CLINICAL AND GENETIC STUDIES IN INHERITED
CARDIOVASCULAR MALFORMATIONS

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Ingrid van de Laar

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CLINICAL AND GENETIC STUDIES IN INHERITED CARDIOVASCULAR MALFORMATIONS

KLINISCH EN GENETISCH ONDERZOEK VAN ERFELIJKE CARDIOVASCULAIRE MALFORMATIES

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ABBREVIATIONS

AAA	abdominal aortic aneurysm
AF	atrial fibrillation
AI	aortic valve insufficiency
AOS	aneurysms-osteoarthritis syndrome
aPWV	aortic pulse wave velocity
ADCL	autosomal dominant cutis laxa
AR	aortic valve regurgitation
ARCL	autosomal recessive cutis laxa
AS	aortic valve stenosis
ASD	atrial septal defect
ATS	arterial tortuosity syndrome
AV	atrioventricular
AVSD	atrioventricular septal defect
BAV	bicuspid aortic valve
BSA	body surface area
CFC	cardio-facio-cutaneous
CHM	congenital heart malformation
CNV	copy number variation
CoA	coarctation of the aorta
CTA	computed tomography angiography
CTGF	connective tissue growth factor
CVM	cardiovascular malformation
DCM	dilated cardiomyopathy
DORV	double outlet right ventricle
EDS	ehlers danlos syndrome
ECG	electocardiogram
EMT	endothelial-to-mesenchymal transdifferentiation
FHF	first heart field
FTAAD	familial thoracic aortic aneurysm-dissection
GWAS	genome wide association studies
GWLA	genome wide linkage analysis
HAA	hypoplastic aortic arch
HCM	hypertrophic cardiomyopathy
Het	heterozygous
HLH	hypoplastic left heart
HLHS	hypoplastic left heart syndrome
HLV	hypoplastic left ventricle
Hom	homozygous

HRFC	hepatorenal fibrocystic
HRV	hypoplastic right ventricle
IAA	interrupted aortic arch
ICV	inferior caval vein
IFT	intraflagellar transport
KV	kupffer's vesicle
LDS	loeys-dietz syndrome
LOD	logarithm (base 10) of the odds
LPM	lateral plate mesoderm
LPV	left pulmonary vein
LR	left-right
LVH	left ventricular hypertrophy
LVNC	left ventricular noncompaction
LVOTO	left ventricular outflow tract obstruction
Mb	megabases
MFS	marfan syndrome
MI	mitral valve insufficiency
MiRNA	microRNA
MO	morpholino oligonucleotides
MR	mitral valve regurgitation
MRA	magnetic resonance angiography
MRI	magnetic resonance imaging
MV	mitral valve
MS	mitral valve stenosis
MVP	mitral valve prolapse
NCCM	noncompaction cardiomyopathy
NGS	next generation sequencing
NPHP	nephronophthisis
NT-proBNP	N-terminal probrain natriuretic peptide
OA	osteoarthritis
OCD	osteochondritis dissecans
PA	pulmonary atresia
PAPVR	partial anomalous pulmonary venous return
PCD	primary ciliary dyskinesia
PCR	polymerase chain reaction
PDA	persistent ductus arteriosus
PPS	peripheral pulmonary stenosis
PS	pulmonary valve stenosis
PTA	persistent truncus arteriosus
PV	pulmonary vein
PVOD	pulmonary veno-occlusive disease

PVS	pulmonary vein stenosis
RPV	right pulmonary vein
SB	stillbirth
SB-MO	splicing blocking morpholino
SCV	superior caval vein
SLSN	Senior-Loken syndrome
SNP	single nucleotide polymorphism
SVAS	supravalvular aortic stenosis
TA	tricuspid atresia
TAA	thoracic aortic aneurysm
TAAD	thoracic aortic aneurysm-dissection
TAPVR	total anomalous pulmonary venous return
TB-MO	translation blocking morpholino
TGA	transposition of the great arteries
TGF	transforming growth factor
TOF	tetralogy of Fallot
TTE	transthoracic echocardiography
UTR	untranslated region
VSD	ventricular septal defect
VSMC	vascular smooth muscle cells
VUS	variant of unknown significance
WES	whole exome sequencing

GENERAL INTRODUCTION

CHAPTER 1

1.1 CARDIOVASCULAR MALFORMATIONS

Cardiovascular malformations comprise a broad spectrum of anomalies of the heart and blood vessels, including congenital heart malformations (CHM) and aortic aneurysms, the two main topics of this thesis. These conditions lead to significant morbidity and mortality both in infancy and adulthood.

A substantial proportion of cardiovascular malformations have a genetic background, including large chromosomal abnormalities, submicroscopic chromosome deletions or duplications, and single gene mutations. However, the majority of cardiovascular malformations is thought to be due to multifactorial inheritance, involving a multitude of mutations in susceptibility genes superposed on unfavorable environmental and life style factors.

In the past decade, great progress has been made in the unravelling of genes involved in cardiovascular malformations. This made it possible to understand the genetic pathways and underlying pathophysiologic mechanisms, and develop therapeutic and preventive measures. It also led to the need for multidisciplinary cardiogenetic clinics in order to improve diagnosis and treatment of cardiovascular diseases. Such a multidisciplinary cardiogenetic clinic has been established in the (Paediatric) Cardiology Department of the Erasmus Medical Center in Rotterdam. In this setting most patients described in this thesis were studied. With the enthusiastic participation of these patients, their families and physicians, scientific studies were initiated to understand the genetic cause of cardiovascular malformations.

1.1.1 Congenital heart malformations

1.1.1.1 Incidence

CHM are the most common birth defects, with an incidence ranging from 6 to 75 out of thousand live births.[1] Accordingly, in the Netherlands every year around 1800 children are born with a CHM. The morbidity and mortality varies with the severity of the CHM, but overall, it is the leading cause of infant death in the Western countries.[2]

1.1.1.2 Classification and genetics

CHM can be classified into syndromic and non-syndromic forms. Syndromic CHM, characterized by additional non-cardiac malformations, is present in about a quarter of children with CHM.[3] The majority of patients has non-syndromic CHM, not associated with other anomalies.

In the past decade tremendous progress has been made in the elucidation of the molecular mechanisms involved in CHM.[2] Monogenic inheritance can be autosomal dominant (most families), or autosomal recessive or X-linked (minority of families).

Studies of monogenic CHM (both syndromic and non-syndromic) usually with autosomal dominant inheritance have proven to be instrumental for the identification of genes involved in CHM.

1.1.1.2.1 Non-syndromic CHM

Whereas monogenic mutations are responsible for many syndromic forms of CHM, mutations in these genes account for only a small proportion of non-syndromic CHM.[4-9] As most cases of non-syndromic CHM occur sporadically, and families with clear monogenic inheritance of non-syndromic CHM are scarce, the identification of genes by a classic positional cloning approach has been hampered. Nevertheless, genetic studies in both human patients and animal models have led to the identification of a significant number of genes that are implicated in non-syndromic CHM, which are discussed briefly below and tabulated in Table 1.[4]

Several signaling pathways have been implicated in non-syndromic CHM (for review: see ref[4]). The first single gene mutation causing non-syndromic CHM was described in the transcription factor gene *NKX2.5* in families with inherited atrial septal defect (ASD) and atrioventricular block.[5] Subsequently, mutations in *GATA4* were found in two kindreds with apparent non-syndromic septal defects.[6] Mutations in the T-box gene *TBX20* have been implicated in cardiomyopathy and septal defects.[7] A single mutation in the *NKX2.6* gene has been associated with persistent truncus arteriosus (PTA).[8] A minority of patients with tetralogy of Fallot (TOF) have a mutation in the *FOG2* gene.[9] *NOTCH1* mutations have been found in patients with left ventricular outflow tract obstruction (LVOTO).[10] Mutations *in JAG1*, another gene of the Notch signal transduction pathway, are occasionally found in patients with non-syndromic TOF or pulmonary valve stenosis (PS).[11] Mutations in genes of the NODAL pathway, including *NODAL, LEFTY2, GDF1, CFC1, TDGF1, ACVR2B, SESN1* and *FOXH1* are involved in cardiac laterality defects. Genes that encode for sarcomeric proteins, including *MYH11, MYH6, MYH7, MYBPC3*, and *ACTC1,* have been implicated in non-syndromic CHM. Other genes involved in non-syndromic CHM include *CITED2, ANKRD1, FLNA, ELN, THRAP2, TLL1, VEGFA, TAB2, CRELD* and *ZIC3* (Table 1).

1.1.1.2.2 Syndromic CHM

CHM occur in many genetic syndromes and have been found to be associated with mutations in a variety of single genes (for reviews: see refs[68-69]). The most common syndromes where CHM form a substantial part of the condition are briefly mentioned here and a more extensive list of syndromic forms of CHM is tabulated in Table 2, although this is not complete.

Table 1 Genes involved non-syndromic CHM

Gene (OMIM) Chromosome	Protein	Location protein	Cardiac phenotypes	Refs
Nodal signaling				
CFC1 (605194) 2q21.1	CRYPTIC	Transmembrane protein	Heterotaxy, TGA, TOF, TA, AVSD, ASD, VSD	4,12-16
ACVR2B (602730) 3p22-p21.3	Activin receptor IIB	Transmembrane protein	Heterotaxy	17-18
TDGF1 (187395) 3p23-p21	CRIPTO	Transmembrane protein	TOF, VSD	15,19
LEFTY2 (601877) 1q42.12	Left-right determination factor 2	Extracellular protein	Heterotaxy	20
GDF1 (602880) 19p13.11	Growth/ differentiation factor1	Extracellular protein	TOF, TGA, DORV, AVSD	21-22
NODAL (601265) 10q22.1	NODAL	Extracellular protein	Heterotaxy, TGA	23-25
FOXH1 (603621) 8q24.3	Forkhead box H1	Cytoplasmic protein	TOF, CHM, TGA, VSD	15,24,26
SESN1 (606103) 6q21	Sestrin 1	Cytoplasmic protein	Heterotaxy	27
CITED2 (602937) 6q24.1	CBP/p300 interacting transactivator with glu/asp rich c-terminal domain	Cytoplasmic protein	VSD, ASD	28-29
Transcriptional network				
NKX2.5 (600584) 5q35.1	NK2 HOMEOBOX 5	Cytoplasmic protein	ASD, VSD, TOF, HLHS, CoA, IAA, heterotaxy, TGA, DORV, Ebstein	4,24,30-35
GATA4 (600576) 8p23.1	GATA-binding protein 4	Cytoplasmic protein	ASD, TOF, ASD, PS, VSD, HRV, PAPVR	4,6,36-37
NKX2.6 (611770) 8p21.2	NK2 HOMEOBOX 6	Cytoplasmic protein	PTA	8
TBX20 (606061) 7p14.2	T-BOX 20	Cytoplasmic protein	ASD, CoA, VSD, PDA, PFO, DCM, MS/HLV, TOF, TAPVR	7,38-39
FOG2 (603693) 8q23.1	Friends of GATA2	Cytoplasmic protein	TOF, DORV	9,24,40
Sarcomere				
MYH11 (160745) 16p13.11	Myosin heavy chain 11	Cytoplasmic protein	PDA	41
ACTC1 (102540) 15q14	Alpha-cardiac actin 1	Cytoplasmic protein	ASD, VSD	42-44

Gene (OMIM) Chromosome	Protein	Location protein	Cardiac phenotypes	Refs
Nodal signaling				
MYH6 (160710) 14q11.2	Myosin heavy chain 6	Cytoplasmic protein	ASD	45
MYH7 (160760) 14q11.2	Myosin heavy chain 7	Cytoplasmic protein	ASD, Ebstein	46
MYBPC3 (600958) 11p11.2	Myosin binding protein C 3	Cytoplasmic protein	ASD, VSD	47-49
Other				
ZIC3 (300265) Xq26.3	Zinc finger protein of cerebellum 3	Cytoplasmic protein	Heterotaxy, TGA, ASD, PS	18,24,50-53
NOTCH1 (190198) 9q34.3	NOTCH1	Transmembrane protein	BAV, AS, HLHS, CoA, VSD, TOF, DORV, MA, TAA	4,10,54-57
ANKRD1 (609599) 10	Ankyrin repeat domain-containing protein 1	Cytoplasmic protein	TAPVR	58
TAB2 (605101) 6q25.1	TAK1-binding protein 2	Cytoplasmic protein	BAV, AS, TAA, arrhytmia	59
VEGFA (192240) 6p21.1	Vascular endothelial growth factor A	Cytoplasmic protein	BAV, AS, CoA, PDA VSD, TAA	60
FLNA (300017) Xq28	Filamin A	Cytoplasmic protein	XMVD	61
TLL1 (606742) 4q32.3	Tolloid-like 1	Cytoplasmic protein	ASD	62
CRELD (607170) 3p25.3	Cysteine-rich protein with EGF-like domains 1	Cytoplasmic protein	AVSD	63-66
THRAP2 (608771) 12q24.21	Thyroid hormone receptor-associated protein 2	Cytoplasmic protein	TGA	67

AS, aortic valve stenosis; ASD, atrial septal defect; AVSD, atrioventricular septal defect; BAV, bicuspid aortic valve; CHM, congenital heart malformation; CoA, coarctation of the aorta; DCM, dilated cardiomyopathy, DORV, double outlet right ventricle; HLHS, hypoplastic left heart syndrome; HLV, hypoplastic left ventricle; HRV, hypoplastic right ventricle; IAA, interrupted aortic arch; MA, mitral valve atresia; MS, mitral valve stenosis; PAPVR, partial anomalous pulmonary venous return; PDA, persistent ductus arteriosus; PPS, peripheral pulmonary stenosis; PS, pulmonary valve stenosis; PTA, persistent truncus arteriosus; SVAS, supravalvular aortic stenosis; TA, tricuspid atresia; TAA, thoracic aortic aneurysm; TAPVR, total anomalous pulmonary venous return; TGA, transposition of the great arteries; TOF, tetralogy of Fallot; VSD, ventricular septal defect; XMVD, X-linked myxomatous valvular dystrophy

Table 2 Genes involved in syndromic CHM

Syndrome (inheritance)	Gene (OMIM) Chromosome	Protein	Location protein	Cardiac phenotypes	Additional features	Refs
Holt-Oram syndrome (AD)	TBX5* (601620) 12q24.21	T-BOX 5	Transcription factor	ASD, VSD, AVSD, TOF, AV conduction defects	Radial ray upper limb defects	70-71,77-78
22q11 deletion syndrome (AD)	TBX1* (602054) 22q11.21	T-BOX 1	Transcription factor	IAA, PTA, TGA, TOF, DORV, VSD	Facial features (dysplastic ears, hooding of eyelids, small alae nasi), velo-pharyngeal insufficiency (VPI), cleft palate, thymic hypoplasia, parathyroid hypoplasia, renal malformations, intellectual disability, growth retardation, psychiatric disorders	79-80
Noonan syndrome and Ras-o-pathies (AD)	*PTPN11* (176876) 12q24.1 And *SOS1, RAF1, KRAS, NRAS, MEK1/2, BRAF, CBL, SHOC2*	SHP2	Cytoplasmic protein	HCM, PS	Facial features (hypertelorism, downslanting palpebral fissures, low set ears), broad webbed neck, pectus, cryptorchidism, intellectual disability, short stature	72
Alagille syndrome (AD)	*JAG1** (601920) 20p12 *NOTCH2* (600275) 1p12-p11	JAGGED1 NOTCH2	Transmembrane protein	PS, PPS, TOF	Facial features (broad forehead, deep-set eyes, pointed chin), posterior embryotoxon, intrahepatic bile ducts paucity, renal abnormalities, abnormal vertebral segmentation	73
Williams syndrome (AD)	*ELN** (130160) 7q11.2	Elastin	ECM protein	SVAS, AS, PPS, PS, CoA	Facial features (periorbital fullness, malar flattening, full cheeks and lips), endocrine abnormalities, connective tissue abnormalities, intellectual disability, specific cognitive profile, short stature	74
CHARGE syndrome (AD)	*CHD7* (608892) 8q12.1	Chromodomain helicase DNA-binding protein 7	Cytoplasmic protein	TOF, PDA, DORV, ASD, VSD	Facial features (facial asymmetry), external ear anomalies, ocular coloboma, choanal atresia, cranial nerve dysfunction, semicircular canal hypoplasia, genital hypoplasia, intellectual disability, short stature	81-82

Syndrome (inheritance)	Gene (OMIM) Chromosome	Protein	Location protein	Cardiac phenotypes	Additional features	Refs
Ellis van Creveld syndrome (AR)	*EVC* (604831) 4p16.2	EVC EVC2	Transmembrane protein	AVSD, common atrium, systemic/ pulmonary venous abn.	postaxial polydactyly, mesomelic shortening of the limbs, short ribs, dysplastic nails and teeth, oral frenula	83 84
Kabuki syndrome (AD)	*MLL2* (602113) 12q13.12	Myeloid/lymphoid or mixed lineage leukemia 2	Cytoplasmic protein	CoA, LVOTO, VSD, ASD	Facial features (high-arched eyebrows, long palpebral fissures, eversion of lower eyelids, flat nasal tip), hypodontia, fetal pads, genitourinary and gastrointestinal anomalies, cleft lip and/or palate, hypotonia, intellectual disability, short stature	85-86
CHAR syndrome (AD)	*TFAP2B** (601601) 6p12	Transcription factor AP2-beta	Cytoplasmic protein	PDA	Facial features (flat midface, broad flat nasal tip, hypertelorism, downslanting palpebral fissures, ptosis, thickened everted lips), a-/hypoplasia of middle phalanges of the fifth fingers.	87
Mowat Wilson syndrome (AD)	*ZEB2* (605802) 2q21-q23	Zinc finger E box-binding homeobox 2	Cytoplasmic protein	PDA, PS, VSD, ASD	Facial features (hypertelorism, medially flared and broad eyebrows, pointed chin, uplifted earlobes), corpus callosum agenesis, eye defects, Hirschsprung disease, genitourinary anomalies, intellectual disability, seizures, microcephaly, short stature	88
Smith-Lemli Opitz syndrome (AR)	*DHCR7* (602858) 11q12q13	7-dehydrocholesterol reductase	Cytoplasmic protein	ASD, VSD, PDA, AVSD	Facial features (ptosis, broad nasal bridge, anteverted nares, micrognathia), microcephaly, corpus callosum agenesis, 2/3 toe syndactyly, genitourinary and gastrointestinal anomalies, hypotonia, intellectual disability, short stature	89

* genes also involved in non-syndromic CHM

AS, aortic valve stenosis; ASD, atrial septal defect; AV, atrioventricular; AVSD, atrioventricular septal defect; CoA, coarctation of the aorta; DORV, double outlet right ventricle; HCM, hypertrophic cardiomyopathy; IAA, interrupted aortic arch; LVOTO, left ventricular outflow tract obstruction; PDA, persistent ductus arteriosus; PPS, peripheral pulmonary stenosis; PS, pulmonary valve stenosis; PTA, persistent truncus arteriosus; SVAS, supravalvular aortic stenosis; TGA, transposition of the great arteries; TOF, tetralogy of Fallot; VSD, ventricular septal defect

Septal defects are present in Holt-Oram syndrome, which is caused by mutations in the *TBX5* gene.[70-71] PS is a characteristic of Noonan syndrome, caused by mutations in the *PTPN11* gene and other genes involved in the RAS-MAPK signaling pathway, including *SOS1, RAF1, KRAS, NRAS, MEK1/2, BRAF, CBL,* and *SHOC2.*[72] PS also is a feature of Alagille syndrome, which is caused by mutations in components of the Notch signaling pathway, including *JAG1* and *NOTCH2.*[73] Supravalvular aortic stenosis occurs in Williams syndrome and is associated with *ELN* haploinsufficiency due to a deletion of chromosome band 7q11.2.[74] Conotruncal defects in 22q11 deletion syndrome result from haploinsufficiency of the transcription factor *TBX1* usually due to deletion of chromosome band 22q11.[75-76]

In this thesis, families with uncommon forms of CHM are described, including laterality defects, abnormal pulmonary vein development, and abnormal valvulogenesis.

1.1.1.3 Laterality disorders

1.1.1.3.1 Definition wand incidence

Laterality disorders refer to a broad group of disorders caused by the embryonic disruption of normal left- right (LR) patterning, including situs inversus totalis and heterotaxy. Situs inversus totalis is the mirror image reversal of all visceral organs including the lungs, spleen, liver and stomach, whereas heterotaxy is the abnormal orientation of one or more organs along the LR axis. The CHMs common in laterality disorders include atrioventricular septal defects (AVSD), transposition of the great arteries (TGA), double outlet right ventricle (DORV), abnormal systemic and/or pulmonary venous connection, abnormal position of the heart (dextrocardia), and isomerism of the atrial appendages. Heterotaxy can also be part of well-defined syndromes, referred to as hepatorenal fibrocystic (HRFC) syndromes. Many of these patients have other, noncardiac malformations, including retinal degeneration, renal cystic disease, congenital fibrocystic diseases of the liver, postaxial polydactyly, cerebral anomalies, diabetes, obesity and skeletal dysplasias.[90] The incidence of laterality defects is estimated at 1:10.000.[91]

1.1.1.3.3 Pathophysiology

Patients with heterotaxy typically have complex cardiac and vascular abnormalities that are thought to originate from partial or complete reversal of heart tube looping. Cardiovascular LR patterning of vertebrate embryos is established early in embryogenesis. The first steps in the establishment of asymmetry is signaling upstream of the node, a transient embryonic organizer, resulting in early intracellular asymmetry. Subsequently, the node and functioning motile and sensory cilia are

formed. The genes involved in node formation are largely unknown, but there is evidence that Notch signaling is necessary for proper node structure. Movement of the cilia at the node produces leftward flow leading to asymmetric expression of Nodal, a ligand of the TGF-β signaling pathway. Subsequently, asymmetric nodal signaling is transferred from the node to the lateral plate mesoderm (LPM). In the latest stage asymmetric signaling is propagated from the LPM to the developing organs for proper morphogenesis.[92] In any of these developmental steps defects can occur that can result in heterotaxy.

1.1.1.3.4 Genetics

Most laterality defects seem to occur sporadically, but familial occurrence has been described, which suggests monogenic inheritance in at least a proportion of patients. Different modes of inheritance have been described, including autosomal dominant, autosomal recessive and X-linked inheritance.[93] Animal studies have identified over 80 genes implicated in left-right patterning mainly involving the nodal signaling pathway, but only a few human orthologs of the Nodal-signaling genes identified in the animal studies have been associated with human laterality defects.[92,94] The only gene unequivocally associated with heterotaxy is *ZIC3*, which causes X-linked laterality defects.[50] Additionally, variants have been found in autosomal genes, including *ACVR2B, CFC1, NODAL, SESN1*, *CRELD1, FOXH1*, *TDGF1*, *GDF1* and *NKX2.5* and *LEFTYA*.[12,15,17,20,22-23,27,64,95] As some of these variants are missense variants inherited from an asymptomatic parent, these variants should be considered as variants of unknown significance (VUS), although they might prove to be pathogenic variants with reduced penetrance. Also, variants in more than one gene have been identified in patients with heterotaxy, suggesting di- or oligogenic inheritance of susceptibility genes with variants showing reduced penetrance.[15,24] All together, variants in these genes account for a small minority of all patients with heterotaxy or cardiac laterality defects.[18,92]

HRFC syndromes, including nephronophthisis (NPHP) , Senior-Loken syndrome, Joubert syndrome, and Meckel-Gruber syndrome, are associated with mutations in 18 cilia-related genes causing a dysfunction of ciliary proteins.[96-97] Mutations in two of these genes, *NPHP2/INVS* and *NPHP3*, can lead also to heterotaxy, situs inversus and isolated CHM.[98-100]

In **chapter 2** we describe the clinical and genetic studies in an Iranian consanguineous family with three affected family members with (cardiac) laterality defects. We performed whole genome linkage analysis, and sequencing of positional candidate genes identified homozygous variants in the *NPHP4* gene. Screening of this gene in sporadic patients with laterality defects identified heterozygous *NPHP4* variants. No other cilia-related features were seen in any of these patients. Since we showed

that *nphp4* is also essential for normal L-R patterning in zebrafish, we propose that *NPHP4* mutations are associated with cardiac laterality defects and heterotaxy in humans and zebrafish.

1.1.1.4 Pulmonary vein anomalies

1.1.1.4.1 Definition and incidence

Congenital pulmonary vein (PV) anomalies include pulmonary vein stenosis (PVS) and abnormal connection of the pulmonary vein system to the left atrium, also referred to as total or partial anomalous venous return (TAPVR/PAPVR).

PVS may appear as a longer segment of narrowing, or complete and diffuse hypoplasia of the pulmonary veins at or near the venous-atrial junction.[101-102] PVS can be classified into primary (or congenital) or secondary PVS. Because of the rare nature of PVS, the incidence of primary PVS has not been reported. Secondary PVS is far more common and occurs in 5-15% of patients as a complication following surgical repair for PAPVR/TAPVR[103] and in 1-9% of patients after pulmonary vein isolation by radiofrequency ablation for atrial fibrillation.[104]

PAPVR/TAPVR is defined as a CHM with one or all pulmonary veins (that normally return oxygenated blood to the left atrium) anomalously connected to the systemic venous system, resulting in persistence of (one of) the cardinal veins draining the pulmonary venous blood to the systemic venous circulation.[103,105]

Both PVS and TAPVR/PAPVR can occur as an isolated trait or in association with other congenital malformations, both cardiac and non-cardiac (syndromic forms). Although up to 80% of patients with primary PVS show a variety of other CHM such as patent ductus arteriosus and septal defects[106], syndromic PVS has not been described. We are the first to describe familial occurrence of primary PVS in association with lymphatic anomalies (**Chapter 3**).[107] TAPVR/PAPVR can be associated with other CHM, especially laterality disorders[103,108], and is also a common feature of cat eye syndrome, which is caused by a partial tetrasomy of chromosome 22q11. Isolated TAPVR is a rare malformation and occurs in only 1 in 15.000 live births.[109] PAPVR is much more common and has been reported in 1 in 160 autopsies but clinical studies have noted fewer occurences.[105]

1.1.1.4.2 Pathophysiology

Controversy still exists on the relation between the pulmonary vein and the systemic venous tributaries: some believe they are connected via the tributaries of the sinus venosus and some are convinced that the pulmonary veins directly connect to the left atrium.[110-111] The latter theory is supported by a recent study in chick embryos which has shown that the pulmonary veins develop by separation from a greater vascular

plexus, which is located within the splanchnic mesoderm.[112] The pulmonary vein is sleeved by myocardium and mice studies have suggested that Pitx2c and Nkx2-5 play a key role in the formation and identity of the pulmonary myocardium.[113] In situ hybridization analysis performed on murine embryos showed ANKRD1 expression in the developing pulmonary veins, suggesting a possible role for this gene in TAPVR pathogenesis.[58] Bleyl et al have shown that dysregulation of the *pdgfra* gene in mice and chick causes TAPVR with low penetrance supporting a role for PDGF-signaling in pulmonary vein development.[109]

In both the primary and secondary PVS, histopathological findings include a variable degree of eccentric abnormal intimal proliferation of spindle-shaped cells leading to occlusion of the lumen of one or more of the pulmonary veins.[114-116] In some cases pure hypoplasia of pulmonary veins is reported, although no histopathology was performed in these patients.[102,117] The underlying pathological mechanism for PVS is currently unknown.

1.1.1.4.3 Genetics

TAPVR/PAPVR is often part of laterality disorders, and many genetic factors that play a role in determining left-right asymmetry, are involved in these conditions (see section 1.1.1.3.4). TAPVR/PAPVR is also common in cat eye syndrome. Familial occurrence of isolated TAPVR/PAPVR is extremely rare and has been reported in a few families with reduced and variable expressivity.[118] Homozygosity mapping in extended TAPVR kindreds revealed a locus in the intergenic region between the *PDGFRA* and *KIT* gene and by performing mutation analysis in sporadic TAPVR patients variants in the *PDGFRA* gene were identified.[109] In two patients with TAPVR a disruption/mutation of the *ANKRD1* gene was found.[58]

Congenital (or primary) PVS is a very rare condition and has never been reported in families. Furthermore, no animal models of PVS have been reported. This precluded the identification of a disease gene involved in PVS.

In **chapter 3** we describe the clinical and genetic studies in a consanguineous Turkish family with four affected siblings with primary PVS in association with prenatal lymphatic abnormalities.[107] Linkage analysis in this family revealed a large homozygous region on chromosome 2q with a significant multipoint logarithms (base 10) of odds (LOD) score of 3.6. This finding might eventually contribute to the identification of a gene implicated in PVS.

1.1.1.5 Valvular anomalies

1.1.1.5.1 Definition and incidence

Anomalies of the atrioventricular and semilunar heart valves account for 25-30% of all CHM.[119] Hereditary valve disease can be classified anatomically as either left-sided, right-sided, or bilateral.[120] Left-sided valve abnormalities are part of a larger spectrum of cardiac abnormalities referred to as LVOTO. LVOTO comprises a spectrum of CHMs, including bicuspid aortic valve (BAV), aortic valve stenosis (AS), coarctation of the aorta (CoA) and hypoplastic left heart syndrome (HLHS).

1.1.1.5.2 Pathophysiology

Animal model studies have elucidated many genetic pathways that play an important role in cardiac valve malformation. In humans, a variety of signaling pathways and transcription factors have been implicated in endocardial cushion formation, valve remodelling, and extracellular matrix stratification.[121] The most relevant and well-established ligands and signaling pathways include the VEGF, NFATc1, NOTCH, Wnt/β -catenin, BMPs, TGF-β, ErbB, and RAS-MAPK pathways.[122]

1.1.1.5.3 Genetics

Cardiac valve anomalies can be syndromic and part of well-defined clinical syndromes with autosomal dominant inheritance, such as Noonan syndrome, and Alagille syndrome.

Non-syndromic valvular anomalies occur mainly sporadic, reflecting a limited contribution of single gene mutations and it is thought that most of these are due to multifactorial inheritance with a combination of genetic and environmental factors, each with reduced penetrance. Only a limited number of pedigrees with monogenic inheritance of non-syndromic heart valve anomalies, nearly all showing autosomal dominant inheritance, have been described.[120,123-126] This has hampered the identification of genes responsible for human non-syndromic valvular anomalies, and only a limited number of genes with high penetrance mutations have been identified. This includes *GATA4, JAG1, NOTCH1, TAB2, VEGFA, FLNA, CRELD1 and ELN.*

As for right-sided valve anomalies, pulmonary valve anomalies usually in combination with atrial septal defects can be due to *GATA4* mutations.[6] *JAG1* mutations, which are usually involved in Alagille syndrome, are present in a small percentage of apparently non-syndromic right-sided valve disease.[11]

As for left-sided valve abnormalities, *NOTCH1* mutations have been identified in both sporadic and familial LVOTO.[10,54-57] Mutations in the *TAB2* gene have been found in 0.4% of patients with CHM, and are mainly associated with left-sided

valvular defects.[59] Mutations in the *VEGFA* gene are identified in 1.6% of LVOTO patients.[60]

A few genes with combined left- and right-sided heart valve anomalies have been identified. Although in patients with a *NOTCH1* mutation often the aortic and/or mitral valve are involved, also right-sided heart malformations have been described, including DORV, pulmonary atresia and TOF (see[10] and personal observation). Mutations in the *FLNA* gene encoding Filamin A are associated with bilateral (myxomatous) valve disease.[127] *CRELD1* mutations are involved in atrioventricular septal defects (also known as endocardial cushion defects).[64] Mutations in the *ELN* gene encoding elastin have been described in patients with non-syndromic autosomal dominant supravalvular aortic stenosis (SVAS) and/or (peripheral) pulmonary stenosis (PS).[128-129]

In **chapter 4** we describe the clinical and genetic studies in two large pedigrees with autosomal dominant inheritance of isolated CHM. The anomalies in the reported families show mainly LVOTO abnormalities in combination with thoracic aortic aneurysms, right-sided heart defects and septal defects.[120] These families might be instrumental in identifying genes involved in cardiac valve morphogenesis and malformation.

1.1.2. Aortic aneurysms

1.1.2.1 Incidence

An aneurysm is, by definition, a permanent dilatation of 50% or more compared with the expected normal diameter of the vessel.[130]

Aortic aneurysms are the most common aneurysms present in the vascular tree and can be categorized based on their anatomic position (thoracic aortic aneurysm: TAA versus abdominal aortic aneurysms: AAA) or their clinical phenotype (syndromic versus non-syndromic).

AAA are by far the most common, comprising more than 80% of all aortic aneurysms and at the age of 80 years almost 6% of males have developed AAA.[131] TAA is less common with an annual incidence estimated at 16 per 100.000 in men and 9 per 100.000 in women.[132] The incidence is increasing in the past years, and it is an issue of debate whether this is due to a true increase in incidence, improved diagnostics (e.g. the increased use of computed tomography (CT) scan), or increased life expectancy. The true incidence is likely to be underestimated since aortic aneurysms can be present asymptomatically. This is certainly the case in AAA, but also TAA can remain asymptomatic until a rupture or dissection occurs leading to death either before reaching a hospital or before the diagnosis is made.[132-133] In contrast to AAA, TAA often leads to dissections, hence the term thoracic aortic

aneurysms and dissections (TAAD) is commonly used. Aortic dissection is defined as the separation of aortic media caused by the flow of extraluminal blood within the layers of the aortic wall, which creates an extraluminal channel called the false lumen. Aortic dissections are classified either by the Debakey or the Stanford classification. In this thesis, we use the Stanford classification system because it is used in the majority of the selected publications. A dissection involving the ascending aorta is termed Stanford type A. Dissections that only affect the aorta distal to the left subclavian artery are termed Stanford type B. Stanford type A dissections are the most frequent and occur in over two-thirds of TAAD cases.[134] Although aortic dilatation is a well established risk factor for dissections, most ascending aortic dissections occur when the aortic diameter is less than 5,5 cm.[135] Aortic rupture or dissection is associated with a significant morbidity, mortality, and medical expenditure. The in-hospital mortality rate associated with acute Stanford type A dissection is 25-30%, and for a Stanford type B dissection 13%.[135] There are dramatic differences in treatment outcomes between intact and ruptured aortic aneurysms.[136-137] Therefore, the detection of aortic aneurysms prior to rupture is critical, and motivated the search for early diagnosis and (preventative) treatment.

Most frequently TAADs occur in the aortic root or the ascending aorta in the sixth or seventh decade of life. Hypertension is an important risk factor which is present in over 60% of sporadic TAAD and AAA patients.[131]

There frequently exists co-occurrence of TAA and AAA, and up to 25% of TAA patients will also have an AAA and vice versa.[136,138] Also aneurysms of other arteries coexist with aortic aneurysms: multiple aneurysms occur in 3-13% of patients with TAA and in about 12% of those with AAA.[139]

1.1.2.2 Classification and genetics

1.1.2.2.1 Abdominal aortic aneurysms

AAA is associated with risk factors such as advanced age, male gender, atherosclerosis, cigarette smoking, and hypertension. In addition, there is a limited genetic component.[138] Evidence of genetic predisposition to the development of AAA has been clear, as 12-19% of AAA patients report one or more first-degree relatives with an aneurysm.[140-141] Several genome-wide association studies and family-based DNA linkage studies identified different single nucleotide polymorphisms (SNPs) that were associated with AAA.[142] Functional studies are now needed to establish the mechanisms by which these genes contribute toward AAA pathogenesis.

1.1.2.2.2 Thoracic aortic aneurysms

TAAD has a stronger genetic component than AAA, and often shows a classic mendelian monogenic inheritance pattern.[143] Many families with monogenic inheritance, suggesting full penetrance of 1 (autosomal dominant) or 2 (autosomal recessive) single gene mutations, have been described. Syndromic forms of TAAD (e.g. Marfan syndrome) are nearly always the consequence of mutation(s) in a single gene, but also a considerable fraction of non-syndromic TAAD shows monogenic inheritance.

1.1.2.2.3 Non-syndromic aortic aneurysms

It has been estimated that 19% to 20% of patients with non-syndromic TAAD have a first-degree relative with TAAD.[144-145] Non-syndromic familial TAAD (FTAAD) most often segregates as an autosomal dominant condition with variable expression and reduced penetrance.[146] FTAAD tends to occur at a much younger age and grows at a higher rate, and therefore seems to be more aggressive.[144]

Several loci and genes have been indentified by genetic studies in FTAAD families. These include 8 loci with 6 genes: TAA1 at chromosome 11q23.2-q24[147], TAA2 at 5q13-q14[148], *TGFBR2* gene (TAA3) at 3p24.1[149], *TGFBR1* gene at 9q22.33[150], *MYH11* gene (TAA4) at 16p13[41], *ACTA2* gene (TAA6) at 10q22-q24[151], *MYLK* gene (TAA7) at 3q21.1[152] and *SMAD3* gene at 15q22.33[153]. As some families show FTAAD not linked to any of these loci, it is likely that additional TAA loci exist.[154] The different genes implicated in non-syndromic FTAAD are discussed below and tabulated in Table 3.

ACTA2

Non-syndromic TAAD can be caused by autosomal dominant mutations in the *ACTA2* gene, which is located on chromosome 10q22-q24 and encodes for the vascular smooth muscle cell (SMC)-specific isoform of α-actin. Mutations in *ACTA2* have been identified in 10-16% of patients with non-syndromic familial TAAD, and in 2.5% of young-onset sporadic TAAD patients.[151,155-157]

The most common feature of patients with an *ACTA2* mutation is aortic aneurysms at the level of the aortic root, although the descending aorta can also be involved. Both type A and type B dissections have been reported, occasionally in minimally dilated aortas with a maximal diameter of 40 mm.[151,158] Rarely, aneurysms/dissections in other arteries are reported.[155,158] In addition to aortic disease, also premature onset of coronary artery disease and ischemic stroke at a young age is reported in patients with an *ACTA2* mutation. Ischemic strokes are often classified as Moya Moya disease, which is characterized by occlusion of the upper portion of the

Table 3 Genes involved in non-syndromic FTAAD

***Gene* (OMIM) Chromosome**	**Protein**	**Location protein (pathway)**	**Associated features**	**Contribution to FTAAD**	**Refs**
ACTA2 (102620) 10q22-q24	Smooth muscle α-actin	Cytoplasmic protein (IGF-1, Ang II)	PDA, BAV, livedo reticularis, iris flocculi, multisystemic smooth muscle dysfunction	10-16%	151,155-157
MYH11 (160745) 16p13.1	Myosin heavy chain 11	Cytoplasmic protein (IGF-1, Ang II)	PDA	~1%	41,157,162
MYLK (600922) 3q21	Myosin light chain kinase	Cytoplasmic protein (unknown)	Gastrointestinal complications	rare	152
TGFBR1 (190181) 9q22.33	Transforming growth factor β receptor type 1	Transmembrane protein (TGF-β)	Generalized aneurysm and tortuosity	~1%	150
TGFBR2 (190182) 3p24.1	Transforming growth factor β receptor type 2	Transmembrane protein (TGF-β)	Generalized aneurysm and tortuosity	4%	149
SMAD3 (603109) 15q22.33	SMAD3	Transmembrane protein (TGF-β)	Generalized aneurysm and tortuosity	2%	153
NOTCH1 (190198) 9q34-35	NOTCH1	Transmembrane protein (NOTCH)	BAV, LVOTO	rare	55

BAV, bicuspid aortic valve; LVOTO, left ventricular outflow tract obstruction; PDA, persistent ductus arteriosus

internal carotid arteries as a result of fibrocellular proliferation in the arteries and the formation of an abnormal vascular network in the vicinity of the arterial occlusion.[155] Overall, *ACTA2* mutations are very rare (0,2%) in patients with isolated premature strokes and coronary artery disease.[155] Also patent ductus arteriosus (PDA) and bicuspid aortic valve (BAV) are described in some patients.[151] Although classified under non-syndromic TAAD, patients with *ACTA2* mutations sometimes show additional clinical features such as livido reticularis and iris flocculi, and although these signs are not present in all patients, they may serve as diagnostic clues.[151,156-157] Also hypotonic bladder, malrotation, hypoperistalsis of the gut, pulmonary hypertension and even prune-belly sequence can occur.[159-160]

Up to date at least 29 unique *ACTA2* mutations have been identified.[157] The penetrance of *ACTA2* mutations is not complete. A reduced penetrance of nearly 50% is reported for aortic disease alone, but combining all vascular disease the penetrance rises to 80%.[155]

Histopathology of the aorta of patients with an *ACTA2* mutation shows cystic media degeneration, and focal areas of SMC proliferation with marked disarray

of SMC.[158] In several small arteries, such as the aortic vasa vasorum, coronary arteries and intracardiac arteries, medial thickening due to SMC proliferation has been documented, leading to stenosis and occlusion.[151,155]

The vascular SMC-specific isoform of α-actin is a major protein in the actin filaments of the contractile unit in vascular SMCs. Mutations are predicted to exert a dominant-negative effect resulting in reduced assembly and incomplete and disorganized actin filaments.[151] Immunofluorescence analysis of fibers in cultivated SMCs with a *ACTA2* mutation revealed a reduced amount of ACTA2-containing fibers. Guo *et al* also found that explanted SMCs and myofibroblasts harbouring *ACTA2* mutations proliferate more rapidly than control cells, suggesting that *ACTA2* mutations lead to occlusive disease through increased proliferation of SMCs.[155] Renard *et al* showed increased Transforming Growth Factor-β (TGF-β) signaling in aortic tissue sections of *ACTA2* patients and hypothesized that this could be explained by defective actin filaments assembly with incorrect fibrillin-1 assembly into microfibrils. As in Marfan syndrome (see below) the latter could result in loss of the ability to sequestrate latent TGF-β, leading to increased expression of the downstream members of the TGF-β pathway (including nuclear phosphorylated SMAD2 (pSMAD2) and connective tissue growth factor (CTGF)).[157] *Acta2* null mice exhibit abnormal vascular contractility, tone, and blood flow, supporting the concept that SMC α-actin has a central role in regulating vascular contractility.[161]

MYH11

Non-syndromic TAAD can be due to autosomal dominant mutations in the Myosin Heavy Chain 11 (*MYH11*) gene which is located on chromosome 16p13.1 and encodes for the smooth muscle myosin heavy chain (SM-MHC).[41,162] *MYH11* mutation carriers have a lower aortic compliance and a higher aortic pulse wave velocity (aPWV) as compared to controls indicating increased aortic stiffness.[41] The aneurysms are located in the ascending aorta, but occlusive vascular disease can also be present, resulting in skin signs such as livedo reticularis, coronary artery disease, peripheral vascular disease and strokes. In all families reported with familial TAAD and a *MYH11* mutation, PDA was present in one or more affected family members.[41]

Up to date only five *MYH11* mutations have been reported.[41,157,162] Variable expressivity and penetrance of *MYH11* mutations has been reported, ranging from TAAD in combination with PDA, solely TAAD or PDA, solely occlusive vascular disease, to non-penetrance.

Histopathology of the aortic media and vasa vasorum from patients with a *MYH11* mutation reveals SMC disarray and focal regions of hyperplasia due to proliferation

of the SMCs, areas of SMC loss and typical medial degeneration. Upregulation of insulin-like growth factor-1 (IGF-1) and angiotensin-converting enzyme (ACE) in SMCs from mutant aortas might be involved in the pathogenesis.[162] A recent paper by Renard *et al* also suggested upregulation of TGF-β signaling in aortic tissue of *MYH11* patients.[157]

Smooth muscle myosin is composed of two smooth muscle myosin heavy chains (SM-MHC), two essential light chains (SM-MLC) and two regulatory light chains (SM-RLC). These units then assemble into thick filaments that slide along adjacent α-actin-containing thin filaments to contract SMCs. One SM-MHC dimer has two N-terminal globular heads and one coiled-coil rod assembled from two SM-MHC α-helical C-terminal tails.[163] All five reported *MYH11* mutations have a dominant-negative effect by causing a conformational change of the α-helical coiled coil domain of the SM-MHC and impair its assembly with a homodimeric counterpart, leading to disruption of the SMC contractile function.

Myh11 null mice exhibit several abnormalities including a delay in closure of the ductus arteriosus, giant thin-walled bladder and abnormal intestinal movement.[164]

MYLK

Autosomal dominant mutations in the *MYLK* gene, which is located on chromosome 3q21 and encodes for myosin light chain kinase (MLCK), can cause non-syndromic FTAAD.[152] The phenotype presented by patients with a *MYLK* mutation is characterized by aortic dissections with minimal or no aortic enlargement prior to dissection. There is a wide phenotypic variability ranging from a type B dissection in an 18-years-old patient to a normal phenotype at age 69. Only two mutations have been described until now.[152]

Histologic studies show aortic media degeneration, characterized by increased proteoglycan deposition and mild elastic fiber thinning and fragmentation. Similar to histopathology of the aortic wall of *MYH11* mutation carriers, there is a significant increase in the presence of small arteries in the medial layer of the aorta.

MLCK is highly expressed in SMCs. Arterial contraction occurs as a result of stretch activation of calcium channels, triggering an influx of calcium that binds to calmodulin. The association of the calcium/CaM complex to MLCK activates the kinase, leading to regulatory light chain phosphorylation. Phosphorylation of SM-RLC favours actin–myosin interaction, thereby initiating the contraction of smooth muscle within hollow organs.[165]

The mutations reported thus far have an effect on kinase activity or calmodulin-binding properties of MLCK.[152] Smooth muscle-specific MCLK knockout mice show

reduced regulatory light chain phosphorylation resulting in severe gut dysmotility with dilatation of the digestive tract, abnormal urinary bladder function and hypotension.[166] Pathology of the ascending aorta in these mice showed no overt aneurysms but did reveal some initial pathogenic abnormalities observed in the progression of aortic disease like increased proteoglycan deposition and increased expression of lumican, decorin, COL3A1, and MMP2.[152] In humans some gastrointestinal complications have been reported including diverticulosis, polyps, duodenal ulcers, adenocarcinoma of the colon, and irritable bowel syndrome.[152]

TAA ASSOCIATED WITH BAV

Bicuspid aortic valve (BAV) disease is the most common CHM with an estimated prevalence between 0.5% and 2%.[167] Half of individuals with BAV have associated TAAD, even if the BAV is functioning normally.[167] Recent studies have shown a lower risk for aortic dissections as previously thought.[168] Because of the high prevalence of BAV as compared to Marfan syndrome, dissections due to BAV are equal to or more common than in Marfan syndrome. The predilection site for aortic aneurysms is the ascending aorta at the sinotubular junction, in contrast to localisation at the aortic root in Marfan syndrome and other syndromic TAAD.[169] Also, pulmonary root aneurysms have been described in association with BAV.[169] Aortic elasticity is reduced in non-stenotic BAV patients.[170]

Several studies have revealed an increased prevalence of BAV and/or TAA in first degree relatives of BAV suggesting a genetic predisposition.[169,171] Therefore, regular screening of first degree relatives of patients with BAV is recommended, regardless of the presence or absence of BAV in these relatives.[169]

Histopathology in patients with BAV shows, like in many other syndromic and non-syndromic forms of TAAD, cystic media degeneration.[172-173] Patients with BAV have thinner elastic lamellae of the aortic media and greater distances between elastic lamellae than patients with tricuspid aortic valves.[174]

In 10-11% of patients with a BAV and aortic aneurysm an autosomal dominant mutation in the *NOTCH1* gene located on chromosome 9q34-35 and encoding NOTCH1 has been identified.[54-55] Mutations in the *NOTCH1* gene are also identified in patients with LVOTO, ranging from hypoplastic left heart syndrome to progressive aortic valve calcification and asymptomatic BAV.[10,57] *NOTCH1* mutations are rarely found in patients with isolated aortic aneurysms.[55] No histopathologic examination of aortic tissue in *NOTCH1* mutation carriers has been performed thus far.

The exact pathogenic mechanism of TAA in BAV patients remains to be elucidated. Recent studies have shown increased TGF-β activity, which has also been reported in

syndromic TAAD[175-176], and there is increasing evidence of interaction between the NOTCH pathway and TGF-β pathway.[177-178] BAV is also seen in syndromic TAAD, such as Loeys-Dietz syndrome (see below).

Notch1 knockout mice show malformation of the large blood vessels in the anterior of the embryo, indicating that NOTCH signaling is required for angiogenic vascular remodelling during embryonic development.[179]

As is illustrated by our family described in **chapter 4**, BAV can coexist with other CHM mainly including LVOTO abnormalities but also right-sided heart valve malformations and aortic aneurysms.[120] Since we excluded *NOTCH1* in these families, it seems plausible that other genes play a role in the co-occurrence of BAV with other CHM/aortic aneurysms.

Copy number variations in non-syndromic TAAD

Recent array-comparative genomic hybridization (CGH) studies demonstrated a significantly larger number of heterozygous duplications on chromosome 16p13.1 in a large cohort of sporadic and familial TAAD patients (1%) as compared to controls (0.09%).[180] However, individuals with this duplication can be asymptomatic.[180] Clinical records of these patients revealed that almost all aneurysms evolve to an aortic dissection. Other studies have shown that 16p13.1 duplications are also associated with autism, mental retardation, schizophrenia, ADHD, although these features were not reported in the TAAD cases with 16p13.1 duplications.[181-183] The duplicated region of chromosome 16p13.1 contains an unusually high number of segmental repeats which make it prone to deletions and duplications. The duplication encompasses the *MYH11* gene, involved in non-syndromic TAAD. Increased *MYH11* expression was found in aortic tissues from TAAD patients with 16p13.1 duplications compared with control aortas.[180] Histopathologic assessment of the ascending aorta revealed abnormalities similar to *MYH11* patients.

Occasionally, mutations in the syndromic TAAD genes, like *TGFBR1/2* and *SMAD3*, are also identified in non-syndromic FTAAD patients (Table 3).

1.1.2.4.1 Syndromic aortic aneurysms

Syndromic aortic aneurysms are associated with additional (non-vascular) features, and most often these features are part of a systemic connective tissue disorder. Most syndromic aortic aneurysms are TAAs but also AAAs occurs, separately or in combination with TAA. The identification of syndromic TAAD genes has led to an increased knowledge of the molecular mechanism involved. Furthermore, it made the use of DNA testing possible in the identification of individuals and family members at risk

for developing an aortic aneurysm, allowing for correct risk assessment, screening advices and opportunities for early preventative surgery.

The most common syndromic aortic aneurysms are discussed below and tabulated in Table 4.

Marfan syndrome

Marfan syndrome (MFS) is the most common syndromic form of aortic aneurysms, affecting one in 5,000 to one in 10,000 individuals.[184]

The clinical diagnosis of MFS is based on diagnostic criteria (the revised Ghent nosology).[185-186] MFS is characterized by cardiovascular, ocular, and skeletal features. The ocular anomalies include ectopia lentis and high myopia. The most important skeletal abnormalities are pectus deformities, dolichostenomelia, scoliosis, and hindfoot deformities. Cardiovascular abnormalities mainly include aortic root dilatation or dissection, but mitral valve prolapse and pulmonary artery dilatation are also frequently observed. Aortic root dilatation, mainly occurring at the level of the sinus of valsalva, usually develops early in MFS and is present in 35% of individuals by the age of 5 years and in 68–80% of individuals by the age of 19 years.[187] In addition, 20% of patients with MFS develop aortic enlargement or dissection at other segments of the aorta, including the proximal descending and abdominal aorta, and possibly these aneurysms are more frequently seen in time as a consequence of prolonged survival due to improved therapeutic management of disease.[188-189] Therefore, intermittent imaging of the complete aorta is indicated in adult patients.[188] It is known that aortic distensibility and stiffness are abnormal in MFS patients and predict aortic dilatation.[190]

Histopathology of the aortic wall in MFS reveales cystic mucoid degeneration, characterised by pools of glycosaminoglycans and focal interlamellar degeneration, local loss of SMCs and degradation of extracellular proteins.[191]

In 1991, Dietz *et al* identified the first heterozygous autosomal dominant mutations in the *FBN1* gene, located on chromosome 15q21.1 encoding fibrillin 1. Since then more than 1000 *FBN1* mutations have been described.[192-193]

FBN1 mutations in exons 24–32 tend to predict a more severe phenotype with neonatal Marfan, representing the most severe end of the spectrum. The majority of mutations in *FBN1* are missense mutations (60%) in the epidermal growth factor (eGF)-like domains of the protein and affecting cysteine residues or amino acids implicated in calcium binding. Premature truncation codon (39%) mutations, seem to be associated with severe skeletal and skin manifestations of disease and lower risk of ocular manifestation.[193] *De novo FBN1* mutations occur in 25% of MFS patients and are more frequently seen in the severe MFS cases.[194]

Table 4 Syndromic forms of aortic aneurysms

Syndrome (inheritance)	Gene (OMIM) Chromosome	Protein	Location protein	Associated features	Prevalence	Refs
TGF-β pathway						
Marfan syndrome (AD)	*FBN1 (*134797) 15q21.1	Fibrillin-1	ECM protein	Mitral valve prolapse, facial features, ectopia lentis*, myopia, striae, increased arm/height ratio, arachnodactyly, pectus, hindfoot deformity, protrusion acetabuli, reduced elbow extension, scoliosis	1:5.000-10.000	186
Loeys-Dietz syndrome (AD)	*TGFBR1* (190181) 9q22.33 *TGFBR2* (190182) 3p24.1	Transforming growth factor-β receptor type I Transforming growth factor-β receptor type II	Transmembrane protein	Generalized arterial tortuosity and aneurysms, hypertelorism, bifid uvula/cleft palate*, craniosynostosis, translucent skin, easy bruising, arachnodactyly, pectus, scoliosis, talipes equinovarus, joint laxity	unknown	215-216
Aneurysms-Osteoarthritis syndrome (AD)	*SMAD3* (603109) 15q22.33	SMAD3	Cytoplasmic protein	Generalized arterial tortuosity and aneurysms, CHM, atrial fibrillation, ventricular hypertrophy, hypertelorism, abnormal uvula, varices, velvety skin, striae. early-onset osteoarthritis*, arachnodactyly, pectus, pes planus, scoliosis	unknown	274-275
Arterial tortuosity syndrome (AR)	*SLC2A10* (606145) 20q13.1	Glucose transporter type 10	Transmembrane protein	Arterial tortuosity* and aneurysms, elongated face, downslanting palpebral fissures, beaked nose, micrognathia, soft & hyperextensible skin, arachnodactyly, pectus, joint laxity, contractures	unknown	235
Hereditary hemorrhagic telangiectasia (AD)	*ENG* (131195) 9q34.1 *ACVRL1* (601284) 12q11-q14 *SMAD4*	Endoglin Activin A receptor type II-like 1 SMAD4	Transmembrane protein Transmembrane protein Cytoplasmic protein	Arteriovenous malformations (AVMs) mainly in the liver, lung or brain. Small AVMs (telangiectases) on lips, tongue, face, fingers, and the nasal, buccal, and gastrointestinal mucosa.	1:10.000	276-278
Autosomal recessive cutis laxa (AR)	*EFEMP2* (604633) 11q13	Fibulin-4	ECM protein	Hypertelorism, downslanting palpebral fissures, emphysema, diaphragmatic and inguinal hernia, cutis laxa*, bone fragility	extremely rare	243

Syndrome (inheritance)	Gene (OMIM) Chromosome	Protein	Location protein	Associated features	Prevalence	Refs
Collagen metabolism						
Vascular type EDS (AD)	*COL3A1* (120180) 2q31	α1 Type III procollagen	ECM protein	Arterial dissection not preceded by aneurysm*, sunken or bulging eyes, prominent cheekbones, thin/pinched nose, thin lips, digestive and/or uterine rupture, thin and translucent skin	1:75.000	279
Kyphoscoliotic type EDS (AR)	*PLOD1* (153454) 1p36.22	Type 1 lysyl hydroxylase	ECM protein	Scleral fragility, rupture of the ocular globe* , hypotonia*, generalized joint laxity, scoliosis	unknown	231
Osteogenesis imperfecta (AD)	*COL1A1* (120150) 17q21.31 *COL1A2* (120160) 7q22.1	α1 Type I procollagen α2 Type I procollagen	ECM protein	Blue sclerae, hearing loss, osteopenia, susceptibility to bone fractures*, dentogenesis imperfecta, short stature	1:15.000	258
COL4A1-related disorders (AD)	*COL4A1* (120130) 13q34	α1 Type IV procollagen	ECM protein	Cerebral small vessel disease*, cerebral aneurysms, porencephaly, retinal arterial tortuosity, Axenfeld-Rieger anomaly, cataract, cardiac arrhythmia, renal cysts, muscle cramps, Raynaud phenomenon	unknown	261-262
X-linked Alport syndrome (XL)	*COL4A5* (303630) Xq22.3	α5 Type IV procollagen	ECM protein	Progressive sensorineural hearing loss (SNHL), ocular abnormalities (anterior lenticonus, maculopathy, corneal endothelial vesicles, and recurrent corneal erosion), renal disease*	1:50.000	280
Lysyl hydroxylase 3 deficiency (AR)	*PLOD3* (603066) 7q22	Type 3 lysyl hydroxylase	ECM protein	Arterial rupture, deafness, bone fragility with contractures*	extremely rare	281
Ras-MEK-ERK pathway						
Noonan syndrome (AD)	*PTPN11* (176876) 12q24.1	Protein-tyrosine phosphatase 2C or SHP2	Cytoplasmic protein	CHM (mainly PS, HCM), facial features* (hypertelorism, downslanting palpebral fissures, low set ears, broad neck), developmental delay, pectus, cryptorchidism, short stature	1:1.000-2.500	72,282
Neurofibromatosis type 1 (AD)	*NF1* (162200) 17q11.2	Neurofibromin-1	Cytoplasmic protein	Café au lait spots*, fibroadenomas, axillary freckling, learning disability	1:4.000	283-284

Syndrome (inheritance)	Gene (OMIM) Chromosome	Protein	Location protein	Associated features	Prevalence	Refs
Other						
Autosomal dominant cutis laxa (AD)	*ELN* (130160) 7q11.2	Elastin	ECM protein	Cutis laxa*, typical facial appearance (long philtrum, large ears, beaked nose)	extremely rare	251
Alagille syndrome (AD)	*JAG1* (601920) 20p12	JAGGED1	Transmembrane protein	CHM (mainly PPS, TOF), broad forehead, deep-set eyes, pointed chin, posterior embryotoxon, intrahepatic bile duct paucity*, renal abnormalities, abnormal vertebral segmentation	1:70.000	73,285
Autosomal dominant polycystic disease (AD)	*PKD1* (601313) 16p13.3-p13.12 *PKD2* (173910) 4q21-q23	Polycystin 1 Polycystin 2	Transmembrane protein	Intra-/ extracranial aneurysms and dissections, bilateral renal cysts*, cysts in other organs (liver, seminal vesicles, pancreas, arachnoid membrane)	1:400-1.000	286
Tuberous sclerosis (AD)	*TSC1* (605284) 9q34 *TSC2* (191092) 16p13.3	Hamartin Tuberin	Cytoplasmic protein	(sub)Cortical tubers*, subependymal nodules, seizures, hypomelanotic macules, facial angiofibromas, ungual fibromas, angiomyolipomas, renal cysts, rhabdomyomas, arrhythmias, lymphangioleiomyomatosis, intellectual disability	1:6.000	287
Turner syndrome	- (partial) monosomy X	-	-	CHM (mainly CoA, BAV), webbed neck, broad thorax, lymphedema, premature ovarian failure*, nonverbal learning disability, short stature	1:2.500	268-269

*discriminating feature

AD: autosomal dominant; AR: autosomal recessive; BAV, bicuspid aortic valve; CHM, congenital heart malformation; CoA, coartation of the aorta; ECM, extracellular matrix; EDS, ehlers-danlos syndrome; HCM, hypertrophic cardiomyopathy; PS, pulmonary valve stenosis; TOF, tetralogy of Fallot; XL: X-linked.

Three hypotheses have been proposed to explain the molecular consequences of a *FBN1* mutation.[195] In the haploinsufficiency model the abnormal fibrillin molecule is not synthesized or is rapidly destroyed. In the dominant-negative model the abnormal fibrillin 1 molecules, polymerising with other (normal) fibrillin 1 molecules, cause an abnormal polymer. In the TGF-β model fibrillin-1 deficiency results in deficient matrix sequestration of TGF-β and subsequent activation of TGF-β and its signaling pathway.

The fibrillin 1 monomer can homopolymerize in the presence of calcium to form microfibrils which are important for formation and homeostasis of both elastic and non-elastic tissues and interact with a large number of ECM components.

Several studies have shown the involvement of the TGF-β pathway in the pathophysiology of Marfan syndrome. TGF-βs are secreted in an inactive form by binding to latent TGF-β-binding protein 1 (LTBP-1) forming a larger complex, named large latent complex (LLC). LTBPs target the TGF-β LLC to the extracellular matrix- an event that controls TGF-β signaling. It is thought that fibrillin 1 deficiency results in failed or improper matrix sequestration of TGF-β and subsequent activation of TGF-β and its signaling pathway.[196] The TGF-β signaling pathway will be discussed in more detail at the end of the introduction.

Fibrillin 1 deficient mice show that many manifestations of MFS, including aortic root aneurysm, are associated with increased TGF-β signaling, and were improved or prevented by TGF-β antagonism.[197-200] Although many studies have shown excessive canonical (Smad-dependent) TGF-β signaling in MFS, recent studies in Marfan mice also suggest a role for non-canonical (Smad-independent) TGF-β signaling such as the extracellular-signal regulated kinases (ERK1/2), the Jun N-terminal kinase–1 (JNK1), phosphatidylinositol-3-OH kinase (PI(3)K)/AKT, the Rho-associated protein kinase (ROCK) and the mitogen-activated protein kinase (MAPK) cascade.[143,201-202] In addition, angiotensin II type 2 receptor (AT2R)-dependent activation of ERK1/2 is shown in the aorta of fibrillin-1-deficient mice.[203] Besides upregulation of TGF-β activity, a potential role of metalloproteinase, the fibrinolytic/coagulation system, is being suggested in the pathogenesis of aortic disease in MFS.[204] Merk *et al* showed that microRNA(miR)-29b might play a role in the aneurysm formation in MFS mice.[205]

Medical treatment of MFS patients with β-adrenoreceptor blockers is common, although its effectiveness is still a matter of debate.[206-208] Habashi *et al* revealed that losartan, an angiotensin II type 1 receptor blocker, prevents aortic aneurysm in a mouse model of Marfan syndrome.[203] In humans, one small study in MFS children treated with losartan showed prevention of progressive aortic root enlargement[209], and clinical trials in adults with MFS are currently being performed.[210] Also doxycycline (a nonspecific inhibitor of matrix metalloproteinases (MMPs), Pravastatin (a statin), and ERK inhibitors seem to have effect in the prevention of aortic root dilation

in MFS mice.[203,211-214] Currently no clinical trials with any of these drugs are being performed.

Loeys-Dietz syndrome

Loeys-Dietz syndrome (LDS) is an autosomal dominant condition caused by mutations in the transforming growth factor β receptor type I (*TGFBR1*) gene located on chromosome 9q22.33 and transforming growth factor β receptor type 2 (*TGFBR2*) gene located on chromosome 3p24.1.[215] LDS is mainly characterized by aneurysms and/or dissections of the aortic root, although 50% of LDS patients have aneurysms/ dissections in other arteries, including cerebral, thoracic and abdominal arteries. In most patients arterial tortuosity of head and neck arteries is present. In addition to the cardiovascular abnormalities, skeletal (pectus, scoliosis, joint laxity, arachnodactyly, talipes equinovarus), craniofacial (hypertelorism, bifid uvula/ cleft palate, craniosynostosis) and cutaneous (translucent skin, easy bruising, dystrophic scars) abnormalities occur.[215] Despite the phenotypic overlap with MFS there are some clear distinctive features in LDS, including more general involvement of the arteries, arterial tortuosity, bifid uvula/cleft palate and craniosynostosis. More importantly, the natural history of patients with LDS tends to be more aggressive than those with MFS. In LDS, aortic dissections often occur at a younger age (mean age of death 26.1 years) or at smaller aortic dimensions (less than 40 mm) compared to MFS, and the incidence of pregnancy-related complications (death and uterine rupture) is particularly high.[216]

The histopathology of the aortic wall in LDS is characterized by a more diffuse medial degeneration, characterized by fragmentation and/or loss of predominantly intralamellar elastic fibers. Increased collagen deposition, and elastic fiber disarray is present. In many other forms of TAA, such as MFS and non-syndromic familial TAAD, medial degeneration is more focally.[173]

Several hundreds of inactivating mutations have been identified in *TGFBR1* or *TGFBR2*, of which circa 75% are located in *TGFBR2* and circa 25% in *TGFBR1*.[217] These are mostly missense mutations located in the intracellular serine threonine kinase domain. *TGFBR1/2* mutations have been identified in typical LDS patients, but also in patients with a clinical spectrum varying from non-syndromic TAAD to MFS.[218-221] Also intrafamilial variation is large, and there exists no clear genotype-phenotype correlation.[184] It has been proposed that patients with *TGFBR1/2* mutations who lack outward discriminating features of LDS should be designated LDS type 2.[186]

Inactivating *TGFBR1/2* mutations in LDS lead to a paradoxically enhanced TGF-β signaling which is demonstrated by the nuclear accumulation of pSMAD2 in VSMCs and increased output of TGF-β-driven gene products such as collagens and connective tissue growth factor (CTGF) in VSMCs.[215-216]

No aortic defects have been reported in mice heterozygous for null mutations in either the *Tgfbr2* or *Tgfbr1* gene.[222] However, in mice with VSMC-specific deletion of *Tgfbr2*, half of the embryos showed heart malformations and all of them dilatation of descending thoracic aorta and elastic fiber disarray in the aortic wall.

LDS patients are typically managed medically with betablockers and exercise restriction to reduce hemodynamic stress.[217] The effect of drugs such as losartan, has only been investigated incidentally and needs to be investigated in the future.[223]

Ehlers-Danlos syndrome

Six subtypes can be differentiated in Ehlers-Danlos syndrome (EDS): the classical, hypermobility, vascular, kyphoscoliotic, arthrochalasia, and dermatosparaxis type.[224] Arterial aneurysms occur most often in the vascular type of Ehlers-Danlos syndrome.

Vascular type Ehlers-Danlos syndrome

Vascular type EDS (EDS IV) represents approximately 5 to 10% of all EDS cases, and is caused by mutations in the *COL3A1* gene, which is located on chromosome 2q31 and encodes collagen 3.[225]

The cardinal features include arterial, digestive and/or uterine fragility or rupture, a thin and translucent skin, extensive bruising, and a characteristic facial appearance. Affected individuals are at risk for rupture of often non-dilated arteries, gastrointestinal perforation or rupture, and uterine rupture during pregnancy.

Mainly medium-sized and small arteries are affected. Arterial rupture may be preceded by aneurysm, arteriovenous fistula or dissection, but may also occur spontaneously. In particular, dissections of the vertebral arteries and the carotids in their extra- and intracranial segments have been reported.[225] In contrast to most syndromic forms of TAAD, where the aortic root is mainly involved, aortic aneurysms in vascular type EDS mainly involve the aortic arch, descending, and abdominal aorta.

Histopathology of the aortic wall reveals significant transmural tears, but otherwise relative nonspecific findings such as minimal medial degeneration with partial disruption of elastic laminae and intervening organized fibrous tissue.[173] Electron microscopy can be of diagnostic value as irregularities in the diameter of collagen fibers and an unidentified fibrinogranular substance within the extracellular matrix is seen.[226]

Vascular type EDS is caused by autosomal dominant mutations in the *COL3A1* gene, which encodes for the α1 type III procollagen which forms homotrimers by linking three α1(III) chains. Type 3 collagen is widely distributed in the skin, blood vessels, and hollow organs and is one of the most abundant proteins in the aortic

media and adventitia. Together with type I collagen it provides tensile strength and rigidity to the aorta.[227]

So far, about 170 mutations have been described in the *COL3A1* gene.[228] A role for the TGF-β pathway in the pathogenesis in vascular type EDS is unclear as this pathway has not yet been studied in the arterial wall tissue of vascular EDS patients.[229]

The surviving *Col3a1* null mice show a phenotype that closely resembles the clinical manifestations of patients with the vascular type EDS, including the rupture of large blood vessels.[230]

Kyphoscoliotic type Ehlers-Danlos syndrome

The autosomal recessive disorder kyphoscoliotic type EDS syndrome (EDS VIA) is caused by mutations in *PLOD1* which is located on chromosome 1p36.22 and encodes for the type 1 lysyl hydroxylase enzyme (LH1). The major clinical characteristics are severe muscle hypotonia at birth, generalized joint laxity, scoliosis at birth, and scleral fragility and rupture of the ocular globe.[231] In addition, these patients are also at risk for arterial rupture both perinatally and in adulthood. Rupture of small, medium and large vessels is reported and is seldom preceded by aneurysms formation.[231-232] Although the vascular complications are by far the most life-threatening complication in this disorder, it seems less frequent than in the vascular type Ehlers-Danlos syndrome.

The kyphoscoliotic type EDS can be diagnosed by abnormally elevated ratio of urinary lysyl pyridinoline to hydroxylysyl pyridinoline crosslinks, a deficiency of LH1 enzyme activity in cultured fibroblasts and/or directly by mutation analysis of *PLOD1*.[231] LH1 is involved in hydroxylation of lysines destined for the triple helical region of collagens. A deficiency of LH1 perturbs the formation of intra- and intermolecular collagen crosslinks with consequent mechanical instability of the affected tissues.[231]

No studies in arterial walls have been performed in humans. However, *Plod1* knock-out mice died at the age of 1-4 months in 15% of cases due to aortic dissection and rupture.[233] Ultrastructural analysis of the aortic wall of these mice showed degenerated SMCs and abnormal collagen fibrils and biochemical analyses demonstrated a deficiency in collagen hydroxylysine content and changes in the collagen cross-linking pattern.

Other types of Ehlers Danlos syndrome

In other types of EDS, mainly the classical type EDS, aneurysms are an extremely rare complication.[234] The Ehlers-Danlos like syndrome with nodular heterotopia will be discussed separately.

Arterial tortuosity syndrome

Arterial tortuosity syndrome (ATS) is a rare autosomal recessive disorder caused by mutations in the *SLC2A10* gene on chromosome 20q13.1 encoding the facilitative glucose transporter GLUT10.[235] ATS is mainly characterized by widespread arterial involvement with elongation, tortuosity, and aneurysms of the large and middle-sized arteries. Other typical features include dysmorphic features and connective tissue manifestations such as a soft, hyperextensible skin, arachnodactyly, pectus deformity, joint laxity, and contractures.[236-237]

Histopathology of affected arterial walls shows a markedly thickened intima due to fibrosis and disruption of the elastic fibers in the media and disorganization of fibronectin extracellular matrix (ECM) and actin cytoskeleton.[173,236,238]

So far, over 17 *SLC2A10* mutations have been reported in 32 families.[239]

Loss of function of GLUT10 results in upregulation of TGF-β signaling in VSMCs.[235] Recent animal studies have shown that GLUT10 is highly expressed in mitochondria of aortic VSMCs, and translocates to mitochondria in response to insulin stimulation.[240] Loss of GLUT10 results in defective transport of dehydroascorbic acid (DHA), the oxidized form of vitamin C, into mitochondria, leading to increased sensitivity of cells to oxidative stress.[240] This inhibits proper extracellular matrix formation, in particular elastogenesis.[240-241] Willaert *et al* suggested that the upregulation of the TGF-β pathway might be explained by dependency of the TGF-β signaling on mitochondrial function.[242] However, Segade *et al* found GLUT10 to be localized to the endoplasmic reticulum (ER), and suggested that GLUT10 is a DHA transporter in the ER.[241] GLUT10 deficiency in ATS might impair the vitamin C-dependent prolyl- and lysyl-hydroxylases. This might result in a reduction in the number of hydroxyproline and hydroxylysine residues in collagens and elastin, leading to an abnormal structure of these essential arterial wall proteins.

Autosomal recessive cutis laxa type Ib

Autosomal recessive cutis laxa (ARCL) type Ib is a rare disorder caused by mutations in the *EFEMP2* gene (also known as *FBLN4)* on chromosome 11q13 encoding the fibulin-4 protein. The main features are cutis laxa and arterial anomalies, including aneurysms, dissections, tortuosity and stenosis.[243] Aneurysms and tortuosity are mainly present in the aorta, but also in the middle sized arteries throughout the body. In addition, craniofacial abnormalities, bone fragility, developmental emphysema, and diaphragmatic and inguinal hernia have been described. Severity ranges from perinatal lethality as a result of cardiopulmonary failure to manifestations limited to the vascular and craniofacial systems. The disorder shows considerable phenotypic overlap with LDS and ATS, and cutis laxa is not always present.[243]

Up till now only six patients with ARCL type Ib have been described.[243-246] The elastic fibers in the tunica media of large arteries of patients with *FBLN4* mutations are markedly decreased in density, fragmented, and shortened.

Fibulin-4 is located in microfibril bundles of the media which anchor elastic fibers to SMCs and mutant fibulin-4 protein leads to impaired elastogenesis. An increased TGF-β signaling is seen in aortic tissue of patients with *FBLN4* mutations, thereby confirming the key role of this signaling pathway in the pathogenesis of arterial aneurysms and tortuosity.[243]

Fbln4 mutant mice models show predominantly aortic aneurysms with upregulation of the TGF-β and ERK1/2 signaling pathway in the aortic wall.[247-248] Mice with a VSMC–specific *Fbln4* deletion and *in vitro* studies revealed that the VSMCs failed to fully differentiate, and show reduced expression of SM-specific contractile genes. Therefore, it has been proposed that aneurysm formation in ARCL type Ib not only results from defective elastic fiber formation but also from disturbed regulation of smooth muscle cell differentiation.[248] In the *Fbln4* mouse model the AT1 receptor blocker losartan prevents aortic media degeneration.[249]

Autosomal dominant cutis laxa

Autosomal dominant cutis laxa (ADCL) can be caused by autosomal dominant mutations in the *ELN* gene, which is located on chromosome 7q11.23 and encodes elastin. This disorder is characterized by generalized loose skin and a typical facial appearance.[250] Although described as a relative benign cutaneous disorder, mild to severe aortic aneurysms leading to aortic rupture early in adulthood can be present.[251-252]

The aortic pathology is, like other forms of thoracic aortic aneurysms, characterized by cystic medial degeneration. Electron microscopy reveals elastic fiber fragmentation and diminished dermal elastin deposition.[252]

At least 17 mutations in ELN have been described up to date.[251]

In most patients frameshift mutations at the 3'-end of *ELN* are found, predicted to result in a mutant tropoelastin (fmTE) protein with an extended carboxy-terminal missense peptide sequence. The mechanism by which fmTE disrupts elastin assembly is thought to result from a combination of both increased aggregation of fmTE and its decreased binding to microfibrils which leads to a poor integration of the elastin and microfibrils in the elastic fibers of patients with ADCL. Increased pSMAD2 staining in fibroblasts indicated enhanced TGF-β signaling, and it has been speculated that due to improper elastic fiber organization, a more compliant ECM results in higher amounts of released TGF-β which clinically manifests in aortic dilatation and pulmonary emphysema.[251]

A transgenic mouse model for ADCL shows emphysematous pulmonary airspace enlargement but no cardiovascular abnormalities.[253]

Type I Collagenopathies

Osteogenesis imperfecta (OI) is caused by mutations in the *COL1A1* and *COL1A2* genes encoding the proα1 and 2 chains of type I collagen.[254] OI is characterized by osteopenie and a susceptibility to bone fractures, and can be classified by clinical and radiological findings in six subtypes.[255] Secondary features can be dentogenesis imperfecta, short stature, blue sclerae and hearing loss at adult age but these are not present in all individuals. In a substantial proportion of OI patients cardiovascular abnormalities are reported, of which aortic regurgitation is most commonly found but also aneurysms and dissections of the thoracic, abdominal and cerebral arteries, are reported.[256-258] Therefore regular cardiovascular screening in patients with *COL1A1/COL1A2* mutations is advised.

Malfait *et al* reported three non-glycine substitutions in *COL1A1* in three patients with arterial aneurysms and dissection of large and middle-sized arteries at a young adult age. In addition, these patients had features of classic EDS and isolated osteopenia.[259] In mice it is shown that the production of type I collagen is important for maintaining aortic strength and integrity.[260]

COL4A1-related disorders

The COL4A1-related disorders cover a spectrum of overlapping phenotypes including autosomal dominant type 1 porencephaly, brain small-vessel disease with hemorrhage, and Hereditary Angiopathy with Nephropathy, Aneurysms, and Muscle cramps (HANAC) syndrome.[261-264] The spectrum of these disorders is characterized by varying degrees of small vessel disease variably combined with porencephaly, cerebral aneurysms, eye defects (including retinal arterial tortuosity, Axenfeld-Rieger anomaly, cataract) and systemic features (including kidney involvement, muscle cramps, Raynaud phenomenon, and cardiac arrhythmia). Cerebral small vessel disease manifests on imaging as diffuse periventricular leukoencephalopathy, lacunar infarcts, microhemorrhage, dilated perivascular spaces, and deep intracerebral hemorrhages. In HANAC patients large arteries can also be affected, causing asymptomatic intracranial aneurysms (ICAs).[263] Single or multiple aneurysms usually affect the intracranial portion of the internal carotid artery.[263,265] In addition, bilateral retinal arteriolar tortuosity can be observed in all patients with HANAC and occasionally in patients with other COL4A1-related disorders.

The *COL4A1* gene encodes for the α1 chain of type IV collagen which together with the α2 chain form $\alpha1\alpha1\alpha2$(IV) heterotrimers, a major component of basement membranes of arteries and veins.

In vascular basement membrane, COL4A1 forms a sheetlike network separating the endothelium from the VSMCs in the media. In HANAC patients ultrastructural examination of the wall of small dermal arteries revealed that VSMCs are dissociated, due to abnormal spreading of the basement membrane.[263] Heterozygous missense mutations in *Col4a1* in mice have been shown to lead to a complex vascular phenotype encompassing defects in maintenance of vascular tone, endothelial cell function and blood pressure regulation.[266]

Turner syndrome

The Turner syndrome is a chromosomal condition caused by a complete or partial monosomy of the X-chromosome. Turner syndrome is fairly common and occurs in 1 out of 2500 woman.[267] It is characterized by short stature, premature ovarian failure, usually normal intelligence with nonverbal learning disability, physical features like webbed neck, shield thorax, and lymphedema. A variety of cardiovascular anomalies are detected in pediatric and young adult patients including, aneurysms (30%), elongation of the transverse arch (31%), BAV (39%), aortic coarctation (16%) and PAPVR (16%).[268] Aneurysms occur most commonly at the level of the sinus of valsalva. Aortic dissection is common in Turner syndrome, and the risk is increased by more than 100-fold.[269] The dissections occur at a median age of 35 years (range 18-61 years) and is more prevalent in (assisted) pregnancy.[270]

Histopathology of the affected aortic wall of Turner patients shows cystic media degeneration.[271-272] The pathogenic mechanism underlying the vascular abnormalities in Turner syndrome is currently unknown although arterial hypertension may also play a role. However, cases of aortic aneurysm and dissection have been described in Turner patients without any other risk factors.[273]

Other disorders with aortic aneurysms

There are many syndromes with aortic aneurysms, including Alport syndrome (*COL4A5* mutations), Alagille syndrome (*JAG1* mutations), autosomal dominant polycystic disease (*PKD1* or *PKD2* mutations), hereditary hemorrhagic telangiectasia (*ENG*, *ACVRL1-ALK1 or SMAD4* mutations), Tuberous sclerosis (*TSC1* or *TSC2* mutations), Neurofibromatosis type 1 (*NF1* mutations), and Noonan syndrome (mutations in genes of the RAS-MAPK pathway). These are tabulated in Table 4.

1.1.2.2.5 TGF-β signaling pathway

The TGF-β signaling pathway is involved in a variety of cellular processes including cell proliferation, differentiation, adhesion, migration and apoptosis. Alterations of specific components of the TGF-β signaling pathway may contribute to a broad

range of inherited and non-inherited conditions such as cancer, and cardiovascular pathology, fibrosis and congenital diseases.[288]

The general mechanism of the TGF-β pathway is relatively simple and well conserved in evolution and will be summarized below.

Mechanism TGF-β pathway

Perturbation of TGF-β signaling has been implicated in the pathogenesis of many disease status including aortic aneurysms. This complex pathway has been studied extensively in the past years in context of this disease, not the least because of its utility as a therapeutic target.[289-290]

TGF-β signaling involves different processes including ligand binding of TGF-β, receptor recruitment and phosphorylation, SMAD phosphorylation, coSMAD binding, and transcription of multiple TGF-β-driven genes.

1. Ligand binding

TGF-β is a ligand of the TGF-β superfamily and is present in three TGF-β isoforms in human, namely TGF-β1, TGF-β2, TGF-β3. TGF-βs are secreted in an inactive form; they are synthesized as propeptide precursors containing a prodomain (also named latency associated peptide, LAP) and the mature domain, a complex that is called small latent complex (SLC). By disulfide-bonding of latent TGF-β-binding protein 1 (LTBP-1) to the LAP of the SLC, a larger complex, named large latent complex (LLC) is formed. LTBP1 possesses domains that interact with matrix molecules such as microfibrils, thereby targeting the LLC to the extracellular matrix. The associations between TGF-β1 and LTBP-1 and between LTBP-1 and matrix proteins determine the sequestration/release of TGF-β1 within the extracellular matrix, which is an important regulating mechanism of TGF-β signaling.

2. Receptor recruitment and phosphorylation

The TGF-β receptors have a cysteine rich extracellular domain, a transmembrane domain and a cytoplasmic serine/threonine rich domain. Seven type I receptors or ALKs (activin-like receptor kinases), among which TGFBR1, and five different type II receptors, including TGFBR2, have been described. The TGF-β ligand binds to the constitutively active TGF-β type II receptor dimer, which recruits a TGF-β type I receptor dimer forming a complex that facilitates the phosphorylation and subsequent activation of the type I receptor.[290]

3. SMAD phosphorylation and coSMAD binding

Smads are a well conserved family of transcriptional factors and members of the Smad family can be classified into three groups: (i) receptor-associated Smads (R-

Smads, namely SMAD1, SMAD2, SMAD3, SMAD5, and SMAD9); (ii) co-operating Smads (Co-Smads, namely SMAD4) and (iii) inhibitory Smads (I-Smads, namely SMAD6, and SMAD7).

The type I receptor phosphorylates the serine residue of the R-SMAD (e.g. SMAD3). Phosphorylation induces a conformational change in the MH2 domain of the R-SMAD and its subsequent dissociation from the receptor complex. The phosphorylated R-SMADs dimerize and this complex has a high affinity for a coSMAD (e.g. SMAD4) and forms a heterotrimeric complex.

4. Transcription

The phosphorylated R-SMAD/co-SMAD complex enters the nucleus of VSMCs where it binds transcription promoters/cofactors and regulates the expression of multiple TGF-β-driven gene targets such as matrix proteins (collagen, fibronectin), matrix metalloproteinases (more specifically MMP2 and MMP9), connective tissue growth factor (CTGF), and members of the fibrinolytic system (plasminogen activator inhibitor-1, PAI-1).

The role of the TGF-β pathway and other signaling pathways in aneurysm formation

The pathogenic mechanisms leading to the remodelling process in TAA are induced by different signaling pathways including the TGF-β pathway and Angiotensin-II (AngII) pathway.

The first insight in de role of the TGF-β pathway in aneurysm formation came from studies in fibrillin-1 deficient mouse model which showed evidence for enhanced TGF-β signaling in most affected tissues.[197] The role of the TGF-β pathway in aneurysm formation was confirmed by the detection of mutations in different members of the TGF-β pathway, in particular *TGFBR1/2* and *SMAD3* in patients with syndromic forms of TAAD [215,274], and paradoxically, studies in the aortic walls of these patients show enhanced TGF-β signaling.

Possible mechanisms that could explain this TGF-β paradox include altered receptor trafficking, impaired autoregulation of TGF-β signaling, alternative signaling cascades or non-autonomous cellular events.[291]

Further evidence for a roll of increased TGF-β the pathogenesis of aortic aneurysms was presented by the observation of enhanced TGF-β signaling in the vessel wall of patients with other syndromic forms of TAAD, including ATS and ARCL, and non-syndromic forms of TAAD (BAV, and *MYH11* and *ACTA2* related TAAD) and degenerative aortic aneurysms.[196]

Several studies both in humans and animal models report on attenuation of TGF-β signaling in aneurysm formation.[218,292,293,242] These studies illustrate the complexity of the roll of TGF-β signaling in aortic disease and were recently discussed by Dietz *et al.*[289]

Downstream of TGF-β

The pathogenic mechanism downstream of TGF-β is not fully elucidated. TGF-β signaling is extensively studied by measuring components of the canonical pathway (SMAD dependent), such as pSMAD2 and its downstream targets such as CTGF. However, TGF-β can also induce non-canonical (Smad-independent) pathways. Recent studies have reported the involvement of non-canonical Smad signaling (e.g. ERK1/2) in the progression of vascular disease in syndromic TAA such as MFS and ARCL.[143] (Fig. 1) These studies are described more extensively in the sections referring to these conditions. However, also in AAA and other syndromic and non-syndromic TAA patients the role of non-canonical Smad signaling is becoming more evident.[143]

Another pathway known to be involved in aneurysm formation is the Ang II signaling pathway. AngII mediates its effects via AngII type 1 receptors (AT1R) and type 2 receptors (AT2R). Mice with Ang II-induced aneurysms are widely used as a model to study the relationship between Ang II and AA progression. There is extensive crosstalk between the TGF-β pathway and the Ang II pathway (Fig. 1). Ang II signaling through AT1R has the capacity to enhance TGF-β signaling by inducing the expression of ligands, receptors and activators.[294] Losartan, an AT1R blocker can reduce TGF-β signaling in the aortic media and has beneficial effects on aortic wall thickness, elastic fiber fragmentation and aortic root growth in MFS and Fbln4 mice.[199,249] The exact mechanism by which losartan antagonizes TGF-β signaling remains to be elucidated. In vascular smooth muscle cells Ang II can activate the Smad pathway, rather than the entire TGF-β signaling pathway, via a TGF-β-independent manner possibly by ERK1/2 and/or MAPK activation.[295-296]

Abnormal VSMCs development and phenotypic switching of VSMCs may be involved in the pathogenesis of AA formation. Each cell type within the aortic wall is capable of responding differently to TGF-β. In addition, the diverse embryonic origins of the VSMCs along the aorta may cause lineage-specific differences in TGF-β signaling capacity.[143]

Despite the great progress that has been made in the understanding of the mechanisms leading to aneurysm formation many uncertainties and conflicting data remain. Great effort will be done to unravel these issues because of the high probability that

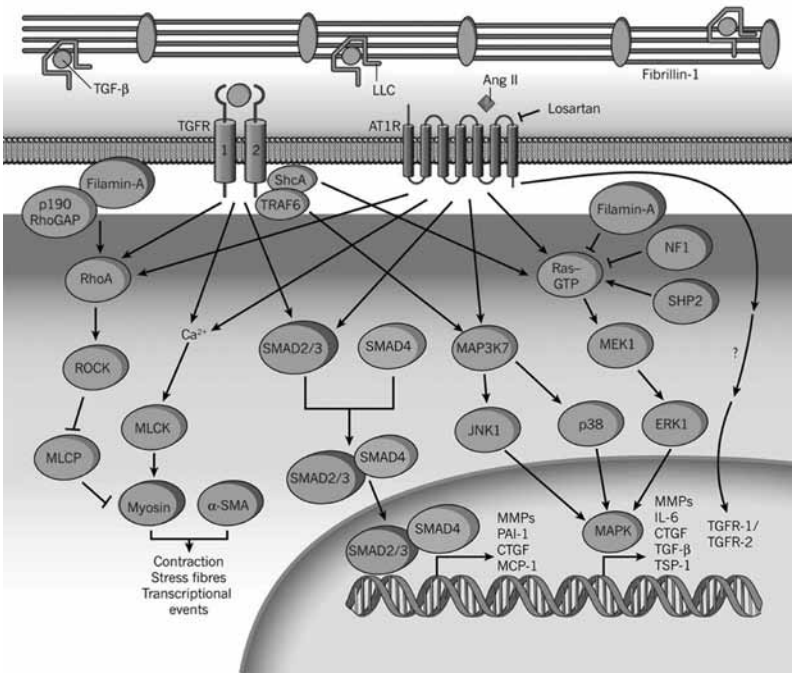

Figure 1 The TGF-β and Ang II signaling pathways. Signaling cascades of TGF-β and Ang II have been implicated in the pathogenesis of aneurysm. Fibrillin-1, the major component of extracellular microfibrils, binds and sequesters the large latent complex (LLC) of TGF-β. After TGF-β activation (release), ligand binds to the TGF-β receptor (TGFR) and activates both canonical (grey) and non-canonical (blue) signaling cascades. The extensive crosstalk between the TGF-β and Ang II type 1 receptor (AT1R) signaling pathways is indicated. Key terminal events in the pathogenesis of aneurysm may include MMP-mediated proteolysis, CTGF-mediated epithelial-to-mesenchymal transition and tissue remodelling, or IL-6- and MCP-1-mediated inflammation. Proteins indicated in purple have been directly implicated in human hereditary aneurysmal disease (see Table 3 and 4). MAP3K7, mitogen-activated protein kinase kinase kinase 7 (also known as TAK1); MEK1, MAP kinase kinase 1; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; p190 RhoGAP, Rho GTPase-activating protein 5; SHP2, protein tyrosine phosphatase 2C; α-SMA, α-smooth muscle actin.
From ref [143] with permission from Prof. H. Dietz

these insights will lead to treatment strategies for aneurysms and perhaps other related disorders.

1.1.2.3. Aneurysms-osteoarthritis syndrome

1.1.2.3.1 Definition and incidence

Aneurysms-osteoarthritis syndrome (AOS; MIM 613795) is caused by heterozygous mutations in the *SMAD3* gene located on chromosome 15q22.33 which encodes for a receptor-activated SMAD protein that plays a role in signal transmission in the TGF-β pathway. It is a newly identified autosomal dominant syndromic form of TAAD

Table 5 cardiovascular phenotype in most common syndromic and non-syndromic TAAD

Condition (gene or chromosomal aberration)	Aneurysms	dissections	tortuosity	CHM (type)	Main location	histopathology	Comments on arterial disease
Syndromic							
Marfan syndrome (*FBN1*)	+++	++	+	+/-	Sinus of Valsalva	CMD/ EFF	Surgical repair when the aorta reaches 5.0cm
Loeys-Dietz syndrome (*TGFBR1/2*))	+++	+++	++	++ (ASD/ PDA)	Sinus of Valsalva and medium sized arteries	DMD/EFF	Aortic dissections documented at aortic diameters <5.0 cm. Surgical repair at aortic diameter ≥ 4.2 cm (by TEE) or 4.4 to ≥ 4.6 cm
Aneurysms-Osteoarthritis syndrome (*SMAD3*)	+++	+++	++	+ (ASD/ PDA/PS)	Sinus of Valsalva and medium sized arteries	CMD/EFF	Aortic dissections documented at aortic diameters <5.0 cm. Surgical repair at aortic diameter ≥ 4.2 cm (by TEE) or 4.4 to ≥ 4.6 cm
Arterial tortuosity syndrome (*SLC2A10*)	+/-	+/-	+++	+ (PS)	Aortic root and medium-sized arteries	EFF	Focal or widespread stenosis in aorta or pulmonary arteries
Autosomal recessive cutis laxa (*EFEMP2*)	++	+/-	+	-	Ascending aorta and medium-sized arteries	DMD/ EFF	-
Vascular type EDS (*COL3A1*)	+/-	+++	-	-	Small and medium sized arteries; aortic arch, descending/abdominal aorta	Minimal CMD	Often dissections not preceded by arterial aneurysms. Surgical repair is complicated by friable tissue. Noninvasive imaging recommended
Kyphoscoliotic type EDS (*PLOD1*)	+	+++	-	-	Small, medium and large sized vessels	u	Often dissections not preceded by arterial aneurysms
Autosomal dominant cutis laxa (*ELN*)	+	+	-	+ (BAV)	Sinus of Valsalva	CMD/EFF	-
Osteogenesis imperfecta (*COL1A1/2*)	+	+/-	-	-	Aortic root	u	Surgical repair is complicated by fragile tissue and propensity for bleeding

Condition (gene or chromosomal aberration)	Aneurysms	dissections	tortuosity	CHM (type)	Main location	histopathology	Comments on arterial disease
COL4A1-related disorders (*COL4A1*)	+	-	+		Intracranial small and medium-sized arteries	Dissociated VSMC	Rupture/dissection rarely described
Turner syndrome ((partial) monosomy X)	+	+	-	+++ (LVOTO)	Sinotubular junction	CMD	Dissection risk is increased in patients with BAV, CoA, hypertension, or pregnancy
Non-syndromic							
TAA6 (*ACTA2*)	+++	+++	-	+ (PDA/ BAV)	Aortic root	CMD, focal VSMC proliferation	Aortic dissections documented at aortic diameters <5.0 cm. Occlusive vascular disease leading to CAD and strokes
TAA4 (*MYH11*)	+++	+	-	++ (PDA)	Ascending aorta	CMD, focal VSMC proliferation	Occlusive vascular disease leading to CAD and strokes
TAA7 (*MYLK*)	+/-	+++	-	-	Ascending aorta	CMD, increase in medial small arteries	Aortic dissection with minimal or no aortic enlargement
TAAD associated with BAV (multiple, among which *NOTCH1*)	++	++	-	+++ (BAV)	Sinotubular junction	CMD	Low risk of aortic dissection

u: unknown

ASD, atrial septal defect; BAV, bicuspid aortic valve; CAD, coronary artery disease; CMD, cystic media degeneration; CHM, congenital heart malformation; DMD, diffuse media degeneration; EDS, ehlers-danlos syndrome; EFF, elastic fiber fragmentation, LVOTO, left ventricular outflow tract obstruction; PDA, persistent ductus arteriosus; PS, pulmonary valve stenosis; TEE, transesophageal echocardiography; VSMC, vascular smooth muscle cell

characterized by the presence of arterial aneurysms and tortuosity, mild craniofacial, skeletal and cutaneous anomalies and early-onset osteoarthritis.[274] Although clinical overlap with LDS exists, early-onset joint abnormalities including osteoarthritis and osteochondritis dissecans seem to be discriminating clinical features and often the patient's presenting symptom.[275] As in MFS and LDS aortic aneurysms and dissections at the aortic root are the main cardiovascular abnormalities in AOS. In addition, aneurysms of other large and medium-sized arteries throughout the body and the brain are often present, indicating AOS is a generalized vascular disease. Tortuosity of the arterial tree is present in a majority of patients and mainly involves the carotid, vertebral, and/or subclavian arteries. The aneurysms in AOS tend to rupture or dissect at smaller sizes as in MFS patients or other non-syndromic TAAD patients. Cardiac abnormalities such as mitral valve prolapse, atrial fibrillation, left ventricular hypertrophy and congenital heart defects are found in some patients. Most of the patients developed joint abnormalities, including OCD, meniscal lesions, intervertebral disc degeneration, and osteoarthritis. These abnormalities were already present at a young age. Great intra- and interfamilial variability is reported.[275]

The incidence of AOS seems to be rare since the frequency of *SMAD3* mutations found in our two cohorts of (not necessarily familial) TAAD patients was 1-2%.[274-275] This is comparable with the frequency of 2% mutations in a cohort of non-syndromic familial TAAD patients reported by Regalado *et al.*[153]

1.1.2.3.3 Pathophysiology

Histopathology of aortic tissue of AOS patients shows disorganization of the tunica media with fragmentation and loss of elastic fibers, mucoid medial degeneration and accumulation of collagen in the media.[274] Heterozygous mutations lead to increased expression of several key players of the TGF-β pathway, including pSMAD2, SMAD3, upstream ligands such as TGF-β1, and downstream targets (CTGF and collagen) in the thoracic aortic wall of the AOS cases which is similar to patients with other syndromic and non-syndromic aneurysms.[274] This clearly indicates the existence of common (TGF-β–related) pathogenic mechanisms leading to arterial wall disease. Studies in *Smad3* knock-out mice display a phenotype resembling human osteoarthritis including abnormal calcification of the synovial joints with osteophytes (knee, vertebral bones, sternum), loss of articular cartilage, intervertebral disc degeneration, and hypertrophic differentiation of articular chondrocytes.[297-298] These studies confirm that Smad3-mediated signals are essential for cartilage maintenance. The vascular phenotype in *Smad3* knock-out and heterozygous mice has yet to be explored, but until now no vascular abnormalities have been described.

1.1.2.3.4 Genetics

Up to date fourteen different *SMAD3* mutations have been identified in AOS patients and one missense variant in *SMAD3* is identified in a patient with osteoarthritis who was not evaluated for other AOS features.[153,275,299] The most likely effect of these mutations is loss of function, with TGF-β signals not being propagated via SMAD3.

The TGF-β pathway seems the primary target for the development of new treatment.

However, our limited understanding of the physiological functioning of this complex pathway warrant further studies before clinical trials with TGF-β antagonists such as Losartan can commence.

In **chapter 5**, **6** and **7** we identified eight pathogenic mutations in the *SMAD3* gene and characterized the clinical spectrum of AOS. These studies revealed that AOS should be regarded as an aggressive aneurysm syndrome, demanding early recognition, surveillance of the entire arterial tree, and prophylactic, early surgical intervention. Molecular diagnosis will allow early and reliable identification of patients at risk for major cardiovascular complications and makes personalized counselling, follow-up and treatment in each patient possible. Our observations open further avenues towards a better understanding and possible treatment of both arterial wall anomalies and osteoarthritis.

Discrimination of both syndromic and non-syndromic forms of TAAD can at times be challenging since many of the associated syndromic features are frequently seen in the general population, have an age-dependent expression and reveal substantial phenotypic variability. Table 5 summarizes the cardiovascular characteristics and histopathology of both syndromic and non-syndromic forms of TAAD.

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1.2 AIM AND OUTLINE OF THE THESIS

This thesis presents the clinical and molecular studies in patients and families with various cardiovascular malformations (CVM), including congenital heart malformations (CHM) and aortic aneurysms. Most patients and their families were encountered at the cardiogenetic clinic of the Paediatric and Adult Cardiology Department of the Erasmus Medical Center in Rotterdam.

The aim of this thesis was to study families with different monogenic forms of CVM in order to delineate the phenotypes, to identify the disease genes and unravel the underlying molecular pathways.

In this thesis, families with rare forms of CHM are described, including laterality defects, abnormal pulmonary vein development, and abnormal valvulogenesis (chapter 2-4). The second part of the thesis (chapter 5-7) describes the identification and characterization of a new syndromic form of aortic aneurysms, specifically Aneurysms-osteoarthritis syndrome (AOS).

Chapter 1 reviews the definitions and incidences, pathophysiology, and genetics of congenital heart malformations and aortic aneurysms.

In **Chapter 2** the clinical and molecular studies in patients with laterality defects is reported. A genome-wide linkage analysis and positional gene sequencing identified variants in *nephronophthisis-4* (*NPHP4*) in patients with cardiac laterality defects and heterotaxy. We used zebrafish as a model vertebrate to characterize the role of *nphp4* in establishing L-R asymmetry.

Chapter 3 delineates the clinical features of a large consanguineous family with primary PVS and reports identification of the first locus for primary PVS found by genome-wide linkage analysis.

In **Chapter 4** two families with autosomal dominant inheritance of both left- and right-sided cardiac valve anomalies are described, and possible pathogenetic pathways and disease genes involved in these cardiac valve malformations are presented.

Chapter 5 reports on the discovery of a new syndromic form of aneurysms, named Aneurysms-osteoarthritis syndrome. By genome-wide linkage analysis the genetic locus is mapped to chromosome 15q22.2-24.2 and mutations in the *SMAD3* gene, a member of the TGF-β pathway, are identified.

Chapter 6 delineates the phenotypic spectrum of *SMAD3*-related Aneurysms-osteoarthritis syndrome in eight families with a total of 45 affected individuals.

In **Chapter 7** the cardiovascular consequences of Aneurysms-osteoarthritis syndrome are described and the first clinical recommendations are provided.

Chapter 8 comprises the general discussion of this thesis, encompassing the significance and implications of our studies and outlining future perspectives

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NPHP4 GENETIC VARIANTS ARE ASSOCIATED WITH PLEIOTROPIC HEART MALFORMATION

CHAPTER 2

ABSTRACT

Rationale: Congenital heart malformations are a major cause of morbidity and mortality especially in young children. Failure to establish normal left-right (L-R) asymmetry often results in cardiovascular malformations and other laterality defects of visceral organs.

Objective: To identify genetic mutations causing cardiac laterality defects.

Methods and Results: We performed a genome-wide linkage analysis in patients with cardiac laterality defects from a consanguineous family. The patients had combinations of defects that included dextrocardia, transposition of great arteries, double outlet right ventricle, atrio-ventricular septal defects and caval vein abnormalities. Sequencing of positional candidate genes identified mutations in *NPHP4*. We performed mutation analysis of *NPHP4* in 146 unrelated patients with similar cardiac laterality defects. Forty-one percent of these patients also had laterality defects of the abdominal organs. We identified eight additional missense variants that were absent or very rare in controls. To study the role of *nphp4* in establishing L-R asymmetry, we used antisense morpholinos to knockdown *nphp4* expression in zebrafish. Depletion of *nphp4* disrupted L-R patterning as well as cardiac and gut laterality. Cardiac laterality defects were partially rescued by human *NPHP4* mRNA, whereas mutant *NPHP4* containing genetic variants found in patients failed to rescue. We show that *nphp4* is involved in the formation of motile cilia in Kupffer's vesicle (KV), which generate asymmetric fluid flow necessary for normal L-R asymmetry.

Conclusions: *NPHP4* mutations are associated with cardiac laterality defects and heterotaxy. In zebrafish, *nphp4* is essential for the development and function of KV cilia and is required for global L-R patterning.

INTRODUCTION

Laterality defects refer to a broad group of disorders caused by the disruption of normal left-right (L-R) asymmetry of the thoracic or abdominal visceral organs.[1] *Situs inversus totalis* is the mirror image reversal of all visceral organs, whereas heterotaxy is the abnormal orientation of one or more organs along the L-R axis.[2] In heterotaxy, congenital heart malformations result in major morbidity and mortality.[3] Although heterotaxy most often occurs as a sporadic condition, familial clustering has been documented with pedigrees suggesting autosomal recessive, autosomal dominant and X-linked inheritance.[4-7]

L-R patterning of vertebrate embryos occurs prior to organ formation and is conducted by a conserved signaling cascade that includes asymmetric expression of the *NODAL*, *LEFTY,* and *PITX2* genes in left lateral plate mesoderm (LPM).[8] Motile cilia are involved in establishing this L-R asymmetric signaling. Laterality defects have been linked to ciliary motility by the observation that 48% of individuals with primary cilia dyskinesia also had *situs inversus totalis* and 6% had heterotaxy.[9]

Animal models have assisted our understanding of L-R patterning and the role of cilia. The *inversus viscerum* (*iv*) mouse has a mutation in the ciliary *left-right dynein* (*lrd*) gene and often develops laterality defects.[10] *Lrd* was found to be required for normal motility of monocilia on an embryonic structure called the node. These node cilia generate a leftward fluid flow that is necessary for normal asymmetric Nodal-Lefty-Pitx2 signaling.[11] In zebrafish, Kupffer's vesicle is a ciliated organ analogous to the mouse node that is essential for normal L-R patterning.[12] Asymmetric fluid flow generated by the monocilia may move signaling factors[11,13] and/or bend mechanosensory cilia[14] to initiate asymmetric signaling.

Dysfunction of ciliary proteins gives rise to a wide range of human disorders known as ciliopathies. They can lead to a variety of defects including craniofacial, skeletal, respiratory, reproductive, renal, visual, olfactory and auditory abnormalities.[15-17] The nephronophthisis (NPHP) and associated ciliopathies - Senior-Loken syndrome, Joubert syndrome, Meckel-Gruber syndrome - are characterized by cilia-related defects, including cystic kidney disease, retinal degeneration, liver fibrosis and brain malformations.[18-19] Mutations in 18 genes are known to cause nephronophthisis and associated ciliopathies.[20-21] Interestingly, mutations in *NPHP2/INVS* and *NPHP3* can also lead to heterotaxy, *situs inversus* and isolated congenital heart malformations.[22-24]

Protein network analysis has shown that several of these proteins form an interaction network organized in at least three connected modules: NPHP1-4-8, NPHP5-6 and MKS.[25] Ciliary localization analysis of eight nephrocystins (NPHP1-6, 9 and

10) indicates that they are present in the primary cilia, the basal body and/or the centrioles and suggest that they participate in ciliary assembly and trafficking.[25-28]

In this study, a genome-wide linkage analysis identified *nephronophthisis-4* (*NPHP4*) variants in patients with cardiac laterality defects. Functional studies indicated that loss of zebrafish *nphp4* resulted in cardiac laterality defects. In addition, *nphp4* depletion disrupted asymmetric Nodal expression in the LPM, indicating *nphp4* is required for global L-R patterning of the embryo. Analysis of cilia in Kupffer's vesicle revealed that loss of *nphp4* reduced cilia length and disrupted asymmetric fluid flow. Our results establish the importance of *nphp4* in cilia development and function. Furthermore, our findings suggest that malfunction of *NPHP4* contributes to a wide range of congenital heart malformations and more complex defects within the heterotaxy spectrum.

MATERIALS AND METHODS

Ethics Statement

Patients and relatives from the index family gave and signed informed consent for participation in the research. DNA samples collection has been performed in accordance with the guidelines of the institutional review boards; the link to patient identifiers is retained by the host institution.

Animal work (zebrafish) was conducted according to national (USA and The Netherlands) and international guidelines**.**

Patients and controls

We studied a large consanguineous family of Iranian origin (Fig. 1a) with complex consanguinity loops. This family was personally followed by us during many years. The healthy parents of branch 1 and 2 are double first degree cousins; the mothers of these parents are sisters and the fathers are brothers. The healthy parents of branch 3 are first degree cousins (their mothers are sisters) and related to the other two branches via the mothers (all mothers are sisters). In this family five patients were born with congenital cardiac defects of which three had cardiac laterality defects (IV-1, IV-8, IV-12).

All patients had extensive cardiologic examinations including electrocardiograms, echocardiograms and cardiac catheterizations. In addition, three patients (IV-1, IV-8, IV-12) with cardiac laterality defects had ultrasound of the abdomen, X-rays and/or MRI. Karyotyping was performed on peripheral blood lymphocytes from two parents and two patients with normal results (III-3, III-4, IV-8, IV-12). A fluorescent *in situ* hybridisation (FISH) of chromosome 22q11 was carried out on one patient (IV-8)

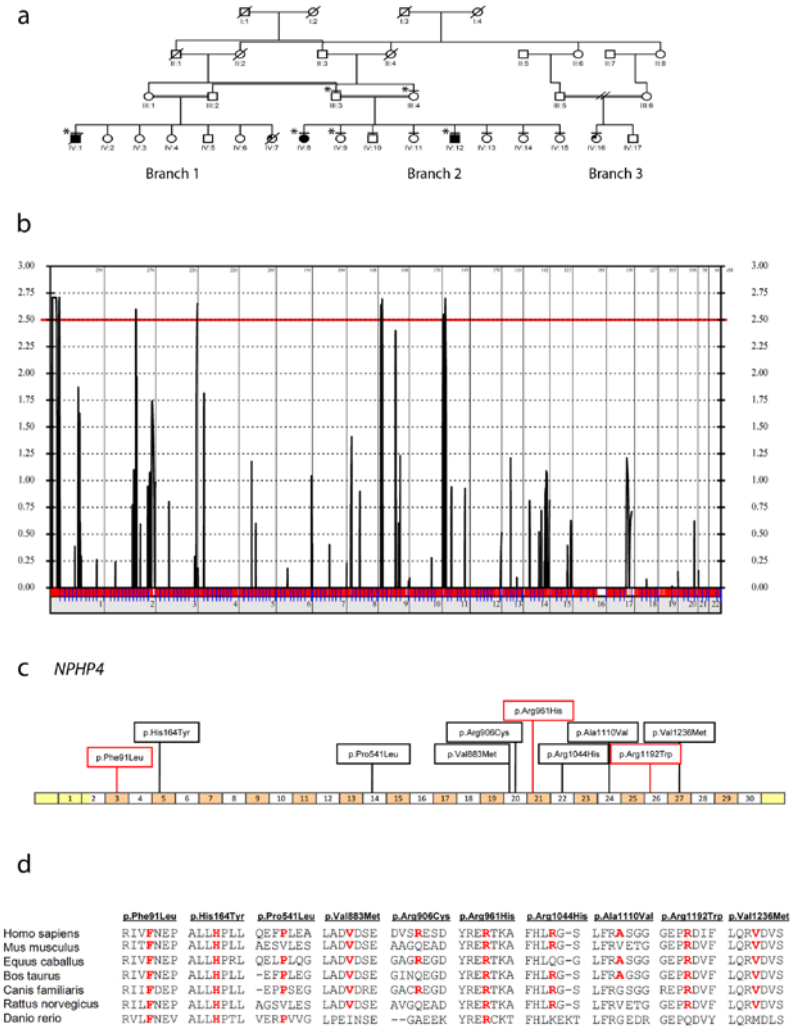

Figure 1 Genome-wide Linkage Analysis (GWLA) and *NPHP4* variants identification.
(a) Simplified genealogical tree of the index family. Squares represent males, circles represent females. A horizontal line above the symbol indicates medical examination. Open symbols indicate healthy individuals, solid black symbols indicate patients with cardiac laterality defects and quarter-filled symbols indicate presence of other (mild) congenital heart malformations. The double line between individuals indicates consanguinity and the diagonal line through a symbol is a deceased family member. Individuals labeled with an asterisk (*) were included in the GWLA. (b) Multipoint LOD scores: X-axis represents all human autosomes and Y-axis corresponds to the LOD scores. Chromosomal regions with LOD scores above 2.5 (red horizontal line) were further investigated. (c) Summary of the *NPHP4* variations identified in patients of the index family, the Dutch and the American cohorts. White and peach colored boxes represent exons and yellow boxes represent untranslated regions. Red-boxed variants were previously described in SLSN4 or NPHP4 patients. (d) Alignment of *NPHP4* protein with several species. The illustrated protein segments were derived from Ensembl reference sequences. Red letters indicate amino acid residues identical to those of human.

which showed no deletion. The asymptomatic siblings and both parents from branch 2 had cardiologic examination (8 individuals), ultrasound of the abdomen and chest X-ray, all with normal results. Two of these siblings (IV-11, IV-14) also had MRI scans of the thorax and abdomen with normal results.

A total of 146 DNA samples from patient cohorts with heart (and other organs) laterality defects were collected. These patients had extensive cardiological evaluation. Clinical evaluation included also X-rays of the thorax, ultrasounds, MRI and CT scans of the thorax and abdomen. Patient samples were collected at the Erasmus Medical Center, Rotterdam, The Netherlands (15 samples), the Department of Clinical Genetics, University Hospital Leuven, Belgium (36 samples) and from the Baylor College of Medicine, Houston, USA (95 samples).

Control DNA samples from each of the patient populations were collected. The Iranian control samples consisted of 532 Kurdish individuals originating from the same province in Iran as the index family. Also, 90 DNA samples (controls with mixed ethnicity) from Iran were included. A total of 180 Dutch control samples were available. The US cohort consisted of 90 Caucasian and 89 Hispanic samples.

Genome-wide scan

The genome-wide search was conducted using DNA samples from six members of the index family including parents, three patients and one unaffected sibling (Fig. 1a). Affymetrix GeneChip Mapping 50K HindIII Arrays containing 57,244 SNP markers were used. Samples were processed according to the manufacturer's instructions (Affymetrix GeneChip Mapping Assay).

Linkage analysis and loci identification

Linkage analysis was performed with Allegro v1.2c (incorporated in the EasyLinkage Plus v5.08 package).[29] LOD scores were obtained using a recessive model of inheritance, with a penetrance of 90%. Map order and genetic inter-SNPs distances were taken from the Affymetrix website (Marshfield sex averaged genetic map) and co-dominant allele frequencies were used.

Since closely spaced SNP markers were used for the linkage analysis, the genome analyses were performed with predefined spacing of 0.1 to 0.4 cM. Single chromosomes showing positive linkage signals were independently analyzed under the same conditions and haplotypes were constructed. Genomic regions with LOD scores above 2.5 were considered as candidate intervals. To facilitate inspection and analyses, graphic visualizations were performed with HaploPainter v029.5.[30]

Microsatellite markers mapping to the identified genomic regions were selected based on their information content. DNAs from all available individuals in the index family were genotyped. PCR products were run on an ABI Prism 3100 genetic se-

quencer (Applied Biosystems) and analyzed using the GeneMapper software v.3.0 (Applied Biosystems). Haplotypes were constructed based on the minimum number of recombinations.

Sequencing analysis

Direct bidirectional sequencing of the entire coding region and the exon-intron boundaries of positional candidate genes was undertaken using PCR primers designed by Primer3 software. Amplified PCR products were purified and sequenced using BigDye Terminator chemistry v3.1 on an ABI Prism 3100 and 3130xl genetic analyzers (Applied Biosystems). Sequences were aligned and compared with consensus sequences obtained from the human genome databases (NCBI, UCSC) using the Applied Biosystems software package SeqScape v2.5. Patient and control samples were sequenced using the same methodology.

Five computer programs (PMut, SHIFT, PolyPhen, SNP3Ds, and HOPE) were used to investigate the potential effect of the amino acid changes on the protein structure and/or function.

TaqMan assays

The *NPHP4* c.3131G>A and c.3706G>A variants in exons 22 and 27 (p.Arg1044His and p.Val1236Met) were screened by an allelic discrimination method (Custom TaqMan SNP genotyping assay, Applied Biosystems). Heterozygote samples were confirmed by direct sequence analysis.

Zebrafish

Zebrafish (*Danio rerio*) embryos were collected and cultured as previously described.[31]

Identification of zebrafish nphp4

A zebrafish *nphp4* ortholog was assembled by combining two overlapping transcripts, ENSDART00000100063 and ENSDART00000111181. The first 10 exons of ENSDART00000100063 were sequenced using wildtype zebrafish cDNA and gDNA to corroborate the reference sequence spanning the morpholino oligonucleotides (MO) design regions. To assess whether zebrafish *nphp4* has two transcripts we used quantitative real-time-PCR (qPCR). Primers were designed to target both transcripts and embryos were injected with splice-blocking SB-MO2, targeting the ENSDART00000100063 transcript only. Knockdown efficiency of both *nphp4* transcripts was between 44-58% (data not shown). Therefore, we refer to the two database transcripts as a single *nphp4* ortholog.

Morpholino Injections and rescue experiments

Antisense morpholino oligonucleotides (MO) were purchased from Gene Tools, LLC. To knockdown *nphp4*, we designed a MO to bind to the start codon and block translation: *nphp4* TB-MO 5′-GCTTCTCCACTCAGACATCAGAGGT-3′ and two MOs to interfere with RNA splicing: *nphp4* SB-MO1 5′-CGGTCAGAGTTGCACT-TACACTGCA-3′ and *nphp4* SB-MO2 5′-TGTGTGTGGTCCATCATTACCTGCT-3′. All MO sequences were aligned with the Danio rerio genome using UCSC Blast and NCBI Blast to confirm specificity to the intended *nphp4* genomic region. Different amounts of each MO were injected to determine the optimum dose. Subsequent experiments were carried out using 4.5 ng *nphp4* TB-MO, 10ng SB-MO1 and 0.8 ng SB-MO2 unless noted otherwise. A standard control MO (5′-CCTCTTACCTCAGT-TACAATTTATA-3) obtained from Gene Tools, LLC, was used for TB-MO and SB-MO2 experiments.

For MO rescue experiments, wild type human NPHP4 cDNA was cloned into pcDNA 3.1-V5-His vector (Invitrogen) and the point mutations c.3131G>A (p.Arg1044His) and c.3706G>A (p.Val1236Met) were generated by using QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies). Single nucleotide changes were confirmed by sequencing. Wild-type and mutant mRNAs were synthesized with mMESSAGE mMACHINE® T7 ULTRA kit (Ambion Inc). 100pg mRNA was co-injected with 4.5ng *nphp4* TB-MO in 1-cell stage embryos. Heart looping and body curvature phenotypes were evaluated at 48 hour-post-fertilization.

RNA Isolation and qPCR

Pooled embryos were quickly frozen in liquid nitrogen and stored at –80°C. Total RNA was isolated from 80 embryos, at 48-53hpf, using an RNA-Bee (Tri-Test, Inc.) protocol. To remove any remaining genomic DNA, the RNA was treated with RNase-free DNase for 30 minutes at 37°C. To synthesize cDNA, 5µg of RNA was reverse-transcribed with oligo-dT primers and Superscript III reverse transcriptase (Invitrogen, California, USA). To measure mRNA levels, qPCR on cDNA samples was carried out using the KAPA SYBR® FAST qPCR Kit (Kapa Biosystems, Inc., MA, USA). Samples were analyzed on the Bio-Rad CFX96 qPCR detection system.

All the primers used for qPCR, including the reference gene *SDHA*, were designed using Primer Express software (version 2.0.0). Primer pairs used to measure knock-down efficiency of *nphp4* with the two splice-blocking morpholinos SB-MO1 and SB-MO2 were 5′- GACCCTCTGCAGTTGAATGC-3′ (forward) and 5′-GAGCTG-GATGTGGCTGTATGG-3′ (reverse); and 5′-GATGAGGGACGTGGATTTCAG-3′ (forward) and 5′-GCGCACACACTGCATAAACG-3′ (reverse), respectively.

In situ hybridization and Immunohistochemistry

Two fragments (<900 bp) of zebrafish *nphp4* cDNA were amplified by RT-PCR and ligated into the pCRII-TOPO vector (Invitrogen). Positive clones confirmed by DNA sequencing were used to generate antisense and sense probes to detect *nphp4* mRNA. Whole-mount *in situ* hybridization was carried out as previously described.[32] A DIG RNA labeling kit (Roche) was used to generate digoxygenin-labeled riboprobes against *nphp4*, *cmlc2*[33], *foxa3*[34], *spaw*[35], *ntl*[36] and *shh*[37]. Stained embryos were mounted in 70% glycerol and imaged with either a Leica DFC300 FX digital colour camera mounted on a Leica MZ16 FA Fluorescence Stereomicroscope or a Nikon DS-Fi1 camera on a Nikon SMZ800 microscope. Images were edited using Photoshop (Adobe) software.

Whole-mount fluorescent immunostaining of cilia was performed using anti-acetylated Tubulin primary antibodies (Sigma) and goat anti-mouse Alexa 488 secondary antibodies (Molecular Probes), as previously described.[12] Embryos were mounted in Slow Fade Reagent (Molecular Probes) and analyzed with a Zeiss Axiocam HSm digital camera on a Zeiss AxioImager M1 AX10 microscope. Images were captured with Zeiss AxioVision software and assembled using ImageJ (NIH) and Photoshop (Adobe) software. Cilia length was measured using ImageJ software.

Visualisation of fluid flow in Kupffer's vesicle

Live embryos were immobilized in 1% low melting point agarose at 6-10 somite stage (SS) and fluorescent beads (Polysciences, Inc.) were injected into Kupffer's vesicle to analyze fluid flow as described.[12] Beads were visualized using a 63X water dipping objective on a Zeiss AxioImager M1 AX10 microscope. Movies were captured with a Zeiss Axiocam HSm digital camera and Zeiss Axiovision software and edited using Quicktime (Apple) software. Fluid flow in each embryo KV was classified as strong, reduced or absent by blind scoring of the movies.

RESULTS

Clinical studies

We identified a consanguineous Iranian family including five patients with congenital heart malformations. Three patients (IV-1, IV-8 and IV-12; Fig. 1a) were born with similar cardiac laterality defects (Table 1). Patient IV-1 had dextrocardia, atrial situs solitus, complete atrioventricular septal defect and discordant ventriculo-arterial connection with dextro-transposition of the great arteries (d-TGA). In addition, he had an interrupted inferior caval vein and a severe pulmonary valve stenosis (PS). He had no surgical correction and died suddenly at the age of 22 years. No autopsy was

Table 1. *NPHP4* variants found in patients with cardiac laterality defects with or without other organs asymmetry. All variants are absent or rare (less than 1%) in control populations

Patient details				Clinical features			*NPHP4* genotypes				
Family	**Origin**	**Patient**	**Sex**	**Cardiovascular abnormalities**	**Other organs asymmetry**	**Other features**	**Coding variant**	**Protein effect**	**Doses**	**Prediction[1]**	**Freq. in controls[2]**
1	Iranian	IV-1	M	Dextrocardia, D-TGA, ASD, VSD, AVSD, PS, interrupted ICV	-	-	c.3131G>A	p.Arg1044His	Hom [5,6]	Pathogenic	0.2% (2/1232) 0.01% (1/6887)
							c.3706G>A	p.Val1236Met	Hom [5,6]	Neutral	0.1% (1/1232) 0%
1	Iranian	IV-8	F	Dextrocardia, ASD, VSD, interrupted right ICV with azygous continuation, persistent left SCV and ICV, cor triatriatum	Left lung isomerism	-	c.3131G>A	p.Arg1044His	Hom [5,6]	Pathogenic	0.2% (2/1232) 0.01% (1/6887)
							c.3706G>A	p.Val1236Met	Hom [5,6]	Neutral	0.1% (1/1232) 0%
1	Iranian	IV-12	M	D-TGA, DORV, VSD, PDA	-	-	c.3131G>A	p.Arg1044His	Hom [5,6]	Pathogenic	0.2% (2/1232) 0.01% (1/6887)
							c.3706G>A	p.Val1236Met	Hom [5,6]	Neutral	0.1% (1/1232) 0%
1	Iranian	IV-16	F	VSD, PS, PDA	-	-	c.3131G>A	p.Arg1044His	Het	Pathogenic	0.2% (2/1232) 0.01% (1/6887)
							c.3706G>A	p.Val1236Met	Het	Neutral	0.1% (1/1232) 0%
2	Dutch, Chinese	02D3049	F	VSD, PA	Right lung isomerism, abdominal situs inversus	Large splenic cyst	c.3329C>T	p. Ala1110Val	Het	Neutral	0.6% (2/318) 0.8% (56/6872) rs139767853

Patient details				Clinical features			*NPHP4* genotypes				
Family	**Origin**	**Patient**	**Sex**	**Cardiovascular abnormalities**	**Other organs asymmetry**	**Other features**	**Coding variant**	**Protein effect**	**Doses**	**Prediction[1]**	**Freq. in controls[2]**
3	Dutch, Cape Verde	07D2466	F	Dextrocardia, AVSD, PS, L-TGA, DORV	Right lung isomerism, midline liver, asplenia	-	c.1622C>T	p. Pro541Leu	Het	Pathogenic	0% (0/182) 0.3% (30/9810)[7] rs145255635
4	Caucasian	LAT0025	M	Mesocardia	Abdominal situs inversus. Midline liver and intestinal malrotation	Cholelithiasis, omphalocele, small spleen	c.271T>C	p. Phe91Leu [3]	Het	Pathogenic	0% (0/180) 0.1% (10/6766)
5	Caucasian	LAT0033	F	Mesocardia, atrial inversion, VSD, ASD, PA, D-TGA, DORV, severe aortic root dilation	Abdominal situs inversus. Midline liver	-	c.490C>T	p. His164Tyr	Het [5]	Pathogenic	0% (0/180) 0%
6	Caucasian	LAT1268	M	HLHS, VSD, ASD, MV hypoplasia, BAV, CoA, PDA, interrupted ICV with azygous continuation	Polysplenia,transverse liver, midline gall bladder and portal vein, intestinal malrotation	Right hydronephrosis	c.2647G>A	p.Val883Met	Het [6]	Neutral	0% (0/122) 0.01% (1/6897)
7	Caucasian	LAT0079	M	Common atrium, right atrial isomerism, single ventricle, AVSD, PA, azygous continuation to right SCV	Abdominal situs inversus	-	c.2716 C>T	p.Arg906Cys	Het	Pathogenic	0% (0/122) 0%

Patient details				Clinical features			*NPHP4* genotypes				
Family	**Origin**	**Patient**	**Sex**	**Cardiovascular abnormalities**	**Other organs asymmetry**	**Other features**	**Coding variant**	**Protein effect**	**Doses**	**Prediction[1]**	**Freq. in controls[2]**
8	Caucasian	LAT1168	M	Right atrial isomerism, single ventricle, ASD, MV atresia, PS, DORV, VSD, infra-diaphragmatic TAPVR, absent ICV with azygous continuation to ipsilateral SCV, persistent left SCV to coronary sinus, right aortic arch with a common brachio-cephalic trunk, abnormal branching pattern of the abdominal vessels from the aorta	Abdominal situs inversus, asplenia and intestinal malrotation	-	c.2882G>A	p.Arg961His [4]	Het [5]	Pathogenic	0.5% (1/182) 0.4% (30/6952)
9	Hispanic	LAT0145	M	HLHS, AVSD, D-TGA, DORV, bicuspid PV, PS, supracardiac TAPVR	Midline liver, asplenia and intestinal malrotation	Mild hepatormegaly with calcifications, cholelithiasis. Hypothyroidism	c.3574C>T	p.Arg1192Trp [4]	Het [6]	Pathogenic	0% (0/182) 0.3% (21/6625) rs139022622
10	Caucasian	LAT1034	M	Single ventricle, MV atresia, VSD, ASD, subvalvular AS, PS, L-TGA, DORV, PDA	-	-	c.3329C>T	p. Ala1110Val	Het	Neutral	0.6% (2/318) 0.8% (56/6872) rs139767853

performed. Patient IV-8 had dextrocardia, dextrorotation and atrial situs solitus. She had an azygos continuation of the right infrahepatic part of the inferior caval vein draining into the right superior caval vein and the suprahepatic part of the inferior caval vein draining into the right atrium. She had a cor triatriatum with the right pulmonary veins draining into the right part of the left atrium and the left pulmonary veins into the left part of the left atrium. A persistent left inferior and superior caval vein also drained into the left part of the left atrium. She had a secundum atrial septal defect (ASD) and perimembranous ventricular septal defect (VSD). She had also left bronchial isomerism. Patient IV-12 had atrial situs solitus, atrio-ventricular concordance and ventriculo-arterial discordance namely, double outlet right ventricle and d-TGA. He also had a subpulmonary VSD, patent foramen ovale and patent ductus arteriosus (PDA).

In addition, patient IV-7 died shortly after birth due to an unspecified congenital heart malformation (Fig. 1a). The fifth patient (IV-16) had mild congenital heart malformations consisting of a small VSD, PS and PDA, which was ligated at one year of age (Table 1).

Physical examination revealed no dysmorphisms and all patients had normal psychomotor development. CT/MRI or ultrasound of the abdomen revealed no kidney cysts and all individuals have reached adulthood at the time of their last evaluations. No abdominal laterality defects such as asplenia or polysplenia, malrotation of the gut or midline liver were detected in any of these cases. None of the patients had signs of abnormal mucociliary clearance. No visual problems or night blindness were reported.

Genome-wide linkage analysis

The genome-wide linkage analysis was performed using Affymetrix SNP arrays. Two unaffected parents, three patients and one healthy sibling (Fig. 1a) were included in

Table 1 continued.

Abbreviations: ASD, atrial septal defect; VSD, ventricular septal defect; AVSD, atrioventricular septal defect; MV, mitral valve; TGA, transposition of great arteries (dextro or levo); DORV, double outlet right ventricle; PDA, persistent ductus arteriosus; BAV, bicuspid aortic valve; AS, aortic stenosis; PA, pulmonary atresia; PV, pulmonary valve; PS, pulmonary valve stenosis; HLHS, hypoplastic left heart syndrome; CoA, coarctation of the aorta; TAPVR, total anomalous pulmonary venous return; ICV, inferior caval vein; SCV, superior caval vein; Het, heterozygous; Hom, homozygous

[1]prediction of the genetic variant effect on protein level (Pmut, SNPs3D, SIFT, PolyPhen, HOPE), [2]based on ethnically matched (in house) control chromosomes and the frequencies reported by the NHLBI Exome Sequencing Project, [3]reported in patients with SLSN4, [4]reported in patients with NPHP4, [5]inherited from father, [6]inherited from mother, [7]total allele counts include European and African American population.

the analysis. Multipoint linkage analysis revealed five regions on chromosomes 1, 2, 3, 9 and 11 with LOD scores above 2.5 (Fig. 1b). The maxLOD scores (2.7) were located on chromosome 1p36 and 11p15. Subsequently, microsatellite markers mapping to all candidate regions were tested. The loci on chromosomes 2, 3, and 9 were excluded, based on heterozygosity observed in the patients (data not shown).

On the chromosome 1p36 locus, all three patients with cardiac laterality defects (IV-1, IV-8 and IV-12) shared a homozygous region covered by 59 SNPs from rs4845835 to rs1203695. Haplotype analysis showed recombinations that delimited the borders of the region from rs2722782 (5.26 Mb) to rs1203696 (14.21 Mb) (Fig. 2). Thus, the candidate region spanned 9 Mb and contained 152 genes (NCBI build 37.1).

These patients also showed homozygous genotypes for 49 consecutive SNPs on chromosome 11, from rs16905816 to rs10500752. Further fine mapping in the 11p area delineated the borders of the linkage region between markers D11S4188 (telomeric) and rs2896598 (centromeric) (Fig. 3). The chromosome 11 locus extended only 3 Mb (9.1-12.1 Mb), containing 34 genes (NCBI build 37.1).

Haplotypes from both loci were examined in all available family members. A healthy person (IV-14, with normal MRI of the thorax/abdomen) had homozygous haplotypes on the chromosome 1 locus (Fig. 2). In addition, individuals IV-3 and IV-4 were homozygotes for the chromosome 11 locus (Fig. 3); both persons were reported as unaffected, but medical examinations could not be performed. Patient IV-16, exhibiting a mild cardiac phenotype and no laterality defects, carried heterozygous haplotypes at both loci (data not shown). Only the three patients with laterality heart defects had homozygous haplotypes on both loci. Since these were the only genomic regions where the three patients showed extended homozygosity, we further investigated these loci.

Sequence analysis

A total of 109 genes on the chromosome 1p36 locus had a known reference sequence (NCBI build 37.1). Selection of genes for sequence analysis was based on available expression and/or functional information. The data was analyzed through the use of Ingenuity pathway analysis (Ingenuity® Systems). Thirty-six candidate genes were selected from the chromosome 1 locus. Direct sequencing of their coding regions identified two novel homozygous missense variants in the *NPHP4* gene present in three patients from the index family: c.3131G>A (p.Arg1044His) and c.3706G>A (p.Val1236Met) (Table 2). These non-synonymous variants are extremely rare in the Iranian (Kurdish) population (allele frequency of 0.2% and 0.1% in 1232 control chromosomes). Moreover, the variants were absent in 270 Caucasian/Dutch and 178 Hispanic control chromosomes.

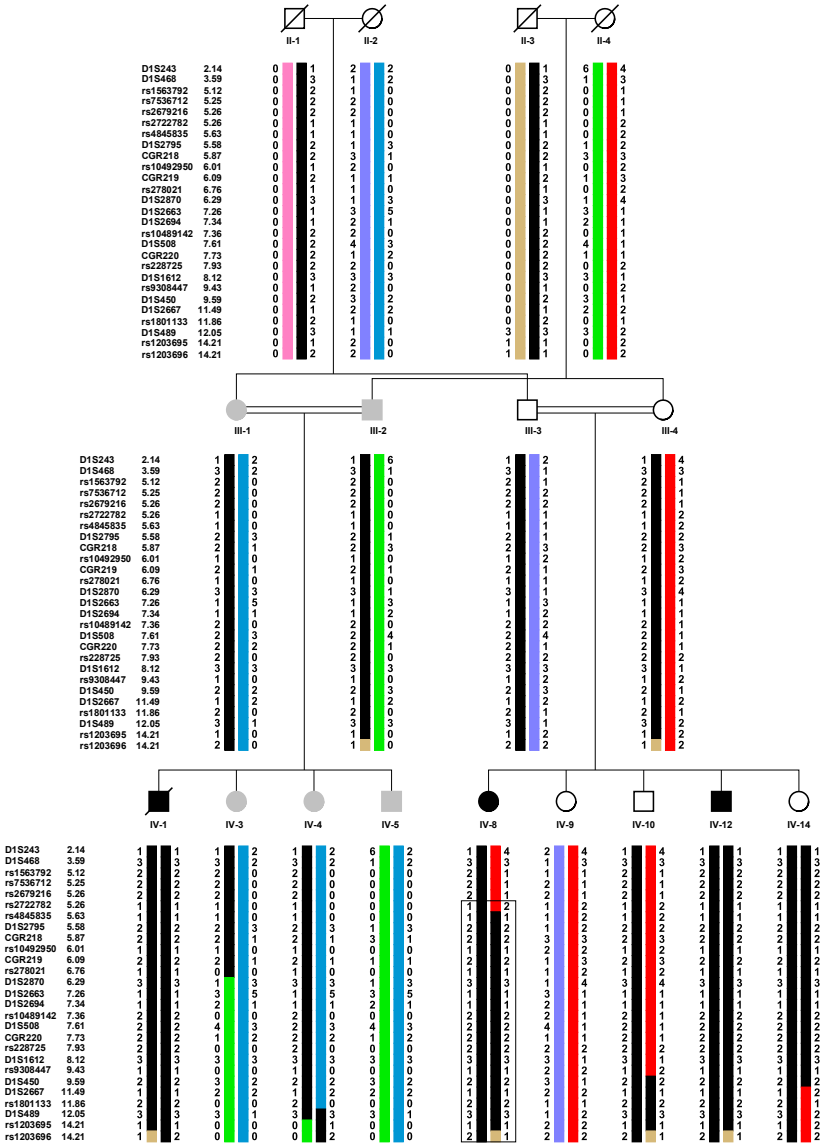

Figure 2 Haplotypes with SNP and microsatellite genotypes corresponding to chromosome 1p36 locus. Summarized genealogical tree of the index family. Persons II-1 and II-3 are brothers; II-2 and II-4 are sisters. Open symbols indicate healthy individuals, solid black symbols indicate patients with cardiac laterality defects and grey symbols indicate asymptomatic (clinically unexamined) individuals. Note that the patients share homozygous haplotypes extending from rs2722782 (5.26 Mb) to rs1203696 (14.21 Mb). The borders of the homozygous region are shown (boxed haplotypes) in person IV-8. Person IV-14 (unaffected, normal clinical examination) has homozygous haplotypes overlapping the same region. Genotypes from generation II were inferred. Physical position (Mb) of each marker is shown according to NCBI build 37.1.

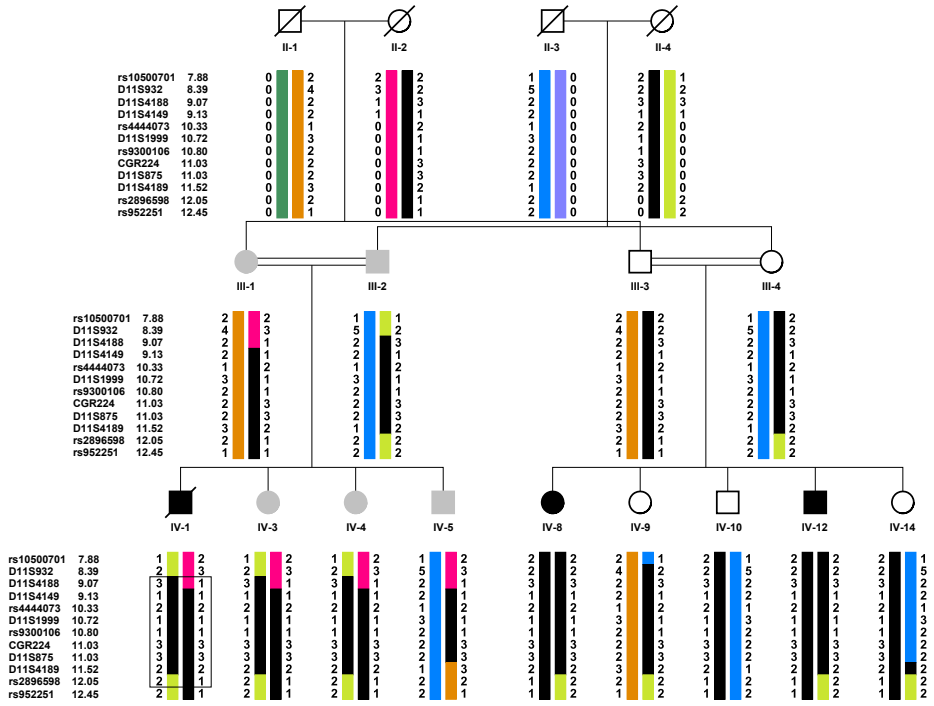

Figure 3 Haplotypes with SNP and microsatellite genotypes corresponding to chromosome 11p15 locus. Symbols are the same as described in Figure 2. The patients show homozygous haplotypes (depicted in black) in a region delimited by markers D11S4188 (9.07 Mb, telomeric) and rs2896598 (12.05 Mb, centromeric). The borders of the homozygous region are shown (boxed haplotypes) in person IV-1. Note that individuals IV-3 and IV-4 (asymptomatic, clinically unexamined) share the same homozygous region. Genotypes from generation II were inferred. Physical position (Mb) of each marker is shown according to NCBI build 37.1.

From the 34 genes mapping to the chromosome 11 locus, 19 genes had a well annotated reference sequence. Sequence analysis of their coding regions and exon-intron boundaries revealed only one novel DNA missense variant (Table 2). In the *AMPD3* gene (*adenosine monophosphate deaminase 3*), the homozygous c.2240 G>A (p.Arg747Gln) variant was found. This variant was not present in 626 control chromosomes. Mutations in the *AMPD3* gene lead to (asymptomatic) deficiency of erythrocyte AMP deaminase (OMIM 612874).[38]

NPHP4 variants in patients with laterality defects (heterotaxy)

We sequenced all 30 exons of *NPHP4* in three cohorts of patients with cardiac laterality defects - with or without other situs abnormalities. Patient samples were collected at the Erasmus Medical Center, Rotterdam, the Department of Clinical Genetics, Leuven and from the Baylor College of Medicine, Houston. All 146 patients

Table 2. All novel genetic variants found in the chromosome 1 and chromosome 11 loci

Locus	Gene	DNA Variant	Patients	Controls	Type of variant	Protein effect
1	*NPHP4*	c.3131G>A	Hom A	Het G/A	Missense	p.Arg1044His
1	*NPHP4*	c.3706G>A	Hom A	Het G/A	Missense	p.Val1236Met
1	*DNAJC11*	IVS9 -100 T>C	Hom C	Het T/C	Intronic	unknown
1	*CHD5*	IVS37 +29 delC	Hom delC	Het delC	Intronic	unknown
1	*KCNAB2*	IVS12 +44 C>T	Hom T	Het C/T	Intronic	unknown
1	*SLC2A7*	c.848 G>A	Hom A	Hom G	Synonymus	p.Gln283Gln
1	*SLC2A5*	c.1321 C>A	Hom A	Hom C	Synonymus	p.Ala446Ala
11	*AMPD3*	c.1701 C>T	Hom T	Het C/T	Synonymus	p.Tyr566Tyr
11	*AMPD3*	c.2240 G>A	Hom A	Het G/A	Missense	p.Arg747Gln
11	*SBF2*	IVS1 -187 delT	Hom delT	Hom delT	Intronic	unknown

had a variety of cardiac laterality defects. Transposition of the great arteries was the most frequently found (49% of the patients). In addition, complete atrio-ventricular septal defect, double outlet right ventricle and abnormal pulmonary venous return were often reported. Dextrocardia was present in 33% of patients. Moreover, 41% had documented laterality defects of the abdominal organs, including abdominal *situs inversus*, asplenia or polysplenia, midline liver and intestinal malrotation.

Nine missense variants were found in 10 patients (Fig.1c, d and Table 1). The population frequency of each allele was tested by sequencing ethnically matched controls. A variant was considered as likely non-pathogenic if the allele frequency in healthy individuals was higher than 1%. Thus, p.Pro1160Leu with a frequency of 2.1% in control chromosomes was excluded from further analysis. In addition, we investigated the frequency of these variants in available databases (dbSNP135, 1000Genomes, NHLBI exome project). All variants were very rare or absent in controls (allele frequency $\leq$0.8%, Table 1).

These rare *NPHP4* variants were significantly more frequent in heterotaxy cases (6%, 9 of 146 cases) than in controls (1.2%, 3 of 250, Fisher's exact test p=0.006). *In silico* evaluation was performed using five prediction computer programs. This assessment predicted the impact of amino acid substitutions on the structure and function of human proteins. The variants were classified as probably pathogenic if at least 3 programs considered them as damaging (Table 1). Seven variants satisfied this criterion. Interestingly, p.Phe91Leu, p.Arg961His and p.Arg1192Trp have been reported in patients with Senior-Loken syndrome type 4 or nephronophthisis type 4.[39]

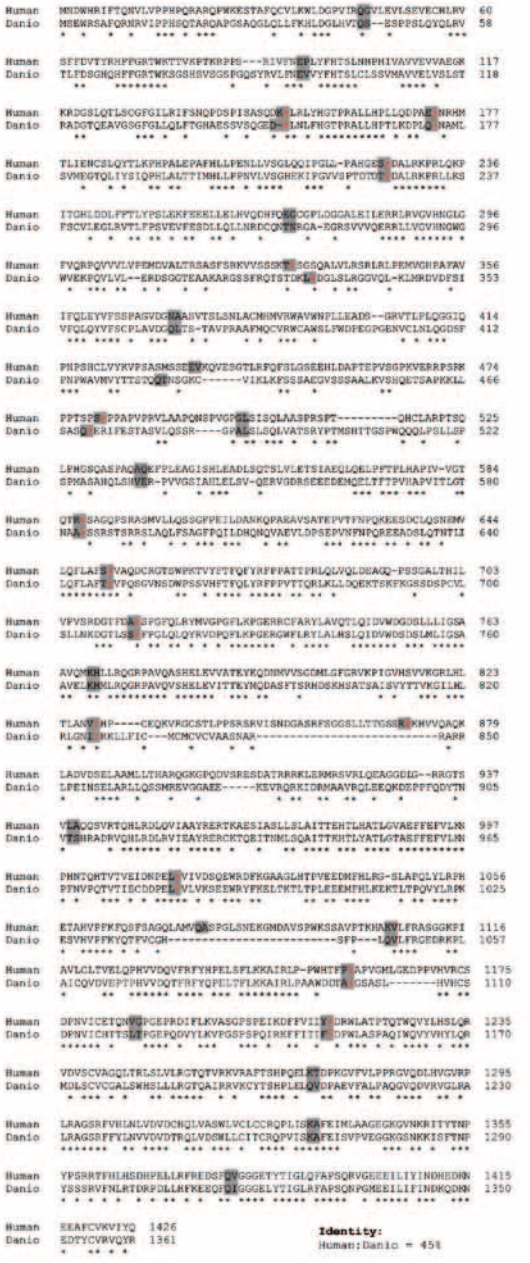

Figure 4 Comparison of human and zebrafish NPHP4 proteins. ClustalW protein alignment of human NPHP4 (ENSP00000367398) with zebrafish (Danio) (ENSDARP00000090835 and ENSDARP00000101221 combined) orthologs. The number of amino acids is indicated at the end of each line. Amino acids indicated with a star (*) are identical among human and zebrafish. The amino acids marked in grey indicate the exon-exon boundaries.

Identification of zebrafish nphp4 and characterization of its expression during embryogenesis

A zebrafish *nphp4* ortholog was taken from the Ensembl database (Fig. 4). To determine the pattern of *nphp4* expression during embryogenesis, we performed reverse transcription PCR (RT-PCR) and RNA *in situ* hybridization experiments at several developmental stages. Consistent with a recent report [40] we found *nphp4* expression was maternally supplied and ubiquitously expressed during the first 24 hours of zebrafish development (Fig. 5). RT-PCR detected *nphp4* expression at all stages tested between 4-cell stage and 100 hours post-fertilization (hpf) (Fig. 5). This

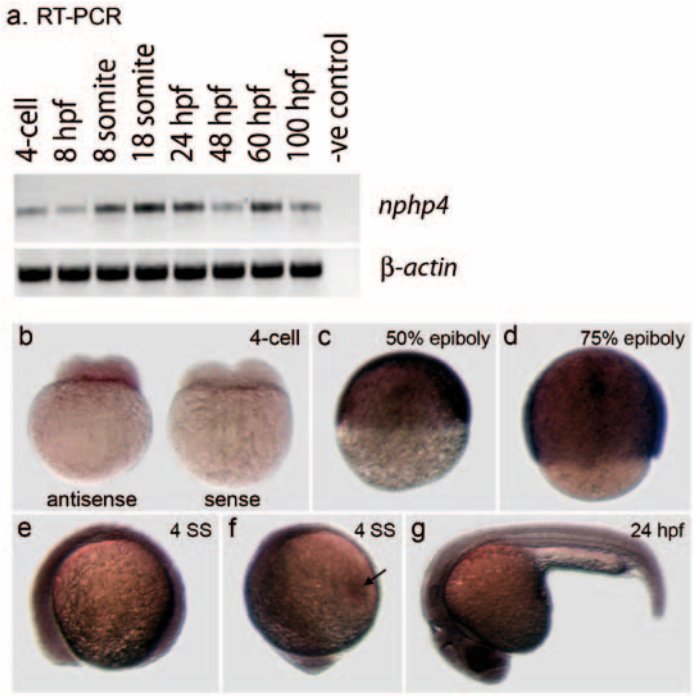

Figure 5 Temporal and spatial *nphp4* expression patterns in the zebrafish embryo. (a) The expression of *nphp4* mRNA at multiple developmental stages between the 4-cell stage and 100 hours post-fertilization (hpf). Amplification of β-actin was used as an internal control. Negative (-ve) controls lacked template. (b-g) in situ hybridization of *nphp4* mRNA expression. Maternal *nphp4* expression was detected at the 4-cell stage using an antisense nphp4 probe, whereas a sense probe showed reduced or absent staining (b). *nphp4* expression was ubiquitous during epiboly stages (c, d) and somite stages (SS) (e-f) and at 24 hpf (g). Enriched staining was detected in the tailbud at 4 SS (arrow in f) where Kupffer's vesicle develops and in anterior regions at 24 hpf (g). A second probe for the *nphp4* gene showed identical results (data not shown).

early and ubiquitous expression pattern suggested a role for *nphp4* during early development.

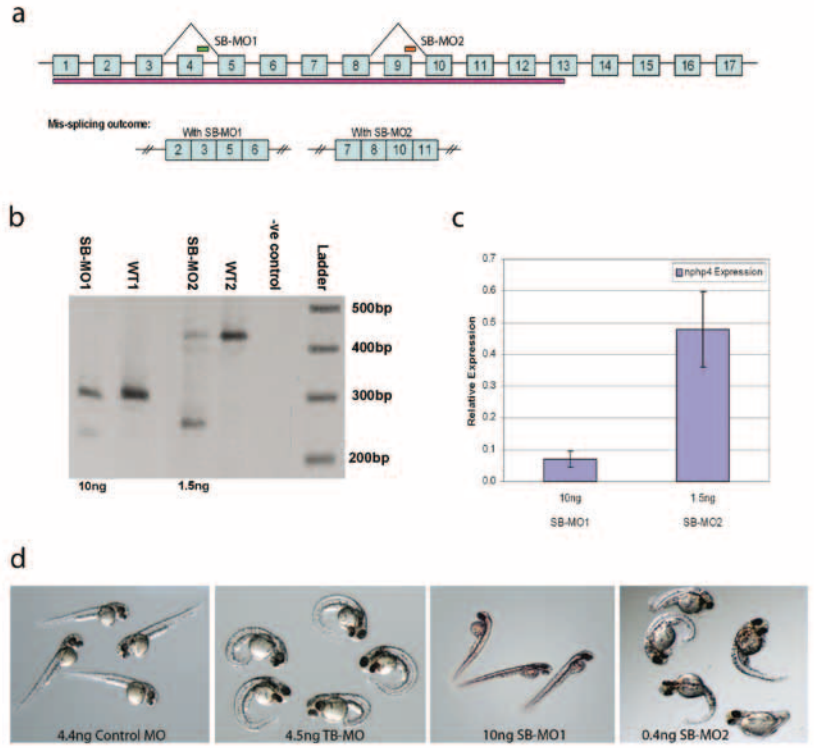

Figure 6 Zebrafish nphp4 MO knockdown. (a) Schematic of the zebrafish *nphp4* gene showing the location of *nphp4* SB-MO1 targeting the exon4/intron4 splice donor site and *nphp4* SB-MO2 targeting the exon9/intron9 splice donor site. The pink bar represents the exons and exon/intron boundaries we confirmed by re-sequencing cDNA and gDNA from wildtype zebrafish. The mis-splicing outcomes represent splicing defects in *nphp4* SB-MO1 or SB-MO2 injected embryos. (b) PCR was performed on cDNA from wild-type embryos and embryos injected with *nphp4* SB-MO1 or SB-MO2. Injecting SB-MO1 or SB-MO2 resulted in the excision of exon 4 or exon 9, respectively. Mis-splicing was confirmed by direct sequencing. Negative (-ve) control lacked template. (c) Quantitative PCR analysis of nphp4 in 50 hpf wildtype and injected embryos confirmed gene knockdown in response to SB-MO1 and SB-MO2. All sample expressions were normalized to the control gene sdha. Relative expression was calculated by setting the wildtype expression level at 1. Error bars represent standard error of the mean. (d) Relative to control MO injected embryos, *nphp4* TB-MO and SB-MO2 injected embryos showed a curved body axis phenotype at 48 hpf, whereas *nphp4* SB-MO1 embryos had normal axial development.

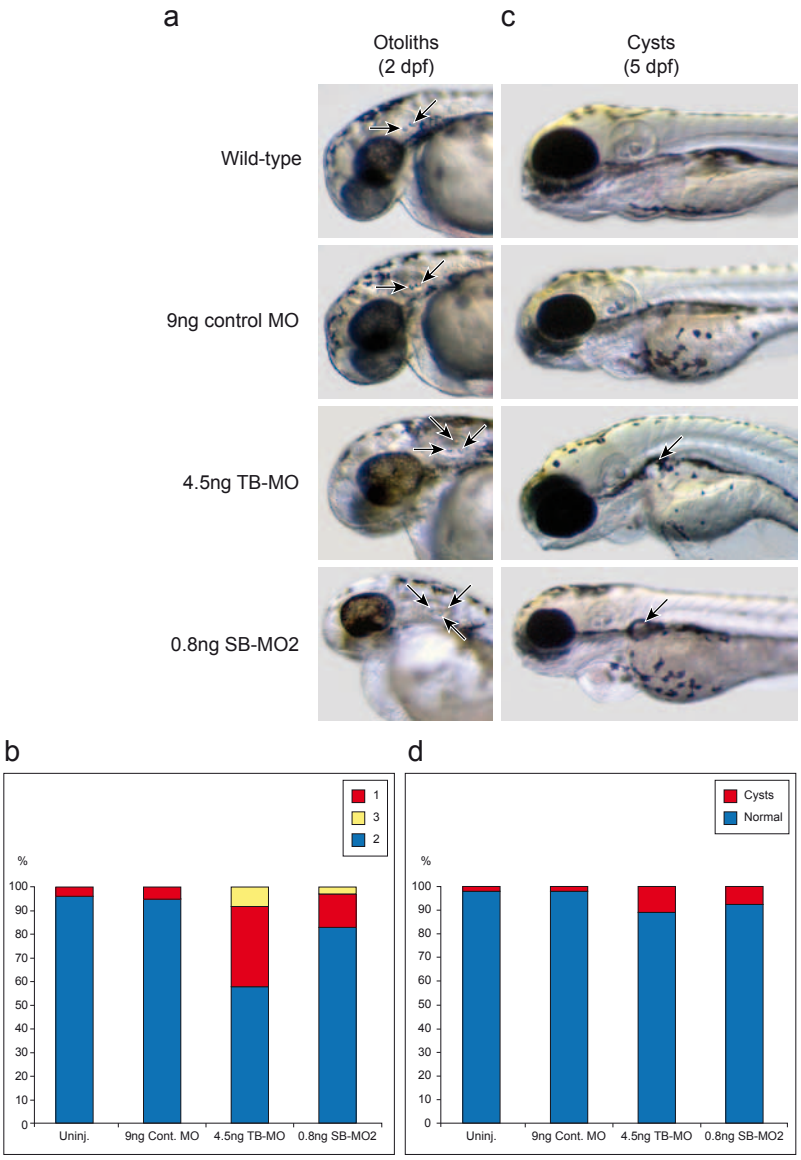

Figure 7 *nphp4* knockdown leads to otoliths defects and (low penetrance) pronephric cysts. (a) Uninjected and control MO injected embryos show normal otoliths (arrows) while a proportion of *nphp4* TB-MO and *nphp4* SB-MO2 injected embryos displayed abnormal number (1 or 3) of otoliths. (b) The distribution of number of otoliths observed in uninjected (n=147), control MO (n=130), *nphp4* TB-MO (n=88) and *nphp4* SB-MO2 (n=96) injected embryos. (c) Kidney cysts (arrows) were observed in a small proportion of the *nphp4* TB-MO and SB-MO2-injected embryos. No cysts were observed in the SB-MO1 injected embryos. (d) Graph shows the distribution of kidney cysts observed in uninjected (n=124), control MO (n=109), *nphp4* TB-MO (n=89) and *nphp4* SB-MO2 (n=87) embryos at 5 dpf.

nphp4 is required for normal cardiac laterality in zebrafish

To assess the function of *nphp4* during embryonic development, we utilized antisense morpholino oligonucleotides (MO) to knockdown expression of zebrafish Nphp4 protein. Embryos injected with a MO designed to block *nphp4* mRNA translation (*nphp4* TB-MO) developed dose-dependent morphological abnormalities reminiscent of embryos with cilia defects [41-43], including a curved body axis (Fig. 6d) and otolith formation defects at 2 days post-fertilization (dpf) (Fig. 7a, b).

In addition, RNA *in situ* hybridization staining of the heart-specific marker *cmlc2* revealed heart laterality defects. Uninjected controls and embryos injected with a standard control MO showed normal rightward looping of the heart at 2 dpf (Fig. 8a, b). However, heart looping in *nphp4* TB-MO injected embryos was significantly altered, as the heart often looped in the reverse orientation or failed to loop (Fig. 8a, b).

To test whether heart laterality phenotypes were specific to knockdown of *nphp4*, we designed two additional MOs to interfere with *nphp4* mRNA splicing at exon 4 (*nphp4* SB-MO1) or exon 9 (*nphp4* SB-MO2) (Fig. 6a, b). Quantitative real time PCR (qPCR) analysis indicated *nphp4* SB-MO1 reduced *nphp4* mRNA levels by 90% (Fig. 6c) and caused heart laterality defects without inducing body axis defects (Fig. 6d and Fig. 8a, b). This indicates heart L-R phenotypes are separable from axial defects. *nphp4* SB-MO2 reduced the amount of normally spliced *nphp4* mRNA by 50% (Fig. 6c) and resulted in curved body axis and heart looping defects (Fig. 6d and Fig. 8a, b, respectively), similar to *nphp4* TB-MO injected embryos. Injecting a lower dose of *nphp4* SB-MO2 (0.4 ng) also altered heart looping, but with reduced penetrance (Fig. 8b), suggesting partial loss of *nphp4* can cause cardiac laterality defects.

Other abnormalities such as hydrocephalus or gross eye defects were not observed. At 5 dpf, pronephric cysts were observed with a low penetrance in embryos injected with TB-MO (11%) or SB-MO2 (8%) (Fig. 7c, d). No pronephric cysts were observed in SB-MO1 injected embryos. Our results using three independent MOs suggested a role for *nphp4* that is required for normal heart laterality in zebrafish.

To further confirm that defects observed in zebrafish embryos were specifically due to *nphp4* depletion, we conducted rescue experiments using human wild-type (wt) *NPHP4* mRNA. Co-injecting *nphp4* TB-MO with wt *NPHP4* mRNA resulted in a partial, but significant, rescue of heart looping defects (% of normal embryos improved from 43% to 60%, p=0.03; Fig. 8c). Next, we co-injected *nphp4* TB-MO with human *NPHP4* mRNA containing either the c.3131G>A (p.Arg1044His) or c.3706G>A (p.Val1236Met) missense variant identified in the index family. In contrast to wt *NPHP4*, these *NPHP4* variants failed to rescue heart looping defects (Fig. 8c). These results suggest that these variants are pathogenic and are involved in human laterality defects.

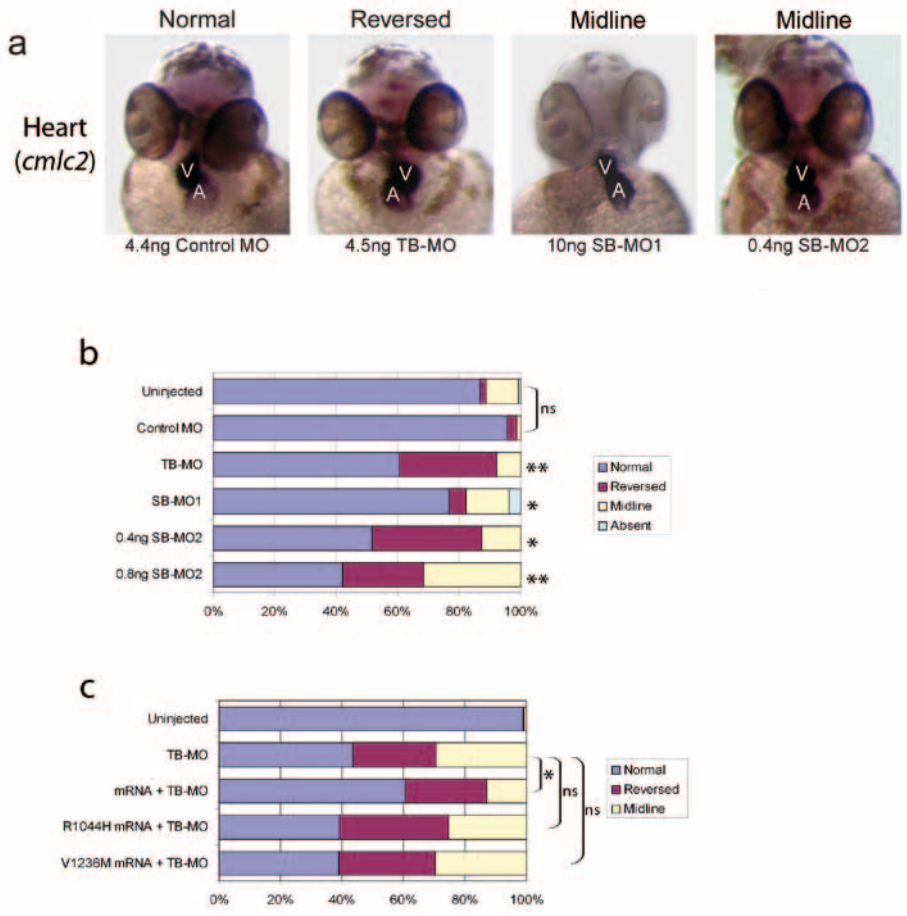

Figure 8 *nphp4* knockdown alters zebrafish heart laterality. Wild-type, but not mutant, human *NPHP4* mRNA partially rescues zebrafish *nphp4* phenotype. (a) *in situ* hybridizations using a heart-specific probe (*cmlc2*) showed that embryos injected with a control MO had predominantly normal cardiac looping. In contrast, heart laterality was often reversed or remained along the midline in *nphp4* MO injected embryos. V=ventricle, A=atrium, (b) The distributions of heart orientation observed in uninjected embryos (n=242), Control MO (n=71), *nphp4* TB-MO (n=89), *nphp4* SB-MO1 (n=106) and *nphp4* SB-MO2 (0.8ng, n=76 and 0.4ng n=95) embryos. (c) Human wt *NPHP4* mRNA partially rescued heart laterality defects; the graph shows the distribution of normal and abnormal heart looping in uninjected embryos (n=239), embryos injected with 4.5ng TB-MO (n=154) and injected with both 100pg human wt *NPHP4* mRNA and 4.5ng TB-MO (n=249). In contrast, mutant *NPHP4* containing either the p.Arg1044His (n=189) or p.Val1236Met (n=188) missense variants failed to rescue the phenotype (4.5ng TB-MO and 100pg mutant *NPHP4* mRNA). *$p \leq 0.038$; ** $p \leq 2.56 \times 10^{-4}$, ns: not significant.

nphp4 controls global L-R patterning of the zebrafish embryo

To determine whether *nphp4* plays a role in heart laterality specifically or is involved in establishing global L-R patterning of the embryo, we analyzed additional markers of L-R asymmetry. RNA *in situ* hybridization, using *foxa3* probes to label the embryonic gut, showed that *nphp4* knockdown significantly altered laterality of the liver and pancreas in *nphp4* MO injected embryos (Fig. 9a, b). We next analyzed expression of the Nodal-related gene *southpaw* (*spaw*), the earliest asymmetrically expressed gene in lateral plate mesoderm (LPM) in zebrafish [35]. Control embryos exhibited normal left-sided *spaw* expression (Fig. 9c, d). In contrast, *nphp4* MO injected embryos showed a significant disruption of *spaw* expression, which was often reversed, bilateral or absent (Fig. 9c, d). Altered asymmetric gene expression can result from defects in the embryonic midline [44]. However, analysis of the midline markers *no tail* and *sonic hedgehog* revealed that midline structures were intact in *nphp4* MO injected embryos (Fig. 10). These results indicate *nphp4* functions independent of midline development to control *spaw* expression and global L-R patterning of the embryo.

nphp4 is required for normal cilia length and directional fluid flow in Kupffer's vesicle

In zebrafish, Kupffer's vesicle (KV) is a transient organ that generates cilia-driven asymmetric fluid flow necessary to bias *spaw* expression to the left LPM. Examination of live embryos at the 8 somite stage showed that the KV organ appeared normal in control MO (Fig. 11a) and *nphp4* MO injected embryos (Fig. 11b, c). However, analysis of cilia in KV by fluorescent immunostaining with acetylated Tubulin antibodies revealed that the cilia were significantly shorter in *nphp4* MO injected embryos (Fig. 11e-g) as compared to controls (Fig. 11d, g). We did not observe a significant difference of KV cilia number between control and *nphp4* MO injected embryos (Fig. 11h). To analyze KV cilia function, we injected fluorescent beads into KV of live embryos and used video microscopy to record fluid flow.[12] Most control embryos showed strong counter-clockwise asymmetric fluid flow (Fig. 11i, l). In contrast, flow was often absent (Fig. 11j, l) or reduced (Fig. 11k, l) in *nphp4* MO injected embryos. Consistent with dose-dependent effects of *nphp4* SB-MO2 on heart looping (Fig. 8b), we observed more severe flow defects in embryos injected with a higher *nphp4* SB-MO2 dose (Fig. 11l). Together, these results show that *nphp4* knockdown results in short KV cilia and compromises asymmetric fluid flow that is necessary for normal L-R patterning.

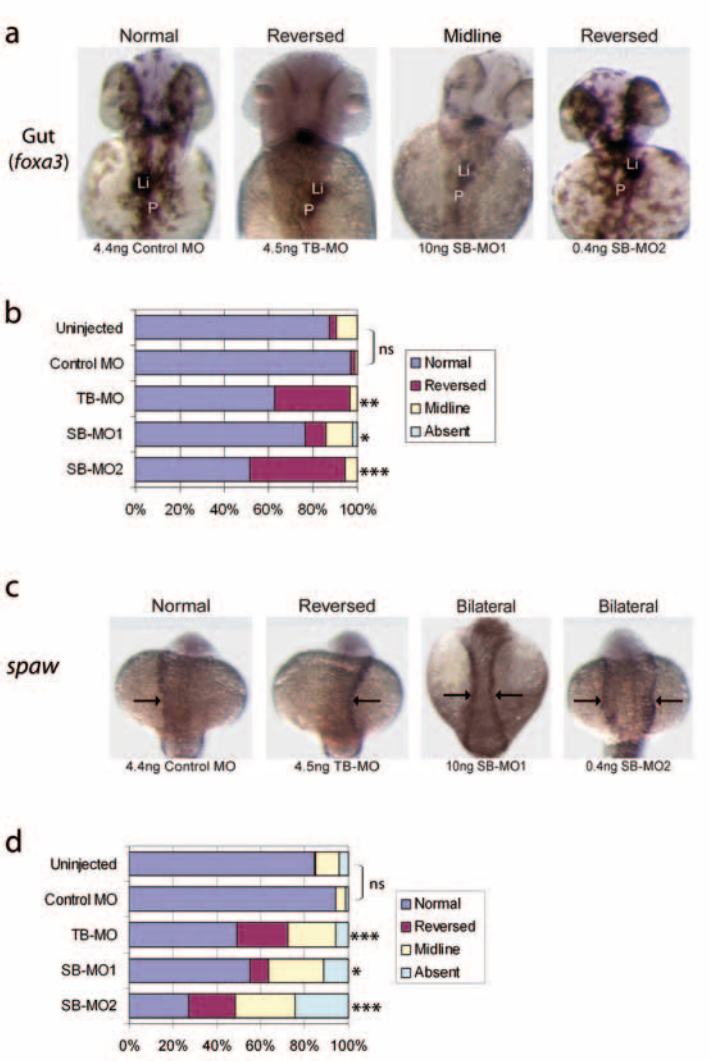

Figure 9 *nphp4* knockdown alters zebrafish gut laterality and disrupts global asymmetric gene expression. (a) *in situ* hybridizations using a gut-specific probe (*foxa3*) showed that embryos injected with a control MO had predominantly normal liver and pancreas orientation. In contrast, gut laterality was often reversed or remained along the midline in *nphp4* MO injected embryos. Li=liver, P=pancreas (b) The distributions of gut orientation observed in uninjected embryos (n=242), Control MO (n=71), *nphp4* TB-MO (n=89), *nphp4* SB-MO1 (n=106) and *nphp4* SB-MO2 (n=76) injected embryos. (c) *in situ* hybridization staining of *southpaw* (*spaw*) expression (arrows) in lateral plate mesoderm at 16-18 SS. *spaw* expression, which is normally left-sided in controls was often reversed, bilateral or absent in *nphp4* MO injected embryos. (d) The distributions of *spaw* expression patterns in uninjected embryos (n=217), Control MO (n=88), *nphp4* TB-MO (n=134), *nphp4* SB-MO1 (n=116) and *nphp4* SB-MO2 (n=70) embryos. *$p \leq 0.028$; **$p \leq 0.0012$ and ***$p \leq 5.9 \times 10^{-4}$, ns: not significant.

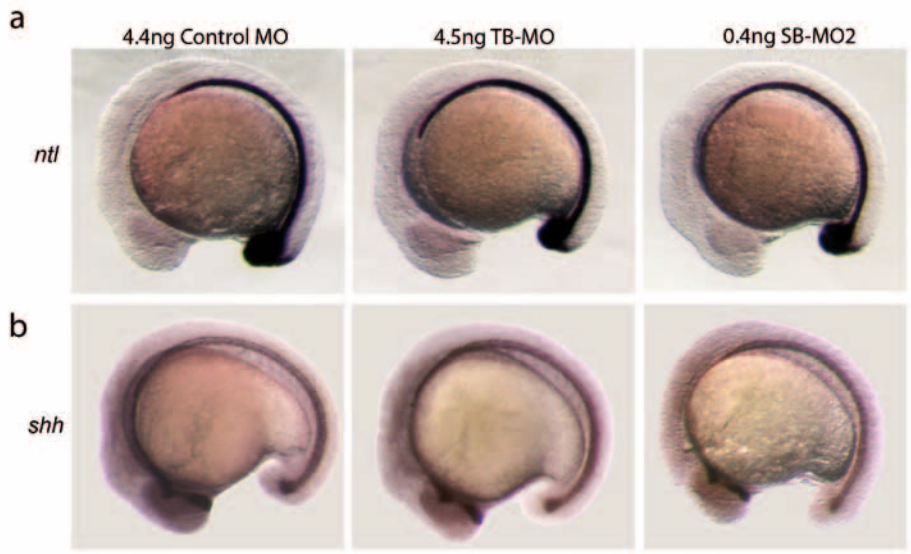

Figure 10 *nphp4* MO knockdown does not disrupt embryonic midline development.
(a, b) Whole-mount *in situ* hybridization analysis of *no tail* (*ntl*) expression in the notochord (a) and *sonic hedgehog* (*shh*) expression in the notochord and floorplate of the neural tube (b). Embryos were injected with control MO, *nphp4* TB-MO or *nphp4* SB-MO2.

DISCUSSION

We found homozygous missense *NPHP4* variants in a consanguineous family containing three patients with cardiac laterality defects, bronchial isomerism and normal abdominal situs.

Interestingly, though *NPHP4* is a cilia related gene that is mutated in patients with autosomal recessive juvenile nephronophthisis (NPHP type 4, OMIM 606966) [45] and Senior-Loken syndrome (SLSN4, OMIM 606996) [46], our patients did not show signs of nephronophthisis or retinitis pigmentosa, which are distinctive features of these diseases.

Because of the known interaction between NPHP1, NPHP2/INVS, NPHP3 and NPHP4 proteins [23-24,45], it is obvious that mutations in one or more of these genes disrupt the same pathway(s) and can lead to similar phenotypes (i.e. nephronophthisis). Conversely, mutations within the same gene can lead to various phenotypic outcomes in different patients. Mutations in *NPHP2* result in nephronophthisis with or without *situs inversus* and mild cardiac defects [23] whereas *NPHP3* mutations lead to isolated nephronophthisis or retinal degeneration.[47] Alternately, *NPHP3* mutations can cause a broad clinical spectrum of early embryonic patterning defects comprising of *situs inversus*, congenital heart defects, central nervous system malformations

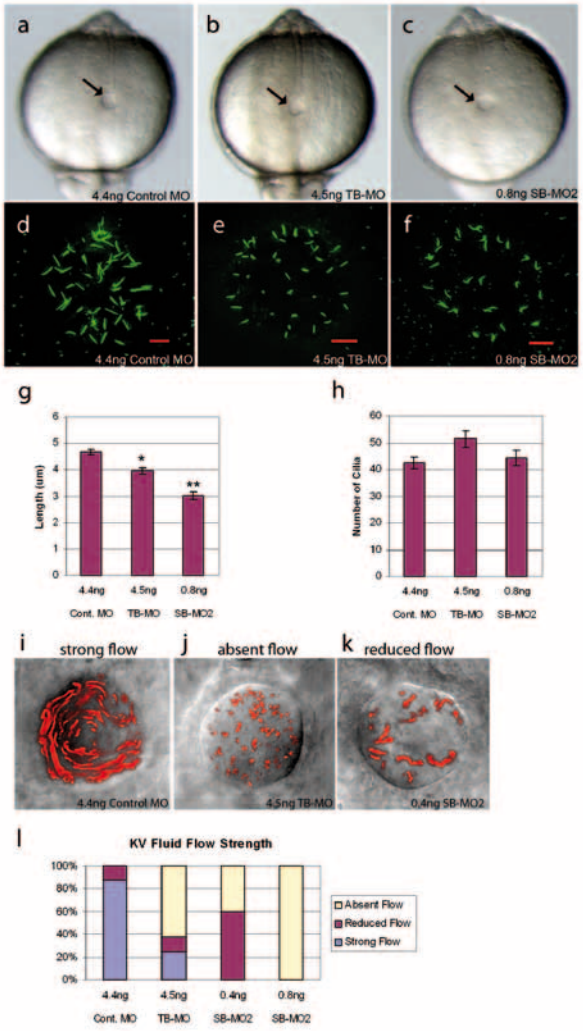

Figure 11 *nphp4* knockdown shortens cilia and disrupts fluid flow in Kupffer's vesicle (KV). (a-c) KV (arrow) appeared similar in live control MO (a), *nphp4* TB-MO (b) and *nphp4* SB-MO2 (c) embryos at 8 SS. (d-f) Visualization of KV cilia using anti-acetylated tubulin antibodies at 8 SS revealed shorter cilia in *nphp4* MO embryos (e,f), relative to controls (d). Red scale bar represents 10 μM. (g-h) Graphs show the average KV cilia length (g) and number (h) in control MO (n=38), *nphp4* TB-MO (n=21) and *nphp4* SB-MO2 (n=25) embryos at 8 SS. Error bars represent standard error of the mean. $*p<0.0001$ and $**p<3x10^{-12}$ when compared to control MO embryos (Student's t-test). (i-k) Fluorescent bead paths (red) superimposed on images of KV of representative embryos at 8 to 10 SS. Strong directional flow (i) was observed in most control MO injected embryos (i), whereas flow was often absent (j) or reduced (k) in *nphp4* TB-MO and *nphp4* SB-MO2 embryos. (l) The percentage of embryos classified with a strong, reduced or absent fluid flow. Embryos were injected with control MO (n=17), *nphp4* TB-MO (n=8), 0.4 ng (lower dose) *nphp4* SB-MO2 (n=5) or 0.8 ng *nphp4* SB-MO2 (n=4).

and renal-hepatic-pancreatic dysplasia.[24] The *NPHP6* gene (*CEP290*) is another good example. The phenotypic spectrum of the mutations ranges from isolated blindness, SLSN, nephronophthisis, Joubert syndrome, Bardet-Biedl syndrome, to the lethal Meckel-Grüber syndrome. [48].

We investigated the presence of *NPHP4* variants in 146 sporadic patients having cardiac laterality defects, with or without involvement of other thoracic or abdominal organs. In 6% of the patients, we identified heterozygous missense variants compared to 1.2% of the ethnically matched controls, indicating mutation excess in the patients ($p<0.006$). No compound heterozygous or homozygous variants were detected in these sporadic cases. Similarly, single heterozygous *NPHP4* variants were found in the majority of patients with autosomal recessive nephronophthisis type 4.[39] A second mutation might be located in an area not covered by exon sequencing or in another (cilia-related) gene. The latest, a complex genetic model with combined effects of multiple genes seems the most plausible explanation. In fact, di- or oligogenic inheritance have been demonstrated in several ciliopathies, including the nephronophthisis [21,49], Joubert syndrome [50] and Bardet Biedl syndrome.[51-52]

The findings in our study are entirely consistent with a complex, oligogenic disease model. The rare heterozygous variants identified in the sporadic cases have probably an epistatic effect with additional genetic modifiers. Even in the index consanguineous family, we cannot exclude the existence of other genetic variants that explain the complex cardiovascular malformations and heterotaxy and the lack of renal/visual disease.

In congenital heart malformations and heterotaxy, the NODAL signaling pathway is a paradigm for oligogenic inheritance. Some patients with heterotaxy and/or conotruncal defects such as double outlet right ventricle (DORV) and transposition of great arteries (TGA), show several mutations in genes belonging to the NODAL signaling pathway.[53-54] As functional significance of mutations in these genes were demonstrated, the cumulative effects of multiple mutations may lead to reduced NODAL signaling eventually resulting in congenital heart malformations. In addition, a combinatorial role between the NODAL signaling pathway and *ZIC3* gene has been demonstrated in familial TGA patients.[55] These studies support the notion that genetic variants or susceptibility alleles within one or more developmental pathways may dysregulate signaling in a synergistic fashion and cause congenital heart malformations or heterotaxy.

Studies in humans, zebrafish and mice indicate that *NPHP2* and *NPHP3* play a role in L-R axis determination.[22-24,47] To investigate the role of *NPHP4* in establishing L-R asymmetry, we used antisense MOs to knockdown expression of zebrafish *nphp4*. Depletion of *nphp4* in zebrafish resulted in abnormal heart and gut orientation, closely resembling the (cardiac) laterality defects observed in the patients.

Co-injection of *nphp4* TB-MO and human wt *NPHP4* mRNA significantly ameliorated the phenotypic spectrum due to *nphp4* depletion. In contrast, co-injection of *nphp4* TB-MO and human *NPHP4* mRNA containing genetic variants found in patients failed to rescue the laterality defects suggesting that these variants are pathogenic. Furthermore, analysis of asymmetric gene expression revealed that *nphp4* knockdown alters asymmetric Nodal expression in the LPM without affecting expression of midline markers.

Our analyses in zebrafish have confirmed that knockdown of *nphp4* results in shortened motile cilia.[56] For first time we show that *nphp4* depletion leads to disruption of cilia-driven fluid flow within KV which most likely cause laterality defects. Similarly, *nphp3* knockdown in zebrafish leads to *situs inversus* and heterotaxy due to defective (fewer and shorter) KV cilia.[57]

In conclusion, we identified *NPHP4* mutations in patients with cardiac laterality defects and other malformations within the heterotaxy spectrum. In zebrafish, our results demonstrate that *nphp4* is required for global L-R patterning of the embryo via regulation of Nodal signaling and plays a role that is essential for the development and function of KV cilia.

The linking of *NPHP4* to L-R axis determination and laterality defects will help dissect the complex genetic composition of heterotaxy and related cardiovascular malformations.

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FIRST LOCUS FOR PRIMARY PULMONARY VEIN STENOSIS MAPS TO CHROMOSOME 2Q

CHAPTER 3

ABSTRACT

Aims: Primary pulmonary vein stenosis (PVS) is a rare cardiac abnormality that exhibits a high morbidity and mortality rate. The disease is characterized by obstruction of the pulmonary venous blood flow owing to congenital hypoplasia of individual extra-pulmonary veins. We describe a consanguineous Turkish family with four affected siblings with primary PVS in association with prenatal lymphatic abnormalities. We aimed to map the first gene for primary PVS.

Methods and results: Patients had extensive cardiologic examinations including electrocardiograms, echocardiograms, ventilation-perfusion scans and cardiac catheterizations. All patients died before the age of 16 months because of severe progressive primary PVS. Chromosomal analysis revealed normal karyotypes.

We performed a genome-wide linkage analysis using 250K single nucleotide polymorphism arrays and found the first locus for primary PVS on chromosome 2q35-2q36.1 (multipoint logarithms (base 10) of odds (LOD) scores 3.6). By fine-mapping with microsatellite markers, we confirmed the homozygous region that extended 6.6 Mb (D2S164-D2S133). Sequencing 12 (188 exons) of the 88 genes from the region revealed no disease-causing sequence variations.

Conclusions: Our findings open perspectives for the identification of the genetic cause(s) leading to PVS, which might contribute to elucidate the pathologic mechanisms involved in this disorder.

INTRODUCTION

Primary pulmonary vein stenosis (PVS) is a rare cardiac abnormality which occurs in about 0.4% of all cardiovascular malformations.[1] It has a high morbidity and mortality rate. It is characterized by obstruction of the pulmonary venous blood flow owing to congenital hypoplasia of individual extrapulmonary veins or constriction of the intima at or near the venous-atrial junction.[2]

The condition can be an isolated anomaly but in up to 80% of patients it coexists with a variety of other congenital heart malformations such as patent ductus arteriosus and septal defects.[3-5]

The pulmonary venous obstruction can involve one or multiple (normally connected) veins. Symptoms present usually during the first months to years of life and are determined by the extensiveness of the PVS.[4-6] The most frequent presenting symptoms are failure to thrive, dyspnoea and recurrent pneumonias. Later, pulmonary hypertension, pulmonary oedema, haemoptysis and congestive heart failure may develop. It is difficult to differentiate the symptoms from chronic lung disease and from other causes of pulmonary venous hypertension.[7-8] Currently, most surgical therapies are unsuccessful due to restenosis and progression of the disease[9] and the majority of the patients die during the first years of life.

Secondary or acquired PVS is a similar condition that has various causes at different ages. In children it can be a complication following surgery for correction of partial or total anomalous pulmonary venous return and occurs in about 5-15% of operated patients.[10] In adults it is a well-recognized complication after radiofrequency ablation for atrial fibrillation in patients refractory to treatment with antiarrhythmic drugs.[11]

In both the primary and secondary forms of PVS, histological findings are similar and show variable manifestations of neointimal proliferation leading to occlusion of the lumen of one or more of the pulmonary veins.[12-14] The underlying mechanism for the pathologic manifestations has never been elucidated.

So far only sporadic patients with PVS have been reported. We describe a consanguineous Turkish family with four affected siblings with primary PVS in association with prenatal lymphatic anomalies. We performed a genome-wide linkage analysis and identified the first locus for primary PVS.

METHODS

Clinical studies

Both parents (third cousins) were healthy. Out of six pregnancies, three children were born alive and all died before the age of 16 months because of severe progressive primary PVS (Fig.1). In two children transient increased (septated) nuchal translucency and signs of fetal hydrops were present at prenatal ultrasound examinations. Furthermore, one pregnancy was terminated at 22 weeks because of severe fetal hydrops, indicating an affected foetus. Two pregnancies resulted in early spontaneous abortions.

Chromosomal analysis revealed a normal karyotype in three affected patients and the healthy parents. Detailed post-mortem macroscopic and microscopic examina-

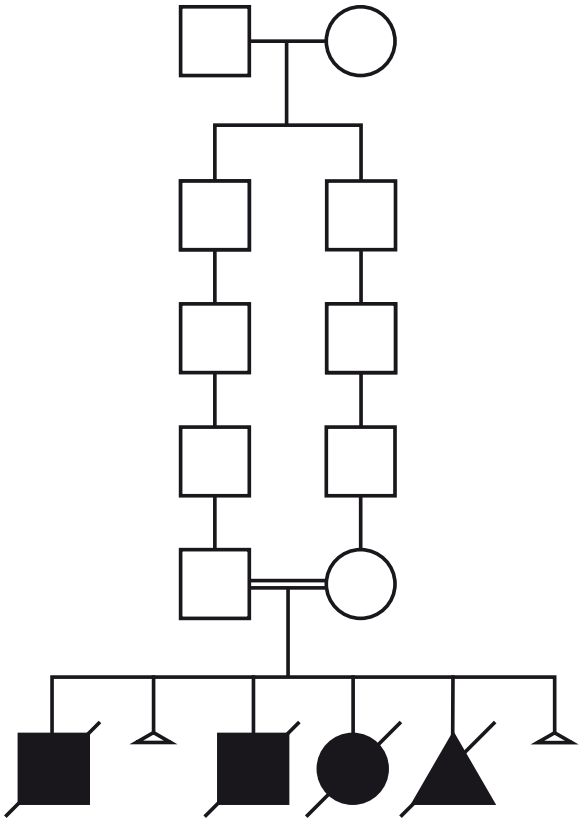

Figure 1: Simplified genealogical tree of the consanguineous family with pulmonary vein stenosis (PVS). Squares indicate males, circles represent females and triangles indicate miscarriages (small triangle) or stillborn (regular sized triangle). Filled symbols represent individuals presenting PVS and/or prenatal lymphatic abnormalities. Unfilled symbols: normal phenotype.

tion of the heart and lungs of the 22-weeks-old foetus was performed. The parents declined autopsy in all three live born patients. Both parents had a cardiologic examination that included echocardiogram and electrocardiogram. Informed consent for DNA studies was obtained from the parents.

Molecular studies

Genotyping

Genomic DNA was isolated from peripheral blood using the Puregene DNA purification kit (Gentra Systems) and from chorion villi cells (one foetus) using standard procedures. A DNA sample from another deceased patient was obtained from stored tissue material (lung biopsy, paraffin-embedded tissue). The genome wide search was conducted using DNA from five members of the family including both parents and three patients (two live born children and one foetus).

The Affymetrix GeneChip Mapping 250K *Nsp* Array containing 262 264 single nucleotide polymorphism (SNP) markers was used. Samples were processed according to the manufacturer's instructions (Affymetrix GeneChip Mapping Assay). Affymetrix GCOS v1.4, and GTYPE software v4.1 were used.

Microsatellite markers

Microsatellite markers mapping to the identified genomic region were selected. Polymerase chain reaction (PCR) products were run on an ABI Prism 3100 genetic sequencer (Applied Biosystems) and analyzed using the GeneMapper software v.3.0 (Applied Biosystems).

Sequencing analysis

Bidirectional sequencing of the coding region and the exon-intron boundaries of candidate genes was undertaken using PCR primers designed by Primer3 software. PCR products were purified and sequenced using BigDye Terminator chemistry v3.1 on an ABI Prism 3130*xl* genetic analyzer (Applied Biosystems). Sequences were aligned and compared with consensus sequences obtained from the human genome databases using the Applied Biosystems software package SeqScape v2.5.

Linkage analysis and loci identification

Genetic linkage analysis was performed to estimate whether two loci (in this case the disease gene and a set of SNPs) are in close proximity to each other on a chromosome. Logarithms (base 10) of odds (LOD) scores, a measure of the likelihood of genetic linkage between loci, were calculated. The statistical package EasyLinkage Plus v5.08[15] designed to perform automated linkage analyses using large-scale SNP

data, was used to perform all analyses. All SNPs showing inconsistency in transmission were removed from further analyses. Allegro v1.2c software (incorporated in the EasyLinkage Plus v5.08 package) was used to perform fully automated single point and multipoint linkage analysis. LOD scores were obtained using a recessive model of inheritance, with a penetrance of 99% and a disease allele frequency of 1:10 000. Allele frequencies of genotyped SNPs were set to codominant. Map order and genetic inter-SNPs distances were taken from the Affymetrix website.

Since closely spaced SNP markers were used for the linkage analysis, the genome analyses were performed with predefined spacing of 0.2 to 0.4 cM, in blocks of 90 and 100 SNPs. Then, single chromosomes showing positive linkage signals were independently analysed under the same conditions and haplotypes were constructed.

Graphical visualization of haplotypes to facilitate inspection and analyses was performed with HaploPainter v029.5[16]; a tool for drawing pedigrees with complex haplotypes.

RESULTS

Clinical studies

The family (Fig. 1) was referred to the Department of Clinical Genetics for genetic counselling after the death of their first child because of primary PVS. During a period of 9 years, three other affected children were born.

Patient 1

The first child was born after an uneventful pregnancy. At the age of 10 months the boy presented with a failure to thrive. Physical examination revealed no abnormalities. However, an X-ray of the thorax showed an increased pulmonary vessel pattern suggesting pulmonary venous obstruction. Electrocardiogram (ECG) was normal but Doppler-echocardiography revealed an increased flow (2 m/s; normal < 1.6 m/s) in the left pulmonary vein (LPV) and a patent foramen ovale with a minimal left-right shunt. Angiography by cardiac catheterisation revealed a severe stenosis of the LPVs near the entrance of the left atrium and the right pulmonary veins (RPVs) could not be visualised. A ventilation-perfusion scintigraphy revealed no perfusion of the right lung. A magnetic resonance imaging (MRI) scan revealed two normally connected LPVs which were stenotic about 2 cm from the entrance of the left atrium and an atretic remnant of the RPVs near the entrance of the left atrium (Fig. 2). At the age of 13 months, the boy was operated and a venoplasty was performed on the LPVs and RPVs. In the consecutive months the child developed respiratory insufficiency due to

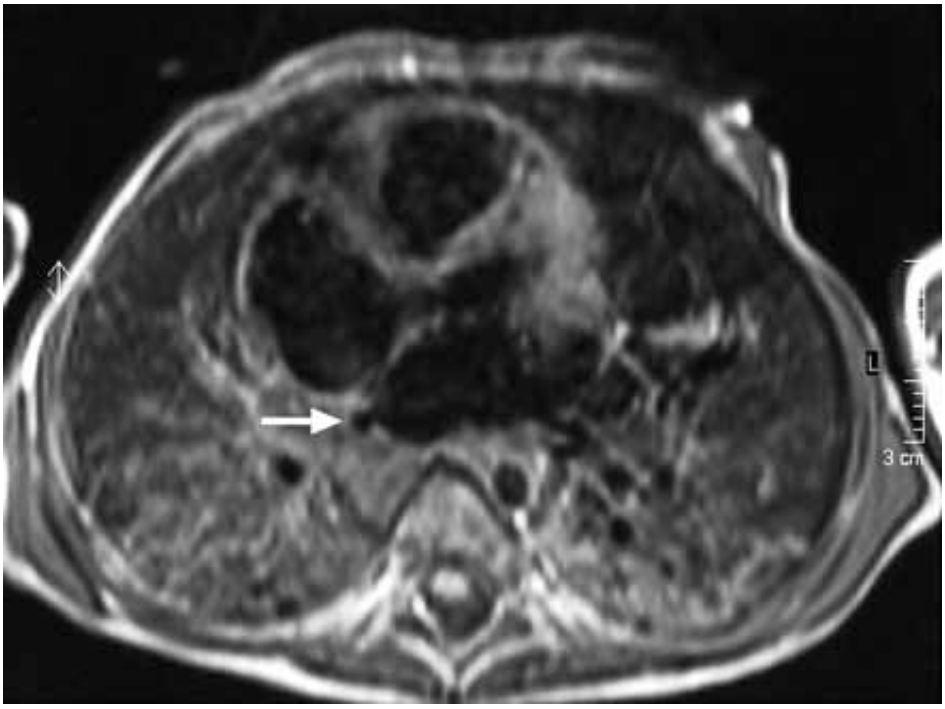

Figure 2: Magnetic resonance image scan of heart and lungs of patient 1 with the atretic remnant of the right pulmonary veins (white arrow).

progressive PVS. The boy had recurrent haemoptysis and subsequent melena. He died at the age of 15.5 months due to respiratory insufficiency.

Patient 2

A prenatal ultrasound revealed an increased nuchal translucency of 11 mm and mild generalized skin oedema at a gestational age of 14 weeks (Fig. 3a). At gestational ages of 19 and 31 weeks, the nuchal translucency and skin oedema were no longer observed. Advanced ultrasound examinations revealed no structural abnormalities and a normal four-chambered view. Normal connecting pulmonary veins with normal venous Doppler signals were observed. A boy was born at term with normal birth measurements. No association between the transient prenatal findings including nuchal translucency and skin oedema and PVS was suspected yet.

At the age of 2.5 months, the child had feeding problems and tachypnoea. Physical examination and cardiologic examination, including ECG and two-dimensional echocardiography revealed no abnormalities. Transoesophageal Doppler-echocardiography showed an increased inflow velocity (1.8 m/s) at the entrance of the LPV and a patent foramen ovale with a haemodynamically insignificant left-right shunt. Angiography by cardiac catheterisation displayed dilated pulmonary veins

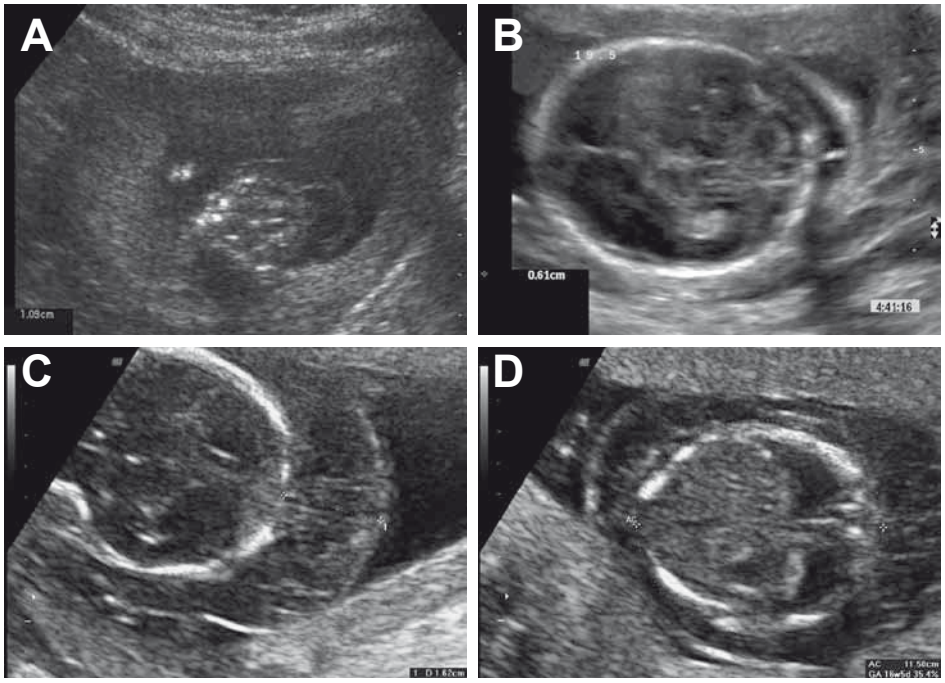

Figure 3: Prenatal ultrasounds performed in (a) patient 2 (II-5) at 14 weeks of gestation showing increased nuchal translucency of 11 mm. (b) patient 3 (II-6) at 19 weeks of gestation showing septated nuchal cystic hygroma of 6 mm. (c) and (d): Patient 4 (II-7) 19 weeks of gestation showing a large nuchal cystic hygroma (c) and bilateral hydrothorax (d).

with severe stenotic lesions near the entrance into the left atrium. The right upper vein was not displayed. A ventilation-perfusion scintigraphy revealed complete absence of perfusion of the right upper and a partial abnormal perfusion of the left lower pulmonary lobe. At the age of 6 months, he was operated and a complete stenosis of the left lower and right upper pulmonary veins until the lung parenchyma was confirmed. A stenosis of the left upper and right lower pulmonary vein near the entrance of the left atrium was seen. A venoplasty of both the left and right PVS was performed. One month after surgery, the boy was admitted to the hospital owing to feeding problems and progressive tachypnoea due to restenosis of the pulmonary veins. He died at the age of 7.5 months because of respiratory insufficiency.

Patient 3

In the fourth pregnancy a septated nuchal translucency of 11 mm was detected at a gestational age of 10 weeks. At 19 weeks, the nuchal translucency evolved and a septated sonolucency with a thickness of 6 mm was seen, most likely representing a cystic hygroma (Fig. 3b). Advanced ultrasound examinations revealed no structural abnormalities, in particular no heart abnormalities. A girl was born at a gestational

age of 39 weeks with normal birth measurements. Soon after birth, she was admitted at the hospital due to haemoptysis and subsequent melena. A cardiac catherisation was performed and revealed a PVS at the left side. There was thrombocytopaenia and anaemia.

At the age of 1.5 months, a cardiac catheterization was performed which revealed a progression of the LPV stenosis and appearance of RPV stenosis. A balloon dilatation was performed on the left side. At the age of 2 months, the girl died due to respiratory insufficiency caused by the PVS.

Patient 4

In the fifth pregnancy, a septated nuchal translucency of 7 mm and fetal hydrops was detected at gestational age of 13 weeks. A chorionic villus sampling was performed at a gestational age of 13.5 weeks, revealing a normal male karyotype. At 19 weeks of gestation there was massive hydrops with a large nuchal cystic hygroma, bilateral hydrothorax, pericardial effusion and ascites (Fig. 3c and d). Different causes of fetal hydrops including feto-maternal blood group antagonism and maternal infections (TORCHS and Parvovirus) were excluded. Normal peak velocity of systolic blood flow in the middle cerebral artery was obtained by Doppler ultrasonography indicating no signs of foetal anaemia. Because of the poor prognosis, the pregnancy was terminated at a gestational age of 22 weeks. A severely hydropic foetus was born with a nuchal hygroma. Autopsy of the heart and lungs revealed no macroscopic abnormalities, in particular, normally connecting pulmonary veins with no signs of stenosis. Microscopic evaluation showed no structural abnormalities except for a dilatation of the lymphatic vessels in the lungs.

Genome wide linkage analysis (GWLA)

The linkage analysis revealed a large homozygous region on chromosome 2q (199-232 cM) with a significant multipoint LOD score of 3.6. This locus was detected through all the analysis models applied (SNP spacing varying from 0.2 to 0.4 cM and different block sizes containing 90-100 SNPs).

We constructed haplotypes on chromosome 2q (199-232 cM) using all informative SNPs in the area. A recombination event occurring in the maternal haplotype determined the upper border of the region between rs2372938 and rs722082 (217.9 Mb), the first informative SNP that was homozygous for all three patients. A recombination occurring in patient II-6 (rs2043566, 237.51 Mb) delimited the telomeric border (data not shown).

We observed an area of approximately 1820 consecutive SNPs for which the patients were (mostly) homozygotes. We noticed that seven of the 1,820 SNPs were heterozygous for at least two of the patients. However, direct DNA sequencing of the

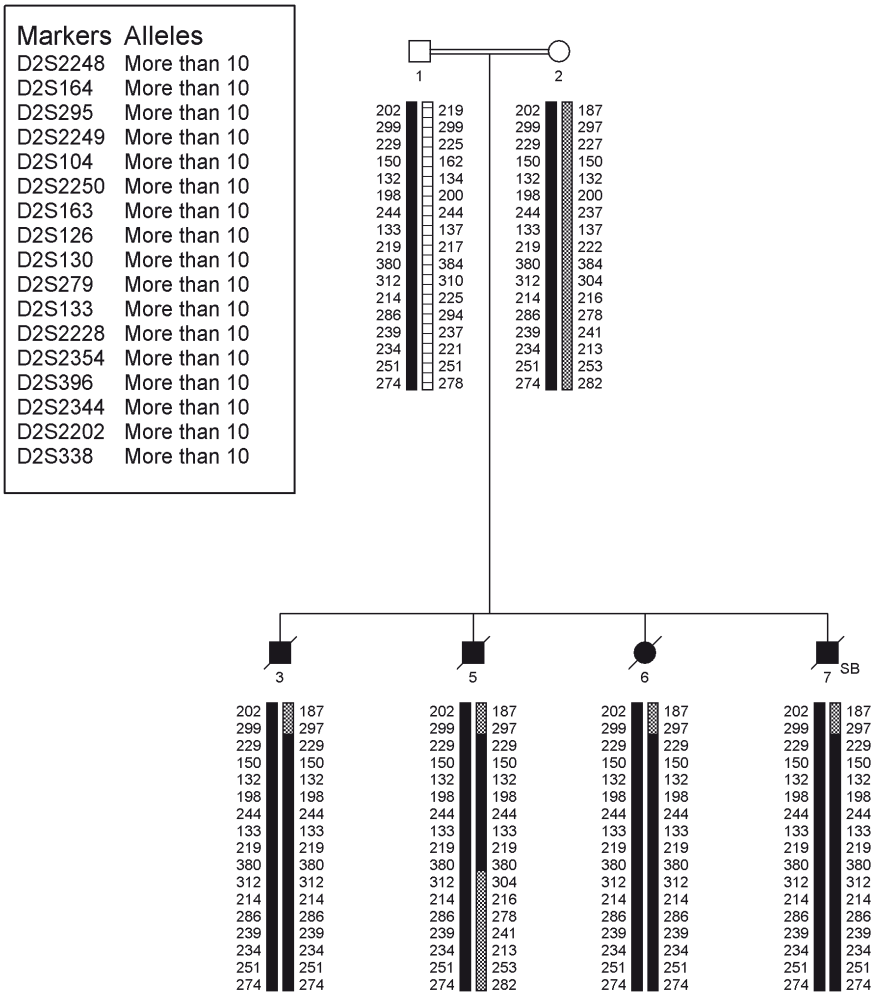

Figure 4: Family tree showing microsatellite haplotypes corresponding to the 2q35-2q36.1 region. The most informative markers are shown; allele numbers are indicated in base pairs. A black bar represents the haplotype segregating with the disease. The maximum candidate region is extending 9.6 cM (6.6 Mb) from the marker D2S164 (214.7 cM) until D2S133 (224.3 cM) containing 88 genes (NCBI build 36.3). SB: stillbirth

region revealed only homozygous genotypes indicating that the previously observed heterozygous SNPs (from array raw data) were likely incorrect genotype calls. In order to confirm these results, we tested 28 microsatellite markers mapping to the 2q35-2q36.1 area. Then we could include DNA samples from all four affected individuals (Fig. 4). Because of a recombination event observed in patient II-5, the maximum candidate region was considerably reduced to 6.6 Mb (9.6 cM) from marker D2S164 (217.7 Mb, 214.7 cM, very close to the border indicated by the SNP analysis) until D2S133 (224.3 Mb, 224.3 cM) containing 88 genes from which 14 have been associated to distinct phenotypes (National Center for Biotechnology Information, NCBI build 36.3). The minimum region was covering 5.9 Mb (8.5 cM) between D2S295 (218 Mb, 215.8 cM) and D2S279 (223.9 Mb, 224.3 cM) after combining genotypes (SNPs and microsatellites) from all patients.

We selected 12 positional candidate genes for further sequence analysis based on protein and molecular function, expression data, and animal models (Table 1). Unfortunately, no novel sequence changes were found.

We also used the SNP data to investigate possible disease causing copy number variations (CNVs). Genome-wide analysis did not reveal any CNV co-segregating with the PVS phenotype.

DISCUSSION

Primary PVS is a rare congenital heart malformation that has so far only been described in sporadic cases. We describe a family with PVS associated with prenatal lymphatic anomalies in four affected siblings suggesting a monogenic cause of the disease. An autosomal recessive mode of inheritance is most likely since the parents are consanguineous and have no signs or symptoms of PVS on cardiological examination.

This is the first report of prenatal lymphatic abnormalities in patients with congenital PVS. We propose that the prenatal lymphatic abnormalities are part of the PVS phenotype like it has been reported before for other syndromic and non-syndromic forms of congenital heart malformations, such as Noonan syndrome and Turner syndrome.[17-19] The disease might be underdiagnosed because of the high intrauterine demise and neonatal death that is associated with hydrops fetalis and owing to the evanescent nature of the increased nuchal translucency.

Increased nuchal translucency, nuchal cystic hygroma and hydrops fetalis have numerous causes of which chromosome abnormalities, infections and congenital malformations, in particular cardiovascular diseases, are the most frequent.[17,20-21] In our patients, both chromosomal abnormalities and infections were excluded. Two of

Table 1. Candidate functional genes sequenced in one patient of the pulmonary vein stenosis family

Gene	No. of exons	Reason for selection	Reference Pubmed no.
Tensin 1 (TNS1)	33	Highly expressed in heart. Focal adhesion protein. Transmembrane junction between extracellular matrix and cytoskeleton. Tyrosine phosphorylation of tensin 1 among others induced by platelet derived growth factor (PDGF), thrombin and angiotensin.	11792844
Angio-associated migratory cell protein (AAMP)	11	High level of expression in endothelial cells. Function in the regulation of endothelial tube formation. Important functional role in the migration of smooth muscle cells during development.	7743515, 10329261, 18634987
Serine/threonine kinase 16 (STK16)	8	*STK16* is involved in *VEGF* expression regulation.	16310770
Desmin (DES)	9	A muscle-specific cytoskeletal protein found in smooth, cardiac, and skeletal muscles. Desmin is required to strengthen and maintain the integrity of these tissues.	8626040
Striated muscle preferentially expressed gene (SPEG)	41	Highly expressed in differentiated vascular smooth muscle cells which may have a role in regulating growth and differentiation of this cell type.	8663449, 10973969
Ephrin receptor A4 (EPHA4)	18	*Ephrin-Eph* signaling is required not only for embryonic vascular development but also for angiogenesis by modulating endothelial cell migration and/or proliferation.	12775584, 12615978
Sphingosine-1-phosphate phosphotase 2 (SGPP2)	5	Highly expressed in heart. *SGPP2* regulates local concentration of *sphingosine-1-phosphate (S1P)*. S1P is a signaling molecule that affects cell proliferation and migration, in a variety of cardiovascular cell types including vascular smooth muscle cells, cardiomyocytes, and endothelial cells. S1P signaling is also important for cardiac development.	12411432, 16434032
Potassium voltage-gated channel, Isk-related family, member 4 (KCNE4)	2	KCNE4 is highly expressed in the atria. KCNE4 exerts dominant effects on slowly activating delayed rectifier potassium current. K(+) channels are present and functionally important in rat pulmonary veins. Pulmonary vein cardiomyocytes form sphincters rich in K(ir) channels, which may modulate venous return both physiologically and in disease states.	15698834, 11350792

Secretogranin II (SCG2)	2	The secretoneurin peptide derived from SCG2(human SCG2165 – 187) stimulates migration and proliferation of vascular smooth muscle cells and acts as an endothelial cytokine to promote angiogenesis and vasculogenesis providing a direct link to vascular development and remodelling.	15326074, 14970115
C-terminal domain of small phosphatase 1 (CTDSP1)	7	*CTDSP1* regulates the *BMP* and the *TGFbeta* pathways by dephosphorylating the linker regions of *Smad1* and *Smad2* but do so with different outcomes depending on the pathway.	17085434
Serine/threonine protein kinase 36 (STK36)	27	*STK36* is able to enhance the gene activator function of the Gli transcription factors.	10806483
Serine/threonine protein kinase 11 interacting protein (STK11IP)	25	*LIP1* interacted with the *TGF-beta*-regulated transcription factor *SMAD4.*	11741830

the three patients with prenatal lymphatic abnormalities developed severe stenosis of the pulmonary veins. In the third patient, the foetus, abnormal dilatation of the lymphatic vessels in the lungs was found on microscopic examination, without gross abnormalities of the pulmonary veins. This foetus was considered affected, as the prenatal presentation (nuchal cystic hygroma and features of hydrops) was similar to the abnormalities observed in the previous affected children. The absence of pulmonary veins occlusion at autopsy was not unexpected, as symptoms or signs of PVS were absent at birth in his affected siblings but developed postnatally.

The dilatation of the lymphatic vessels might suggest that they are not secondary to venous obstruction in the lungs, but owing to abnormalities of the lymphatic vessels and/or lymphatic vessel connections itself. Pulmonary venous flow is limited during foetal life and lymphatic abnormalities or hydrops are usually not present in other conditions in which pulmonary venous obstruction occurs in prenatal life, such as partial or total anomalous pulmonary venous return. Another option is that the dilatation of the lymphatic vessels might be owing to increased drainage of fluid from the lungs by this route.

The mechanism by which increased nuchal translucency and hydrops in both syndromic and non-syndromic patients with congenital heart malformations are produced remains unclear. A mechanistic theory is that there is a primary disorder of (lymph) angiogenesis or endothelial function which could cause both collections of nuchal fluid and also cardiac malformations.[22] Since the lymphatic system originates from lymphatic endothelial cells that sprout from the embryonic veins and then migrate, it might be possible that genetic defects in proteins essential for these lymphatic and venous endothelial cells lead to a disturbed venous-lymphatic phenotype.[23]

It remains obscure why the pulmonary veins were apparently normal at birth and PVS developed only after birth. As the pulmonary veins of the aborted foetus were normal at 22 weeks of gestation and all affected neonates had initially no clinical abnormalities suggesting PVS, the disease might have been induced by the introduction of oxygen in the pulmonary venous circulation, or hemodynamic changes in the pulmonary circulation such as increased blood flow.

The mechanism by which both primary and secondary PVS develop might be similar. Histopathologic examination of specimen of patients with isolated primary PVS revealed that the disease is caused by eccentric abnormal intimal proliferation of spindle-shaped cells in the pulmonary veins. The lesional cells stain positive for smooth muscle actin and muscle-specific actin and have the histologic appearance of myofibroblasts.[13] Further immunohistochemical analysis of the lesional cells in primary PVS showed expression of receptor tyrosine kinases.[24] Defining the origin of these smooth muscle-like cells is challenging. Pathological studies of specimens of patients with secondary PVS are obviously rare but some studies revealed neointimal fibromuscular proliferation both in areas close to and remote from the site of operation or catheter ablation.[12,14] The fact that the pathological findings in secondary PVS are similar to primary PVS are pointing to a comparable disease pathogenesis.

PVS should be differentiated from (intra)pulmonary veno-occlusive disease (PVOD), that is characterized by obstruction of small pulmonary veins and usually develops in adulthood.[25] In contrast, PVS is caused by obstruction of the extrapulmonary veins at the venoatrial junctions. It might be possible that PVOD develops secondary to chronic PVS due to pulmonary hypertension as is described both in patients with primary and secondary PVS.[7,14,26] Heterozygous mutations in the *bone morphogenetic protein receptor type II (BMPR2*) have been described in patients with PVOD. Mutation analysis of *BMPR2* in one of our patients revealed no pathogenic mutations.

We performed a GWLA and found a candidate interval on chromosome 2q35-2q36.1 comprising maximum 9.6 cM (6.6 Mb) and containing 88 genes. This interval includes some interesting candidate functional genes (Table 1). Among them, *sphingosine-1-phosphate phosphotase 2*, (*SGPP2,* involved in controlling cell proliferation and migration in a variety of cell types including vascular smooth cells, cardiomyocytes and endothelial cells) seemed particularly promising. Unfortunately, sequencing of these genes revealed no disease causing sequence variations so far. Finding other patients with homozygosity on the chromosome 2q region may help reduce the number of genes to investigate.

Our findings open perspectives for the identification of the genetic cause(s) leading to PVS, which might contribute to elucidate the pathologic mechanisms involved in this disorder. The contribution of this gene to other cases with primary or secondary PVS remains to be elucidated.

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WEB RESOURCES

The URLs for data presented herein are as follows: http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi; http://genome.ucsc.edu; http://www.ncbi.nlm.nih.gov

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AUTOSOMAL DOMINANT INHERITANCE OF CARDIAC VALVES ANOMALIES IN TWO FAMILIES: EXTENDED SPECTRUM OF LEFT-VENTRICULAR OUTFLOW TRACT OBSTRUCTION

CHAPTER 4

ABSTRACT

Only a limited number of families with clear monogenic inheritance of nonsyndromic forms of congenital valve defects have been described. We describe two multiplex pedigrees with a similar nonsyndromic form of heart valve anomalies that segregate as an autosomal dominant condition. The first family is a three-generation pedigree with 10 family members affected with congenital defects of the cardiac valves, including six patients with aortic stenosis and/or aortic regurgitation. Pulmonary and/or tricuspid valve abnormalities were present in three patients, and ventricular septal defect (VSD) was present in two patients. The second family consists of 11 patients in three generations with aortic valve stenosis in seven patients, defects of the pulmonary valves in two patients, and atrial septal defect (ASD) in two patients. Incomplete penetrance was observed in both families. Although left ventricular outflow tract obstruction was present in most family members, the co-occurrence with pulmonary valve abnormalities and septal defects in both families is uncommon. These families provide evidence that left-sided obstructive defects and thoracic aortic aneurysm may be accompanied by right-sided defects, and even septal defects. These families might be instrumental in identifying genes involved in cardiac valve morphogenesis and malformation.

INTRODUCTION

Anomalies of the atrioventricular and semilunar heart valves and associated structures account for 25 –30% of all congenital cardiovascular malformations (CVM).[1] Most occur sporadically in a single patient without affected family members, and unassociated with other malformations (non-syndromic). On the other hand, well-defined syndromes with autosomal dominant inheritance, such as Noonan syndrome (caused by *PTPN11*, *KRAS*, *SOS1* or *RAF1* mutations), and Alagille syndrome (caused by *JAGGED1* and *NOTCH2* mutations) are often associated with valve defects.[2-4]

Only a limited number of families with clear monogenic inheritance of nonsyndromic forms of congenital valve defects have been described. Familial nonsyndromic valve anomalies often include either the left-sided heart valves (aortic and mitral valve) or the right-sided heart valves (tricuspid and pulmonary valve). Left-sided valve anomalies can be part of a spectrum of anomalies of the left ventricular outflow tract referred to as LVOTO (left ventricular outflow tract obstruction; also known as obstructive anomalies of the left heart and aorta)[5-7], but in some families they occur without other LVOTO anomalies.[8-10] In two families with LVOTO anomalies including bicuspid aortic valve (BAV) with calcification, Garg *et al* documented truncating mutations in NOTCH1.[7] Two patients in these families also exhibited right-sided heart malformations, including double outlet right ventricle and tetralogy of Fallot. Bicuspid aortic valve underlies the majority of patients with aortic valve disease and familial BAV has been described in single families suggesting autosomal inheritance.[11-14] Studies on heritability of BAV support that genetic factors play a major role in BAV and demonstrate that BAV is often associated with other cardiovascular malformations, in particular LVOTO anomalies and thoracic aortic aneurysm (TAA).[5,15-16] Locus heterogeneity for familial BAV has been established in several studies[17-18], and genome-wide scans in families with BAV and/or associated CVM has demonstrated linkage to chromosomes 15q25-26, 18q, 5q and 13q.[18]

Another frequent left-sided heart valve anomaly, mitral valve prolapse, can be inherited as an autosomal dominant trait, and has been linked to chromosome 13.[19-20] Familial right-sided valve anomalies frequently represent syndromic forms of CVM and only a few nonsyndromic families are reported. Pulmonary stenosis (PS) is common in families with Noonan syndrome (*PTPN11* mutations), Watson syndrome (*NF1* mutations) and Alagille syndrome (*JAGGED1* mutations). Mutations in *PTPN11*, however, have not been convincingly shown to be present in patients with non-syndromic PS[21], although a few cases of possible nonsyndromic PS with *JAGGED1* mutations have been described.[22] Nonsyndromic familial right-sided valve anomalies have only been described in a few small families. PS has been reported in some families with clear autosomal dominant inheritance[23-24], and in some families

with unknown mode of inheritance.[25-30] PS combined with ASD in a large pedigree has been shown to be due to a mutation in the *GATA4* gene.[31] Familial pulmonic valve atresia and familial occurrence of tricuspid anomalies are very uncommon, and have only been described in a few families.[32-38]

Only a few families with combined left- and right-sided heart valve anomalies with monogenic inheritance have been described. A large family with autosomal dominant inheritance of mainly atrioventricular valve defects including Ebstein anomaly and atrioventricular canal has been described by Schunkert *et al*.[39] Atrioventricular septal defects (AVSD) (also known as endocardial cushion defects) can be inherited as an autosomal dominant trait with variable expression and incomplete penetrance. These valve anomalies can be due to mutations in the gene encoding the cell adhesion molecule *CRELD1*[40], and a second locus is located on chromosome 1p31-p21. A few families with X-linked valvular dysplasia, a condition characterized by myxomatous degeneration, valvular regurgitation and secondary calcification affecting all four heart valves have been described.[41-42] Recently mutations in the *FLNA* gene encoding filamin A were identified in these families.[43]

The paucity of multiplex families with a clear Mendelian inheritance pattern of nonsyndromic cardiac valve malformation has precluded the identification of human genes specifically involved in cardiac valve morphogenesis and malformation. We describe two families with a similar autosomal dominant form of congenital heart malformation mainly consisting of cardiac valve anomalies.

CLINICAL REPORTS

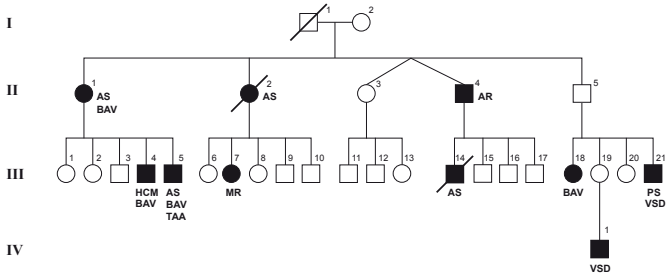
Family 1

The pedigree of family 1 is shown in Figure 1.

Patient 1. The index patient (II-1) visited our Department of Clinical Genetics for genetic counseling. She had aortic valve replacement at the age of 42 because of a severely stenotic BAV. She received two new aortic valve prostheses in the following 30 years, and at the age of 60 she developed atrial fibrillation. Three of the five children of patient II-1 were healthy, and cardiologic examination including ECG and echocardiography revealed no abnormalities. Two other children (patient 2 and 3) are affected.

Patient 2. One of the five children (III-4) of patient II-1 was asymptomatic until he presented with progressive dyspnea at the age of 44. Echocardiography revealed a BAV, a dilated left ventricle with thickening of the posterior wall, and left ventricular dysfunction.

Family 1



Family 2

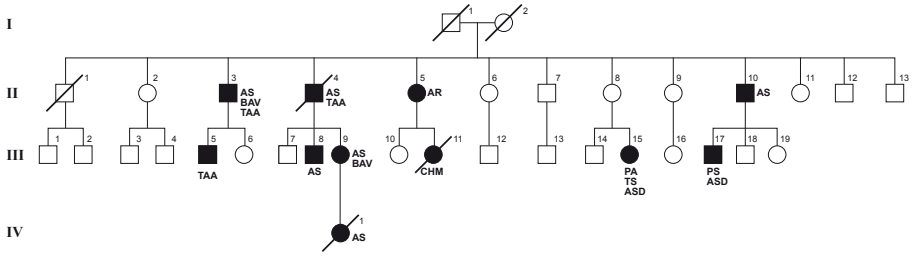


Figure 1 Pedigrees of two families with autosomal dominant inheritance of congenital valve anomalies. AR, aortic valve regurgitation; AS, aortic valve stenosis; ASD, atrial septal defect; BAV, bicuspid aortic valve; CVM Cardiovascular malformation; HCM, hypertrophic cardiomyopathy; MR, mitral valve regurgitation; PS, pulmonary valve stenosis; PA, pulmonary atresia; TAA, thoracic aortic aneurysm; VSD, ventricular septal defect.

Patient 3. Another son (III-5) of patient II-1 was diagnosed with a BAV with mild stenosis and regurgitation, and mild dilatation of the aorta at the age of 26 years. An X-ray of the thorax showed an elongated aorta. At the age of 36 years the aortic valve was stenotic, calcified and thickened, and mild tricuspid regurgitation was present. His left ventricular function remained good. Chromosomal analysis showed a normal male karyotype. A microdeletion of the 22q11 (TBX1 gene) and 7q11.23 (elastin gene) region was excluded by fluorescent in situ hybridization (FISH).

Patient 4. A sister (II-2) of patient II-1 was diagnosed with valvular aortic stenosis and regurgitation, mitral stenosis with regurgitation, and tricuspid regurgitation. She underwent three operation for aortic and mitral valve prostheses between the age of 40 and 44 years. At the age of 45 years a tricuspid valve correction was performed.

She died at the age of 50. In four of her five children cardiologic examination including ECG and echocardiography revealed no abnormalities.

Patient 5. A daughter (III-7) of patient II-2 was diagnosed with moderate mitral valve regurgitation at the age of 50 years.

Patient 6. A brother (II-4) of patient II-1 was diagnosed with aortic valve regurgitation and received an aortic valve replacement.

Patient 7. The son (patient III-14) of patient II-4 was diagnosed with aortic stenosis. Surgical correction was performed at the age of 9 years. He died one day after surgery.

Patient 8. One of the three daughters of patient II-5 (patient III-18) underwent valvulotomy for a severely stenotic BAV at the age of 9 years. She also received a mitral valve prosthesis for severe mitral valve regurgitation.

Patient 9. The brother of patient III-18 (patient III-21) was operated at the age of 5 years for a VSD and valvular PS. He developed a re-stenosis and pulmonary valve regurgitation. Patient 10. A son (IV-1) of an asymptomatic sister of patient III-18 was diagnosed with a perimembranous VSD at the age of 4 months.

Family 2

The pedigree of family 2 is shown in Figure 1.

Patient 1. The parents of patient (III-15) visited our Department of Clinical Genetics for genetic counseling when their daughter was born with pulmonary atresia with intact ventricular septum, double-chambered right ventricle and ASD. The aortic valve showed no abnormalities. Surgical correction was performed in the first year and again at the age of 5 years. At the age of 7 years an obstructive fibromuscular bundle in the right ventricle was removed, and a small tricuspid valve with prolapse of the leaflets was found during operation. The chordae were abnormally long and attached directly to the ventricle wall without papillary muscles. Chromosomal analysis showed a normal female karyotype. A microdeletion of the 22q11 region and 7q11.23 region were excluded by fluorescent in situ hybridization (FISH). Cardiac evaluation of both parents of patient 1 showed no abnormalities, the asymptomatic mother (II-8) was 53 years at examination.

Patient 2. The son (III-17) of a brother of the mother of patient III-15 was diagnosed at birth with a severe valvular and infundibular PS with intact ventricular septum and a secundum ASD. Surgical correction was performed at the age of 1 year. After a second operation at the age of 6 he died from hypoxic encephalopathy.

Patient 3. The father (II-10) of patient III-17 underwent cardiologic evaluation at the age of 35 years because of the familial CVM, and a valvular aortic stenosis was diagnosed. Chromosomal analysis and FISH of the 22q11 region showed no abnormalities.

Patient 4. A brother (II-4) of patient II-13 was diagnosed at the age of 31 years with a severe valvular aortic stenosis and regurgitation and dilatation of the ascending aorta. At the age of 32 years his severely calcified aortic valve was replaced by a Bjork Shiley prosthesis. Chromosomal analysis and FISH of the 22q11 region showed no abnormalities. He died suddenly at the age of 50 years.

Patient 5. The daughter (III-9) of patient II-4 was evaluated at the age of 7 years for a cardiac murmur, but was thought to have no abnormality. After giving birth to an affected child (IV-1) she was reevaluated and was diagnosed as having a stenotic BAV.

Patient 6. Patient III-9 gave birth to a girl (patient IV-1) with critical valvular aortic stenosis who died several days after birth.

Patient 7. A son (III-8) of patient II-4 was diagnosed with a severe valvular aortic stenosis and received aortic valve replacement.

Patient 8. A brother (II-3) of patients II-4 and II-10 was examined at the age of 47 years because of the familial CVM: echocardiography revealed a mildly stenotic, thickened BAV, with mild dilatation of the ascending aorta. At the age of 58 years the aortic root diameter was 53 mm and there was an ascending aorta aneurysm measuring 61 mm. There was left ventricular hypertrophy. A Bentall procedure including replacement of the ascending aorta and proximal aortic arch was performed. A severely calcified aortic valve was replaced. Pathological examination showed a calcified aortic valve and wall. Intima fibrosis was present and mild medial degeneration. The elastin fibers showed no abnormalities. The daughter of this patient (III-11) showed no abnormalities on cardiologic examination, including echocardiography.

Patient 9. A son (III-5) of patient II-3 was examined by the cardiologist and had mild dilatation of the ascending aorta (43 mm) without valvular abnormalities, left ventricular dysfunction or hypertrophy.

Patient 10. A daughter (III-11) of a sister (II-5) of patients II-8 died 12 days after birth with an enlarged heart.

Patient 11. Cardiologic evaluation of the mother (II-5) of patient 10 at the age of 43 years revealed no abnormalities, but reevaluation at the age of 57 years showed a sclerotic aortic valve with moderate regurgitation.

Additional family members. Cardiologic evaluation (echocardiography and ECG) of healthy family members in generation II (II-2, II-6, II-7, II-8, II-9 and II-11) showed no abnormalities. These healthy familymembers were between 39 and 64 years of age at the time of examination. The grandparents in generation I were not investigated. The grandfather died of a cardiac arrest at the age of 57 years.

MOLECULAR STUDIES

Linkage analysis using polymorphic microsatellites D9S1826, D9S158, and D9S1838 flanking the *NOTCH1* gene was performed in both families. This excluded *NOTCH1* as the disease gene in both families since multiple recombinants were found. Additionally, sequence analysis of all coding exons and intron-exon boundaries of the *NOTCH1* gene was normal in one affected patient in each family.

DISCUSSION

We describe two families with autosomal dominant inheritance of isolated CVM mainly involving the cardiac valves. None of the affected family members showed signs of a connective tissue disorder or malformation syndrome. Among the 10 affected family members of the first family, 6 were diagnosed with an abnormal stenotic and/or insufficient aortic valve. In three patients, regurgitation of the mitral valve was present. Pulmonary valve abnormality was diagnosed in one family member. In the second family, abnormal semilunar valves were present in nine family members, seven with abnormal aortic valves and two with defects of the pulmonary valves (Table 1). Different cardiologists evaluated patients and BAV may not always be reported if present. In both families, septal defects were present in several patients, which we believe may be part of the spectrum since they co-existed with valve anomalies in several family members. In the first family, one patient had a VSD and PS. In the second family, an ASD was present in a patient with PS and in a patient with pulmonary atresia. No other congenital abnormalities or dysmorphic features were present in any of the patients, indicating that the CVM in these families is nonsyndromic. In both families, autosomal dominant inheritance is well supported since there are three affected generations with male-male inheritance and expression in both females and males. Nonpenetrance is present in one obligate carrier in both families. In family 2, patient II-2 was unaffected at the age of 43 years but showed moderate aortic valve regurgitation 14 years later at the age of 57. Patient II-8 showed no abnormalities at echocardiography at the age of 53. She has a tri-leaflet aortic valve and normal function of cardiac valves and the left ventricle.

Autosomal dominant inheritance of nonsyndromic congenital valve anomalies has only been described in a limited number of families. In most cases consistence of valve anomalies of either predominantly left- sided or right- sided structures of the heart is present. In both presented families predominantly aortic valve anomalies were observed, although right-sided malformations such as pulmonary and tricuspid valve anomalies were present in some patients. This observation is also documented

Table 1. Families with cardiac valve abnormalities

	Family 1										Family 2										
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Anomaly	II-1	III-4	III-5	II-2	III-7	II-4	III-14	III-18	III-21	IV-1	III-15	III-17	II-10	II-4	III-9	IV-1	III-8	II-3	III-5	III-11	II-5
Aortic stenosis	+		+	+			+	+					+	+	+	+	+	+			
Bicupid aortic valve	+	+	+					+							+			+			
Thoracic aortic aneurysm			+											+				+	+		
Aortic regurgitation			+	+		+															+
Pulmonary atresia											+										
Pulmonary stenosis									+			+									
Mitral stenosis				+																	
Mitral regurgitation				+	+			+													
Tricuspid stenosis											+										
Tricuspid regurgitation				+																	
Atrial septal defect											+	+									
Ventricular septal defect									+	+											
Cardiomyopathy		+																			
Unspecified CHM																				+	

in other studies with smaller families (for instance only two persons affected) where patterns of inheritance are not always clear.[18,44-45] In a recent study demonstrating high heritability of hypoplastic left heart syndrome (HLHS) a high percentage of HLHS probands had both left- and right-sided valve dysplasia suggesting that HLHS is a severe form of valve malformation and anomalies of the left- and right-sided valves may have a common etiology.[46]

Human genes known to be involved in valvulogenesis include genes associated with elastogenesis and collagen synthesis, as elastin and collagen are major components of semilunar and atrioventricular valves. As mutations in most of these genes are associated with syndromic forms of CVM they are unlikely to be involved in our families with non-syndromic CVM. Familial BAV and aortic valve stenosis in association with TAA is a well-recognized entity. Loscalzo *et al* suggested that altered TGF-β signaling might play a role in BAV with TAA as several aneurysm syndromes, including Marfan syndrome[47], Loeys Dietz syndrome[48] and arterial tortuosity syn-

drome[49] are associated with upregulation of the TGF-β pathway leading to loss of elastic fiber integrity. However, *TGFBR1* and *TGFBR2* gene analysis in 13 families with BAV and TAA revealed no mutations.[16] Recently mutations in the gene ACTA2, encoding vascular smooth muscle cell α- actin, were identified as a major cause of autosomal dominant inherited TAA. Interestingly multiple familymembers in 4 out of 14 described families with *ACTA2* mutations showed BAV, indicating that genes encoding sacromeric proteins might be good candidate genes for a subset of familial LVOTO.[50]

Elastin mutations in our families are unlikely as they predominantly cause supravalvular aortic stenosis, although aortic valve stenosis can also occur. Furthermore, right-sided valve anomalies and ASD/VSD are rarely described in patients with *elastin* mutations.[51-52]

Only a few genes have been involved in nonsyndromic CVM with a monogenic mode of inheritance; these include the *NOTCH1*, *Elastin*, *NKX2.5*, *NKX2.6*, *GATA4*, *CRELD1*, *MYH6*, *ACTA2*, *TBX20* and *FLNA* genes [for reviews:[53-55]]. Mutations in *NOTCH1* have been found in two families with BAV, aortic valve stenosis, aortic valve calcification and other LVOTO anomalies.[7] Interestingly one patient with AS and BAV also had ascending aortic dilatation. In both our families *NOTCH1* was excluded as the disease gene by linkage analysis using polymorphic microsatellites (D9S1826, D9S158, D9S1838) flanking the *NOTCH1* gene that showed multiple recombinants in both families. Additionally, sequence analysis of all coding exons and intron-exon boundaries was normal in an affected family member in both families.

Human *NKX2.5* mutations can cause a number of different cardiac phenotypes[56-57], including ASD/VSD and LVOTO, but anomalies of the semilunar valves, as observed in 17/21 of the patients in our families, are not often described.[58] A *NKX2.5* mutation was excluded in both our families by mutation analysis in 1 affected family member. *GATA4* mutations can lead to nonsyndromic ASD and other CVM including PS[31], whereas gross deletions of the 8p23 region encompassing the *GATA4* gene are associated with a variety of cardiac anomalies, mainly PS and ASD. However, in contrast to our families left-sided cardiac anomalies are uncommon in these patients, although aortic/mitral regurgitation was reported in 1/18 patient of the families described by Garg *et al*.[31] So far, no good candidate genes have been reported for our families, therefore a genome-wide linkage analyses has been initiated in both families.

Mature valve structures arise from endothelial cells of the endocardial cushions.[59] The endocardial cushions are formed by endothelial-mesenchymal transdifferentiation of a subset of endothelial cells that invade the extracellular matrix and differentiate into mesenchymal cells.[60] Valve leaflets eventually consist of a single endothelial cell layer and a central layer consistent of collagen, elastin and glycosaminoglycans.[61]

The endocardial cushion tissue contributes not only to the formation of valves, but also to the formation of membranous septa.[62] This might explain why some patients in our families have septal defects apart from valve anomalies.

In contrast to the sparse knowledge about the genes involved in human valve formation and malformation much more is known about valvulogenesis in mice. In mice signal transduction pathways including Wnt/β - catenin, Vegf, Notch, Bmp - Tgfβ, and Erb, and transcription factors including different GATA, FOX and SOX transcription factors have been implicated in heart cushion/valve formation (Table 2, Figure

Table 2. Cardiac valve anomalies caused by gene defects in mice

Gene	Cardiac anomaly	Reference
Wnt/ β-Catenin signaling		
Has2	Absence of cardiac jelly/endocardial cushions	63
Hdf (Cspg2 ,versican)	Absence of endocardial cushion swelling	64
ß-catenin	Lack of heart cushion formation	65
Notch signaling		
Notch1	Hypoplastic cardiac cushions	66
Hesr2	Dysplastic AV valves, ASD, VSD	67
Hey 1/HeyL	dysplastic atrioventricular and pulmonary valves	68
Hey2	TA, VSD, TOF	69
EphrinB2	Thickened aortic, pulmonary and mitral valve	70
Fgf8	single AV valve, hypoplastic arch arteries, DORV	71
Ece1/Ece2	abnormal AV valve formation, truncus arteriosus	72
Vegf signaling		
Cx45	Endocardial cushion defects	73
Nfatc1	Absent semilunar valves	74
Nf1	Hyperplastic valve tissue	75
Tie 2 (TEK)	Endocardial cushion defects	76
eNos	BAV, AS, ASD, VSD	77
Hhex	AV valve dysplasia	78
Bmp-Tgf-β signaling		
Bmpr2	Absent semilunar valves, truncus arteriosus	79
Bmp4	variable	80
Bmp6/7	Hypoplastic cardiac cushions	81
Alk3	Hypoplastic cardiac cushions	82
Madh6 (Smad6)	Thickened valves	83
Perlecan (HSPG2)	Malformed semilunar valves, TGA	84

Gene	Cardiac anomaly	Reference
Fibulin-4	thickened aortic valvular leaflets	85
Erb signaling		
ErbB1 (Egfr , Her1)	Enlarged thickened semilunar and AV valves	86-87
ErbB3 (Her3)	Hypoplastic cardiac cushion	88
HB-EGF	Enlarged malformed semilunar and AV valves	87,89
Tace (Adam17)	Enlarged semilunar and AV valves	87
β *Meltrin (Adam19)*	immature valves, VSD	90-91
Egfr/Ptpn11	Semilunar valve hyperplasia	92
GATA transcription factors		
GATA4	Common AV valve	93
Fog 1	Common AV valve, DORV	94
Fog 2	TA, PS, AV canal, ASD, VSD, TOF	95-96
Nkx2.5	BAV, AS, ASD	97
Pitx2	Enlarged endocardial cushion	98
Sox transcription factors		
Sox4	Semilunar valve defects, truncus arteriosus	99
Sox9	Hypoplastic endocardial cushions	100
Fox transcription factors		
Foxp1	Thickened endocardial cushion	101
Foxc1	Valve anomalies, IAA, CoA, VSD	80
Foxc2	Valve anomalies, IAA, CoA, VSD	80
Various		
EphA3	Hypoplastic endocardial cushions	102
Pdgf-a(patch mutation)	Septal and valve defects	103
Pdgf-b	AV valve malformation, VSD	104
Apoe	Sclerotic, stenotic aortic valves	105
Ccn1	Atrioventricular septal defects	106
Periostin	AV valve abnormalities, ASD	107
Chm1	Thickened, calcified, stenotic aortic valves	108
Cxcr7	Semilunar valve malformation, VSD	109

AV, atrioventicular; AS, aortic valve stenosis; ASD, atrial septal defect; BAV, bicupid aortic valve; CoA, coarctation of the aorta; DORV, double outlet right ventricle; IAA, interrupted aortic arch; PS, pulmonary stenosis; TA, tricupid atresia; TGA, transposition of the great arteries; TOF, tetralogy of Fallot; VSD, ventricular septal defect

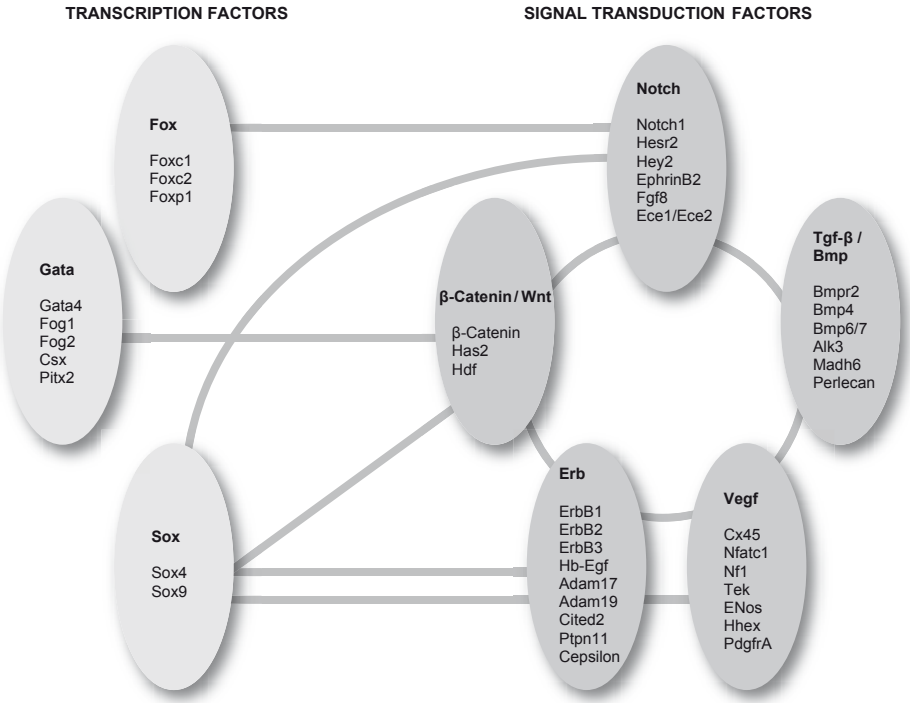


Figure 2 Molecular pathways and their cross-talking involved in cardiac morphogenesis.

2). These different signaling pathways exhibit extensive cross-talking, resulting in a complex integrated process of cardiac valve morphogenesis [for review:[60,62]]. These mouse models could provide functional candidate genes for families with cardiac valve anomalies once positional genetics approaches have localised the human disease gene.

The two multiplex families in this report may facilitate identification of human genes specifically involved in cardiac valve morphogenesis and aortic wall disease. The co-occurrence of aortic and pulmonary valve abnormalities and aortic aneurysms in these families supports a common genetic etiology in some forms of left- and right-sided valve anomalies, and expands the phenotype of LVOTO.

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MUTATIONS IN *SMAD3* CAUSE A SYNDROMIC FORM OF AORTIC ANEURYSMS AND DISSECTIONS WITH EARLY-ONSET OSTEOARTHRITIS

CHAPTER 5

ABSTRACT

Thoracic aortic aneurysms and dissections are a main feature of connective tissue disorders, such as Marfan syndrome and Loeys-Dietz syndrome.

We delineated a new syndrome presenting with aneurysms, dissections and tortuosity throughout the arterial tree in association with mild craniofacial features and skeletal and cutaneous anomalies. In contrast with other aneurysm syndromes, most of these affected individuals presented with early-onset osteoarthritis. We mapped the genetic locus to chromosome 15q22.2-24.2 and show that the disease is caused by mutations in *SMAD3*. This gene encodes a member of the TGF-β pathway that is essential for TGF-β signal transmission.[1-3] *SMAD3* mutations lead to increased aortic expression of several key players in the TGF-β pathway, including SMAD3. Molecular diagnosis will allow early and reliable identification of cases and relatives at risk for major cardiovascular complications. Our findings endorse the TGF-β pathway as the primary pharmacological target for the development of new treatments for aortic aneurysms and osteoarthritis.

INTRODUCTION

Aortic aneurysms represent a common vascular condition with life-threatening complications, including aortic dissection or rupture.[4] Aneurysms may be a manifestation of multisystem disorders such as Marfan syndrome (MFS), Loeys-Dietz syndrome (LDS) or arterial tortuosity syndrome (ATS). A common pathological feature in these disorders is increased TGF-β pathway signaling in the arterial wall. This discovery led to the identification of a key role for TGF-β and downstream signal transducers in the pathogenesis of aortic aneurysms.[5-8]

METHODS

Clinical studies

We investigated a four-generation family of Dutch origin with 12 individuals presenting with arterial aneurysms and dissections compatible with autosomal dominant inheritance and variable expression (family 1; Fig. 1). The index case was initially referred to the Department of Clinical Genetics (Erasmus Medical Center, Rotterdam, The Netherlands) by his cardiologist. As the family history revealed multiple (potentially) affected relatives, family members were approached by the index case and invited for genetic counselling. After information about the medical condition was provided, at risk relatives and 5 unrelated spouses were enrolled in the family study, and provided written informed consent for participation in clinical and genetic studies. Cases also provided written permission for publications of photographs. Medical records (when available) from deceased cases were revised.

Seventeen family members had an extensive physical examination by clinical geneticists and were scored for features in five major systems (cardiovascular, skeletal, joints, craniofacial and skin/integument) affected by connective tissue disorders.

Eighteen family members had an extensive cardiologic examination including physical examination, electrocardiography, transthoracic echocardiography and imaging of the entire aorta by computed tomography or magnetic resonance imaging (MRI). The diameters at the aortic root, at the level of the annulus, the sinus of Valsalva, the sinotubular junction, the proximal ascending aorta, the aortic arch, the descending aorta, the abdominal aorta and other large arteries (for example splenic artery and iliac artery) were measured at the maximum systolic dimensions. Cross-sectional echocardiography images were obtained in the parasternal long-axis orientation and plotted against normograms derived from normal individuals' measurements.[9] Magnetic resonance angiography (MRA) or computed tomography angiography (CTA) of the thoracic and abdominal vascular tree was performed

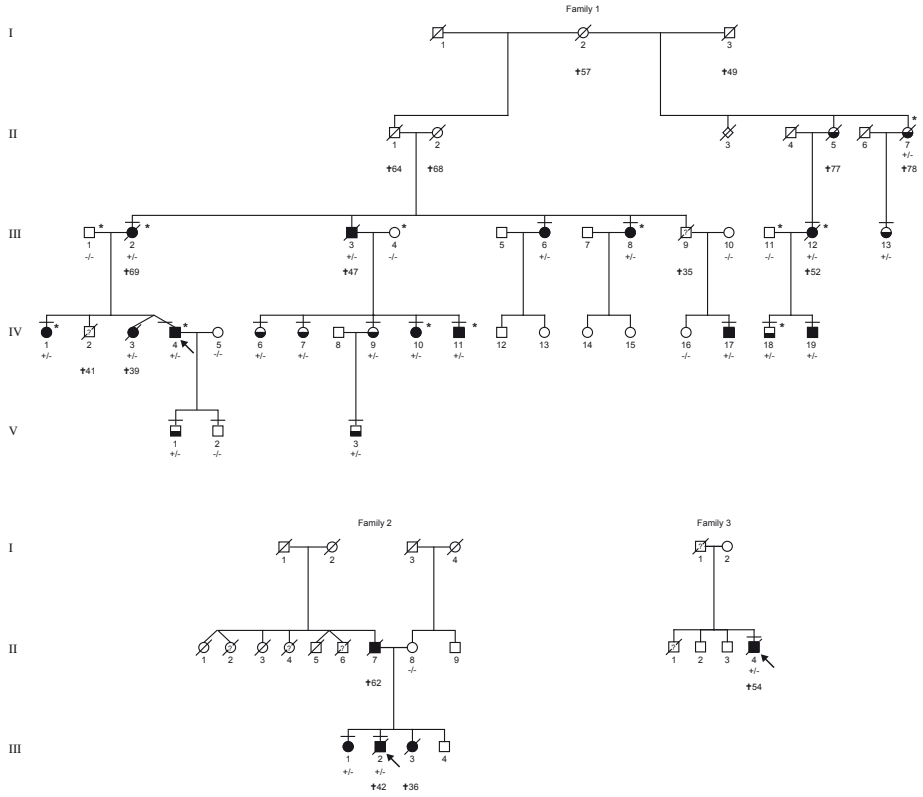

Figure 1 Simplified genealogical trees of three unrelated families with Aneurysms-Osteoarthritis syndrome (AOS). Squares indicate males, circles represent females. A horizontal line above the symbol indicates medical examination. An arrow points to the index patient. Filled symbols represent individuals with arterial aneurysms. Half-filled symbols represent cases with skeletal and/or cutaneous abnormalities, but no arterial aneurysms. Open symbols are individuals with a normal or unknown phenotype. An asterisk indicates that the individual was included in the genome-wide linkage analysis (only family 1). The presence (+/-) or absence (-/-) of a SMAD3 mutation is indicated underneath. A question mark (?) indicated sudden death of unknown cause, with age of death below the symbol.

using a 1.5 Tesla whole-body MRI scanner and the multidetector computed tomography equipment. The images from both computed tomography and MRI were plotted against normograms derived from normal individuals' measurements corrected for body surface area (BSA). The BSA was calculated by the DuBois and DuBois formula (BSA (m^2)= 0.007184 x Height (cm) 0.725 x Weight (kg) 0.425).

The individuals were identified as affected if an arterial aneurysm was present or the BSA-corrected aortic diameter at any level was greater than the 95% CI.[9-10] Also, individuals with operated aortic aneurysms and individuals who died from an aneurysm or dissection, confirmed by autopsy, were considered affected.

A radiographic skeletal survey of the total spine, hips, knees, hands, and feet was performed in 17 family members. Osteoarthritis in the extremities was scored with the Kellgren and Lawrence grades (0-4) for radiographic severity.[11] To evaluate spine osteoarthritis, the presence of disc degeneration and osteophytes or joint space narrowing at the intervertebral joints and uncovertebral joints were scored (grade 0-3).[11-12]

The Kellgren-Lawrence Grading Scale is as follows:

Grade 1: doubtful narrowing of joint space and possible osteophytic lipping.

Grade 2: definite osteophytes, definite narrowing of joint space.

Grade 3: moderate multiple osteophytes, definite narrowing of joints space, some sclerosis and possible deformity of bone contour.

Grade 4: large osteophytes, marked narrowing of joint space, severe sclerosis and definite deformity of bone contour.

The diagnosis of osteoarthritis was made when a score of grade 2 (moderate severity) or more was reached. Joint replacement due to osteoarthritis was also considered as definite outcome of osteoarthritis. In addition, the presence of spondylolysis or spondylolisthesis, meniscal pathology and osteochondritis dissecans (OCD) was scored.

Survival analysis

The SPSS (version 15) software was used to construct Kaplan-Meier curves and to estimate median survival.

Linkage analysis

Genomic DNA was isolated from peripheral blood using standard procedures (Gentra Systems). DNA samples from deceased patients were obtained from stored tissue (frozen or paraffin embedded tissue). A genome-wide search was conducted using DNA from 12 members from family 1 (Fig. 1) with the Affymetrix GeneChip Mapping 250K *Nsp* Array containing 262 264 SNP markers as described previously.[13]

Microsatellite markers mapping to the identified genomic regions were selected. DNA samples from 27 family members from family 1 were genotyped using an ABI Prism 3130*xl* genetic sequencer (Applied Biosystems) as described previously.[13]

The statistical package easyLINKAGE Plus v5.08[14], designed to perform automated linkage analyses using large-scale SNP data, was used to perform all analyses. Allegro v1.2c software was used to perform single point and multipoint linkage analysis as previously described.[13] LOD scores were obtained using a dominant model of inheritance, with a penetrance of 90% and a disease allele frequency of 1:1000. A phenocopy rate of 1% was considered. Allele frequencies of genotyped

SNPs were set to codominant. Map order and genetic inter-SNP distances were taken from the Affymetrix website.

Sequence analysis

Sequencing of all coding exons and exon-intron boundaries of candidate genes was undertaken using PCR primers designed by Primer3 software. PCR products were purified and sequenced using BigDye Terminator chemistry v3.1 on an ABI Prism 3130*xl* genetic analyzer (Applied Biosystems). Sequences were aligned and compared with consensus sequences obtained from the human genome databases (SeqScape v2.5 software, Applied Biosystems). For annotation of DNA and protein changes the Mutation Nomenclature guidelines from the Human Genome Variation Society (HGVS) were followed. To describe mutations at the cDNA level, the A from the ATG start codon of the reference sequence was numbered as 1.

Molecular modeling and predictions

The effects of the mutations on the protein structure and function were predicted using molecular graphics, modeling and simulation programs from YASARA NOVA[15] and WHAT IF Twinset.[16] All figures of the protein structures were made using the same programs and POV-Ray for raytracing. The alterations are located in the MH2 domain of the protein that was solved experimentally.[17] We used the re-refined PDB-file 1U7F downloaded from the PDB_REDO website. This file contains a SMAD3/SMAD3/SMAD4 trimer, of which each monomer contains the linker and the MH2 domain.

Histology and immunohistochemistry

Paraffin-embedded autopsy tissues from four individuals who died from aortic dissections or rupture (family 1, individual IV-3 and family 2, individuals II-7, III-2 and III-3; Fig. 1) were available. Fragments from the ascending aorta taken during surgical procedures were available from two patients (family 1, individuals IV-4 and IV-11; Fig. 1). Tissues from ascending aortas from three age-matched donors were used as controls. All samples were histologically examined after Hematoxylin-Eosin, Verhoeff-van Gieson (elastin), Alcian blue (proteoglycans) and Masson's trichrome (collagen) staining using standard techniques.

For immunohistochemistry, sections (7 μM) were deparaffinized, followed by antigen retrieval using microwave treatment in 0.01 M sodium citrate solution. Endogenous peroxidase activity blocking and immunoincubation were performed as described previously.[18] Primary antibodies against total SMAD3 (ab28379), TGF-β1 (ab53169), CTGF (ab5097) were all obtained from Abcam, phosporylated-Smad2

(3108) was from Cell Signaling Technology and Collagen III was from Biogenex (MU167UC).

RESULTS AND DISCUSSION

We delineated a new aneurysm syndrome inherited as an autosomal dominant trait with variable clinical expression in three unrelated Dutch families. In the largest family, we ascertained 22 individuals with arterial aneurysms and/or skeletal or cutaneous abnormalities (Fig. 1; family 1). The craniofacial abnormalities were mild and included hypertelorism (widely spaced eyes), abnormal palate and/or uvula and dental malocclusion (Fig. 2a). We did not observe craniosynostosis (premature closure of cranial sutures) and mental retardation. Twelve individuals presented with aneurysms of the aorta, mainly at the level of the sinus of Valsalva (Fig. 2b), but also affecting the abdominal aorta and/or other arteries such as the splenic, common iliac, mesenteric, renal, vertebral (Fig. 2b) and main pulmonary artery. This family has a strong history of sudden death occurring between 35 and 69 years of age. These deaths were mainly due to dissection and/or rupture of the aorta, which also occurred in mildly dilated aortas (maximal ascending aortic diameter of 4.0-4.5 cm; Fig. 2b). Arterial tortuosity of the cerebral, thoracic and/or abdominal arterial tree was present in a majority of the cases.

We found other (congenital) heart diseases, such as persistent ductus arteriosus, atrial septal defect, pulmonary valve stenosis and atrial fibrillation. Mitral valve abnormalities, ranging from mild valve prolapse to severe regurgitation requiring valve replacement, were frequently reported. In addition, five normotensive patients had idiopathic mild-to-moderate predominantly concentric left ventricular hypertrophy.

In contrast with other aneurysm syndromes, most of our cases presented with early-onset osteoarthritis. All these individuals had radiologically proven osteoarthritis of one or more joints at a mean age of 42 years, primarily involving the knees, spine and/or thumb base. We commonly observed intervertebral disc degeneration of the cervical and lumbar discs and detected this degeneration as early as 12 years of age (Fig. 2c). The osteoarthritis seen in the hand and wrist only involved the scapho-trapeziotrapezoidal, first carpometacarpal and, occasionally, metacarpophalangeal joints (Fig. 2c). In contrast with classical hand osteoarthritis, the distal and proximal interphalangeal joints were not affected. Osteochondritis dissecans (OCD; Fig. 2c) and/or meniscal lesions not preceded by major injury or trauma were also frequently observed at young ages.

In addition, umbilical and/or inguinal hernias, velvety skin and striae were recurring features in cases. Varices or thread veins presented at a young age and were

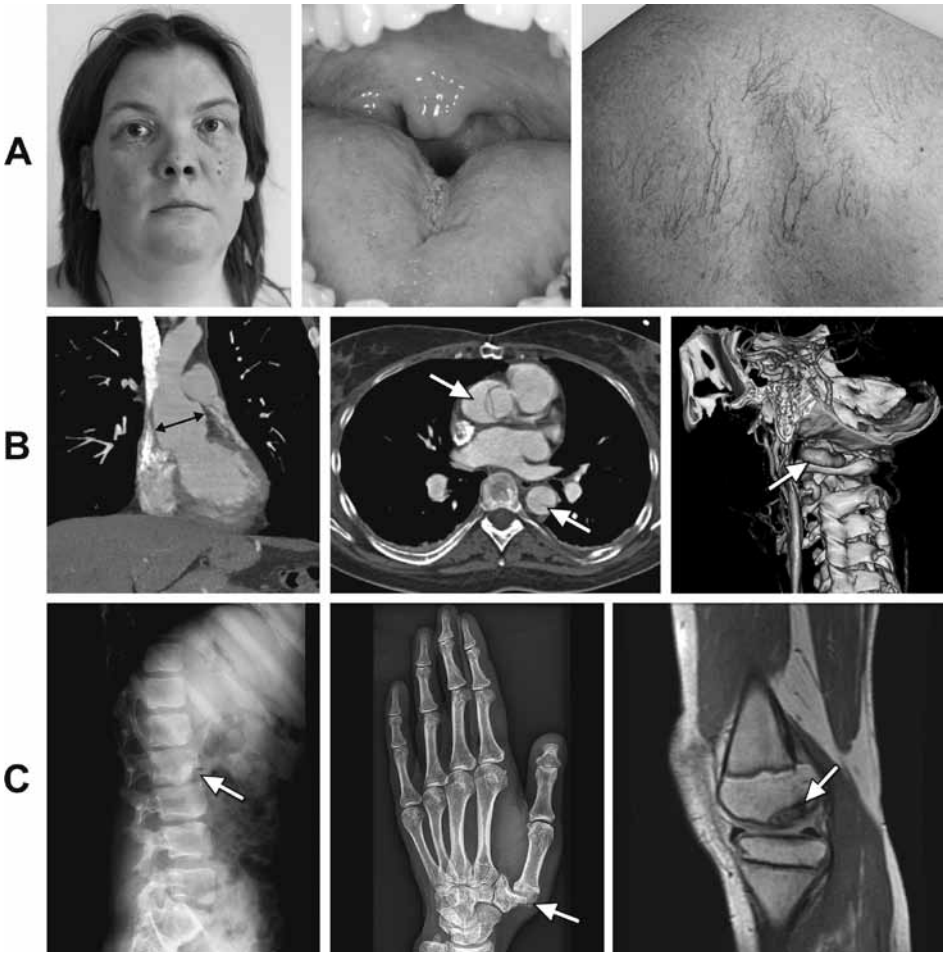

Figure 2 Clinical features of cases from family 1. Written consent was obtained for publication of these images. (a) Physical examination (from left to right). Facial features including a long face, hypertelorism, flat orbital ridges and malar flattening in individual IV-10; bifid uvula in individual IV-9; and translucency of the skin with visible thread veins in individual III-6. (b) Aneurysms and dissections (from left to right). Multi-slice computed tomography angiogram of thoracic aorta showing an aneurysm of the aortic root (45 mm, arrow) of individual IV-11 at the age of 29 years; multi-slice computed tomography angiogram in 50-year-old female (individual III-12) shows a Stanford type A aortic dissection (arrows) with dissection flap extending from the aortic valve into the descending aorta at a maximal aortic diameter of 40 mm; and a 3D-reconstructed computed tomography angiogram (left posterior view) shows a fusiform aneurysm of the left vertebral artery (arrow) in a 46-year old female (individual IV-1). (c) Osteoarthritis and osteochondritis (from left to right). A 12-year-old boy (individual V-3) with early degenerative abnormalities of the lumbar spine, deformations of the vertebral body (arrow) and narrowing of the intervertebral disc space at multiple levels; advanced osteoarthritis of the first carpometacarpal joint of the left hand (arrow) in a 46-year old female (individual IV-1); and magnetic resonance imaging of the left knee of 12-year-old individual (individual V-3) shows a large osteochondral lesion without separation in the medial femoral condyle (arrow).

resistant to therapy (Fig. 2a). Ophthalmologic examination in ten cases revealed no abnormalities. The main clinical characteristics of all 27 cases from three families are summarized in Table 1 and Table 2.

Table 1. Clinical findings in 27 individuals with aneurysms-osteoarthritis syndrome (AOS)

Features	No.	Percentage
Cardiovascular	23/26	85
Aortic aneurysm/dissection	15/26	58
Aneurysms of other vessels	7/17	41
Arterial tortuosity	9/17	53
Mitral valve prolapse	10/22	45
Mitral regurgitation	6/22	27
Aortic insufficiency	4/22	18
Pulmonary valve stenosis	1/22	5
Persistent ductus arteriosus	1/22	5
Left ventricular hypertrophy	5/22	23
Atrial fibrillation	5/22	23
Skeletal		
Dolichostenomelia	5/19	26
Arachnodactyly	9/17	53
Pectus deformity	3/19	16
Scoliosis	9/21	43
Camptodactyly	3/19	16
Joint laxity (Beighton score ≥5/9)	3/16	19
Protrusio acetabulae	5/17	29
Pes planus	17/17	100
Osteoporosis	2/17	12
Joints		
Osteoarthritis in one or more joints	21/21	100
Osteoarthritis feet/ankle	6/18	33
Osteoarthritis hand/wrist	7/17	41
Osteoarthritis knee	13/21	62
Osteoarthritis hip	2/18	11
Osteoarthritis facet joints	11/18	61
Osteoarthritis uncovertebral joints	9/18	50
Intervertebral disc degeneration	18/20	90
Spondylysis	5/20	25
Spondylolisthesis	4/20	20
Meniscal lesions	6/18	33

Osteochondritis dissecans	11/18	61
Craniofacial		
Hypertelorism	7/19	37
Abnormal uvula	5/16	31
Cleft palate	1/19	5
High arched palate	7/19	37
Dental malocclusion	8/15	53
Skin/integument		
Velvety skin	12/18	67
Easy bruising	5/18	28
Atrophic scars	5/18	28
Striae	11/18	61
Umbilical/inguinal hernia	9/18	50
Other		
Bladder/uterus/bowel prolapse	7/15	47
Dural ectasia	4/8	50
Varices	14/22	64

No., number of cases with the feature/number of cases examined for the feature.

Aiming to map the disease gene, we performed a genome-wide linkage analysis (GWLA) using 250k SNP arrays in family 1. We obtained a significant multipoint logarithms (base 10) of odds (LOD) score of 3.6 on chromosome 15q. Haplotype analysis confirmed that all 12 individuals with arterial aneurysms and 10 individuals with skeletal and/or cutaneous anomalies (not included in the GWLA) shared the disease-associated haplotype. Further fine mapping in the 15q area mapped the gene locus between markers D15S155 (centromeric) and D15S980 (telomeric). This 12.8-Mb candidate region (60.4-73.2 Mb) contains 157 genes (NCBI build 37.1).

Based on their roles in the TGF-β signaling pathway, we selected the *SMAD3* and *SMAD6* genes (*SMAD* family members *3* and *6*) for sequence analysis. We found no pathogenic mutations in *SMAD6*. In family 1, a heterozygous *SMAD3* mutation, c.859C>T, was found to segregate with the phenotype. This mutation leads to the substitution of arginine for tryptophan at position 287 of SMAD3 (p.Arg287Trp; Fig. 3a).

To evaluate the frequency of *SMAD3* mutations among individuals with aneurysm, we sequenced all *SMAD3* exons in 99 individuals with thoracic aortic aneurysms and dissections (TAAD) and Marfan-like features but without *FBN1*, *TGFBR1* and *TGFBR2* mutations. We found heterozygous *SMAD3* mutations in 2 out of 99 cases. One of the mutations (within family 2) was a deletion of two nucleotides (c.741-742delAT) found in exon 6 and leading to a frameshift in the DNA sequence and a premature

Table 2. Individual clinical features of 27 AOS cases

	Fam 1																						Fam 2			Fam 3
	II-5	**II-7**	**III-2**	**III-3**	**III-6**	**III-8**	**III-9**	**III-12**	**III-13**	**IV-1**	**IV-3**	**IV-4**	**IV-6**	**IV-7**	**IV-9**	**IV-10**	**IV-11**	**IV-17**	**IV-18**	**IV-19**	**V-1**	**V-3**	**II-7**	**III-1**	**III-2**	**III-3**	**II-4**
Sex	F	F	F	M	F	F	M	F	F	F	F	M	F	F	F	F	M	M	M	M	M	M	M	F	M	F	M
Age (d: age of death)	d77	d78	d69	d47	66	63	d35	d52	58	47	d39	42	44	42	41	38	33	34	31	28	14	12	d62	49	d42	d36	d54
Cardiovascular																											
Aortic aneurysm/ dissection	-	-	+	+	-	+	u	+	-	+	+	+	-	-	-	+	+	+	-	-	-	-	+	+	+	+	+
Aneurysms of other vessels	u	u	+	u	+	-	u	+	-	+	u	+	-	-	-	-	-	-	-	+	u	u	u	u	-	u	+
Arterial tortuosity	u	u	+	u	+	+	u	+	+	+	u	+	-	-	-	-	-	-	-	-	u	u	u	u	+	u	+
Mitral valve abnormalities	+	+	+	u	-	-	u	+	+	+	u	+	-	-	-	-	+	-	-	+	+	+	u	+	-	u	+
Congenital heart disease	-	-	-	u	-	-	u	+	-	-	u	-	-	-	-	-	-	-	-	+	-	-	u	-	-	u	-
Other heart disease	+	+	+	u	-	+	u	+	+	-	u	-	-	-	-	-	-	-	-	+	-	-	u	-	-	u	-
Skeletal																											
Pectus deformity	u	u	U	u	-	-	u	-	-	-	u	-	-	-	-	+	-	+	-	-	+	-	u	-	-	u	-
Scoliosis	u	+	+	u	+	+	u	+	-	-	u	-	-	-	-	-	+	+	+	+	-	-	u	-	-	u	-
Joint laxity (Beighton score)	u	u	U	u	0/9	2/9	u	u	0/9	5/9	u	1/9	1/9	1/9	3/9	6/9	0/9	0/9	2/9	6/9	u	4/9	u	2/9	0/9	u	u
Joints																											
Osteoarthritis ≥ 1 joint	u	+	+	u	+	+	+	+	+	+	u	+	+	+	+	+	+	+	+	+	+	+	u	+	u	u	+
Intervertebral disc degeneration	u	+	+	u	+	u	u	-	+	+	u	+	+	+	+	+	+	+	+	+	+	+	u	+	-	u	+
OCD or meniscal abnormalities	u	u	U	u	+	+	+	+	+	-	u	+	-	+	+	+	+	+	+	-	-	+	u	-	u	u	u

	Fam 1																							Fam 2			Fam 3
	II-5	II-7	III-2	III-3	III-6	III-8	III-9	III-12	III-13	IV-1	IV-3	IV-4	IV-6	IV-7	IV-9	IV-10	IV-11	IV-17	IV-18	IV-19	V-1	V-3	II-7	III-1	III-2	III-3	II-4
Craniofacial																											
Hypertelorism	u	u	U	u	-	-	u	-	+	+	u	+	-	-	-	+	-	+	+	+	-	-	u	-	-	u	-
Abnormal palate/uvula	u	u	U	u	-	+	u	-	+	+	u	+	+	-	+	-	+	+	+	+	-	-	u	-	-	u	+
Skin/integument																											
Velvety skin	u	u	u	u	-	+	u	u	+	+	u	+	+	+	+	+	+	-	-	+	-	+	u	+	-	u	-
Umbilical/inguinal hernia	u	u	u	u	+	-	u	u	-	-	u	+	+	+	+	-	+	+	-	+	-	-	u	-	-	u	+
Other																											
Bladder/uterus/bowel prolaps	+	+	-		+	+		-	-	+	-		-	+	-	-								+		-	
Varices	+	+	+	u	+	-	u	+	+	+	u	+	-	-	+	+	-	+	-	-	-	-	u	+	+	u	+

+: feature is present; -: feature is absent; u: data unknown or unavailable; blank: not applicable

protein termination at codon 309 in exon 7 (p.Thr247Pro*fs*X61) (Fig. 3a, b,c). The heterozygous deletion was present in two affected siblings and was not found in the healthy mother, thus it is likely the deletion originated from the affected siblings' deceased father (Fig. 1; family 2). Sequencing of complementary DNA (cDNA) from individuals III-1 and III-2 carrying the heterozygous c.741-742delAT mutation revealed a normal *SMAD3* allele and a very weak signal of the mutated allele. In addition, treatment of fibroblast cultures with cycloheximide (a known inhibitor of nonsense-mediated RNA decay) markedly increased the signal of the mutant allele (data not shown). Altogether, the data indicate that most of the abnormal RNA was subjected to nonsense messenger RNA decay and that a truncated, abnormal SMAD3 protein could barely be formed. The third mutation, in family 3, was a novel missense mutation, c.782C>T, leading to the substitution of threonine for isoleucine (p.Thr261Ile) that was found in the index case.

Several findings support that these three mutations are pathogenic: i) none of the mutations were found in 544 Dutch control chromosomes; ii) the c.741-742delAT deletion is a truncating mutation that removes nearly the complete MH2 domain including the TGFBR1 target site for SMAD phosphorylation and the residues involved in homomer and heteromer formation with SMAD3 and SMAD4; iii) both missense mutations affect evolutionary highly conserved amino acids within the MH2 domain of SMAD3 and other receptor-SMAD and co-SMAD proteins (Fig. 3d); iv) four computer programs which predict the possible impact of amino acid substitutions on the structure and function of human proteins (PMut, SIFT, PolyPhen and SNPs3D; see URLs) indicated that both missense mutations are pathogenic; v) our molecular modeling based on the known three-dimensional (3D) SMAD3 structure predicted that the mutations have detrimental structural effects that disturb protein trimerization (Supplementary note can be viewed in the online issue, which is available at www.nature.com/ng). Both altered amino acids are located on or very close to the protein interfaces (Fig. 3e). Introduction of isoleucine (p.Thr261Ile) will lead to conformational changes and disturbed monomer interactions (Fig. 4a-d). In a similar way, the replacement of a positively charged by a neutral amino acid (p.Arg287Trp) will cause a rearrangement of the residues and loops that interact with other SMAD3 and SMAD4 monomers (Fig. 4e-f).

In addition, we sequenced *SMAD3* in 29 index cases with familial TAAD and 50 cases with abdominal aneurysms (49% of which had a positive family history), but we found no mutations.

We investigated the effect of *SMAD3* mutations on the aortic wall by histology and immunohistochemistry of aorta fragments obtained during surgery (two cases with the p.Arg287Trp alteration) or postmortem (four cases, one with the p.Arg287Trp and three with the p.Thr247Pro*fs*X61 alteration). The findings were similar in all

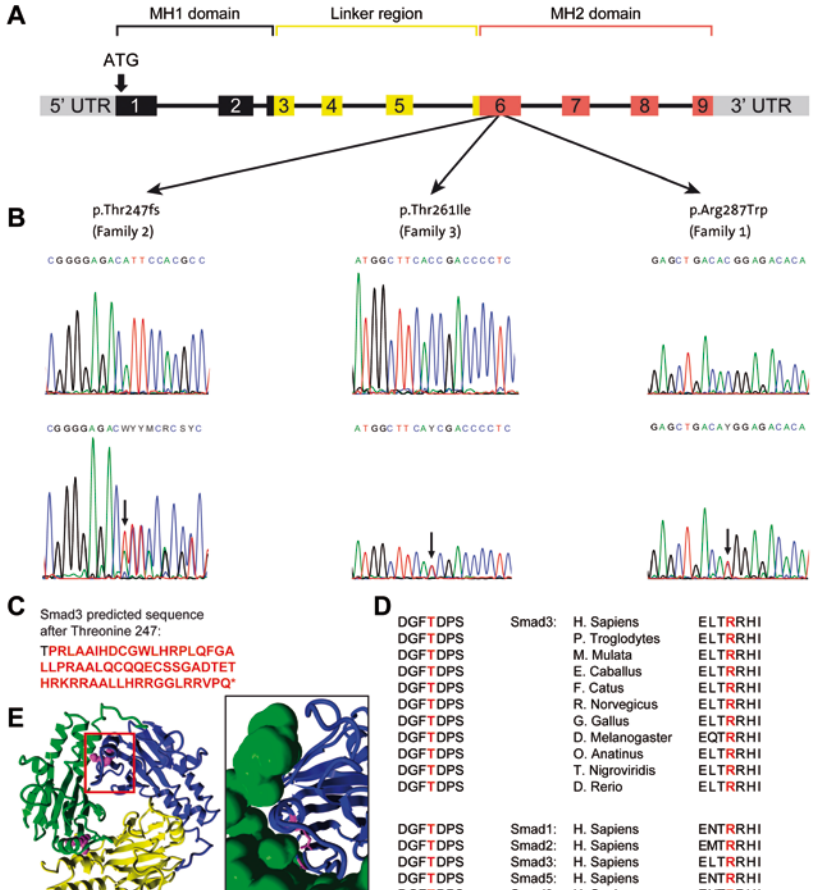

Figure 3 SMAD3 and mutations. (a) Schematic representation of the *SMAD3*. Boxes represent exons 1-9 with the untranslated regions (UTRs) and the open reading frame. The three main functional domains, MH1, MH2 and the linker region, are indicated. The mutations (resulting in p.Thr247*fs*X61, p.Thr261Ile and p.Arg287Trp) are located in the MH2 domain, which mediates oligomerization of SMAD3 with SMAD4 and SMAD-dependent transcriptional activation. (b) Chromatogram showing the normal (upper) and mutated (lower) *SMAD3* sequences, corresponding nucleotides and three mutations (arrows). (c) Predicted SMAD3 protein sequence from the mutation resulting in p.Thr247*fs*X61. Premature protein termination occurs after 61 aminoacids. (d) Cross-species protein conservation of SMAD3 and protein conservation of human receptor and co-SMAD proteins around the altered amino acids p.Arg287 and p.Thr261. (e) Overview of the heterotrimeric complex formed by SMAD3/SMAD3/SMAD4 MH2-domains. The proteins are shown in ribbon presentations. The SMAD3 domains are colored green and blue, whereas the SMAD4 domain is shown in yellow. The altered amino acids (p.Thr261 and p.Arg287) are colored magenta and are shown in ball representation. Note that the two mutated residues are located on and close to the interaction surface of the SMAD monomers. A close-up of a SMAD3-SMAD3 interaction site with the two mutated residues p.Thr261 and p.Arg287 shown (one SMAD3 monomer is displayed with its surface, the other monomer is displayed in ribbon representation).

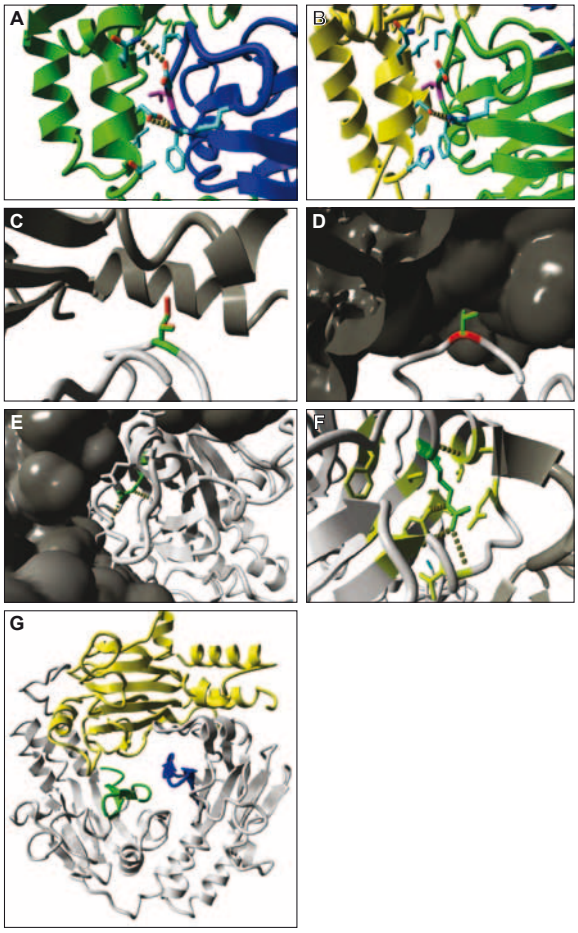

Figure 4 Overview of all the interactions made by residues surrounding Thr261 and Arg 287. (a) the SMAD3/SMAD3 interface; (b) the SMAD3/SMAD4 interface. SMAD3 is colored green in both plots; in panel A the second SMAD3 monomer is colored blue. In panel B, SMAD4 is shown in yellow. The proteins are shown as ribbon presentation with relevant side chains added as stick model and colored by element (carbon=cyan, oxygen=red, nitrogen=blue). The mutated threonine is colored magenta. Hydrogen bonds are indicates with yellow dotted lines. (c) Close-up of the p.Thr261Ile mutation. The two monomers are shown in different shades of grey. The original threonine residue is colored green; the mutant isoleucine is colored red. In panel c, both monomers are shown in ribbon representation. In panel d, the surface of the other monomer is shown. This illustrates clearly that the threonine side chain fits exactly at the interface while the isoleucine side chain clashes with the other monomer. This effect is similar at the SMAD3/SMAD3 and the SMAD3/SMAD4 interface. (e) Overview of Arg287 in the core of a domain that has multiple contacts with the other SMAD3 monomer. Both monomers are different shades of grey. Arginine 287 is shown in green. Hydrogen bonds are shown as yellow dotted lines. Note that arginine is a key residue that provides hydrogen bonds that are required for the stability and correct formation of the loops. Residues in those loops interact with the other monomer. The role of this arginine is similar at the SMAD3/SMAD3 and the SMAD3/SMAD4 interface. (g) Overview of the SMAD3/3/4 trimer. The residues that are lost by the frameshift mutation are colored grey.
See the website (http://swift.cmbi.ru.nl/hanka/smad3/) for more details.

individuals studied. Disorganization of the tunica media with fragmentation and loss of elastic fibers was observed with variable severity, as well as characteristic mucoid medial degeneration and accumulation of collagen in the media (Fig. 5).

We studied the expression of several members of the TGF-β pathway including total SMAD3 (non-phophorylated and phosphorylated forms), phosphorylated SMAD2, TGF-β1 and connective tissue growth factor (CTGF), by immunohistochemistry in two individuals with the mutation resulting in p.Arg287Trp. We observed increased labeling intensity of all these markers. TGF-β1 expression was present throughout the aneurismal aortic media, whereas the controls only showed substantial expression

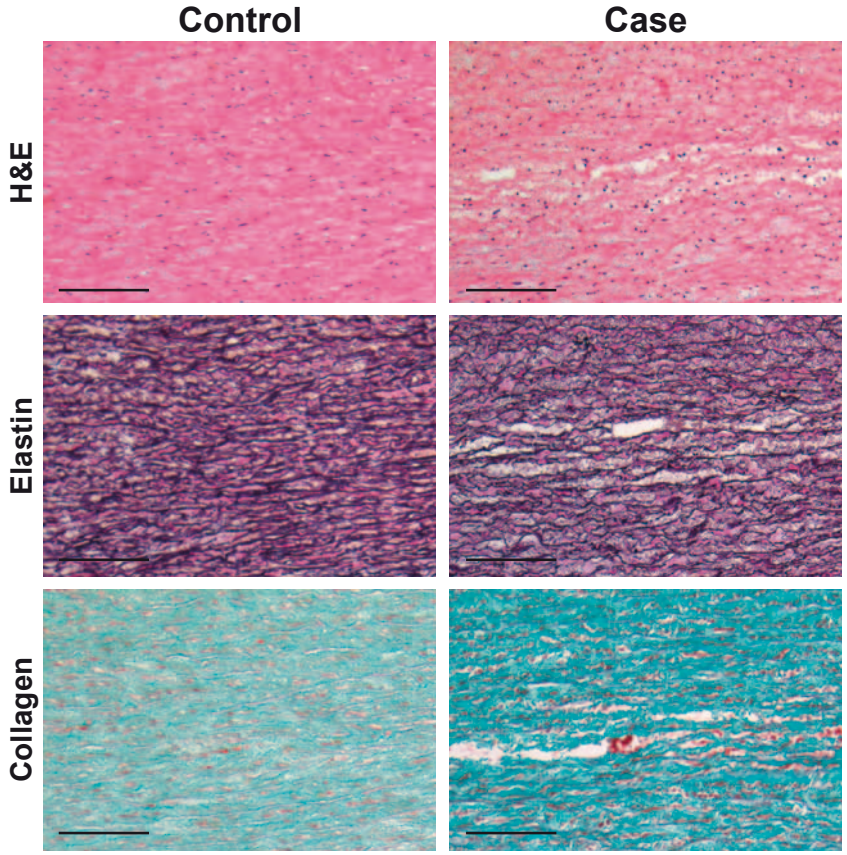

Figure 5 Histology of aortic media in Aneurysms-Osteoarthritis syndrome. Aortic media from a control (donor, left column) and case (right column) with a *SMAD3* mutation resulting in p.Thr247*fs*X61 (III-2, family 2). Scale bars correspond to 100 μm. Hematoxylin-eosin (H&E) staining displaying abnormal architecture of the aortic media and a dissection tear in the case. A Verhoeff-van Gieson staining for elastin (dark purple fibers); note the disarray, fragmentation and loss of elastic fibers in case versus control. A dissection tear is shown. A Masson's trichrome staining for collagen (green) showing intense collagen staining and disruption the medial architecture in the case.

in the media adjacent to adventitia layer, which normally shows the highest activity (Fig. 6). CTGF showed a markedly increased cytoplasmatic expression in the medial vascular smooth muscle cells (VSMCs) of the cases (Fig. 6). Because CTGF stimulates

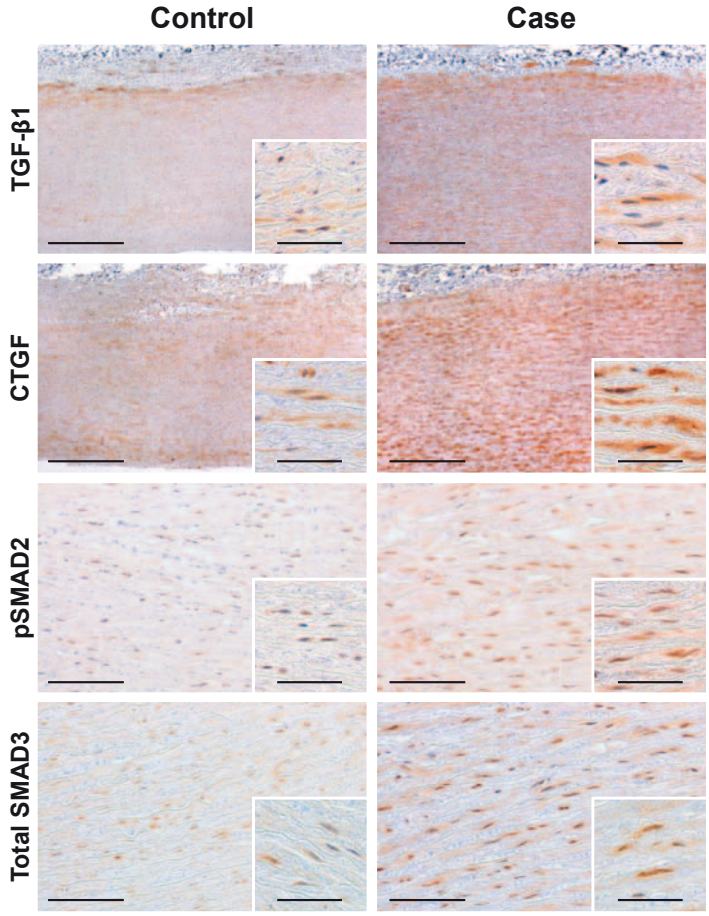

Figure 6 Immunohistochemistry in Aneurysms-Osteoarthritis syndrome. Aortic wall (from top to bottom: adventitia, media and intima layers) from a control (donor) and case with a *SMAD3* mutation resulting in p.Arg287Trp (IV-3, family 1). TGF-β1 immunostaining; note the increased TGF-β1 expression through the aortic media, whereas the control only shows significant expression in the outer media adjacent to the adventitia. Connective tissue growth factor (CTGF) immunolabeling is shown. CTGF is a TGF-β responsive product which normally induces collagen synthesis. Note the increased labeling in the cytoplasm of media cells from the case compared to the donor. Scale bars, 200 μm; inset scale bars, 50 μm. Photomicrographs from the middle section of the aortic media showing phosphorylated SMAD2 (pSMAD2) immunolabeling with marked nuclear staining in the case. Total SMAD3 (phosphorylated and non-phosphorylated SMAD3) immunostaining showing increased nuclear and cytoplasmatic labeling in the case as compared to the donor. Scale bars, 100 μm; inset scale bars, 50 μm.

production of collagen III by the VSMCs[19], we investigated the expression of collagen III, a known TGF-β and SMAD3 target[20], in the aortic wall. We observed increased collagen III labeling in the aortic media of the cases (data not shown).

We observed enhanced phosphorylated SMAD2 (pSMAD2) labeling through the medial VSMCs of the cases. Whereas in the controls pSMAD2 showed a weak nuclear signal, we observed a strong nuclear and moderate cytoplasmatic staining in the cases (Fig. 6). Total SMAD3 revealed increased cytoplasmatic and nuclear immunostaining in the cases (Fig. 6). This is consistent with excessive activation and nuclear translocation of SMAD2 and SMAD3. The overall increased expression of all these members of the TGF-β pathway is indicative of enhanced TGF-β signaling in the aortic wall of the cases.

Individuals with *SMAD3*-related disease show a phenotypic spectrum with remarkable intra- and interfamilial variability. Although some cases present with arterial aneurysms and dissections occurring at a young age (below 40 years), others have only skeletal or cutaneous features. We propose to refer to this disorder as aneurysms-osteoarthritis syndrome (AOS).

Our cases show a clear phenotypic overlap with other aneurysm syndromes such as LDS and MFS (Table 3). The main difference with these syndromes is the invariable presence of osteoarthritis at a young age in all cases with *SMAD3* mutations. In fact, in many AOS cases symptomatic osteoarthritis, or OCD, was the presenting feature to seek medical advice.

The median survival in this cohort of 27 cases was 62 years, which is higher than the median survival in patients with LDS (37 years)[21] but is lower than the median survival in individuals with MFS (70 years, with treatment).[23] Similar to LDS, the arterial aneurysms in individuals with *SMAD3* mutations extend beyond the aortic root and tend to rupture or dissect at a smaller size than in individuals with MFS or non-syndromic TAAD. Therefore, we recommend that *SMAD3*-associated disease should be regarded as an aggressive aneurysm syndrome, demanding early diagnosis, surveillance of the entire arterial tree and prophylactic surgical intervention. The virtual absence of a genotype-phenotype correlation makes personalized counseling, follow-up and treatment of each individual with a *SMAD3* mutation necessary.

SMAD3 encodes for a key regulator in the TGF-β pathway that activates or represses gene transcription.[2,17] All *SMAD3* mutations identified here are located in the MH2 domain of the protein, a region that is extremely well conserved among other species and other SMAD proteins. Whereas one truncating mutation deletes nearly the complete MH2 domain (Fig. 4g), the missense mutations substitute amino acids essential for trimer formation with SMAD3 and SMAD4, which is crucial for the translocation of the SMAD complex to the nucleus and for gene regulation. Mammalian cells where the polar p.Arg287 is replaced by a non-polar residue were

Table 3. Comparison of the main clinical features in aneurysm syndromes

Features	AOS (%) *N*=27	LDS type I[21] (%) *N*=40	LDS type II[21] (%) *N*=12	MFS[22] (%) *N*=1013
Cardiovascular				
Aortic root aneurysm/dissection	58	98	100	77
Aneurysms of other vessels	41	52	73	7
Arterial tortuosity	53	84	67	-
Mitral valve abnormalities	59	29[b]	-	54
Congenital heart disease	9	35	-	-
Other heart diseases	32[a]	-	-	-
Skeletal				
Pectus deformity	16	68	-	59
Scoliosis	43	50	-	53
Joint laxity (Beighton score ≥5)	19	68	100	63
Joints				
Osteoarthritis ≥ 1 joint	100	-	-	-
Intervertebral disc degeneration	90	-	-	-
OCD or meniscal abnormalities	72	-	-	-
Craniofacial				
Hypertelorism	37	90	0	-
Abnormal palate/uvula	58	90	25	-
Craniosynostosis	0	48	0	-
Skin/integument				
Velvety skin	67	28	82	-
Herniae	50	-	36	10
Other				
Ectopia lentis	0	0	-	54

[a] Left ventricular hypertrophy and atrial fibrillation. [b] Based on 16 cases[5]. -: not reported in referred articles.

unable to form homomers with SMAD3 or heteromers with SMAD4.[24] If SMAD3 heterotrimer formation is affected, TGF-β signals cannot be propagated via SMAD3. Other compensatory or alternative mechanisms may be also activated (for example, by SMAD2 and/or the normal SMAD3 allele) that could lead to additional dysregulation of the pathway.

These heterozygous mutations lead to increased expression of several key players of the TGF-β pathway including phosphorylated SMAD2, SMAD3, upstream ligands such as TGF-β1, and downstream targets (CTGF and collagen) in the thoracic aortic wall of the cases. Activation of the TGF-β signal transduction pathway has been observed before in other diseases with arterial wall anomalies, including MFS, LDS,

ATS, aneurysms associated with bicuspid aortic valve and degenerative aneurismal aortic disease.[5,19,25] This clearly indicates the existence of common (TGF-β related) pathogenic mechanisms leading to arterial wall disease.

AOS is characterized in all cases by joint abnormalities including osteochondritis dissecans (OCD), meniscal abnormalities, intervertebral disc degeneration and early-onset osteoarthritis. We were unable to study the TGF-β pathway in joints from individuals with *SMAD3* mutations. However, in *Smad3* knockout mice, progressive loss of articular cartilage, formation of osteophytes and intervertebral disc degeneration were observed.[26-27] These mouse anomalies closely resemble the human joint abnormalities resulting from *SMAD3* mutations.

Studies in the Smad3 knockout mice show a reduction of TGF-β signaling in the intervertebral discs.[27] Without TGF-β mediated inhibition, chondrocytes undergo abnormal terminal differentiation leading to the progressive loss of articular cartilage and formation of large osteophytes, as is typically observed in human osteoarthritis.[26] However, in heterozygote animals, these studies did not asses the status of TGF-β signaling. Although the detailed molecular pathology in both Smad3 knockout mice and AOS remains to be elucidated, the osteoarthritic anomalies are similar, suggesting that *SMAD3* and the TGF-β pathway are essential for cartilage integrity. To our knowledge there is no data indicating or excluding a vascular phenotype in the Smad3 knockout mice.

Our findings implicate the TGF-β signaling pathway as the primary pharmacological target for the development of new treatment strategies for arterial wall anomalies and osteoarthritis. However, our limited understanding of the physiological functioning of this complex pathway and the paradoxical increased TGF-β signal propagation caused by mutations in key molecules within the pathway (SMAD3 and the TGF-β receptors) warrant further studies before clinical trials with TGF-β antagonists such as Losartan[28] can commence.

URLS:

PMut, http://mmb.pcb.ub.es/PMut/; PolyPhen, http://genetics.bwh.harvard.edu/pph/; SIFT, http://sift.jcvi.org/; SNP3D, http://www.snps3d.org/. PDB_REDO, http://www.cmbi.ru.nl/pdb_redo.; PovRay, www.povray.org. Accession numbers: RefSeq NM_005902.3 (SMAD3 mRNA) and NP_005893.1 (Smad3 protein).

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PHENOTYPIC SPECTRUM OF THE SMAD3-RELATED ANEURYSMS-OSTEOARTHRITIS SYNDROME

CHAPTER 6

ABSTRACT

Background: Aneurysms-osteoarthritis syndrome (AOS) is a new autosomal dominant syndromic form of thoracic aortic aneurysms and dissections characterised by the presence of arterial aneurysms and tortuosity, mild craniofacial, skeletal and cutaneous anomalies, and early-onset osteoarthritis. AOS is caused by mutations in the *SMAD3* gene.

Methods: A cohort of 393 patients with aneurysms without mutation in *FBN1*, *TGFBR1* and *TGFBR2* was screened for mutations in *SMAD3*. The patients originated from The Netherlands, Belgium, Switzerland and USA. The clinical phenotype in a total of 45 patients from eight different AOS families with eight different *SMAD3* mutations is described. In all patients with a *SMAD3* mutation, clinical records were reviewed and extensive genetic, cardiovascular and orthopaedic examinations were performed.

Results: Five novel *SMAD3* mutations (one nonsense, two missense and two frameshift mutations) were identified in five new AOS families. A follow-up description of the three families with a *SMAD3* mutation previously described by the authors was included. In the majority of patients, early-onset joint abnormalities, including osteoarthritis and osteochondritis dissecans, were the initial symptom for which medical advice was sought. Cardiovascular abnormalities were present in almost 90% of patients, and involved mainly aortic aneurysms and dissections. Aneurysms and tortuosity were found in the aorta and other arteries throughout the body, including intracranial arteries. Of the patients who first presented with joint abnormalities, 20% died suddenly from aortic dissection. The presence of mild craniofacial abnormalities including hypertelorism and abnormal uvula may aid in the recognition of this syndrome.

Conclusion: The authors provide further insight into the phenotype of AOS with *SMAD3* mutations, and present recommendations for a clinical work-up.

INTRODUCTION

Aortic aneurysm is a common condition, with high mortality from dissections and ruptures.[1] Whereas abdominal aortic aneurysms usually occur sporadically, thoracic aortic aneurysms and dissections (TAAD) can be inherited in an autosomal dominant manner with decreased penetrance and variable expression.[2] Familial TAAD is subdivided into non-syndromic forms, sometimes associated with bicuspid aortic valve and/or patent ductus arteriosus,[3-5] and syndromic forms with features of a systemic connective tissue disorder. Non-syndromic familial TAAD can be caused by mutations in genes encoding proteins of the contractile unit of the vascular smooth muscle cell such as the *ACTA2, MYH11* and *MYLK* genes.[3-5] However, in the majority of patients, the genetic cause is still unknown.

Syndromic familial TAAD includes several systemic connective tissue disorders such as Marfan syndrome (MFS), caused by mutations in the *FBN1* gene; Loeys-Dietz syndrome (LDS), caused by mutations in the *TGFBR1* or *TGFBR2* gene; arterial tortuosity syndrome (ATS), caused by mutations in the *SLC2A10* gene and autosomal recessive cutis laxa type I (ARCL), caused by mutations in the *FBLN4* gene.[6-11] As all these syndromes are characterized by increased transforming growth factor (TGF)-β signaling in the arterial wall, it has become evident that TGF-β signaling plays a central role in the pathogenesis of arterial aneurysms.[6-11]

Recently, we described a new syndromic form of autosomal dominant TAAD characterized by the presence of arterial aneurysms and tortuosity, mild craniofacial features, skeletal and cutaneous anomalies, and osteoarthritis at a young age.[12] As arterial aneurysms and early-onset osteoarthritis are the cardinal features of this new disorder, the term aneurysms-osteoarthritis syndrome (AOS) was coined. Patients with AOS show aneurysms throughout the arterial tree and a high risk of early dissection/rupture resembling patients with LDS. Interestingly, early-onset joint abnormalities, including osteoarthritis, intervertebral disc degeneration, osteochondritis dissecans (OCD) and meniscal anomalies are present in almost all patients with AOS, whereas they are uncommon in LDS, MFS, or ATS. This establishes early-onset joint abnormalities as key features of this new syndrome.

We previously showed that AOS in three different families is caused by heterozygous mutations in the *SMAD3* gene encoding SMAD3, which is a key protein in the TGF-β pathway.[12] Here we identified five novel *SMAD3* mutations and present an extensive clinical description of 45 patients from eight families with SMAD3-related AOS.

METHODS

Patient collection

DNA from 393 patients (95 Dutch, 158 Belgian, 133 Swiss and seven North-American) with TAAD but without mutation in the coding region of the *FBN1*, *TGFBR1* and *TGFBR2* genes was analysed for mutations in the coding region of the *SMAD3* gene. When a *SMAD3* mutation was found, clinical data of the patient were collected, clinical investigations were performed, and a family tree was constructed or extended through family histories, whereby possibly affected relatives were studied and screened for the *SMAD3* mutation found in the index.[12]

A total of 34 patients with a mutation in *SMAD3* were interviewed and examined by a clinical geneticist, six of whom have subsequently died. All had extensive clinical investigations, with scoring of five major systems implicated in connective tissue disorders, including the cardiovascular, joint, skeletal, craniofacial, and cutaneous systems. Medical records from 11 deceased patients were reviewed. This study was approved by the medical ethics committee of the Erasmus Medical Center Rotterdam (Erasmus MC), and all patients gave written informed consent for this study.

Cardiovascular studies

Extensive cardiovascular studies were performed in 29 patients with AOS with a *SMAD3* mutation, and included physical examination, ECG, transthoracic echocardiography, and imaging of the thorax and abdomen by CT angiography (CTA) or magnetic resonance angiography (MRA) as described previously.[12] Aortic root dilatation was defined as a Z-score ≥ 2 at any level. Z-scores were calculated based on the body surface area-corrected normal values published by Roman et al.[13] For the other arteries, aneurysm is defined as a 50% or greater increase in diameter compared with the expected normal diameter of the vessel. CTA of the cervical and intracranial arteries was performed in 17 AOS patients with a *SMAD3* mutation. Tortuosity of the thoracic, abdominal and cerebral arteries was scored by a radiologist.

Joint studies

Twenty-five patients were evaluated by an orthopaedic surgeon. An extensive physical examination for signs of osteoarthritis, intervertebral disc degeneration, spondylolysis or spondylolisthesis, OCD, meniscal lesions and joint laxity was performed.

Nineteen patients filled out a questionnaire about joint complaints. A radiographic skeletal survey of the total spine, hips, knees, hands, and feet was performed in 26 patients. Osteoarthritis in the extremities is characterized by the degradation of articular cartilage and subchondral bone of joints and was scored as described previously.[12] In addition, the presence of spondylolysis or spondylolisthesis was

scored. OCD, defined as a separation of an articular cartilage subchondral bone segment from the remaining articular surface, was scored in all patients that were radiologically evaluated. MRI of the joint was performed when abnormalities were seen on radiography or if patients had symptoms. Every patient who had surgery for meniscal pathology, OCD, or joint replacement because of osteoarthritis was considered affected for the respective feature.

Phenotypic studies

Physical examination was performed by a clinical geneticist. Hypertelorism was defined as an inner canthal distance ≥ +2 SD without lateral displacement of the inner canthi.[14] Dolichostenomelia was defined as an arm span/height ratio of ≥ 1.05. Arachnodactyly was scored when the middle finger length exceeded the palm length, as described by Hall et al.[14] Scoliosis was radiographically defined as a lateral curvature of the spine greater than 20 degrees in the coronal plane accompanied by vertebral rotation in the axial plane measured on standing x-rays. Hypermobility was scored when the Beighton score was ≥ 5. Acetabular protrusion was scored on pelvic radiographs or CT scans when the acetabular line crossed the normal oval shape formed by the two iliopectineal lines.

Molecular studies

Genomic DNA was isolated from peripheral blood using standard procedures (Gentra Systems, Minneapolis-USA). DNA samples from deceased patients were obtained from stored autopsy tissue (frozen or paraffin-embedded tissue). Bidirectional sequencing of all coding exons and exon-intron boundaries of the *SMAD3* gene was undertaken as previously described.[12] For annotation of cDNA and protein changes the Mutation Nomenclature guidelines from the Human Genome Variation Society (HGVS) were followed (the A from the ATG start codon and Met of the reference sequence NM_005902.3 and NP_005893.1 respectively, were numbered 1).

If *SMAD3* missense mutations were identified in patients with AOS, the possible presence in controls was investigated by direct sequencing in at least 342 ethnically matched control chromosomes. The putative pathogenicity of missense variants was investigated *in silico* using the prediction programs PolyPhen-2, HOPE and SIFT.

RESULTS

Identification of eight families with *SMAD3* mutations

SMAD3 sequence analysis in 393 patients with TAAD (without mutations in the *FBN1*, *TGFBR1* and *TGFBR2* genes) revealed five novel heterozygous *SMAD3* mutations:

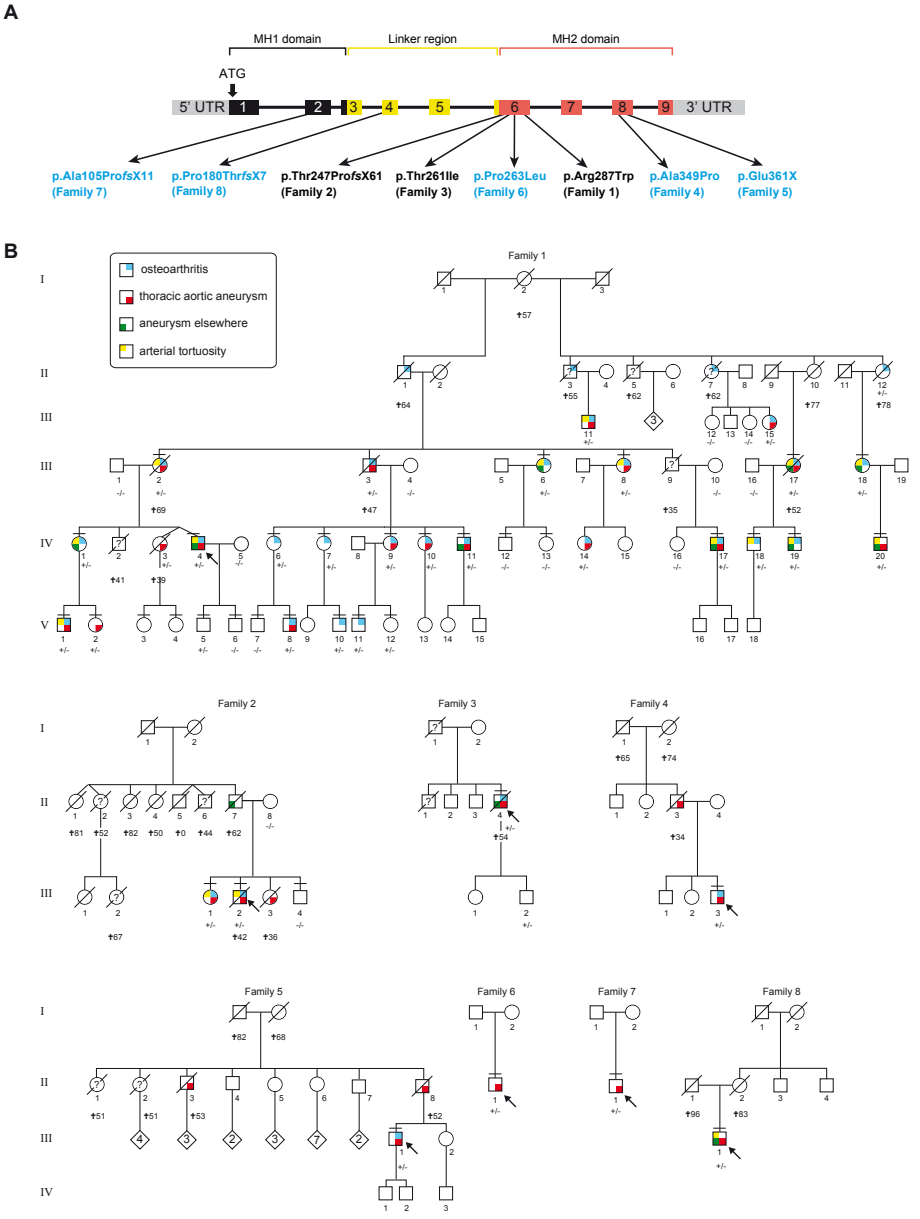

Figure 1 *SMAD3* mutations in eight families with aneurysms-osteoarthritis syndrome (AOS). (A) Schematic representation of the *SMAD3* gene. Boxes represent exons 1-9 with the untranslated regions (UTRs). The three main functional domains MH1, MH2 and the linker region are indicated. Mutations previously identified in the AOS syndrome[12] are depicted in black font, and mutations identified in this study are depicted in blue. (B) Simplified family trees of eight unrelated families with AOS. Squares indicate males, circles represent females. A horizontal line above the symbol indicates medical examination by one of us.

c.313delG (p.Ala105ProfsX11), c.539_540insC (p.Pro180ThrfsX7), c.788C>T (p.Pro263Leu), c.1045G>C (p.Ala349Pro), c.1080dupT (p.Glu361X) (Fig. 1A). Three other mutations have previously been reported by our group: c.741_742delAT (p.Thr247fsX61), c.782C>T (p.Thr261Ile) and c.859C>T (p.Arg287Trp) (Fig. 1A).[12]

The eight families with *SMAD3* mutations are unrelated and originate from the Netherlands (four families), Belgium (two families), Spain (one family) and the USA (one family). After molecular screening, 45 patients with a *SMAD3* mutation were identified. The genealogical trees of these AOS families are shown in figure 1b. In four families multiple patients were reported (Fig. 1B; families 1, 2, 4, and 5). In three families the parents were unavailable for testing and no medical records were available.

The mutations are located in exons 2, 4, 6 or 8 of the *SMAD3* gene (Fig. 1A). Four mutations introduced a frame-shift (p.Ala105ProfsX11, p.Pro180ThrfsX7 and p.Thr247fsX61) or stop codon (p.Glu361X), and were considered to be pathogenic. Four missense mutations (p.Thr261Ile, p.Pro263Leu, p.Arg287Trp and p.Ala349Pro) were probably pathogenic, based on the following observations: i) all involved residues that are highly conserved throughout evolution (from primates to zebrafish, data not shown); ii) *in silico* analysis predicts that these missense variants are likely to be pathogenic; iii) in two familial cases the *SMAD3* mutation co-segregated with AOS; iv) these four mutations were absent in at least 342 ethnically matched control chromosomes. All variants are absent in the 1094 individuals from the 1000Genomes project.

Initial clinical features

Clinical data for 45 patients with a *SMAD3* mutation were collected. The mean age of these patients with AOS was 45 years, including six children aged 17 (n=3), 15, 13, and 9 years. The main clinical characteristics of all 45 individuals from the eight families are summarized in Table 1. All patients with a *SMAD3* mutation had one or more signs of AOS.

Figure 1 continued

Due to lack of space, generation III from family 1 is split into two levels. An arrow points to the index patient. The upper right blue square indicates the presence of osteoarthritis, the lower right red square the presence of a thoracic aortic aneurysm, the lower left green square the presence of an aneurysm in any other artery, and the upper left yellow square the presence of arterial tortuosity. Open symbols are individuals with a normal or unknown phenotype. Four individuals with open symbols (family 1, patient II-10, V-5, V-12 and family 2, patient III-2) had other signs of AOS, not indicated in the legend. A question mark (?) indicates sudden cardiovascular death possibly from an arterial rupture or dissection without autopsy. Age of death is displayed below the symbol. The presence (+/-) or absence (-/-) of a *SMAD3* mutation is indicated underneath.

Table 1. Clinical findings in 45 patients with aneurysms-osteoarthritis syndrome

Feature	No	Percentage
Cardiovascular anomalies	40/45	89
Arterial anomalies	33/40	83
Thoracic aortic aneurysm	28/39	72
Abdominal aortic aneurysm	4/33	12
Aortic dissection/rupture	13/39	33
Aneurysm(s) of thoracic/abdominal arteries	9/25	36
Aneurysm(s) of cerebral arteries	6/16	38
Aortic tortuosity	10/26	38
Arterial tortuosity of thoracic/abdominal arteries	8/21	38
Arterial tortuosity of cerebral arteries	8/16	50
Ventricular hypertrophy	6/33	18
Atrial fibrillation	8/33	24
Mitral valve anomalies	18/36	50
Congenital heart malformation*	3/33	9
Joint anomalies		
Osteoarthritis of ≥1 joint	25/26	96
Osteoarthritis feet/ankle	8/26	31
Osteoarthritis hand/wrist	14/26	54
Osteoarthritis knee	13/26	50
Osteoarthritis hip	4/26	15
Osteoarthritis facet- and/or uncovertebral joints (spine)	20/26	77
Intervertebral disc degeneration	34/37	92
Spondylysis/spondylolisthesis	10/26	38
Meniscal lesions	7/25	28
Osteochondritis dissecans	14/25	56
Painful joints	23/27	85
Joint laxity	3/31	10
Skeletal anomalies		
Dolichostenomelia	7/33	21
Long slender fingers	13/33	39
Camptodactyly	4/30	13
Pectus deformity	12/33	36
Scoliosis	22/36	61
Protrusio acetabulae	7/20	35
Pes planus	30/33	91
Other phenotypic anomalies		
Hypertelorism	10/32	31
Abnormal palate	15/28	54

Abnormal uvula	13/25	52
Velvety skin	18/29	62
Striae	17/32	53
Easy bruising	10/28	36
Hernia inguinalis/umbilicalis	17/40	43
Prolapse of bladder/uterus/bowel	7/17	41
Migraine/severe headache	15/30	50
Varices	18/31	58
Chronic fatigue	11/28	39

* Congenital heart malformations included atrial septal defect, persistent ductus arteriosus, pulmonary valve stenosis and bicuspid aortic valve

Adults

All but three adult patients had consulted different physicians because of AOS symptoms prior to this study. In 19/35 (54%) of the adult patients joint complaints were the initial symptom for which medical advice was sought (age range 18-61 years). In none of them was a (aneurysm) syndrome suspected. In these patients with AOS, joint abnormalities mainly consisted of OCD, osteoarthritis, and meniscal lesions.

Cardiovascular abnormalities were the presenting feature in 46% (16/35) of the adult patients (age range 20-66 years). Sudden death from aortic dissections, aortic aneurysms and severe mitral valve insufficiency was the most common presentation. In three patients, the diagnosis of MFS was made at the time of presentation on the basis of the revised Ghent criteria.[15]

One patient (Fig. 1; family 1, patient II-1) died suddenly at the age of 64 years from an unknown cause.

Children

All six children (aged 9-17 years) were referred for initial check-up after AOS was diagnosed in the family. Radiological studies were performed in three patients (family 1, patients V-8, V-10 and V-11). A 12-year-old patient presented with knee and lower back pain. Radiography and MRI showed agenesis of the anterior cruciate ligaments, OCD of the knee and severe intervertebral disc degeneration. A 17-year-old boy with mild back pain had severe intervertebral disc degeneration at multiple levels. One 16-year-old boy had a tenodesis of the first metacarpophalangeal joint.

All six children had cardiovascular examinations, which revealed an aortic aneurysm at the level of the sinus of Valsalva in two patients. These aneurysms were first diagnosed at the age of 14 and 16 years. Two children had mitral valve prolapse.

Cardiovascular anomalies

Cardiovascular abnormalities were documented in 89% (40/45) of our patients with AOS. These included thoracic aortic aneurysm and/or dissection, aneurysm of other arteries, tortuosity of the arterial tree, left ventricular hypertrophy, atrial fibrillation and congenital heart malformation. Arterial anomalies were present in 83% of patients.

Thoracic aortic aneurysms (TAA) were present in 28 of 39 patients who had aortic root measurements. They were mainly present at the level of the sinus of Valsalva and ranged from 36 mm (Z-score 2,9) to 63 mm (Z-score 13,2) with a mean age at diagnosis of 39 years (range 14-65 years) (Fig. 2A). Eleven patients had been

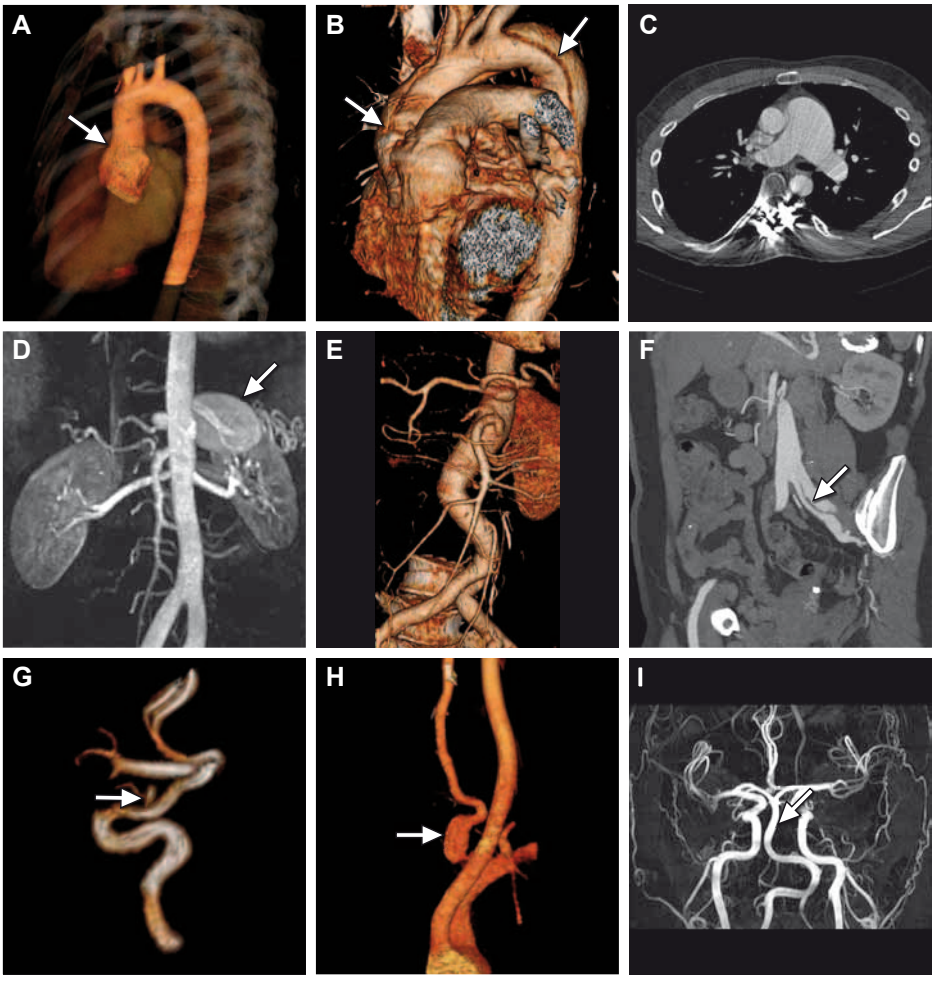

Figure 2 Arterial anomalies, including aneurysms, dissections and tortuosity.

successfully operated on by elective aortic root replacement at maximum aortic diameters between 40 and 63 mm. Mean age at surgery was 41 years (range 20-64 years). In four patients, an abdominal aortic aneurysm was reported, at ages 49, 50, 61 and 62 years (Fig. 2E).

In total, 13 patients had an aortic dissection. A Stanford type A dissection was present in 11 patients; in five of them, the aortic root diameters could be determined before dissection occurred and ranged between 40 and 63 mm (mean 51 mm). In two patients, aortic dissections occurred while the aorta was only mildly dilated (Fig. 1B; family 1, patients III-2 and III-17) with maximal ascending aortic diameters of 45 mm and 40 mm, respectively (Fig. 2B). Five patients with a Stanford type A dissection had a successful aortic root replacement at a mean age of 46 years (38-52 years). Four patients had a Stanford type B dissection, and in two of them the dissection occurred in only mildly or non-dilated abdominal aortas (Fig. 2F). Two patients had both a Stanford type A and B dissection. Three patients had dissections in other non-dilated arteries, namely the coronary, common and internal iliac, and superior mesenteric artery.

Fifteen patients with AOS died suddenly between 34 and 69 years of age. Autopsy was performed in six patients and confirmed a Stanford type A dissection in five patients and a Stanford type B dissection in one patient. In seven patients no autopsy was performed, but three of them were previously known to have aortic aneurysms/dissections. Other arterial aneurysms were detected in nine of 25 (36%) patients studied, mainly involving the vertebral, pulmonary, splenic, iliac and mesenteric arteries (Fig. 2C-E). One patient (Fig. 1B; family 1, patient IV-4) had an aneurysm of

Figure 2 continued

(A) Three-dimensional (3D) reconstructed CT angiography (CTA) of a 20-year-old man (family 1, patient V-1) shows an aneurysm at the level of the sinus of Valsalva of 45 mm (arrow). (B) 3D reconstructed CTA of a 50-year-old woman (family 1, patient III-17) showing a Stanford type A aortic dissection (arrows) extending into the brachiochephalic trunk at a maximal aortic diameter of 40 mm. (C) CTA of a 29-year-old man (family 1, patient IV-19) shows an aneurysm of the truncus pulmonalis of 50 mm. (D) Magnetic resonance angiograophy (MRA) of a 38-year-old man (family 1, patient IV-4) shows an aneurysm of the splenic artery of 40mm (arrow) and marked arterial tortuosity of the splenic artery. (E) 3D reconstructed CTA of a 50-year old woman (family 1, patient III-17) showing tortuosity of abdominal aorta, suprarenal aneurysm of the abdominal aorta of 30mm, and aneurysms of the coeliac trunk, and left common iliac artery. (F) CTA of a 45-year-old woman (family 1, patient IV-1) shows a Stanford type B aortic dissection at a maximal abdominal aortic diameter of 24 mm with dissection flap extending into the left common iliac artery (arrow). (G) MRA of a 34-year-old man (family 1, patient IV-17) shows a saccular aneurysm of the right ophthalmic artery of 3.5 mm (arrow). (H) 3D reconstructed CTA of a 29-year old man (family 1, patient IV-19) shows a fusiform aneurysm of the left vertebral artery of 11 mm (arrow). (I) MRA of a 41-year-old man (family 4, patient III-3) showing the cerebral arteries. The calibre of the basilar artery is similar to that of the internal carotid arteries, indicating fusiform dilation.

the splenic artery of 40 mm (Fig. 2d) and another patient (Fig. 1B; family 2, patient II-7) showed bilateral internal iliac aneurysms of 80 mm, as well as an abdominal aortic aneurysm of 100 mm. Imaging of the cerebral arteries revealed both intra- and extracranial aneurysms in 38% of patients involving the vertebral, carotid, basilar and ophthalmic arteries (Fig. 2G-I). In two patients (Fig. 1B; family 1, patient II-10 and II-12) a stroke was reported, at 56 and 76 years. The family history of family 2 revealed two 50% risk carriers with a stroke at 52 and 67 years (Fig. 1B; family 2, patients II-2 and III-2).

Tortuosity of the large- or medium-sized arteries was present in the majority of patients. Aortic tortuosity was found in 38% (Fig. 2E), tortuosity of other thoracic and abdominal arteries (mainly the subclavian and splenic arteries) in 38% (Fig. 2D), and tortuosity of the cerebral arteries (including the vertebral, internal carotid, cerebral and pericallosal arteries) in 50% of our patients with AOS.

Left ventricular hypertrophy was diagnosed in 18% of patients. It was mild to moderate, mainly concentric, and was not the consequence of hypertension as most patients were normotensive without treatment. Atrial fibrillation was a common finding, with 24% (8/33) of patients having at least one episode. The age of onset ranged between 23 and 76 years. Three out of eight patients had a single episode of atrial fibrillation after surgery. Mitral valve abnormalities were reported in half of the patients, the youngest being 14-years old. These anomalies ranged from mild prolapse to severe regurgitation requiring valve replacement. Congenital heart malformations were found in 9% (3/33) of our AOS patients, and included bicuspid aortic valve, pulmonary valve stenosis, persistent ductus arteriosus, and atrial septal defect. Of 13 women having a total of 23 pregnancies, one patient had a severe postpartum hemorrhage, but no other vascular complications or uterine ruptures were reported. In more than 30% of patients who initially presented with cardiovascular anomalies, joint abnormalities were reported later in life.

Joint anomalies

Almost all (96%) patients studied had radiologically proven osteoarthritis, with 75% of these in two or more joint types. Eighty-five percent of these patients had painful joints. The mean age at osteoarthritis diagnosis was 42 years and the youngest patient with osteoarthritis was detected at 12 years of age. The joints that were mostly affected were spine, hands and/or wrists, and knees, but osteoarthritis was also reported in all other joints including feet and/or ankle, hip and shoulder (Fig. 3H-J). Hand/wrist osteoarthritis was present in 14 patients and in half of them the first carpometacarpal joint was involved (Fig. 3H). Other affected joints were the scaphotrapeziotrapezoidal, distal interphalangeal, proximal interphalangeal joints and occasionally metacarpophalangeal I joints. Furthermore, intervertebral disc

degeneration mainly involving the cervical and lumbar discs was present in 92% (34/37) of patients (Fig. 3E-G) on retrospective evaluation of x-rays and CT scans. In addition, vertebral bodies showed shape irregularities located in the region of the anterior growth plates. These abnormalities were in some documented cases already present at a young age (youngest: 12 years).[12] Spondylolysis and/or spondylolisthesis (Fig. 3F) were common (38%).

More than half of the patients (56%) had non-traumatic OCD even at young age (Fig. 3a,c,d). OCD occurred mainly in the knee and occasionally in the ankle or hip. Patients with OCD were operated before the age of 40 years- the youngest patient at the age of 10 years. OCD was asymptomatic in some cases (Fig. 3B). Seven patients with AOS (28%) had meniscal lesions, one of whom had bilateral meniscectomy at the age of 13 years. In one patient, a congenital absence/agenesis of the anterior cruciate ligament was seen on MRI of the knee at the age of 12 years (Fig. 3B). Three patients had an arthroplasty of the knee at an average age of 64 (range 61-68 years), and one patient had an arthroplasty of the thumb base at the age of 58 years. Joint laxity, defined as a Beighton score of ≥ 5, was seen in a minority (10%) of patients.

In the 19 patients who initially presented with joint abnormalities, extensive cardiovascular workup was performed in the following years because of their family history or cardiovascular symptoms. In 64%, cardiovascular abnormalities were reported. More importantly, four out of 19 died suddenly from an aortic dissection.

Skeletal anomalies

Approximately 40% of the patients had long and slender fingers and toes, but overt arachnodactyly (as defined above) was not present. A positive thumb sign was seen in seven patients and a positive wrist sign in one patient. Dolichostenomelia was present in 21% of patients.

Twelve patients (36%) had pectus carinatum, pectus excavatum or asymmetry of the costosternal junction. Scoliosis was present in 61% of our patients, and three of them were operated for severe scoliosis. One patient had foraminal stenosis requiring foraminotomy of L5-S1 with spondylodesis of L4-S1. Protrusio acetabulae was present in one-third (35%) of patients and was usually mild. Over 90% of patients had pes planus. Camptodactyly was present in four out of 30 (13%) patients.

Craniofacial abnormalities

Figure 4 illustrates the facial features of 20 patients with AOS. Facial characteristics included high forehead, hypertelorism, long face, flat supraorbital ridges, and malar hypoplasia, but were generally mild. Uvular anomalies (raphe, broad or bifid) were common in our series (52%). Of the 13 patients with uvular abnormalities, 62% had

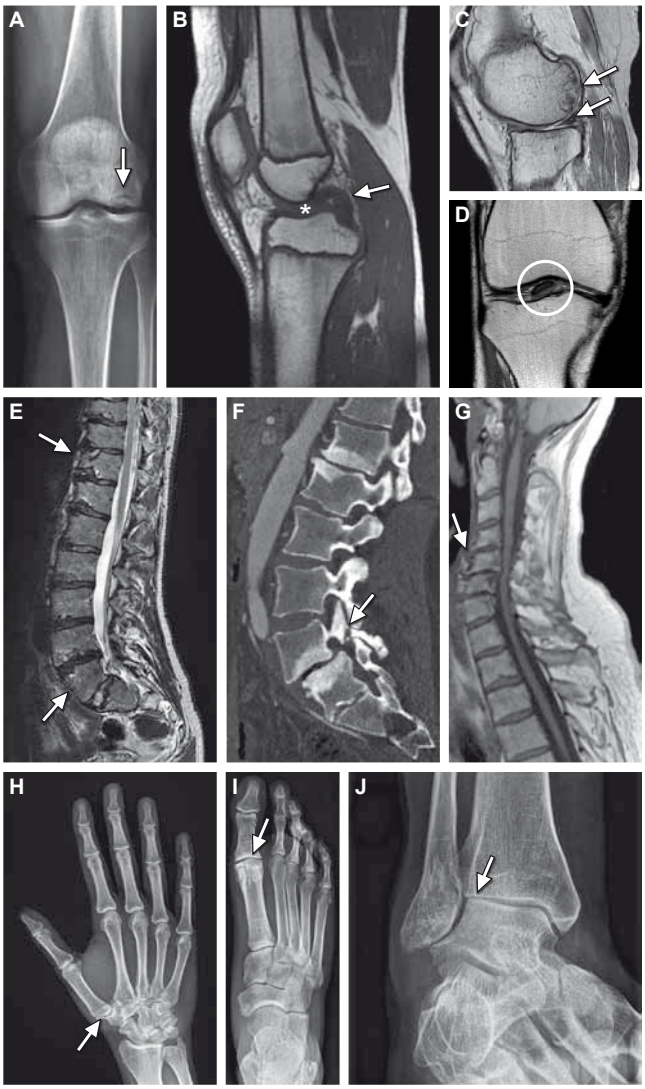

Figure 3 Osteoarthritis and osteochondritis.
(A) X-ray of the left knee of a 41-year-old patient (family 4, patient III-3) shows a large osteochondral lesion without separation in the lateral femoral condyle (arrow). (B) MRI of the right knee of a 12-year-old patient (family 1, patient V-10) shows congenital absence of the anterior cruciate ligament (ACL). Asterisk: no ACL is seen in its expected location. The arrow points to the normal posterior cruciate ligament. (C) MRI of the knee of a 48-year-old woman (family 2, patient III-1) shows a large osteochondral lesion without separation in the medial femoral condyle (upper arrow). There is also a horizontal tear of the medial meniscus (lower arrow). (D) MRI of the right knee of a 17-year-old man (family 1, patient V-1) with a loose intraarticular body (encircled) due to a large osteochondral lesion of the medial femoral condyle (not shown). (E) MRI of the thoracic and lumbar spine of a 17-year-old man (family 1, patient V-8) with marked irregularity and impression of the antero-inferior endplates at multiple levels (see arrows for examples).

Figure 4 Facial features of 20 patients with aneurysms-osteoarthritis syndrome from four different families. Photographs are boxed in red (family 1), green (family 2), blue (family 4), and yellow (family 5). Facial features include hypertelorism, a long face, flat orbital ridges, a high forehead and malar flattening. Overall, the most prominent facial feature is hypertelorism. Written consent was obtained for publication of these images.

a broad uvula with or without a raphe and 38% had a true bifid uvula. Uvular abnormalities may be an easy diagnostic clue, as they only occur in LDS, but not in other syndromic or non-syndromic forms of TAAD. High-arched palate was common and one patient was operated for a cleft palate. Dental malocclusion and retrognathia were occasionally seen. No craniosynostosis was observed or reported. There was a marked inter- and intra-familial variability in facial features (Fig. 4).

Additional features

Some features that are common in connective tissue disorders are also frequent in AOS. Umbilical and/or inguinal hernias were present in 17/40 (43%) of patients (age range 1-50 years). Pelvic floor prolapse occurred in seven of 17 adult women

Figure 3 coninued

F: CT scan of a 44-year-old woman (family 1, patient IV-6) with severe multilevel degenerative disc disease and a spondylolisthesis at the L4-L5 level due to a bilateral spondylolysis (arrow). (G) MRI scan of a 50-year-old man (family 3, patient II-4) with marked degenerative abnormalities of the lower cervical spine (arrow) with narrowing of the spinal cord. (H) X-ray of the right hand and wrist of a 31-year-old man (family 1, patient IV-18) with moderate osteoarthritis of the first carpometacarpal joint (arrow). (I) X-ray of the right foot of a 31-year-old man (family 1, patient IV-18) with moderate osteoarthritis of the first metatarsophalangeal joint (arrow). (J) X-ray of the ankle of a 40-year-old woman (family 1, patient IV-9) with marked degenerative changes of the talocrural joint with severe lateral joint space narrowing (arrow).

and mainly involved the uterus (6/7) and occasionally the bladder (2/7) and bowel (1/7). The mean age at operation for pelvic floor prolapse was 50 years (range 43-64). Varices or thread veins were reported or observed in 18 of 31 patients, usually already presented at a young age (youngest patient 17 years) and were therapy(surgery)-resistant. Velvety skin and striae were present in the majority (62% and 53%) of the patients. Other recurrent findings included easy bruising and atrophic scars. Recurrent severe headaches or migraine was present in half (15/30) of the patients and did not co-occur with the cerebrovascular abnormalities.

Some additional features occurred sporadically in the eight families, but were not systematically evaluated in all patients. Diverticulosis was reported in four patients, and dural ectasia in seven patients. Two patients had unexplained severe lung emphysema, at the age of 63 and 54 years; one patient did not smoke but no details on smoking or other risk factors for emphysema were available for the other patient. In three patients xanthelasmata around the eye were reported, although no dyslipidaemia was found. Almost 40% (11/28) of patients complained of chronic or intermittent increased fatigue. Ophthalmological examination in 29 patients revealed no lens luxation. One patient had cataract surgery and multiple procedures for retinal detachment; one patient had mild cataract at the age of 54 years, and amblyopia was present in two patients. Hydrocephaly was not found. No moderate or severe developmental delay was reported in any patient, although no IQ tests were performed.

DISCUSSION

SMAD3 mutations

We have identified here five new and private heterozygous *SMAD3* mutations in five unrelated AOS families. As we screened 393 patients with TAAD (negative for *FBN1*, *TGFBR1* and *TGFBR2* mutations), *SMAD3*-associated TAAD represents a small fraction of TAAD. Because patients in our cohort were initially analysed for syndromic TAAD genes, this cohort may be enriched for patients with MFS and LDS features.

In total, we have identified eight *SMAD3* mutations, six of which were located in the MH2 domain, which mediates oligomerisation of SMAD/SMAD4 and Smad-dependent transcriptional activation. Two frame-shift mutations were located upstream within the MH1 or proline-rich linker region. They led to truncated transcripts, which were probably subjected to nonsense-mediated mRNA decay, as shown before for the p.Thr277ProfsX61 mutation.[12] The most likely effect of these mutations is loss of function, with TGF-β signals not being propagated via SMAD3. Notably, we have

previously shown that this leads to a paradoxical increase in TGF-β signaling in the aortic wall[12], which has also been found in other syndromes characterised by arterial wall anomalies, such as MFS, LDS, ATS, and FBLN4-related AR-CL.[7,10-11,16]

Regalado *et al*[17] recently described four different *SMAD3* mutations (p.Ala112Val, p.Asn218fs, p.Glu239Lys and p.Arg279Lys) in patients with TAAD and aneurysms affecting other vessels, including cerebral arteries and osteoarthritis. The frequency of *SMAD3* mutations in their cohort of nonsyndromic familial TAAD patients was 2%, which is comparable to that in our cohort of (not necessarily familial) TAAD patients.

In addition, a p.Asn197Ile missense variant was found in a patient with osteoarthritis who was not evaluated for other AOS anomalies.[18]

Patients with AOS

We present the clinical and molecular data for 45 patients with *SMAD3* mutations from eight unrelated families with AOS. The patients come from families of Dutch, Belgian, Spanish and American ancestry.

All patients with a *SMAD3* mutation exhibited symptoms or signs of AOS, with the youngest patient being 9 years old. Although not all families could be completely evaluated, the penetrance of the mutations is nearly 100%. The expression varied from very mild (isolated bifid uvula in a 9-year-old girl, family 1, patient V-12) to severe (multiple aneurysms and dissections in a 50-year-old woman, family 1, patient III-17) disease. Age-dependant progression of the phenotype is evident, as aneurysms and osteoarthritis were encountered mainly during adulthood, although this study only included six children. The cardiovascular abnormalities at a young age were generally mild and mainly include mitral valve prolapse or congenital heart malformations. The youngest patient diagnosed with an aortic aneurysm was 14 years old. All dissections occurred in adulthood, the youngest patient was 34 years of age.

AOS is mainly characterised by a combination of arterial anomalies with early-onset osteoarthritis, but mild craniofacial anomalies and other features reminiscent of connective tissue disorders are also present. The AOS phenotype with typical cardiovascular and orthopaedic anomalies was present in at least five out of eight families. Two families were not screened for joint abnormalities, and in only one family joint problems were not reported. Similarly, Regalado *et al* reported osteoarthritis or joint disease in four of their five families (37% of their cases) with *SMAD3* mutations, although radiological investigations to asses osteoarthritis were not performed.[17]

Intrafamilial variability, as illustrated by the clinical findings in a large family of 33 patients with AOS, was significant: while some patients presented mainly with arterial aneurysms and dissections, others only had joint abnormalities. Therefore the genotype-phenotype correlation, if present, will be difficult to establish.

Cardiovascular anomalies

The vast majority (89%) of patients with AOS had cardiovascular abnormalities. Aneurysms and tortuosity were found throughout the complete arterial tree studied, in both large and medium-size vessels, including the cerebral arteries. Despite the presence of intracranial aneurysms, stroke has rarely been reported in AOS. Dissections occurred in the aorta and in medium-/small-sized arteries, including a coronary artery. Arterial dissection and rupture occurred occasionally in aortas that were only mildly dilated; therefore early preventive surgery with resection of the aneurysms is advised.

Apart from arterial aneurysms and tortuosity, there were also other cardiovascular anomalies present in many patients with AOS, including mitral valve anomalies, congenital heart malformations, ventricular hypertrophy and atrial fibrillation. The congenital heart malformations were significantly more common than expected in the general population ($p<0,0001$). It is very likely that *SMAD3* mutations also lead to cardiac hypertrophy and atrial fibrillation via TGF-β upregulation. It is currently unclear how loss-of-function mutations in *SMAD3* lead to a paradoxical increase in TGFβ signaling[12] and the congenital and age-related cardiovascular anomalies described above.[19]

Joint anomalies

Most of the patients developed joint abnormalities, including OCD, meniscal lesions, intervertebral disc degeneration, and osteoarthritis. These abnormalities were already present at a young age. Interestingly, joint complaints were the first symptom for which clinical advice was sought in the majority of patients.

OCD was present in more than half of patients, mainly in the medial, but also in the lateral, femoral condyle of the knee. Interestingly, mutations in the *ACAN* gene encoding the proteoglycan aggrecan have been described in families with autosomal dominant inheritance of OCD.[20] Aggrecan is a downstream effector of the TGF-β signaling pathway, and may mediate *SMAD3*-associated OCD in AOS.

Intervertebral disc degeneration was present in most (92%) patients and mainly involved the cervical and lumbar spine. Mice studies have shown that TGF-β is essential for promoting and/or maintaining the intervertebral disk during development.[21]

Osteoarthritis was present in almost all patients with AOS, and primarily involved the joints of the knee, spine, hand and foot. In SMAD3-related disease, osteoarthritis could be secondary to OCD, joint laxity or disc degeneration, which are present in many patients with AOS. However, OCD of the medial femoral condyle (most commonly observed in patients with AOS) rarely results in osteoarthritis. This area is non-weight-bearing, and therefore less prone to osteoarthritis.[22] In addition, osteoarthritis is also present in joints not affected by OCD or meniscal lesions. Joint laxity may

also play a role in development of osteoarthritis: pes planus, scoliosis, and joint hypermobility may indicate ligamental insufficiencies. Therefore, in addition to intrinsic abnormalities of the hyaline cartilage of the joints, early-onset osteoarthritis in these families may be enhanced by overload based on abnormal menisci, intervertebral discs and/or ligaments. Spinal osteoarthritis at the intervertebral and uncovertebral joints may be the result of the early disc degeneration.

It is likely that osteoarthritis in AOS is not only due to secondary changes but also to abnormal development of the cartilage directly caused by the *SMAD3* mutation. TGF-β has a dual role in chondrocytes, primarily acting as a stimulator in chondrocyte differentiation and, in later stages of development, blocking chondrocyte terminal differentiation.[23-25] SMAD3 has an important function in this TGF-β mediated growth inhibition and maintenance of the articular cartilage.[25-26] This is corroborated in *Smad3* knock-out mice, which show premature chondrocyte maturation and subsequent premature osteoarthritis.[25,27] A direct role for SMAD3 in osteoarthritis is further supported by the identification of a *SMAD3* mutation in a patient with knee osteoarthritis[18], the recent association between a single-nucleotide polymorphism in intron 1 of *SMAD3* and the risk of both hip and knee osteoarthritis[28], and several *in vitro* studies.[29]

Other phenotypic anomalies

A MFS habitus with dolichostenomelia, long slender fingers, pectus deformity and scoliosis was present in a minority of patients. Aspecific cutaneous anomalies commonly seen in connective tissue disorders, including velvety skin with striae and easy bruising, were present in the majority of patients with AOS. Craniofacial features were often mild or absent and mainly included hypertelorism (Fig. 4) and a broad or bifid uvula. Overall, the phenotypic anomalies in many patients with AOS were discrete, and missed on consultation for cardiovascular anomalies, whereby the patients were classified as non-syndromic TAAD.

Comparison with other TGFβ-related syndromes

Although many cases of AOS were classified as non-syndromic TAAD, their phenotype overlaps with that of aneurysm syndromes such as MFS and LDS. Some patients had a MFS habitus, whereas others had craniofacial anomalies with features such as hypertelorism and broad/bifid uvula reminiscent of LDS.

The cardiovascular features in patients with AOS are similar to those of LDS, including thoracic aortic aneurysms at the level of the sinus of Valsalva, and aneurysms and tortuosity throughout the arterial tree. However, involvement of the entire arterial tree, including the intracranial arteries, is rare in patients with MFS. Similarly to LDS, the aortic aneurysms of patients with AOS tend to rupture at smaller aortic diameters

than in MFS. Aneurysms are less common in ATS than in AOS, although a similar tortuosity of the entire arterial tree is found.

Atrial fibrillation and ventricular hypertrophy (24% and 18%, respectively) have not yet been reported in LDS and are both uncommon in MFS[30], whereas some patients with ATS show ventricular hypertrophy.[31] Mitral valve anomalies, mainly prolapse, are equally common in AOS (50%) and MFS (54%)[32] and less common in LDS (35%).[33]

Congenital heart malformations are found in only 9% of patients with AOS, in contrast with 22-35% in LDS.[8] The nature of these defects- that is persistent ductus arteriosus and atrial septal defect- are similar in both disorders.[8] In only 1% of patients with MFS have congenital heart malformations been reported.[33]

Joint anomalies with osteoarthritis, OCD and meniscal lesions are key features of AOS, being present in almost all patients. Such anomalies are rarely described in LDS, MFS or ATS. None of the 90 patients with LDS type I or II described by Loeys *et al* were reported to have osteoarthritis, OCD or meniscal abnormalities, although cervical dislocation or instability, spondylolisthesis and intervertebral disc degeneration have been occasionally described.[8,34] Interestingly, arthralgia, osteoarthritis of the hand, hip and/or spine was reported in several patients from a large family with LDS due to a *TGFBR2* mutation.[35] Also in MFS, osteoarthritis, OCD and meniscal abnormalities have only sporadically been described[36], although spondylolisthesis is present in 6% of MFS patients.[37] Further joint studies in patients with MFS and LDS are warranted to establish the frequency of osteoarthritis and OCD in these related syndromes.

In conclusion, joint anomalies such as osteoarthritis, OCD or meniscal abnormalities may be a useful discriminating feature from other forms of TAAD. Therefore the syndrome is named AOS. X-ray examinations of knees, total spine and hands, particularly in TAAD patients with a medical or family history of joint complaints or abnormalities, is recommended. Furthermore, as these typical joint anomalies may be the presenting feature of AOS before symptoms or signs of the cardiovascular features become obvious, we recommend imaging of the heart and complete arterial tree including cerebral arteries, to exclude arterial anomalies in patients with early onset osteoarthritis in combination with OCD or a family history of aortic aneurysm or sudden death.

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AGGRESSIVE CARDIOVASCULAR PHENOTYPE OF ANEURYSMS-OSTEOARTHRITIS SYNDROME CAUSED BY PATHOGENIC *SMAD3* VARIANTS

CHAPTER 7

ABSTRACT

Objectives: To describe the cardiovascular phenotype of the Aneurysms-Osteoarthritis syndrome (AOS) and provide clinical recommendations.

Background: AOS, caused by pathogenic *SMAD3* variants, is a recently described autosomal dominant syndrome characterized by aneurysms and arterial tortuosity in combination with osteoarthritis.

Methods: AOS patients in participating centers underwent extensive cardiovascular evaluation, including imaging, arterial stiffness measurements and biochemical studies.

Results: We included 44 AOS patients from 7 families with pathogenic *SMAD3* variants (age 42±17 years). In 71% an aortic root aneurysm was found. In 33% aneurysms in other arteries in thorax and abdomen were diagnosed and in 48% arterial tortuosity. In 16 patients cerebrovascular imaging was performed and cerebrovascular abnormalities were detected in 56%. Fifteen deaths occurred at a mean age of 54 ± 15 years. Main cause of death was aortic dissection (9/15;60%), which occurred at mildly increased aortic diameters (range 40-63 mm). Furthermore, cardiac abnormalities were diagnosed, such as congenital heart defects (6%), mitral valve abnormalities (51%), left ventricular hypertrophy (19%) and atrial fibrillation (22%). N-terminal brain natriuretic peptide (NT-proBNP) was significantly higher in AOS patients compared to matched controls ($p<0.001$). Aortic pulse wave velocity was high-normal (9.2±2.2 m/s), indicating increased aortic stiffness, which strongly correlated with NT-proBNP ($r=0.731$, $p=0.005$).

Conclusions: AOS predisposes patients to aggressive and widespread cardiovascular disease, and is associated with high mortality. Dissections can occur at relatively mildly increased aortic diameters, therefore early elective repair of the ascending aorta should be considered. Moreover, cerebrovascular abnormalities were encountered in the majority of patients.

INTRODUCTION

Aortic aneurysms and dissections were ranked as the 19th common cause of death in the US in 2007.[1] The true incidence is probably much higher, since many aortic aneurysms are silent. Thoracic aortic aneurysms and dissections (TAAD) are often found in the context of genetic syndromes, such as Marfan syndrome (MFS) and Loeys-Dietz syndrome (LDS), but are also associated with bicuspid aortic valves (BAV).[2-4] MFS is one of the most common hereditable connective tissue disorders with abnormalities predominantly in the skeletal, ocular, pulmonary and cardiovascular

system.[2] LDS shows some similarities with MFS, but exhibits widespread arterial aneurysms and tortuosity.[3]

Recently our group found that pathogenic *SMAD3* variants cause Aneurysms-Osteoarthritis Syndrome (AOS).[5] AOS is inherited as an autosomal dominant disorder and is found to be responsible for 2% of familial TAAD.[5-6] Aneurysms, dissections and tortuosity throughout the arterial tree are the main cardiovascular features.[5] In addition, early-onset osteoarthritis is present in almost all patients and is often the first reason to seek medical advice.[5] Mild craniofacial abnormalities, such as hypertelorism and bifid uvula, are also associated with AOS.[5] Furthermore, umbilical and/or inguinal hernias, varices, velvety skin and striae are common findings.[5]

The aim of this clinical article is to describe the cardiovascular consequences of AOS and provide clinical recommendations.

METHODS

Clinical studies

From 2009 on, all AOS patients with a pathogenic *SMAD3* variant in participating centers were included in this ongoing cohort study. Genetic identification methods have previously been described.[5] Patients underwent comprehensive clinical evaluation, including risk factor assessment, physical examination, biochemical measurements, 12-lead electrocardiography (ECG), transthoracic echocardiography (TTE) and computed tomography angiography (CTA) of thorax and abdomen. Due to logistical reasons, not all examinations could be performed in every patient. In a subset of patients CTA of the cerebral vessels and arterial stiffness measurements were also performed. Patients were monitored for occurrence of cardiovascular events, especially dissection or mortality. Autopsy was requested in case of death and performed when possible. Biochemical and arterial stiffness measurements were compared 1-to-1 with age-, sex- and smoking status matched controls. Apparently healthy controls were recruited from hospital personnel and their acquaintances; and only underwent biochemical and arterial stiffness measurements and smoking status assessment.

Transthoracic echocardiography (TTE)

Aortic root dimensions were measured at the level of the aortic annulus, Valsalva sinus, sinotubular junction, and proximal ascending aorta. Aortic dilatation was considered when normalized diameters for gender and body surface area (BSA) were greater than the mean + 2 standard deviations.[7] Left ventricular mass was calculated using the Devereux-modified formula.[8] Left ventricular hypertrophy (LVH)

was defined by a BSA-indexed threshold of >134 g/m2 for men and >110 g/m2 for women.[9] Mitral valve regurgitation was quantified into 4 grades: trace, mild, moderate and severe.[10] Mitral valve prolapse was defined by a coaptation occurring 2 mm behind the mitral annulus in the parasternal long-axis view.[11]

Computed tomography angiography (CTA)

Presence of aneurysms, dissections and arterial tortuosity throughout the body was systematically evaluated by an experienced cardiovascular radiologist. Aortic root dimensions were measured at the level of the aortic annulus, Valsalva sinus, sinotubular junction, proximal ascending aorta, aortic arch, thoracic descending aorta and abdominal aorta. We considered the aorta dilated if the Z-score was ≥2 according to gender and BSA.[12] Tortuosity was defined as a severe (pigtail-like) curve or multiple curves in an artery. A trained neuroradiologist and neurologist evaluated presence, location, type and diameter of intracranial aneurysms, dissections or tortuosity.

Cardiovascular risk factor assessment

Information on cardiovascular risk factors was collected during the outpatient clinic visit at the Department of Cardiology. Smoking was classified as never, former, or current smoking. Height and weight were measured and body mass index was calculated (kg/m2). Sitting blood pressure was measured during 30 minutes at 5-minute intervals using an automated device (Dynamap, Critikon Inc, model 8101) in a private room.

Biochemical measurements

Blood samples for laboratory measurements were obtained intravenously after a 30-minute rest period. Plasma renin concentration was measured by an immunoradiometric assay (Cisbio), aldosterone by radioimmunoassay (Coat-A-Count, Siemens), endothelin-1 by achemiluminescent ELISA (QuantiGlo, R&D Systems), and N-terminal probrain natriuretic peptide (NT-proBNP) by a radioimmunoassay (Phoenix Pharmaceuticals, Inc).

Aortic pulse wave velocity (aPWV)

Measurements of aortic stiffness were carried out in a controlled environment at 22±1 °C after subjects had reclined at rest for 15 minutes. The aPWV was assessed by ECG-gated applanation tonometry (SphygmoCor® system, ArtCor, Sydney, Australia) with patients in supine position.[13] Time delay between the rapid upstroke of the feet of recorded pulse waves in the carotid and femoral arteries was measured. The distance between the carotid artery and groin was measured with calipers. The

aPWV was calculated as the ratio between the distance and foot-to-foot time delay and was expressed in meters per second.

Carotid distensibility

Carotid diameters and intima-media thickness (IMT) were measured on the distal wall of the right common carotid artery, 1 to 2 cm beneath the bifurcation, with a high-precision echo-tracking device (Wall Track System, Pie-medical Esaote).[14] End-diastolic diameter (D) and absolute stroke change in diameter during systole (ΔD) were computed as the mean of 5 cardiac cycles of 3 successive recordings. Cross-sectional arterial wall distensibility coefficient was calculated according to the following equation: distensibility coefficient (DC) = $2\Delta D/(D*\text{central pulse pressure})$ (10-3/kPa).[15] Wall cross-sectional area and Young's elastic modulus were calculated according to previously described formulas.[16]

The study was approved by the institutional review board and ethical committee of the Erasmus MC in Rotterdam. Written informed consent was obtained from each patient.

Data analysis

SPSS 15.0 (SPSS Inc., Chicago, Illinois) was used for the statistical analyses. *P<0.05* was considered statistically significant. The one-sample Kolmogorov-Smirnov Test and histograms were used to check normality. Normally distributed continuous data are presented as mean ± standard deviation and categorical variables as frequency (n) and percentages. Non-normal distributed data are presented as median with interquartile range (interquartile range (IQR), 25th and 75th percentiles). For comparison between the control and patient groups, a student's *t* test taking into account the 1-1 pairing or the signed-ranks Wilcoxon test was used. Biochemical measurements were also compared with reference values from the clinical chemical laboratory of the Erasmus MC, Rotterdam. For correlation analysis, the Pearson r correlation coefficient and Spearman correlation test were used.

RESULTS

We here describe the cardiovascular features of 44 AOS patients from 7 families. Genetic mutations are specified in Table 1. Twenty-seven patients from 3 families were previously described briefly in the first report on AOS.[5] Table 2 presents the baseline characteristics of the study population. Two patients (62 and 64 years) had hypertension and used anti-hypertensive drugs.

Table 1 Cardiovascular abnormalities for each individual patient

		Vascular abnormalities in thorax/abdomen				Intracranial and brachiocephalic vascular abnormalities		Intracardiac abnormalities				Mortality	Osteo-arthritis (OA), joint abnormalities	Pathogenic SMAD3 variants
Age (yr)	Gender	Aortic root aneurysm	Aneurysms other arteries	Dissection (type)	Tortuosity	Aneurysm	Tortuosity	CHD	MV abn.	AF	LVH	No /yes, age (yr) cause		
72	F	-	-	-	-	-	--	no	yes	yes	yes	77, heart failure *	--	c.859C>T (p.Arg287Trp)
74	F	no	no	no	-	-	--	no	yes	yes	yes	78, heart failure	feet, hands, knees, hip, spine	c.859C>T (p.Arg287Trp)
67	F	yes	no	A	yes	-	--	no	no	no	yes	69, AD	feet, hands, knee, spine	c.859C>T (p.Arg287Trp)
45	M	yes	no	A	-	-	--	no	-	-	--	47, AD	knee, OCD knee	c.859C>T (p.Arg287Trp)
63	F	no	abdominal aorta, common, external iliac artery	no	no	yes	yes	no	no	no	--	no	feet, hands, knee, spine, OCD knee	c.859C>T (p.Arg287Trp)
62	M	yes	no	no	no	no	yes	no	yes	no	no	no	feet, hands, knee	c.859C>T (p.Arg287Trp)
57	F	yes	no	no	no	-	--	no	no	no	no	no	hands, knees, hip, spine	c.859C>T (p.Arg287Trp)
30	F	yes	no	no	-	-	--	no	yes	no	no	no	OA	c.859C>T (p.Arg287Trp)
34	M	-	-	-	-	-	--	-	-	--	--	35*, SCD, no autopsy	-	c.859C>T (p.Arg287Trp)
46	F	yes	descending aorta, brachiocephalic, iliac, superior mesenteric artery	A and B	yes	-	--	PDA and ASD	yes	yes	yes	52, AD	knee, spine	c.859C>T (p.Arg287Trp)
56	F	no	no	no	yes	yes	yes	no	yes	yes	no	no	feet, hands, knee	c.859C>T (p.Arg287Trp)

		Vascular abnormalities in thorax/abdomen				Intracranial and brachiocephalic vascular abnormalities		Intracardiac abnormalities				Mortality	Osteo-arthritis (OA), joint abnormalities	Pathogenic SMAD3 variants
Age (yr)	Gender	Aortic root aneurysm	Aneurysms other arteries	Dissection (type)	Tortuosity	Aneurysm	Tortuosity	CHD	MV abn.	AF	LVH	No /yes, age (yr) cause		
31	M	yes	no	no	yes	yes	yes	no	no	no	no	no	--	c.859C>T (p.Arg287Trp)
44	F	no	common, internal, external iliac, splenic, celiac artery	B	yes	yes	yes	no	yes	no	no	no	hands, spine	c.859C>T (p.Arg287Trp)
20	M	yes	no	no	yes	-	--	no	yes	no	no	no	knee, hip, OCD knee	c.859C>T (p.Arg287Trp)
37	F	yes	no	A	-	-	--	-	-	-	--	39, AD	--	c.859C>T (p.Arg287Trp)
39	M	yes	splenic and hepatic artery	Proximal LAD coronary artery	yes	no	no	no	yes	yes	yes	no	hands, knee, OCD knee	c.859C>T (p.Arg287Trp)
13	M	no	no	no	-	-	--	no	yes	--	no	no	--	c.859C>T (p.Arg287Trp)
41	F	no	no	no	no	no	no	no	no	no	no	no	knee, spine	c.859C>T (p.Arg287Trp)
16	M	yes	no	no	no	no	no	no	yes	no	no	no	-	c.859C>T (p.Arg287Trp)
39	F	no	no	no	no	no	no	no	no	no	no	no	feet, knee, spine, OCD knee	c.859C>T (p.Arg287Trp)
34	F	yes	no	no	no	no	no	no	no	no	no	no	knee, spine, OCD knee	c.859C>T (p.Arg287Trp)
33	F	yes	no	no	-	no	no	no	no	no	no	no	spine, OCD knee	c.859C>T (p.Arg287Trp)

		Vascular abnormalities in thorax/abdomen				Intracranial and brachiocephalic vascular abnormalities		Intracardiac abnormalities				Mortality	Osteo-arthritis (OA), joint abnormalities	Pathogenic SMAD3 variants
Age (yr)	**Gender**	**Aortic root aneurysm**	**Aneurysms other arteries**	**Dissection (type)**	**Tortuosity**	**Aneurysm**	**Tortuosity**	**CHD**	**MV abn.**	**AF**	**LVH**	**No /yes, age (yr) cause**		
30	M	yes	pulmonary, celiac and superior mesenteric artery	no	no	no	yes	no	yes	no	yes	no	hands, OCD knee	c.859C>T (p.Arg287Trp)
29	M	yes	no	no	no	yes	yes	no	no	no	no	no	hands, OCD	c.859C>T (p.Arg287Trp)
26	M	no	pulmonary, superior mesenteric, common iliac and vertebral artery, PDA	no	no	yes	no	PS, PDA	yes	yes	no	no	spine	c.859C>T (p.Arg287Trp)
26	M	yes	no	no	yes	no	no	no	yes	no	no	no	feet, hands, spine, OCD	c.859C>T (p.Arg287Trp)
58	F	yes	--	no	–	–	–	no	yes	no	no	no	feet, hands, knee, hip, spine	c.859C>T (p.Arg287Trp)
42	M	yes	no	A	yes	–	–	no	no	no	–	42, AD	–	c.741-742delAT (p.Thr247fsX61)
44	F	yes	no	no	yes	no	yes	no	yes	no	no	no	knee, spine, OCD knee	c.741-742delAT (p.Thr247fsX61)
35	F	yes	no	A	–	–	–	--	–	–	–	36, AD	–	c.741-742delAT (p.Thr247fsX61)
60	M	--	abdominal aorta, iliac arteries	B	–	–	–	--	–	–	–	62, AD	–	c.741-742delAT (p.Thr247fsX61)
54	M	yes	abdominal aorta, pulmonary artery	A and B	no	–	–	--	no	yes	no	54, AD	OCD hip	c.782C>T (p.Thr261Ile)
15	F	yes	no	no	–	–	–	no	yes	no	no	no	–	c.859C>T (p.Arg287Trp)

		Vascular abnormalities in thorax/abdomen				Intracranial and brachiocephalic vascular abnormalities		Intracardiac abnormalities				Mortality	Osteo-arthritis (OA), joint abnormalities	Pathogenic SMAD3 variants
Age (yr)	Gender	Aortic root aneurysm	Aneurysms other arteries	Dissection (type)	Tortuosity	Aneurysm	Tortuosity	CHD	MV abn.	AF	LVH	No /yes, age (yr) cause		
17	M	no	no	no	–	–	--	no	no	no	no	no	hands, spine	c.859C>T (p.Arg287Trp)
13	M	no	no	no	–	–	--	no	yes	no	no	no	OA, OCD knee	c.859C>T (p.Arg287Trp)
9	F	no	no	no	–	–	--	no	no	no	no	no	--	c.859C>T (p.Arg287Trp)
55	M	–	–	–	–	–	--	–	–	–	--	55*, SCD, no autopsy	OA	c.859C>T (p.Arg287Trp)
68	M	–	–	–	–	–	--	–	–	--	--	68*, bowel rupture, no autopsy	--	c.859C>T (p.Arg287Trp)
62	F	–	–	–	–	–	--	–	–	--	--	62*, SCD, no autopsy	OA	c.859C>T (p.Arg287Trp)
54	M	yes	no	A	–	–	--	no	no	--	--	no	--	c.313delG (p.Ala105ProfsX11)
48	M	yes	no	A	yes	–	--	no	no	no	no	no	--	c.1080dupT (p.Glu361X)
56	M	yes	no	A	–	–	--	no	no	–	--	no	--	c.788C>T (p.Pro263Leu)
41	M	yes	no	no	no	–	–	no	no	–	no	no	OA, OCD knee	c.1045G>C (p.Ala349Pro)
34	M	yes	no	A	–	–	–	–	–	–	–	34, AD	--	c.1045G>C (p.Ala349Pro)

n -- indicates there data were not available; LVH, left ventricular hypertrophy;AD, aortic dissection; AF, atrial fibrillation; ASD, atrial septal defect; CHD, congenital heart disease; LAD, left anterior descending; MW, mitral valve; PDA, persistent ductus arteriosus; PS, pulmonary stenosis; OA, osteoarthritis; OCD, osteochondritis dissecans; SCD, sudden cardiac death. * These patients were obligate carriers.

Table 2. Baseline characteristics.

Covariates	AOS patients (n=44)
Age, years	42 ± 17
Male, n (%)	24 (55)
Height, cm	181 ± 13
Weight, kg	78 ± 15
Body mass index, kg/m^2	24 ± 4
Blood pressure, mmHg	
Systolic blood pressure	124 ± 14
Diastolic blood pressure	74 ± 8
Mean arterial pressure	92 ± 11
Oxygen saturation,%	98 ± 1
Smoking, n (%) *	
Never	24 (73)
Current	6 (18)
Former	3 (9)
Creatinine, µmol/l *	72 ± 11

AOS indicates Aneurysms-Osteoarthritis Syndrome.
* Smoking status and creatinine measurements could only be obtained from 33 patients.

Survival

Fifteen deaths in AOS patients with confirmed pathogenic *SMAD3* variants occurred at a mean age of 54 ± 15 years. Autopsy confirmed an aortic dissection as cause of death in 6 patients. In the 9 other patients no autopsy was performed, but 3 patients were previously known with aortic aneurysms/dissections. Causes of death with age at time of death are specified in Table 1. No intracranial hemorrhage as cause of death has been reported.

Aneurysms, dissections and arterial tortuosity in thorax and abdomen

In 27/38 patients (71%) an aortic root aneurysm was found (range 36-63 mm; Z-score 2.9-13.2; Fig. 1A). For 6 patients, we did not have aortic dimension data because they died before TTE or CTA could be performed. In 8/24 patients (33%) aneurysms in other arteries in thorax and abdomen were diagnosed: descending thoracic and abdominal aorta (100 mm), pulmonary trunk (50 mm), superior mesenteric, splenic (40 mm), celiac, hepatic, and common, external and internal iliac artery (80 mm) (Fig. 1B; Table 1). Arterial tortuosity throughout the great vessels of the abdomen and thorax was present in 48% (11/23).

Mean aortic diameters measured by CTA and TTE are shown in Table 3. The aorta was most often dilated at the level of the sinus of Valsalva. CTA aortic diameter measurements correlated well with TTE (sinus of Valsalva: r=0.939, p<0.001). Two

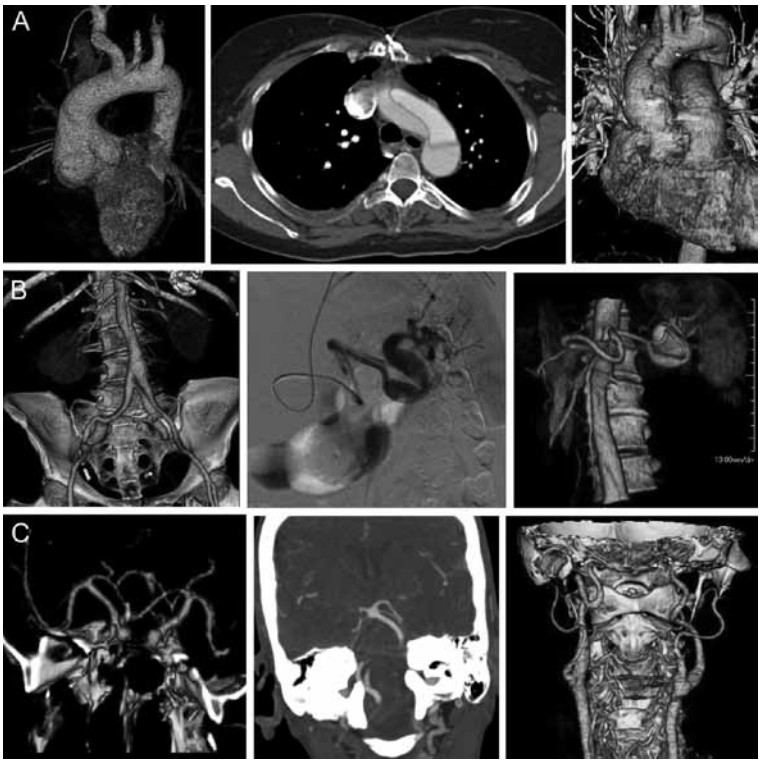

Figure 1 Cardiovascular abnormalities throughout the body in patients with AOS. **(**A) Thorax (from left to right). Aneurysm of the aortic root (54 mm) in 31-year-old male. Stanford type A aortic dissection at a maximal aortic diameter of 40 mm in a 50-year-old female. (B) Abdomen (from left to right). Aortic dissection at a maximal abdominal aortic diameter of 24 mm with dissection flap extending into the left common iliac artery (true lumen in internal iliac artery and false lumen in external iliac artery) and aneurysm in the right common iliac artery (27 mm) and right external iliac artery (16 mm) in 45-year-old female. Tortuosity and aneurysm in left splenic artery (21 mm) in same 45-year-old female. (C) Head and neck (from left to right). Two saccular aneurysms in the left and right carotid siphon in a 31-year-old male. Fusiform aneurysm of the top of the basilar artery in a 26-year-old male. Tortuosity of the internal carotid artery in a 34-year-old male.

out of 6 evaluated children (33%) presented with aortic diameter Z-scores above normal range according to age (Z+2.9 in a male 16-year-old and Z+3.3 in a female 15-year-old).

Thirteen patients, age 46 ± 10 years, were diagnosed with 1 or more aortic dissections. Stanford type A aortic dissection was diagnosed in 11 patients (Fig. 1A). In 8 patients this was the first manifestation of the disease. Range of sinus of Valsalva diameter measured before aortic root dissection occurred was 40 - 63 mm (reliable aortic measurements prior to dissection were available for 5 patients only). Stanford type B aortic dissection was diagnosed in 2 patients (Fig. 1B). In addition, 2 patients were diagnosed with both a type A and B dissection at different time

points. None of these aortic dissections occurred during the 23 pregnancies and deliveries in our AOS cohort. In 1 patient a dissection in a non-dilated proximal left anterior descending coronary artery was found.

Elective cardiovascular operations and interventions

Fifteen patients underwent ≥1 elective cardiovascular interventions at a mean age of 41 ± 11 years: 12 valve-sparing aortic root replacements, 1 Bentall procedure, 2 splenic artery coiling, 1 abdominal aneurysm repair, and 1 patient had aortic repair surgeries in thorax and abdomen and mitral valve repair. In 2 patients post-operative complications occurred: one developed painful splenic ischemia for which re-operation was necessary and another patient developed a total AV block after valve sparing aortic root replacement for which pacemaker implantation was necessary.

Aneurysms and tortuosity of brachiocephalic and intracranial vasculature

CTA of the brachiocephalic and intracranial vasculature was performed in 16 patients, age 37 ± 14 years. In 56% (9/16) we found cerebrovascular abnormalities (Table 3). Six patients (38%) were diagnosed with ≥1 intracranial aneurysms (Fig. 1C). Tortuosity of brachiocephalic and intracranial vessels was found in 50% (8/16) of the patients (Fig. 1C). Thirty-one percent of patients (5/16) had a combination of aneurysms and tortuosity. In addition, 1 patient showed multiple caliber changes of both intra- and extracranial vessels. In 7 patients no cerebrovascular abnormalities were found. Two patients reported to have suffered from a non-fatal stroke at respectively 56 and 76 years old, but it is unclear from their medical history whether these were ischemic or hemorrhagic strokes.

Cardiac abnormalities

In 18/35 patients (51%) ≥1 mitral valve abnormalities were diagnosed (5 prolapse, 5 billowing, and respectively 5 mild, 2 moderate and 3 severe mitral valve regurgitation). In 2 patients structural congenital heart defects were found: 1 patient had an atrial septal defect and persistent ductus arteriosus (PDA) and another patient had mild congenital pulmonary valve stenosis (peak velocity 1.82 m/s) and PDA. A remarkable finding in this patient was a saccular aneurysm within the PDA. In addition, 1 patient was found to have a BAV during surgery.

Left ventricular systolic function and mitral inflow patterns were normal in all patients (Table 4). LVH was present in 19% (6/31), with a mean interventricular septal thickness of 12 ± 2 mm, left ventricular posterior wall thickness of 12 ± 2 mm, left ventricular mass of 296 ± 84 gram and BSA-indexed left ventricular mass of 146 ±

Table 3. Detailed information about 9 AOS patients with brachiocephalic and intracranial vascular abnormalities.

Gender	Age (years)	Aneurysm	Aneurysm location(s)	Diameter (mm)	Fusiform or saccular	Anterior or posterior	Intra- / extracranial	Tortuosity	Tortuosity location(s)	Anterior or posterior	Intra- or extracranial
Female	44	Yes, *	Basilar artery (bilateral)	4.5	Fus	Post	Intra	Yes	Internal carotid arteries (bilateral)	Ant	Extra
Female	63	Yes, one	Left arteria communicans anterior A2 origo	3	Fus	Ant	Intra	Yes	Origo vertebral arteries (bilateral)	Post	Extra
Female	56	Yes, two	1: Top of basilar artery 2: Origo of basilar artery	1: 7 2: 5.5	1: Fus 2: Fus	1: Post 2: Post	1: Intra 2: Intra	Yes	1: Origo right vertebral artery 2: Vertebrobasilar system	1: Post 2 : Post	1: Extra 2: Intra
Male	29	Yes, two	1: Basilar artery 2: Right ophthalmic artery	1: 5 2: 4	1: Fus 2: Sac	1: Post 2: Ant	1: Intra 2: Intra	Yes	Internal carotid artery, and arteria cerebri anterior and media (bilateral)	Ant	Extra and intra
Male	31	Yes, two	1: Right carotid siphon 2: Left carotid siphon	1: 5 2: 5	1: Sac 2: Sac	1: Ant 2: Ant	1: Extra 2: Intra	Yes	Left vertebral artery	Post	Intra
Male	26	Yes, two	1: Top of basilar artery 2: Proximal vertebral artery	1: 5 2: 11	1: Fus 2: Fus	1: Post 2: Post	1: Intra 2: Extra	No			
Male	34	No						Yes	1: Internal carotid (bilateral) 2: Vertebrobasilar	1: Ant 2: Post	1: Extra 2: Intra
Male	62	No						Yes	1:Internal carotid (bilateral) 2: Vertebrobasilar system	1: Ant 2: Post	1: Extra 2: Intra
Female	44	No						Yes	Vertebral arteries (bilateral)	Post	Intra

Abbreviations: ant = anterior; post = posterior; intra = intracranial; extra = extracranial; fus = fusiform; sac = saccular.
* In addition, multiple caliber changes intracranial and extracranial anterior and posterior were found.

34 gram/m^2. None of these patients had hypertension, aortic coarctation, or aortic stenosis.

Rhythm disturbances

ECG revealed sinus rhythm in all patients (Table 4). In 5 patients premature ventricular contractions (≥3) were found. Seven out of 31 patients (22%) had a history of at least one episode of documented atrial fibrillation (AF).

Aortic stiffness and biochemical measurements

Table 5 shows aortic stiffness and biochemical measurements for healthy controls and AOS patients. The aPWV tended to be higher in AOS patients compared to controls (9.2 ± 2.2 versus 7.8 ± 1.8 m/s; p=0.076). Compared to reference values

Table 4. Outcome measurements.

Covariates	AOS patients
Electrocardiography (n=31)	
Heart rate, bpm	67 ± 12 (50 – 90)
PR-interval, msec	159 ± 24 (136 – 204)
QRS-duration, msec	101 ± 10 (90 118)
Echocardiography (n=31)	
Left atrial diameter, mm	37 ± 5 (26 – 49)
Interventricular septal thickness, mm	10 ± 2 (9 – 15)
Left ventricular posterior wall thickness, mm	10 ± 2 (8 – 14)
Left ventricular wall mass, gram	204 ± 75 (135 – 342)
Left ventricular end-diastolic diameter, mm	52 ± 8 (40 – 64)
Left ventricular end-systolic diameter, mm	33 ± 5 (25 – 42)
Fractional shortening,%	36 ± 7 (28 – 48)
Peak E velocity, m/s	0.6 ± 0.2 (0.3 – 0.9)
Peak A velocity, m/s	0.5 ± 0.1 (0.3 – 0.7)
Transmitral E/A ratio	1.5 ± 0.5 (0.5 – 2.2)
E wave decelaration time, msec	233 ± 82 (134 – 420)
Aortic diameters, mm	
Annulus	26.8 ± 3.1 (20 – 31)
Sinus of Valsalva	40.1 ± 8.2 (30 – 50)
Sinotubular junction	31.9 ± 4.8 (27 – 38)
Proximal ascending aorta	32.8 ± 4.5 (27 – 46)
Computed tomography angiography (n = 38)	
Aortic diameters, mm	
Annulus	29.8 ± 5.9 (23 – 38)
Sinus of Valsalva	41.4 ± 8.2 (30 – 63)
Sinotubular junction	32.0 ± 4.9 (27 – 38)
Ascending thoracic aorta	32.4 ± 5.2 (28 – 39)
Aortic arch	25.6 ± 5.4 (19 – 34)
Descending thoracic aorta	24.9 ± 4.5 (20 – 32)
Diaphragmatic level aorta	22.2 ± 5.0 (16 – 28)
Abdominal aorta	22.3 ± 5.3 (15 – 100)

AOS indicates Aneurysms-Osteoarthritis Syndrome. Values are expressed as mean ± standard deviation (absolute range).

Table 5. Aortic stiffness and biochemical measurements in AOS patients and controls.

Variable	Reference Values	AOS patients (n=21)	Control group (n=21)	p-value
Age, years		38.6 ± 14.8	38.8 ± 10.4	0.975
Male, n (%)		9 (43)	9 (43)	1.000
Smoker, n (%)				0.347
Never		16 (76)	18 (86)	
Current		2 (10)	0 (0)	
Former		3 (14)	3 (14)	
Heart rate, bpm		65.8 ± 15.6	68.4 ± 10.0	0.555
Pulse wave velocity, m/s *		9.2 ± 2.2	7.8 ± 1.8	0.076
Transit time, msec		169 ± 61	154 ± 29	0.383
Augmentation index @HR75,%		23 ± 18	18 ± 17	0.220
Systolic blood pressure, mmHg				
Brachial		127 ± 15	128 ± 12	0.790
Central		116 ± 14	112 ± 15	0.386
Diastolic blood pressure, mmHg				
Brachial		74 ± 9	74 ± 8	0.968
Central		75 ± 9	74 ± 6	0.883
Mean arterial pressure, mmHg				
Brachial		92 ± 11	93 ± 8	0.669
Central		92 ± 11	87 ± 8	0.076
Pulse pressure, mmHg				
Brachial		53 ± 7	54 ± 11	0.735
Central		41 ± 8	37 ± 10	0.129
Carotid intima-media thickness, µm		621 ± 193	620 ± 149	0.981
Carotid end-diastolic diameter, mm		6.8 ± 1.3	6.7 ± 0.7	0.774
Carotid stroke change, µm		383 ± 142	444 ± 214	0.325
Wall cross-sectional area, mm^2		5.8 ± 2.2	6.8 ± 2.1	0.184
Distensibility coefficient, 10^{-3}/kPa		26.2 ± 9.9	29.3 ± 13.4	0.438
Young's elastic modulus, kPa x 10^3		0.18 ± 0.11	0.22 ± 0.10	0.351
NT-proBNP, pg/ml	<115	94.1 (52.5 – 172.9)	12.7 (8.5 – 55.1)	<0.001
Renin, pg/ml	6 – 30	6.8 (4.0 – 16.2)	9.2 (5.9 – 10.6)	0.917
Aldosteron, pg/ml	50 – 200	56.5 (36.0 – 70.0)	50.5 (33.5 – 71.0)	0.598
ET-1, pg/ml	<2.5	1.7 (1.5 – 2.0)	0.7 (0.6 – 1.0)	<0.001

AOS indicates Aneurysms-osteoarthritis syndrome; NT-proBNP, N-terminal Pro Brain Natriuretic Peptide; ET-1, endothelin-1. * Pulse wave velocity measurements could only be obtained in 19 AOS patients.

controlled for age and blood pressure, 6/18 patients (33%) had an aPWV value >2 standard deviations.[17] Aortic diameter and aPWV were not correlated (r=-0.278; p=0.357). NT-proBNP was higher in AOS patients than in matched controls (94.1 (52.5 – 172.9) versus 12.7 (8.5 – 55.1) pg/ml; p<0.001) and correlated with aPWV (r=0.731, p=0.005).

Associated findings of AOS

Osteoarthritis was confirmed by X-rays in 25 out of 26 (96%) patients who underwent orthopedic evaluation, while 85% exhibited painful joints. Mean age at osteoarthritis diagnosis was 42 years, while the youngest patient was 12 years old. Spine, hands/wrists and knees were mostly affected (Table 1). Pes planus was present in 91% of patients and scoliosis in 61%. Other associated anomalies included hypertelorism (31%), abnormal palate (54%), abnormal uvula (52%), hernia inguinalis or umbilicalis (43%) and uterus, bladder or bowel prolapse (41%). More detailed information about these associated findings will be reported separately.

DISCUSSION

AOS is a recently described autosomal dominant connective tissue disorder characterized by aneurysms, dissections and tortuosity throughout the arterial tree in combination with osteoarthritis and mild craniofacial features. The AOS phenotype may resemble that of other connective tissue disorders such as MFS and LDS (Table 6). The main site of aortic aneurysms in AOS is the sinus of Valsalva. Similar to LDS, AOS is an aggressive disease with substantial mortality and a high risk of aortic rupture and dissection in mildly dilated aortas.[18] AOS and LDS are both associated with widespread arterial tortuosity and aneurysms in thorax and abdomen.[18] In contrast to MFS, cerebrovascular abnormalities frequently occur in AOS and LDS.[19] Identification of the underlying genetic defect in TAAD patients is crucial, considering the variability in prognosis, treatment strategy and risk assessment in family members.

Cardiac abnormalities in AOS

In addition to the aneurysms and tortuosity of the arterial tree, we also found cardiac abnormalities. A remarkable finding in about one fifth of the patients was left ventricular hypertrophy in the absence of hypertension or aortic stenosis. Primary cardiomyopathy is reported in one quarter of Marfan patients showing mainly a reduced left ventricular ejection fraction, but only in a minority (2.9%) LV mass was increased.[20] Mice studies have determined that TGF-β induces proliferation of cardiac fibroblasts and hypertrophic growth of cardiomyocytes.[37] Furthermore, TGF-β

Table 6. Comparison of cardiovascular findings in different disorders affecting the aorta.

Cardiovascular features	**Aneurysms-osteoarthritis syndrome**[5]	**Marfan syndrome** [2,20-26]	**Loeys-Dietz syndrome** [3,18-19,25-26]	**Vascular Ehlers-Danlos syndrome** [25,27-29]	**Turner syndrome** [30-32]	**Bicuspid aortic valve** [4,33-36]
Genetic defect or mutation	*SMAD3*	*FBN1*	*TGFBR1 / TGFBR2*	*COL3A1*	45,X0	Multiple, among which *NOTCH1*
Thoracic aortic aneurysm	71%	77%	98-100%	25-50%	20-30%	15-45%
Main location of aortic dilatation	Sinus of Valsalva	Sinus of Valsalva	Sinus of Valsalva	Descending thoracic and abdominal aorta	Sinotubular junction	Ascending aorta
Aneurysms of other arteries in abdomen/thorax	18%	7%	52-73%	48-63%	4-29%	None
Arterial tortuosity abdomen/thorax	48%	40%	67-84%	NR	NR	NR
Intracranial aneurysms	38%	6.5%	32%	25-33%	5%	10%
Intracranial tortuosity	50%	NR	67-84%	NR	NR	NR
Mitral valve abnormalities	51%	60-80%	29%	6%	2%	NR
Atrial fibrillation	22%	8%	NR	NR	1%	NR
Congenital heart disease *	6%	1%	22-35%	0%	16-49%	15%
Left ventricular mass	Increased in 19%	Increased in 2.9%	NR	NR	Increased	Increased
Median survival (years)	62 †	70 †	37	48	70	Comparable to population estimates
NT-proBNP	Elevated	Elevated	NR	NR	Normal	NR
Pulse wave velocity	Increased	Increased	NR	Normal	Increased	Increased
Intima-media thickness	Normal	Normal	NR	Decreased	Increased	NR
Carotid distensibility	Normal	Decreased	NR	Increased	NR	NR

*NR indicates not reported; NT-proBNP, N-terminal Pro Brain Natriuretic Peptide. * Including: atrial septal defect, persistent ductus arteriosus, pulmonary stenosis, coarctation of the aorta, partial anomalous pulmonary venous return. † With elective interventions.*

neutralizing antibodies were able to attenuate LV hypertrophy and losartan reduced non-myocyte proliferation, implying possible therapeutic implications in humans as well.[38]

Similar to MFS, mitral valve abnormalities were common in AOS patients; and 22% of AOS patients had a history of AF. Mice studies have shown that TGF-β*1* induced myocardial fibrosis in the atria plays an important role in predisposing to AF.[39] Atrial fibrogenesis in patients with AF occurs in two phases: an early increase, but later loss of responsiveness to TGF-β*1*, while the fibrosis progresses.[40]

Furthermore, evidence from mouse studies suggests that TGF-β signaling is essential in the embryogenesis of the heart, valvular pathogenesis and organization of the aortic wall.[41-42] In many mouse models with disrupted TGF-β signaling activities congenital heart defects are present.[41] In the future, SMAD3 knockdown mice will help to explore the mechanism behind the cardiac abnormalities in AOS.

Aortic stiffness and NT-proBNP in AOS

NT-proBNP in AOS patients was elevated compared to controls, although none of the patients had extremely high NT-proBNP levels >250 pg/ml. In vivo and in vitro studies have shown that treatment with BNP can attenuate cardiac hypertrophy via the TGF-β*1* pathway.[43] One might hypothesize that the elevated NT-proBNP levels in AOS patients are in fact a protective mechanism against emergence of LV hypertrophy. Since (mild-to-moderately) elevated NT-proBNP levels in other patient groups are reported to predict cardiovascular outcome and AF recurrence, evaluation of the prognostic value of NT-proBNP in AOS patients with respect to clinical outcome may be important.[44-45]

The aPWV as a measure of aortic stiffness, was high-normal in AOS patients, as was previously described in for instance MFS and BAV.[21,33] Ascending aortic diameter and aPWV were not correlated, suggesting that arterial stiffness occurs independently of aneurysm formation. In MFS patients an augmentation index >11% has reported to predict progression of aortic diameters, so further research is warranted to test whether this also holds true for AOS patients.[46]

Clinical suggestions for cardiologists treating AOS patients

Although AOS is a recently discovered aneurysm syndrome and the full spectrum of the disease and its progression need to be clarified, some preliminary suggestions may be derived from the current findings. Because multisystem involvement is frequently observed, cooperation in a multidisciplinary team with clinical geneticists, cardiologists, orthopedic surgeons, radiologists, neurologists and when necessary (vascular/cardio-thoracic) surgeons is important.

Monitoring and screening

Cardiologists should suspect AOS in every TAAD patient without molecular diagnosis or known cause and test these patients for *SMAD3* mutations. Furthermore, we suggest that clinicians treating patients with arterial aneurismal disease in any large artery (intracranial, iliac, splenic artery etcetera) should at least ask whether these patients exhibit joint complaints. In the physical examination one must pay special attention to presence of AOS-associated findings, such as joint anomalies and abnormal uvula.

Extensive cardiovascular evaluation using echocardiography and CTA or magnetic resonance imaging (MRI) (head to pelvis) is recommended in every adult AOS patient. Initially, these diagnostic investigations should be performed annually to determine rate of progression. Thereafter, frequency of imaging should be guided by the findings, for instance annually if the aortic diameter is >35 mm, or if the aortic diameter shows significant growth (>5 mm/year).

The phenotype seems to be age-dependent as aneurysms mainly and dissections only occurred in adulthood, however our series only included 6 children with AOS. Concerning screening in childhood, clear suggestions are difficult to formulate at this time. We suggest that frequency of cardiologic evaluation with TTE and/or MRI must be guided by the aortic root Z-score and presence of other cardiac abnormalities.

Although in our cohort no dissections occurred during pregnancy or delivery, pregnancy should be considered high risk in AOS patients with aneurysms, as in MFS and LDS.[47]

Treatment

The implication of TGF-β signaling in the pathogenesis of aortic aneurysm syndromes suggests a TGF-β antagonist as specific pharmaceutical target.[48] Although losartan showed promising results in MFS mouse models, we have to await the results of randomized clinical trials in MFS, SMAD3 knockdown mice and consequently AOS clinical trials.[48] At the moment, attention should be focused on genetic counseling, screening of relatives, interventional or surgical treatment. Medical treatment with losartan and/or beta blockade might be beneficial. Stringent control of hypertension to limit aortic wall stress is recommended.[12]

Since dissections in AOS patients can occur at relatively small aortic diameters, early elective surgical intervention is indicated in order to reduce the risk of mortality. Since data are limited and rate of progression is unknown, we suggest applying surgical recommendations for Loeys-Dietz syndrome.[12] Valve-sparing aortic root replacement using the re-implantation technique is the intervention of choice.[49] For peripheral aneurysms, individual size or rate of growth and location must determine the treatment strategy.

Currently, the risk of rupture of intracranial aneurysms associated with AOS is unknown. No deaths due to intracranial hemorrhage occurred in our series. Life

expectancy, and size, location and rate of growth of the aneurysm are the most important determinants to decide whether intervention is needed.

Study limitations

First, the number of subjects included in the present study is relatively small, since AOS is only recently discovered. Second, the population is quite heterogeneous, particularly in disease severity and age, and due to logistical reasons and mortality it was not possible to perform every examination in all 44 patients. Further research is necessary to confirm our findings and gain more insight in the disease mechanisms and progression.

CONCLUSIONS

AOS is an aggressive, inherited, connective tissue disorder characterized by arterial tortuosity, aneurysms and osteoarthritis. Aortic root enlargement is the most common cardiovascular finding in our series, but cerebrovascular abnormalities were also present in >50% of patients. Aortic dissections occur at smaller diameters than observed in for instance MFS, and as such need early elective surgical treatment. Larger prospective follow-up studies are warranted to determine progression over time and clinical relevancy of the cardiac and intracranial abnormalities.

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SUMMARY AND DISCUSSION

CHAPTER 8

Cardiovascular malformations (CVM), including congenital heart malformations (CHM) and aortic aneurysms, are a relatively common cause of mortality and morbidity. In the recent years enormous progress has been made in the discovery of many genetic factors that contribute to the development and homeostasis of the heart and large vessels (**Chapter 1**). An important contribution to this understanding has been made by the identification of disease genes in familial cases of CVM. As described in this thesis, we studied families with different monogenic forms of CVM in order to delineate the phenotypes, to identify the disease genes and unravel the underlying molecular pathways. Although monogenic (syndromic and nonsyndromic) forms of CVM are relatively scarce, studies in these kindreds can provide knowledge that is also important to understand the etiology of sporadic cases of CVM.

The findings of our studies on heterotaxy (**Chapter 2**) suggest that heterotaxy is a complex, oligogenic disease in the majority of patients. Linkage analysis performed in a consanguineous Iranian family with laterality defects identified homozygous variants in the cilia-related *NPHP4* gene and rare heterozygous *NPHP4* variants in sporadic heterotaxy patients. The rare heterozygous *NPHP4* variants identified in the sporadic heterotaxy cases have probably a major effect with either additional genetic modifiers, environmental, or stochastic factors. These genetic modifiers could be present in other genes in the same or an interacting signaling pathway. Notably, most cases of CVM are non-syndromic and sporadic and the etiology seems to be the result of a complex interplay of mostly unknown factors, including several genes with high penetrance mutations, modifying effects of susceptibility alleles, environmental exposures and gene-environment interactions.

In **Chapter 3** we describe in a consanguineous family with autosomal recessive inheritance of primary pulmonary vein stenosis (PVS) with prenatal lymphatic abnormalities. Families with multiple siblings affected with PVS have not been described so far. Homozygosity mapping studies resulted in the identification of the first locus for PVS to chromosome 2q. Whole exome sequencing (WES) and whole genome sequencing of the linkage region did not identify novel or rare exonic variants. WES covers only the coding regions which constitute only 1% of the entire human genome. It is therefore possible that the PVS mutation is located in important regulatory sequences such as promotors, enhancers, microRNAs, or evolutionary conserved non-coding sequences. Proving the pathogenicity of variants found in this region will require functional studies. Genetic studies in additional families could help in the identification the causative gene but will be difficult due to the rarity and high mortality of the disorder.

In **Chapter 4** we report on two large multiplex families with autosomal dominant inheritance of Left Ventricular Outflow Tract Obstruction (LVOTO) in association with right-sided valve anomalies and septal defects thereby extending the spectrum of cardiac anomalies seen in LVOTO. LVOTO is a genetic heterogeneous condition, with so far only one disease gene identified (*NOTCH1*). It is anticipated that different genes play a role in families with similar LVOTO phenotypes, and therefore these often small multiplex families cannot be pooled in linkage studies. As the two families described here were small only suggestive linkage to several large chromosomal regions could be obtained. Next generation sequencing (NGS) will hopefully facilitate the identification of LVOTO disease genes. After NGS the exomes of multiple affected family members can be compared to identify shared novel or rare exonic variants.

In **Chapter 5** we demonstrate how classical genome-wide linkage studies followed by positional cloning is a successful approach for gene finding in monogenic disorders. We studied a large family with autosomal dominant inheritance of a syndromic form of aortic aneurysms. We mapped the genetic locus to chromosome 15q22.2-24.2 and identified pathogenic mutations in the *SMAD3* gene, which encodes for a receptor-activated SMAD protein that plays a role in signal transmission in the TGF-β pathway. Immunohistochemistry revealed upregulation of several members of the TGF-β signaling pathway in the aortic wall of *SMAD3* patients, which is indicative of enhanced TGF-β signaling.

The condition is characterized by a combination of arterial aneurysms and tortuosity with early-onset osteoarthritis, and the presence of mild craniofacial, skeletal and cutaneous anomalies. Although patients in these *SMAD3* families showed features similar to both Loeys-Dietz syndrome and Marfan syndrome, the frequency of osteoarthritis is much higher than described in both of these syndromes. We named this separate clinical syndrome as Aneurysms-Osteoarthritis Syndrome (AOS).

Identification of this gene provides the opportunity to identify patients who are at risk of an aggressive vascular disease and could benefit from cardiovascular follow up and early surgical intervention. As the phenotype of this syndrome was found to be highly variable, a molecular diagnosis is helpful in identifying family members with often minor features, but at risk for aggressive aortic disease.

In **Chapter 6** the contribution of *SMAD3* mutations was studied in sporadic or familial TAAD patients, and a *SMAD3* mutation was found in 2%. Extensive clinical studies in 45 patients with *SMAD3* mutations from eight unrelated AOS families revealed a highly penetrant age-dependant phenotype with great intra- and inter-familial variability. In the majority of patients joint anomalies such as osteoarthritis,

OCD or meniscal abnormalities are the presenting feature of AOS before signs of the cardiovascular abnormalities become obvious. These observations lead to the clinical recommendations to perform X-rays of the joints in TAAD patients with a medical or family history of joint abnormalities, and to perform cardiovascular imaging in patients with early-onset osteoarthritis in combination with OCD or a family history of aortic aneurysm or sudden death.

In **Chapter 7** the results of extensive cardiovascular evaluation by imaging, arterial stiffness measurements and biochemical studies in 44 patients with AOS are presented. AOS predisposes patients to aggressive and widespread cardiovascular disease and is associated with high mortality as is indicated by the mean age of death of 54 years. Tortuosity and aneurysms occurred in large and medium-sized arteries throughout the body but was also frequently observed in the brachiocephalic and intracranial vasculature. Dissections occasionally occurred in relatively mildly increased aortic diameters indicating the need for early preventative surgery.

Moreover, cardiac abnormalities such as mitral valve prolapse, atrial fibrillation, left ventricular hypertrophy and congenital heart malformations were encountered in a substantial proportion of patients. Studies in humans and animal models have shown that TGF-β signaling plays a critical role in the pathogenesis of cardiac hypertrophy and atrial fibrillation.[1-3]

In AOS patients increased TGF-β signaling is present in the aortic wall. It could be speculated that *SMAD3* mutations also cause increased TGF-β-signaling in cardiomyocytes and cardiac fibroblasts and thereby inducing cardiac hypertrophy and atrial fibrosis leading to fibrillation. However, it is currently unclear how presumed loss-of-function mutations in *SMAD3* lead to a paradoxical increase in TGF-β signaling and the congenital and age-related cardiovascular anomalies described above.

Further studies of the role of *SMAD3* in the cardiovascular phenotype are ongoing in the *smad3* knock-out mouse model. In addition, we will evaluate the role of *smad3* in embryonic development by using antisense morpholinos to knockdown *smad3* expression in zebrafish.

Identification of the molecular defect in AOS families may also contribute to new therapeutic strategies interfering with the molecular pathways involved in aneurysms. The identification of excessive TGF-β signaling related to the development of aortic aneurysms in patients with MFS and successful treatment of MFS mouse with TGF-β neutralizing antibodies turned modulation of the TGF-β pathway into one of the most promising therapeutic strategy in thoracic aortic disease. Upregulated TGF-β signaling in the aortic wall is also found in other human disorders with aortic aneurysms, including Marfan syndrome (*FBN1* gene), Loeys-Dietz syndrome (*TGFBR1*

and *TGFBR2* genes), arterial tortuosity syndrome (ATS) (*SLC2A10* gene), autosomal recessive cutis laxa (*EFEMP2* gene), the aneurysms-osteoarthritis syndrome (*SMAD3* gene), and non-syndromic forms of familial aortic aneurysms (*ACTA2* and *MYH11* genes). For these separate syndromes further (animal) studies are needed to understand if and how therapeutic intervention the TGF-β pathway will alter the course of the disease. This is of particular importance as conflicting data are reported indicating, decreased activity of the TGF-β signaling pathway in aneurysm formation. Mizuguchi et al reported lowering of TGF-β signaling in MFS patients owing to TGFBR2 mutations using cell transfection assay.[4] Loss of TGF-β signaling in aneurysm formation is also reported in several animal models. In apolipoprotein E knockout (ApoE–/–) mice, supplementation of TGF-β1 prevented aortic root dilatation.[5] Wang et al showed that inactivation of TGF-β signaling increases the susceptibility to Ang II-induced aneurysm formation in mice.[6] Recently, studies in an ATS animal model has shown that treatment of *slc2a10* zebrafish knockdowns with tgfbr1 inhibitors led to an aggravated phenotype.[7] Although it is difficult to compare complex signaling pathways such as the TGF-β pathway in different developmental stages in different species, these studies are a warning for premature clinical trials in humans with (genetically-determined) aortic aneurysms using pharmacological inhibitors of the TGF-β pathway, despite early successes with such drugs like Losartan in patients with Marfan syndrome.

FUTURE PERSPECTIVES

Currently, the era of Sanger sequencing of individual (candidate) disease genes is changing rapidly into the era of NGS, which is massive parallel sequencing of very large amounts of sequence at very low cost.

In the diagnostic setting, Sanger sequencing of individual disease genes is being replaced by NGS sequencing of large panels of genes, certainly in case of heterogenic disease that can be caused by mutations in multiple genes. More and more diagnostic labs offer multigene NGS panels for cardiovascular disease (cardiomyopathies, arrhythmias, aortic aneurysms) or disease in general (deafness, retinitis pigmentosa, dementia, cancer, etc).

In the research setting, the classical "positional genetics" approach with linkage studies in multiplex families and Sanger sequencing of individual functional candidate genes in the linkage region is replaced by NGS sequencing of the whole exome or genome. Over the last 3 years this whole exome approach has led to the discovery of an increasing number of new disease genes. Certainly in small families not amenable to positional cloning or isolated patients the whole exome NGS ap-

proach is the most cost-effective approach to gene identification. Most congenital CVM occur sporadically, and extended families with clear monogenic inheritance are scarce, thereby precluding the identification of diseases genes involved in CVM by a classical positional genetics. It is therefore expected that the integration of whole exome or genome NGS within the research lab will yield a large crop of new cardiovascular disease genes. Also for the individual patient who wants a molecular diagnosis in a diagnostic setting whole exome or genome NGS holds great promise, and the first genetic labs have launched diagnostic whole exome sequencing in 2011.

Although currently both research and diagnostic whole exome or genome NGS are focusing on monogenic disease, it is anticipated that this will also constitute the most cost-effective approach to the identification of risk factor in susceptibility genes in multifactorial (complex) disease.

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SAMENVATTING

Cardiovasculaire malformaties (CVM), inclusief aangeboren hartafwijkingen en aorta aneurysmata, zijn een relatief veel voorkomende oorzaak van ziekte en sterfte. De laatse jaren is enorme vooruitgang geboekt in onze kennis over het ontstaan van CVM door de ontdekking van vele genetische factoren die betrokken zijn bij de normale en abnormale ontwikkeling van het hart en de grote bloedvaten (**hoofdstuk 1**). In dit proefschrift hebben we families met monogene overerving van CVM bestudeerd om zo de fenotypes te omschrijven, ziektegenen te identificeren en de onderliggende moleculaire netwerken te ontrafelen. Alhoewel monogene (syndromale en niet-syndromale) vormen van CVM relatief zeldzaam zijn, kunnen studies in deze families onze kennis over de etiologie van sporadische gevallen van CVM verbeteren.

In **hoofdstuk 1** wordt de incidentie, classificatie en genetica van congenitale hartafwijkingen en aorta aneurysmata samengevat met speciale aandacht voor bepaalde monogene vormen van CVM, namelijk lateralisatie-afwijkingen, longvenestenosen, hartklepaandoeningen en Aneurysma-osteoarthritis syndroom.

In **hoofdstuk 2** worden onze studies in patienten met lateralisatie-afwijkingen gerapporteerd. Deze data suggereren dat lateralisatie-afwijkingen ofwel heterotaxie complexe, oligogene aandoeningen zijn in de meerderheid van de patienten. Middels DNA koppelingsonderzoek en DNA sequentieanalyse in een consanguine familie met een monogene vorm van lateralisatie afwijkingen werden homozygote varianten in het cilia-gerelateerde *NPHP4* gen gevonden. In sporadische heterotaxie patienten werden heterozygote *NPHP4* varianten gevonden die wellicht in combinatie met additionele genetische modificerende factoren, omgevingsfactoren of stochastische factoren tot heterotaxie leiden.

In **hoofdstuk 3** beschrijven we een consanguine familie met autosomaal recessief overervende vorm van primaire longvenestenose (PVS) waarbij prenataal tevens lymfatische afwijkingen werden vastgesteld. Families met meerdere siblings met PVS zijn tot op heden niet eerder geschreven. Homozygotie mapping studies resulteerde in de identificatie van het eerste locus voor PVS op chromosoom 2q.

In **hoofdstuk 4** rapporteren we over twee grote families met autosomaal dominant overervende linkszijdige obstructieve aangeboren hartafwijkingen (LVOTO). In deze families was LVOTO geassocieerd met rechtszijdige klepafwijkingen en septale

defecten waardoor het spectrum van hartaandoeningen dat gezien wordt bij LVOTO wordt uitgebreid. LVOTO is een genetisch heterogene aandoening. Verwacht wordt dat verschillende genen een rol spelen in families met vergelijkbare LVOTO fenotypes, en daardoor kunnen deze, vaak kleine families, niet samengevoegd worden in koppelingsstudies. Aangezien de twee families die hier beschreven werden klein zijn, werd alleen suggestieve koppeling tot verschillende grote chromosomale regionen verkregen. Next generation sequencing (NGS) zal hopelijk de identificatie van LVOTO ziektegenen faciliteren.

In **hoofdstuk 5** beschrijven we een nieuwe autosomaal dominante syndromale vorm van aorta aneurysmata gekenmerkt door een combinatie van arteriële aneurysmata en arteriële kronkeling (tortuositeit) met vroegtijdige artrose, en de aanwezigheid van milde craniofaciale, skelet- en huidafwijkingen. We hebben dit nieuwe syndroom de naam Aneurysma-Osteoarthritis Syndroom (AOS) gegeven. Alhoewel AOS patienten kenmerken toonden die vergelijkbaar zijn met Loeys-Dietz syndroom en soms ook Marfan syndroom, is de frequentie van vroege arthrose veel hoger dan is beschreven voor deze beide syndromen.

In een grote AOS familie lokaliseerden we het AOS locus op chromosoom 15q22.2-24.2. Vervolgens identificeerden we pathogene mutaties in het *SMAD3* gen, dat codeert voor een receptor-activerend SMAD eiwit dat een rol speelt in the signaal overdracht van het TGF-β signaaltransductie netwerk. Immunohistochemie toonde een upregulatie van verschillende componenten van het TGF-β netwerk in de aortawand van *SMAD3* patienten, hetgeen een indicatie is voor toegenomen TGF-β signalering.

In **hoofdstuk 6** wordt een uitgebreide klinische studie beschreven van 45 patiënten met *SMAD3* mutaties uit acht niet-verwante AOS families. In een meerderheid van de patienten waren gewrichtsafwijkingen zoals arthrose, osteochondritis dissecans (OCD), or meniscusafwijkingen het eerste kenmerk van AOS, nog voordat symptomen van cardiovasculaire afwijkingen duidelijk werden. Deze observaties leiden tot de klinische aanbevelingen om 1) röntgenfoto's te verrichten bij patiënten met een (thoracaal) aneurysma en een medische voorgeschiedenis of familiegeschiedenis van gewrichtsafwijkingen, en om 2) cardiovasculaire beeldvorming te verrichten in patienten met vroegtijdige arthrose in combinatie met OCD of een familiegeschiedenis van aorta aneurysmata of plotse dood. Uit deze studie met patiënten met *SMAD3* mutaties blijkt dat AOS een leeftijdsafhankelijk maar hoog penetrant fenotype heeft met grote intra- en interfamiliale variabiliteit.

In **hoofdstuk 7** worden de resultaten van een uitgebreide cardiovasculaire evaluatie door middel van beeldvorming, arteriele stijfheidsmetingen en biochemische studies in 44 patienten met AOS gepresenteerd. AOS kenmerkt zich door uitgebreide en agressieve cardiovasculaire afwijkingen en is geassocieerd met een hoge mortaliteit, met een mediane leeftijd van overlijden op de leeftijd van 54 jaar. Aneurysmata en kronkeling kwamen voor in grote en middelgrote arteriën in het gehele lichaam, inclusief de brachiocephale en intracraniële vaten. Dissecties traden soms op bij relatief mild toegenomen aortadiameters, wat duidelijk maakt dat vroegtijdige preventieve chirurgie noodzakelijk is. Tevens werden hartaandoeningen zoals mitralis klep prolaps, atriumfibrilleren, linker ventrikel hypertrofie en aangeboren hartafwijkingen waargenomen in een aanzienlijk aantal AOS patiënten.

In **hoofdstuk 8** worden de resultaten van de studies in dit proefschrift besproken en in perspectief gezet. Voor adequaat genetisch advies voor patiënten en hun familieleden, is het van groot gelang om de oorzaak van CVM vast te stellen. Daarom blijft de identificatie van de genetische oorzaak van cardiovasculaire aandoeningen een grote uitdaging. Alhoewel is aangetoond dat de “positionele genetica” technologie met koppelingsonderzoek en sequencing van kandidaat genen gelegen in het koppelingsinterval zeer waardevol is voor de identificatie van genen betrokken bij cardiovasculaire aandoening, is deze technologie enkel mogelijk in grote families met monogene vormen van CVM. De grootste fractie CVM is echter sporadisch of komt voor in kleinere families: hier bieden nieuwe technieken zoals NGS grote mogelijkheden: sequencing van grote linkage intervals in families zoals gepresenteerd worden in dit proefschrift wordt mogelijk zodat nieuwe ziektegenen geïdentificeerd kunnen worden. Bovendien kan NGS van het gehele exoom of genoom tot de identificatie leiden van het moleculaire defect in sporadisch voorkomende CVM.

CURRICULUM VITAE

Ingrid van de Laar werd op 25 mei 1976 geboren te Liempde. Zij behaalde haar VWO Gymnasium diploma aan het Jacob-Roelantslyceum te Boxtel, waarna zij Geneeskunde studeerde aan de Universiteit van Utrecht. Ter afsluiting van haar doctoraal fase deed zij een half jaar wetenschappelijk onderzoek bij de afdeling Hematologie van de Universiteit van Minnesota, Minneapolis, in de Verenigde Staten. In 1999 behaalde zij haar doctoraal examen en begon zij aan haar co-schappen waarvan het co-schap Neurologie en Oogheelkunde in het buitenland werden volbracht, respectievelijk in Melbourne (Australië) en Tunbridge Wells (UK). Het artsexamen werd in 2002 behaald waarna zij twee jaar als ANIOS (arts niet in opleiding tot specialist) ging werken op de afdeling Klinische Genetica in het ErasmusMC te Rotterdam. Vanaf januari 2004 was zij in opleiding tot klinisch geneticus in het Erasmus MC (opleiders Prof. H. Meijers -Heijboer, Drs A.J.M. Hoogeboom, Dr. J.A. Maat-Kievit). In 2005/2006 heeft zij vier maanden klinische stage gelopen bij het Centrum voor Medische Genetica, Leuven, België (Prof. J.P. Fryns). In 2006 ontving zij de Dr E. Dekker stipendium van de Nederlandse Hartstichting voor een arts in opleiding tot specialist. In die tijd werd de start gemaakt met het promotieonderzoek dat heeft geleid tot dit proefschrift. In juli 2009 vond registratie plaats als klinisch geneticus. Momenteel is zij werkzaam als klinisch geneticus op de afdeling Klinische Genetica van het Erasmus MC (prof. dr. R. Hofstra). Zij is getrouwd met Jochem de Graaf en heeft twee kinderen, Taeke (5 jaar), en Kiki (3 jaar).

PHD PORTFOLIO SUMMARY

Name PhD student:	Ingrid van de Laar
Erasmus MC department:	Clinical Genetics
PhD period:	2006-2012
Promotors:	Prof. Dr. B.A. Oostra
	Prof. Dr. J.W. Roos-Hesselink
Copromotors:	Dr. A.M. Bertoli-Avella
	Dr. M.W. Wessels
Date of defence thesis:	June 27th, 2012
Title thesis:	Clinical and genetic studies in cardiovascular malformations

Professional academic skills

2004-2009	Residency Clinical Genetics Erasmus MC, Rotterdam
2006	Clinical fellowship at the Center for Human Genetics, Leuven, Belgium
2009	Board certified registration as Clinical Geneticist

In-depth courses

2011	Fourth European course in Clinical Dysmorphology "what I know best" Rome, Italy
2010	Hands-on course on Morphology of Congenital Heart Disease, Leiden
2008	Cardiogenesis and Congenital Cardiopathies: from developmental models to clinical applications, Bologna, Italy
2006	Cursus rekenen aan genen, Amsterdam
2006	19th course in Medical Genetics, Bertinoro, Italy

Teaching

2011	Genetisch Consulenten In Opleiding, Utrecht "Aangeboren hartafwijkingen"
2011	3e Jaars studenten Geneeskunde, Rotterdam Minor aangeboren hartafwijkingen
2010	Cardiovasculair verpleegkundigen, Utrecht "Klinische Genetica voor cardiovasculair verpleegkundigen"
2008	HLO studenten, Albeda College, Rotterdam "Klinische genetica voor HLO"
2008	Obstetrische verpleegkundigen, Albeda College, Rotterdam "Klinische genetica en prenatale diagnostiek"
2007+2008	3e Jaars studenten Geneeskunde, Rotterdam "Haplotypering en genetisch onderzoek in families"

Symposia and conferences

Oral presentations

2011	European Human Genetics Conference, Amsterdam "SMAD3 mutations in new aneurysm syndrome"
2011	Symposium Thoracale Aortapathologie, Rotterdam "Geen Marfan, wat dan?"
2011	Landelijk Overleg Genetische Counseling (LOG), Utrecht "SMAD3 mutations identified in a new Aortic Aneurysm syndrome with early onset Osteoarthritis"
2010	European Human Genetics Conference, Gothenburg, Sweden "A new locus for syndromic Thoracic Aortic Aneurysms and Dissections maps to chromosome 15q"
2009	Medisch-Genetisch Centrum Zuid-West Nederland (MGC) symposium, Rotterdam "First locus for primary pulmonary vein stenosis maps to chromosome 2q: first steps to gene finding"
2008	Joint meeting UK/ Dutch Clinical Genetics Societies, Liverpool, UK, "Delineation of the 5q35 deletion: a new microdeletion syndrome associated with congenital heart disease and microcephaly"
2007	Assistentenvoordrachten LOG Groningen "New locus for primary pulmonary vein stenosis"
2007	Najaarsvergadering sectie Kindercardiologie, VUMC, Amsterdam "Cardiogenetica in de kindercardiologische praktijk"
2006	17th European Dysmorphology meeting in Straatsburg "Severe limb anomalies in CHARGE syndrome"

Poster presentations

2010 Weinstein Cardiovascular Development Conference, Amsterdam, The Netherlands
"A new locus for a syndromic form of Thoracic Aortic Aneurysms and Dissections (TAAD) maps to chromosome 15q"

2009 Weinstein Cardiovascular Development Conference, San Francisco, USA
"Genome-wide linkage analysis reveals new locus for laterality defects of the heart"

2008 European Human Genetics Conference, Nice, France
"Primary pulmonary vein stenosis and lymphatic anomalies: a new syndrome?"

2008 Nederlandse Hartstichting, Amsterdam, The Netherlands
"Genome-wide linkage analysis reveals new loci for malformations of cardiac valves"

Attended

2006 European Human Genetics Conference, Amsterdam, The Netherlands

Awards

2006-2009 Persoonsgebonden Dr E. Dekkers beurs van de Nederlandse Hartstichting voor arts in opleiding tot specialist

LIST OF PUBLICATIONS

Srebniak M, Boter M, Oudesluijs GO, Cohen-Overbeek T, Govaerts LCP, Diderich KEM, Oegema R, Knapen MFCM, **van de Laar IMBH**, Joosten M, Van Opstal D, Galjaard RH
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