Next generation sequencing in clinical practice

Illustrated by studies in hereditary connective tissue disorders

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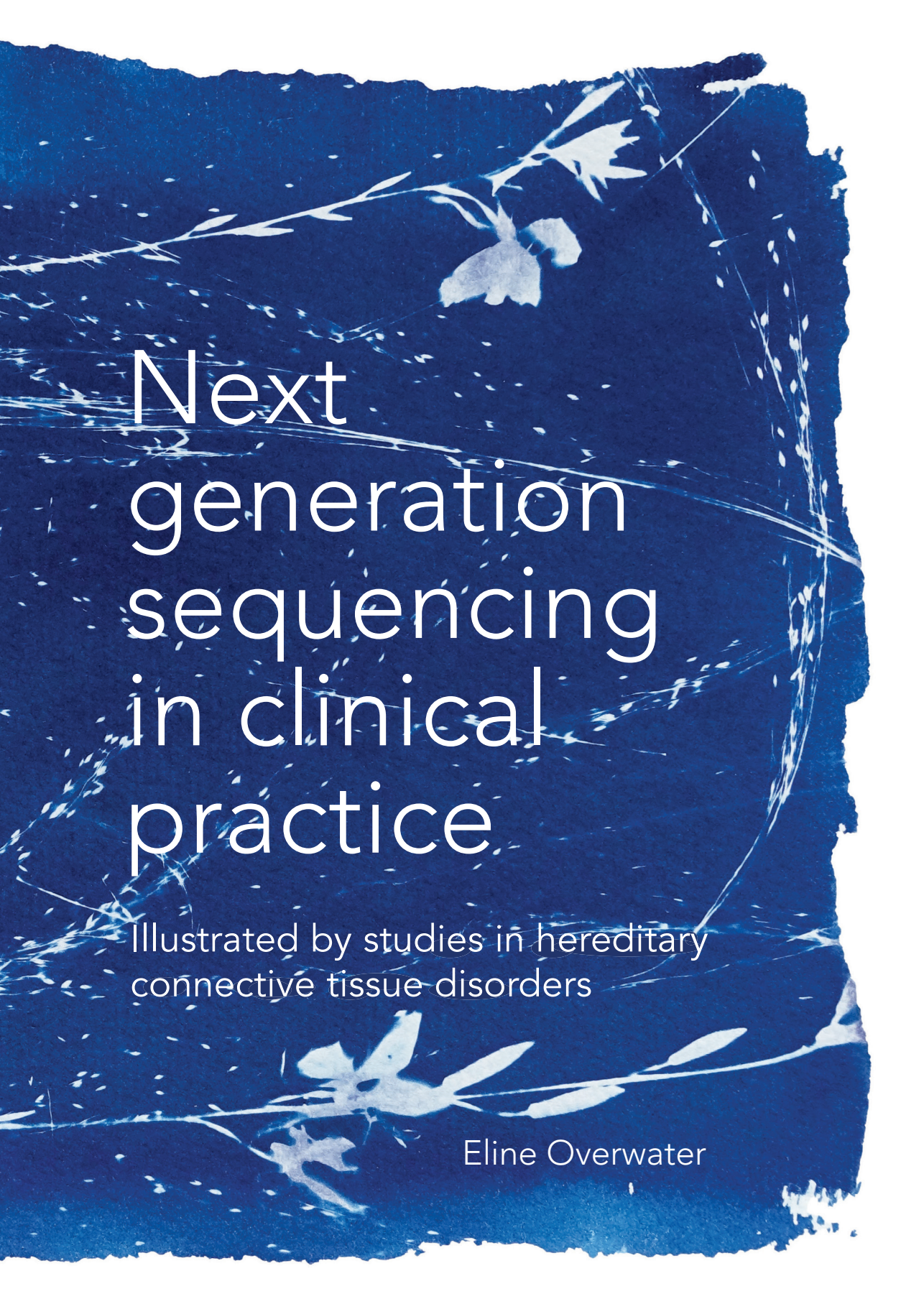
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Next generation sequencing in clinical practice

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in het openbaar te verdedigen in de Agnietenkapel

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General introduction

History

Due to an increased understanding of hereditary disorders, clinical genetics was officially recognized as a medical specialty in the Netherlands in 1987. Since then, the field continued to evolve at an unprecedented pace. In 1989 the first disease-causing gene (the *CFTR* gene involved in cystic fibrosis) was published. Only twelve years later the approximately 22.300 protein coding genes were identified through the "human genome project", a project that took over a decade costing an estimated 2.7 billion dollars [1-3]. At that time, it was unimaginable that whole genome sequencing would be feasible in a single day, a feat that was achieved within 20 years after publication of the landmark papers on the human genome project [4]. Nowadays, next generation sequencing (NGS), a technology that allows for massively parallel sequencing of the entire genome or specific genes of interest, is part of daily practice. Clinical genetics services have become an important part of the diagnostic process, treatment and prevention of numerous diseases [5].

For many hereditary diseases gene-specific recommendations, pharmacological and reproductive options, and presymptomatic testing allow for individualized health care. This underlines the potential benefits of establishing a genetic diagnosis for both patients and their relatives. Raising awareness of a potential genetic cause of diseases is therefore highly relevant. Examples of diseases where establishing a genetic diagnosis may be lifesaving, in patients and their relatives, include hereditary forms of cancer and hereditary aortic diseases. For these disorders, the options for genetic testing have also increased dramatically in recent years [6, 7].

The increasing number of patients referred for genetic counseling and the implementation of DNA testing in clinical guidelines reflects the appreciation of the added value of genetic testing in patient care. In the Netherlands the number of patients evaluated by a clinical geneticist has increased steadily during the past years (from approximately 12.000 in 2000 to almost 40.000 in 2015). However, it remains challenging to ensure all patients at risk for a genetic disorder are referred for genetic counseling [8]. Especially, in rare genetic disorders or in patients with a non-specific presentation of a genetic disease, the treating physician may not be aware of the possibility of an underlying genetic cause. In addition, the presentation (e.g. age at diagnosis) of a pathogenic variant in a high-risk gene may overlap with that of a common disease in the general population. This overlap is likely the result of an interplay between non-genetic (e.g. environmental factors) and genetic factors

[9]. This ads to the difficulty in determining which patients may benefit from genetic testing [10]. In families and patients without the classical presentation of a high-risk genetic predisposition, it is more likely that DNA testing is not performed, resulting in morbidity and mortality that could, in retrospect, have been prevented. These observations emphasize the need for increasing awareness and improved evidence-based guidelines for genetic testing to increase detection of at-risk patients. This is for example demonstrated by the recent evaluation of existing guidelines in breast cancer patients, which indicated that nearly half the (likely) pathogenic variants are missed when applying current guidelines for DNA testing [10].

The focus of this thesis is on the genetic aspects of connective tissue disorders and their associated clinical features (among others aortic disease, bicuspid aortic valve and ectopia lentis), with emphasis on the opportunities and challenges provided by NGS [11].

Recent developments and opportunities in hereditary connective tissue disorders illustrated by hereditary aortic disease

In the majority of patients suffering from a thoracic aortic aneurysm or dissection, the disease does not occur in multiple relatives, and is associated with older age and hypertension. However, in approximately 20% of patients the disease occurs in multiple relatives [12]. In approximately 25% of familial aortic disease, an underlying genetic defect can currently be identified [7]. Aortic disease was traditionally considered to be either non-syndromic (limited to a thoracic aortic aneurysm or dissection, e.g. aortic disease caused by pathogenic variants in the *ACTA2* gene MIM* 102620, the *MYH11* gene MIM* 160745, or the *MYLK* gene MIM* 600922) or syndromic (part of a generalized disease affecting multiple organ systems, e.g. Marfan syndrome MIM# 154700 caused by pathogenic *FBN1* variants MIM* 134797, vascular Ehlers Danlos syndrome MIM# 130050 caused by pathogenic *COL3A1* variants MIM* 120180 and Loeys-Dietz syndrome [13] caused by pathogenic variants in *SMAD2, SMAD3, TGFB2, TGFB3, TGFBR1* or *TGFBR2*). Nowadays, non-syndromic and syndromic aortic disease are often considered to be a phenotypic spectrum which can be caused by pathogenic variants in the same gene as shown by several studies and case reports [14-16].

The clinical applications of advances in the field of human genetics in aortic disease are illustrated here by genetic testing in suspected Marfan syndrome. Marfan syndrome is the most common hereditary connective tissue disorder caused by pathogenic variants in the *FBN1* gene. This gene encodes the major element of extracellular microfibrils, fibrillin, which is found in connective tissue throughout the body [17]. Marfan syndrome is characterized by, among others, skeletal and ocular features and an increased risk for aortic aneurysms and dissections [18]. The sequence of the *FBN1* gene was first published in 1991 [19]. Since 2000, sequencing of the *FBN1* gene is performed by our diagnostic DNA laboratory, initially using denaturing high performance liquid chromatography (DHPLC) and Sanger sequencing. Analysis of the gene at that time took approximately 6 months. In addition, the lack of large genomic patient- and control databases such as the gnomAD database (gnomad.broadinsitute.org), resulted in a significant risk of finding variants of unknown significance. Therefore, genetic testing was initially only considered in severely affected patients with classical features of the syndrome and a high prior risk of carrying a pathogenic variant. Nowadays, over 20 genes have been associated with hereditary aortic disease which can be analyzed simultaneously by NGS, including copy number variation analysis, in patients presenting with aortic disease in several weeks [7].

The decrease in time, costs and number of variants of unknown significance associated with DNA diagnostics has resulted in a lower threshold for genetic testing in aortic disease [7, 20]. As mentioned above, for many diseases, there is an overlap in the disease severity and onset between the general population and patients carrying a pathogenic variant in a high-risk gene. This overlap is likely the result of a combination of additional genetic and non-genetic factors (e.g. diet, smoking) in an interplay with the pathogenic variant [21]. This phenomenon is also observed in Marfan syndrome which is characterized by marked intra- and inter familial variability. This makes it difficult to determine which patients may benefit from genetic testing.

Outline of the thesis

In **part one** the outcomes and applications of next generation sequencing (NGS) in different hereditary connective tissue disorders are illustrated.
In **chapter 2**, an overview is provided of the outcomes of genetic testing in a cohort of 810 patients with suspected hereditary aortic disease using NGS analysis including copy number variation analysis. In **chapter 3**, the outcomes of genetic testing in patients diagnosed with ectopia lentis are discussed. Ectopia lentis is often isolated, but sometimes associated with, for example, Marfan syndrome. An algorithm for clinical screening and genetic testing in patients and families presenting with a bicuspid aortic valve is proposed in **chapter 4**. Although bicuspid aortic valve often occurs as a familial condition, a pathogenic variant in a known high-risk gene is rarely identified nowadays. In **chapter 5** the results of a retrospective cross-sectional multicenter study of patients carrying a pathogenic *TGFB3* variant are shown, which contributes to the knowledge of the genotypic and phenotypic spectrum of *TGFB3* associated disease.

In **part two** the importance of raising awareness for genetic diseases is illustrated. Hereditary disorders of connective tissue may be recognizable by specific disease associated symptoms such as iris flocculi in patients carrying a pathogenic variant in the *ACTA2* gene. When these symptoms are very rare, they are often not recognized as an indication for a genetic disease. This is illustrated in **chapter 6**. In addition, the extreme variability that can be associated with vascular Ehlers Danlos syndrome and Marfan syndrome is further illustrated in **chapters 7, 8 and 9**. The presented cases highlight the clinical value of genetic testing in diagnosing hereditary aortic disease and the opportunities provided by the rapid advances in the field of human genetics.

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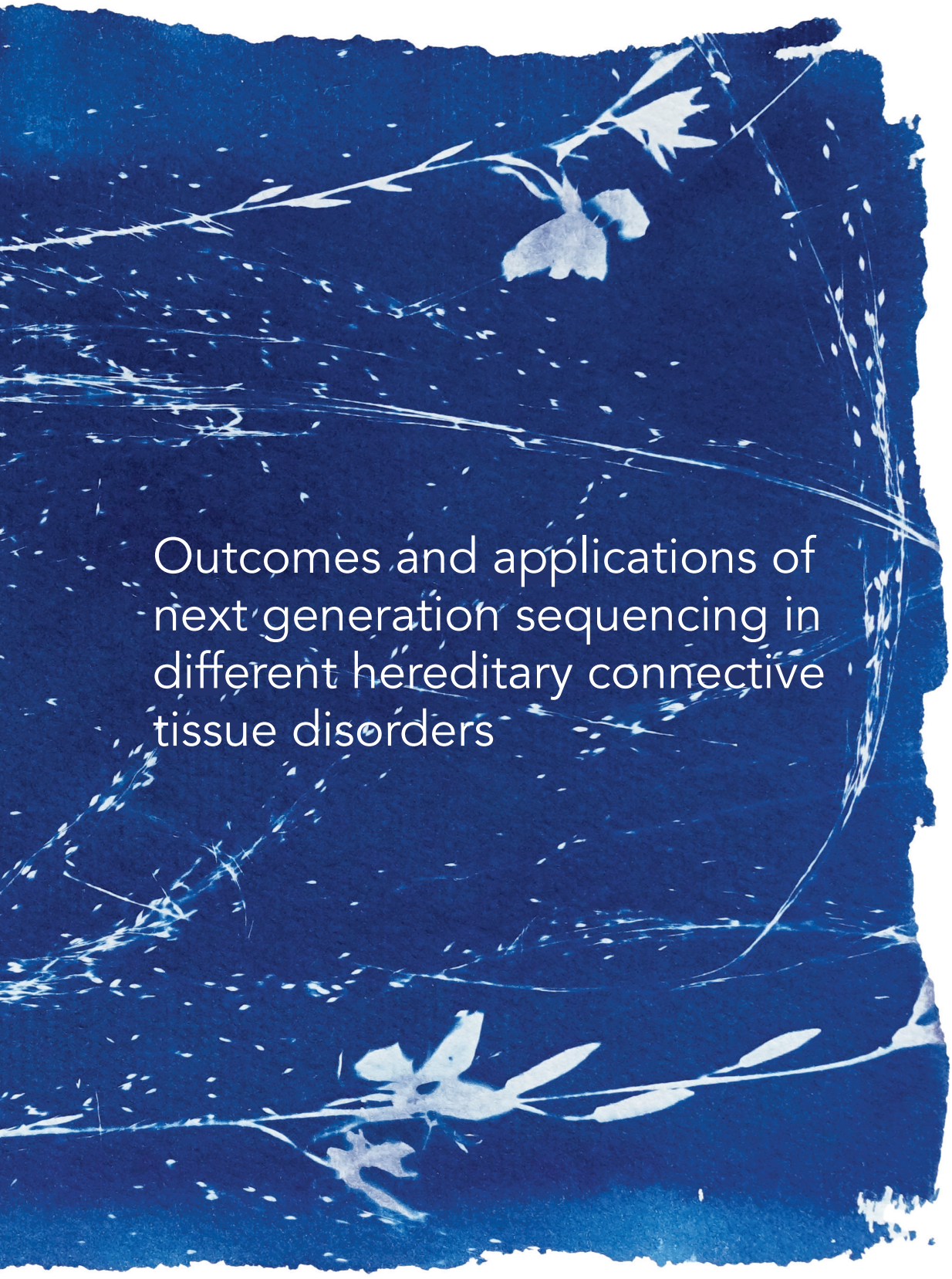
Part 1



Outcomes and applications of next generation sequencing in different hereditary connective tissue disorders


2

Results of next generation sequencing gene panel diagnostics including copy number variation analysis in 810 patients suspected of heritable thoracic aortic disorders

E. Overwater, L. Marsili, M.J.H. Baars, A.F. Baas, I. van de Beek, E. Dulfer, J.M. van Hagen, Y. Hilhorst-Hofstee, M. Kempers, I.P. Krapels, L.A. Menke, J.M.A. Verhagen, K.K. Yeung, P.J. Zwijnenburg, M. Groenink, M.M. Weiss, E. Voorhoeve, J.P. van Tintelen, A.C. Houweling*, A. Maugeri*

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Human Mutation 2018

Abstract

Simultaneous analysis of multiple genes using next generation sequencing (NGS) technology has become widely available. Copy-number variations (CNVs) in disease-associated genes have emerged as a cause for several hereditary disorders. CNVs are, however, not routinely detected using NGS analysis. The aim of this study was to assess the diagnostic yield and the prevalence of CNVs using our panel of Hereditary Thoracic Aortic Disease (H-TAD) associated genes. Eight hundred ten patients suspected of H-TAD were analysed by targeted NGS analysis of 21 H-TAD associated genes. In addition, the eXome Hidden Markov Model (XHMM; an algorithm to identify CNVs in targeted NGS data) was used to detect CNVs in these genes. A pathogenic or likely pathogenic variant was found in 66 out of 810 patients (8.1%). Of these 66 pathogenic or likely pathogenic variants, six (9.1%) were CNVs not detectable by routine NGS analysis. These CNVs were four intragenic (multi-)exon deletions in *MYLK*, *TGFB2*, *SMAD3* and *PRKG1* respectively. In addition, a large duplication including *NOTCH1* and a large deletion encompassing *SCARF2* were detected. As confirmed by additional analyses, both CNVs indicated larger chromosomal abnormalities, which could explain the phenotype in both patients. Given the clinical relevance of the identification of a genetic cause, CNV analysis using a method such as XHMM should be routinely incorporated into the clinical diagnostic care for H-TAD patients.

Background

Over the last decade, advances in clinical genetics have led to the identification of disease-associated genes at a rapid pace. Especially when surveillance, early detection and/or treatment provide health benefits for the index patient and at-risk relatives, identification of an underlying genetic cause is highly relevant. Therefore, recommendations for genetic counselling and DNA testing are increasingly being incorporated into clinical guidelines [1, 2]. Thoracic aortic aneurysms and aortic dissections (TAAD) are a significant cause of sudden death at young age and is an example of a disease where screening of at-risk relatives can be lifesaving [3, 4]. Because aortic aneurysms are often asymptomatic and aortic dissections are often fatal and preventable by timely surgical intervention, the identification and clinical screening of at-risk relatives are clinically highly relevant and recommended [5]. In the majority of cases, TAAD is a sporadic occurrence, associated with, among others, hypertension, bicuspid aortic valve and older age. However, in approximately 20% of cases TAAD is reported to be familial (FTAAD), often with an autosomal dominant pattern of inheritance with incomplete penetrance [6-8]. TAAD that is caused by a pathogenic variant in one of the disease associated genes (Hereditary Thoracic Aortic Disease (H-TAD)), can be subdivided in nonsyndromic and syndromic aortic disease. The phenotypic manifestations of both syndromic and nonsyndromic H-TAD are highly variable, both within and between families. Syndromic H-TAD is only diagnosed in a minority of cases and includes, among others, Marfan syndrome (MIM# 154700), Loeys-Dietz syndrome (MIM# 609192, MIM# 610168, MIM# 613795, MIM# 614816, MIM# 615582) and vascular Ehlers Danlos syndrome (MIM# 130050). The genes most frequently associated with nonsyndromic H-TAD are involved in smooth-muscle cell function (*ACTA2*; MIM# 611788, *MYH11*; MIM# 132900, *MYLK*; MIM# 613780). Of note, variants in genes originally associated with syndromic H-TAD have also been reported in patients presenting with apparently nonsyndromic H-TAD [9-11]. Given the incomplete penetrance and the highly variable age of onset within both heritable and sporadic TAAD [7, 8, 12-14], follow-up of at-risk relatives with normal aortic diameters at initial cardiologic screening is important. The identification of a pathogenic variant in a TAAD patient allows for targeted screening of relatives and enables prenatal and preimplantation genetic diagnosis. In addition, specific recommendations on imaging, surgical, and pharmacological treatment based on the underlying genetic cause are emerging [15-17]. A causative variant can be identified in approximately 20% of FTAAD families [18]. Next generation sequencing (NGS) allows for the rapid analysis of multiple genes in a diagnostic setting at relatively low costs. Therefore, DNA testing

is increasingly offered to TAAD patients. The majority of the detected variants are single-nucleotide changes. CNVs have emerged as a relevant cause for several genetic disorders including cancer, intellectual disability, and neuropsychiatric disorders [19-21]. Routine diagnostic variant-calling analysis by (short reads-) NGS technology is not suitable for detecting CNVs. Therefore, CNVs may be missed unless additional testing is performed, for example by multiplex ligation-dependent probe amplification (MLPA) or targeted array analysis. However, these tests are often not routinely performed and/or do not include all the relevant genes. The detection of CNVs in NGS sequencing data using statistical and computational tools is an alternative approach. The eXome hidden Markov model (XHMM) is one of several algorithms developed for the detection of CNVs through NGS data [22, 23]. XHMM has identified (potential) causative CNVs in, for example, patients with Parkinson's disease, autism spectrum disorders and rare diseases like Joubert syndrome and very early onset inflammatory bowel disease [24-27]. The aim of this study was to assess both the diagnostic yield of our panel of H-TAD-associated genes and the prevalence of CNVs in these genes. Here, we present the results of routine NGS analysis (variant-calling analysis) and XHMM analysis on the NGS sequencing data of the largest series of TAAD patients described so far (n=810) referred for analyses of the H-TAD panel. In addition, we provide an overview of the clinical data of patients with a pathogenic or likely pathogenic variant, with a special focus on patients with CNVs. The results of this study underline the importance of CNV analysis in routine diagnostic testing in patients with H-TAD.

Methods

Genetic data

DNA diagnostics was performed at the Department of clinical genetics at the VU University Medical Center (VUmc, Amsterdam, the Netherlands) from March 2015 to June 2017. The routine NGS panel included *ACTA2, COL3A1, EFEMP2, ELN, FBN1, FBN2, MYH11, MYLK, NOTCH1, PLOD1, PRKG1, SCARF2, SKI, SLC2A10, SMAD2, SMAD3, SMAD4, TGFB2, TGFB3, TGFBR1* and *TGFBR2*. Since October 2016 the *BGN* gene was added to the panel (analyzed in 166 patients), while *SCARF2*, which was not associated with TAD but had previously been selected in view of a possible differential diagnosis 'Congenital contractural arachnodactyly' and 'Van den Ende-Gupta syndrome' was excluded from routine analysis. The previously described bioinformatics read-depth based tool XHMM (https://atgu.mgh.harvard.edu/xhmm/) was used for CNV detection in the NGS sequencing data. CNV confirmation was performed using

either a home-made MLPA test, in combination with the P300 or the P200 MLPA kit of MRC Holland, or an SNP array. Detailed information on the analyzed genes and applied methodologies are available in the Supporting Materials and Methods.

Clinical data

Informed consent for NGS gene panel analysis was obtained from all 810 patients after genetic counseling by the referring physician. The main reasons for analysis of this gene panel include familial or early onset aortic aneurysms or dissections or signs of generalized connective tissue disorders. The majority of patients was referred by a clinical geneticist who frequently participated in a multidisciplinary team specialized in connective tissue disorders. A standardized survey was sent to the referring physicians in order to collect the medical data of patients carrying an identified genetic variant (including ophthalmologic and cardiologic findings, family history, and physical examination). Written informed consent was obtained from the patients and/or their parents with an aberration detected by XHMM, as more detailed medical data were published. Under Dutch law, assessment of the study protocol by our ethics committee was not indicated since only genetic and clinical data collected during regular patient care were used.

Results

A pathogenic or likely pathogenic variant in an H-TAD-associated gene was identified in 66 of 810 index patients (8.1%). Of these, 60 (90.9%) were identified using routine NGS panel analysis (variant-calling analysis). In the other six cases (9.1%), a pathogenic or likely pathogenic CNV was detected using XHMM. In 84 patients (10.4%) only variants of unknown significance (VUS) were identified. No pathogenic or likely pathogenic variants and/or VUS were identified in 660 patients (81.5%). The mean age at DNA diagnostics of index patients with a pathogenic or likely pathogenic variant was 36 years (median 36, range 0-77). The mean age of the remaining patients was 46 years (median 49, range 0-78). There was a male preponderance in index patients with a pathogenic or likely pathogenic variant, VUS or without a VUS or pathogenic variant (68%, 64% and 67% respectively).

Genetic and clinical data in patients with variants identified by variant-calling analysis

Table 1 provides an overview of the molecular data of the 60 pathogenic or likely pathogenic variants identified by variant-calling analysis. Of these variants, 37 (62%) have not been described previously and all of them were unique. Heterozygous pathogenic or likely pathogenic variants were identified in *FBN1* (N = 18, 30%), *ACTA2* (N = 8, 13.3%), *SMAD3* (N = 7, 11.7%), *COL3A1* (N = 6, 10%), *TGFB2* (N = 4, 6.7%), *TGFBR1* (N = 3, 5%), *TGFBR2* (N = 3, 5%), *FBN2* (N = 3, 5%), *MYH11* (N = 2, 3.3%), *TGFB3* (N = 2, 3.3%), *PRKG1* (N = 1, 1.7%) and *NOTCH1* (N = 1, 1.7%). Homozygous pathogenic *SLC2A10* variants were identified in two patients (3.3%). No (likely) pathogenic variants were found in *BGN*, *EFEMP2*, *ELN*, *PLOD1*, *SKI*, *SMAD2*, and *SMAD4*. In addition, 90 VUS were identified (patients 9, 52, 67-150, Table 1 and Supporting Information Table S1). In six patients (patients 9 and 52 in Table 1 and Supporting Information Table S1; and patients 69, 75, 90, and 127 in Supporting Information Table S1) two VUS (in different genes) were identified. An overview of the clinical data of all 60 patients with a pathogenic or likely pathogenic variant identified by variant-calling analysis is provided in Table 2. The clinical data of patients 67-150 with a VUS are available in Supporting Information Table S2.

Genetic and clinical data in patients with a CNV identified by XHMM analysis

The results of the XHMM analysis in the six patients with a CNV (patients 61-66) are depicted in Figure 1 and are summarized in Table 3.

In patient 61, a deletion of two exons in the *MYLK* gene was identified (NM_053025.3: c.(2390+1_2391-1)_(3448+1_3449-1)del). This deletion is predicted to generate an *out-of-frame* deletion in the long transcript of the *MYLK* gene (NM_053025.3) and a loss of the first 682 coding nucleotides, including the alternative translation initiation codon in the smooth-muscle cell specific transcript encoding isoform 5 (Uniprot Q15746-7). This male patient was diagnosed with a type B dissection at the age of 60 years and developed a type A dissection at the age of 65 years. He was treated surgically (Bentall procedure). Medical history and physical examination did not reveal any other signs of a connective tissue disorder. Pedigree analysis revealed that his sister suddenly died at the age of 53 years. No medical records, autopsy or DNA were available. The 35-year-old son of the index patient did not carry the two-exon deletion of *MYLK*. Until now, no other relatives opted for genetic testing.

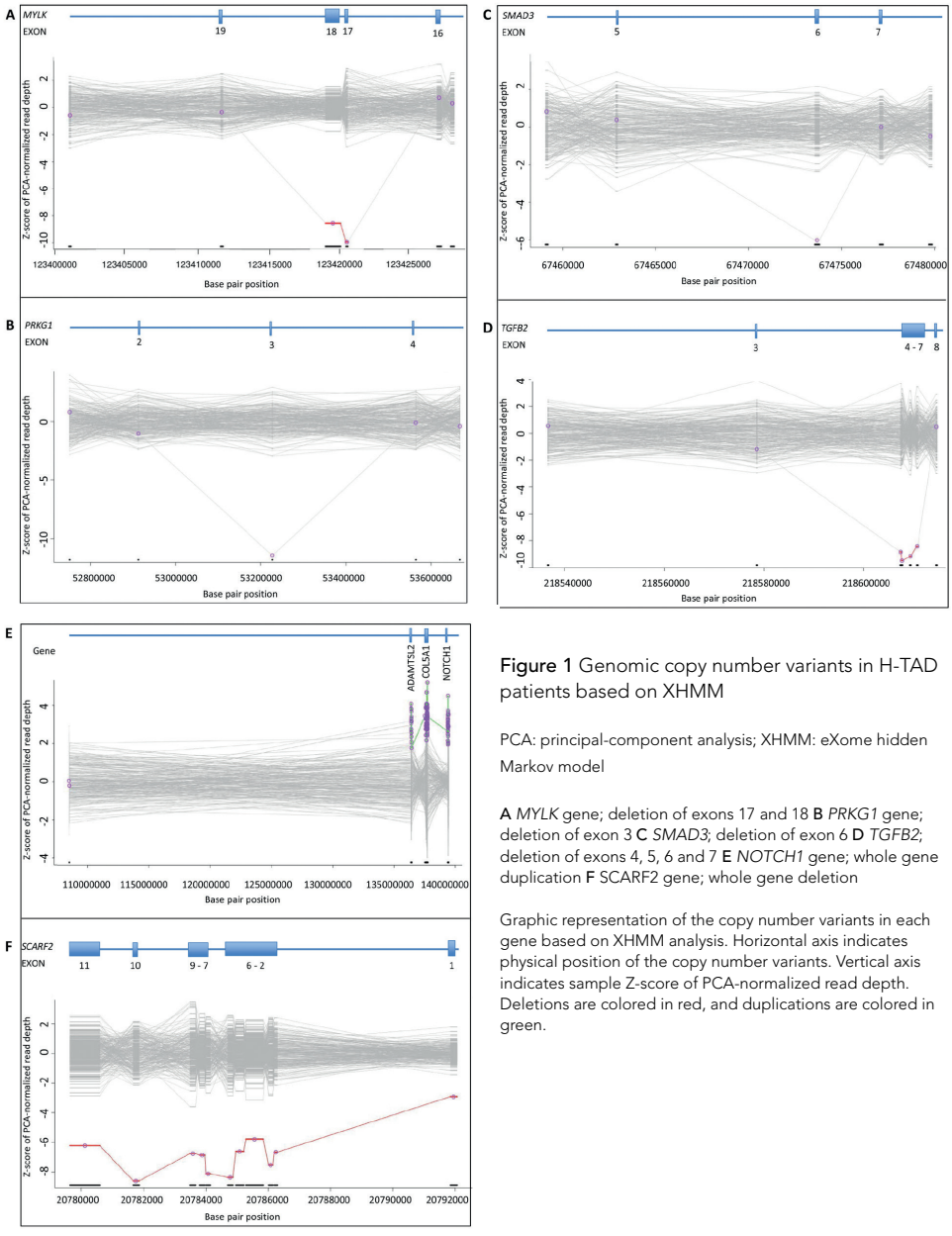

Figure 1 Genomic copy number variants in H-TAD patients based on XHMM

PCA: principal-component analysis; XHMM: eXome hidden Markov model

A *MYLK* gene; deletion of exons 17 and 18 **B** *PRKG1* gene; deletion of exon 3 **C** *SMAD3*; deletion of exon 6 **D** *TGFB2*; deletion of exons 4, 5, 6 and 7 **E** *NOTCH1* gene; whole gene duplication **F** SCARF2 gene; whole gene deletion

Graphic representation of the copy number variants in each gene based on XHMM analysis. Horizontal axis indicates physical position of the copy number variants. Vertical axis indicates sample Z-score of PCA-normalized read depth. Deletions are colored in red, and duplications are colored in green.

Table 1 Summary of the genetic features of patients with a pathogenic or likely pathogenic variant detected by variant-calling analysis of 21 H-TAD genes

Patient	Gene	Nucleotide change	Protein change	Effect	Domain	Conservation	SIFT / MutationTaster / Polyphen-2 / Grantham distance	MAF ExAC	Segregation analysis[a]	Reference
1	*ACTA2*	c.115C>T	p.(Arg39Cys)	Missense	Actin	Baker's yeast[b]	+ / + / - / 180	Absent	yes	[35]
2	*ACTA2*	c.116G>A	p.(Arg39His)	Missense	Actin	Baker's yeast[b]	+ / + / - / 29	Absent	yes	[36]
3	*ACTA2*	c.179C>A	p.(Ala60Glu)	Missense	Actin	Baker's yeast[b]	+ / + / + / 107	Absent	n.a.	Novel
4	*ACTA2*	c.419C>T	p.(Ala140Val)	Missense	Actin	Baker's yeast[b]	+ / + / ±/ 64	Absent	yes	[31]
5[c]	*ACTA2*	c.445C>T	p.(Arg149Cys)	Missense	Actin	Baker's yeast[b]	+ / + / + / 180	Absent	yes	[37]
6	*ACTA2*	c.835A>G	p.(Thr279Ala)	Missense	Actin	Baker's yeast[b]	+ / + / - / 58	Absent	n.a.	Novel
7	*ACTA2*	c.854T>C	p.(Met285Thr)	Missense	Actin	Baker's yeast[b]	+ / + / ± / 81	Absent	n.a.	Novel
8	*ACTA2*	c.1120C>T	p.(Arg374Cys)	Missense	Actin	C. elegans (FCUT Baker's yeast)	+ / + / - / 180	1 / 121346	n.a.	Novel
9[d]	COL3A1	c.318_325del	p.(Pro107Argfs*13)	Frameshift (NMD expected)	NA	NA	NA	Absent	n.a.	Novel
10	COL3A1	c.555del	p.(Gly186Valfs*36)	Frameshift (NMD expected)	NA	NA	NA	Absent	yes, incomplete penetrance	[38, 39]
11	COL3A1	c.971G>A	p.(Gly324Asp)	Missense	Triple helix	Chicken[b]	+ / + / ± / 94	Absent	de novo[e]	Novel
12	COL3A1	c.2050G>A	p.(Gly684Arg)	Missense	Triple helix	Chicken[b]	+ / + / + / 125	Absent	yes	Novel
13	COL3A1	c.3219_3222dup	p.(Ala1075Trpfs*20)	Frameshift (NMD expected)	NA	NA	NA	Absent	Maternally inherited	Novel
14	COL3A1	c.3446G>A	p.(Gly1149Asp)	Missense	Triple helix	Chicken[b]	+ / + / + / 94	Absent	n.a.	[40]
15	FBN1	c.32T>G	p.(Leu11Arg)	Missense	Signal peptide	Dog[b]	+ / + / + / 102	Absent	n.a.	[41]
16	*FBN1*	c.439C>T	p.(Gln147*)	Nonsense (NMD expected)	NA	NA	NA	Absent	n.a.	Novel
17	*FBN1*	c.986dup	p.(Asp330Argfs*18)	Frameshift (NMD expected)	NA	NA	NA	Absent	n.a.	Novel

18	*FBN1*	c.2177A>G	p.(Glu726Gly)	Missense	EGF-like 11	Tetraodon[b]	+ / + / + / 98	Absent	n.a.	[42]
19	*FBN1*	c.2645C>T	p.(Ala882Val)	Missense	TB 4	Tetraodon[b]	+ / + / + / 64	Absent	n.a.	[43-48]
20	*FBN1*	c.2660G>A	p.(Cys887Tyr)	Missense	TB 4	Tetraodon[b]	+ / + / + / 194	Absent	n.a.	Novel
21	*FBN1*	c.2668T>C	p.(Cys890Arg)	Missense	TB 4	Tetraodon[b]	+ / + / + / 180	Absent	n.a.	[49, 50]
22	*FBN1*	c.2953G>A	p.(Gly985Arg)	Missense	TB 5	Tetraodon[b]	+ / + / + / 125	Absent	n.a.	[45, 51-55]
23	*FBN1*	c.3152T>G	p.(Phe1051Cys)	Missense	EGF-like 15	Tetraodon[b]	+ / + / + / 205	Absent	n.a.	Novel
24	*FBN1*	c.3373C>T	p.(Arg1125*)	Nonsense (NMD expected)	NA	NA	NA	Absent	yes	[42, 44, 46, 53, 56-58]
25	*FBN1*	c.4987T>C	p.(Cys1663Arg)	Missense	EGF-like 28	Zebrafish[b]	+ / + / + / 180	Absent	n.a.	[42, 55, 59]
26	*FBN1*	c.5015del	p.(Cys1672Leufs*10)	Frameshift (NMD expected)	NA	NA	NA	Absent	n.a.	Novel
27	*FBN1*	c.5699G>C	p.(Cys1900Ser)	Missense	EGF-like 32	Zebrafish[b]	+ / + / + / 112	Absent	n.a.	[42]
28	*FBN1*	c.6031T>C	p.(Cys2011Arg)	Missense	EGF-like 34	Zebrafish[b]	+ / + / + / 180	Absent	*de novo*[e]	Novel
29	*FBN1*	c.6942C>G	p.(Tyr2314*)	Nonsense (NMD expected)	NA	NA	NA	Absent	*de novo*[e]	Novel
30	*FBN1*	c.7708G>A	p.(Glu2570Lys)	Missense	EGF-like 45	Tetraodon[b]	+ / + / + / 56	Absent	n.a.	[56, 60, 61]
31	*FBN1*	c.8188C>T	p.(Arg2730Trp)	Missense	C-terminal domain	Tetraodon[b]	+ / + / + / 101	Absent	n.a.	Novel
32	*FBN1*	c.8578_8579dup	p.(Asp2860Glufs*4)	Frameshift (NMD not expected)	Asprosin chain	NA	NA	Absent	n.a.	Novel
33	*FBN2*	c.3812G>C	p.(Gly1271Ala)	Missense	EGF-like 19	Chicken[b]	+ / + / + / 60	Absent	n.a.	[62]
34	*FBN2*	c.3889G>A	p.(Gly1297Ser)	Missense	EGF-like 20	Chicken[b]	+ / + / + / 56	2 / 121372	Paternally inherited	Novel
35	*FBN2*	c.7526_7527del	p.0	Frameshift (NMD confirmed)	NA	NA	NA	Absent	n.a.	Novel
36	*MYH11*	c.3315-5G>A	p.?	Splice (NMD not expected)	Coiled coil region	NA	NA	Absent	n.a.	Novel
37	*MYH11*	c.5293C>T	p.(Arg1765Trp)	Missense	Coiled coil region	Zebrafish[b]	+ / + / + / 101	1 / 115948	n.a.	Novel

38	*NOTCH1*	c.2123A>G Mosaic	p.(Tyr708Cys)	Missense	EGF-like 18	Tetraodon (FCUT Fruitfly)	+ / + / + / 194	Absent	*de novo* (inferred)	Novel
39	*PRKG1*	c.530G>A	p.(Arg177Gln)	Missense	cGMP-binding, high affinity	C. elegans[b]	- / + / + / 43	Absent	n.a.	[28]
40	*SLC2A10*	c.510G>A[f]	p.(Trp170*)	Nonsense (NMD expected)	NA	NA	NA	Absent	n.a. (consaguineous parents)	[63, 64]
41	*SLC2A10*	c.1276G>T[f]	p.(Gly426Trp)	Missense	Transmem-brane helical region 10	Tetraodon[b]	+ / + / + / 184	3 / 116638	confirmed parental carriership	[65]
42	*SMAD3*	c.1A>T	p.(Met1?)	Loss of initiation codon	Initiator methionine	C. elegans[b]	NA	Absent	n.a.	Novel
43	*SMAD3*	c.391_394dup	p.(Thr132Argfs*35)	Frameshift (NMD expected)	NA	NA	NA	Absent	n.a.	Novel
44	*SMAD3*	c.492dup	p.(Asn165*)	Frameshift (NMD expected)	NA	NA	NA	Absent	Yes	Novel
45	*SMAD3*	c.802C>T	p.(Arg268Cys)	Missense	MH2	C. elegans[b]	+ / + / + / 180	Absent	Yes	Novel
46	*SMAD3*	c.893A>G	p.(Tyr298Cys)	Missense	MH2	Fruitfly	- / + / + / 194	Absent	Yes	Novel
47	*SMAD3*	c.1010-2A>G	p.?	Splice (NMD not expected)	MH2	NA	NA	Absent	n.a.	Novel
48	*SMAD3*	c.1179dup	p.(Cys394Leufs*4)	Frameshift (NMD not expected)	MH2	NA	NA	Absent	Yes	[66]
49	*TGFB2*	c.709G>T	p.(Glu237*)	Nonsense (NMD expected)	NA	NA	NA	Absent	n.a.	Novel
50	*TGFB2*	c.979C>T	p.(Arg327Trp)	Missense	Transforming growth factor beta-2 chain	Frog	+ / + / + / 101	Absent	n.a.	[67, 68]
51	*TGFB2*	c.989G>A	p. (Arg330His)	Missense	Transforming growth factor beta-2 chain	Tetraodon	+ / + / + / 29	Absent	*Incomplete penetrance?*	Novel

52[d]	***TGFB2***	**c.1017-1G>T**	**p.?**	**Splice (NMD possible)**	**Transforming growth factor beta-2 chain**	**NA**	**NA**	**Absent**	***de novo***	**Novel**
53	***TGFB3***	**c.899G>A**	**p.(Arg300Gln)**	**Missense**	**Latency-associated peptide chain**	**Fruitfly[b]**	**+ / + / + / 43**	**Absent**	**Yes**	**[69]**
54	*TGFB3*	c.1075A>C	p.(Ser359Arg)	Missense	Transforming growth factor beta-3 chain	Fruitfly[b]	+ / + / + / 110	Absent	n.a.	Novel
55	*TGFBR1*	c.790G>A	p.(Ala264Thr)	Missense	Protein kinase	Fruitfly[b]	+ / + / + / 58	Absent	yes (incomplete penetrance)	Novel
56	*TGFBR1*	c.1255+2T>C	p.(Tyr378Asnfs*3)	Splice (exon 7 skipping partially stable at RNA level)	Protein kinase	NA	NA	Absent	yes	Novel
57	***TGFBR1***	**c.1460G>A**	**p.(Arg487Gln)**	**Missense**	**Protein kinase**	**Fruitfly[b]**	**+ / + / + / 43**	**Absent**	***de novo*[e]**	**[70-75]**
58	*TGFBR2*	c.1565G>A	p.(Arg522Gln)	Missense	Protein kinase	Zebrafish[b]	+ / + / + / 43	1 / 121046	Paternally inherited	Novel
59	***TGFBR2***	**c.1630G>T**	**p.(Glu544*)**	**Nonsense (NMD not expected)**	**Protein kinase**	**NA**	**NA**	**Absent**	**n.a.**	**Novel**
60	***TGFBR2***	**c.1669C>T**	**p.(Gln557*)**	**Nonsense (NMD not expected)**	**Not in functional domain/region**	**NA**	**NA**	**Absent**	**n.a.**	**Novel**

Used RefSeq transcripts (based on Genome build: GRCh37/hg19): *ACTA2*: NC_000010.10(NM_001141945.2), *COL3A1*: NC_000002.11(NM_000090.3), *FBN1*: NC_000015.9(NM_000138.4), *FBN2*: NC_000005.9(NM_001999.3), *MYH11*: NC_000016.9(NM_001040113.1), *NOTCH1*: NC_000009.11(NM_017617.3), *PRKG1*: NC_000010.10(NM_001098512.2), *SLC2A10*: NC_000020.10(NM_030777.3), *SMAD3*: NC_000015.9(NM_005902.3), *TGFB2*: NC_000001.10(NM_001135599.2), *TGFB3*: NC_000014.8(NM_003239.4), *TGFBR1*: NC_000009.11(NM_004612.2), *TGFBR2*: NC_000003.11(NM_001024847.2).

Pathogenic variants (class 5) are depicted in bold.
FCUT, functionally conserved up to; n.a., not available; NA, not applicable; NMD, nonsense mediated mRNA decay
[a]Yes, segregation analysis performed in (at least) one family member, variant segregated accordingly.
[b]No further alignment available.
[c]This family is recently described in literature (Overwater & Houweling, 2017).
[d]A variant of unknown significance was identified in these patients as well (Supporting Information Table S1).
[e]Paternity and maternity not confirmed.
[f]Homozygous variant.
- Tolerated (SIFT), polymorphism (MutationTaster), and benign (Polyphen-2) predictions.
± Possibly damaging (Polyphen-2) prediction.
+ Deleterious (SIFT), Disease-causing (MutationTaster), probably damaging (Polyphen-2) predictions.

Alignment, SIFT, MutationTaster, Polyphen-2, Grantham distance: Alamut GRCh37 accessed July 2017.

Table 2 Summary of the clinical features of patient with a pathogenic or likely pathogenic variant detected by variant-calling analysis of 21 H-TAD genes

Patient	Involved gene	Sex, Age[a]	Cardiovascular feature(s)	Systemic feature(s)	Family history Genotype/Relative/Phenotype		
1	*ACTA2*	♀, 16	PDA	None	+ - ? ?	F PU PA PGF	Dis (B, 51y), CVD Clinically not affected Dis, unconfirmed (deceased) Dis, unconfirmed (deceased)
2	*ACTA2*	♂, 28	Dis (A and B, 26y), BAV	None	+[b] -	F Sib	An (AoR 42mm, AAo 49mm, AA, 61y) BAV Clinically not affected
3	*ACTA2*	♂, 46	Dis (A, 45y)	None	?		No relatives clinically affected
4	*ACTA2*	♀, 69	Dis (B, 61y; A, 65y)	None	- + +	B (2) Si N	Clinically not affected Rup (AA, 62y) An (AA, 35mm)
5[c]	*ACTA2*	♂, 36	Dis (B, 36y)	Iris flocculi, livedo reticularis	+ +	M D	Dis (B, deceased, 30y), iris flocculi Iris flocculi
6	*ACTA2*	♂, 73	An (AoR, 52mm, 69y)	None	?		No relatives clinically affected
7	*ACTA2*	♂, 22	Dis (A, 21y), BAV	PP, SS, Myopia -5/-5 dpt	?		No relatives clinically affected
8	*ACTA2*	♂, 57	Dis (B, 57y), An (AoR 41mm, 57y)	Myopia -4 dpt, pneumothorax	?	B	SUD (58y)
9[d]	*COL3A1*	♂, 59	Rup (AoA, 54y), An (AA, 59y)	None	? ? ?	B B N	Rup (AoA, deceased, 59y) An (AA) An (AA, severe, 40y)
10	*COL3A1*	♂, 52	Dis (A, 47y), An (subclavian and vertebral artery, 52y)	Increased AHR	?		No relatives clinically affected
11	*COL3A1*	♀, 44	Dis (B, 44y)	NA	 -	 Si	*De novo*[e] Borderline An (AoR, 40mm, 51y), HT
12	*COL3A1*	♀, 31	An (renal and carotid artery), Dis (mammary-, subclavian- and iliac artery), occlusion (brachial artery)	None	- ? +	F M Si	Clinically not affected Gastric perforation Dis (iliac artery)
13	*COL3A1*	♂, 42	Dis (A, 38y)	Hyperkyphosis, hypermobile fingers	+ - -	M PU PGF	Clinically not affected Rup (AA, 55y), CVD Rup (AA, 63y), CVD
14	*COL3A1*	♂, 45	Dis (coronary artery, 42y), An (AAo, 47mm, 45y)	Soft skin	?		No relatives clinically affected
15	*FBN1*	♂, 66	Dis (B, 49y), An (subclavian artery, AA, 54y)	NA	?	So	Clinical features of MFS

16	*FBN1*	♀, 27	An (AoR, 41mm, 27y), MVP	Arachnodactyly	? +	M D	Clinical features of MFS No clinical features of MFS (5 months)
17	*FBN1*	♂, 35	An (AoR, 50mm, 35y), ASD, atrial flutter (23y)	Growth inhibiting treatment, HAP, crowding, retrognathia SS, IH	? ? ?	F PA PCo	SUD (44y), clinical features of MFS SUD (43y), clinical features of MFS Clinical features of MFS
18	*FBN1*	♂, 5	An (AAo, 27mm, Z-score +2.7, 5y), VSD	PP, hyperkyphosis, wrist sign +, dolichocephaly, malar hypoplasia, EL, BS 8/9	?		No relatives clinically affected
19	*FBN1*	♂, 53	An (thoracic aorta, 80mm, 53y)	Wrist and thumb sign +, IH	?	PF	Multiple relatives with An and/or Dis
20	*FBN1*	♀, 36	An (AoR, severe, 35y), MVP	Scoliosis, PC, Myopia -6.5 dpt, SS	?		No relatives clinically affected
21	*FBN1*	♂, 11	NA	Increased AHR, PD, clinical features of MFS			NA
22	*FBN1*	♂, 32	Dis (A, 15y), MVP	Marfanoid habitus, PP, reduced elbow extension, arachnodactyly, HAP, crowding, myopia -5/-3 dpt, SS	?		No relatives clinically affected
23	*FBN1*	♀, 0	An (AoR,0y), MI, TI	PC, joint contractures, arachnodactyly, dysmorphic facial features	?		No relatives clinically affected
24	*FBN1*	♂, 3	None	Height +3.4 SD, arachnodactyly, HAP, ptosis, epicanthal folds, delayed speech	+ ? ?	M MF MU	Arachnodactyly, tall stature Anamnestic MFS Premature birth, intracranial bleeding, epilepsy, spasticity, developmental delay
25	*FBN1*	♀, 29	An (AoR, 41mm, 29y), MI	Arachnodactyly, HAP, dolichocephaly, EL, RD	?	F	SD (42y), myocardial infarction
26	*FBN1*	♀, 11	MVP	Marfanoid habitus, PP, wrist and thumb sign +, joint luxations, SS, recurrent hematomas	?		Clinically not affected
27	*FBN1*	♀, 9	None	Increased AHR, PC, club foot, PP, thumb sign +, downslanting, malar hypoplasia, myopia, recurrent hematomas	?		No relatives clinically affected
28	*FBN1*	♂, 5	None	Tall stature, arachnodactyly, PP, PC, wrist sign +, HAP, hypermobility, macular degeneration			*De novo*[e]
29	*FBN1*	♀, 10	An (AAo, 31mm, Z-score +2.7, 10y)	PD, PP, arachnodactyly, HAP, dolichocephaly, myopia			*De novo*[e]
30	*FBN1*	♂, 54	Dis (A, 54y)	Pneumothorax, NA	+	So(2)	Clinically not affected
31	*FBN1*	♀, 46	An (AAo, 46mm, 46y), cerebral infarction (33y), stenosis (axillary-, brachial- and subclavian artery, 36y)	Hypermobile fingers	?		No relatives clinically affected
32	*FBN1*	♂, 0	MI, TI	PC, PP, dolichocephaly, downslanting, enophthalmos, floppy ears	?		No relatives clinically affected
33	*FBN2*	♂, 10	TI	Tall stature, PE, HAP, crowding	?	MF	An (aorta), hypermobility

34	*FBN2*	♂, 55	Borderline An (AAo, 39mm, 54y)	PE, hyperkyphosis, hammer toes, downslanting, myopia	+	F	Clinically not affected
35	*FBN2*	♂, 65	An (AAo, 45mm, 64y)	Hammer toes, HAP, enophthalmos, prominent eyes and nose, malar hypoplasia	? -	F B	An (AA, at older age) An (AAo, 45 mm, 39y)
36	*MYH11*	♂, 71	Dis (A and B, 70y), An (AA, 54mm, 71y)	None	?	M	Rup (aorta, deceased)
37	*MYH11*	♂, 59	Aneurysm (AAo, 46mm, 58y), BAV, PFO	PP, malar hypoplasia, cutaneous hyperextensibility	?		No relatives clinically affected
38	*NOTCH*	♂, 77	An (AAo and AoA, 85mm, 77y)	None	?		*De novo* (inferred, mosaic) No relatives clinically affected
39	*PRKG1*	♂, 52	Dis (subclavian-, iliac- and brachiocephalic artery, 42y), borderline an (AAo, 40mm, 52y)	SS	?		No relatives clinically affected
40	*SLC2A10*	♀, 15	Arterial tortuosity (aorta, pulmonary artery, carotid arteries), MI, ASD	PP, hypermobile fingers, hypermobility, thumb sign +, clinodactyly, hypertelorism, periorbital fullness,	?		No relatives clinically affected
41	*SLC2A10*	♂, 0	An (AoR, 17mm, Z-score +3.3, 5 months), PFO, abnormal course AoA and pulmonary vessels	Arachnodactyly, abnormal thumb position, downslanting, hypertelorism, HAP, retrognathia diaphragmatic hernia	HE HE	F M	Clinically not affected Clinically not affected
42	*SMAD3*	♀, 62	Dis (A, 60y), MI	PP, early onset arthrosis, myopia -2.5/-4 dpt	?	F	An (AA, deceased, 67y)
43	*SMAD3*	♂, 68	An (thoracic aorta)	Tall stature, PE, scoliosis, early onset arthrosis, mild myopia	+	D	Tall stature, arachnodactyly
44	*SMAD3*	♀, 37	Dis (coronary artery, 32y), VSD	Brachydactyly type E, hypertelorism, prominent venous pattern, varicose veins, recurrent hematomas, myopia -6 dpt, IH, UH	? ?	M MGF	SUD (cause unknown, 50y) SUD (cause unknown, 51y)
45	*SMAD3*	♀, 76	Dis (B, 63y), An (AoA, 60mm, 70y)	Arthralgia, genu valgum, hypermobility, IH	? + + +	So So GSo GDa	Dis (aorta, deceased, 44y) Skeletal features fitting SMAD3 Borderline An (AoR, 40) Clinically not affected
46	*SMAD3*	♂, 17	None	Scoliosis, PE, flat cornea	+ ? ?	F PA PGM	An (cerebral, 49y), PC SUD (anamnestic aneurysm AA, 40y) SUD (anamnestic aneurysm AA, 60y)
47	*SMAD3*	♀, 51	Dis (A, B, 51y)	Scoliosis, arthralgia, early onset arthrosis	+	So	Clinically not affected
48	*SMAD3*	♀, 40	Borderline an (AoR, 40y), MVP, MI	Wrist and thumb sign +, SS	+ ? ?	F PGM PF	Dis (A, 57y), aneurysm (aorta, 40y), HT Dis (thoracic aorta, 71y) Several relatives with SUD (cause unknown)
49	*TGFB2*	♀, 19	None	Patellofemoral pain syndrome, wrist sign +, BS 7/9, downslanting, varicose veins	- -	M B	Clinically not affected Clinically not affected
50	*TGFB2*	♂, 39	An (AoR, 55mm, 25y), MVP	Scoliosis, PD, wrist and thumb sign +, hypermobility, recurrent hematomas in iliopsoas muscle, dural ectasia	?		No relatives clinically affected

51	*TGFB2*	♂, 0	None	Arachnodactyly, joint contractures, retrognathia	+ +	F PA	No clinical information available Dis (thoracic aorta)
52[d]	*TGFB2*	♂, 32	An (AoR, 44mm, 32y)	PC, PP, arachnodactyly, HAP, dolichocephaly, enophthalmos, malar hypoplasia, crowding, myopia -6.5 dpt, pneumothorax	 - -	 F B	*De novo*[e] An (AAo, 52mm, 65y), BAV PD, PP, myopia
53	*TGFB3*	♂, 43	None	Increased AHR, PD, thumb sign +, BS 6/9	+ +	Si So	Clinical features of connective tissue disorder Clinical features of connective tissue disorder
54	*TGFB3*	♂, 59	AVI (25y), An (AoR, 46mm, 25y; AoR, 55mm, AAo 48mm, 57y)	PP, HAP, downslanting, UH	-	So	Clinically not affected
55	*TGFBR1*	♂, 56	Dis (A and B, 56y)	Scoliosis, PE, dolichocephaly, enophthalmos, malar hypoplasia	+	M	Clinically not affected
56	*TGFBR1*	♂, 33	An (AoR, 43mm, 31y)	SS, dural ectasia	+ + ?	M MA MGF	An (AoR, 44mm, AAo, 44mm, 58y) An (thoracic aorta, 55y) SUD (cause unknown, 64y)
57	*TGFBR1*	♂, 16	Dis (thoracic aorta, deceased, 16y)	PE, tall stature, scoliosis, arachnodactyly			*De novo*
58	*TGFBR2*	♂, 14	An (AoR, 40mm, Z score +4.3, 14y), VSD, DCRV	None	+	F	An (AoR, 42mm, 52y)
59	*TGFBR2*	♂, 15	None	PD, hyperkyphosis, arthralgia, myopia -3 dpt			NA
60	*TGFBR*	♀, 16	An (AoR, 44mm, 16y), MVP	PP, arachnodactyly, hypermobility, luxations of hips and knees, bifid uvula, hypertelorism, blue sclerae			NA

AA, abdominal aortic; AAo, ascending aorta; AHR, arm / height ratio; An, aneurysm; AoA, aortic arch; AoR, aortic root; ASD, atrial septal defect; AVI, aortic valve insufficiency; B, brother; BAV, bicuspid aortic valve; BS, Beighton score; CVD, cardiovascular disease; D, daughter; DCRV, double chambered right ventricle; Dis, dissection; dpt, dioptre; EL, ectopia lentis; F, father; GDa, granddaughter; GSo, grandson; HAP, highly arched palate; HE, heterozygous carrier; HT, hypertension; IH, inguinal hernia; M, mother;MF, maternal family; MFS, Marfan syndrome;MGF, maternal grandfather; MI, mitral valve insufficiency; MU, maternal uncle;MVP,mitral valve prolapse; N, nephew; NA, no further information available; PA, paternal aunt; PC, pectus carinatum; PCo, paternal cousin; PD, pectus deformity;PDA, patent ductus arteriosus; PE, pectus excavatum; PF, paternal family; PFO, patent foramen ovale; PGF, paternal grandfather; PGM, paternal grandmother; PP, pes plani; PU, paternal uncle; RD, retinal detachment; Rup, rupture; SD, standard deviation; Si, sister; Sib, sibling; So, son; SS, skin striae; SUD, sudden death; TI, tricuspid valve insufficiency; UH, umbilical hernia; VSD, ventricular septal defect

[a]Age (in years) at DNA diagnostics.
[b]Low-grademosaicism detected by NGS analysis in the father of the index patient.
[c]This family is recently described in literature (Overwater & Houweling, 2017).
[d]A variant of unknown significance was identified in these patients as well (Supporting Information Table S1).
[e]Paternity and maternity not confirmed.
+ variant present
− variant absent
? unknown

Table 3 Summary of the genetic features of six patients with a pathogenic or likely pathogenic CNV

Patient	Gender, Age[a]	Involved gene, exon(s) based on XHMM analysis	Loss/ gain	Protein change	Effect	Confirmed CNV	Validation technique	Variant classification
61	M, 66	*MYLK*; exon 17 and 18	Loss	Isoform 1 (NM_053025.3): p.(Asn798Leufs*13) Isoform 5 (smooth-muscle cell specific): p.(0)	Frameshift (NMD expected) Loss of initiation codon (no protein expected)	*MYLK*, deletion exon 17 and 18[b]	MLPA	5
62	M, 36	*PRKG1*; exon 3[c]	Loss	p.(Asp145_Thr183delinsAla)	*in-frame* deletion-insertion	*PRKG1*, deletion exon 3[c]	MLPA	4
63	M, 31	*SMAD3*; exon 6[d]	Loss	p.(Asp220_Ile290del)	*in-frame* deletion	*SMAD3*, deletion exon 6[d]	MLPA	5
64	M, 17	*TGFB2*; exons 4 – 7[e]	Loss	p.(Ile199_Arg390del)	*in-frame* deletion	*TGFB2*, deletion exons 4-7[e]	MLPA	5
65	F, 0	Duplication *NOTCH1*; whole gene[f]	Gain	NA	NA	unbalanced translocation: 46,XX,der(7)t (7;9)(p22.3;q33.3)[f]	SNP array and karyotyping	5
66	M, 0	Deletion *SCARF2*; whole gene[g]	Loss	NA	NA	22q11.2 deletion: arr[hg19] 22q11.2(207796 45_20792061)x1[g]	SNP array	5

CNV, copy number variation; MLPA, multiplex ligation-dependent probe; NA, not applicable; NMD, nonsense mediated mRNA decay; XHMM, eXome hidden Markov model.

[a]Age (in years) at DNA diagnostics.
[b]HGVS nomenclature:NC_000003.11(NM_053025.3)(MYLK): c.(2390+1_2391-1)_(3448+1_3449-1)del.
[c]HGVS nomenclature:NC_000010.10(NM_001098512.2)(PRKG1): c.(433+1_434-1)_(547+1_548-1)del.
[d]HGVS nomenclature:NC_000015.9(NM_005902.3)(SMAD3): c.(658+1_659-1)_(871+1_872-1)del.
[e]HGVS nomenclature:NC_000001.10(NM_001135599.2)(TGFB2): c.(594+1_595-1)_(1170+1_1171-1)del.
[f]ISCN nomenclature after additional SNP array and karyotyping.
[g]ISCN nomenclature after additional SNP array

In patient 62, a deletion of one exon of *PRKG1* was detected (NM_001098512.2: c.(433+1_434-1)_(547+1_548-1)del). This deletion is predicted to lead to an *in-frame* deletion of 39 amino acids and the insertion of an Alanine residue and encompasses a large part of the high-affinity cGMP-binding domain of the PRKG1 protein including Arginine177. A recurrent substitution of this arginine for glutamine has been reported in patients with H-TAD and shown to have a gain- of-function effect[28]. At the age of 35 years this male patient was diagnosed with an aortic root dilatation, a type A dissection, aortic valve insufficiency, and dilated cardiomyopathy. He was treated surgically (Bentall procedure). His skin showed stretch marks on the shoulders and chest. Medical history, ophthalmological evaluation and physical examination did not reveal any other features of a connective tissue disorder. A cardiomyopathy gene panel analysis (50 genes) did not result in the identification of a genetic cause for his dilated cardiomyopathy. Family history showed no clinically affected relatives. No relatives were available for cardiologic evaluation and DNA diagnostics.

In patient 63, a deletion of one exon in *SMAD3*, predicted to result in an *in-frame* deletion of part of the MH2 domain, was found (NM_005902.3: c.(658+1_659-1)_(871+1_872-1)del). This male patient was followed up from the age of eight years, after his father, who was diagnosed with a chronic dissection of the ascending aorta at the age of 33 years, suddenly died at the age of 37 years. The paternal grandmother died at the age of 39 years, possibly caused by an aortic dissection as well. The patient was diagnosed with an aortic root dilatation with a maximal diameter of 48 mm and a dilated left coronary artery at the age of 30 years. He was treated surgically (David procedure). Physical examination revealed pes plani, a prominent venous pattern on the chest and arms, and several dysmorphic facial features including dolichocephaly, hypertelorism and retrognathia. He had no signs of earlyonset osteoarthritis.

In patient 64, a four-exon deletion was detected in the *TGFB2* gene (NM_001135599.2: c.(594+1_595-1)_(1170+1_1171-1)del). This deletion is predicted to result in an *in-frame* deletion of a large part of the TGFB2 protein. This 17-year-old male patient was under regular cardiologic surveillance because of TAAD in his father and paternal grandfather. At the age of 17 years cardiologic evaluation revealed an aortic root dilatation of 39 mm (Z-score +3.28). Moreover, he had inguinal hernia repair at the age of one year, recurrent patellar dislocation, an asymmetric pectus deformity, and mild dysmorphic facial features including a long face, downslanting palpebral fissures and a highly arched palate. The intragenic *TGFB2* deletion was also present in his clinically affected father (clinical features include aortic root aneurysm requiring surgery at

age 31 and aortic dissection at age 46) and his 11-year-old sister (features consisted of pectus deformity and highly arched palate and mild myopia). The phenotypes of all family members will be described in more detail elsewhere (Vliegenthart *et al.*, manuscript in preparation). All intragenic deletions were confirmed by MLPA analysis (Supporting Information Figure S1).

In patients 65 and 66, XHMM findings were suggestive of a larger chromosomal abnormality.

In patient 65, a duplication of the entire *NOTCH1* gene was detected. *COL5A1* and *ADAMTSL2*, which are located in the same chromosomal region (9q) and are present in our NGS platform, were also duplicated in this newborn female patient who presented after birth with several dysmorphic features. Facial features included frontal bossing, deep-set eyes, low set ears with overfolded helices and a crumpled left ear with a preauricular tag, micrognathia and a small mouth. In addition, flexion contractures of elbows, wrists and knees and striking arachnodactyly were noticed. Based on these features, she was initially suspected to have neonatal Marfan syndrome or Beals syndrome. Because XHMM analysis indicated a large 9q duplication, an SNP array was performed. A copy-number gain at 9q33.3-q34.43 (11.8Mb; hg19; chr9:129172353-141020389) and a copy-number loss at 7p22.3 (2Mb; hg19; chr7:43360-2067625) were found. Subsequent karyotyping revealed an unbalanced translocation 46,XX,der(7)t(7;9)(p22.3;q33.3). Parental cytogenetic studies showed that her father carried a balanced reciprocal translocation; 46,XY,t(7;9)(p22.3;q33.3). Results of the array and karyotyping are shown in Figure 2A. In the literature, overlapping phenotypic manifestations such as similar craniofacial features, joint contractures and arachnodactyly have been described in the 9q duplication syndrome [29]. During follow-up, she was treated for bleeding esophageal varices probably caused by portal vein thrombosis, which have not been described in patients with a 9q duplication syndrome and/or 7p22.3 deletion previously.

Finally, a deletion of the entire *SCARF2* gene, located at 22q11, was detected in patient 66. This newborn male patient presented with severe perinatal problems, including asphyxia and the need for resuscitation, after an uncomplicated pregnancy. Furthermore, initially a connective tissue disorder was suspected based on the presence of a relative dilatation of the aortic root in relation to the body surface area (16 mm, Z-score +3) and a strangulated inguinal hernia. Physical examination revealed unilateral postaxial polydactyly without any other dysmorphic features. Simultaneous

analysis of the NGS H-TAD gene panel and SNP array revealed that the heterozygous deletion of *SCARF2* was part of a 22q11.2 deletion (i.e., DiGeorge syndrome) (3.2Mb; hg19; chr22:20779645_20792061). A normal male karyotype (46,XY) was seen. Parental fluorescence in situ hybridization (FISH) revealed that his mother also carried the 22q11.2 deletion (ish del(22)(q11.2q11.2)(HIRA-)). Results of array and FISH are shown in Figure 2B. Except for delayed motor and speech development at childhood and complaints of fatigue and recurrent infections, his mother had no medical problems. Cardiac ultrasound showed no abnormalities. Most clinical features of the index patient, including inguinal hernia and postaxial polydactyly, were consistent with the established diagnosis. During follow-up the relative dilatation of the aortic diameter was normalized.

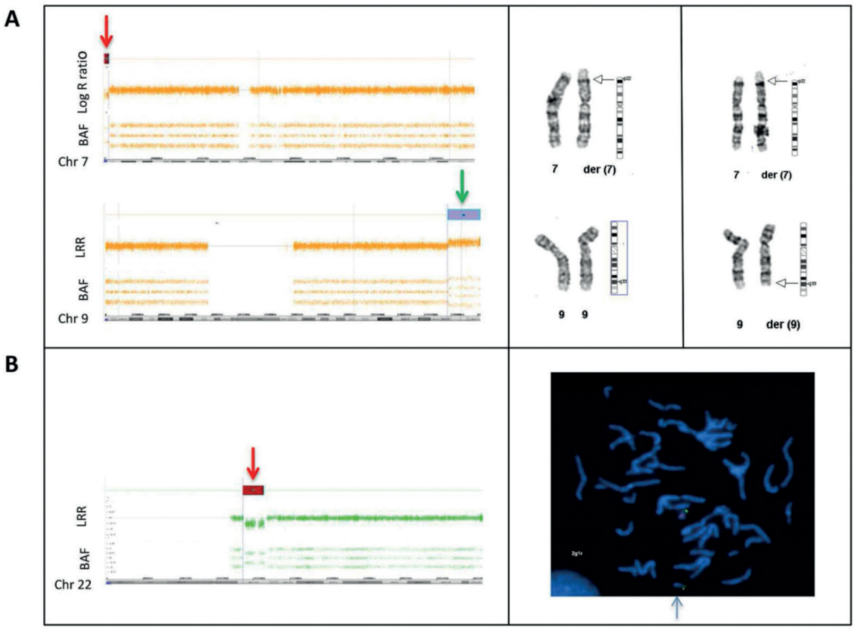

Figure 2 Further characterization of XHMM results by additional (cyto-) genetic testing
BAF, B allele frequency; Chr, chromosome; der, derivate chromosome; LLR, log R ratio; FISH, fluorescence in situ hybridization.

A SNP array profile of chromosomes 7 and 9 are shown on the left. The top plot of each image shows the LRR, which provides an estimation of the copy number for each marker aligned to its chromosomal position. The bottom plot of each image shows the BAF for each SNP aligned to its chromosomal position. SNP array analysis revealed a terminal copy number loss at 7p22.3 (2Mb; GRCh37; chr7:43360-2067625) indicated with a red arrow and a terminal copy number gain at 9q33.3-q34.43 (11.8Mb; GRCh37; chr9:129172353-141020389) indicated with a green arrow.

Chromosomes 7 and 9 from the index (left) with the unbalanced translocation and the father (right) carrying the balanced translocation are shown on the right. The breakpoints of the reciprocal translocation are indicated with an arrow. The index has the derivative chromosome 7 lacking a short segment from the short arm of chromosome 7 that is replaced by an extra copy of a terminal segment of chromosome 9q. The father has two derivative chromosomes 7 and 9 each carrying a segment of the other chromosome.

B SNP array profile of chromosome 22 is shown on the left. SNP array analysis revealed a copy number loss at 22q11.2 (3.2Mb; GRCh37; chr22:20779645_20792061) indicated with a red arrow.

The results of metaphase FISH on blood from the mother is presented on the right. The 22q11.2 region is recognized by the HIRA probe, producing a red signal. The green signal is from the ARSA probe hybridizing with the ARSA gene on chromosome band 22q13.33. The 22q11.2 deletion is indicated by a blue arrow. Metaphase FISH analysis revealed that the mother is also a carrier of the 22q11.2 deletion (ish del(22)(q11.2q11.2)(HIRA-)).

Discussion

This study provides the results of the molecular and clinical findings in the largest cohort of patients suspected of H-TAD reported in the literature to date. In addition, this is the first report describing CNV analyses of 21 H-TAD- associated genes using variant-calling analysis combined with XHMM analysis. In this cohort of 810 patients, a pathogenic or likely pathogenic variant was identified in 66 patients (8.1%). Overall, we identified a relatively low number of pathogenic or likely pathogenic variants in our H-TAD cohort compared to previous studies that identified mutations in 10.3% to 35.5%. [12, 30-34]. This wide range is likely to be explained by differences in clinical and demographic characteristics of the study populations and different inclusion criteria used for genetic testing. In general, DNA testing in the Netherlands is increasingly offered at a lower threshold to TAAD patients (e.g., not only to very young patients or patients with a positive family history for H-TAD) which may explain the relatively low mutation detection yield.

Using routine NGS analysis (variant-calling analysis) pathogenic or likely pathogenic variants were identified in *FBN1*, *ACTA2*, *SMAD3*, *COL3A1*, *TGFB2, TGFBR1*, *TGFBR2, FBN2, MYH11, TGFB3*, *SLC2A10*, PRKG1 and *NOTCH1*. As expected, most of the pathogenic and likely pathogenic variants were detected in *FBN1* (n=18, 30%). Of these, at least 14 (78%) fulfilled the revised Marfan criteria. However, the proportion of pathogenic *FBN1* and *COL3A1* variants in this cohort is biased because single-gene analysis of these two genes is still offered in our institute and variants in these genes detected using single-gene analysis were not included in this study. Therefore, it is likely that in patients with a highly suggestive phenotype of vascular Ehlers Danlos syndrome, single-gene analysis of *COL3A1* was requested instead of NGS panel analysis. This might explain the high proportion of *COL3A1* variants predicted to result in haploinsufficiency detected in this study (3 of 6 = 50%, compared with approximately 4% of nonsense/frameshift variants currently reported in the *COL3A1* LVOD database; https://eds.gene.le.ac.uk/home.php?select_db=COL3A1), as the phenotype in patients with COL3A1 haploinsufficiency is often confined to vascular events.

Of the pathogenic and likely pathogenic variants identified, 37 (67%) have not been described previously. None of these variants were identified more than once in our patient cohort. This emphasizes the extreme allelic heterogeneity of H-TAD- related disorders. Young age at diagnosis, a positive family history, and presence of syndromic features were shown to be the strongest predictors for the identification of a disease-

causing variant in the literature (p = 0.001 to 0.01) [12]. The observation that the mean age at DNA testing in the group of patients with a pathogenic or likely pathogenic variant was 11 years lower than the mean age in the groups without a pathogenic or likely pathogenic variant is in line with this. However, 10 of the 66 patients with a pathogenic or likely pathogenic variant were over the age of 60 years at the time of DNA testing (15.2%). Of these, three patients (30%) had a negative family history for aortic disease, sudden death <45 years, or systemic features of a connective tissue disorder. These observations underscore the reduced and age-dependent penetrance with a high degree of clinical heterogeneity in H-TAD. In five patients, with an identified pathogenic or likely pathogenic variant, DNA testing of both parents suggested a *de novo* occurrence, while in one case a *de novo* occurrence was inferred as the variant was detected in mosaic status. This was in line with the negative family history for aortic disease in these families.

Of the 66 pathogenic or likely pathogenic variants, six were CNVs detected by XHMM analysis. These aberrations account for an incremental yield of 9.1% of the identified pathogenic or likely pathogenic variants, underscoring the relevance of adding a technique to identify CNVs in TAAD patients. The CNVs included (multi-) exon deletions in *MYLK*, *PRKG1*, *SMAD3,* and *TGFB2*. To the best of our knowledge, intragenic (multi-)exon deletions have not been reported in these genes before. The clinical features of the patients with these (multi-)exon deletions did not differ notably from the known phenotypic manifestations related to variants in these genes. Moreover, a large duplication including the whole *NOTCH1* gene and a large deletion encompassing *SCARF2* were detected by XHMM analysis. These aberrations were part of an unbalanced translocation (46,XX,der(7)t(7;9)(p22.3;q33.3)) and a 22q11.2 deletion (22q11.2(20779645_20792061)x1), respectively and were classified as the cause of the clinical features of the patients.

The results of this study underline the importance of CNV analysis using a bioinformatics tool such as XHMM in the clinical diagnostic care for TAAD patients. As CNV analysis is often not routinely performed for most genes included in this NGS platform, these CNVs would not have been detected by regular genetic analysis. Four of the six detected CNVs in this study were small intragenic deletions (two single-exon deletions, one 2-exon and one 4-exon deletion). These are generally not detected by routine CGH or SNP array analysis. This highlights the importance of using a CNV detection tool which allows detection of CNVs with (small) single-exon resolution. Based on the results of this study, single-exon-sensitive deletion/duplication analysis on a routine basis should be recommended in patients suspected of H-TAD.

Conclusion

In 66 of 810 (8.1%) patients suspected of H-TAD, a pathogenic or likely pathogenic variant was identified using our NGS gene panel in combination with XHMM analysis. Six of these 66 pathogenic or likely pathogenic variants (9.1%) were a CNV, not detectable by routine NGS analysis. This study is the first to describe the incremental yield of CNV analysis in patients suspected of H-TAD. Our study underscores the importance of CNV analysis using a bioinformatics tool such as XHMM in the clinical diagnostic care for H-TAD patients.

Online Supplement

For the online supplement, containing supporting information please access through the QR-code below.

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NGS panel analysis in 24 ectopia lentis patients; a clinically relevant test with a high diagnostic yield

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Abstract

Background: Several genetic causes of ectopia lentis (EL), with or without systemic features, are known. The differentiation between syndromic and isolated EL is crucial for further treatment, surveillance and counseling of patients and their relatives. Next generation sequencing (NGS) is a powerful tool enabling the simultaneous, highly-sensitive analysis of multiple target genes. **Objective:** The aim of this study was to evaluate the diagnostic yield of our NGS panel in EL patients. Furthermore, we provide an overview of currently described mutations in *ADAMTSL4*, the main gene involved in isolated EL. **Methods:** A NGS gene panel was analysed in 24 patients with EL. **Results:** A genetic diagnosis was confirmed in 16 patients (67%). Of these, four (25%) had a heterozygous *FBN1* mutation, 12 (75%) were homozygous or compound heterozygous for *ADAMTSL4* mutations. The known European *ADAMTSL4* founder mutation c.767_786del was most frequently detected. **Conclusion:** The diagnostic yield of our NGS panel was high. Causative mutations were exclusively identified in *ADAMTSL4* and *FBN1*. With this approach the risk of misdiagnosis or delayed diagnosis can be reduced. The value and clinical implications of establishing a genetic diagnosis in patients with EL is corroborated by the description of two patients with an unexpected underlying genetic condition.

Introduction

Ectopia lentis (EL) is a rare condition characterized by displacement or malposition of the lens caused by a partial or complete disruption of the zonular fibers. Currently several genetic causes have been identified underlying EL with systemic associations (i.e. syndromic EL) and/or EL without systemic associations (i.e. isolated EL). Both syndromic and isolated EL are genetically heterogeneous. The main syndromic forms of EL include Marfan syndrome (MFS, *FBN1* gene; MIM# 154700), homocystinuria (HCU, *CBS* gene*;* MIM# 236200), Weill-Marchesani syndrome (WMS, *ADAMTS10, FBN1* and *LTBP2* gene*;* MIM# 277600, MIM# 608328 and MIM# 614819), Weill-Marchesani-like syndrome (*ADAMTS17*; MIM# 613195) and sulfite oxidase deficiency syndrome (SOD, *SUOX* gene*;* MIM# 272300). In addition to Weill-Marchesani syndrome, the *LTBP2* gene is also associated with a complex rare recessive ocular disorder, including EL, microspherophakia, megalocornea and/or secondary glaucoma. Moreover, some of the reported *LTBP2* patients had marfanoid skeletal findings (MIM# 251750). Isolated EL (et pupillae) can be inherited in both an autosomal recessive (*ADAMTSL4* gene; MIM# 610113 and MIM# 225200) and an autosomal dominant manner (*FBN1* gene; MIM# 129600). Most of the genes associated with EL are reported to be expressed in the zonular fibers (*ADAMTS10*, *FBN1* and *LTBP2*) and the ciliary muscle (*ADAMTSL4*) of the eye. The zonular fibers position the ocular lens in the center of the optic path and conduct contraction of the ciliary muscle. The zonular fibers contain microfibrils, mainly composed of fibrillin-1 molecules. It is hypothesized that ADAMTSL4, ADAMTS10, ADAMTS17 and LTBP2 are involved in the formation and/or maintenance of the zonular fibers (Figure 1).

Current literature suggests that (apparently) isolated EL caused by *FBN1* mutations may not be a distinct diagnosis, but a mild manifestation of a broader clinical spectrum of MFS with both inter- and intrafamilial variability and potentially associated with life-threatening aortic disease [7-9]. Since in some cases, the presenting clinical feature of syndromic EL may be the luxation of the lens (e.g. in homocystinuria and MFS), DNA testing (and metabolic screening) has become an indispensable diagnostic tool in making the distinction between syndromic and isolated EL [10,11]. Rapid simultaneous analysis of multiple genes with a high accuracy has become available with the introduction of multi-gene panel testing using next generation sequencing (NGS). This targeted approach has become widely available at relatively low costs and is incorporated into standard clinical practice at a rapid pace.

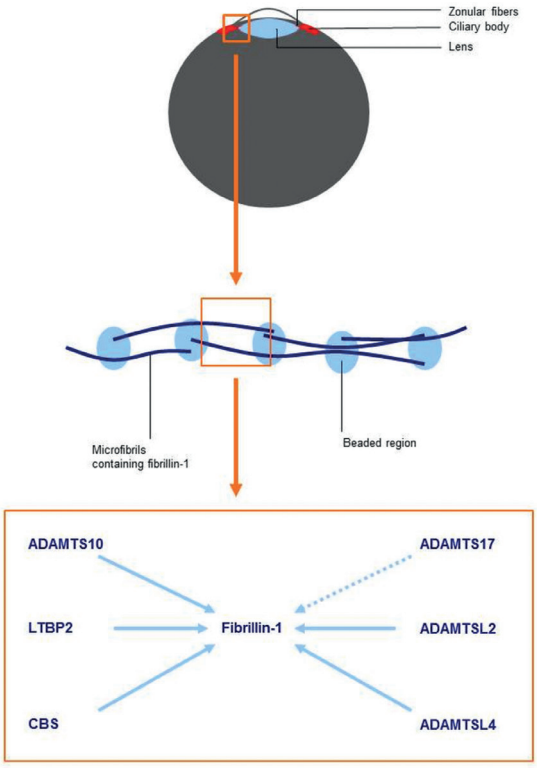

Figure 1 Schematic representation of the eye; the microfibrils in the zonular fibers and the proteins encoded by the genes associated with EL are depicted

ADAMTS10, ADAMTSL2, ADAMTSL4 and LTBP2 can bind either directly or indirectly to fibrillin-1 through a complex of other proteins [1-5]. The exact mechanism by which cystathionine beta synthase (CBS) deficiency results in EL remains unknown. Patients with CBS deficiency have elevated levels of homocysteine, since the CBS enzyme cannot convert homocysteine to cysteine. The resulting lack of cysteine has been suggested to interfere with fibrillin-1 deposition resulting in zonular fiber disruption [6]. The role of ADAMTS17 in relation to fibrillin-1 has not been defined yet and remains elusive [3], this is indicated with a dashed line.

Distinguishing isolated EL from syndromic EL has several clinical implications including the patients' prognosis, the options for surveillance and prevention of potentially life-threatening associated complications such as aortic dissection. In addition, molecular confirmation of the underlying genetic cause is essential in establishing the mode of inheritance and recurrence risk in relatives. Furthermore, knowledge of the genetic defect enables the patient to consider reproductive options and allows (presymptomatic) DNA testing in relatives.

We designed a connective tissue NGS platform enriched for a panel of the main genes involved in isolated EL and syndromic EL (*ADAMTSL4*, *FBN1*, *ADAMTS10* and *ADAMTS17*). In addition, we included *ADAMTSL2,* associated with Geleophysic Dysplasia (GD; MIM# 231050; a condition with a high degree of clinical overlap to WMS) [12]. *ADAMTSL2* was included to enable distinguishing between WMS and GD in patients suspected of these rare disorders, even though mutations in this gene have not been reported in EL patients. We present the diagnostic yield of our NGS gene panel in 24 patients referred for genetic analysis of EL. An enlarged gene panel was subsequently analysed in the patients with no detected genetic cause. Furthermore, we provide an overview of reported mutations in *ADAMTSL4*, the assumed main cause of isolated EL. Finally, the value of gene panel testing in EL is corroborated by two patients diagnosed with an unexpected underlying genetic condition of EL with important clinical implications.

Patients and Methods

Genetic data

In 24 index patients with EL DNA diagnostics was performed at the Department of clinical genetics at the VU University Medical Center (VUmc, Amsterdam, the Netherlands) from February 2013 to February 2015. The patients and/or their parents gave informed consent for genetic testing after counseling. The routine EL protocol included targeted NGS analysis of *ADAMTS10*, *ADAMTS17*, *ADAMTSL2*, *ADAMTSL4* and *FBN1* and MLPA analysis of the *FBN1* gene. Additionally, deletion/duplication analysis of all included genes was performed using XHMM, a bio-informatics read-depth based tool for the detection of copy number variations in NGS data (https://atgu.mgh.harvard.edu/xhmm/). In six patients where no pathogenic mutations were identified, whole exome sequencing (WES) was performed and an extended panel of genes including *LTBP2*, *THSD4* (*ADAMTSL6)*, *ADAMTS18*, *CBS*, *SUOX*, *PAX6* and *VSX2* was analysed (see supplemental data for detailed information regarding material and methods). Since this study was limited to the retrospective use of information previously collected in the course of regular patient care, review of the study protocol by our ethics committee was not required.

To obtain an overview of all reported *ADAMTSL4* mutations we used the LOVD Eye diseases database (https://grenada.lumc.nl/LOVD2/eye/home.php?select_db=ADAMTSL4) and PubMed. Our search strategy in PubMed was performed using

the terms "ectopia lentis" and "*ADAMTSL4*". Inclusion criteria were: studies describing *ADAMTSL4* mutations, English language and published before May 2017.

Clinical data

The clinical data of the 24 included EL patients were retrospectively collected after sending a standardized questionnaire to the referring physicians (all clinical geneticist or pediatricians). The clinical findings are reported as described by the physician who submitted the case for DNA diagnostics. The collected data comprise the available records of patient's medical- and family history, physical examination (including dysmorphologic features), ophthalmologic findings and additional investigations (e.g. genetic testing, metabolic screening and cardiological evaluation).

Results

Genetic data

In 16 out of the 24 EL patients (67%) pathogenic mutations were identified that confirm the molecular diagnosis (Table 1). Of these 16 patients, 12 (75%) (patients 1-12) had EL due to homozygous or compound heterozygous *ADAMTSL4* mutations, and 4 (25%) (patients 13-16) had EL caused by a heterozygous *FBN1* mutation. In one of the six patients who underwent the analysis of the extended gene panel, a heterozygous pathogenic mutation was found in *LTBP2* (patient 20). XHMM analysis on a subsequent targeted NGS platform in this patient failed to identify a copy number variant (CNV) in the second *LTBP2* allele. Since mutations in this gene inherit in an autosomal recessive manner, the molecular diagnosis could not be confirmed with this finding. No (presumably) pathogenic mutations were found in *ADAMTSL2, THSD4 (ADAMTSL6), ADAMTS10, ADAMTS17, ADAMTS18*, *CBS*, *PAX6*, *SUOX*, *VSX2* and no deletions or duplications were found with XHMM and MLPA testing.

The pathogenic *ADAMTSL4* mutations were homozygous in nine and compound heterozygous in three patients (Table 1). All identified *ADAMTSL4* mutations introduce a premature stop codon. The known European founder mutation c.767_786del [13] was homozygous in six EL patients and compound heterozygous in three patients. We found two mutations, c.1del p.(0?) and c.1937dup p.(Arg647Alafs*49), which have not been reported previously. A schematic overview of the previously published *ADAMTSL4* mutations, as well as the mutations identified in our study, is shown in Figure 2. Table 2 provides an overview of the four identified *FBN1* mutations in our study, including assessed or predicted effect at protein level, molecular properties and classification.

Table 1 Summary of the genetic features of the 24 EL patients

Patient	Gender, Age#	Origin	Involved gene	Zygosity	Nucleotide change(s)^	Protein change(s)^	Consanguinity	Family history
1	M, 19	Dutch	*ADAMTSL4*	HOM	c.767_786del	p.(Gln256Profs*38)	-	+[a]
2[b]	M, 29	Dutch	*ADAMTSL4*	HOM	c.767_786del	p.(Gln256Profs*38)	+	-
3	M, 0	Dutch	*ADAMTSL4*	HOM	c.767_786del	p.(Gln256Profs*38)	-	-
4	M, 1	Dutch	*ADAMTSL4*	HOM	c.767_786del	p.(Gln256Profs*38)	-	-
5	M, 0	Dutch	*ADAMTSL4*	HOM	c.767_786del	p.(Gln256Profs*38)	-	-
6	F, 56	French	*ADAMTSL4*	HOM	c.767_786del	p.(Gln256Profs*38)	-	-
7	M, 37	Dutch	*ADAMTSL4*	HOM	c.925C>T	p.(Arg309*)	-	+[c]
8	M, 4	Turkish	*ADAMTSL4*	HOM	c.1937dup	p. (Arg647Alafs*49)	+	+[d]
9	F, 7	Moroccan	*ADAMTSL4*	HOM	c.1del	p.(0?)	+	+[e]
10	F, 9	Dutch	*ADAMTSL4*	HET, HET	c.745del, c.767_786del	p. (Leu249Tyrfs*21), p. (Gln256Profs*38)	-	-
11[f]	F, 24	Dutch	*ADAMTSL4*	HET, HET	c.767_786del, c.2254C>T	p.(Gln256Profs*38), p.(Gln752*)	-	+[g]
12	F, 10	Dutch	*ADAMTSL4*	HET, HET	c.767_786del, c.2254C>T	p.(Gln256Profs*38), p.(Gln752*)	-	-
13	M, 33	Dutch	*FBN1*	HET	c.1766A>T	p.(Asn589Ile)	-	-[h]
14	M, 5	Dutch, Indonesian	*FBN1*	HET	c.4817-1G>A	p.Ile1607_Asp1648del	-	-[i]
15	F, 4	Dutch	*FBN1*	HET	c.1837+5G>A	p.Lys612_Ile614ins22	-	-
16[j]	F, 48	Dutch	*FBN1*	HET	c.1633C>T	p.(Arg545Cys)	-	-
17[k,j]	M, 69	Dutch	Unknown	NA	NA	NA	-	-
18[l]	M, 25	Surinamese	Unknown	NA	NA	NA	-	-
19	F, 35	Dutch	Unknown	NA	NA	NA	-	-
20[m]	M, 60	Dutch	L*TBP2?*	HET	c.2377C>T	p.(Gln793*)	-	-
21[b]	F, 12	Dutch	Unknown	NA	NA	NA	-	-
22[k]	M, 45	Dutch	Unknown	NA	NA	NA	-	-
23	M, 3	Moroccan	Unknown	NA	NA	NA	+	-
24	F, 21	Dutch	Unknown	NA	NA	NA	-	-

EL, ectopia lentis; HET, heterozygous; HOM, homozygous; NA, not applicable
Age (in years) at DNA diagnostics. ^Ref. seq. : *ADAMTSL4*: NM_019032.5; *FBN1*: NM_000138.4; *LTBP2*: NM_000428.2
[a] One second-degree relative (paternal grandfather) with EL and glaucoma. No medical records available.
[b] An array CGH was performed in addition to the EL gene panel. No pathogenic copy number variations were found.
[c] Monozygotic twin brother with both EL and congenital unilateral hearing loss.
[d] Dizygotic twin brother with bilateral EL, also homozygous for the c.1937dup in *ADAMTSL4*. Possibly also other family members with EL, no medical records available.
[e] Brother is also said to be affected. No medical records available and no DNA diagnostics performed.
[f] Sequencing of *TGFBR1*, *TGFBR2*, TWIST, *FGFR1*, *FGFR2* and *FGFR3*, and SNP array and karyotype analysis did not reveal any pathogenic abnormalities.
[g] Monozygotic twin sister with both EL and craniosynostosis.
[h] The brother of the father of the index suddenly died at the age of 45 years. The mother of the index was not a carrier of the *FBN1* mutation, the father refused DNA diagnostics.
[i] DNA diagnostics in the parents of the index revealed that the *FBN1* mutation occurred de novo.
[j] DNA diagnostics of *POLG* and mitochondrial DNA was performed in addition to the EL gene panel. No pathogenic mutations were found.
[k] DNA diagnostics of *LTBP2* was performed by Sanger sequencing, in addition to the EL gene panel. No pathogenic mutations were found.
[l] Not enough DNA available for WES.
[m] Extended gene panel analysis on WES data identified a single pathogenic heterozygous *LTBP2* mutation in this patient. Additional CNV detection on a targeted analysis did not reveal an additional *LTBP2* aberration.

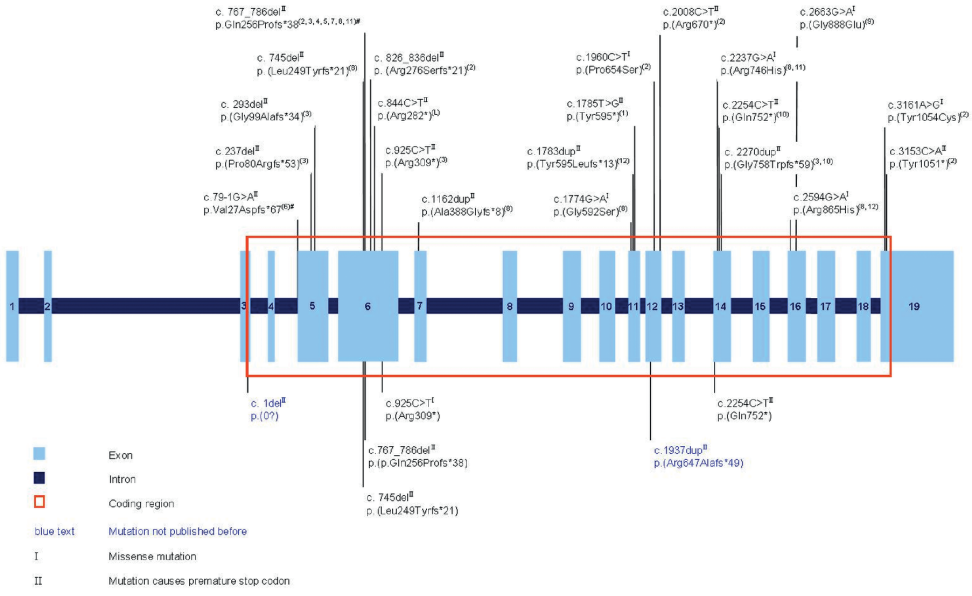

Figure 2 Schematic representation of *ADAMTSL4*, isoform 1 (NM_019032.5)

Lines above the schematic representation of the gene indicate the approximate position of mutations found in literature; lines below indicate the mutations found in our study.
[1]: [14]; [2]: [15]; [3]: [8]; [4]: [16]; [5]: [17]; [6]: [18]; [7]: [13]; [8]: [19]; [9]: [20]; [10]: [21], [11]: [22]; [12]: [23];
L, LOVD database, (www.lovd.nl); # In some publications this mutation is not described according to the recommendations of the Human Genome Variation Society (HGVS; http://www.hgvs.org/mutnomen/), the nomenclature was corrected in these cases.

Table 2 Overview of identified *FBN1* mutations in our EL patient cohort (NM_000138.4)

Patient	*FBN1* mutation	Protein	Expected effect	Domain	Conservation	SIFT, PP2, MT	Aortic involvement reported in literature	Pathogenicity
13	c.1766A>T	p.(Asn589Ile)	Dominant negative	cb-EGF-like	Up to fruit fly and among all FBN1 cb-EGF -like domains	++, ++, ++	No[a]	Likely pathogenic
14	c.4817-1G>A	p.Ile1607_Asp1648del[b]	In frame deletion	NA	NA	NA	No[c]	Pathogenic
15	c.1837+5G>A	p.Lys612_Ile614ins22[b]	In frame insertion	NA	NA	NA	No[d]	Pathogenic
16	c.1633C>T	p.(Arg545Cys)[e]	Disturbance disulphide bridges forming due to extra Cys[5]	cb-EGF-like	Up to zebrafish	++, ++, ++	Yes[f]	Pathogenic

cb-EGF-like, calcium binding EGF-like domain; EGF-like, epidermal growth factor-like domain; EL, ectopia lentis; MFS, Marfan syndrome; MT, Mutation Taster variant classification tool; NA, not applicable; PP2, PolyPhen-2 variant classification tool; SIFT, SIFT variant classification tool; ++ predicted as (likely) pathogenic. To our knowledge none of these mutations have been identified in population databases (dbSNP and ExAC).

[a] Another *FBN1* mutation at the same position (c.1766A>G) has been described previously by Pepe et al. [7] in a family with EL and several systemic features, but without aortic dilatation or dissection. Based on the available data this family did not fulfill the revised Ghent criteria for MFS.

[b] The predicted effect of the mutation was confirmed by sequencing analysis of cDNA in other patients with the same variants (clinical data not available, unpublished data).

[c] No aortic involvement has previously been reported to be associated with this mutation in literature. Rommel et al. [24] described this mutation (indicated as IVS38-1G>A) in a 7-year-old girl with EL, mitral valve prolapse and typical skeletal features. Biggin et al. [25] identified this mutation in a 14-year-old patient with EL, striae atrophica and several skeletal features, no cardiovascular involvement was described. However, patient 14, described in our study, fulfilled the revised Ghent criteria (EL and aortic root dilation).

[d] Biggin et al. [25] described this mutation in a patient with ectopia lentis and mild skeletal manifestations, no cardiovascular involvement was described.

[e] Each cb-EGF-like domain of *FBN1* contains six cysteine residues, paired to form three disulphide bridges. Introduction of a cysteine in a cb-EGF-like domain affects the pairing of the cysteines and the forming of the disulphide bridges within the domain. These type of mutations are therefore generally considered to be pathogenic.

[f] This specific mutation has been described before in patients with MFS [26].

Clinical data

The clinical features of the 24 EL patients are summarized in Table 3. Median age at diagnosis of EL was three years; mean 12, range 0-69 years (for one patient the age at diagnosis was unknown). In 22 out of 24 patients (92%) the index patient had bilateral EL and surgery was performed in 10 out of 23 patients (43%, for one patient this information was not available). Ocular findings that were reported in at least two patients were refractive errors (n=22, 92%), cataract (n=6, 25%), amblyopia (n=5, 21%), ectopia pupillae (n=5, 21%), iridodonesis (n=4, 17%), coloboma of the iris or lens (n=3, 12.5%), flat cornea (n=3, 12.5%) and increased intraocular pressure (n=2, 8.3%). Cardiological evaluation was performed in 17 patients (71%) to rule out cardiac manifestations of a potential underlying connective tissue disorder (e.g. aortic dilatation, mitral valve prolapse, calcification of the mitral valve). The results of the cardiological evaluation, as well as the presence of skeletal and other systemic features in the three main groups (EL caused by *ADAMTSL4* (n=12), EL caused by *FBN1* (n=4) and EL with unknown cause (n=8)), are summarized in Table 3. Metabolic screening (for homocystinuria and/or sulfite oxidase deficiency) was performed in 18 out of 24 patients (75%). No abnormalities were found.

The median age at diagnosis of EL in the *ADAMTSL4* patients was three years (mean 3, range 0-9 years; based on the 11 index patients with available data). In the EL patients with a *FBN1* mutation the median age at diagnosis of EL was four years (mean 10, range 0-33 years). In the group of EL patients with an unknown cause the median age at diagnosis was 18 years (mean 26, range 0-69 years). Ectopia Pupillae (EP) was reported in five out of 12 *ADAMTSL4* patients (42%) and in none of the patients with a *FBN1* mutation or with an unknown cause.

In seven out of 12 patients (58%) with *ADAMTSL4* mutations cardiological evaluation was performed. No abnormalities were identified except for minimal mitral valve prolapse in patient 12 (Table 3). Based on the revised Ghent criteria none of the patients with *ADAMTSL4*-associated EL fulfilled the criteria for MFS [9]. According to those criteria, two out of four patients (50%, patients 14 and 16, Table 2 and 3) of the EL patients with a *FBN1* mutation fulfilled the criteria for MFS. In patient 14 the presence of an aortic root dilatation (Z score ≥2) and EL allowed the unequivocal diagnosis of MFS. In patient 16 this diagnosis could be established based on the combination of EL and a *FBN1* mutation previously associated with aortic disease. Patients 13 and 15 were both diagnosed with isolated ophthalmological abnormalities. The *FBN1* mutations identified in patients 13 and 15 have not been reported in literature in

Table 3 Summary of clinical features of the 24 EL patients

	Patient	Gender, Age (y)	Age at EL (y)	Unilateral/ bilateral EL	Surgery for EL	Other ocular features	Cardiovascular	Other
ADAMTSL4	1	M, 19	9	Bilateral	-	Membrana pupillaris persistens	-	Facial features (3/5)[a]
	2	M, 29	0	Bilateral	+	Amblyopia, cataract, EP, increased intraocular pressure	-	Facial features (1/5)[b]
	3	M, 0	0	Bilateral	-	EP, iridodonesis	NP	-
	4	M, 1	1	Bilateral	-	Iridodonesis	NP	-
	5	M, 0	0	Bilateral	-	Coloboma iris, EP, increased intraocular pressure, iridodonesis	NP	-
	6	F, 56	<20	Bilateral	+	Amblyopia, coloboma iris, EP, retinal detachment	NP	-
	7	M, 37	4	Bilateral	+	Cataract	-	AHR 1.06, unilateral hearing loss, facial features (2/5)[c]
	8	M, 4	2	Bilateral	-		NP	-
	9	F, 7	±6	Bilateral	+	Amblyopia, anisometropia, EP	-	-
	10	F, 9	5	Bilateral	-		-	Pes planus, pectus carinatum
	11	F, 24	3	Bilateral	+	Cataract, iridodonesis	-	Multiple skeletal and systemic features[d]
	12	F, 10	3	Bilateral	+	Translucent irides	Minimal MVP	Pes planus
FBN1	13	M, 33	33	Bilateral	-		-	-
	14	M, 5	5	Bilateral	-		Aortic root 25.6mm Z +2.9	-
	15	F, 4	3	Bilateral	+	Amblyopia, coloboma lens	-	-
	16	F, 48	0	Bilateral	-	Achromatopsia, only light perception	-	AHR 1.05, slightly reduced extension of elbows
	17	M, 69	69	Bilateral	+	Cataract	-	Pes planus, pneumothorax, inguinal hernia
Unknown	18	M, 25	0	Bilateral	+	Cataract, nystagmus, uveitis	NP	Scoliosis
	19	F, 35	32	Unilateral	-	Translucent irides	-	Multiple skeletal features[e]
	20	M, 60	59	Bilateral	-	Cataract, flat cornea	Aortic root 45mm Z > 2	Facial features (2/5)[f]
	21	F, 12	0	Bilateral	NA	Amblyopia	-	Arthritis, mild developmental delay
	22	M, 45	40	Bilateral	-	Flat cornea	-	-
	23	M, 3	0	Unilateral	-	Amblyopia	NP	-
	24	F, 21	4	Bilateral	+	Flat cornea	-	Multiple skeletal and facial features (4/5)[g]

AHR, arm span to height ratio; EL, ectopia lentis; EP, ectopia pupillae; MVP, mitral valve prolapse; NA, not applicable; NP, not performed; -, no abnormalities
Refractive error was reported in all patients, except for patients 18 and 23. Refractive error frequently comprised moderate to high myopia oftentimes in combination with astigmatism.

[a] Dolichocephaly -, enophtalmus -, downslanting palpebral fissures +, malar hypoplasia +, retrognathia +
[b] Dolichocephaly -, enophtalmus -, downslanting palpebral fissures -, malar hypoplasia -, retrognathia +
[c] Dolichocephaly -, enophtalmus -, downslanting palpebral fissures -, malar hypoplasia +, retrognathia +
[d] Craniosynostosis, scoliosis, hyperlaxity of elbows and knees, hirsutism, hypoplastic toenails
[e] AHR 1.06, length > 2.5 SD, scoliosis, arachnodactyly, positive wrist sign, pes cavus, joint problems
[f] Dolichocephaly -, enophtalmus -, downslanting palpebral fissures -, malar hypoplasia +, retrognathia +
[g] Congenital dysplasia of the hip, mild scoliosis, arachnodactyly, pes planus, positive wrist and thumb sign, high arched palate. Dolichocephaly +, enophtalmus -, downslanting palpebral fissures +, malar hypoplasia +, retrognathia +

patients fulfilling the criteria for MFS. Following the revised Ghent criteria, one patient without an identified mutation (patient 20) was clinically diagnosed with MFS. At the age of 59 years bilateral EL was diagnosed, and cardiological examination revealed a dilatation of the aortic root (45 mm) in this normotensive patient.

In patients 1, 2, 7, 10, 11 and 12 with *ADAMTSL4* mutations the presence of one or more systemic features in addition to EL were reported, i.e. mild skeletal abnormalities, dysmorphic facial features, congenital unilateral hearing loss, craniosynostosis and developmental delay (Table 3). The reported additional systemic features in the EL patients with an unknown genetic cause are summarized in Table 3.

Two cases illustrating the value of NGS panel testing

We present two patients diagnosed with EL several years before the availability of NGS analysis. The first patient visited our outpatient clinic at the age of 46 years. At childhood both the index patient and his brother were diagnosed with bilateral lens luxation. By lacking any of the systemic features fitting a syndromic condition he was suspected to have isolated EL. He was not under regular (cardiological) surveillance and had not been referred to a clinical genetics department following the diagnosis of EL (over 30 years ago). At the age of 45 he was evaluated by a cardiologist because of increasing dyspnea. This revealed an aneurysm of the aortic root and ascending aorta with a maximal diameter of eight centimeters, aortic valve regurgitation and a dilated left ventricle with an ejection fraction of 18% (Figure 3). He underwent a successful Bentall procedure and was referred to our clinical genetics department for DNA testing of *FBN1*. A pathogenic heterozygous missense mutation (c.1380T>G) in *FBN1* was identified, resulting in the substitution of a cysteine to tryptophan (p.Cys460Trp) in one of the non-calcium binding EGF-like domains of *FBN1*.

The second patient (patient 1, Table 1 and 3), a 19-year-old male, was diagnosed with bilateral lens luxation at the age of nine years. He was referred to our department for analysis of suspected MFS. Although he did not fulfill the clinical criteria of MFS, there was a suspicion of MFS. He had mild dysmorphic features, including mild downslanting palpebral fissures, malar hypoplasia and retrognathia, and a seemingly autosomal dominant inheritance pattern with reduced penetrance of lens luxation. The deceased paternal grandfather was also reported to have had a lens luxation, yet no medical records were available. DNA diagnostics of *FBN1* did not confirm the molecular diagnosis of MFS. At that time, NGS analysis was not yet available. Cardiological evaluation revealed normal aortic diameters. A diagnosis could not be

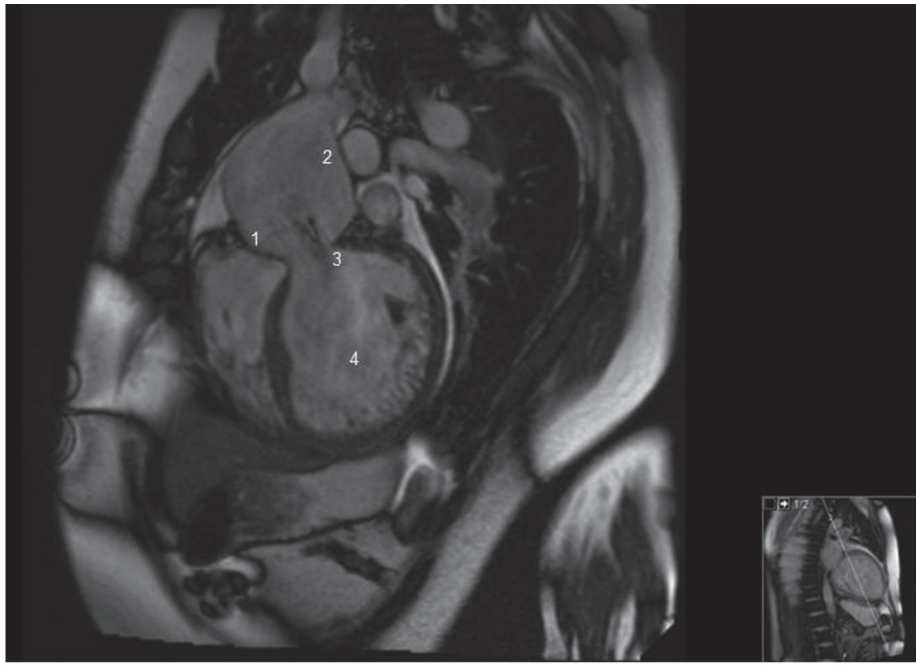

Figure 3 Cardiac magnetic resonance imaging in a patient initially suspected to have isolated EL

1: Aortic root aneurysm (75 mm); **2:** Ascending aortic aneurysm (80 mm) with normal diameter of the aortic arch; **3:** Severe aortic regurgitation; **4:** Severe dilatation of the left ventricle (EDV 520 ml).

established at that time. Several years later, NGS analysis of our EL gene panel revealed a homozygous pathogenic c.767_786del p.(Gln256fs*38) mutation (patient 1, Table 1) in *ADAMTSL4*. Because currently cardiac symptoms have not been clearly associated with *ADAMTSL4* mutations [19], the patient and his relatives could be reassured and discharged from cardiological surveillance.

Discussion

The diagnostic yield of the NGS panel was 67%: in 16 out of the 24 EL patients a genetic cause of EL could be identified. In our cohort, causative mutations were exclusively found in *ADAMTSL4* and *FBN1* (in 50% and 17% of the 24 EL patients respectively). The predominance of *ADAMTSL4* mutations is in line with the findings of Neuhann *et al*. and Chandra *et al*. who report *ADAMTSL4* mutations to be the most frequent cause of isolated EL (in 80% and 53% of their EL patient cohort respectively)

[19, 8]. In the cohort of Aragon-Martin *et al.* however, *FBN1* mutations were a more frequent cause of EL than *ADAMTSL4* mutations (64% *vs.* 17%) in EL patients without cardiac involvement and no or only minor skeletal involvement [15]. Furthermore, Li *et al.* identified a pathogenic *FBN1* mutation in 34 out of 40 Chinese patients presenting with EL, whereas no *ADAMTSL4* mutations were identified (85% *vs.* 0%) [27]. Potential explanations for this difference may be regional influences (including founder effects), relatively small numbers of included patients and different inclusion criteria in these studies. For example; patients with a high initial suspicion of MFS were possibly not included in our cohort, since analysis of the *FBN1* gene may have been requested separately instead of the NGS EL panel. Meanwhile, *ADAMTSL4* analysis was not offered separately. This might explain the relatively high percentage of *ADAMTSL4* mutations in our study.

At the time this study has been performed, our targeted NGS panel only included four EL-related genes (*ADAMTSL4*, *ADAMTS10*, *ADAMTSL17*, and *FBN1*); later, the *LTBP2* and the *CBS* genes have been included. In six of the eight patients with no identified causative mutations an additional panel of EL-related genes was therefore analysed on WES data. This panel included *LTBP2, COL18A1* [28], *PAX6* [29], *VSX2* [30] and *ADAMTS18* [31]. Mutations in these genes are reported to be involved in a clinical spectrum of different ocular phenotypes (e.g. high myopia, microcornea, cone-rod dystrophy and/or aniridia, rarely in combination with EL). *THSD4 (ADAMTSL6)* was also included in the additional gene panel. This gene is evolutionary closely related to *ADAMTSL4*, and has been shown to bind to fibrillin-1 microfibrils and enhance their formation. Therefore, this gene is a promising candidate gene for EL. To our knowledge, *THSD4 (ADAMTSL6)* mutations have not been reported in EL patients [3, 4]an autosomal dominant disorder manifesting with skeletal overgrowth, aortic aneurysm, and lens dislocation (ectopia lentis. In this extended gene panel, only one heterozygous pathogenic mutation was found in *LTBP2* (patient 20). Deletion/duplication analysis using a targeted approach in this patient failed to reveal an aberration on the second allele. Since mutations in this gene inherit in an autosomal recessive manner, the molecular diagnosis could not be confirmed in this patient. Possibly a deep intronic mutation or a variation in a regulatory element of this gene accounts for the phenotype in this patient. No (likely) pathogenic *ADAMTSL2, ADAMTS10, ADAMTS17, THSD4 (ADAMTSL6), ADAMTS18*, *CBS*, *PAX6*, *SUOX*, or *VSX2* variants were identified in this study. As such, no evidence was found for one of these genes being a common genetic cause of EL. In this study, no genetic cause could be identified in 33% of the patients. Though deep intronic mutations or a variation in a regulatory element in the analysed genes cannot be excluded, our results

suggest that mutations in other, so far unknown, genes may be involved in EL. Also, multifactorial origin of a proportion of these unexplained EL cases cannot be excluded.

As expected, the most common *ADAMTSL4* mutation identified was the European founder c.767_786del [13]. Nine out of 10 *ADAMTSL4* patients with European ancestry carried this mutation either homozygous (n=6) or compound heterozygous (n=3). The reported allele frequencies range from 0.002 (Exome Aggregation Consortium (ExAC), http:// exac.broadinstitute.org) to 0.0079 [17]DNA sequencing, and RT-PCR analysis.\n\ nRESULTS: Ocular signs in affected persons were increased median corneal thickness and astigmatism, angle malformation with prominent iris processes, displacement of the pupil and lens, lens coloboma, spherophakia, loss of zonular threads, early cataract development, glaucoma, and retinal detachment. No cardiac or metabolic abnormalities known to be associated with ectopia lentis were detected. Affected persons shared a 0.67 cM region of homozygosity on chromosome 1. DNA sequencing revealed a novel mutation in ADAMTSL4, c.767_786del20. This deletion of 20 base pairs (bp. Probably, different allele frequencies in different ethnic populations are responsible for this broad range of reported allelic frequencies of this mutation. Unsurprisingly, both EL patients with a non-European background (Turkish and Moroccan) carried *ADAMTSL4* mutations previously unreported in European patients. In general, most *ADAMTSL4* mutations identified cause a premature stop codon in the ADAMTSL4 protein. This may result in a deficiency of the ADAMTSL4 protein due to nonsense mediated mRNA decay, or in the production of a truncated ADAMTSL4 protein [17,18]. In literature, five *ADAMTSL4* missense mutations have been reported in EL patients (c.1774G>A p.(Gly592Ser), c.1960C>T p.(Pro654Ser), c.2237G>A p.(Arg746His), c.2594G>A p.(Arg865His), and c.3161A>G p.(Tyr1054Cys)) [15, 19, 23]. These mutations were classified as (likely) pathogenic, but functional and/or segregation analyses were however not performed.

The mean age at diagnosis was three years in the group of EL patients with *ADAMTSL4* mutations (n=12), 10 years in the patients with a *FBN1* mutation (n=4), and 26 years in the group without an identifiable mutation (n=8). In line with these findings, Chandra *et al*. previously suggested that mutations in *ADAMTSL4* may cause a more severe ocular phenotype than *FBN1* mutations with a significantly earlier onset [8].

Cardiological evaluation was performed in seven of 12 patients carrying *ADAMTSL4* mutations and revealed no aortic dilatation (Table 3). This is in accordance with observations reported in literature [19]. One patient without an identifiable *FBN1* mutation (12.5%) fulfilled the revised Ghent criteria for MFS (EL and aortic root dilatation

with a Z score ≥2), underscoring the importance of cardiological evaluation in case of EL with an unidentifiable genetic cause. Two patients with a *FBN1* mutation (50%) fulfilled the revised Ghent criteria for MFS (patients 14 and 16, Table 2 and 3), while the other two patients with a *FBN1* mutation appeared to have isolated EL. However, it has been reported that patients initially diagnosed with isolated EL caused by a *FBN1* mutation may later develop additional symptoms resulting in the diagnosis of (mild) MFS [7, 11]. A recent study by Chandra *et al*. presented that 46% of the patients initially diagnosed with isolated EL, had to be reclassified as MFS after the *FBN1* mutation they carried was reported in patients with aortic aneurysms or dissection [26]. As was already recommended in the revised Marfan nosology [9], we therefore stress the importance of cardiological follow-up in patients with apparently isolated EL caused by a *FBN1* mutation.

In several EL patients with *ADAMTSL4* mutations systemic features were reported (Table 3). In a recent study, it was concluded that there is insufficient evidence for major systemic involvement as a part of the *ADAMTSL4* mutation spectrum [19]. Interestingly, patient 11 in our group, and her monozygous twin sister both had craniosynostosis in addition to EL (these patients were reported previously [32]. Extensive DNA testing of craniosynostosis genes was performed and did not reveal any mutations (Table 1). Craniosynostosis has been reported in two other patients with *ADAMTSL4* mutations and may therefore be a rare manifestation of the clinical spectrum [16, 17]. As also concluded by Neuhann *et al*. [19] additional studies in larger cohorts are required to further elucidate the phenotypic spectrum associated with *ADAMTSL4* mutations.

We further illustrate the value of the current NGS EL panel by the clinical history of the two presented patients. The first of these two patients was initially not referred for genetic analysis based on the lack of systemic features. More than 30 years later, however, he was diagnosed with MFS after cardiological examination revealed a large aortic aneurysm. Nowadays, even before the patient fulfilled the clinical criteria for MFS, this diagnosis could have been established by DNA testing. The second patient (patient 1, Table 1 and 3) was initially suspected for MFS based on mild systemic features consistent with this syndrome and a seemingly autosomal dominant inheritance (with reduced penetrance) of lens luxation. This patient was found to carry a homozygous *ADAMTSL4* mutation. Since currently cardiac symptoms have not been clearly associated with *ADAMTSL4* mutations [19], the patient and his relatives could be reassured and discharged from cardiological surveillance.

Conclusions

Making the distinction between isolated and syndromic EL by identifying the underlying genetic cause is crucial for the further treatment, surveillance and counseling of patients and their relatives. By using a NGS gene panel the risk of misdiagnosis or delayed diagnosis can be reduced. The diagnostic yield of our NGS panel was high; in 16 out of the 24 EL patients (67%) a genetic cause of EL could be identified. In our cohort, causative mutations were found in *ADAMTSL4* and *FBN1*, and our results confirm that *ADAMTSL4* mutations are a frequent cause of (isolated) EL. Since the molecular diagnosis could not be confirmed in 33% of the patients, our results suggest that unknown additional genes are likely involved in EL.

Online supplement

For the online supplement, containing supporting information please access through the QR-code below.

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4

Clinical and genetic aspects of bicuspid aortic valve: a proposed model for family screening based on a review of literature

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Abstract

Introduction: Bicuspid aortic valve (BAV) is the most common congenital cardiac defect causing serious morbidity including valvular dysfunction and thoracic aortic aneurysms (TAA) in around 30% of BAV patients. Cardiological screening of first degree relatives is advised in recent guidelines given the observed familial clustering of BAV. However, guidelines regarding screening of family members and DNA testing are not unequivocal. **Aim:** To provide an overview of the literature on echocardiographic screening in first degree relatives of BAV patients and to propose a model for family screening. In addition, we provide a flowchart for DNA testing. **Methods:** We performed a PubMed search and included studies providing data on echocardiographic screening in asymptomatic relatives of BAV patients. **Results:** Nine studies were included. In 5.8-47.4% of the families BAV was shown to be familial. Of the screened first degree relatives 1.8-11% was found to be affected with BAV. Results regarding a potential risk of TAA in first degree relatives with a tricuspid aortic valve (TAV) were conflicting. **Conclusions:** The reported familial clustering of BAV underlines the importance of cardiological screening in relatives. After reviewing the available family history, patient characteristics and the results of cardiological screening in relatives, follow-up in relatives with a TAV and/or DNA testing may be advised in a subset of families. In this study we propose a model for the clinical and genetic work-up in BAV families, based on the most extensive literature review on family screening performed until now.

Introduction

With an estimated prevalence of 0.5-2% bicuspid aortic valve (BAV) (MIM# 109730) is the most common congenital cardiac defect associated with an increased risk of serious complications, including thoracic aortic aneurysms (TAA) [1-6]. As BAV, either with or without associated TAA (+/-TAA), often is a familial condition, cardiological screening of first degree relatives of BAV patients has been advised in recent guidelines of the American College of Cardiology and American Heart Association (ACC/AHA) [7-9]. However, screening is currently largely dependent on local initiatives and several important questions remain to be addressed. For example, it remains unclear whether relatives with a tricuspid aortic valve (TAV) are at an increased risk for the development of TAA and if DNA testing is a useful tool in the identification of families with a high risk for TAA at young age. The aim of this study is to provide an overview of the literature on family screening in first degree relatives, to provide an overview of the results of DNA testing in BAV (+/- TAA) families and to propose a model for clinical and genetic workup in BAV (+/-TAA) families. The results of the literature search and our proposed screening model are presented after a general overview of the clinical and genetic aspects of BAV (+/-TAA). The current understanding of the pathology, clinical aspects and management of BAV disease and genetic syndromes associated with BAV were recently reviewed and are outside the scope of our study [10-13].

Bicuspid aortic valve: clinical and genetic aspects

The bicuspid aortic valve, in most cases, consists of two unequal sized leaflets. The larger leaflet typically has a central raphe or ridge resulting from a fusion of the commissures (in ~70% fusion of the right and left coronary cusp, the remainder mostly from fusion of the right and non coronary cusp, and rarely the left and non coronary cusp) resulting in a functionally bicuspid aortic valve [10, 14]. A central raphe is absent in the less frequently occurring true bicuspid valve [15-17]. BAV can be an isolated congenital anomaly, but can also be associated with other abnormalities such as aortic coarctation, ventricular septal defects and hypoplastic left ventricle [6, 9, 12].

In most cases, BAV can be diagnosed and hemodynamically assessed using transthoracic echocardiography. In a minority of patients, particularly in calcific valve disease, higher resolution imaging techniques such as cardiac magnetic resonance imaging, computed tomography or trans-esophageal echocardiography may be required [16, 18, 19].

Although BAV may retain normal function throughout adult life, around 30% of the people with BAV will develop clinical complications [3-6]. Therefore, patients with BAV are advised to remain under regular surveillance by a cardiologist [9]. Taking into account the high incidence, BAV may be responsible for more morbidity and mortality than other congenital cardiac defects combined [5, 6]. In patients with BAV aortic stenosis and/or insufficiency is more frequent. In addition, BAV patients are more prone to aortic dilatation [16]. Dilatation of the thoracic aorta, most commonly located in the ascending aorta, has been reported in 35-80% of adult BAV patients and has rarely been observed as early as childhood [11-14, 20-25]. In adults TAA is defined as an aortic diameter with a z-score of ≥2, corresponding to an observed value >1.96 standard deviations above the predicted value for age, gender, and body surface area (BSA) [26]. An aortic root >4.0 cm in adults is considered to be dilated irrespective of age, gender or BSA [27-29]. Aortic dilatation in BAV may be restricted to the ascending aorta, but can also include the aortic root [25, 30]. In a retrospective study among 241 BAV patients referred for cardiac surgery, mean age 56 (range 16-85) years, aortic dilatation was seen in 97 patients (40%). The aortic root was involved in 9 (9.3%), the ascending aorta in 68 (70.1%), both the root and the ascending aorta in 14 (14.4%), the ascending aorta and the aortic arch in 5 (5.2%) and the root, ascending and aortic arch in 1 of the patients (1%) [25]. The relatively rare dilatation restricted to the aortic root is most commonly observed in men below the age of 40 and is reported to be associated with an increased risk of aortic dissection. This 'root' phenotype has been proposed to be the form of bicuspid aortopathy most likely to be associated with a genetic cause [3, 13, 20, 24, 31-33].

Although rare, the most feared complication in BAV patients is thoracic aortic dissection, which has been reported at young age [34]. Whereas the lifetime risk of aortic dissection in BAV patients was initially reported around 5%, recent studies show a lifetime risk of aortic dissection of less than 1% in BAV patients and a normal life expectancy [4, 6, 35-36]. This difference can potentially be explained by increased surveillance and timely surgical intervention in recent years. Aortic dissection is, in the majority of cases, preceded by slowly progressive asymptomatic aortic dilatation. This allows for screening and preventive surgery when indicated. Surgery is recommended in BAV patients with a diameter of the aortic sinuses or ascending aorta >5.5 cm, or >5.0 cm in the presence of an additional risk factor (growth ≥0.5 cm/year or a family history of aortic dissection) [37, 38]. Although the risk of aortic dissection is lower than initially estimated, cardiovascular surgery was performed in 22-27% of the BAV patients during follow-up [3, 4].

Familial BAV associated with dilatation of the aorta was first reported in literature over 40 years ago with the observation of a BAV in both a father and his son, who died in his sleep at the age of 19. No features of Marfan syndrome were observed. Autopsy confirmed an aortic dissection and a BAV [39]. The first systematic study on cardiological screening in asymptomatic relatives of BAV patients, to our knowledge, was published in 1978 [40]. The authors screened 188 first degree relatives of 41 BAV patients by auscultation and eccentricity index. BAV was diagnosed in 3.7-9.6% of the relatives and was familial in 14.6-31.7% of the 41 families (depending on the inclusion of doubtful cases). The familial clustering of BAV (+/-TAA) has since been confirmed by a number of studies, indicating a high heritability of BAV [41]. In addition, 20% of pediatric patients with a left ventricular outflow tract obstruction have (an) affected first degree relative(s), frequently a previously undetected BAV further illustrating a strong genetic contribution to the origin of BAV [42]. However, to date, a genetic cause has been identified in only a minority of BAV families, mostly showing an autosomal dominant inheritance pattern with reduced penetrance. In 2005 *NOTCH1* mutations were reported to be involved in a spectrum of developmental aortic valve anomalies, including BAV and severe aortic valve calcification [43]. Sequence analysis of *NOTCH1* indicated a potential overrepresentation of non synonymous missense variants among BAV (+/-TAA) patients, however *NOTCH1* mutations were found only in <5% of BAV cases in several subsequent studies [43-49]. Interestingly, *NOTCH1* mutations were recently shown to cause Adams Oliver syndrome. This is a rare developmental disorder with aplasia cutis of the scalp and transverse limb defects, frequently associated with cardiac defects, including BAV [50]. Based on studies in individual patients, linkage analysis in families and animal studies, several other genes and candidate loci have been implicated to be potentially involved in BAV [51-58]. These studies emphasize the genetic as well as phenotypic heterogeneity of BAV.

Whether dilatation of the proximal aorta in patients with BAV is a primary manifestation of an underlying genetic disorder, or secondary to hemodynamic effects related to the abnormal aortic valve remains controversial [21, 32, 59, 60]. Martin *et al* performed bivariate genetic analyses between aortic dimensions and BAV. Their results did not support a shared underlying genetic basis for BAV and aortic measures [60]. However, Loscalzo *et al*. conclude that BAV and TAA might be independent manifestations of a single gene defect with an autosomal dominant pattern of inheritance with incomplete penetrance. They studied segregation of BAV and TAA in 13 TAA families referred for analysis of known aneurysm, dissection or rupture. In total 110 first degree relatives of index patients were included. In 15 (13.6%) BAV was seen, in 10 cases associated with

TAA. Twenty four of the relatives (22%) were diagnosed with TAA in the presence of a normal tricuspid aortic valve [32]. In addition, Keane *et al.* reported that aortic size in BAV patients was larger than in control patients. They observed comparable degrees of aortic regurgitation, stenosis or mixed lesions, and concluded that intrinsic pathology appears to be responsible for aortic enlargement [21]. These observations support the hypothesis that BAV and TAA are independent manifestations of a single gene defect in a subset of BAV families [21, 32, 61]. The risk of dilatation of the thoracic aorta might therefore be increased in some first degree relatives of BAV patients, even in the absence of a bicuspid aortic valve. Vice versa, a limited number of studies suggest that BAV might be more prevalent in familial thoracic aneurysms and dissections (FTAAD) and in some hereditary connective tissue disorders associated with an increased risk of aortic aneurysms (for example Marfan syndrome) [33, 62-64]. Several studies illustrate the value of DNA testing in families with BAV (+/- TAA) by the identification of pathogenic mutations in known TAAD genes in FTAAD families with affected individuals with BAV. This provides further evidence for the hypothesis that both TAA and BAV may be independent phenotypic manifestations of a single mutation [31, 33, 62]. BAV was identified in four FTAAD patients in three of 14 studied families with an autosomal dominant pattern of thoracic aortic dissections on the basis of a mutation in smooth muscle aortic alpha actin (*ACTA2*) gene [62]. A mutation in transforming growth factor-beta receptor type II (*TGFBR2*) gene, associated with Loeys-Dietz syndrome, was found in a 48-year-old woman with BAV and a proximal aortic aneurysm including the aortic root, measuring 56 mm. Her brother died suddenly at the age of 42 immediately after the onset of excruciating chest pain. Her father, who also carried the mutation, had a history of elective surgical replacement of the aortic valve and ascending aorta for BAV and ascending aortic aneurysm at the age of 72 [33]. In several subsequent studies no *TGFBR1* or *TGFBR2* mutations were found in sporadic and familial BAV [32, 44, 64]. Recently, *FBN1*-mutations were linked to BAV as well by the identification of three *FBN1* mutations in two BAV patients with TAA (age at diagnosis 15 and 19 years) who did not fulfill the Marfan syndrome criteria according to the revised Ghent criteria [64]. In both patients the aortic diameter exceeded the threshold for surgery and the aortic size was largest at the level at the sinuses of Valsalva. Two of the three mutations (pArg529Gln and Arg2726Trp) were previously identified to be associated with variable/incomplete Marfan phenotype. In addition, the prevalence of BAV was reported to be increased in a cohort of 257 unrelated patients diagnosed with Marfan syndrome according to the updated Ghent criteria. Echocardiography showed BAV in 12 patients (4.7%) [63]. In three of these 12 patients DNA testing was performed revealing a pathogenic *FBN1* mutation in two cases, supporting the hypothesis that

FBN1 mutations may not only be associated with an increased incidence TAA, but also with BAV.

Overview of the literature on family screening in first degree relatives of BAV patients

We performed a PubMed search using the term "bicuspid aortic valve" in combination with at least one of the following terms in title and/or abstract: "gene(s), genetic(s), syndrome(s), family, relatives, family screening, pedigree analysis, inherited, aortic aneurysm, aortic dilatation or aortic dissection". Inclusion criteria were: studies providing data on systematic cardiological screening using echocardiography in first degree relatives of at least 20 BAV index patients, English language, published before November 2014. The number of at least 20 probands was arbitrarily chosen as we estimated that smaller cohorts or case series might introduce uncontrollable bias.

Using the abovementioned search strategy, we identified 683 articles. These articles were assessed for eligibility by reading title, abstract and/or full text. Of these, 27 contained data on family screening. Nine studies met our inclusion criteria. The results of these studies are summarized in Table 1. In 5.8-47.7% of the families BAV was shown to be familial (defined as BAV diagnosed in at least one first degree relative of the index patient). Of the screened first degree relatives of BAV patients 1.8-11% was found to be affected with BAV. The results regarding the risk of TAA with TAV in first degree relatives are conflicting. One study reported the presence of TAA in 32% of first degree relatives with TAV [61]. In this study, 53% of the BAV index patients had a dilated aortic root. Dilatation of the aortic root is described to be relatively rare in BAV and is proposed to be the form of bicuspid aortopathy most likely to be associated with a genetic cause [3, 13, 20, 24, 31-33]. The other studies reported percentages of TAA in the first degree TAV relatives of 3-4% [54, 65-68]. This is around population risk since 2.3% of the general population, by definition, is expected to have z scores >2 [28, 69, 70]. In the large study by Robledo-Carmona *et al*. only mild aortic dilatation (<4 cm) at older age (>50 years), was observed among nine out of 270 first degree relatives with a TAV, comparable to the observations in the control cohort [68]. They concluded that if their findings are confirmed by other studies, echocardiographic follow-up of the aortic dimensions of TAV first degree relatives might not be necessary [68]. In addition, in a recent study by Dayan *et al* among first degree relatives of BAV patients without TAA, normal aortic dimensions were seen in all relatives with a tricuspid valve [71].

Table 1. Overview of literature on family screening

Studies	BAV index patients (n)	Phenotype	Familial BAV/ families screened (%)	First degree relatives with BAV/ first degree relatives screened (%)	First degree relatives with BAV+TAA / first degree relatives with BAV (%)	TAA definition	First degree relatives with TAV+TAA / first degree relatives with TAV (%)
Huntington [66]	30	Adults, isolated BAV (n=18), BAV+ TAA (n=7), BAV + CVM (n=5)	11/30 (36.7%)	17/186 (9.1%)	N/A	N/A	5/169 (3.0%)
Cripe [41]	50	Pediatric patients, BAV (n=38), BAV+CVM (n=12)	16/50 (32%)	24/259 (9.3%)	N/A	-	N/A
Martin [54]	38	Pediatric patients, BAV (n=24), BAV +CMV (n=14)*	18/38 (47,4%)	36/315 (11%)	3/36 (8%)	N/A	7/279 (2.5%)
Bine [61]	49	Adults °	N/A	5/53 (9.4%)	N/A	Maximal dimension at any level of the root 95% CI of the diameter at the sinuses of Valsalva of a normal reference population [26]	14/ 44 (32%)
Kerstjens-Frederikse [42]	50	Pediatric patients, BAV/aortic valve stenosis	14/50 (28%)	N/A	N/A	-	N/A
Panayotova [67]	24	Adults, undergoing surgery for BAV or BAV + TAA	4/24 (16.7%)	4/52 (8%) #	2/4 (50%)	Definition TAA: increase in aortic size >50% of the upper limit of normal as per standard nomograms taking into account BSA, age and gender	2/48 (4.2%)
Demir [65]	66	Pediatric patients, isolated BAV (n=52)	3/52 (5.8%)	3/168 (1.8%)	N/A	z score >2	5/163 (3.1%)
		Pediatric patients, BAV + CoA (n=14)	1/14 (7.1%)	1/38 (2.6%)	N/A	z score >2	4/37 (11%)
Robledo-Carmona [68]	100	Adults, BAV+ CMV (n=13), BAV+TAA (n=42)	15/100 (15%)	16/348 (4.6%)	2/13 (15.4%)	Indexed sinus diameter>2.1 cm/m2 and tubular diameter >2 cm/m2 [28]	9/270 (3.3%) ^
Hale [72]	181	Pediatric patients	N/A	21/207 (10.1%)	N/A		N/A

BAV, bicuspid aortic valve; TAA, thoracic aortic aneurysm; TAV, tricuspid aortic valve; CVM, cardiovascular malformation; N/A, not available; CI, confidence interval; BSA, body surface area; CoA, coartaction of the aorta.
*Second degree relatives were included in some families, we only included families with 2 affected first degree relatives to calculate the % familial BAV. Several families were reported previously in Cripe *et al*. [41]; °5 first degree relatives diagnosed with BAV were added to the BAV index group; #on the basis of family history aortic valve disease was present in a further four relatives (8%); ^only mild dilatation (<4 cm) not significantly different to control group.

A proposed model for clinical and genetic work-up in BAV (+/-TAA) families based on literature

Based on our review of the literature and our clinical practice, we propose the model shown in Figure 1 as a tool for the clinical and genetic screening of BAV patients and their relatives. The main purpose of this model is identifying at-risk relatives for BAV and TAA. Following the ACC/AHA guidelines and the recommendations of the authors of the nine studies on family screening, we recommend cardiological screening including echocardiography in all first degree relatives (> 18 years) [7-9]. We do not perform screening in asymptomatic children given the low chance of significant abnormalities not detected during the routinely performed prenatal ultrasound at 20 weeks gestation and cardiac auscultation after birth. After reviewing the available family history, patient characteristics and the results of cardiological screening in relatives, follow-up in relatives with a TAV and/ or DNA testing may be advised (Figure 1). In case of sporadic or familial cases of BAV without TAA or aortic dissection in relatives, we do not recommend follow-up in first degree relatives with a tricuspid valve and normal aortic diameters. In these families, we only recommend DNA diagnostics in the index patient and echocardiographic follow-up in the first degree relatives (e.g every five years, depending on the age and echocardiographic findings) when dilatation of the aortic root in the BAV index patient develops before the age of 60 years. Based on our review of literature, with most studies showing only a slightly increased risk of TAA in relatives with TAV, we advise clinical follow-up of first degree relatives with TAV only in the following situations; sporadic BAV with aortic root involvement before the age of 60 years, TAA in a relative with TAV before the age of 60, and in families with two or more persons with (suspected) TAA and a TAV irrespective of age. The cut off at the age of 60 years is more or less arbitrary and is chosen since increased aortic diameters are more likely to be associated with older age and hypertension then with genetic factors when observed later in life [73]. In contrast, aortic root involvement at young age is relatively rare in BAV, but relatively frequent in FTAAD and syndrome associated TAAD, and might be associated with mutations in TAAD genes [33, 62, 64, 74, 75]. First degree relatives of young BAV patients with dilatation of the aortic root may therefore have an increased risk of TAA in absence of BAV [22, 32, 61, 62]. In our clinical genetics department DNA diagnostics of 13 TAAD genes (*ACTA2, COL3A1, EFEMP2, ELN, FBN1, FBN2, MYH11, MYLK, PLOD1, SLC2A10, SMAD3, TGFBR1, TGFBR2*) and *NOTCH1* is offered in the following three situations: BAV patients with aortic root dilatation (z>2 or an aortic diameter >4.0 cm) before the age of 60, in case of TAA in a relative with TAV before the age of 60 years, and in familial TAA.

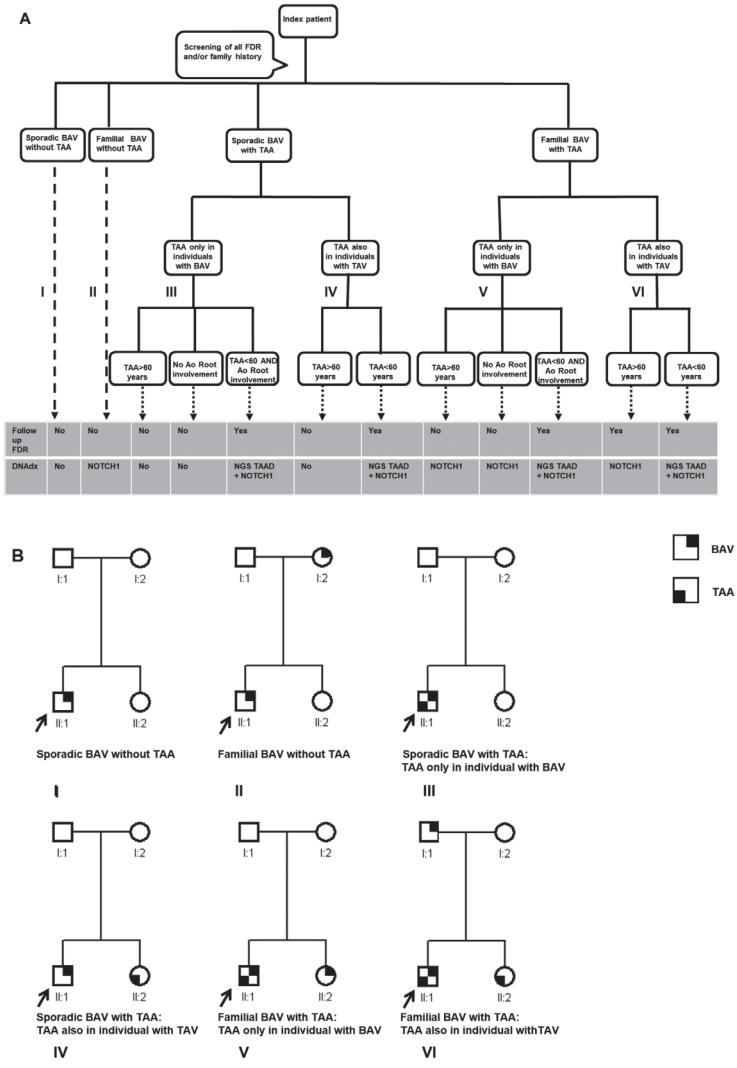

Figure 1 Ao root involvement, aortic root involvement; NGS, next generation sequencing; TAA, thoracic aortic aneurysm; TAV, tricuspid aortic valve.

A Flow chart illustrating a proposed model for genetic testing, and clinical work-up of first degree relatives (FDR) of patients with bicuspid aortic valve (BAV). Clinical follow-up of first degree relatives comprises echocardiographic screening at a low frequency (e.g., every five years) starting at the age of 18 years. In our center NGS TAAD analysis includes: *ACTA2, COL3A1, EFEMP2, ELN, FBN1, FBN2, MYH11, MYLK, PLOD1, SLC2A10, SMAD3, TGFBR1 and TGFBR2.*

B Simplified pedigrees illustrating examples of the different familial situations in the flow chart. Men are encoded with squares, women with circles. Index patients are indicated with an arrow.

Furthermore, in confirmed familial BAV cases not fulfilling the aforementioned criteria, sequencing of the *NOTCH1* gene may be considered. In sporadic cases of BAV, or sporadic BAV patients with TAA <60 years not involving the aortic root, the chance of identifying a pathogenic mutation in *NOTCH1* is likely to be low. *NOTCH1* mutation screening in these patients may be considered, depending on the social context of the patient, but is not routinely recommended by us (Figure 1). In familial BAV without TAA, *NOTCH1* mutation screening can be helpful to identify at-risk relatives, especially in patients with calcified bicuspid valves [43]. We currently advise follow-up of all relatives carrying a pathogenic *NOTCH1* mutation irrespective of the presence or absence of BAV given a potentially increased risk of TAA [48]. Future phenotype-genotype studies may potentially enable the identification of specific *NOTCH1* mutations associated with an increased risk for TAA. When using our proposed model it is important to consider all available clinical data of a family in its entirety. In case of new information during follow-up one should reconsider the situation using the model and adjust the clinical and genetic work-up accordingly (for example in case of the development of an aortic root aneurysm in a BAV patient or a newly diagnosed family member with BAV). This model is meant as a tool for non syndromic BAV (+/-TAA) patients and their families. In case of evidence for a hereditary connective tissue disorder we recommend a custom multidisciplinary work-up and targeted analysis of candidate genes for the suspected underlying syndrome instead of using the presented model. For example in cases of extreme degrees of aortic dilatation and/or the presence of other symptoms or features. The individual work-up in patients with (suspected) genetic syndromes associated with an increased occurrence of BAV, such as Turner syndrome, is outside the scope of our study.

Discussion

We propose a model for the clinical and genetic screening in non-syndromic BAV (+/-TAA) families based on a review of the literature. The percentage of BAV in first degree relatives of BAV index patients was 1.8-11% and BAV was found to be familial in 5.8-47.4% of the families in the nine included studies. The different percentages reported in literature might be explained by the small number of index patients and screened first degree relatives in some studies. Furthermore, the participation rates of the first degree relatives in cardiological screening varied between studies and were not mentioned in all papers. This could have resulted in a selection bias. In addition, different patient groups with potentially different prevalences of BAV were included

(e.g pediatric patients with/without additional congenital cardiac defects versus adult index patients). Furthermore, the prevalence of (familial) BAV might differ between geographical regions as suggested by Robledo-Carmona *et al.* [68]. In all studies, the importance of screening in first degree relatives of BAV patients was underlined by the authors. One study addressed the cost-effectiveness of cardiological screening in siblings of children with BAV. It was concluded that screening is effective and inexpensive and should be incorporated into clinical care [72]. We feel confident that, using our model, the majority of high-risk families can be identified. In our opinion, repeated follow-up in all first degree relatives of all BAV patients poses an unjustifiable burden on health resources. Although based on a few studies only, DNA sequencing appears to be a promising tool in the identification of a subset of high-risk families. Aortic dissections at a young age are suggestive for a connective tissue disorder or FTAAD, whereas dissections in BAV patients usually occur later in life. The mean age at presentation (either detection of an aneurysm or after presentation with a complication) was lowest in syndrome associated and monogenetic aneurysm patients at around 25-27 years versus 55-57 years in familial aneurysms and 64-66 years in the sporadic aneurysm group, and 55 years in the BAV group [76]. However, the ages at presentation within these groups were highly variable and largely overlapping, further complicating differentiating between sporadic BAV, familial BAV (+/-TAA), FTAAD and syndromal TAAD when faced with an individual patient without screening of relatives [75-78]. The reported location of aortic dilatation in the families with an identifiable pathogenic mutation in *FBN1*, *ACTA2* or *TGFBR2* included the aortic root and dissections were observed at young age. Mutations in these genes are associated with a high risk of aortic dilatation and dissection at young age, in most cases in patients with tricuspid aortic valves. Although evaluation in larger cohorts is required, DNA diagnostics appears to be a promising and valuable tool in identifying a minority of high-risk families presenting with an index patient with BAV (+/-TAA). The identification of a pathogenic mutation enables genetic testing of family members and selective clinical follow-up of at-risk relatives. Furthermore, DNA testing is becoming widely available at rapidly declining costs and is increasingly incorporated into standard clinical practice [74, 75]. Novel techniques such as whole exome- and whole genome sequencing are likely to be valuable in the identification of novel genes in BAV and TAA. Therefore, it is likely that DNA testing will take a more prominent part in risk stratification in BAV (+/-TAA) patients and their families in the near future. In the Netherlands, genetic counseling and DNA testing in patients with suspected inherited cardiovascular disease is performed mainly by clinical geneticists and genetic counselors working in multidisciplinary teams also including pediatric cardiologists, cardiologists and social

workers specialized in cardiogenetic disorders. These outpatient clinics provide well equipped setting for the coordination of family screening, collecting of clinical data and, when indicated, performing dysmorphologic examination and DNA testing. BAV patients can be referred to these outpatient clinics when there is an indication for DNA testing and/or clinical follow-up of first degree relatives on the basis of our flowchart or when a genetic syndrome is suspected. Other reasons for referral can for example be questions about the inheritance of BAV, including potential implications for family members and/ or (future) offspring. Further insight into the genetic and pathophysiological mechanisms leading to BAV and/or TAA is required to enable the identification of novel factors causing health risks in BAV patients and their relatives. These include aortic medial degeneration, vascular smooth muscle cell apoptosis and the interplay between BAV morphology, sheer stress and valvular dysfunction and TAA in BAV patients,

Limitations of our model include the limited number of studies, different patient groups and the absence of DNA testing in the majority of publications. When using our proposed model it is important to consider all available clinical data of a family in its entirety. This may be difficult due to loss of follow-up of the index patient (e.g. after moving or due to non compliance) and because of difficulties to obtain all relevant medical records of relatives (e.g. because not all first degree relatives are informed by the index patient and/or not all relatives are participating in cardiological screening). Long term follow-up in well-characterized BAV cohorts is required to test the feasibility, sensitivity and specificity of our model which should be adjusted accordingly when necessary.

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5

Phenotypic spectrum of *TGFB3* disease-causing variants in an international cohort and first report of a homozygous patient

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Abstract

Disease-causing variants in *TGFB3* cause an autosomal dominant connective tissue disorder which is hard to phenotypically delineate because of the small number of identified cases. The purpose of this retrospective cross-sectional multicentre study is to elucidate the genotype and phenotype in an international cohort of *TGFB3* patients. Eleven (eight novel) *TGFB3* disease-causing variants were identified in 32 patients (17 families). Aortic root dilatation and mitral valve disease represented the most common cardiovascular findings, reported in 29% and 32% of patients, respectively. Dissection involving distal aortic segments occurred in two patients at age 50 and 52 years. A high frequency of systemic features (65% high-arched palate, 63% arachnodactyly, 57% pectus deformity, 52% joint hypermobility) was observed. In familial cases, incomplete penetrance and variable clinical expressivity were noted. Our cohort included the first described homozygous patient, who presented with a more severe phenotype compared to her heterozygous relatives. In conclusion, *TGFB3* variants were associated with a high percentage of systemic features and aortic disease (dilatation/dissection) in 35% of patients. No deaths occurred from cardiovascular events or pregnancy-related complications. Nevertheless, homozygosity may be driving a more severe phenotype.

Introduction

A heritable connective tissue disorder due to *TGFB3* variants was first described in 2013 and listed in OMIM as Loeys-Dietz syndrome-5 (MIM# 615582), although controversy exists regarding this nomenclature [1,2]. Its clinical manifestations mainly involve the skeletal, ocular and cardiovascular systems.

Given the rarity of the disorder, with no more than 50 cases described so far, a precise delineation of its phenotype is yet to be determined [3-10].

Here we report the clinical and genetic findings of 32 patients from 17 families, and we give an overview of all reported *TGFB3* disease-causing variants. Our aim is to achieve a better understanding of the phenotype related to *TGFB3* disease-causing variants.

Methods

Patients were identified through the diagnostic laboratories at the department of clinical genetics, Amsterdam UMC, Vrije Universiteit Amsterdam, the Netherlands, and at the département de génétique, Hôpital Bichat, Paris, France (Supporting Information). Referring physicians were invited to participate in this study by a written invitation and were mailed questionnaires in order to retrospectively collect clinical data. The data collection period was from April 2018 to May 2019. All genetic and clinical data were acquired during standard patient care. Informed consent for publication was obtained from all patients, or their legal representatives, in accordance with the Declaration of Helsinki and national legal regulations.

In order to obtain an overview of all known disease-causing *TGFB3* variants, databases and previously published articles were consulted (Supporting Information).

Results

TGFB3 variants

Eleven (eight novel) variants were detected in our cohort: seven missense, one nonsense, and three splice site variants (Table 1 and 2). Four variants (36%) were located in the RKKR motif. According to the AMCG criteria [10], all variants are classified as

pathogenic or likely pathogenic except for p.(Asp303Ala) and p.(Ser359Arg) that are variants of unknown significance (Supporting Information). Twenty-six other variants were extrapolated from databases and literature (Table 2).

Clinical data

Our cohort consisted of 32 *TGFB3* patients (56% males) from 17 families. Mean age at last evaluation was 32 years (range 4-60 years).

Aortic dilatation or dissection was observed in 11/31 (35%) of patients and exhibited age-related penetrance (Figure 1). Aortic root dilatation occurred in 9/31 (29%) patients and was associated with dilatation of distal aorta in three patients. Two patients presented with distal aortic dilatation in the absence of aortic root dilatation. Dissection of the aorta occurred in two patients at age 50 and 52. Two patients underwent preventive aortic surgery, both after age 40. Two patients displayed arterial tortuosity. Mitral valve disease (prolapse and/or insufficiency) and aortic valve insufficiency were observed in 9/28 (32%) and 2/28 (7%) of patients, respectively. Neither atrial/ventricular septal defects nor atrioventricular block were noted. Varices of lower limbs were seen in 5/24 (21%) of patients.

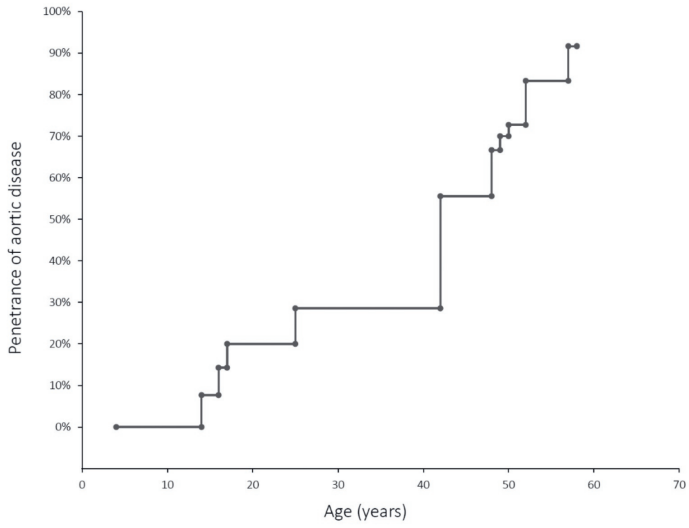

Figure 1 Inverted Kaplan–Meier curve indicating the age-related penetrance of aortic disease, with age at first aortic event (dilatation/dissection/surgery) as the survival variable

Table 1 Summary of the relevant clinical and genetic features of patients in our cohort

ID	Rel	Sex, age[a]	Cardiovascular feature(s)	Systemic feature(s)	FH	Variant	Effect[b]
1	IC	♀, 52	Dissection (AA, 50y), dilatation (iliac artery, 51y), tortuosity (internal carotid and vertebral arteries), varices	Height +0.5 SD, retrognathia, myopia (-7 dioptres), DPF, crowded teeth, thin upper lip, pointed nose, kyphosis, color blindness, symptomatic hypokalemia (52y)	+	c.170dup p.(Glu58*)	P (Ic)
2	Si	♀, 50	Tortuosity (internal carotid and vertebral arteries)	Hypertelorism, PE, JH, Ara, PP, EB	+	c.170dup p.(Glu58*)	P (Ic)
3	IC	♀, 51	Dilatation (thoracic aorta, 48y), varices (17y)	Height + 4 SD, retrognathia, long face, smooth philtrum, BU, HAP, asymmetric pectus, bilateral coxa valga, Ara, PP, EB	+	c.463C>T p.(Arg155Trp)	LP (II)
4	IC	♀, 16	None	Height +1 SD, HAP, PE, breast asymmetry, scoliosis, Ara	?	c.463C>T p.(Arg155Trp)	LP (II)
5	IC	♂, 21	Dilatation (AoR, 16y)	Height +3.5 SD, dolicocephaly, retrognathia, myopia, DPF, HAP, cleft palate, PC, kyphoscoliosis, JH, Ara, PP, sternoclavicular subluxation, SS, autoimmune thrombocytopenia, chronic diarrhea	?	p.516+1G>A p. ?	LP (I)
6	IC	♂, 50	Dilatation (AoR, Bentall procedure 42y), mitral valve insufficiency, DCM, arrhythmia	Midface hypoplasia, HAP, scoliosis, JH, increased AHR, Ara, EB, delayed wound healing, thin translucent skin, soft velvety skin, subcutaneous fat markedly reduced	?	c.517-3_517-2delinsAG p.?	LP (I)
7	IC	♀, 10	Mitral valve prolapse and insufficiency, billowing of the tricuspid valve	Height +1.5 SD, retrognathia, long face, hypertelorism, DPF, prominent eyes, HAP, scoliosis, JH, Ara, PP, camptodactyly, hammertoes, thin translucent skin, subcutaneous fat markedly reduced, decreased muscle mass	*De novo*	c.889A>G p.(Arg297Gly)	LP (V)
8	IC	♂, 58	Dilatation (AAo, AoR), aortic valve insufficiency, David procedure (57y), varices	Height +3 SD, dolichocephaly, retrognathia, long face, midface hypoplasia, unilateral DPF, HAP, Ara, subcutaneous fat markedly reduced	-	c.898C>T p.(Arg300Trp)	P (IIIb)
9	IC	♂, 6	Mitral valve prolapse	BU, PE, JH, Ara, thoracic kyphosis, foot eversion	*De novo*	c.898C>T p.(Arg300Trp)	P (IIIb)
10	IC	♂, 27	Upper normal limit of AoR diameter, mitral valve insufficiency, moderate dilatation of the left ventricle	Height +1.5 SD, exotropia, downslanting palpebral fissures, BU, HAP, PC, scoliosis, dolichostenomelia, Ara, PP, SS, dural ectasia	-	c.898C>T p.(Arg300Trp)	P (IIIb)
11	IC	♂, 54	Dissection (type B extending to the AA, 52y), dilatation (AoR, 52y)	Height +1 SD, cataract (bilateral, surgery 52y and 53y), myopia, BU, pneumothorax (14y), JH, PP, hyperelasticity of the skin, subdural hematoma, IH (53y), angioneurotic edema	+	c.898C>T p.(Arg300Trp)	P (IIIb)
12	So	♂, 14	Dilatation (AoR, 14y)	Height +0.5 SD, midface hypoplasia, myopia, BU, HAP, PE, increased AHR, Ara, PP	+	c.898C>T p.(Arg300Trp)	P (IIIb)

13	IC	♀, 12	Mitral valve prolapse	Height +0.5 SD, long face, hypertelorism, blue sclerae, BU, HAP, posterior cleft palate, JH, Ara, PP, EB, delayed wound healing, thin translucent skin, soft velvety skin	+	c.898C>T p.(Arg300Trp)	P (IIIb)
14	Mo	♀, 35	None	Height +0.5 SD, long face, smooth philtrum, hypertelorism, blue sclerae, DPF, HAP, hiatal henia (18y), JH, increased AHR, Ara, PP, hallux valgus, EB, delayed wound healing, thin translucent skin, soft velvety skin	+	c.898C>T p.(Arg300Trp)	P (IIIb)
15	Si	♀, 6	Mitral valve prolapse	Height +1 SD, metopic ridge, retrognathia, long face, hypertelorism, blue sclerae, DPF, ptosis, HAP, JH, Ara, PP, hallux valgus, EB, delayed wound healing, thin translucent skin, soft velvety skin	+	c.898C>T p.(Arg300Trp)	P (IIIb)
16	MU	♀, 40	Mitral valve prolapse and insufficiency	Height +2.3 SD, long face, hypertelorism, flat cornea, PC, osteoarthritis, kyphoscoliosis, JH, Ara, PP, patellar tendon rupture, fatigue, EB, thin translucent skin, soft velvety skin, skin hyperextensibility	+	c.898C>T p.(Arg300Trp)	P (IIIb)
17	MCo	♂, 8	Mitral valve insufficiency (7y)	Height +2.5 SD, dolicocephaly, long face, blue sclerae, DPF, ptosis, HAP, hypermetropia, strabismus, PE, JH, Ara, PP, genu valgum, EB, thin translucent skin, soft velvety skin, congenital hypotonia, delay of motor development, frequent falls	+	c.898C>T p.(Arg300Trp)	P (IIIb)
18	IC	♂, 30	Incomplete RBBB	Dolichocephaly, BU, PE, scoliosis, JH, increased AHR, Ara, acetabular protrusion, cavus foot, SS, dural ectasia, IH	?	c.898C>T p.(Arg300Trp)	P (IIIb)
19[c]	IC	♂, 44	Varices	Height +3 SD, PC, JH, increased AHR, clubfeet, coxarthrosis	+	c.899G>A p.(Arg300Gln)	P (IIIb)
20	So	♂, 4	Mitral valve prolapse	Height +2 SD, dolichocephaly, long face, hypertelorism, myopia, BU, high forehead, frontal bossing, short philtrum, PE, JH, PP, congenital hypotonia (mild), delay of motor development	+	c.899G>A p.(Arg300Gln)	P (IIIb)
21	Si	♀, 52	None	Height +3 SD, long face, prominent eyes, PC, bilateral coxa valga, JH, increased AHR, Ara, left pes planus, right pes cavus, clubfeet, EB, soft velvety skin, decreased muscle mass	+	c.899G>A p.(Arg300Gln)	P (IIIb)
22	Ne	♂, 8	None	Height +2 SD, HAP, anomaly of teeth position, PP, IH (unilateral)	+	c.899G>A p.(Arg300Gln)	P (IIIb)
23	IC	♂, 52	Dilatation (AoR, 52y), mitral valve prolapse and insufficiency	Height -0.5 SD, PE, osteoarthritis, increased AHR, osteoporosis (51y), kyphosis, pinching vertebral discs, subcutaneous fat markedly reduced.	-	c.908A>C p.(Asp303Ala)	VUS
24	IC	♂, 18	None	Height +2 SD, dolicocephaly, BU, HAP, cleft palate, PE, spondylolisthesis, JH, Ara, PP, decreased muscle mass	*De novo*	c.952C>T p.(Arg318Cys)	LP (V)
25[c]	IC	♂, 60	Dilatation (AoR 46mm, 25y ; AoR 55mm, 57y; AAo 48mm, 57y), aortic valve insufficiency (25y), paroxysmal supraventricular tachycardia (55y)	Height 0 SD, myopia, amblyopia, DPF, HAP, PP (during childhood), UH	+	c.1075A>C p.(Ser359Arg)	VUS

26	Dau	♀, 33	None	Height -1 SD, hypermetropia	+	c.1075A>C p.(Ser359Arg)	VUS
27	Si	♀, 58	None	Height -0.6 SD, cataract (unilateral, surgery at 49y), myopia	+	c.1075A>C p.(Ser359Arg)	VUS
28	IC	♀, 22	Dilatation (AoR, 17y)	Height +1.5 SD (target height -0.3SD), retrognathia, long face, hypertelorism, myopia, DPF, prominent eyes, low set ears, overbite, BU, cleft palate, PE, spondylolisthesis, scoliosis, increased AHR, Ara, PP, thin translucent skin, subcutaneous fat markedly reduced, decreased muscle mass, diaphragmatic hernia, UH	+	c.1081-2A>T[d] p.?	P (Ic)
29	Br	♂, 18	NA	Height -1 SD, BU, dental crowding, PP, congenital hip dysplasia	+	c.1081-2A>T p.?	P (Ic)
30	Si	♀, 10	None	Height +1 SD, myopia (-5.5/-5.75 doptres), HAP	+	c.1081-2A>T p.?	P (Ic)
31	Mo	♀, 41	None	Height 0 SD, 163.5 cm, DPF, HAP, broad uvula	+	c.1081-2A>T p.?	P (Ic)
32	F	♂, 49	Dilatation (AoR 39 mm, 49y), varices	Height -0.5 SD, hypertelorism, cataracts (47y), myopia (-16 dioptres), broad uvula, PC, increased AHR, osteoporosis, EB, thin translucent skin, IH (unilateral, 34y), UH	+	c.1081-2A>T p.?	P (Ic)

Note: Used TGFB3 RefSeq transcripts (based on Genome build: GRCh37/hg19): NC_000014.8(NM_003239.4).
Abbreviations: AA, abdominal aorta; AAo, ascending aorta; AHR, arm/height ratio; AoA, aortic arch; AoR, aortic root; Ara, arachnodactyly; Br, brother; BU, bifid uvula; Dau, daughter; Dao, descending aorta; DCM, dilated cardiomyopathy; DPF, downslanting palpebral fissures; EB, easy bruising; F, father; FH, family history; HAP, highly arched palate; JH, joint hypermobility; IC, index case; IH, inguinal hernia; LP, likely pathogenic; MA, maternal aunt; MCo, maternal cousin; MFS, Marfan syndrome; Mo, mother; MU, maternal uncle; NA, no information available; Ne, nephew; Ni, niece; P, pathogenic; PA, paternal aunt; PC, pectus carinatum; PE, pectus excavatum; PP, pes plani; Rel, relationship; RBBB, right bundle branch block; Rel, relationship; SD, standard deviation; Si, sister; So, son; SS, skin striae; UH, umbilical hernia; VUS, variant of unknown significance; y, age at diagnosis (years); +, known family history; −, no family history; ?, unknown.

[a] Age (in years) at last evaluation.
[b] According to the AMCG criteria (Richard et al [11]).
[c] This patient is recently described in literature (Overwater et al [7]).
[d] Homozygote.

Table 2. Overview of all reported *TGFB3* disease-causing variants

Nucleotide change	Protein change	Domain	gnomAD allele count	Reference(s)
c.106A>T	p.(Lys36*)	LAP	0	Schepers *et al.* [8]
c.170dup	p.(Glu58*)	LAP	0	Current study
c.321dup	p.(Phe108Ilefs*18)	LAP	0	ClinVar
c.353-1G>C	p.?	LAP	0	ClinVar
c.427A>T	p.(Arg143*)	LAP	0	Ziganshin *et al.* [9]
c.437del	p.(Leu146Hisfs*68)	LAP	0	Schepers *et al.* [8]
c.463C>T	p.(Arg155Trp)	LAP	0	Current study
c.516+1G>A	p.?	LAP	0	Current study
c.517-3_517-2delinsAG	p.?	LAP	0	Current study
c.517-1G>C	p.?	LAP	0	ClinVar
c.704del	p.(Asn235Metfs*11)	LAP	0	Bertoli-Avella *et al.* [3]
c.754+2T>C	p.Glu216_Lys251del	LAP	0	Bertoli-Avella *et al.* [3]
c.787G>C	p.(Asp263His)	LAP	0	Bertoli-Avella *et al.* [3]; Schepers *et al.* [8]
c.796C>T[a]	p.(Arg266Cys)	LAP	3	Schepers *et al.* [8]
c.826C>T	p.(Pro276Ser)	LAP	0	ClinVar
c.883_884del	p.(Gly295Serfs*28)	LAP	1	ClinVar
c.889A>G	p.(Arg297Gly)	RKKR motif	0	Current study
c.898C>T	p.(Arg300Trp)	RKKR motif	0	Bertoli-Avella *et al.* [3]; Schepers *et al* .[8]; Current study
c.898C>G	p.(Arg300Gly)	RKKR motif	0	Kuechler *et al.* [5]
c.899G>A	p.(Arg300Gln)	RKKR motif	0	Matyas *et al.* [6]; Kim *et al.* [4]; Schepers et al [8]; Overwater *et al.* [7]; Current study[b]
c.908A>C[a]	p.(Asp303Ala)	Cytokine	4	Current study
c.916del	p.(Tyr306Thrfs*63)	Cytokine	0	ClinVar
c.927-1G>C	p.?	Cytokine	1	ClinVar
c.952C>T	p.(Arg318Cys)	Cytokine	0	Current study
c.965T>C	p.(Ile322Thr)	Cytokine	2	Bertoli-Avella *et al.* [3]
c.979G>T	p.(Asp327Tyr)	Cytokine	1	Schepers *et al.* [8]
c.989G>A	p.(Trp330*)	Cytokine	0	ClinVar
c.1020T>A	p.(Tyr340*)	Cytokine	0	ClinVar
c.1034C>G	p.(Ser345*)	Cytokine	0	ClinVar
c.1075A>C[a]	p.(Ser359Arg)	Cytokine	0	Overwater *et al.* [7]; Current study[b]
c.1081-2A>T[c]	p.?	Cytokine	0	Current study
c.1095C>A	p.(Tyr365*)	Cytokine	0	Bertoli-Avella *et al.* [3]
c.1102_1105del	p.(Leu368Thrfs*18)	Cytokine	0	ClinVar
c.1157del	p.(Leu386Argfs*21)	Cytokine	0	Bertoli-Avella *et al.* [3]
c.1195G>T	p.(Glu399*)	Cytokine	0	ClinVar
c.1202T>C	p.(Leu401Pro)	Cytokine	0	Bertoli-Avella *et al.* [3]; Schepers *et al.* [8]
c.1226G>A	p.(Cys409Tyr)	Cytokine	0	Rienhoff *et al.* [1]

Note: Used *TGFB3* RefSeq transcript (GRCh37/hg19): NC_000014.8(NM_003239.4)
[a]Variant of unknown significance.
[b]Same patient is reported by Overwater *et al.* [7] and in the current study.
[c]Index case is homozygote.

Average height was +1.3 SD (range -1 SD to +4 SD). The most common systemic signs were high-arched palate (65%), arachnodactyly (63%), pes planus (63%), pectus deformity (57%), and joint hypermobility (52%), long face (42%), downslanting palpebral fissures (39%), bifid uvula (38%), increased arm span (36%), easy bruising (34%), thin translucent skin (32%), myopia (33%), hypertelorism (32%), and scoliosis (30%). Some of the features previously associated with variants in *TGFB3* were not observed (brachycephaly, wide face, ectopia lentis, scapulae alata, cervical spine instability, contractures of fingers, palmar flexion, transient postnatal pes adductus, low birth weight), or were observed in 10% of patients or less (metopic ridge, midface hypoplasia, smooth philtrum, exotropia, ptosis, cerebral hemorrhage, osteoporosis, camptodactyly of toes, hiatal hernia, osteoarthritis, spondylolisthesis, bilateral coxa valga, congenital hypotonia, delayed motor development, autoimmune disease).

No major vascular or visceral complication occurred in 18 pregnancies (six women).

Homozygous patient

Patient #28 was the first child of consanguineous parents of Moroccan origin. She was born at 38+2 weeks of gestation. Birth weight was 2930 g. Cleft palate, umbilical hernia, and joint contractures (involving elbows, knees and feet, which improved significantly over time) were diagnosed shortly after birth. Arachnodactyly and dysmorphic craniofacial features, including micrognathia and low-set ears, were also observed in the neonatal period. She was later diagnosed with a leftsided bochdalek hernia and developed pectus excavatum, L5-S1 spondylolisthesis, scoliosis, severe myopia, and dilatation of the aortic root (Z-score 2.14 at age 17 years). She received medroxyprogesterone and ethinylestradiol for anti-growth purposes, and she was treated by long-term nocturnal gastrostomy feedings to improve her nutritional status. Both motor and cognitive developments were normal. Genetic testing revealed a homozygous c.1081-2A>T *TGFB3* variant (Supporting Information). Both parents (patients #31 and #32), a brother (patient #29), and a sister (patient #30) were heterozygous and were less severely affected. Another sibling did not carry the variant and displayed no sign of the disease.

Discussion

Our findings are largely in line with the most comprehensive report on *TGFB3* variants published before [8]. Aortic disease (dilatation or dissection) affected 35% of patients

showing age-related penetrance (Figure 1). No aortic dilatation occurred before the age of 14 years. Arterial dissections occurred at dilated aortic segments after the age of 50 years in two patients who displayed marked systemic features. No deaths related to cardiovascular or pregnancy complications were observed. Men were more likely to be diagnosed with aortic disease, but difference between men and women was not significant. Arterial tortuosity was observed in two patients. Two patients were diagnosed with dural ectasia, a clinical feature which was not previously described in association with *TGFB3* variants. As *TGFB3* patients are not systematically screened for dural ectasia, whether this is a coincidental finding or an underestimated clinical feature remains unclear.

Compared to Schepers et al [8], we identified a higher rate of downslanting palpebral fissures (39% vs. 15%), and a lower rate of joint hypermobility (52% vs. 80%), scoliosis (30% vs. 69%), and osteoarthritis (8% vs. 46%). Clubfoot was reported by Schepers and colleagues in 25% (3/12) of patients [8]. Two (7%) related patients in our cohort presented with clubfoot. However, clubfoot in this family may not be related to the *TGFB3* variant since a family member, who did not have the *TFGB3* variant, exhibited clubfoot in the absence of any other systemic feature (cardiac investigation not performed). Differences in results between previous studies and ours may reflect differences in clinical assessment and/or in reason of referral of genetic testing.

Consistently with data from literature and databases, in our cohort we observed missense, splice site, and truncating variants spanning the entire gene, with a mutation hotspot in the RKKR motif. Finally, we described the first homozygous *TGFB3* patient, who presented with aortic dilatation at a younger age and more marked systemic features, compared to her heterozygous relatives.

Limitations of the study

This study is limited by small patient numbers, relatively young age of patients, possible ascertainment bias due to recruitment, and retrospective nature of the data.

Conclusion

No more than 50 patients carrying disease-causing *TGFB3* variants have been reported so far [1, 3-9]. Here, we described genetic and clinical data from 32 *TGFB3* patients from 17 families including the first homozygous individual. In our cohort, variants in

TGFB3 were associated with a high frequency of systemic features. Aortic disease (dilatation or dissection) was revealed in 35% of patients, but no increased overall mortality nor pregnancy complications were observed. Nevertheless, homozygosity might be associated with a more severe phenotype.

Finally, we suggest that regular surveillance of distal aorta is appropriate in TGFB3 patients.

Online supplement

For the online supplement, containing supporting information please access through the QR-code below.

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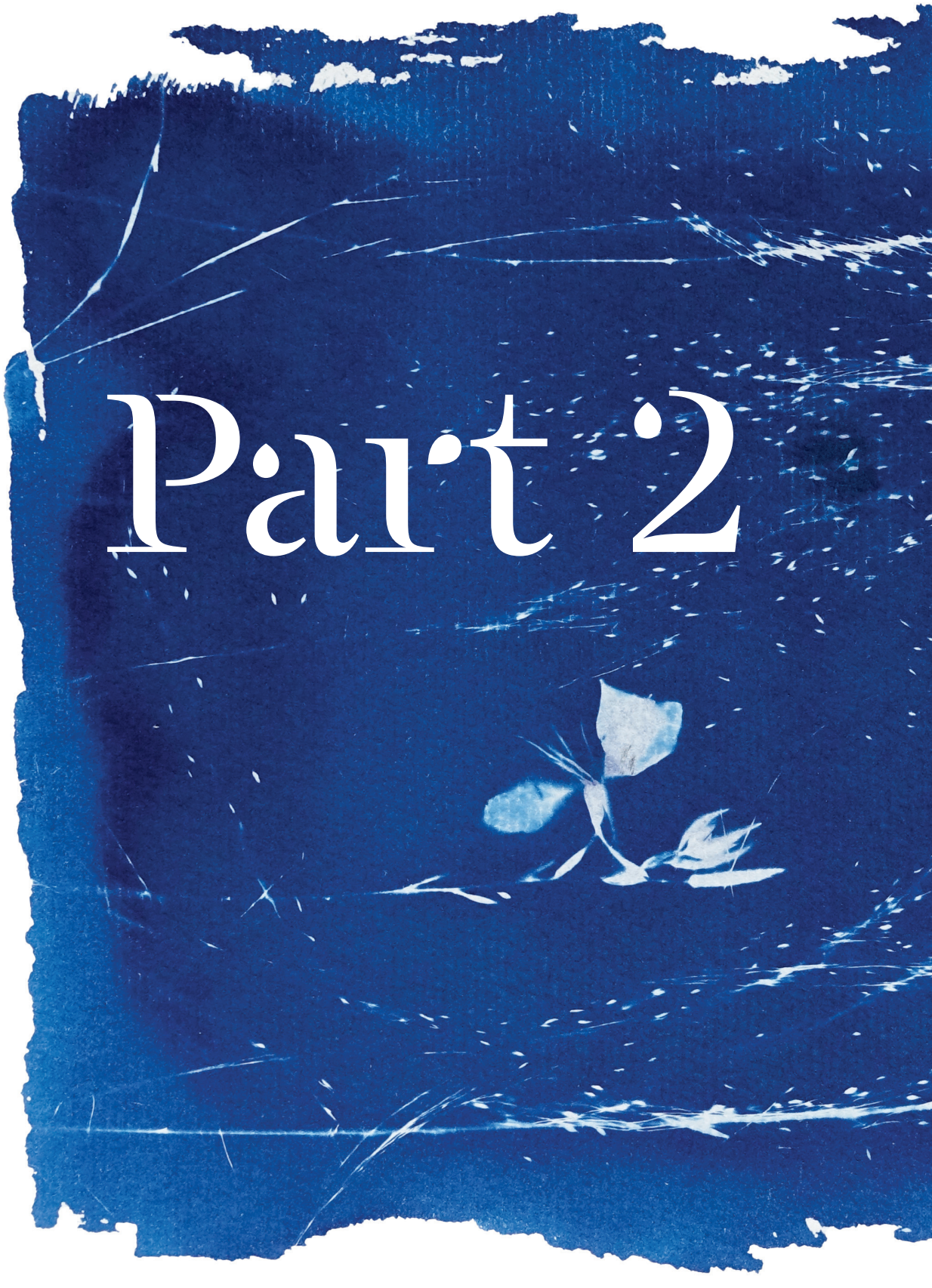
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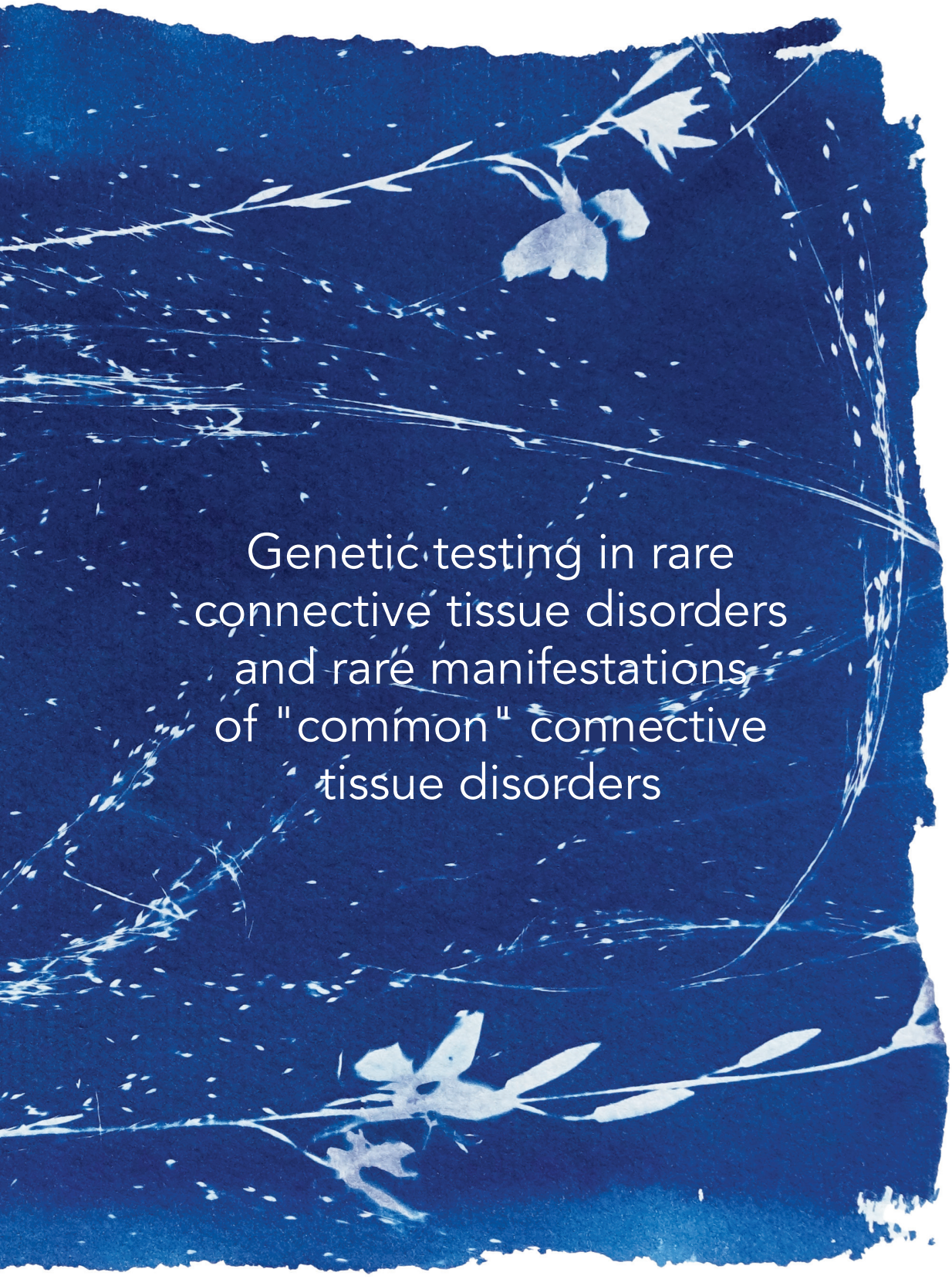

Genetic testing in rare connective tissue disorders and rare manifestations of "common" connective tissue disorders


6

Iris flocculi and type B aortic dissection

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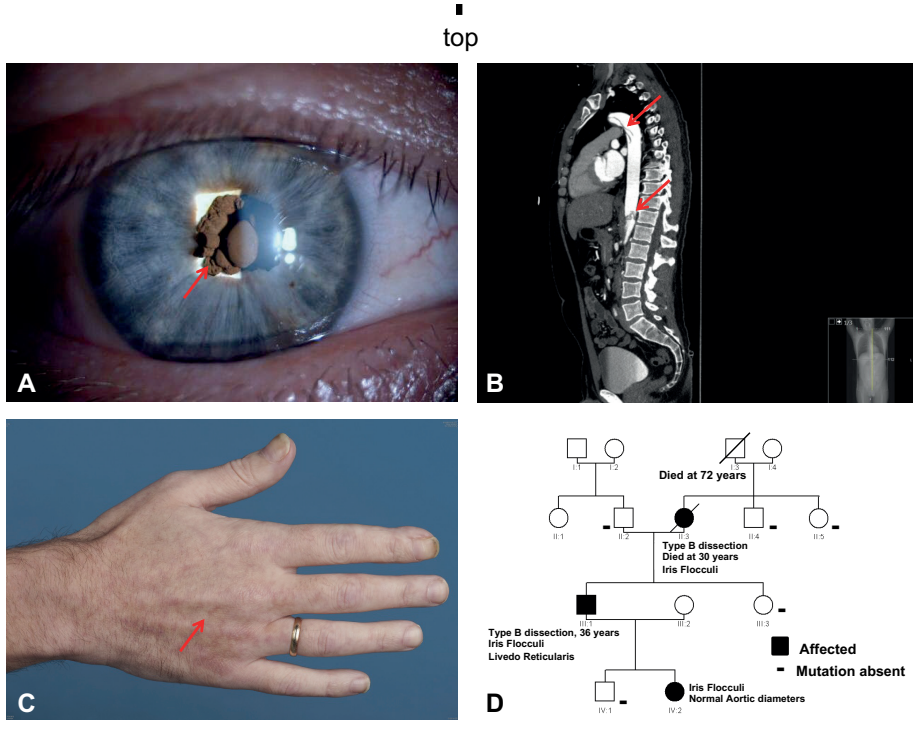
Ophthalmology 2017

A 36-year-old man with bilateral iris flocculi since childhood, developed a type B aortic dissection (Figure 1**A** and **B**, indicated with red arrow). Physical examination revealed livedo reticularis on his extremities (Figure 1**C**, indicated with red arrow). The medical history of his relatives is depicted in the pedigree (Figure 1**D**). DNA testing revealed mutation c.445C>T, p.(Arg149Cys) in the smooth muscle a-actin-2 gene (*ACTA2*, Online Mendelian Inheritance in Man MIM# 102620). Red flags for this *ACTA2* mutation are iris flocculi, thoracic aortic aneurysms and dissections, and livedo reticularis. Early recognition of this disorder by the ocular phenotype can be lifesaving, as often



Autosomal dominant Marfan syndrome caused by a previously reported recessive *FBN1* variant

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Abstract

Background: Pathogenic variants in *FBN1* cause autosomal dominant Marfan syndrome, but can also be found in patients presenting with apparently isolated features of Marfan syndrome. Moreover, several families with autosomal recessive Marfan syndrome caused by pathogenic variants in *FBN1* have been described. The aim of this report is to underline the clinical variability that can be associated with the pathogenic variant c.1453C>T, p.(Arg485Cys) in *FBN1*. **Methods:** We provide the clinical details of two autosomal dominant families with this specific *FBN1* variant, which was previously associated with autosomal recessive Marfan syndrome. **Results:** Clinical data of 14 individuals carrying this variant from these two families were collected retrospectively. In both families the diagnosis of autosomal dominant Marfan syndrome was established based on the characteristics of the variant and the phenotype which includes aortic aneurysms and dissections. Of interest, in one of the families, multiple relatives were diagnosed with early onset abdominal aortic aneurysms. **Conclusion:** In conclusion, *FBN1* variant c.1453C>T, p.(Arg485Cys) is a pathogenic variant that can cause autosomal dominant Marfan syndrome characterized by a high degree of clinical variability and apparently isolated early onset familial abdominal aortic aneurysms.

Introduction

Marfan syndrome (MFS, MIM# 154700) is a multisystem disorder with an estimated prevalence of 1 in 5,000-10,000. MFS is caused by pathogenic variants in *FBN1* (MIM# 134797), encoding fibrillin-1, and is classically characterized by autosomal dominant inheritance [1]. However, several MFS families with an apparently autosomal recessive mode of inheritance have been reported [2-5]. A large proportion of pathogenic *FBN1* variants causing MFS are missense variants, commonly occurring in EGF-like domains and involving cysteine residue substitutions with a predicted dominant negative effect [6]. MFS is classically characterized by skeletal features, ectopia lentis (EL) and thoracic aortic aneurysms and dissections. The diagnosis is based on the revised Ghent criteria [7]. Diagnosing MFS is essential since cardiological surveillance and, when indicated, timely aortic surgery is lifesaving [8]. The most feared complication of MFS, aortic dissection, is reported in up to 50% of undiagnosed MFS patients and may be the presenting feature of unrecognized MFS [9]. Aortic aneurysms and dissections in MFS are typically located in the aortic root and ascending aorta; however, the descending and abdominal aorta may be involved as well [7, 10-12] . Pathogenic variants in *FBN1* may result in classical MFS but have also been reported in families presenting with, for example, apparent isolated thoracic aortic aneurysms and dissections [13].

The clinical features of two families with autosomal dominant MFS caused by *FBN1* variant c.1453C>T, p.(Arg485Cys) and a high rate of abdominal aneurysms is presented here. Homozygosity for this variant was previously reported to cause autosomal recessive MFS in a consanguineous family [2]. In addition, this variant was reported in a heterozygous state in one patient in a Taiwanese MFS cohort [14]. Only limited clinical information was provided in this publication. Our report illustrates the importance of clinical follow-up in *FBN1* mutation carriers, irrespective of previously reported phenotypes associated with that specific variant and suggested mode of inheritance.

Material and methods

We retrospectively collected the clinical data of two families (n=14 patients) with the heterozygous c.1453C>T variant in *FBN1* (NC_000015.9(NM_000138.4):c.1453 C>T p.(Arg485Cys)). The families were referred for DNA diagnostics by their clinical geneticists from VU University Medical Center and Leiden University Medical Center, the Netherlands. Informed consent for DNA diagnostics was obtained from all

patients. Next generation sequencing (NGS) gene panel diagnostics including 13 genes associated with hereditary thoracic aortic disease (*ACTA2*, *COL3A1*, *FBN1*, *FBN2*, *MYH11*, *MYLK*, *PLOD1*, *SLC2A10*, *SMAD3*, *TGFBR1*, *TGFBR2*, *EFEMP2* and *ELN*) was performed. Assessment of the study protocol by our ethics committee was not required since only anonymized data collected during regular patient care were used. Both pedigrees have been slightly adapted in order to ensure privacy.

Results

Family 1 (Figure 1A indicates the pedigree at initial presentation of the family, Figure 1B indicates the pedigree after several years follow-up, Table 1): The proband (III:2) and her daughter (IV:2) were referred for genetic analysis because of the familial occurrence of abdominal aortic aneurysms (AAA) and a type B aortic dissection at older age. Both were diagnosed with an AAA (4.0 cm at the age of 62 years and 5.0 cm at the age of 38 years, respectively). Ophthalmological and physical examination did not reveal any signs of MFS. NGS gene panel diagnostics in IV:2 revealed *FBN1* variant c.1453C>T, p.(Arg485Cys) which was confirmed by Sanger sequencing in III:2. This variant substitutes an arginine by a cysteine in a calcium-binding(cb)-EGF-like domain of fibrilline 1. Introduction of a cysteine in a cb-EGF-like domain likely affects the formation of disulfide bridges within the domain. This type of alteration is generally considered to be pathogenic [7]. However, because of the nonspecific phenotype and the fact that this variant had been reported in a family with autosomal recessive MFS [2], the heterozygous variant was initially classified as likely pathogenic. In order to clarify the clinical significance of this variant in heterozygote state, we offered combined clinical and genetic screening to first degree relatives of family members with an aneurysm or dissection. During follow-up, the proband, her daughter and several other family members carrying the *FBN1* variant (III:2, III:4, III:5, IV:1, IV:2) were diagnosed with hallmark cardiovascular features of MFS (Figure 1B, Table 1). Of note, no relatives were diagnosed with significant ocular and/or skeletal involvement. Based on the cosegregation and the associated cardiovascular phenotype during follow-up, the variant was re-classified to a dominant pathogenic variant and the diagnosis of MFS was established in this family.

Family 2 (Figure 1C, Table 1): The proband (III:1) was referred to a clinical genetics outpatient clinic at 39 years of age for genetic counseling after a type A aortic dissection and an aneurysm of a coronary artery. Physical examination revealed downslanting

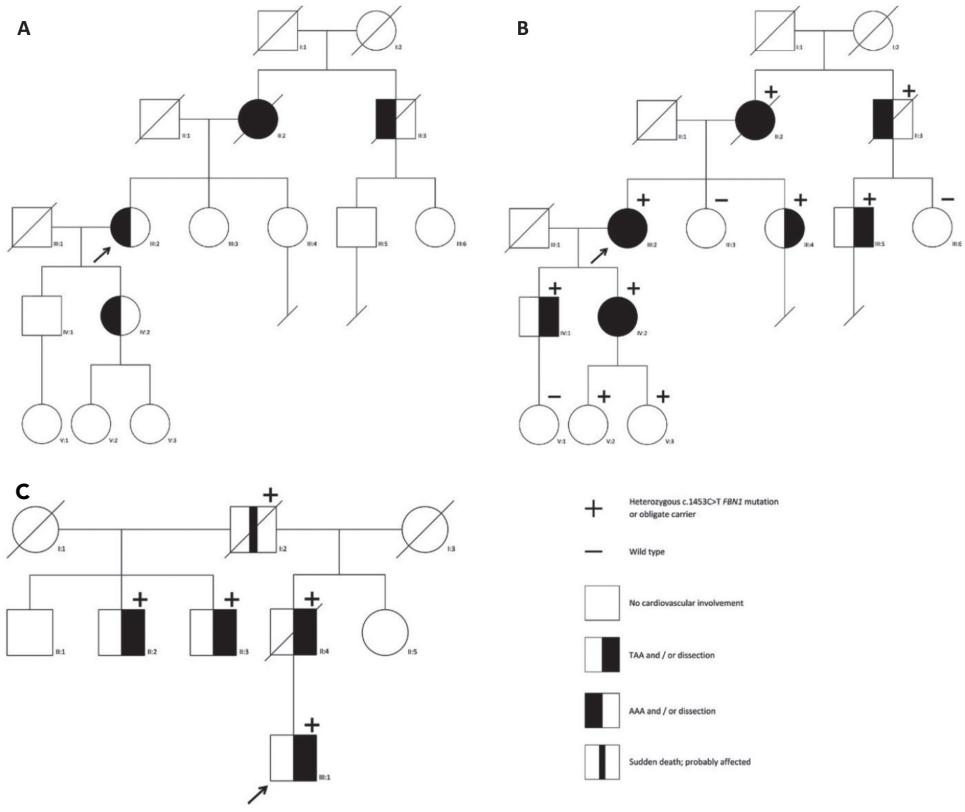

Figure 1 Pedigrees of families 1 and 2.

A indicates the pedigree at initial presentation of family 1,
B indicates the pedigree of this family after several years follow−up.
C Shows family 2. The proband is indicated with an arrow.

palpebral fissures, scoliosis and pes plani. His father (II:4) was diagnosed with a type A dissection at the age of 42 years. He died at the age of 59 years due to heart failure. The paternal grandfather (I:2) died suddenly at the age of 57 years. The c.1453C>T, p.(Arg485Cys) variant in *FBN1* was identified by NGS gene panel diagnostics resulting in the diagnosis MFS. Both the father and the paternal grandfather were obligate carriers, since the paternal half-brothers (II:2 and II:3) of the father were also found to carry the *FBN1* variant. II:2 had an aortic sinus of 4.0 cm and an elongated sinotubular junction at the age of 51 years, whereas II:3 was diagnosed with a thoracic aortic aneurysm of 4.1 cm at the age of 47 years. In addition, both of them had minor signs of MFS at physical and/or ophthalmological examination.

In both families, NGS analysis revealed no other (likely) pathogenic variants or variants of unknown significance.

Table 1 Clinical details of families 1 and 2, and the previously published family (De Vries *et al*. [2])

	Patient	c.1453C>T*	Cardiovascular involvement	Ocular involvement	Skeletal involvement, other features
Family 1	II:2	OC	Type B dissection 63y, rupture AAA 73y	Unknown	Unknown
	II:3	OC	Rupture AAA 80y	Unknown	Unknown
	III:2	Het	AAA 62y (E.S.), bilateral subclavian aneurysm 66y (E.S.), TAA 69y	None	Elongated facies, malar hypoplasia
	III:4	Het	Type A dissection 59y	None	Malar hypoplasia, pectus carinatum, scoliosis
	III:5	Het	Type B dissection 58y	None	None
	IV:1	Het	TAA 46y (E.S.)	None	Pectus excavatum, pes plani
	IV:2	Het	AAA 38y (E.S.), type B dissection 41y	None	None
	V:2	Het	None 18y	None	None
	V:3	Het	None 14y	None	None
	III:3	WT	None 62y	None	None
	III:6	WT	None 48y	None	None
	V:1	WT	Unknown	NP	Unknown
Family 2	I:2	OC	Sudden death 57y	Unknown	Unknown
	II:2	Het	Borderline TAA 51y	Myopia > 3 dpt	Span to height ratio >1.05
	II:3	Het	TAA 47y	None	Downslanting palpebral fissures, elbow contractures, pectus carinatum, pes plani
	II:4	OC	Type A dissection 42y, died at 59y heart failure	Unknown	Unknown
	III:1	Het	Type A dissection 39y, dilatation coronary artery 39y	NP	Downslanting palpebral fissures, scoliosis, pes plani
De Vries et al.	II:1	Het	None 43y	None	Span to height ratio >1.05, high palate
	II:2	Het	None 43y	None	None
	II:3	Het	None 37y	None	Span to height ratio >1.05, high palate
	II:4	Het	Aortic root 40 mm† 40y	None	None
	III:1	Hom	MVP 13y, distal TAA dissection 20y, TAA 22y (E.S.), died 23y	Bilateral lens subluxation, ptosis	Scoliosis, elbow contractures, pectus excavatum, highly arched palate, facial appearance, pneumothorax
	III:4	Hom	None 13y	Bilateral lens subluxation, flat cornea	Highly arched palate, lumbosacral dural ectasia

Given the initial uncertainty about the pathogenicity of the variant, cardiologic and/or ophthalmologic evaluation was also performed in several individuals without the variant in family 1 (patient III:3, III:6 and V:1).

AAA: abdominal aortic aneurysm; E.S.: elective surgery; Het: heterozygous; Hom: homozygous; MVP: mitral valve prolapse; NP: not performed; OC: obligate carrier; TAA: thoracic aortic aneurysm; WT: wild type; y: age in years.
† Considered normal for BSA
* Nomenclature *FBN1* variant according to HGVS:
NC_000015.9(NM_000138.4):c.1453C>T p.(Arg485Cys)

Discussion

In total we present the phenotypic features of 10 genetically confirmed carriers and four obligate carriers of variant c.1453C>T, p.(Arg485Cys) in *FBN1*. These data show that this variant -contrary to earlier observations- is a cause of autosomal dominant MFS. In 2007, de Vries *et al*. reported two cousins with MFS caused by the homozygous c.1453C>T *FBN1* variant, while the four heterozygous parents (ages 37-43 years) did not fulfill the original Ghent criteria for MFS at that time [7]. This variant has not been identified in large population databases (ExAC, gnomAD and GoNL) and has, to our knowledge, only been published in one additional patient from a Taiwanese MFS cohort [14].

Though MFS is generally characterized by a dominant mode of inheritance, several other MFS families with an apparently autosomal recessive mode of inheritance have been reported in literature [3-5]. Prior to the availability of *FBN1* analysis, Fried *et al*. already suggested the possibility of an autosomal recessive mode of inheritance in MFS [5]. Hilhorst-Hofstee *et al*. described three MFS patients homozygous for *FBN1* variant c.7454A>T, p.(Asp2485Val) [3]. In this family, 13 heterozygous relatives were identified, of which only one was diagnosed with MFS based on the original Ghent criteria [7]. Khan *et al*. reported a 3-year-old girl with bilateral lens subluxation and facial features suggestive of MFS carrying *FBN1* variant c.7258A>C, p.(Asn2420His) homozygously [4]. Her heterozygous parents were unaffected. In addition, several families with autosomal dominant MFS have been described in which family members carrying either homozygous or compound heterozygous *FBN1* variants were more severely affected; however this was not always the case [15-18]. Because the c.1453C>T, p.(Arg485Cys) *FBN1* families we describe show a clear autosomal dominant pattern of inheritance, the former report of apparently autosomal recessive MFS due to homozygosity of this variant might be due to age dependent penetrance and clinical variability. The age at evaluation of the heterozygous parents of the apparently autosomal recessive family varied between 37 and 43 years and unfortunately cardiological follow-up data are not available. The age at diagnosis of aortic aneurysms and/or dissections in the two presented autosomal dominant families ranged from 38 to 80 years. Therefore, the cardiological phenotype in the unaffected carriers of the variant might still develop during further follow-up. In the literature, a high degree of clinical variability has been reported concerning the age of onset, the severity and extent of the clinical manifestations. Different genetic mechanisms, including a second pathogenic variant in another gene associated with thoracic aortic aneurysm and a polygenic model

involving multiple modifier loci, are suggested to be a cause of this clinical variability in MFS by recent research [19].

The variability of cardiovascular involvement is also illustrated by family 1 in which the apparent early onset familial AAA was the reason for referral. AAA have been reported as a feature in MFS, and in rare cases even as the presenting feature [12, 20-22]. Family 2 in this report underlines the importance of DNA testing in individuals with a family history of young patients with AAA and the importance of regular imaging of the abdominal aorta in individuals with Marfan syndrome.

Conclusions

This study corroborates the high degree of clinical variability associated with variants in *FBN1* and provides novel insights into the pattern of inheritance of *FBN1* variant c.1453C>T, p.(Arg485Cys). Moreover, it underlines the importance of clinical follow-up in heterozygous *FBN1* mutation carriers irrespective of the previously suggested mode of inheritance related to a specific variant.

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Spontaneous coronary artery dissection as the presenting feature of vascular Ehlers Danlos syndrome

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Cardiogenetics 2021

Abstract

A spontaneous coronary artery dissection as the sole presenting feature of vascular Ehlers Danlos syndrome is an uncommon finding. We present a 33-year-old woman with sudden onset chest pain caused by a spontaneous coronary artery dissection. Genetic testing revealed vascular Ehlers Danlos syndrome as the underlying cause. Specifically, we show the value of genetic testing, which, in some patients, may be the only way of establishing a diagnosis.

Introduction

Spontaneous coronary artery dissection (SCAD) is a rare cause of acute coronary syndrome and sudden cardiac death. SCAD is the result of the formation of a hematoma in the coronary arterial vascular wall, in the absence of a traumatic or iatrogenic cause [1]. The most common cause is atherosclerosis. In addition, SCAD is associated with pregnancy, fibromuscular dysplasia and vigorous exercise. In approximately 3% of SCAD cases a genetic defect can be identified [2]. Genetic causes include polycystic kidney disease and connective tissue disorders. Establishing a genetic diagnosis allows for disease specific follow-up for the patient. In addition, presymptomatic testing and preventive measures in relatives may be lifesaving. Here, we present the case of a previously healthy woman diagnosed with SCAD. Genetic testing resulted in the diagnosis of vascular Ehlers Danlos syndrome (vEDS). vEDS is an autosomal dominantly inherited connective tissue disorder caused by pathogenic variants in the COL3A1 gene encoding type III procollagen. vEDS patients are at risk for arterial, bowel and uterine rupture and pneumothorax. In addition, other recognizable phenotypic features are often present. These include thin, translucent skin, easy bruising, characteristic facial appearance, joint hypermobility and acrogeria. The reported prevalence is 1:50,000-1:200,000. However, this is likely to be an underestimation since prevalence estimates in rare genetic disorders are often based on only the 'classical' severe phenotypes of the disorders [3]. This is not surprising as only a few years ago DNA testing was very time consuming and expensive, and was only performed in patients with a high prior risk of a genetic disease. In 2017, criteria suggestive for vEDS were proposed. Arterial rupture or dissection in individuals younger than 40 years is one of the major criteria [4]. This report underlines that SCAD may be the sole presenting feature of vEDS in the absence of other features associated with the disorder. In addition, this report shows that genetic testing may be the only way of establishing a diagnosis.

Case

A 33-year-old previously healthy woman presented with sudden onset chest pain. She was diagnosed with a myocardial infarction caused by SCAD. She was treated successfully by percutaneous coronary intervention with placement of four stents (Figure 1). Since in a minority of patients a spontaneous arterial dissection is caused by an underlying genetic cause, she was referred for genetic counselling. Her medical history (including two uncomplicated pregnancies), family history and physical

examination did not reveal any further signs indicating a connective tissue disorder or hereditary kidney disease. The prior risk of a genetic cause therefore was low. A previously reported heterozygous pathogenic variant, c.1744G>A p.(Gly582Ser), in the COL3A1 gene was identified using targeted next generation sequencing analysis of 21 genes associated with aortic dilatation and Marfan-like syndromes, confirming the diagnosis vEDS. Establishing the diagnosis allowed for disease specific recommendations including vascular imaging, strict regulation of blood pressure, and additional vascular imaging. Regulation of blood pressure was first attempted with celiprolol, as suggested by the BBEST study, but was switched to atenolol due to side effects (frequent headaches) [5]. MRA was performed and no other arterial aneurysms or dissections were present. The patient remains under regular surveillance. Relatives were offered the option of pre symptomatic genetic testing.

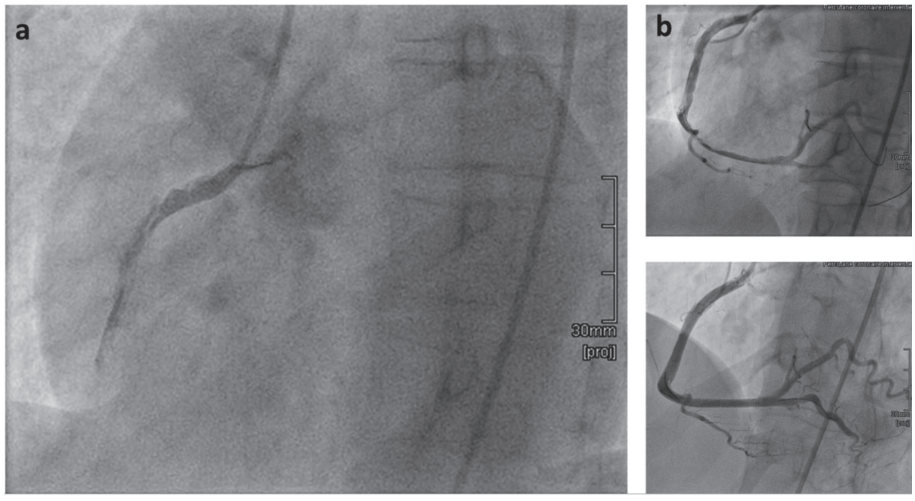

Figure 1 **A** Type F coronary dissection, caused by a Type 4 SCAD, of the right coronary artery (RCA) before intervention; TIMI flow grade 0. **B** Above Restoration of flow after wiring, however widespread coronary dissection and diminished coronary flow remain. Below Result after percutaneous coronary intervention with four drug-eluting stents with restoration of coronary flow.

Discussion

This case underlines that isolated SCAD can be the sole presenting feature of vEDS and further illustrates the clinical variability that can be associated with pathogenic COL3A1 variants. The role of genetic testing in SCAD patients remains to be established. In a recent study, among 384 SCAD survivors from the UK a pathogenic variant was detected in 14 patients (3.6%), including two COL3A1 variants [2]. In many cases, the presence of recognizable skeletal features, age at presentation or family history will provide additional clues for an underlying genetic disease. However, these clues are easily missed, and they may be completely absent as illustrated by this report [6]. In some patients, DNA testing can therefore be the only way to diagnose these genetic disorders. In families and patients without the classical presentation of a genetic predisposition, it is likely that DNA testing is not performed, resulting in preventable morbidity and mortality. This is illustrated by a recent study by our group among 810 patients referred for genetic testing after diagnosis of a thoracic aortic aneurysm or dissection. A (likely) pathogenic variant was detected in 66 of 810 patients (8.1%). The mean age at DNA testing in the group of patients with an identifiable genetic cause was 11 years younger than the mean age in the group without a genetic cause. However, 10 of the 66 patients carrying a pathogenic or likely pathogenic variant (15.2%) were over the age of 60 years at the time of DNA testing. Of these, three had a negative family history for aortic disease and no systemic features of a connective tissue disorder [7]. These observations indicate the need for increasing awareness of these genetic disorders and improvement of evidence-based guidelines for DNA testing. The time and costs associated with DNA testing have decreased rapidly over the recent years. In addition, the availability of large genomic population databases has facilitated the interpretation of detected variants. When combined, these factors have resulted in the possibility of genetic testing at lower thresholds in many countries. Evaluation of existing guidelines in breast cancer patients indicated that nearly half the (likely) pathogenic variants are missed using current guidelines for DNA testing. The option of offering all breast cancer patients the possibility of genetic testing is currently being debated by some experts [8, 9]. This debate is likely to include other diseases, possibly including SCAD in the near future.

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9

Hereditary thoracic aortic disease associated with cysteine substitution c.937T>G p.(Cys313Gly) in *FBN1*

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A 56-year-old male was diagnosed with a type A aortic dissection, followed by a type B dissection 3 years later. There were no other signs indicating a familial connective tissue disorder. Pathogenic variant c.937T> G p.(Cys313Gly) in *FBN1* [(NM_000138.4), MIM* 134797] was identified by DNA testing, consistent with Marfan syndrome (MIM# 154700). The variant was identified in 21 out of 53 tested relatives (Figure 1). A thoracic aortic aneurysm was diagnosed in eight relatives carrying the variant, three of whom met the criteria for preventive surgery. One of the deceased obligate carriers probably had a thoracic aortic aneurysm. Most mutation carriers had a systemic score [1] of zero or one, although the highest score was four. As illustrated by this image, *FBN1* variant c.937T>G p.(Cys313Gly) can cause isolated aortic disease. Timely recognition of individuals with a pathogenic *FBN1* variant is highly important, as it enables the prevention of severe cardiovascular complications [2, 3].

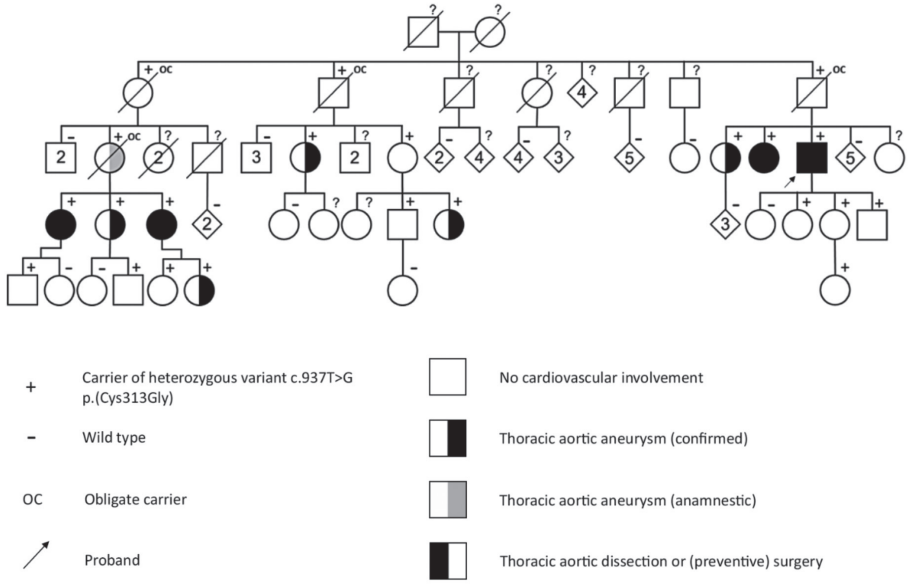

Figure 1 Pedigree depicting thoracic aortic aneurysms and/or dissections

Squares represent males, *circles* indicate females

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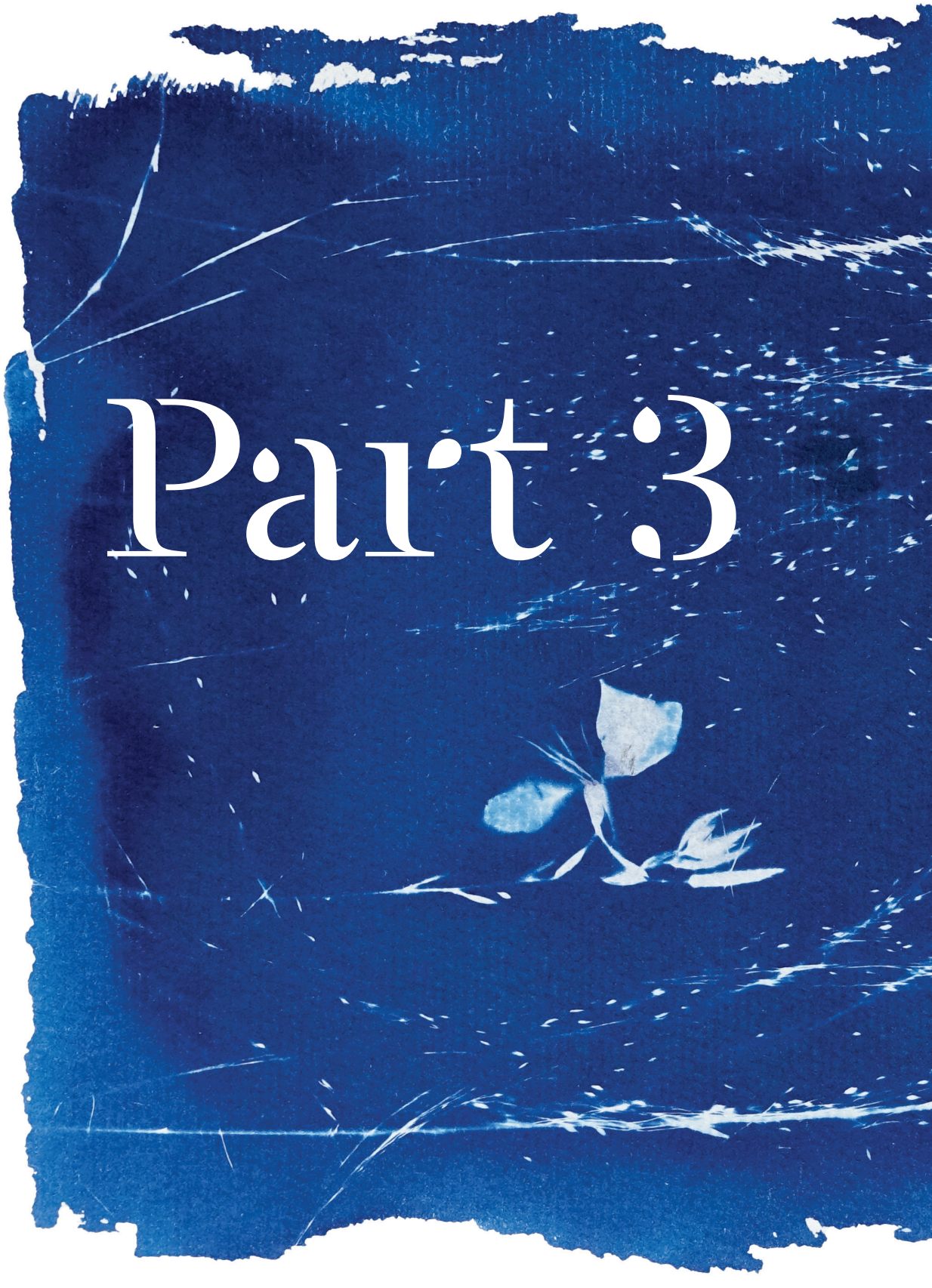
Part 3

General discussion and summary


10

General discussion and future perspectives

General discussion and future perspectives

Recent advances in molecular genetics have resulted in the identification of disease-associated genes at a rapid pace over the past years. Given the decreased cost and time associated with genetic testing, currently, in many cases multiple disease associated genes are analyzed in parallel and even whole exomes or genomes can be sequenced for diagnostic purposes. For many genetic diseases, surveillance, early detection and treatment provide tremendous potential health benefits for not only the index patient, but also for at-risk relatives. In this thesis opportunities provided by these developments are illustrated by our clinical and genetic studies in hereditary connective tissue disease. Notwithstanding these valuable opportunities, the recent developments also bring us with new challenges for the future, several of which will be discussed here.

Genetic disorders associated with aortic aneurysms and/or vascular fragility are examples of conditions where detecting a genetic predisposition may be lifesaving. Patients and their relatives carrying a pathogenic variant in a high-risk gene associated with aortic disease or vascular fragility are at an increased risk of sudden death due to dissection or rupture, often at a young age. Regular imaging of the aorta (and other arteries) often allows for timely preventive surgery. Recommendations for cardiological screening and thresholds for preventive surgical intervention are increasingly based on the specific underlying disease-causing gene [1, 2]. Furthermore, the identification of a pathogenic variant allows reproductive choices to be made (e.g. pre-implantation genetic testing).

The estimated prevalence of genetic disorders is often based on the 'classical' severe presentation and is likely to be an underestimation. This is illustrated in this thesis by the identification of pathogenic *FBN1* variants in patients with aortic disease without any further signs of Marfan syndrome. This is not surprising, as until recently, DNA testing was time-consuming and expensive. In addition to the associated time and costs, the lack of control databases resulted in a relatively high risk of finding variants of uncertain significance and misdiagnosis. Therefore, genetic testing was mostly considered in patients with a severe (classical) phenotype and a relatively high likelihood of finding a pathogenic variant. Due to the lowered threshold for genetic testing, we now know that many of these classical phenotypes represent the most severe end of a clinical

spectrum. The milder part of the spectrum may be unrecognizable without genetic testing.

Genetic testing in connective tissue disorders, including aortic aneurysms and/or vascular fragility, is associated with several challenges.

- It is likely that currently not all disease-associated genes have been identified yet given the relatively low yield of genetic testing in, for example, familial aortic disease. Whole exome and whole genome case control studies in large databases, such as the UK biobank [3], with patients from different backgrounds allow an unprecedented opportunity to identify rare disease associated genes. Potential gene-disease associations of recently discovered genes in these large-scale case-control studies need to be validated and re-evaluated over time. To systematically address this issue, the ClinGen initiative was initiated where experts in the field systematically assess the clinical and experimental evidence to assess gene-disease associations (https://clinicalgenome.org/). These efforts are essential in establishing evidence-based gene panels for genetic disorders.
- Our observations further underline the incomplete penetrance and the sometimes highly variable phenotype associated with hereditary aortic disease [4-9]. Our findings emphasize the need for increasing awareness of the clinical variability of these disorders, the overlap with disease in the general population, and improved evidence-based guidelines for genetic testing to increase the detection rate of high-risk patients. Recognizable features can be completely absent and the age at aortic dissection in hereditary aortic disease can be similar to that observed in sporadic (non-monogenetic) cases in the general population. In some patients, DNA testing is the only way to diagnose a genetic disorder. In families and patients without the classical presentation of a genetic predisposition, it is likely that DNA testing is not performed, resulting in potentially preventable morbidity and mortality, as illustrated by several of our publications. Of course, the prior risk of these patients of having a monogenetic disorder is very low. Genetic counseling is, currently, time consuming and expensive and often associated with a long waiting time before the first appointment. In addition, the possibility of a genetic cause of disease is likely to induce unwarranted anxiety in patients with a very low prior risk. It is therefore likely that initiatives like mainstreaming, where other medical specialists request genetic testing after a brief discussion of the potential outcomes, following a diagnosis of a (sometimes genetic) disease, will increase. An example is breast cancer, where the option of offering genetic testing to all breast cancer patients is currently being debated by some experts in the field [10]. In the near future, this debate is likely to

extend to other diseases, potentially including thoracic aortic disease, ectopia lentis, and spontaneous coronary artery dissection.

- The clinical phenotype in monogenetic disease is not only often highly variable, but in some cases, clues for a genetic disorders can be easily missed since they may be very rare. An example of the latter is the patient with iris flocculi, and a Type B dissection described in this thesis. At the time of presentation only a few patients with iris flocculi and aortic disease caused by a pathogenic variant in *ACTA2* had been reported world-wide. Hopefully, computer-assisted diagnosis will provide a valuable tool in recognizing these very rare disorders in the future. To be effective, these tools should be easily accessible and widely available. A recent example of artificial intelligence (AI) in the recognition of rare genetic disorders is provided by several publications on the recognition of long-QT syndrome, a rare inherited arrhythmia syndrome on ECGs, by AI [11, 12].
- Clinicians are not only tasked with identifying which patient may benefit from DNA testing, but also with providing tailored recommendations to those with an identified disease-causing pathogenic variant. Since it is currently not possible to predict individual risks, often all individuals carrying a pathogenic variant are given the same information about the identified disorder and the same recommendations for surveillance. The complexity of predicting individual risks following the identification of a pathogenic variant is illustrated by the observations in the presented families with pathogenic variants in *TGFB3* and *FBN1*. With the emerging availability of large-scale genomic databases, such as the UK Biobank, it has become clear that many diseases in the general population have a, sometimes large, genetic component. There is increasing evidence that common genetic variants (i.e. minor allele frequency of >1%) can also contribute to the clinical variability in hereditary diseases caused by single rare pathogenic variants [13, 14]. This genetic component consists of many genetic variants of small individual effects. Although these individual genetic variants often convey only a limited effect on disease risk, their effects can be combined in a polygenic risk score (PRS), of which the contribution can be much larger. Interestingly, for several common diseases such as coronary artery disease, the risks in the highest PRS percentiles were shown to be comparable to those in rare monogenetic forms of the disease like familial hypercholesterolemia [15]. It is likely, in the future, that risks for aortic disease for patients carrying a pathogenic variant in *FBN1* identified in a relative with late onset aortic dissection without other features of Marfan syndrome, such as the family presented in this thesis, are considerably lower than those in a family with the classical presentation of Marfan syndrome. The genetic factors constituting the PRS in several diseases have been identified by large scale genome-

wide association studies (GWAS), often including thousands of individuals, in the past few years [16]. It is likely that these efforts will provide us with clinical options for individualized care for many diseases in the years to come.

In conclusion, this thesis illustrates the changes in the field of genetic care for patients with connective tissue disorders as a result of NGS analysis, the clinical applications of this technology and the challenges for the near future.

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11

English summary

English summary

The research in this thesis was performed at the department of Human Genetics of the Amsterdam UMC. At location AMC there is a long-standing history of care for patients with Marfan syndrome and related disorders. In addition, genetic testing for these disorders has been performed at the diagnostic laboratory of the VUmc for years. Combined, we were able to study both clinical and genetic data in a large patient cohort. This proved us with an excellent opportunity for clinical research, both in relatively large patient cohorts and in families with these connective tissue disorders.

In **part one**, the outcomes and applications of next generation sequencing (NGS) in different hereditary connective tissue disorders are illustrated by several cohort studies and/or literature studies. These studies illustrate the clinical variability and the importance of genetic testing. NGS allows for relatively cheap and fast analysis of multiple disease-causing genes simultaneously. This enabled genetic testing in patients with a relatively low prior risk of a monogenetic disease. Based on the outcomes of genetic testing in cohort studies such as those described in part 1, guidelines on whom to offer these tests can be established.

In **chapter 2**, we found a pathogenic or likely pathogenic variant in 66 (8.1%) of patients referred to genetic testing for hereditary thoracic aortic disease in a cohort of 810 patients. The added value of copy number variant (CNV) analysis was underlined by the detection of a disease-causing CNV in six of these patients (9.1%). In **chapter 3**, the clinical relevance of genetic testing in patients presenting with ectopia lentis (EL) is highlighted. In 16 out of 24 patients with EL (67%) a genetic diagnosis was established. The clinical relevance of genetic testing is illustrated by two patients: one with EL without any recognizable features of Marfan syndrome with a pathogenic variant in *FBN1*. The second patient was under surveillance by a cardiologist with suspected Marfan syndrome due to EL and mild skeletal features. He could be reassured and discharged from further cardiological follow-up after the identification of a homozygous pathogenic *ADAMTSL4* variants associated solely with EL. Based on a review of literature we propose a model for clinical and genetic screening in patients with a bicuspid aortic valve (BAV) in **chapter 4.** BAV is the most common congenital cardiac defect, associated with valvular dysfunction and an increased risk of aortic aneurysms. Due to the observed familial clustering of BAV, cardiological evaluation is often recommended in first degree relatives. In a minority of patients, a genetic cause can be identified. In **chapter 5,** the phenotypic spectrum associated with Loeys-

Dietz type 5 (LDS5), caused by pathogenic *TGFB3* variants, in a Dutch-French cohort is described in detail. This cohort included the first report of a patient with LDS5 due to a homozygous pathogenic variant in *TGFB3*, who presented with a more severe phenotype when compared to heterozygous relatives.

In **part two**, the high degree of clinical variability associated with a genetic predisposition for aortic disease is illustrated by several cases with a rare and/or mild manifestation of these disorders. In the (relatively recent) past, genetic testing was often only considered in patients with severe or familial disease (i.e. the classical phenotype). It has become clear that in many cases these diseases are part of a clinical spectrum that may only be detectable by genetic testing, as illustrated here.

In **chapter 6**, we report on a family with a very rare disorder: familial iris flocculi and early onset aortic disease. At the time of publication only a few patients with this disorder, caused by a pathogenic variant in *ACTA2*, had been published worldwide. The recognition of the ocular features can be lifesaving. In **chapter 7** we report a family with autosomal dominant Marfan syndrome associated with a previously reported 'recessive' variant in *FBN1*. This observation highlights the clinical variability associated with this variant. In **chapter 8** a patient with spontaneous coronary artery dissection (SCAD) caused by a pathogenic variant in the *COL3A1* gene, associated with vascular Ehlers Danlos syndrome (vEDS), is reported. The patient had no other features of vEDS. In approximately 5% of patients with a SCAD a genetic cause can be identified, sometimes in absence of any other feature of a hereditary disorder. In **chapter 9** the variability of Marfan syndrome is further illustrated. A patient with relatively late-onset aortic disease caused by a pathogenic variant in the *FBN1* gene, causing Marfan syndrome, is described. Family history was negative for aortic disease at the time of presentation with an aortic dissection in the proband. By cascade screening, 21 relatives carrying the pathogenic variant were identified. In three, preventive aortic surgery to treat a potentially life-threatening aneurysm was performed.


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Nederlandse samenvatting

Nederlandse samenvatting

Het in dit proefschrift beschreven onderzoek vond plaats op de afdeling Humane Genetica van het Amsterdam UMC. Locatie AMC kent een lange historie van zorg voor patiënten met de verdenking op Marfan syndroom en aanverwante bindweefselaandoeningen. En het laboratorium voor DNA-diagnostiek op locatie VUmc heeft van oudsher expertise op het gebied van DNA-diagnostiek van deze aandoeningen. Deze combinatie maakte het mogelijk in relatief grote patiëntcohorten met deze bindweefselaandoeningen de klinische en genetische aspecten in kaart te brengen.

In **deel één** zijn de uitkomsten en toepassingsmogelijkheden van next generation sequencing (NGS) bij erfelijke bindweefselaandoeningen in kaart gebracht aan de hand van verschillende retrospectieve cohortstudies en/of literatuuronderzoek. De grote variatie in klinische manifestaties van deze aandoeningen en het belang van DNA-diagnostiek bij het stellen van deze diagnoses worden geïllustreerd. NGS maakt het mogelijk om relatief snel en goedkoop meerdere ziekteveroorzakende genen te analyseren. Hiermee is het haalbaar geworden om genetische diagnostiek aan te bieden bij patiënten met een lagere voorafkans op een monogenetische aandoening. Cohortstudies zoals beschreven in deel 1 kunnen bijdragen aan de ontwikkeling van richtlijnen voor het aanbieden van DNA-diagnostiek.

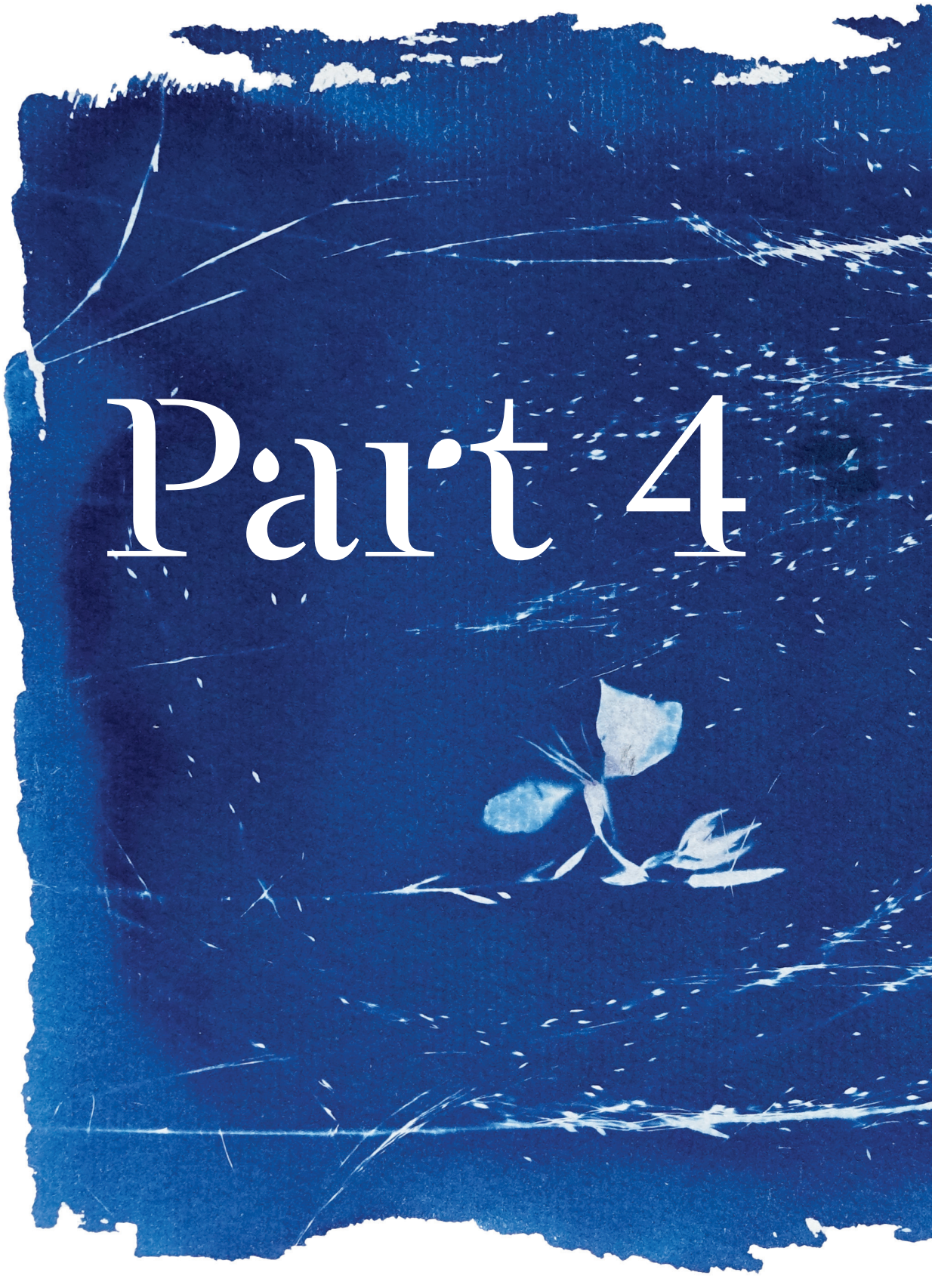
In **hoofdstuk 2** wordt de uitkomst van DNA-diagnostiek beschreven in 810 patiënten met een mogelijk erfelijke aortaziekte. Bij 66 patiënten (8.1%) werd een (waarschijnlijk) pathogene variant aangetoond. Het belang van copy number analyse bij diagnostiek naar erfelijke aortaziekten werd benadrukt met de bevinding dat bij 6 van deze 66 patiënten (9.1%) een deletie of duplicatie als oorzaak werd aangetoond. In **hoofdstuk 3** wordt het klinisch belang van DNA-diagnostiek bij patiënten met een lensluxatie beschreven. Bij 16 van de 24 patiënten (67%) werd een erfelijke oorzaak aangetoond. De klinische relevantie hiervan wordt geïllustreerd aan de hand van twee patiënten. Allereerst een patiënt met een lensluxatie zonder uiterlijke kenmerken bij wie de diagnose Marfan syndroom werd bevestigd op basis van de aanwezigheid van een pathogene *FBN1* variant. De tweede patiënt was sinds jaren onder cardiologische controle vanwege het vermoeden van Marfan syndroom vanwege de lensluxatie en milde skeletkenmerken. Er werd een homozygote pathogene variant in het *ADAMTSL4* gen aangetoond. Hierna waren verdere cardiologische controles niet langer nodig aangezien dit gen alleen geassocieerd is met lensluxaties. Gebaseerd op een overzicht

van de literatuur wordt in **hoofdstuk 4** een voorstel gedaan voor klinische en genetische screening van mensen met de meest frequent voorkomende aangeboren hartafwijking, een bicuspide aortaklep. Een bicuspide aortaklep is geassocieerd met klepdysfunctie en soms met aneurysmata van de thoracale aorta. Aangezien een bicuspide klep veelal bij meerdere mensen binnen een familie aanwezig is, wordt cardiologisch onderzoek bij eerstegraads verwanten geadviseerd. In een kleine minderheid van deze families kan een erfelijke oorzaak worden aangetoond. In **hoofdstuk 5** wordt het klinische spectrum dat geassocieerd is met pathogene varianten in het *TGFB3* gen beschreven. Dit gen is betrokken bij het Loeys-Dietz syndroom type 5. In dit hoofdstuk wordt een cohort Franse en Nederlandse patiënten in detail beschreven. Ook wordt in dit hoofdstuk de eerste patiënt in de literatuur beschreven met een homozygote pathogene variant in het *TGFB3* gen. Deze patiënt had een ernstiger klinisch beeld in vergelijking met haar heterozygote verwanten.

In **deel twee** van het proefschrift wordt de hoge mate van klinische variabiliteit die geassocieerd is met erfelijke aortaziekten beschreven aan de hand van verschillende publicaties over patiënten met een zeldzame en/of milde uiting van deze ziekten. In het (nog vrij recente) verleden werd DNA-diagnostiek in veel gevallen pas overwogen bij patiënten met een zeer ernstig klinisch beeld of een duidelijk belaste familieanamnese (de klassieke presentatie). Het is duidelijk geworden dat veel van deze ziekten onderdeel zijn van een breed klinisch spectrum waarbij soms de diagnose alleen te stellen is met behulp van DNA-diagnostiek, zoals hier wordt beschreven.

In **hoofdstuk 6** wordt een familie beschreven met een zeer zeldzame oogaandoening: familiaire iris flocculi in combinatie met dissecties van de aorta op jonge leeftijd. Ten tijde van de publicatie waren er slechts enkele patiënten met deze aandoening, veroorzaakt door een pathogene variant in het *ACTA2* gen, wereldwijd beschreven. Het herkennen van deze specifieke oogafwijkingen kan levensreddend zijn. In **hoofdstuk 7** beschrijven we een familie met een autosomaal dominant beeld van Marfan syndroom. In deze familie werd een pathogene variant in het *FBN1* gen aangetoond die eerder werd beschreven als oorzaak van autosomaal recessief Marfan syndroom. Deze bevinding benadrukt de hoge mate van klinische variabiliteit geassocieerd met deze *FBN1* variant. In **hoofdstuk 8** beschrijven we een patiënte met een spontane coronair dissectie (SCAD), veroorzaakt door een pathogene variant in het *COL3A1* gen. Dit gen is betrokken bij vasculair Ehlers Danlos syndroom. De beschreven patiënt had geen andere kenmerken van vasculair Ehlers Danlos syndroom. Bij ongeveer 5% van de mensen met een SCAD kan een erfelijke oorzaak worden aangetoond,

soms in afwezigheid van enig ander kenmerk van een erfelijke aandoening. In **hoofdstuk 9** wordt de variabiliteit van Marfan syndroom nogmaals geïllustreerd. Er wordt een patiënt beschreven met een aortadissectie op relatief late leeftijd en een aanvankelijk negatieve familieanamnese. Er werd een pathogene variant in het *FBN1* gen aangetoond waarmee in combinatie met de aortadissectie de diagnose Marfan syndroom kon worden gesteld. Met familieonderzoek werden vervolgens 21 verwanten gevonden die drager bleken te zijn van deze aanleg. Bij drie van hen werd een preventieve operatie vanwege een aneurysma van de aorta verricht.


Part 4

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- **Clinical and genetic aspects of bicuspid aortic valve: a proposed model for family screening based on a review of literature**
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- H.Y. Cheung, Phenotypic spectrum of *TGFB3* disease-causing variants in a Dutch-French cohort and first report of a homozygous patient.	2019	2

Publications

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Eline Overwater was born on the 21st of March 1986 in Amsterdam, the Netherlands. She graduated in 2004 from secondary school (Ignatius Gymnasium, Amsterdam). After she completed her first year of Biomedical Sciences at the University of Amsterdam, she started her medical studies at the University of Amsterdam (AMC). In 2012 she completed her medical degree and started as a resident (ANIOS) at the clinical genetics department at the VUmc. In 2013 she started her clinical genetics training at the AMC. During her work, she became increasingly interested in the genetic aspects of connective tissue disorders. This resulted in the start of her PhD project, which she combined with her training as a clinical geneticist. In 2020 she started as a clinical geneticist at the University Medical Center Groningen.

She lives in Wergea with Nils Knotter and their three children: Toon (2016), Jette (2019) and Onne (2022).

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