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

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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 analyzed 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 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
1 | 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 counseling and DNA testing are increasingly being incorporated into clinical guidelines (Ackerman et al., 2011; Eccles et al., 2016). 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 (Hoyert, Arias, Smith, Murphy, & Kochanek, 2001; Olsson, Thelin, Stahle, Ekstrom, & Granath, 2006). 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 (Hiratzka et al., 2010). 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 (Biddinger, Rocklin, Coselli, & Milewicz, 1997; Coady et al., 1999; Robertson et al., 2016). 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, and 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, and 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 (Gago-Diaz et al., 2014; Regalado et al., 2011, 2016). Given the incomplete penetrance and the highly variable age of onset within both heritable and sporadic TAAD (Campens et al., 2015; Coady et al., 1999; Khalique et al., 2009; Robertson et al., 2016), 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 (den Hartog et al., 2016; Franken et al., 2015; D. Milewicz et al., 2016). A causative variant can be identified in approximately 20% of FTAAD families (D. M. Milewicz, Regalado, Shendure, Nickerson, & Guo, 2014). 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 (Pollack et al., 2002; Shlien & Malkin, 2010; Thapar & Cooper, 2013). 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 (Fromer & Purcell, 2014; Fromer et al., 2012). 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 (Kelsen et al., 2015; Koyama et al., 2017; Poultnay et al., 2013; Spataro et al., 2017). 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.

2 | METHODS

2.1 | 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, TGFBR2, TGFBR3, 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 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.

2.2 | 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 because only genetic and clinical data collected during regular patient care were used.

3 | 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 35 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).

3.1 | 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%), TGFBR2 (N = 4, 6.7%), TGFBR1 (N = 3, 5%), TGFBR2 (N = 3, 5%), FBN2 (N = 3, 5%), MYH11 (N = 2, 3.3%), TGFBR3 (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.

3.2 | 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)_(547+1_549-1)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.

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 (Guo et al., 2013). At the age of 35 years, this male patient was diagnosed with an aortic root dilatation, a type A dissection at the age of 65 years. 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
### 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 | MAF ExAC | Segregation analysisa | Reference |
|---------|------|-------------------|----------------|--------|--------|-------------|---------|------------------------|-----------|
| 1       | ACTA2 | c.115C > T        | p.(Arg39Cys)   | Missense | Actin  | Baker’s yeastb | + / + / − / 180 | Absent | yes                   | (Hoffjan et al., 2011) |
| 2       | ACTA2 | c.116G > A        | p.(Arg39His)   | Missense | Actin  | Baker’s yeastb | + / + / − / 29  | Absent | yes                   | (Guo et al., 2009)    |
| 3       | ACTA2 | c.179C > A        | p.(Ala60Glu)   | Missense | Actin  | Baker’s yeastb | + / + / 107    | Absent | n.a.                  | Novel      |
| 4       | ACTA2 | c.419C > T        | p.(Ala140Val)  | Missense | Actin  | Baker’s yeastb | + / / ± / 64   | Absent | yes                   | (Lerner-Ellis et al., 2014) |
| 5c      | ACTA2 | c.445C > T        | p.(Arg149Cys)  | Missense | Actin  | Baker’s yeastb | + / + / + / 180 | Absent | yes                   | (Guo et al., 2007)    |
| 6       | ACTA2 | c.835A > G        | p.(Thr279Ala)  | Missense | Actin  | Baker’s yeastb | + / + / − / 58  | Absent | n.a.                  | Novel      |
| 7       | ACTA2 | c.854T > C        | p.(Met285Thr)  | Missense | Actin  | Baker’s yeastb | + / / ± / 180   | 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      |
| 9d      | 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 | (Pepin et al., 2014; Schwarze et al., 2001) |
| 11      | COL3A1| c.971G > A        | p.(Gly324Asp)  | Missense | Triple helix | Chickenb | + / / ± / 94 | Absent | de novo<sup>c</sup> | Novel      |
| 12      | COL3A1| c.2050G > A       | p.(Gly684Arg)  | Missense | Triple helix | Chickenb | + / + / + / 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 | Chickenb | + / + / + / 102 | Absent | n.a.                | (Frank et al., 2015) |
| 15      | FBN1  | c.32T > G         | p.(Leu11Arg)   | Missense | Signal peptide | Dogb | + / + / + / 102 | Absent | n.a.                | (Baetens et al., 2011) |
| 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 | Tetraodonb | + / + / + / 98 | Absent | n.a.                | (Steneure et al., 2009) |
| 19      | FBN1  | c.2645C > T       | p.(Ala882Val)  | Missense | TB 4 | Tetraodonb | + / + / + / 64 | Absent | n.a.                | (Aragon-Martin et al., 2010; Comeglio et al., 2007; Howarth, Yearwood, & Harvey, 2007; Hung et al., 2009; B. Loeys et al., 2004; Robinson et al., 2012) |
| 20      | FBN1  | c.2660G > A       | p.(Cys887Tyr)  | Missense | TB 4 | Tetraodonb | + / + / + / 194 | Absent | n.a.                | Novel      |
| 21      | FBN1  | c.2668T > C       | p.(Cys890Arg)  | Missense | TB 4 | Tetraodonb | + / + / + / 180 | Absent | n.a.                | (Colloid-Beroud et al., 2003; Keilty, Rantamaki, Child, Shuttleworth, & Peltonen, 1995) |

(Continues)
| Patient | Gene | Nucleotide change | Protein change | Effect | Domain | Conservation | MAF ExAC | Segregation analysis | Reference |
|---------|------|-------------------|----------------|--------|--------|-------------|----------|---------------------|-----------|
|         |      |                   |                |        |        |             |          |                     |           |
| 22      | FBN1 | c.2953G > A       | p.(Gly985Arg)  | Missense | TB 5   | Tetraodon\(^b\) | + / + / 125 | Absent n.a.          | (Faivre et al., 2009; Howarth et al., 2007; B. Loeys, Nuytinck, Delvaux, De Bie, & De Paepe, 2001; Rommel et al., 2005; Turner et al., 2009; Yoo et al., 2010) |
| 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 | (Attanasio et al., 2008; Comigelio et al., 2007; Hung et al., 2009; Magyar et al., 2009; Rommel et al., 2005; Sheikzadeh et al., 2012; Stheneur et al., 2009) |
| 25      | FBN1 | c.4987T > C       | p.(Cys1663Arg) | Missense | EGF-like 28 | Zebrafish\(^b\) | + / + / 180 | Absent n.a. Novel |          |
| 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. Novel |          |
| 28      | FBN1 | c.6031T > C       | p.(Cys2011Arg) | Missense | EGF-like 34 | Zebrafish\(^b\) | + / + / 180 | de novo \(^d\) Novel |          |
| 29      | FBN1 | c.6942C > G       | p.(Tyr2314*)   | Nonsense (NMD expected) | NA | NA | NA | Absent de novo \(^d\) Novel |          |
| 30      | FBN1 | c.7708G > A       | p.(Glu2570Lys) | Missense | EGF-like 45 | Tetraodon\(^b\) | + / + / 56 | Absent n.a. Novel | (Arbustini et al., 2005; Attanasio et al., 2008; Soylen et al., 2009) |
| 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. Novel | (Buchan et al, 2014) |
| 34      | FBN2 | c.3889G > A       | p.(Gly12975ser) | 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 |          |

(Continues)
| Patient | Gene   | Nucleotide change | Protein change       | Effect                  | Domain                    | Conservation | SIFT/Mutation Taster/ Polyphen-2/ Grantham distance | MAF ExAC | Segregation analysis | Reference                        |
|---------|--------|-------------------|----------------------|-------------------------|---------------------------|--------------|-----------------------------------------------------|---------|----------------------|----------------------------------|
| 37      | MYH11  | c.5293C > T       | p.(Arg1765Trp)       | Missense                | Coiled coil region        | Zebrafish    | +/+/+/+101                                          | 1/115948 | n.a.                 | Novel                            |
| 38      | NOTCH1 | c.2123A > G       | p.(Tyr708Cys)        | Missense                | EGF-like 18               | Tetraodon (Fruitfly)  | +/+/+/+194                                          | Absent  | de novo (inferred)  | Novel                            |
| 39      | PRKG1  | c.530G > A        | p.(Arg177Gln)        | Missense                | cGMP-binding, high affinity | C elegans    | --/+/+/43                                           | Absent  | n.a.                 | (Guo et al., 2013)               |
| 40      | SLC2A10| c.510G > A        | p.(Trp170*)          | Nonsense (NMD expected) | NA                        | NA           | NA                                                  | Absent  | n.a. (consanguineous parents) | (Coucke et al., 2006; Moceri et al., 2013) |
| 41      | SLC2A10| c.1276G > T       | p.(Gly426Trp)        | Missense                | Transmembrane helical region 10 | Tetraodon    | +/+/+/+184                                          | 3/116638 | confirmed paternal carriership | (Callewaert et al., 2008) |
| 42      | SMAD3  | c.1A > T          | p.(Met1?)            | Loss of initiation codon | Initiator methionine      | C elegans    | 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    | +/+/+/+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                  | (Aubart et al., 2014)           |
| 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.                 | (Lindsay et al., 2012; Schubert, Landis, Shikany, Hinton, & Ware, 2016) |
| 51      | TGFB2  | c.989G > A        | p.(Arg330His)        | Missense                | Transforming growth factor beta-2 chain | Tetraodon    | +/+/+/+29                                           | Absent  | Incomplete penetrance? | Novel                            |
| 52d     | TGFB2  | c.1017-1G > T     | p.?                  | Splice (NMD possible)   | Transforming growth factor beta-2 chain | NA          | NA                                                  | Absent  | de novo               | Novel                            |

(Continues)
| Patient | Gene | Nucleotide change | Protein change | Effect | Domain | Conservation | SIFT/Mutation Taster/ Polyphen-2/ Grantham distance | MAF ExAC | Segregation analysis | Reference |
|---------|------|-------------------|----------------|--------|---------|-------------|---------------------------------------------------|---------|----------------------|-----------|
| 53      | TGFB3 | c.899G > A        | p.(Arg300Gln)  | Missense | Latency-associated peptide chain | Fruitfly<sup>b</sup> | + / + / + / 43 | Absent | Yes                  | (Matyas, Naef, Tollens, & Oexle, 2014) |
| 54      | TGFB3 | c.1075A > C       | p.(Ser359Arg)  | Missense | Transforming growth factor beta-3 chain | Fruitfly<sup>b</sup> | + / + / + / 110 | Absent | n.a.                 | Novel     |
| 55      | TGFBR1| c.790G > A        | p.(Ala264Thr)  | Missense | Protein kinase | Fruitfly<sup>b</sup> | + / + / + / 58 | Absent | yes (incomplete penetrance) | Novel     |
| 56      | TGFBR1| c.1255+2T > C     | p.(Tyr378Asnfs*3,0) | 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<sup>b</sup> | + / + / + / 43 | Absent | de novo<sup>d</sup> | (Akutsu et al., 2007; Jondeau et al., 2016; B.L. Loey et al., 2006; Matyas et al., 2006; Melenovsky et al., 2008; Yang et al., 2012) |
| 58      | TGFBR2| c.1565G > A       | p.(Arg522Gln)  | Missense | Protein kinase | Zebrafish<sup>b</sup> | + / + / + / 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_01135599.2), TGFB3: NC_000014.8(NM_032339.4), 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

<sup>a</sup>Yes, segregation analysis performed in (at least) one family member, variant segregated accordingly.
<sup>b</sup>No further alignment available.
<sup>c</sup>This family is recently described in literature (Overwater & Houweling, 2017).
<sup>d</sup>A variant of unknown significance was identified in these patients as well (Supporting Information Table S1).
<sup>e</sup>Paternity and maternity not confirmed.
<sup>f</sup>Homzygous variant.
<sup>g</sup>Tolerated (SIFT), polymorphism (MutationTaster), and benign (Polyphen-2) predictions.
<sup>h</sup>Possibly damaging (Polyphen-2) prediction.
<sup>i</sup>Deleteors (SIFT), Disease-causing (MutationTaster), probably damaging (Polyphen-2) predictions.
Alignment, SIFT, MutationTaster, Polyphen-2, Grantham distance: Alamut GRCh37 accessed July 2017.
| Patient | Involved gene | Sex, age | Cardiovascular feature(s) | Systemic feature(s) | Genotype | Relative | Phenotype |
|---------|---------------|----------|---------------------------|---------------------|----------|---------|-----------|
| 1       | ACTA2         | ♀, 16    | PDA                       | None                | +        | F       | Dis (B, 51 y), CVD |
|         |               |          |                           |                     | –        | PU      | Clinically not affected |
|         |               |          |                           |                     | ?        | PA      | Dis, unconfirmed (deceased) |
|         |               |          |                           |                     | ?        | PGF     | Dis, unconfirmed (deceased) |
| 2       | ACTA2         | ♂, 28    | Dis (A and B, 26 y), BAV  | None                | +        | F       | An (AoR 42 mm, AAo 49 mm, AA, 61 y) BAV |
|         |               |          |                           |                     | –        | Sib     | Clinically not affected |
| 3       | ACTA2         | ♂, 46    | Dis (A, 45 y)             | None                | ?        |        | No relatives clinically affected |
| 4       | ACTA2         | ♀, 69    | Dis (B, 61 y; A, 65 y)    | None                | –        | B (2)   | Clinically not affected |
|         |               |          |                           |                     | +        | Si      | Rup (AA, 62 y) |
|         |               |          |                           |                     | +        | N       | An (AA, 35 mm) |
| 5       | ACTA2         | ♂, 36    | Dis (B, 36 y)             | Iris flocculi, livedo reticularis | +      | M       | Dis (B, deceased, 30 y), iris flocculi |
|         |               |          |                           |                     | +      | D       | Iris flocculi |
| 6       | ACTA2         | ♂, 73    | An (Aor, 52 mm, 69 y)     | None                | ?        |        | No relatives clinically affected |
| 7       | ACTA2         | ♂, 22    | Dis (A, 21 y), BAV        | PP, SS, Myopia – 5/–5 dpt | ?      |        | No relatives clinically affected |
| 8       | ACTA2         | ♂, 57    | Dis (B, 57 y), An (Aor 41 mm, 57 y) | Myopia – 4 dpt, pneumothorax | ?      | B       | SUD (58 y) |
| 9a      | COL3A1        | ♂, 59    | Rup (Aoa, 54 y), An (AA, 59 y) | None               | ?      | B       | Rup (Aoa, deceased, 59 y) |
|         |               |          |                           |                     | ?      | B       | An (AA) |
|         |               |          |                           |                     | ?      | N       | An (AA, severe, 40 y) |
| 10      | COL3A1        | ♂, 52    | Dis (A, 47 y), An (subclavian and vertebral artery, 52 y) | Increased AHR | ?      |        | No relatives clinically affected |
| 11      | COL3A1        | ♀, 44    | Dis (B, 44 y)             | NA                  | -      | Si      | de novo \* |
|         |               |          |                           |                     |        |         | Borderline An (Aor, 40 mm, 51 y), HT |
| 12      | COL3A1        | ♀, 31    | An (renal and carotid artery), Dis (mammary-, subclavian- and iliac artery), occlusion (brachial artery) | None | –      | F       | Clinically not affected |
|         |               |          |                           |                     | ?      | M       | Gastric perforation |
|         |               |          |                           |                     | +      | Si      | Dis (iliac artery) |
| 13      | COL3A1        | ♂, 42    | Dis (A, 38 y)             | Hyperkyphosis, hypermobile fingers | +      | M       | Clinically not affected |
|         |               |          |                           |                     | –      | PU      | Rup (AA, 55 y), CVD |
|         |               |          |                           |                     | –      | PGF     | Rup (AA, 63 y), CVD |
| 14      | COL3A1        | ♂, 45    | Dis (coronary artery, 42 y), An (Aao, 47 mm, 45 y) | Soft skin | ?      |        | No relatives clinically affected |
| 15      | FBN1          | ♂, 66    | Dis (B, 49 y), An (subclavian artery, AA, 54 y) | NA | ?      | So      | Clinical features of MFS |
| 16      | FBN1          | ♀, 27    | An (Aor, 41 mm, 27 y), MVP | Arachnodactyly | ?      | +      | Clinical features of MFS |
|         |               |          |                           |                     |        | M       | No clinical features of MFS (5 months) |
| 17      | FBN1          | ♂, 35    | An (Aor, 50 mm, 35 y), ASD, atrial flutter (23 y) | Growth inhibiting treatment, HAP, crowding, retrogynathia SS, IH | ?      | F      | SUD (44 y), clinical features of MFS |
|         |               |          |                           |                     | ?      | PA      | SUD (43 y), clinical features of MFS |
|         |               |          |                           |                     | ?      | PCo     | Clinical features of MFS |
| 18      | FBN1          | ♂, 5     | An (Aao, 27 mm, Z-score +2.7, 5 y), VSD | PP, hyperkyphosis, wrist sign +, dolichocephaly, malar hypoplasia, EL, BS 8/9 | ?      | F      | No relatives clinically affected |
| 19      | FBN1          | ♂, 53    | An (thoracic aorta, 80 mm, 53 y) | Wrist and thumb sign +, IH | ?      | PF      | Multiple relatives with An and/or Dis |
| 20      | FBN1          | ♀, 36    | An (Aor, severe, 35 y), MVP | Scoliosis, PC, Myopia – 6.5 dpt, SS | ?      |        | No relatives clinically affected |
| 21      | FBN1          | ♂, 11    | NA                       | Increased AHR, PD, clinical features of MFS | NA |        | |

(Continues)
| Patient | Involved gene | Sex, age | Cardiovascular feature(s) | Systemic feature(s) | Family history |
|---------|--------------|----------|---------------------------|---------------------|----------------|
| 22      | FBN1         | ♂, 32    | Dis (A, 15 y), MVP        | Marfanoid habitus, PP, reduced elbow extension, arachnodactyly, HAP, crowding, myopia -5/-3 dpt, SS | ? No relatives clinically affected |
| 23      | FBN1         | ♀, 0     | An (AoR, 0 y), 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 | + MMF MU Arachnodactyly, tall stature Anamnestic MFS Premature birth, intracranial bleeding, epilepsy, spasticity, developmental delay |
| 25      | FBN1         | ♀, 29    | An (AoR, 41 mm, 29 y), MI | Arachnodactyly, HAP, dolichocephaly, EL, RD | ? F SD (42 y), 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 |
| 29      | FBN1         | ♀, 10    | An (AAo, 31 mm, Z-score +2.7, 10 y) | PD, PP, arachnodactyly, HAP, dolichocephaly, myopia | de novo |
| 30      | FBN1         | ♂, 54    | Dis (A, 54 y)             | Pneumothorax, NA | + So(2) Clinically not affected |
| 31      | FBN1         | ♀, 46    | An (AAo, 46 mm, 46 y), cerebral infarction (33 y), stenosis (axillary-, brachial- and subclavian artery, 36 y) | 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, 39 mm, 54 y) | PE, hyperkyphosis, hammer toes, downslanting, myopia | + F Clinically not affected |
| 35      | FBN2         | ♂, 65    | An (AAo, 45 mm, 64 y)     | Hammer toes, HAP, enophthalmos, prominent eyes, and nose, malar hypoplasia | ? – F B An (AA, at older age) An (AAo, 45 mm, 39 y) |
| 36      | MYH11        | ♂, 71    | Dis (A and B, 70 y), An (AA, 54 mm, 71 y) | None | ? M Rup (aorta, deceased) |
| 37      | MYH11        | ♂, 59    | An (AAo, 46 mm, 58 y), BAV, PFO | PP, malar hypoplasia, cutaneous hyperextensibility | ? No relatives clinically affected |
| 38      | NOTCH1       | ♂, 77    | An (AAo and AoA, 85 mm, 77 y) | None | de novo (inferred, mosaic) No relatives clinically affected |

(Continues)
**TABLE 2 (Continued)**

| Patient | Involved gene | Sex, age | Cardiovascular feature(s) | Systemic feature(s) | Family history | Genotype | Relative | Phenotype |
|---------|---------------|----------|---------------------------|--------------------|----------------|----------|----------|-----------|
| 39      | PRKG1         | ♂, 52   | Dis (subclavian-, iliac- and brachiocephalic artery, 42 y), borderline an (AAo, 40 mm, 52 y) | 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, 17 mm, Z-score +3.3, 5 months), PFO, abnormal course AoA, and pulmonary vessels | Arachnodactyly, abnormal thumb position, downslanting, hypertelorism, HAP, diaphragmatic hernia | HE F           | Clinically not affected |
| 42      | SMAD3         | ♀, 62   | Dis (A, 60 y), MI | PP, early onset arthrosis, myopia −2.5/−4 dpt | ? F | An (AA, deceased, 67 y) |
| 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, 32 y), VSD | Brachydactyly type E, hypertelorism, prominent venous pattern, varicose veins, recurrent hematomas, myopia −6 dpt, IH, UH | ? M | SUD (cause unknown, 50 y) |
| 45      | SMAD3         | ♀, 76   | Dis (B, 63 y), An (AoA, 60 mm, 70 y) | Arthralgia, genu valgum, hypermobility, IH | ? + + + | Dis (aorta, deceased, 44 y) |
| 46      | SMAD3         | ♂, 17   | None | Scoliosis, PE, flat cornea | + | Clinically not affected |
| 47      | SMAD3         | ♀, 51   | Dis (A, B, 51 y) | Scoliosis, arthralgia, early onset arthrosis | + So | Clinically not affected |
| 48      | SMAD3         | ♀, 40   | Borderline an (AoR, 40 y), MVP, MI | Wrist and thumb sign +, SS | + F | Dis (A, 57 y), aneurysm (aorta, 40 y), HT |
| 49      | TGFB2         | ♀, 19   | None | Patellofemoral pain syndrome, wrist sign +, BS 7/9, downslanting, varicose veins | − M | Clinically not affected |
| 50      | TGFB2         | ♂, 39   | An (AoR, 55 mm, 25 y), MVP | Scoliosis, PD, wrist and thumb sign +, hypermobility, recurrent hematomas in iliopectos muscle, dural ectasia | ? | No relatives clinically affected |
| 51      | TGFB2         | ♂, 0    | None | Arachnodactyly, joint contractures, retrogastria | + F | No clinical information available |
| 52      | TGFB2         | ♂, 32   | An (AoR, 44 mm, 32 y) | PC, PP, arachnodactyly, HAP, dolichocephaly, enophthalmos, malar hypoplasia, crowding, myopia −6.5 dpt, pneumothorax | − B | de novo |

(Continues)
TABLE 2 (Continued)

| Patient | Involved gene | Sex, age | Cardiovascular feature(s) | Systemic feature(s) | Family history | Genotype | Relative | Phenoype |
|---------|---------------|----------|---------------------------|---------------------|---------------|----------|----------|----------|
| 53      | TGFB3         | ♂, 43    | None                      | Increased AHR, PD, thumb sign +, BS 6/9 | +             | Si       | Clinical features of connective tissue disorder |
|         |               |          |                           |                     | +             | So       | Clinical features of connective tissue disorder |
| 54      | TGFB3         | ♂, 59    | AVI (25 y), An (AoR, 46 mm, 25 y; AoR, 55 mm, AAo 48 mm, 57 y) | PP; HAP, downslanding, UH | -             | So       | Clinically not affected |
| 55      | TGFB1         | ♂, 56    | Dis (A and B, 56 y)       | Scoliosis, PE, dolichocephaly, enophthalmos, malar hypoplasia | +             | M        | Clinically not affected |
| 56      | TGFB1         | ♂, 33    | An (AoR, 43 mm, 31 y)     | SS, dural ectasia | +             | M        | An (AoR, 44 mm, AAo, 44 mm, 58 y) |
|         |               |          |                           |                     | +             | M        | (anterior aorta, 55 y) |
|         |               |          |                           |                     | ?            | MA       | SUD (cause unknown, 64 y) |
| 57      | TGFB1         | ♂, 16    | Dis (thoracic aorta, deceased, 16 y) | PE, tall stature, scoliosis, arachnodactyly | de novo       |          |          |
| 58      | TGFB2         | ♂, 14    | An (AoR, 40 mm, Z-score 4.3, 14 y), VSD, DCRV | None | + | F | An (AoR, 42 mm, 52 y) |
| 59      | TGFB2         | ♂, 15    | None                      | PD, hyperkyphosis, arthralgia, myopia -3 dpt | NA |          |          |
| 60      | TGFB2         | ♀, 16    | An (AoR, 44 mm, 16 y), MVP | PR, 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; PGM, paternal grandmother; PP, pes plani; PU, paternal uncle; RD, retinal detachment; Rup, rupture; SD, standard deviation; S, sister; Sib, sibling; So, son; SS, skin striae; SUD, sudden death; TA, tripe deformity; TH, heterozygous carrier; UH, umbilical hernia; VSD, ventricular septal defect

* Low-grade mosaicism detected by NGS analysis in the father of the index patient.

| Patient | Involved gene | Sex, age | Cardiovascular feature(s) | Systemic feature(s) | Family history | Genotype | Relative | Phenoype |
|---------|---------------|----------|---------------------------|---------------------|---------------|----------|----------|----------|
| 61      | TGFB2         | ♂, 16    | An (AoR, 44 mm, 16 y), MVP | PR, arachnodactyly, hypermobility, luxations of hips and knees, bifid uvula, hypertelorism, blue sclerae | NA |          |          | (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 early onset 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, downslanding palpebral fissures, and a highly arched palate. The intragenic TGFB2 deletion was also present in his clinically affected father (clinical features include aortic
FIGURE 1  Genomic copy-number variants in H-TAD patients based on XHMM analysis. 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 CNVs. Vertical axis indicates sample Z-score of PCA-normalized read depth. Deletions are colored in red, and duplications are colored in green.

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, microglossia, 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-q34.3 (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 (Amarillo, O’Connor, Lee, Willing, & Wambach, 2015). 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.

4 | 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% (Campens et al., 2015; Lerner-Ellis et al., 2014; Poninska et al., 2016; Proost et al., 2015; Wooderchak-Donahue et al., 2015; Ziganshin et al., 2015). 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, TGFBR2, TGFBR1, TGFBR2, FBN2, MYH11, TGFBR3, 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 LOVD 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 (Leistritz, Pepin, Schwarze, & Byers, 2011).

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 = 0.001–0.01) (Campens et al., 2015). 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 TGFβ2. 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

| TABLE 3 | Summary of the genetic features of six patients with a pathogenic or likely pathogenic CNV |
|---------|---------------------------------|
| Patient | Gender, age | 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) | MYLK, deletion exon 17 and 18 | MLPA | 5 |
| 62      | M, 36       | PRKG1; exon 3c | Loss | p.(Asp145 Thr183 delinsAla) | in-frame deletion-insertion | PRKG1, deletion exon 3c | MLPA | 4 |
| 63      | M, 31       | SMAD3; exon 6d | Loss | p.(Asp220 Ile290del) | in-frame deletion | SMAD3, deletion exon 6d | MLPA | 5 |
| 64      | M, 17       | TGFβ2; exons 4–7a | Loss | p.(Ile199_Arg390del) | in-frame deletion | TGFβ2, deletion exons 4–7a | MLPA | 5 |
| 65      | F, 0        | Duplication NOTCH1; whole gene | Gain | NA | NA | unbalanced translocation: 46,XX,der(7)t (7;9)[p22.3;q33.3] | SNP array and karyotyping | 5 |
| 66      | M, 0        | Deletion SCARF2; whole gene | Loss | NA | NA | 22q11.2 deletion: arr[hg19] 22q11.2(20779645_20792061)x1 | SNP array | 5 |
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-)).

encompassing SCARF2 were detected by XHMM analysis. These aberrations were part of an unbalanced translocation (46,XX,der(7)(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.

5 | 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.

DECLARATIONS

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Under Dutch law, assessment of the study protocol by our ethics committee was not indicated because only genetic and clinical data collected during regular patient care were used.
CONSENT FOR PUBLICATION

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. Informed consent for DNA diagnostics was obtained from all 810 patients after genetic counseling by the referring physician.

AVAILABILITY OF DATA AND MATERIAL

All data and protocols used for this study are either included in the article (or in its supporting files) or are available upon request.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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Not applicable

AUTHORS’ CONTRIBUTIONS

EO, JPT, ACH, and AM initiated the project. The genetic tests were validated and/or supervised by LM, PR, MMW, EV, and AM. The clinical data of the patients were collected by EO, MJHB, AFB, IB, ED, JMH, YHH, MK, IPK, LAM, JMAM, KKY, PIGZ, MG, JPT, and ACH. The first draft of the manuscript was written by EO and LM. This was supervised by JPT, ACH, and AM. The manuscript was read and approved by all authors.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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