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ARTICLE

Confirmation of the role of pathogenic *SMAD6* variants in bicuspid aortic valve-related aortopathy

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Abstract

Progressive dilatation of the thoracic aorta leads to thoracic aortic aneurysm (TAA), which is often asymptomatic but predisposes to lethal aortic dissections and ruptures. TAA is a common complication in patients with bicuspid aortic valve (BAV). Recently, rare loss-of-function *SMAD6* variants were shown to contribute significantly to the genetic aetiology of BAV/TAA. Intriguingly, patients with craniosynostosis have also been reported to be explained molecularly by similar loss-of-function *SMAD6* variants. While significantly reduced penetrance of craniosynostosis has been reported for the *SMAD6* variants as such, near-complete penetrance is reached upon co-occurrence with a common *BMP2* SNP risk allele. Here, we report on the results of a *SMAD6*-variant analysis in 473 unrelated non-syndromic TAA patients, of which the *SMAD6*-positive individuals were also studied for the presence of the *BMP2* risk allele. Although only 14% of the TAA patients also presented BAV, all novel likely pathogenic *SMAD6* variants ($N = 7$) were identified in BAV/TAA individuals, further establishing the role of *SMAD6* variants to the aetiology of BAV/TAA and revealing limited contribution to TAA development in patients with a tricuspid aortic valve. Familial segregation studies confirmed reduced penetrance (82%) and variable clinical expressivity, with coarctation of the aorta being a common comorbidity. None of our six *BMP2*+/*SMAD6*+ patients presented with craniosynostosis. Hence, the proposed digenic model for craniosynostosis was not supported in the presented BAV/TAA cohort, suggesting that additional factors are at play. Finally, our data provide improved insights into the clinical spectrum of *SMAD6*-related BAV/TAA and has important implications for molecular diagnostics.

Introduction

Thoracic aortic aneurysm (TAA) is characterized by progressive dilatation of the thoracic aorta. Untreated TAA can lead to life-threatening aortic dissection and/or rupture [1]. TAA can present as an isolated condition or as part of connective tissue disorders such as Marfan syndrome and Loeys-Dietz syndrome [2]. It is also prevalent in individuals with bicuspid aortic valve (BAV), i.e. the most common congenital heart disorder characterized by two aortic valve leaflets instead of the normal three (tricuspid aortic valve, TAV) [3]. At least 10–20% of BAV patients develop TAA at some stage in life and BAV individuals have an eight-fold increased risk of aortic dissection, typically occurring at young age [4, 5]. Besides TAA, patients with BAV are at increased risk for aortic valve stenosis and coarctation (CoA) [5].

Therapies capable of preventing, stopping, or reversing TAA formation are not available yet. Early diagnosis and continuous surveillance of at-risk individuals thus serve as life-saving procedures [3]. The availability of genetic testing would be extremely useful to identify pre-symptomatic individuals [6], but causal variants in the currently known TAA genes explain only about one-quarter of TAA patients

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[7]. A molecular explanation is identified in far fewer patients with BAV/TAA [8]. Hence, acquisition of improved insights into the genetic basis of TAA is indispensable for improved risk stratification.

Over the past couple of years, genetic variability in *SMAD6* has been reported to impinge on the risk of congenital heart disease (CHD) [9–11]. *SMAD6* encodes a negative regulator of the bone morphogenetic protein (BMP) pathway [12]. In 2012, Tan et al. [11] reported heterozygous *SMAD6* variants in two sporadic BAV patients either combined with mild-to-moderate aortic valve stenosis or with CoA. In a study of 2871 paediatric patients with CHD, nine *SMAD6* (likely) pathogenic variants were identified in patients presenting with tetralogy of Fallot, hypoplastic left heart syndrome, dextro-transposition of the great arteries, CoA, or BAV [10]. In 2017, rare *SMAD6* variants were also shown to be significantly enriched in BAV/TAA cases, explaining ~2.5% of patients [9]. The variant spectrum of *SMAD6*-related cardiovascular disease encompasses frameshift, nonsense and MH1/MH2-domain locating missense variants as well as an in-frame deletion (Suppl. Table 1). Prior segregation analysis, performed in two BAV/TAA families, revealed variable expressivity (BAV/TAA, TAV/TAA and CoA) and incomplete penetrance of TAA (at ages 28 and 39) [9].

Intriguingly, rare *SMAD6* variants (i.e. truncating and MH1/MH2-located missense variants) have also been reported to cause non-syndromic midline craniosynostosis (Suppl. Table 1) [13, 14]. Craniosynostosis is the most common congenital craniofacial birth defect and results from the premature fusion of the cranial sutures. Whereas incomplete penetrance of craniosynostosis was observed for the *SMAD6* variants as such, near-complete penetrance was reached upon co-occurrence with a common variant near *BMP2* (rs1884302) [13, 14]. Cardiovascular abnormalities were not reported in the *SMAD6*-related craniosynostosis patients, even not in the patient who carried the identical *SMAD6* variant (p.(Pro152Profs*27), c.455_461del) found in a BAV/TAA patient [9, 14].

Because prior segregation analysis confirmed the presence of a *SMAD6* variant in a TAA patient with a normal aortic valve, we interrogated the spectrum of phenotypical variability of *SMAD6*-positive patients, irrespective of the co-occurrence with BAV, in a cohort of 473 TAA patients. Secondly, since near-complete penetrance for craniosynostosis has been reported to be reached by the co-occurrence of a *BMP2* risk allele (rs1884302, minor allele frequency (MAF) C-allele = 34%) and a *SMAD6* variant, we also genotyped this specific *BMP2* variant in the *SMAD6*-variant carriers presenting with cardiovascular disease. We anticipated the near absence of the *BMP2* single nucleotide polymorphism (SNP) risk allele in our *SMAD6*-positive patients.

Material and methods

Study cohort

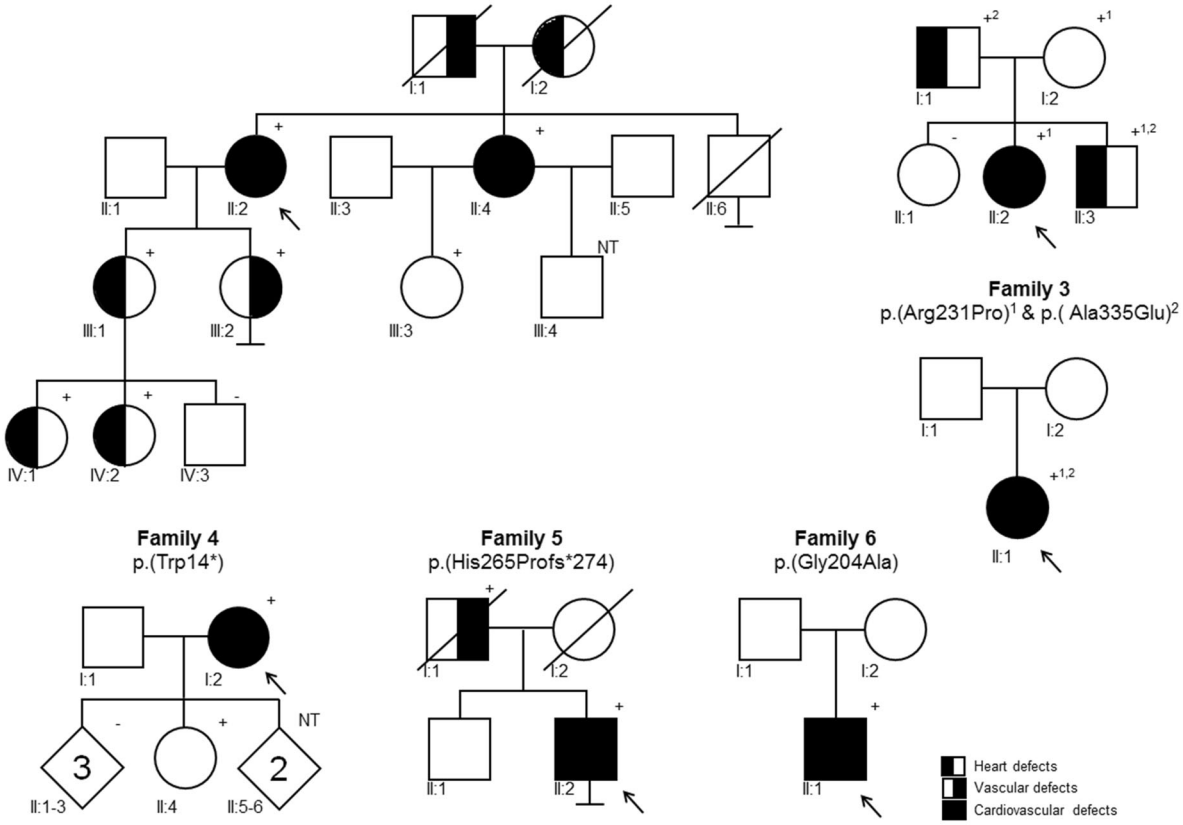
The patient population consisted of 473 non-syndromic TAA patients ascertained from three different research centres: Radboud University Medical Center, Nijmegen, the Netherlands; Center of Medical Genetics, University of Antwerp and Antwerp University Hospital, Antwerp, Belgium; and Institute of Genetic Medicine, Johns Hopkins University, Baltimore, MD, USA. All TAA patients were negative for mutations in known TAA genes. Of these, 65 had BAV. All patients had a *Z*-score exceeding 3 [15, 16] when height and weight were available, or an aortic diameter of at least 40 mm at the level of the sinus of Valsalva or ascending aorta. The number of aortic valve leaflets and the aortic dimensions were determined using echocardiography, computed tomography, or magnetic resonance imaging. A positive family history was defined as having at least one first- or second-degree relative with BAV and/or TAA. The study was approved by the local ethics committees and all study participants gave informed consent at the respective sample-contributing centres.

SMAD6 targeted resequencing and variant validation

Whole blood- or saliva-derived genomic DNA (gDNA) was enriched for all coding *SMAD6* exons (RefSeq transcript NM_005585.4) using centre-dependent TAA and/or CHD gene panels (HaloPlex Target Enrichment System [17]; single molecule Molecular Inversion Probes (smMIP) [18]; Agilent SureSelectXT Human All Exon 50 Mb; Invitae Aortopathy Comprehensive Panel). Subsequent sequencing was performed on an Illumina NextSeq500 or HiSeq1500 instrument (2 × 150 bp or 2 × 100 bp paired-end). Regions of insufficient coverage were complemented with Sanger sequencing as previously described [9]. The sequences of the primers are listed in Suppl. Table 2.

We selected for rare (MAF ≤ 0.1% in gnomAD [19] r2.0.2) heterozygous non-synonymous coding and splice-site (±10 bp from exon–intron boundaries) *SMAD6* variants after applying quality parameters (coverage: ≥10×, allelic balance: 0.25–0.85). Pathogenicity programmes such as Combined Annotation Dependent Depletion (CADD, v1.4) [20], MutationTaster [21], SIFT [22] and PolyPhen-2 [23] were used to evaluate variant deleteriousness, and Alamut Visual 2.8.0 software (Rouen, France) was used to predict altered splicing. In addition, we selected high-confidence copy number variations with a low frequency in healthy individuals (MAF ≤ 0.1% in the Database of Genomic Variants (DGV)) from the available gene panel sequencing

a



b

Chromosomal location (hg19)	Nucleotide change	Protein change	Protein domain	PolyPhen-2	Mutation Taster2	SIFT	CADD	gnomAD
chr15:66995638	c.42G>A	p.(Trp14*)	-	NA	Disease causing	NA	46	Absent
chr15:66996207	c.611G>C	p.(Gly204Ala)	MH1	Benign	Disease causing	Tolerated	21.3	Absent
chr15:66996287	c.691C>G	p.(Arg231Gly)	MH1	Probably Damaging	Disease causing	Damaging	33	Absent
chr15:66996288	c.692G>C	p.(Arg231Pro)	MH1	Probably Damaging	Disease causing	Damaging	29.9	Absent
chr15:66996390	c.794del	p.(His265Pro*fs 274)	MH1	NA	Disease causing	NA	NA	Absent
chr15:67073386	c.1004C>A	p.(Ala335Glu)	MH2	Probably Damaging	Disease causing	Damaging	30	Absent

Chromosomal location (hg19)	Copy Number	Min.-Max. size (kb)	DGV	Involved genes
chr15:g.(66825594_67400490)	1	575-601	Absent	*LCTL, LINC01169, LINC02206, LOC102723493, SMAD6, SMAD3, ZWILCH*

c

data. All variants of interest were validated with an independent technique (i.e. Sanger sequencing or SNP-based microarray). Segregation analysis was performed upon availability of gDNA of relatives. Variants listed in Fig. 1b are submitted to ClinVar (SCV000854413–SCV000854420). And these variants were classified using

◀ **Fig. 1** Variant analysis. **a** Pedigrees of the families with their respective *SMAD6* variant. **b** Overview of the novel *SMAD6* deletion and next-generation sequencing variants in our non-syndromic TAA cohort, and their respective annotations. Prediction programmes were used to evaluate their pathogenic effect (PolyPhen-2, MutationTaster2, SIFT (Sorting Intolerant From Tolerant) and CADD (Combined Annotation Dependent Depletion)). All variants are absent in gnomAD database, and the deletion was not reported in the Database of Genomic Variants (DGV). **c** Conservation of specific residues among species. (Reference build: GRCh37; RefSeq: NM_005585.4)

guidelines of the American College of Medical Genetics [24] (Suppl. Table 3).

BMP2 rs1884302 genotyping

All *SMAD6*-positive patients identified in this cohort and previously published by Gillis et al. [9] were genotyped for the *BMP2* rs1884302 risk allele 'C' using Sanger sequencing as previously described [9].

Results

Phenotypical characterization of patient cohort

In total, 473 unrelated non-syndromic TAA patients were screened for genetic variability in *SMAD6*. The clinical and molecular characteristics of the study participants are summarized in Table 1. The average age at inclusion was 53 ± 17 years and 70% were male, complying with the known 2:1 male preponderance of TAA [25]. In 14% of the TAA patients ($N = 65$) the presence of BAV was noted. A positive family history was known for 30% of patients, whereas the family history was negative or unknown for 70% of the individuals.

Contribution of likely pathogenic *SMAD6* variants to the genetic aetiology of BAV/TAA and TAV/TAA

We identified seven novel likely pathogenic *SMAD6* variants in 473 non-syndromic TAA patients, i.e. 1.5% of the patient population (Fig. 1, Table 1). All *SMAD6* variants were identified in BAV/TAA probands ($N = 7/65$, 10.8%) (Table 2).

The first variant was a heterozygous deletion (hg19 chr15:g.(66,817,545_66,825,594)_(67,400,490_67,418,205)del) spanning the complete coding sequence of *SMAD6* and exon 1 of *SMAD3* (Fig. 1, Table 2). The cardiovascular features of the 67-year-old female proband (1-II:2) included BAV with severe valve stenosis and aortic regurgitation, an aortic root dilatation of 47 mm, and a history of type A dissection extending to the region between the superior mesenteric artery and renal arteries. In addition, she presented with an aneurysm of the arteria basilaris, subconjunctival haemorrhages and mitral valve prolapse (MVP). Emergent repair for the dissection was performed at age 64. Six months later, the proband received Bentall aortic root repair with prosthetic aortic valve. Non-cardiovascular manifestations comprised diverticulitis, joint arthralgia, ventral hernia, anaemia, fatty liver, splenomegaly (due to a post-dissection splenic infarct) as well as Crohn's disease and auto-immune hepatitis. She also tested positive for the antinuclear antibodies. The father of the proband (1-I:1) died of stroke at age 84 and the mother (1-I:2) died of heart failure at age 86. None of the parents were known to have aneurysmal disease but gDNA is not available for genetic testing. The proband's two variant-carrying children presented with either TAV, mild-to-moderate aortic regurgitation and normal aortic diameters (1-III:1, 40-year-old) or a borderline ascending aortic dilatation (37 mm) (1-III:2, 38-year-old), respectively. Two (1-IV:1, age 9; 1-IV:2, age 7) of the three grandchildren tested positive for the familial *SMAD6* variant and presented with cardiac features such as pulmonary valve stenosis (1-IV:1) and patent foramen ovale (1-IV:2). Both individuals had TAV and normal aortic diameters. The variant-positive sister of the proband (1-II:4, age 65) had a type A aortic dissection extending to the abdominal part of the aorta at age 64. The thoracic aorta showed mild-to-moderate atherosclerosis. She also presented with patent foramen ovale and moderate mitral regurgitation with MVP, but a normal aortic valve. Non-cardiovascular features included umbilical hernia, scoliosis, cervical spine fusion (compressed vertebrae), arthritis (with surgery for feet and thumbs). Echocardiography in the variant-carrying niece of the proband (1-III:3, age 45) was normal. She has a history of arthritis, migraines and melanoma. The cardiovascular status of the 41-year-old nephew of the proband (1-III:4) is unknown, but he is also known with arthrosis.

Table 1 Characteristics of the non-syndromic TAA patient cohort

	Cohort ($N = 473$) (%)
Average age (years)	52.5 ± 16.5
Gender	
Male	332 (70.2)
Female	141 (29.8)
BAV[a]	65 (13.7)
Family history	
Familial	141 (29.8)
Unknown	332 (70.2)

CHD congenital heart disease, *BAV* bicuspid aortic valve. *TAA* thoracic aortic aneurysm

[a]Excluding four patients with an unspecified valve replacement (possibly: 14.6% of the cohort with BAV)

Table 2 Clinical data of all *SMAD6*-positive individuals and/or individuals with a cardiovascular feature

Ind.	Gender/ Age	*SMAD6* variant	Clinical features Heart defects	Vascular anomalies	Other clinical data	Remarks
1-II:2	F/67	Deletion encompassing complete *SMAD6* and exon 1 of *SMAD3*	BAV, AS, AR, MVP	Aortic root dilatation (47 mm), type A aortic dissection, brain aneurysm, subconjunctival haemorrhages	Crohn's disease, auto-immune hepatitis, diverticulitis, joint arthralgia, ventral hernia, anaemia, fatty liver, splenomegaly (due to splenic infarct post dissection)	Surgery for dissection, aortic valve replacement and Bentall aortic root repair, ANA test positive
1-I:1	M/84†	NA		Stroke	Alzheimer, arthritis, gastro-oesophageal reflux	Cause of death: stroke
1-I:2	F/86†	NA	MVP, congestive heart failure		MVA, arthritis, osteoporosis, diverticulitis	Cause of death: heart failure
1-III:1	F/40	Deletion encompassing complete *SMAD6* and exon 1 of *SMAD3*	Mild-to-moderate AR			TAV, normal aortic diameters
1-IV:1	F/9	Deletion encompassing complete *SMAD6* and exon 1 of *SMAD3*	Pulmonary valve stenosis			TAV, normal aortic diameters
1-IV:2	F/7	Deletion encompassing complete *SMAD6* and exon 1 of *SMAD3*	PFO		Bladder/Ureter	Surgery for bladder/ureter, TAV, normal aortic diameters
1-III:2	F/38	Deletion encompassing complete *SMAD6* and exon 1 of *SMAD3*		Borderline ascending aortic dilatation (37 mm)		
1-II:4	F/65	Deletion encompassing complete *SMAD6* and exon 1 of *SMAD3*	PFO, moderate mitral regurgitation with MVP	Type A aortic dissection, moderate aneurysm at the level of the aortic root and ascending aorta, mild-moderate atherosclerotic disease of thoracic aorta	Atelectasis, umbilical hernia, scoliosis, cervical spine fusion (compressed vertebrae), polydactyly, arthritis	Bone surgery (thumb joint (arthritis) and feet (accessory navicular bone))
1-III:3	F/45	Deletion encompassing complete *SMAD6* and exon 1 of *SMAD3*			Joint arthritis, migraines, melanoma	TAV, normal aortic diameters
1-III:4	M/41	Not tested			Joint arthritis (manual labour)	No echocardiogram
2-II:2	F/33	p.(Pro257Leu)	BAV, AS	TAA (Z-score >3)		
2-I:1	M/62	p.(Arg231Gly)	AS			Surgery for septal defect and AS (mechanic valve) (61 years)
2-I:2	F/59	p.(Pro257Leu)				TAV, normal aortic diameters
2-II:3	M/31	p.(Pro257Leu), p.(Arg231Gly)	BAV, AR			
3-II:1	F/17	p.(Arg231Pro), p.(Ala335Glu)	BAV	TAA (41 mm) (*Z*-score = 6.48), aortic root (28 mm) (*Z*-score = 1.92)	Scoliosis, radioulnar synostoses, bilateral fusion of capitulum and hamatum, dysmorphism, myopia, developmental delay, mild disability	15q11.2 BP1–BP2 microdeletion

Table 2 (continued)

Ind.	Gender/ Age	*SMAD6* variant	Clinical features Heart defects	Vascular anomalies	Other clinical data	Remarks
4-I:2	F/37	p.(Trp14*)	BAV, mild-to-moderate AR	TAA (46 mm) and thin aortic wall		Surgery for aneurysm
4-II:4	F/17	p.(Trp14*)				Asymptomatic (no echocardiogram)
5-II:2	M/57	p.(His265Profs*274)	BAV	CoA, TAA (30 mm, post-surgery)		Resection and end-to-end anastomosis of CoA, TEVAR for postoperative aneurysm
5-I:1	M/80†	p.(His265Profs*274)		Thoracic-abdominal diffuse aneurysm (90 mm in abdomen), tortuosity of the aorta, calcified vessels		Cause of death: aortic rupture
6-II:1	M/21	p.(Gly204Ala)	BAV	Ascending aorta aneurysm (*Z*-score = 5.44)		

No information about the fusion pattern of the aortic valve phenotype was available. *Ind.* Individual, *MVP* mitral valve prolapse, *ANA* antinuclear antibodies positive, *MVA* mosaic variegated aneuploidy, *BAV* bicuspid aortic valve, *TAV* tricuspid aortic valve, *TAA* thoracic aortic aneurysm, *AS* aortic stenosis, *PFO* patent foramen ovale, *AR* aortic regurgitation, *CoA* coarctation of the aorta, *TEVAR* thoracic endovascular aortic repair, *BP* breakpoint, *F* female, *M* male. Age in years. (Reference build: GRCh37; RefSeq: NM_005585.4)

The proband of the second family, a 33-year-old female (2-II:2), carried the p.(Pro257Leu) *SMAD6* variant. She was previously reported [9] and presented with BAV/TAA (Z-score >3) as well as mild aortic valve stenosis. A prototypical dilatation of the ascending aorta was observed as the diameter of the ascending aorta (39 mm) was bigger than the sinus of the aorta (33 mm). Surprisingly, another heterozygous missense variant of uncertain significance (c.691C > G, p.(Arg231Gly)) in the MH1-domain of SMAD6 was identified in the father (2-I:1; 62-year-old) of the proband (Fig. 1, Table 2). The p.(Arg231Gly) variant is absent from gnomAD and prediction programmes (PolyPhen-2, MutationTaster2 and SIFT) anticipate a deleterious effect with a CADD_phred score of 33 (Fig. 1). The father (2-I:1) underwent surgery (61 years) for a septal defect and received a mechanic valve prosthesis because of severe aortic stenosis. The unaffected 59-year-old mother of the proband (2-I:2) also carried the p.(Pro257Leu) variant. The proband's 31-year-old brother (2-II:3) presented with BAV and aortic regurgitation, and carries both *SMAD6* variants. The unaffected 35-year-old sister (2-II:1) is variant-negative.

Interestingly, two *SMAD6* variants were also identified in family 3 (Fig. 1, Table 2), i.e. p.(Arg231Pro) (c.692G > C, variant of uncertain significance) and p.(Ala335Glu) (c.1004C > A, likely pathogenic), located in the MH1- and MH2-domain, respectively. They are both absent from gnomAD, and a deleterious effect is predicted by different in silico programmes (Fig. 1) (PolyPhen-2, MutationTaster2 and SIFT) with a CADD_phred score of 30 for both variants. In addition to those two *SMAD6* variants, the 17-year-old female proband (3-II:1) also carries a deletion between breakpoint site (BP) 1 and 2 on Chr15q11.2. She presented with a dysplastic asymmetric aortic valve with a hypoplastic left coronary leaflet and a progressive aortic dilatation of the ascending aorta (41 mm) (*Z*-score = 6.48). The aortic root diameter at the level of the sinuses of Valsalva was normal (28 mm) (*Z*-score = 1.92). Additionally, she displayed scoliosis, radioulnar synostoses, bilateral fusion of capitulum and hamatum, dysmorphism, myopia, developmental delay and mild disability. The latter neurological manifestations can most likely be explained by the Chr15q11.2 deletion.

The fifth identified *SMAD6* variant was a nonsense pathogenic variant (c.42G > A, p.(Trp14*)), likely resulting in nonsense-mediated mRNA decay and, as such, haploinsufficiency. The variant is absent from gnomAD and has a CADD_phred score of 40 (Fig. 1). The 37-year-old female proband (4-I:2) displayed BAV with mild-to-moderate aortic regurgitation and an aneurysm of the aorta ascendens (46 mm) for which she underwent prophylactic aortic surgery in the context of the woman's desire for another pregnancy. Four of the proband's six children were checked for presence of the variant, of which one (4-II:4; 17 years)

Table 3 Comparison of studies for evaluation of digenic model for craniosynostosis

Studies	Carriers (probands)			
	SMAD6+/ BMP2− risk allele		*SMAD6+/BMP2+* risk allele	
	Craniosynostosis	No craniosynostosis	Craniosynostosis	No craniosynostosis
Craniosynostosis cohort[a]	6 (5)	20 (0)	15 (12)	1 (0)
BAV/TAA cohort[b,c]	0 (0)	16 (13)	0 (0)	6 (4)

[a]Combining all published papers [13, 14]

[b]Combining this study with the published study of Gillis et al. [9]

[3]Of the 27 *SMAD6*-positive samples, 22 individuals were genotyped for the *BMP2* risk allele because gDNA was not available for testing. The total number of the probands are shown between parenthesis

tested positive. She is asymptomatic. No gDNA of the two remaining children was available.

The next *SMAD6* variant, i.e. c.794del (p.(His265Profs*274), pathogenic variant), is predicted to lead to a C-terminally elongated protein due to loss of the native stop codon. The variant is absent from gnomAD. The 57-year-old male proband (5-II:2) had BAV and CoA, which was treated with a resection and end-to-end anastomosis. Additionally, endovascular aortic repair was performed to treat a postoperative aneurysm of 30 mm located at the former CoA site. The proband's variant-positive father (5-I:1) deceased at age 80 from rupture of a thoracic-abdominal diffuse aneurysm with a maximal abdominal diameter of 90 mm. He was also known with aortic tortuosity and arterial wall calcification. No valve anomalies were reported.

Finally, we identified a missense variant located in the functional MH1-domain (c.611G > C, p.(Gly204Ala), variant of uncertain significance) that is absent from gnomAD, not unequivocally designated as pathogenic by prediction programs and has a CADD_phred score of 21.3. The male 21-year-old proband presented with BAV and an ascending aortic aneurysm (*Z*-score = 5.44), for which losartan treatment was started. Prior to this study, a class 3 splice region variant in *FBN1* (c.5788+4C > A) was also identified in the proband [24]. This variant is reported five times in 245,778 alleles in the gnomAD database (0.002%) and has a CADD_phred score of 9.4. With the exception of Human Splicing Finder, none of the other four prediction programs in Alamut, predicted the creation of a novel acceptor splice-site. Moreover, history and physical exam did only reveal surgery for a bilateral umbilical hernia but no other signs of connective tissue disease. Both parents are unavailable for clinical evaluation or genetic testing.

Investigation of the phenotypic consequence of co-occurrence of a rare pathogenic *SMAD6* variant and the *BMP2* rs1884302 risk allele

The *SMAD6* variant-positive BAV/TAA patients reported previously by Gillis et al. and described here ($N = 22$) were investigated for the presence of the *BMP2* rs1884302 risk allele C (MAF = 34% [13]). The risk allele was identified in 6 out of the 22 *SMAD6*-positive BAV/TAA patients ($N = 6/22$, 27.2%). None of our *SMAD6+/BMP2+* BAV/TAA patients presented with craniosynostosis (retrospective analysis) (Table 3). Although there are only limited data on the fusion pattern of the aortic valve and the location of the aneurysm, no trend for a difference between *BMP2+* and *BMP2−* *SMAD6*-variant-positive patients in our cohort was observed.

Discussion

Loss-of-function variants in *SMAD6* have previously been shown to cause both CHD [9–11] and craniosynostosis [13, 14]. *SMAD6* encodes a negative regulator of the BMP signalling pathway that plays an important role in aortic valve development and skull formation [26, 27]. In a non-syndromic TAA cohort consisting of 473 patients, we found seven novel likely pathogenic *SMAD6* variants in six families, including one family that was previously reported by Gillis et al. [9]. Although only 14% of our patients in our cohort has a BAV in combination with TAA, all probands carrying likely pathogenic *SMAD6* variants presented with BAV/TAA ($N = 7/65$, 10.8%). Thus, our study confirms the prominent contribution of genetic variability in *SMAD6* to the aetiology of BAV/TAA. Rare *SMAD6* variants were not identified in pure TAV/TAA families, suggesting a limited role for *SMAD6* genetic variability in TAV/TAA disease. Previously, *SMAD6* variants were reported to explain 2.5% of BAV/TAA patients [9]. The higher frequency (10.8%) of *SMAD6* variants in this BAV/TAA cohort might be attributed to the more severe cardiovascular phenotype, demonstrated by a younger inclusion age (47.6 ± 17.4 years versus 63.5 ± 14.4 years) and a higher proportion of positive family histories (30% versus 9%). The positive predictive value of a family history for cardiovascular disease is further supported by the observation that 50% of the *SMAD6*-positive patients in this cohort presented with a positive family history ($N = 3/6$).

Similarly to the published rare deleterious *SMAD6* variants, we also identified nonsense, frameshift and missense *SMAD6* variants locating in the MH1/MH2-domain of the protein. Furthermore, we identified the first *SMAD6* gene deletion. *SMAD6* deletions have not yet been reported in the DECIPHER database nor in patients with craniosynostosis [13, 14]. The deleted region also included exon 1 of *SMAD3* and, as such, results in haploinsufficiency for SMAD3. *SMAD3* loss-of-function mutations cause Loeys-Dietz syndrome type 3, a TAA syndrome characterized by MVP, aortic regurgitation, hernias and arterial aneurysms [28]. An increased prevalence of BAV is also observed in patients with Loeys-Dietz syndrome type 3 [29]. We cannot exclude the possibility that this *SMAD3* deletion also contributes to the observed cardiovascular phenotype.

Besides variable BAV and TAA expressivity, segregation analysis also revealed incomplete penetrance as well as the occasional occurrence of extra-cardiovascular features. In our current study, 17 *SMAD6*-variant carriers were identified in six *SMAD6*-positive families, from which only the probands (average age of 38.7 years) presented with BAV/TAA ($N = 6/17$, 35.3%). Non-penetrance for cardiovascular features was observed in 3/17 *SMAD6*-variant carriers (average age of 40 years; 1-III:3, 2-I:2 and 4-II:4) ($N = 3/17$, 17.6%). In total, cardiovascular manifestations were observed in 14 *SMAD6*-variant carriers ($N = 14/17$, 82.4%). In the probands, two presented with BAV/TAA only whereas four BAV/TAA patients also presented with aortic valve stenosis (1-II:2, 2-II:2), aortic regurgitation (1-II:2, 4-I:2), MVP (1-II:2), brain aneurysm (1-II:2), subconjunctival haemorrhages (1-II:2) and CoA (5-II:2). The latter BAV/TAA patient (5-II:2) was treated for CoA with surgery. Intriguingly, CoA was described in two *SMAD6*-related BAV(/TAA) families before [9, 11]. Even though the total number of *SMAD6* variant-positive patients with CHD is still relatively low ($N = 30$), a trend towards a higher prevalence of, and hence increased risk for, CoA (10%) is observed as compared to (BAV/TAA) cases in general (7%) [5]. Of the remaining eight *SMAD6*-positive variant carriers, six displayed a heart defect including BAV (2-II:3), aortic regurgitation (1-III:1, 2-II:3), aortic valve stenosis (2-I:1), which was treated with surgery, patent foramen ovale (1-IV:2, 1-II:4), moderate mitral regurgitation with MVP (1-II:4), and pulmonary valve stenosis (1-IV:1). Additionally, one of these patients (1-II:4) presented with vascular anomalies like type A aortic dissection and moderate aneurysm at the level of the aortic root and ascending aorta, which is potentially aggravated by mild-moderate atherosclerosis. Finally, two out of the 14 *SMAD6*-variant carriers only presented vascular anomalies. One patient (5-1:1) had a diffuse thoracic-abdominal aneurysm, tortuosity of the aorta, and calcified arterial vessels, while the other patient (1-III:2) presented with a mild aortic dilatation.

Non-cardiovascular disease was found in five out of the 17 *SMAD6*-positive patients ($N = 5/17$, 29.4%). However, four out of these five patients ($N = 4/5$, 80%) belonged to the same family and harboured, in addition to the *SMAD6* deletion, a deletion of *SMAD3* exon 1. The clinical features of these patients presented in various systems such as the musculoskeletal system (1-II:2, 1-II:4, 1-III:3), the central nerve system (1-III:3), the immune system (1-II:2, 1-II:4) and the genitourinary system (1-IV:2). The fifth patient (3-II:1) presented extra features like mild disability and developmental delay, most likely accounted for by the Chr15q11.2 microdeletion, as discussed above. Overall, our data suggest that the phenotypical expression of *SMAD6* variants seems confined to the cardiovascular system. Taken together, these observations makes genetic counselling of *SMAD6*-positive patients quite difficult.

Since both cardiovascular features and craniosynostosis are observed as the consequence of an identical type of variant (i.e. loss-of-function), it is tempting to hypothesize that other genetic factors could be involved in modifying the phenotypic outcome of SMAD6 deficiency. In five out of our six BAV/TAA families, a second genetic class 3, 4 or 5 variant was identified, which could potentially increase the penetrance for CHD. Two *SMAD6*-positive families (family 2 and 3) harboured a second rare *SMAD6* missense variant located in a functional domain. Interestingly, in both families one of the variants affected the amino acid at position 231. Unfortunately, segregation analysis was only performed in family 2, but the patient harbouring both *SMAD6* variants did not present with a strikingly more severe cardiovascular phenotype. Moreover, family members presenting either of the two *SMAD6* variants also had a cardiovascular phenotype, suggesting that each of them is at least contributing to the phenotype. Also, two *SMAD6*-positive families carried a second hit in another TGFβ-related gene, i.e. a deletion of exon 1 of *SMAD3* or a potential splice-site variant in *FBN1*. Another family had a Chr15q11.2 microdeletion in addition to the *SMAD6* missense variant. BAV/TAA has not been reported with the 15q11.2 BP1–BP2 microdeletion syndrome but few patients [30] have been described with cardiovascular defects, including ventricular septal defect, patent ductus arteriosus and patent foramen ovale [31, 32].

Current insights into the molecular disease mechanisms of craniosynostosis were solely acquired by studies with patients displaying this skull phenotype, in which incomplete penetrance for rare deleterious *SMAD6* variants was observed. However, near-complete penetrance was reached upon co-occurrence with a variant near *BMP2* (rs1884302) [13, 14]. Hence, absence of this common variant was expected in our *SMAD6*-variant-positive study population. We identified six *SMAD6+/BMP2+* non-syndromic TAA patients without craniosynostosis. This is not in line with

the findings by Timberlake et al. [13, 14]. They showed the presence of the *BMP2* SNP risk allele in 16 out of their 41 *SMAD6*-positive individuals ($N = 16/41$, 39%) and only 1 out of the 16 individuals with the pathogenic *SMAD6+/BMP2+* genotype did not present craniosynostosis (6%) (Table 3) [13, 14]. As such, we were unable to support the proposed digenic model described by Timberlake et al. [13]. Our findings suggest involvement of additional factors that contribute to the phenotypic outcome of patients either causing CHD or craniosynostosis. Supportive evidence for these observations is inferred by mouse studies. Mice depleted for the murine orthologue of *SMAD6*, i.e. *Madh6*$^{-/-}$ mice, presented with a sole cardiovascular [33] (129/SvEv×BALB/cBy; 129/SvEv×C57Bl/6; 129/SvEv inbred) or craniofacial, axial and appendicular skeletal phenotype [34] (C57Bl/6 ×BALB/c) depending on the genetic background of the mouse model. This supports the need for looking into the genetic backgrounds of these patients. Ultimately, more research focussing on gaining insights into the diseases' characteristics will allow better risk stratification strategies as well as patient management. Limitations of our study include the need for further functional validation of the identified *SMAD6* variants and an additional unbiased study to address modifying risk factors that influence the cardiovascular phenotypes of *SMAD6*-variant-positive individuals.

To conclude, our results confirm the role of deleterious *SMAD6* variants in the genetic aetiology of the BAV/TAA pathology, and reveal little contribution to TAV/TAA development. Besides variable clinical expressivity, reduced penetrance and the occasional occurrence of extra cardiovascular features, an increased risk for CoA seems to be emerging. The proposed digenic model of near-complete penetrance for craniosynostosis was not supported by findings in our BAV/TAA cohort, suggesting that other genetic or stochastic factors are at play. Finally, our data provide novel insights into the complex genetic architecture of (BAV/)TAA disease with important implications for molecular diagnostics. Screening of *SMAD6* in young BAV/TAA patients, especially with a positive history for CHD, is recommended.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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