.

Data analysis

Patients will be divided in 2 groups based on the morphologic assessment of the valves at surgery; a bicuspid aortic valve and tricuspid aortic valve group. Candidate genes will be sequenced in both the bicuspid aortic valve group and the tricuspid aortic valve

group. Of all identified genetic variation in the group with a bicuspid aortic valve it will be determined whether this is population specific. Then the predicted effect of all detected variants will be assessed. Synonymous mutations will only be investigated further in case of strong in silico evidence of an effect on splicing. Missense variants will be prioritized, according to the already existing information in databases and the prediction of a deleterious effect (based on conservation criteria and in silico assessment by algorithms like Polyphen and SIFT). Control samples (tricuspid aortic valve patients) will be examined identically. Appropriate statistics will be applied on the functional assays, by using SPSS version 15.0 for windows. For comparing independent means an unpaired t-test or chi-square test for comparing categorical variables will be used. When normality assumptions are violated non-parametric tests (such as the wilcoxon rank sum test) will be used. All morphological consequences and down-stream targets of the gene function in bicuspid aortic valves will be analyzed in valvular tissue and compared with our "healthy" controls. For our sub-group analysis we would like to identify mutations or genetic variation in patients with both a bicuspid aortic valve and aortic dilatation.

Inclusion of patients in this study started in July 2007 and patients will be included until July 2011. Up to July 2009, 80 patients were included in the University Medical Center Utrecht. Approximately 40% of these patients were diagnosed with a bicuspid aortic valve at surgery.

Conclusion

This study aims to determine genetic variation associated with the development of a bicuspid aortic valve. Besides DNA, tissue samples of these patients are snap frozen and stored in the tissue bank of the University Medical Center Utrecht. The collection of these tissue samples is a major advantage. These samples will allow the investigation of morphological consequence and down-stream targets of the gene when pathological mutations or interesting genetic variants are present.

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CHAPTER 7b

Bicuspid stenotic aortic valves: clinical characteristics and morphologic assessment using Magnetic Resonance Imaging and echocardiography

Bicuspid stenotic aortic valves: clinical characteristics and morphologic assessment using Magnetic Resonance Imaging and echocardiography

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Background A bicuspid aortic valve (BAV) is one of the most common congenital heart defects with a population prevalence of 0.5-1.3%. Identifying BAV is clinically relevant seeing that these patients tend to be more susceptible to aortic stenosis, endocarditis and ascending aorta pathology (especially aortic dilatation and dissection).

Methods and results Patients with severe aortic stenosis necessitating aortic valve replacement surgery were included in this study. DNA and the aortic valve were collected at surgery. All aortic valves were stored in the biobank of the University Medical Center Utrecht. Additionally to the morphological assessment of the aortic valve by the surgeon and pathologist, echocardiographic images and MRI of the aortic valve were evaluated. In total, 80 patients were included between July 2007-2009 of whom 32 (40%) were diagnosed with BAV. Patients with BAV were significantly younger (55 vs 71 years of age) and were more frequently male. Typically, the ascending aorta was larger in patients with BAV. Notably, a significant difference between the surgeon and pathologist in determining valve morphology was found. Therefore, for the further analyses the surgeon's assessment was used as golden standard. MRI was performed in 33% of patients. MRI could determine valve morphology in only 96% versus 73% with ECHO. Sensitivity of MRI for BAV in a population of patients with severe aortic stenosis is higher than echocardiography (75% vs 55%), whereas specificity was better with the latter (91% vs 79%).

Conclusion Among unselected patients with severe aortic valve stenosis, a high percentage of patients with BAV are found. Imaging and assessment of the aortic valve morphology when stenotic is challenging. **(Submitted)**

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Introduction

A bicuspid aortic valve (BAV) is one of the most common congenital heart defects with a population prevalence of 0.5-1.3% [1-3]. The defect is considered to be a heritable disorder, with a family recurrence rate of circa 35% [4]. To date only mutations in NOTCH1 have been identified, but other loci have been implicated as well [5-7]. Bicuspid aortic valves progress more rapidly into regurgitation or stenosis of the valve [8]. This results in a higher occurrence of aortic valve replacement, especially at a younger age [8]. Additionally, BAV patients are more susceptible than tricuspid aortic valves to nest bacteria or other organisms, leading to endocarditis [9].

BAV is not only a peculiar valve morphology leading to specific valve pathology, it is also frequently associated with (asymptomatic) ascending aorta dilatation which leads to an increased susceptibility to ascending aortic aneurysms and aortic dissection [10-14]. Aortic elasticity measurements of BAV patients suggest that diminished aortic elasticity is at least part of its causation [15]. Unfortunately, BAV patients frequently remain undiagnosed until the manifestations of symptoms. Therefore, screening and detecting of patients is warranted. In 2007 we started a study, designed for the detection of genetic mutations/ variations in BAV patients in a cohort of patients accepted for aortic valve replacement in the UMC Utrecht and the St Antonius Hospital Nieuwegein. In this report we would like to describe the clinical characteristics of the included patients thus far. Additionally, the morphological assessment of the aortic valve by the pathologist and surgeon were compared. Sensitivity and specificity for the diagnosis BAV in a population of severe aortic stenosis with MRI and echocardiography was determined as well.

Material and methods

Study design

This study was designed as a multicenter observational cross-sectional study. The participating centers are the UMC Utrecht and St. Antonius Hospital Nieuwegein, the Netherlands. The study has been reviewed and approved by the ethical boards of both hospitals. The methodology and privacy regulations are in accordance with Dutch and European privacy protection laws.

Research population

All patients accepted for aortic valve replacement between 18 and 80 years of age were eligible for participation in this study. Patients with endocarditis and prior aortic valve replacement were excluded. As this study was primarily designed for the identification of genetic variation associated with BAV, patients with a genetic diagnosis such as Marfan's disease, Down syndrome, Turner syndrome and Noonan syndrome were excluded from participation as well.

Collecting data and tissue preparation

Informed consent was obtained from patients with aortic stenosis and who were accepted for aortic valve replacement. During valve surgery, 10 ml of blood was obtained for genomic DNA extraction and the aortic valve was photographed *in vivo* and excised. In case of a BAV, the surgeon documented the raphal position, if present, on a standardized form. After excision the aortic valve leaflets were collected on ice by dedicated staff and immediately taken to the department of pathology. Then the aortic valve leaflets were inspected, measured and photographed by the pathologist. Each of the aortic valve leaflets was subsequently bisected. One half of the valve leaflet was snap frozen in liquid nitrogen and stored at -80 ˚C for further analyses. The remaining half was immersion fixed in formalin overnight and embedded in paraffin. Haematoxylin and eosing (H&E) as well as Elastica van Giesson (EvG) stainings was performed on 3 micron slides for routine histopathological purposes. Both frozen and formalin fixed and paraffin embedded tissue are stored in the Biobank of the UMC Utrecht.

Echocardiography and MRI

An MRI investigation of the aorta and aortic valves was made for identification of either a bicuspid or tricuspid aortic valve and for identification of possible aortic root dilatation and coarctation of the aorta before surgery. First of all the aorta was examined in the transverse imaging planes. Then, imaging planes perpendicular to the long axis were used to assess the diameters of the aortic root, sinotubular junction, ascending aortic, aortic arch and the descending aorta accurately. The morphology of the aortic valve was evaluated in specific imaging planes perpendicularly or longitudinally oriented through the aortic valve. All images were evaluated by a radiologist and cardiologist experienced in assessing cardiac MRIs. Echocardiograms from the referring hospitals were reviewed by at least 2

Table 1. Clinical characteristics

	Overall (n=80)	Tricuspid (n=48)	Bicuspid (n=32)	p-value*
Male (%)	54 (65.1)	24 (51)	26 (84)	0.004
Age at operation	68.5 (56.5-74)	71 (68-75)	55 (48-66)	<0.0001
AVA (cm2)	0.76 (0.23)	0.78 (0.23)	0.72 (0.22)	NS
Peak gradient	78 (22)	78 (21)	75 (13)	NS
Hypertension (%)	41 (51)	31 (65)	10 (31)	0.003**
Diabetes (%)	8 (10)	7 (15)	1 (3)	NS
Obese (%)	19 (24)	16 (33)	3 (5)	0.010
Hyperchol. (%)	28 (31)	20 (42)	8 (25)	NS
Smoking (%)				
- never	37 (46)	23 (48)	14 (44)	
- past	15 (19)	7 (16)	8 (25)	
- recently stopped	13 (16)	7 (16)	6 (19)	
- current smoker	15 (19)	11 (23)	4 (13)	NS

AVA: AorticValve Area, Hyperchol: hypercholesterolaemia, NS: non significant, unadjusted p-values. ** when adjusted for age, hypertension is not significantly associated with a tricuspid aortic valve.*

investigators (ICJ and MJC). The aortic valve was examined in the left parasternal long axis view (a bicuspid aortic valve shows a doming configuration when it opens during systole), M-mode was used to determine whether the valve closed centrally and the short axis view was used for determining further valve morphology ("fish-mouth" opening in true bicuspid aortic valve, or somewhat altered with a fused commissure). If the aortic valve was too calcified to determine the valve morphology, then the valve was defined to be "undetermined". The aorta was measured at several positions in the parasternal long axis view. Measurements were made at the level of the annulus, sinus valsalvae (aortic root) sinotubular junction and the ascending aorta measured 4 cm from the annulus in diastole. The aortic root was also evaluated at the M-mode recording.

Data analysis

Patients were divided into 2 groups based on the morphologic assessment of the valves at surgery (golden standard); a bicuspid aortic valve and tricuspid aortic valve group). For comparing clinical characteristics an unpaired t-test was used for continuous variables and a chi-square test for comparing categorical variables. For the comparison of

aortic diameter means between the bicuspid and tricuspid groups, ANCOVA (analysis of covariance) was used with as covariates aortic valve morphology, hypertension and age. A paired t-test was used to compare differences in echocardiographic evaluation of aortic size versus aortic diameters measured with MRI. The McNemar test was used for determining differences between surgical and pathological evaluation of aortic valve morphology. Sensitivity and specificity were calculated with the Clinical Calculator 1 (http://faculty.vassar.edu/lowry/clin1.html). Two-sided p-values below 0.05 were considered statistically significant. SPSS for Windows, (release 15.0.0. 2006, Chicago SPSS inc.) was used for all other statistical analyses.

Results

Clinical characteristics

Of 80 included patients 65% were male and mean age at operation was 68.5 years. 51% of all patients were hypertensive and 25% were obese (Table 1). Diabetes and hypercholesterolemia occurred in respectively 10% and 31% of all patients. Additionally, more males than females were found to have a BAV and on average, patients with a bicuspid aortic valve where younger than patients with a tricuspid aortic valve (55 vs 71 years, $p<0.0001$). Also, hypertension and overweight was significantly more observed in the tricuspid aortic valve patients, although the number of patients is limited. When correcting for the confounding factor age, hypertension was no longer associated with a tricuspid aortic valve. None of the other risk factors associated with typical cardiovascular disease were significantly different between the two groups.

Assessment of aortic valve morphology

Surgeon versus pathologist

It was obvious that there was a significant difference between the surgeon and pathologist in determining valve morphology (Table 2). Especially, when the surgeon diagnosed a tricuspid aortic valve, the pathologist more often assessed the aortic valve to be bicuspid with a raphe (type 1). This difference is not surprising as these valves were very stenotic and therefore it was difficult for the pathologist to determine whether the valve leaflets were congenitally fused (raphe) or fused due to the degenerative disease itself (Figure 1). Additionally, due to severe calcified valve leaflets it was sometimes impossible

Figure 1.

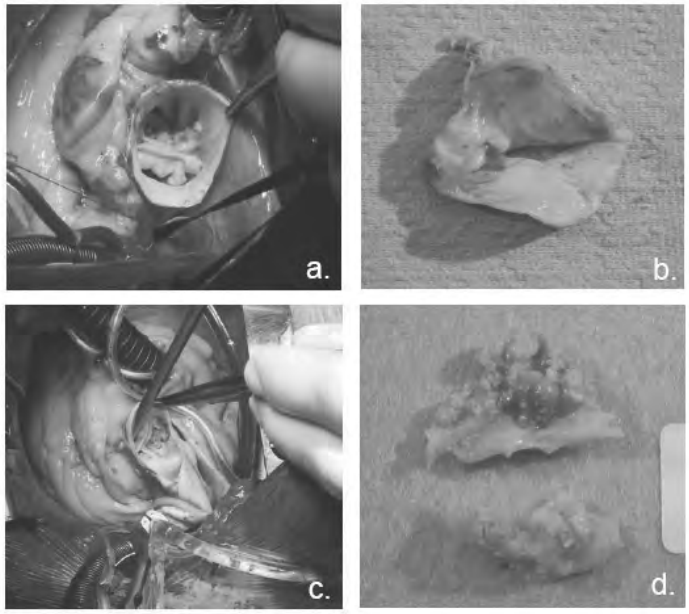

Figure 1. *Photographs of aortic valve at surgery and at pathology.* ***a-b****: patient with severe aortic stenosis and diagnosed with a type 0 bicuspid aortic valve by both surgeon (a) and pathologist (b);* ***c-d****: different patient with a tricuspid aortic valve at surgery (c), this photograph shows a clear view of the presence of 3 separate valve leaflets. (d) the excised valves of the same patient. Evidently, it is hard to identify 3 separate valve leaflets and this aortic valve was determined to be a type 1 bicuspid aortic valve (with raphe) by the pathologist. For figure in colour: Supplement colour figures page 204.*

Figure 2.

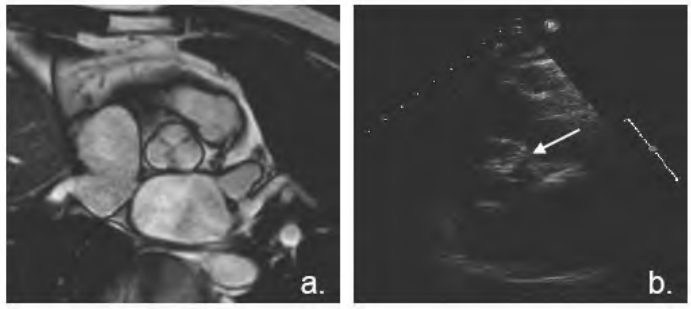

Figure 2. a. *MRI of a patient with a tricuspid aortic valve.* ***b.*** *patient with a tricuspid aortic valve with echocardiography. Although, this echocardiogram was analyzable, the view of the aortic valve is markedly inferior compared with MRI.*

for the surgeon to excise intact valve leaflets. Therefore, for the further analysis the surgeon's assessment of the valve morphology was used as golden standard. In total, 38% of patients were found to have a bicuspid aortic valve, 57% had a tricuspid aortic valve and in the remaining 5% the valve morphology could not be determined.

MRI and ECHO

Diagnostic accuracy of MRI (Figure 2) was 79 %. In addition, MRI was able to correctly identify a bicuspid aortic valve in 75% (95% CI 43-93%) of cases (sensitivity). Specificity of MRI was 0.79 (95% CI 0.49-0.94) (Table 2). In 1 (~4%) patient aortic valve morphology could not be determined with MRI. The positive predictive value was 75% (95% CI 43-93%) and the negative predictive value was 79% (95% CI 49-94%).

In 27% of patients the morphology of the aortic valve could not be determined by echocardiography due to severe calcifications of the aortic valves (~70%) or insufficient window quality (~30%). Of the remaining echocardiograms (Figure 2), the diagnostic accuracy was 66%. Sensitivity, in this population of patients with severe aortic stenosis, was only 0.54 (95% CI 0.25-0.82), which is somewhat smaller than MRI. Echocardiography was, on the other hand, more specific than MRI for excluding the presence of a bicuspid aortic valve (0.91 95% (95% CI 0.25-0.82) vs 0.79 (95% CI 0.49-0.94)) (Table 2). The positive and negative predictive value were 75% (95% CI 36-96%) and 80% (95% CI 60-92%) respectively.

Table 2. Aortic valve morphology; surgery compared with pathologist, ECHO and MRI

		Surgical assessment		
		Bicuspid	Tricuspid	Total
pathologist	Bicuspid	29	14	43
	Tricuspid	2	35	37
	Total	31	49	**80**
ECHO	Bicuspid	6	2	8
	Tricuspid	5	21	26
	Total	11	23	**34**
MRI	Bicuspid	9	3	12
	Tricuspid	3	11	14
	Total	14	12	**26**

Table 3. Aortic dimensions measured with MRI and ECHO

Aortic position	MRI (mean (SD))			ECHO (mean (SD))		
	Tricuspid	Bicuspid	p-value*	tricuspid	Bicuspid	p-value*
Sinus valsalva	32.9 (4.38)	35.3 (7.18)	NS	32.8 (4.20)	36.4 (5.11)	NS
STBJ	29.7 (3.55)	29.3 (3.47)	NS	27.8 (4.11)	32.1 (5.43)	NS
Ascending aorta	33.8 (5.18)	39.5 (7.01)	0.021	29.7 (5.24)	33.4 (5.42)	0.045
Aortic arch	27.0 (3.12)	27.9 (4.85)	NS			
Descending TA	25.7 (0.70)	25.5 (0.75)	NS			
Max diameter	35.7 (4.36)	40.5 (6.40)	0.001	33.2 (4.09)	37.8 (4.80)	0.018

*All diameters are in mm. NS: not significant, STBJ: Sinotubular junction; TA: Thoracic Aorta. * P-values are corrected for hypertension and age.*

The maximum aortic diameter was defined as the maximum aortic diameter measured at 3-5 measuring levels at ECHO and MRI respectively. Measurements were compared between patients with a bicuspid and tricuspid aortic valve. The ascending aorta was significantly wider in the bicuspid compared with the tricuspid patients even when corrected for possible confounders such as hypertension and age (Table 3). Additionally, the maximum diameter measured in any of the five measurement sites was significantly broader in the bicuspid aortic group (p = 0.001) (Table 3). When assessing aortic dimensions with echocardiography similar results were found; the ascending aorta (29.7 (TAV) vs 33.4 (BAV) mm, p = 0.045) and maximum diameter (33.2 vs 37.8 mm, p=0.018) were significantly associated with the presence of BAV. Additionally, the aortic diameters of the sinus valsalvae and the sinotubular junction demonstrated a trend towards a wider aorta in the bicuspid aortic valve group (p= 0.087 and p= 0.067 respectively), even when corrected for hypertension and age (Table 3). The aortic dimensions of the sinus valsalvae and sinotubular junction were comparable between MRI and echocardiography. The ascending aortic dimensions, on the other hand, were systematically underestimated with echocardiography (mean difference 3.5 mm (95% CI 0.574- 6.49 mm, p= 0.023)).

Discussion

To our knowledge, this is the first study which systematically assessed valve morphology by the surgeon and pathologist, MRI and echocardiography in an unselected population of patients with severely stenotic aortic valves. Furthermore, an unknown

significant difference between the assessment of aortic valve morphology between the surgeon and pathologist was identified.

Clinical characteristics

In this report 40% of patients with severe aortic valve stenosis were found to have a BAV, which is comparable with previous reported frequencies [8]. Hypertension was found in 51% of all patients who participated in our study, which is higher than expected considering population prevalence of hypertension in the Netherlands (27-34% for persons >60 years of age) [16, 17]. This difference could be merely due to small numbers, but other reports have also described the increased prevalence of hypertension in patients with aortic stenosis versus a control population of individuals without relevant valve disease [18]. Therefore this difference appears a real finding. These data therefore suggest, that hypertension is implicated in the development of aortic stenosis.

Surprisingly, overweight was significantly associated with a tricuspid aortic valve even after adjustment for age. A high fat/high carbohydrate diet induces aortic valve disease in mice, independently of cholesterol level [19]. Therefore overweight is likely to be a risk factor for the development of aortic stenosis. Bicuspid aortic valves are already more susceptible for aortic valve stenosis than tricuspid aortic valves. Therefore additional risk factors presumably more explain aortic valve stenosis in tricuspid than bicuspid aortic valves. Certainly, as numbers were small, this finding can also be due to chance.

Additionally, significantly more male than female patients were observed with a bicuspid aortic valve (84% vs 51%). Similar gender differences in the incidence and prevalence of BAV have been previously reported [1, 20-22].

Imaging of the aortic valve and aortic dimensions

BAV is associated with aortic dilatation which leads to an increased susceptibility to aortic dissection and ascending aortic aneurysms [10-14]. Therefore, it is important to recognize aortic valve morphology for future follow-up (e.g. regular assessment of the ascending aorta) and possibly intervention. Furthermore, for risk stratification in family members, knowledge of valve morphology can be important as well. Imaging of the aortic valve when severely stenotic is challenging. Due to the severity of stenosis and calcified

nature of the aortic valves, echocardiograms were frequently unable to differentiate between TAV en BAV. As predicted, MRI was able to assess aortic valve morphology more frequently than echocardiography (4% vs 27%).

Additionally, MRI appeared more sensitive for the detecting of BAV in this population than echocardiography (0.75 vs 0.55). Whilst MRI was more sensitive, echocardiography on the other hand appeared more specific (0.91 vs 0.79) in this patient group. Certainly, patients who presented to the cardiologist in a less advanced stage may presumably be better identified with either imaging techniques. From a cost-effectiveness perspective, echocardiography would still be first choice. Namely, in those with adequate echocardiographic images, echocardiography has been reported to be both highly sensitive (0.79-0.92) and specific (0.93-0.96) [23, 24]. When the echocardiograms prove unanalyzable or when in doubt, MRI can be useful to come to a diagnosis.

MRI is thought of as the gold standard for the determination of aortic dimensions. In this report aortic dimensions were assessed with both echocardiography and MRI. Both MRI and echocardiography demonstrated a significant larger ascending aorta in BAV patients compared with tricuspid aortic valve patients. This pattern is comparable with previous reports of aortic diameters in BAV patients [11-13]. When comparing aortic dimensions assessed by means of MRI and ECHO, the ascending aortic dimensions were systematically underestimated with echocardiography. Therefore, when the ascending aorta appears large at echocardiography, it is important to consider evaluating the aorta with MRI as standardized care. The role of other advanced imaging techniques such as CT was not evaluated in this report.

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CHAPTER 8

Summary and future perspectives

Summary and future perspectives

Discovering genes and gene function in human sporadic congenital heart disease

Nowadays, circa 85-95% of children with a congenital heart defect (CHD) survive into adulthood due to better operation techniques [1, 2]. Therefore, the number of adults with a CHD is still growing. As these patients also enter reproductive age, knowledge of heritability of these defects is essential. Isolated congenital heart defects are most frequently sporadic. Despite the sporadic nature, a genetic component is still very likely to contribute to the occurrence of these defects [3]. Not only do genetic factors contribute in 50-95% of all congenital heart defects to their occurrence, but also higher recurrence rates among siblings and offspring of patients with CHD are observed [3, 4].

Even though, the extensive knowledge of the genetic control of cardiogenesis in animals, this has not translated into an equal amount of clinical knowledge of the genetic determinants of CHD in man. This ilargely due to the complex pathogenesis of CHD and the scarcity of large families with multiple affected individuals suitable for conventional genetic analyses as reviewed in **chapter 1** and **chapter 2**. To circumvent this limitation a candidate gene sequencing approach in combination with functional analysis of identified genetic variants and pedigree analysis was chosen in chapter 3 and 4 to detect mutations in genes associated with congenital atrioventricular valve and/or septal defects. Although, the candidate gene sequencing screen as described in **chapter 3** resulted in the identification of 18 genetic variants predicted to alter protein function, this result could not be validated in the majority of pedigree analysis. A possible explanation is that cumulative impairment of developmental pathways at several distinct loci is the most likely cause of isolated CHD, as previously described in laterality cardiac defects [5]. In other words, a certain genetic susceptibility is probably needed before a genetic variant will result in altered cardiac development as is demonstrated in a model with $Smad1^{+/-}$ $Smad5^{+/-}$ double heterozygous mice. These mice have defects that encompass the entire range of disturbances described for Smad1- and Smad5-deficient embryos, while $Smad1^{+/-}$ or $Smad5^{+/-}$ mice have no cardiac defects at all [6, 7].

This is presumably also true for Down syndrome patients as described in **chapter 4.** Although, an increased dosage of chromosome 21 is most likely part of the etiology (especially considering the prevalence of congenital heart defects in these patients) only a proportion of Down syndrome patients exhibit a CHD [8, 9]. Therefore, genetic imbalance caused by the presence of an extra copy of chromosome 21 will seriously disrupt one or more developmental pathways, but predisposing variants at other loci are required for a heart defect to occur, such as the presence of the functionally impaired *ALK2* variant detected in the proband of chapter 4. This further explains why the father without trisomy 21 did not demonstrate a cardiac defect in the presence of this *ALK2* variant.

Besides variation in chromosome copy number, copy number variants (subtle deletions or duplications) might be involved in the etiology of isolated CHD as well [10]. In a recent report *de novo* and inherited rare copy number variants were identified in circa 3.3% of isolated cases of Tetralogy of Fallot. As small copy number variants would be predicted to escape detection by the array platform, these authors suggest that in approximately 10% of all isolated TOF cases (rare) *de novo* copy number variants can be expected [10]. Whether these variants either lead to the observed phenotype or merely increase disease susceptibility is not known. In **chapter 5** we sought to determine whether a *GATA4* copy number variant (duplication) causes CHD. Haploinsufficience and loss of function of *GATA4* have been related to familial incidence of atrial septal defects and duplications in this region including *GATA4* (8p23) have also been reported in patients with other heart defects [11-14]. This suggests that *GATA4* is a dosage sensitive gene that, when present in an additional copy, may also cause congenital heart disease. In this chapter we present a patient with a ventricular and atrial septal defect (plus some other nonspecific congenital abnormalities) in whom we've identified a duplication located in band 8p23.1 including *GATA4* and its promoter exclusively. Pedigree analysis revealed four additional family members with this *GATA4* duplication. In none of these four family members carrying the *GATA4* duplication a cardiac defect was detected. Thus, in this family a chromosomal duplication containing *GATA4* and its promoter appeared not to segregate with a congenital heart defect. Therefore, we concluded that elevated levels of *GATA4* protein, due to a *GATA4* gene duplication are not sufficient to cause CHD. Whether *GATA4* duplications increase disease susceptibility cannot be excluded.

A disadvantage of using a candidate gene screen for identification of mutations in human congenital heart defect is that it is hypothesis driven. Therefore, only genes known to be involved in cardiac differentiation will be investigated further whereas other genes, which might be implicated as well, remain undiscovered. Sometimes certain chromosomal alterations might help identifying novel genes for heart development. Such an example is described in **chapter 6** where a case is presented of a proband with TGA and a *de novo* balanced translocation disrupting a gene of unknown function, *SPOCK3*. The prior change of a gene being disrupted by a balanced translocation is less then 3% and consequently the gene disrupted is an immediate candidate. Future knock-out experiments of SPOCK3 and in situ hybridization in zebrafish and mice will further ascertain the function of SPOCK3 in cardiogenesis.

The last chapter (7) describes the study design and clinical characteristics of another candidate gene sequencing screen in patients with a bicuspid aortic valve.

Future perspectives

It is known that certain environmental factors, such as maternal smoking and diabetes, can increase the risk of CHD [15-18]. Whether a CHD occurs in the setting of these factors might depend on the interaction with genetic variants (gene-environment interaction). A molecular explanation for this is demonstrated in few studies, were gene expression was altered during embryogenesis by environmental (maternal) factors [19, 20]. For both preventive purposes and risk assessment (genetic counseling) the role of gene-environmental interactions should be further established in for example large randomized clinical trials, but also more fundamental studies are necessary to generate new hypotheses.

Additionally, research should focus on the function of microRNAs (miRNAs) in cardiac differentiation. MiRNAs are small transcribed non-protein-coding sequences and are abundantly present in all cells. They are regulators of gene expression during both embryogenesis and postnatal cell-signaling. MiRNAs are important for cardiac differentiation as well. Already, several miRNAs have been found to regulate expression of genes implicated in heart development, such as Nodal and Lefty (miR-15, miR-16 and miR-430) and selective ablation of Mir-1-2 and deficiency of miR-133a in mice results in failure of ventricular septation and prenatal or early postnatal death [21-24]. Additionally a functional

variant in miR-196a2 was found to contribute to the susceptibility of congenital heart disease in a Chinese population [25].

Another important factor for heart defect susceptibility which needs further exploration is the epigenetic control of cardiac development. Epigenetics refers to mitotically and/or meitoically heritable variations in gene expression that are not caused by changes in DNA sequence. Epigenetic mechanisms are essential during early embryogenesis for processes such as cell differentiation, maintenance of a committed lineage and X-chromosome inactivation. Key epigenetic players are DNA methylation and histone post translational modification [26-29]. DNA methylation may affect transcription of genes by physically impeding binding of transcription factors to genes. Additionally, methylated DNA can be bound by methyl-CpG-binding domain proteins (MBDs) which than recruit additional proteins to the locus that can modify histostones and thereby form compact, inactive (silent) chromatin. In cancer research changes in DNA methylation pattern are extensively investigated and have now been associated with the occurrence of the disease [26-29]. Further studies are needed to assess whether DNA methylation patterns are involved in CHD as well.

Future research will adopt more advanced techniques to discover novel genes important for heart development, such as genome wide association studies (GWAs) and whole genome sequencing. Advantages of these approaches are that they're hypothesis free. GWAs are now used to detect genetic factors contributing to common complex diseases such as myocardial infarction. For discovering genes in congenital heart defects large world wide collaborations are necessary as variants associated with congenital heart defects are most likely rare variants with small effect (explained in chapter 1). In the near future whole genome sequencing will not only be achievable, but also come within reach for advanced clinical diagnostic testing. Whole genome sequencing will generate a lot of new information and interpreting these results will be difficult. Tools for the interpretation of such huge amount of data in the context of CHD will not be not readily available. The more complex and heterogeneous the disorder, the more difficult this will be.

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Nederlandse samenvatting

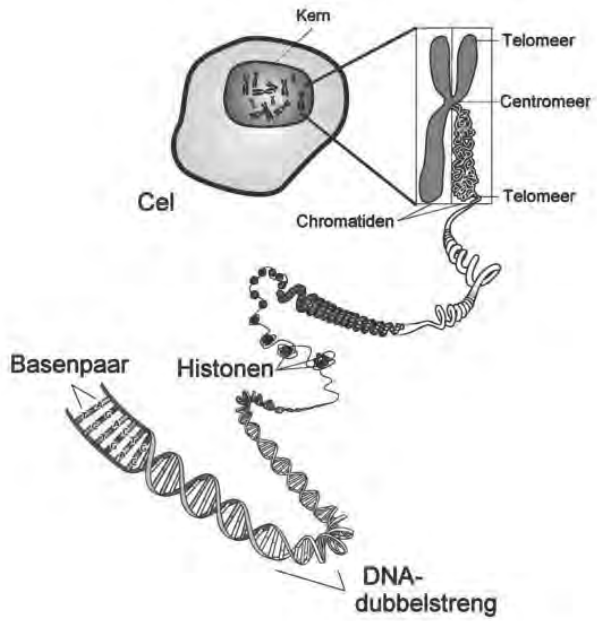
Nederlandse samenvatting

Een aangeboren (congenitale) hartafwijking is een van de meest voorkomende aangeboren afwijkingen met een geboorte incidentie van circa 1-13 per 1000 levendgeborenen. Voorheen, haalden kinderen met een congenitale hartafwijking de volwassen leeftijd vaak niet. Dit is echter veranderd sinds de introductie van betere operatietechnieken, zo'n 20-30 jaar geleden. Daardoor neemt het aantal patiënten met een congenitale hartafwijking op de volwassen leeftijd nog steeds toe. Hierdoor ontstaan er vragen van deze patiënten zoals hoe groot de kans is dat zij zelf een kind krijgen met een congenitale hartafwijking. Kennis van de erfelijkheid van aangeboren hartafwijkingen is dus onmisbaar, om deze vragen te kunnen beantwoorden.

Congenitale hartafwijkingen kunnen voorkomen in het kader van een syndroom, maar ook heel vaak zijn deze hartafwijkingen op zichzelf staand. Het is gebleken dat erfelijke (genetische) factoren een belangrijke rol spelen bij de ontwikkeling van deze hartafwijkingen. Namelijk, in 50-95% van alle patiënten met een congenitale hartafwijking (zonder syndroom) is het gebleken dat genetische factoren hebben bijgedragen aan het ontstaan van deze hartafwijking. Ook is het herhalingsrisico in een familie met een patiënt met een congenitale hartafwijking verhoogd.

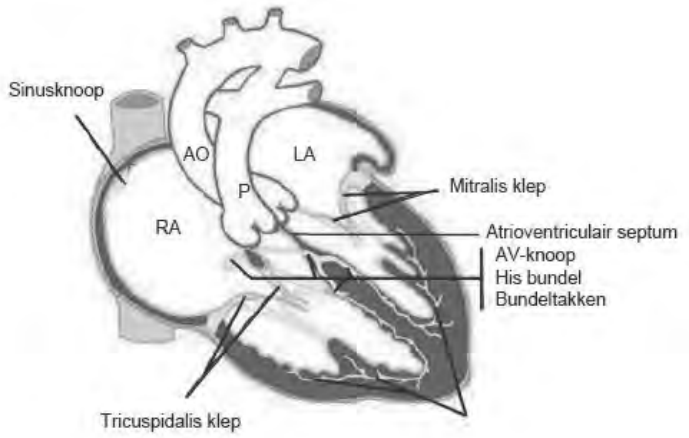
DNA is de opslagplaats van ons erfelijk materiaal. Hierin ligt de blauwdruk voor het ontstaan van bepaalde eigenschappen zoals welke kleur haar iemand krijgt of hoe lang iemand wordt. Naast deze uiterlijke kenmerken ligt hierin ook de informatie voor het ontstaan van de organen en ook het ontstaan van bepaalde aangeboren afwijkingen. DNA zit in iedere cel van ons lichaam in de vorm van chromosomen. Van elk chromosoom hebben we twee exemplaren (in totaal 23 paren). Op een chromosoom bevinden zich tientallen tot honderden genen die bestaan uit een stukje van een DNA sequentie. Elk gen kan coderen voor 1 of meerdere eiwitten, die een belangrijke rol spelen in allerlei processen van het lichaam, inclusief de ontwikkeling van het hart (Figuur 1). Net als bij het chromosoom is elk gen in tweevoud aanwezig, waarvan 1 gen afkomstig is van de moeder en het andere van de vader. Voor filmpje van DNA tot eiwit: http://www.youtube.com/watch?v=D3fOXt4MrOM&feature=related

Figuur 1.



Figuur 1. *Cel met celkern. In de celkern bevinden zich chromosomen. Van elk chromosoom hebben we 2 exemplaren. Het chromosoom bestaat uit compact DNA dat wordt samengepakt door histonen. Het DNA bestaat uit 2 strengen van nucleotiden. De strengen zijn aan elkaar verbonden door basenparen. Een basenpaar verbindt de tegenover liggende nucleotide en vormen gezamenlijk de dubbelstrengse helix.*

Figuur 2.



Figuur 2. *Schematische weergave van het hart. De mitralis en tricuspidalis klep zijn de atrioventriculaire kleppen. AO: aorta; LA: Linker Atrium; P: arteria pulmonalis; RA: Rechter Atrium*

Onderzoek met dieren heeft veel inzicht gegeven over de manier waarop genen de hartontwikkeling controleren. Dit heeft helaas nog niet geleid tot dezelfde hoeveelheid kennis van de ontwikkeling van congenitale hartafwijkingen bij de mens. Dit komt voornamelijk doordat de ontstaanswijze van deze hartafwijkingen zeer complex is, zoals beschreven wordt in hoofdstuk 1 en 2. Bovendien zijn er maar weinig grote families met meerdere aangedane familieleden bij wie het overervingpatroon geschikt is voor conventionele genetische analyses. In hoofdstuk 3 en 4 is er daarom gekozen voor een kandidaat gen *sequencing* screen in een populatie van patiënten met een congenitale hartafwijking van de atrioventriculaire klep en/of septum (Figuur 2). Dit houdt in dat, bij deze patiënten, verschillende genen zijn gesequenced (methode voor het bepalen van de volgorde van nucleotiden in DNA). Al de onderzochte genen bleken in dierstudies belangrijk voor de ontwikkeling van de atrioventriculaire klep en/of het septum. Alle gen varianten die gevonden werden in deze patiënten, werden door een computermodel geëvalueerd op hun voorspelde effect op de eiwitfunctie. Van 18 gen varianten werd een verandering van de eiwitfunctie voorspeld, echter in bijna alle families konden we geen overerving vinden van de aangeboren hartafwijking. De meest aannemelijke verklaring is dat aangeboren hartafwijkingen maar zelden worden veroorzaakt door één pathogene mutatie, maar veel vaker door meerdere genetische varianten die allemaal afzonderlijk een klein effect hebben, maar samen zorgen voor een groot effect en dus samen voor een hartdefect.

Dit geldt waarschijnlijk ook voor patiënten met het syndroom van Down, zoals wordt beschreven in hoofdstuk 4. Het Down syndroom wordt in de meeste gevallen veroorzaakt door een extra chromosoom 21 (trisomie 21). Van alle patiënten met het Down syndroom heeft 40-60% een aangeboren hartafwijking. Het lijkt daarom dat het hebben van een extra kopie van het chromosoom 21 wel een groot verhoogd risico geeft op het ontwikkelen van hartafwijkingen, maar niet alle patiënten met Down syndroom krijgen ook daadwerkelijk een hartdefect. Waarschijnlijk zijn ook andere factoren, naast de trisomie 21, noodzakelijk voor het ontstaan van deze aangeboren hartafwijking, zoals genetische variatie in andere genen. Hoofdstuk 4 geeft een voorbeeld hiervan, waarbij in een Down syndroom patiënt met hartafwijking een genetische belangrijke variatie werd aangetoond van het *ALK2* gen, terwijl zijn vader met dezelfde *ALK2* variant zonder trisomie 21 geen hartafwijking had.

Naast variatie van het aantal chromosomen zou ook variatie van het aantal kopieën van een gen (dus in plaats van het gebruikelijk genpaar, is er 1 gen minder (deletie) of 1 of meer genen meer (duplicatie)) betrokken kunnen zijn bij de ontwikkeling van hartafwijkingen. Dit worden ook wel copy number variants (CNVs) genoemd. Een CNV kan spontaan zijn ontstaan ('de novo') of doorgegeven worden van ouder op kind. Recentelijk, is er een artikel verschenen waarbij in 3.3% van patiënten met een geïsoleerde hartafwijking (tetrologie van Fallot) een CNV werd gevonden. Deze auteurs suggereren verder dat door de gebruikte techniek dit waarschijnlijk een onderschatting is van het daadwerkelijke percentage van patiënten met een CNV. Zij achtten het zelfs aannemelijk dat ongeveer in 10% van deze geïsoleerde hartafwijking een CNV kan worden gevonden. Of deze CNVs ook daadwerkelijk leiden tot een aangeboren hartafwijking of dat alleen de ziekte gevoeligheid hierdoor toeneemt, is nog niet duidelijk.
In hoofdstuk 5 is er gekeken of een duplicatie van het gen *GATA4* mogelijk aangeboren hartafwijkingen kan veroorzaken. Het is reeds bekend dat zowel haploinsufficientie (slechts 1 functionele kopie) en "loss-of-function" van *GATA4* resulteren in het (familiair) voorkomen van met name atrium septum defecten. Omdat er ook hartafwijkingen zijn gevonden bij patiënten met een chromosomale duplicatie inclusief *GATA4*, werd er gedacht dat *GATA4* een "dosage sensitive gene" is, of, in andere woorden, dat zowel teveel als te weinig *GATA4* kan leiden tot hartafwijkingen. In hoofdstuk 5 beschrijven we een patiënt met een ventrikel en atrium septum defect (inclusief nog wat andere niet specifieke congenitale afwijkingen) en een kleine duplicatie gelokaliseerd in chromosoom band 8p23.1 die alleen *GATA4* en zijn promotor bevat. Analyse van de overige familieleden identificeerde nog 4 additionele familieleden met deze *GATA4* duplicatie, maar geen van deze familieleden had een congenitale hartafwijking. Daarom hebben we geconcludeerd dat verhoogde concentraties van het *GATA4* eiwit ten gevolge van een *GATA4* duplicatie niet voldoende is om congenitale hartafwijkingen te veroorzaken. Of *GATA4* duplicaties de ziekte gevoeligheid vergroten, kan niet worden uitgesloten.

Het nadeel van het gebruik van een kandidaat gen sequencing screen voor de identificatie van mutaties bij congenitale hartziekten bij de mens, is dat het gebaseerd is op hypotheses. Hierdoor worden alleen genen bekeken waarvan *bekend* is dat ze betrokken zijn bij cardiale differentiatie en andere genen die mogelijk ook betrokken zijn, blijven onontdekt. Soms kunnen bepaalde chromosomale verandering helpen om nieuwe genen

belangrijk voor de hartontwikkeling te ontdekken. Een voorbeeld hiervan wordt beschreven in hoofdstuk 6. Hier wordt een casus gepresenteerd van een proband (de eerste persoon in een familie waarbij een hartdefect is gevonden) met transpositie van de grote vaten en een 'de novo' gebalanceerde translocatie waarvan het breukpunt precies in een gen waarvan de functie nog niet bekend is ligt, genaamd SPOCK3. De a priori kans dat een gen wordt onderbroken door een gebalanceerde translocatie is kleiner dan 3%. Het gen dat wordt onderbroken is hierdoor meteen een kandidaat gen. Toekomstige knock-out experimenten van SPOCK3 en in situ hybridisatie (een techniek waarbij er wordt gekeken naar de expressie van SPOCK3 in het hart tijdens embryogenesis), moet verder de functie van dit gen voor de hartontwikkeling nog gaan bepalen.

Hoofdstuk 7 beschrijft verder het studiedesign en klinische karakteristieken van een andere kandidaat gen sequencing screen bij patiënten met een bicuspide aortaklep. Deze studie is nog niet afgerond, de data wordt nog steeds verzameld. DNA onderzoek wordt pas gedaan als er minimaal 200 patiënten zijn verzameld uit kosten besparend perspectief. Van de eerst 80 geïncludeerde patiënten hebben we al wel de klinische gegevens op een rij gezet.

Dankwoord

Dankwoord

"Il n'est point de hasard. Nous avons nommé ainsi l'effet que nous voyons d'une cause que nous ne voyons pas. " (voltaire 1694-1778)

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Gelukkig bestaat er meer dan alleen maar onderzoek in het leven, al lijkt dat soms even niet zo. Juist afleiding is dan goed. Daarvoor wil ik alle vrienden, familie, schoonfamilie en bekenden van ver en dichtbij bedanken. In het bijzonder wil ik de volgende personen bedanken:

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Irene

Curriculum vitae

Irene Joziasse was born on august 7th 1979 in Gouda, the Netherlands. She grew up in the city of Waddinxveen where she graduated at the Coenecoop College in 1997. At the University of Nijmegen she studied biomedical research between 1997 and 1999. Thereafter, she went to Medical school at the same University. Between February and July 2002 she participated in a research project quantifying coronary artery dimensions and plaques in patients after myocardial infarction at the Greenlane Hospital, Auckland, New Zealand (Prof. Dr. J French) which was funded by the "Dr. Dekker beurs" from the Netherlands Heart Foundation.

Irene graduated Medical school in July 2004 and started working at the department of Cardiology as resident at the University Medical Center Nijmegen (Prof. Dr. FWA Verheugt). In June 2006 she initiated with the research work as presented in this thesis at the department of Cardiology at the University Medical Center Utrecht (Prof. Dr. PA Doevendans/ Prof. Dr. BJM Mulder). She obtained a master in genetic epidemiology at the Erasmus University Rotterdam in august 2009. In September 2009 she started her training as General Practioner at the University Medical Center Utrecht, while continuing working as researcher at the department of cardiology on a part-time base.

List of publications

Articles

Dieker H, French JK, Joziasse IC, Brouwer MA, West TM, Webber BJ, Verheugt FWA, White HD. Antiplatelet therapy does not slow the progression of coronary artery disease: a placebo-controlled one-year angiographic follow-up study. *American Heart Journal* 2007; 153: 66-.e1-8.

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Smith KA, Joziasse IC, Chocron S, van Dinther M, Verhoeven MC, Rehmann H, van der Smagt JJ, Doevendans PA, Mulder BJ, ten Dijke P, Bakkers J. Identification of a dominant-negative ALK2 allele in a family with congenital heart defects. *Circulation* 2009; 119: 3062-9.

Joziasse IC, Smith KA, Chocron S, v. d. Smagt JJ, Mulder, BJM, Doevendans PA, Bakkers J. ALK2 mutation in Down Syndrome with congenital heart defect. *Submitted*

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Dieker H, French JK, Joziasse IC, Brouwer MA, West TM, Webber BJ, Verheugt FWA, White HD. Antiplatelet therapy does not slow the progression of coronary artery disease: a placebo-controlled one-year angiographic follow-up study. *Neth Heart J* 2004; 12 (suppl.1): 25.

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Bakkers J, Chocron S, Gouriev V, Smith K, Deprez RLD, Joziasse IC, Doevendans PA, Mulder BJ, Simpson M, Barycki J, Cuppen E. Large Scale Mutation Discovery Screen Identifies Functionally Variant UGDH Alleles in Patients with Atrioventricular Valve Defects. *Circulation* 2007; 116: II_604.

Joziasse IC, Vink A, van Oosterhout M, Cramer MJ, Doevendans PA. Accuracy of congenital bicuspid aortic valve diagnosis not as straightforward as anticipated. *NVVC 2008.*

Book chapters

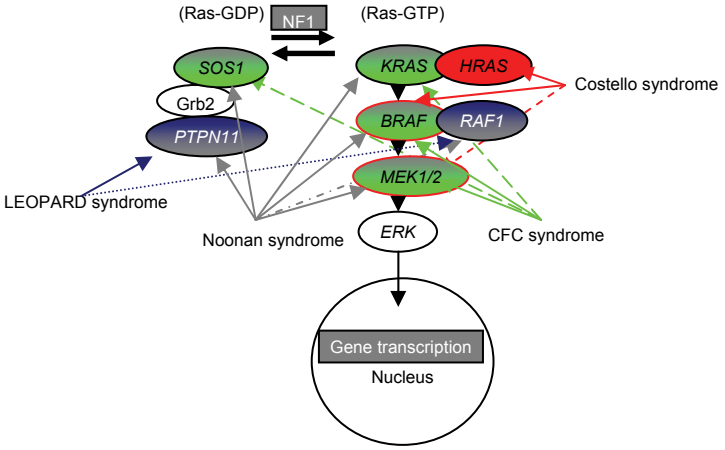
Chapter 5. Cardiomorphogenetics of congenital heart disease. Joziasse IC, Doevendans PA, online published at cardiology.nl.

Chapter 9. Genetics of congenital heart disease. Joziasse IC, Roos-Hesselink JW.

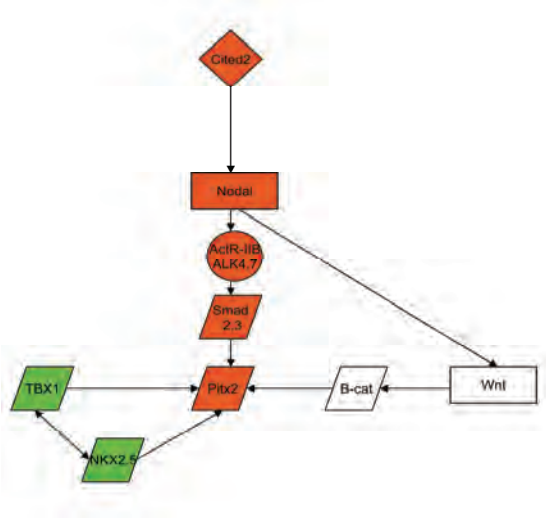
Presentations

ICIN Knowledge day, april 2009; title: Genetic variation in Congenital heart defects

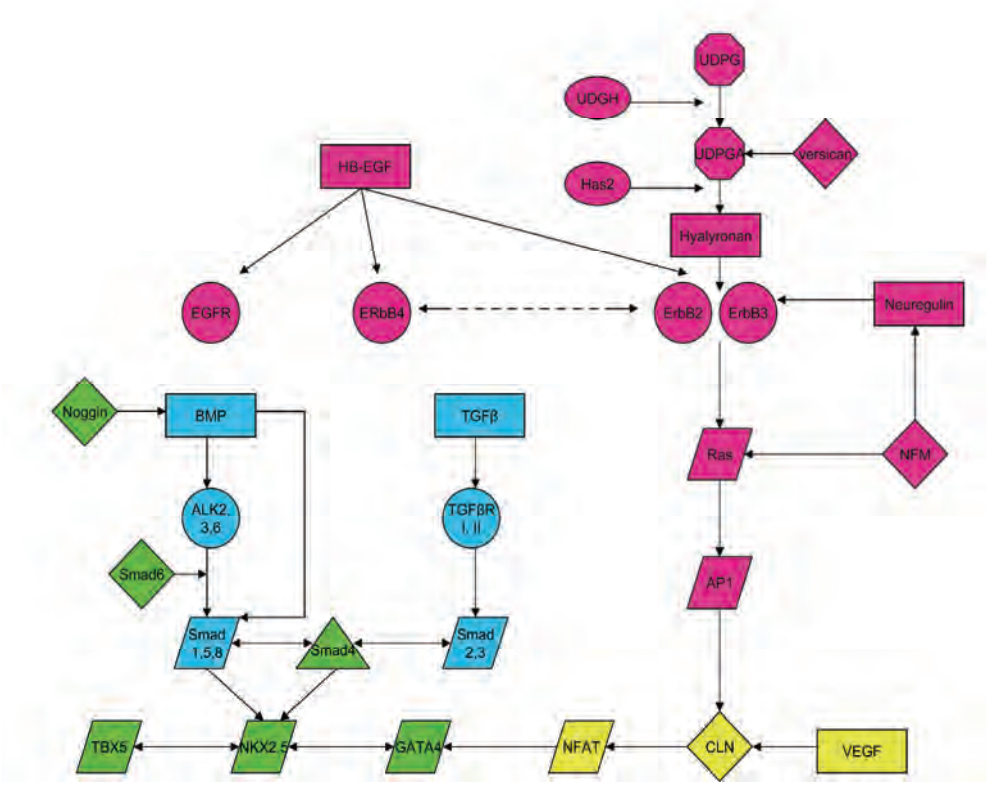
Supplement colour figures



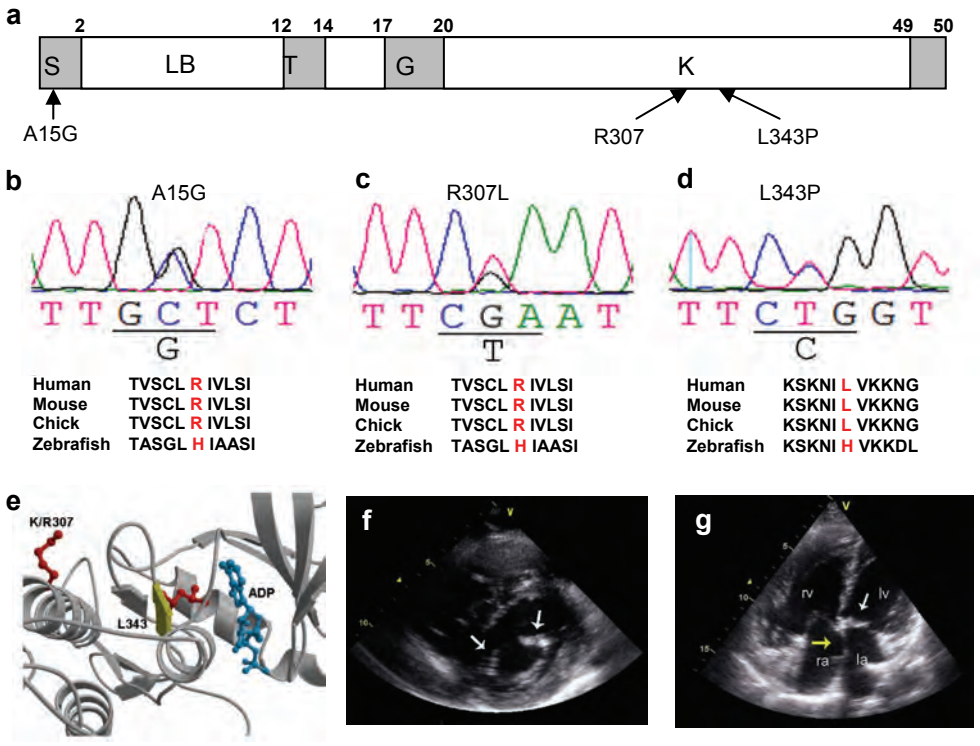
Chapter 1, Figure 3: MAPK- pathway and associated syndromes. *Mutations in genes of the MAPK pathway have been found in individuals with Noonan syndrome, Cardiofaciocutaneous (CFC) syndrome, LEOPARD syndrome and Costello's syndrome. These syndromes phenotypically overlap and as the same genes are implicated, these syndromes probably belong to the same disease entity. PTPN11 and RAF1 can be mutated in Noonan syndrome and in LEOPARD syndrome and are shown in both grey and blue. Mutations have been found in SOS1 and KRAS in patients with both Noonan syndrome and CFC syndrome and are shown in both grey and green, whilst BRAF and MEK mutations have been found in the latter two syndromes plus Costello syndrome (encircled in red). Mutations in HRAS have only been found in patients with Costello syndrome depicted in red here.*



Chapter 2, Figure 2: Nodal interactions and Wnt pathway*. In orange: Cited2/Pitx2 pathway. In green: shared (common) genetic signaling proteins/ Transcription factors. In white/grey: Wnt pathway. Rectangles: Ligands. Circles: receptors. Parallelogram: transcription factors. Diamantes: miscellaneous. B-Cat: β-catenin. Commonly used names: ALK4/ActR-IB.*



Chapter 2, Figure 1: Signaling pathways in atrioventricular valve formation. *In blue, transforming growth factor β (TGFβ) and Bone Morphogenetic Protein (BMP) signaling, through Smad proteins. In pink: ErbB and Ras Signaling. In yellow: Vascular endothelial growth factor (VEGF) - nuclear factor of activated T-cells pathway (NFAT). In green: shared (common) genetic signaling proteins/ Transcription factors. Rectangles: Ligands. Circles: receptors. Parallelogram: transcription factors. Triangle: carrier protein. Diamonds: miscellaneous. Octagon: component of other protein. Dashed arrow: indirect effect. Minus sign: inhibitory effect. HB-EGF: heparin binding- Endothelial Growth Factor; EGFR: Endothelial Growth Factor Receptor; BMP: Bone Morphogenetic protein, ALK: Activine receptor-like Kinase BMPR: Bone Morphogenetic Protein Receptor; TGFβR: Transforming Growth Factor β Receptor; UDPG: UDP-Glucose; UDPGA: UDP-Glucuronic Acid, UDGH: UDP-Glucose dehydrogenase; NFM: Neurofibromin; AP1: Activator Protein 1; CLN: calcineurin. Commonly used alternative names are: ALK2/ActR-I, ALK3/BMPR-IA, ALK5/TβR-I, and ALK6/BMPR-IB.*



Chapter 3, Figure 1. Identification of ALK2 variations in patients with CHDs. ***a***. *Schematic representation of the ALK2 protein and structural domains: signal peptide (SP), ligand binding domain (LBD), transmembrane domain (TM), GS domain (GS) and kinase domain (KD).* ***b-d***. *Sequencing chromatograms showing heterozygous cSNPs A15G (**b**) R307L (**c**) and L343P (**d**) in genomic DNA from affected individuals with accompanying protein alignments below (aligned by ClustalW). The protein alignment shows the poorly conserved nature of the A15 and R307 residues and strong conservation for the L343 residue.* ***e***. *TGFβRI (pdb entry 1PY5) as a model for ALK2, showing R307 and L343 in red, ADP in blue and the β-sheet, where L343 resides in yellow.* ***f,g***. *Echocardiograms of probands carrying variations R307L (**f**; individual III:2) and L343P (**g**; individual III:4).* ***f***. *Echocardiogram of R307L proband (after surgery, four-chamber view). Corrected common atrium, cleft of anterior MV and interruption of inferior caval vein, showing a thickened anterior MV leaflet (white arrow), thickened portion of atrial septum (yellow arrow) and an enlarged right chamber due to pulmonary hypertension. RV: right ventricle, LV: left ventricle, RA: right atrium, LA: Left atrium.* ***g.*** *Echocardiogram of L343P proband (short axis parasternal), showing a cleft MV (arrows), located in the middle of the anterior MV leaflet. Doppler examination showed mild MV regurgitation.*

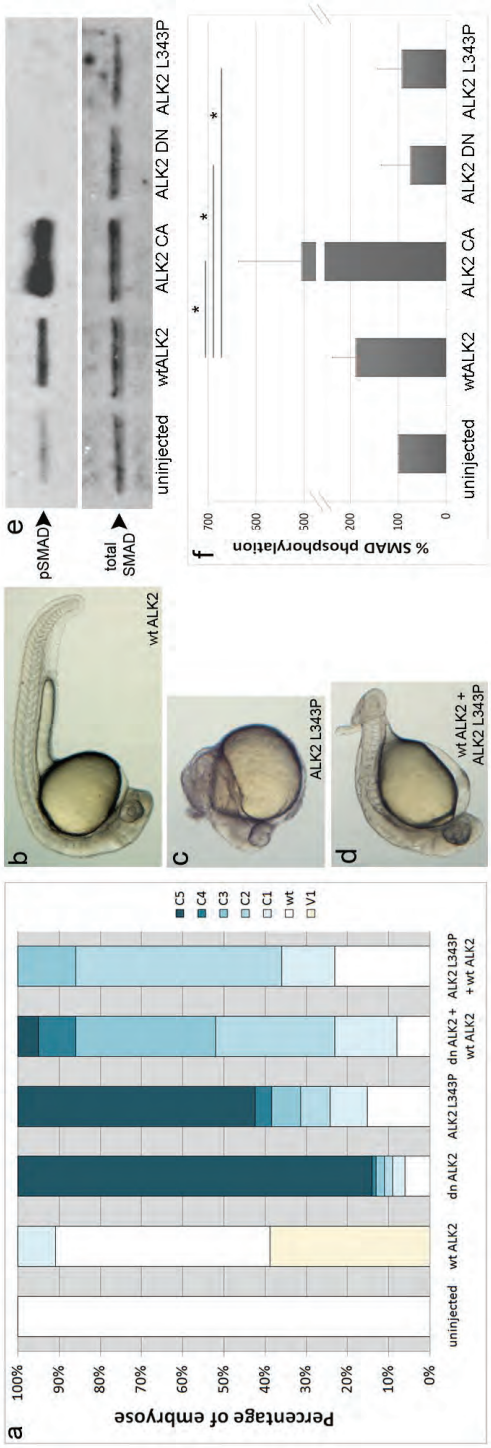


Figure 3. ALK2 L343P acts in a dominant-negative fashion in vivo. *Zebrafish embryos uninjected and injected with variant RNA at the single cell stage.* ***a****. Graphical depiction of the DV phenotype of embryos uninjected or injected with wtALK2, ALK2 DN alone or coinjected with wt ALK2, ALK2 L343P alone or coinjected with wtALK2 at the single cell stage and scored at 28 hpf.* ***b-d****. Images of* ***b****. wtALK2,* ***c****. ALK2 L343P and* ***d****. wtALK2 and ALK2 L343P injected embryos at 28 hpf.* ***a,d****. wtALK2 RNA resulted in a wild type phenotype in the majority of embryos (51% of 292 embryos) whereas the ALK2 DN RNA resulted in the majority of embryos with severe C5 dorsalisation (86% of 149 embryos). ALK2 L343P RNA also severely dorsalised embryos to C5 (c; 57% in 150 embryos) although not as potently as ALK2 DN RNA. Coinjection of wtALK2 with ALK2 L343P RNA partially rescued the dorsalisation phenotype resulting in a more intermediate C2 phenotype in the majority of embryos (****d****; 50% in 70 embryos). A similar affect was observed after coinjection of wtALK2 with ALK2 DN RNA.* ***e,f****. pSMAD levels in wt embryos injected with ALK2 variant RNA.* ***e****. Representative western blots of pSMAD (top) and total SMAD (bottom) proteins.* ***f****. Graphical representation of 4 independent experiments showing significantly lower pSMAD signaling in embryos injected with ALK2 L343P RNA. Bars are mean ± SEM and represent the percentage of SMAD phosphorylation, relative to uninjected embryos. The percentage of pSMAD was determined as a ratio of optical density of the pSMAD band compared with the total SMAD band. Statistical significance was determined by t test, where* $* p < 0.05$.

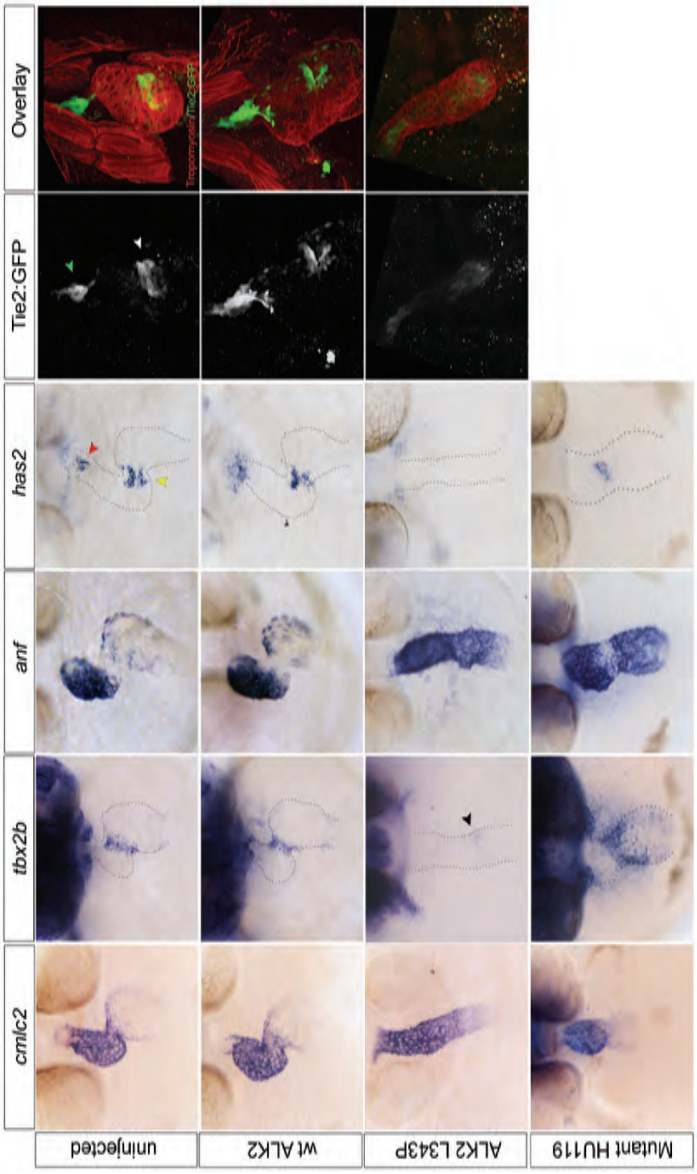
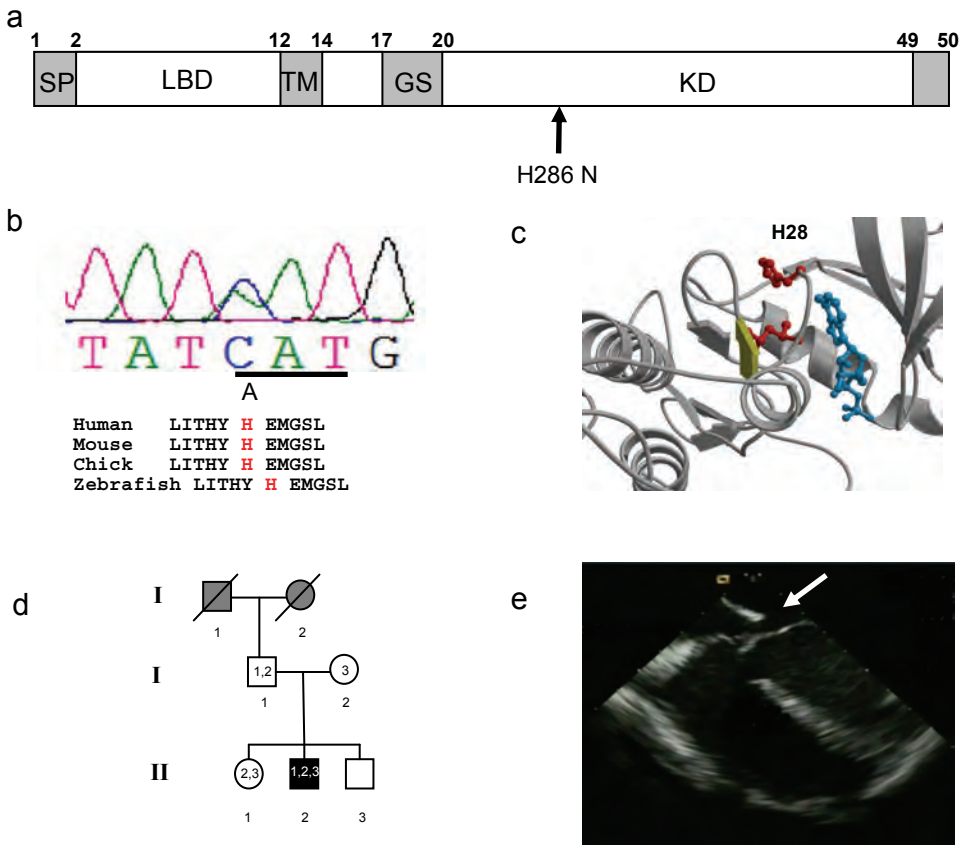
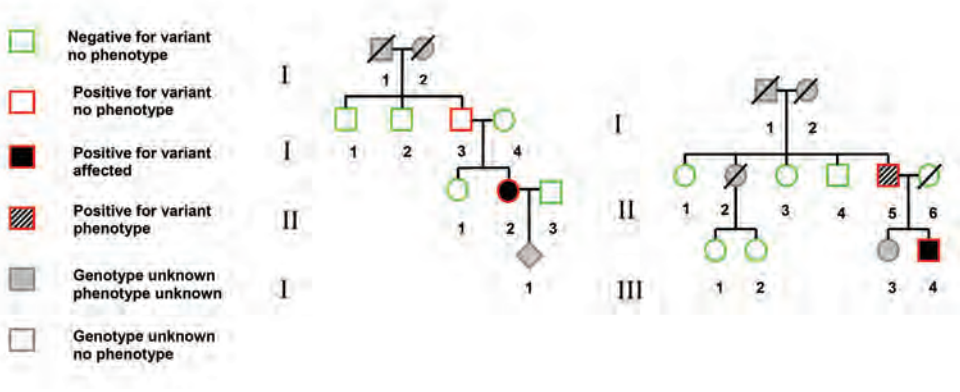


Figure 4. The AVC and ECs are improperly formed in embryos injected with ALK2 L343P RNA. *ISH staining for cardiac myosin (cmlc2), the AVC myocardium (tbx2b), the atrial and ventricular chambers (anf) and the ECs (has2) and immunofluorescent staining for ECs. Cmlc2 staining showed a disruption in the overall morphology of the heart tube in ALK2 L343P injected embryos (33/62 embryos) compared with uninjected (9/67 embryos) and wtALK2 injected embryos (10/69 embryos). A loss of AVC identity was apparent in embryos injected with ALK2 L343P RNA compared with uninjected and wtALK2 RNA injected controls. tbx2b expression was lost or reduced in ALK2 L343P RNA injected embryos (40/81 embryos; arrow head) compared with uninjected (9/62 embryos) and wtALK2 injected embryos (13/57 embryos). anf expression was continuous throughout the heart tube of ALK2 L343P injected embryos (22/32 embryos) compared with uninjected (13/39 embryos) and wtALK2 injected embryos (6/42 embryos). has2 expression was also lost in ALK2 L343P injected (23/124 embryos) compared with uninjected (0/50 embryos) and wtALK2 injected embryos (0/120 embryos)(red arrowhead: OFT, yellow arrowhead: AVC). Disruption of these markers is not due to defective cardiac looping as these markers were patterned normally in a non-looping mutant (HU119). AVC (white arrowhead) and OFT (green arrowhead) cushions were visualised using the transgenic line, Tg(Tie2:EGFP), counterstaining the myocardium with anti-Tropomyosin antibody. Reduced expression of GFP and a loss of EC structure were observed in ALK2 L343P RNA injected embryos.*

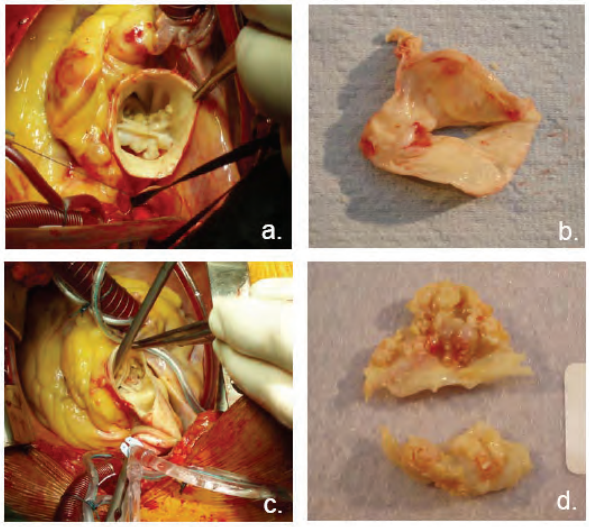


Chapter 4, Figure 1. Identification of ALK2 variant in DS. ***a***. *Schematic representation of the ALK2 protein and structural domains: signal peptide (SP), ligand binding domain (LBD), transmembrane domain (TM), GS domain (GS) and kinase domain (KD). Arrows indicate approximate locations of p.His286Asp in ALK2.* ***b***. *Sequencing chromatogram showing the p.His286Asp variant in genomic DNA from the proband with DS and a primum type atrial septal defect with accompanying protein alignment below (aligned by ClustalW). The protein alignment shows strong conservation between species of the p.His286Asp residue.* ***c***. *TGFβRI (pdb entry 1PY5) as a model for ALK2, showing H268 in red, ADP in blue and the β-sheet in yellow.* ***d***. *Family pedigree of proband (III:2). Only the father, without DS, was in possession of the ALK2 variant and did not have a congenital heart defect at cardiac echocardiography. Carriers of variants are depicted with a number in the squares or circle; 1: ALK2 p.His286Asp variant, 2: ALK3 p.Glu414Lys and 3: ERBB3 p.Thr1169Il and individuals with or without CHDs are shaded black or white, respectively. Grey colouration indicates the genotype or phenotype was unable to be determined. Deceased individuals are annotated by a diagonal strike-through.* ***e***. *Echocardiogram of proband carrying the p.His286Asp variant (III:2) before surgery, demonstrating a large primum type atrial septal defect (arrow) and overriding AV valve.*

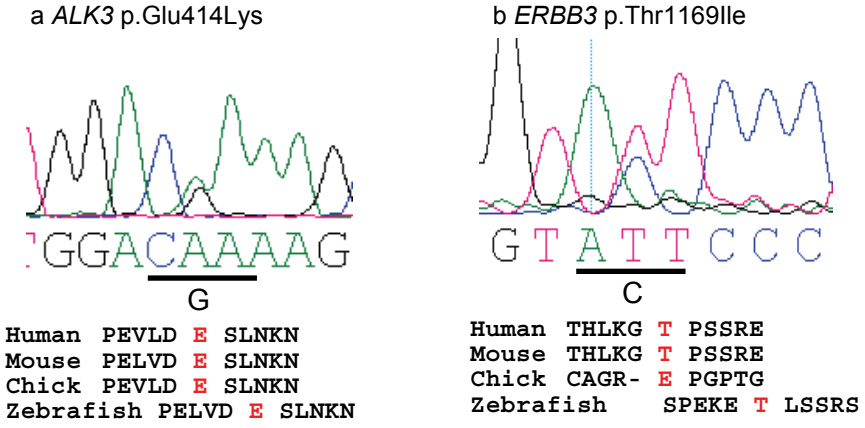
Chapter 4, Figure 3. Rescue experiments in laf mutants. *Rescue experiments in the zebrafish ALK2/8 mutant, lost-a-fin (laf).* ***a.*** *Injection of wt ALK2 or p.His286Asp RNA at the single cell stage in laf/Alk2 mutants. Wt ALK2 RNA rescues phenotype in 65% whilst injection of p.His286Asp rescues phenotype in 51%. This is significantly less effective compared to wt RNA injection. Statistical significance was determined by Student t test, where * p < 0.05.* ***b.*** *laf embryo identifiable by its C1 dorsalisation phenotype; with cardiac edema (black arrow), absent ventral fin (blue arrow).* ***c.*** *rescued laf zebrafish sibling after injection with ALK2 p.His286Asp demonstrating wt phenotype* ***d.*** *wt zebrafish.*



Chapter 3, Supplementary Figure 1. *Family pedigrees of patients (identified by asterisk) with identified mutations.* ***a.*** *The family carrying the R307L variant exhibited no obvious inheritance pattern.* ***b.*** *The L343P pedigree was uninformative for inheritance of the mutation with CHD. The phenotype of II:5 could not be ruled out as degenerative and thus could not unambiguously be classified with a CHD. Carriers of non-carriers of variants are depicted with a red or green outlines, respectively, and individuals with or without CHDs are shaded black or white, respectively. The ambiguous phenotype is represented by hatching. Grey colouring indicates the genotype or phenotype was unable to be determined. The diamond indicates a miscarriage and deceased individuals are annotated by a diagonal strike-through.*



Chapter 7b, Figure 1. *Photographs of aortic valve at surgery and at pathology.* ***a-b****: patient with severe aortic stenosis and diagnosed with a type 0 bicuspid aortic valve by both surgeon (a) and pathologist (b);* ***c-d****: different patient with a tricuspid aortic valve at surgery (c), this photograph shows a clear view of the presence of 3 separate valve leaflets. (d) the excised valves of the same patient. Evidently, it is hard to identify 3 separate valve leaflets and this aortic valve was determined to be a type 1 bicuspid aortic valve (with raphe) by the pathologist.*



Chapter 4, Supplementary figure 1. *Sequencing chromatogram with alignment below (aligned by ClustalW) showing* ***a.*** *the ALK3 p.Glu414Lys. Protein alignment of the ALK3 p.Glu414Lys demonstrates strong conservation between species.* ***b.*** *Sequencing chromatogram of the ERBB3 p.Thr1169Ile variant. Conservation alignment did not show strong conservation of the ERBB3 p.Thr1169Ile region.*