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Predictors of severe aortic involvement in Marfan syndrome

PhD thesis

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List of abbreviations

ACMG - American College of Medical Genetics and Genomics

ACTA2 - actin alpha 2, smooth muscle gene

ATI - aortic tortuosity index

BMI - body mass index

BMP - bone morphogenic protein

bp - base pair

CATI - carotid artery tortuosity index

cbEGF - calcium-binding epidermal growth factor-like domain

CNV - copy number variation

COL3A1 - collagen type III alpha 1 chain gene

CT - computed tomography

CTA - computed tomography angiography

Cys - cysteine

DM - distance metric

DN - dominant negative

DN-CD – dominant negative variant affecting or creating cysteine residues or in-frame deletion variants in the central cbEGF domains (exons 25–36 and 43–49) of the *FBN1* gene

DN Cys - dominant negative mutation eliminating a disulfide-bonding cysteine

DN-nonCD – dominant negative variant not affecting nor creating cysteine residues or in-frame deletion variants in the central cbEGF domains (exons 25–36 and 43–49) of the *FBN1* gene

DN non-Cys - dominant negative mutation not eliminating a cysteine

EACTS - European Association for Cardio-Thoracic Surgery

ECM - extracellular matrix

EGF - epidermal growth factor-like domain

EMMPRIN - extracellular matrix metalloprotease inducer

ERK1/2 - extracellular-signal regulated kinase 1 and 2

ESC – European Society of Cardiology

FBN1 - fibrillin-1 gene

FBP - filtered back projection

HI – haploinsufficient

HTAD - heritable thoracic aortic disease

ICM - inflection count metric

IMA – internal mammary artery

IR - iterative reconstruction

JNK - c-Jun N-terminal kinase

KCNN - potassium calcium-activated channel subfamily N member 1 gene

LAP - latency associated protein

LDS - Loeys-Dietz syndrome

LIMA - left internal mammary artery

LLC - large latent complex

LOX - lysyl oxidase gene

LTBP - latent transforming growth factor-β binding protein

MAD - mitral annular disjunction

MASS - mitral valve prolapse, myopia, mild and non-progressive aortic dilation, nonspecific skin and skeletal manifestations (phenotype)

MLPA - multiplex ligation-dependent probe amplification

MMP - matrix metalloprotease

MMP2 - matrix metalloprotease 2

MMP3 - matrix metalloprotease 3 gene

MMP9 - matrix metalloprotease 9

MRA - magnetic resonance angiography

MRI - magnetic resonance imaging

MYH11 - myosin heavy chain 11 gene

MYLK - myosin light chain kinase gene

NGS - next-generation sequencing

NMD - nonsense-mediated mRNA decay

PCR - polymerase chain reaction

PI3K/AKT - phosphoinositide 3-kinase/AKT

PRKG1 - protein kinase cGMP-dependent 1 gene

PTC - premature termination codon

p38 MAPK - p38 mitogen-activated protein kinase

RIMA - right internal mammary artery

SKI - SKI proto-oncogene gene

SLC - small latent complex

SLC2A10 - solute carrier family 2 member 10

SMAD2 - SMAD family member 2 gene

SMAD3 - SMAD family member 3 gene

SNV - single nucleotid variant

SOAM - sum of angles metric

TB – transforming growth factor-β-binding-like domain

TGF-β - transforming growth factor-β

TGFB2 - transforming growth factor-β 2 gene

TGFB3 - transforming growth factor-β 3 gene

TGFBR1 - transforming growth factor-β receptor 1 gene

TGFBR2 - transforming growth factor-β receptor 2 gene

TS - targeted sequencing

TTE - transthoracic echocardiography

vEDS - vascular Ehlers-Danlos syndrome

VCF - variant call format

VTI - vertebral tortuosity index

VUS - variants of unknown significance

WES – whole-exome sequencing

WGS – whole-genome sequencing

1 Introduction

The first case of Marfan syndrome was reported by Antoine-Bernard Marfan, a French pediatrician, in 1896 [1]. The described patient mainly presented with skeletal abnormalities and was later speculated to rather had suffered from congenital contractural arachnodactyly, a syndrome delineated by Beals and Hecht, known to have several overlapping features with Marfan syndrome [2,3]. Aortic aneurysm and subsequent acute aortic events were first linked to Marfan syndrome about 50 years after the original description of the syndrome [4].

Marfan syndrome is a rare, systemic connective tissue disorder with a prevalence of about 1:5000 and no predilection for either sex. Marfan syndrome is inherited in an autosomal dominant manner and caused by mutations of the fibrillin-1 gene (*FBN1*) [5]. *FBN1* encodes the fibrillin-1 protein, which is an important component of connective tissues, explaining the multisystem involvement in Marfan syndrome. The most characteristic manifestations involve the cardiovascular, musculoskeletal and ocular systems [6].

Early reports have described that the survival of Marfan syndrome patients is about two-thirds of that of unaffected individuals, mainly due to aortic complications [7]. Since then, owing to the accumulating knowledge and thus the progress of disease management, the survival of Marfan syndrome has improved significantly [8]. However, patients with Marfan syndrome are in an increased risk for developing aortic dissection, which is a life-threatening cardiovascular event, necessitating urgent cardiac intervention. The best survival can be reached by the prevention of aortic dissection, which could be achieved by a prophylactic aortic surgery, the mortality of which is significantly lower than that of the operation of an acute type A aortic dissection [9,10]. The indications for a prophylactic aortic surgery are mainly based on the diameter of the aorta, however, an acute aortic event can also occur below the surgical threshold. On the other hand, undergoing the operation as late as possible carries benefits for the patient [11]. Thus, it is of high clinical relevance to optimize the indications and the best timing for a prophylactic aortic surgery in patients with Marfan syndrome.

1.1 Clinical manifestations

1.1.1 Cardiovascular system

Involvement of the cardiovascular system is associated with the highest morbidity and mortality of Marfan syndrome. Out of the cardiovascular manifestations, the most frequently encountered one is the dilation of the ascending aorta at the level of the sinus of Valsalva. A dilated aorta carries the risk of aortic dissection and rupture, which are life-threatening cardiovascular events. Apart from aortic aneurysm formation, patients with Marfan syndrome may also present with a dilated proximal pulmonary artery, although, a consequent dissection or rupture is extremely rare [12]. Another hallmark cardiovascular feature is mitral valve prolapse with or without mitral regurgitation. Although mitral valve prolapse is mostly associated with a benign course, it may lead to heart failure through severe regurgitation, to supraventricular and ventricular arrhythmias and even to sudden death [13]. In severe pediatric cases mitral valve involvement is related to the highest morbidity and mortality by leading to congestive heart failure, requiring cardiac surgery [14]. Mitral annular disjunction (MAD), which is a separation between the mitral valve hinge point and the left ventricular myocardium, has previously been reported to occur more frequently in Marfan syndrome patients and may be associated with a higher rate of arrhythmic events and need for mitral valve intervention [15]. Furthermore, aortic valve dysfunction leading to volume overload, hence left ventricular dilation and failure [14], as well as tricuspid valve prolapse with or without regurgitation can also develop in Marfan syndrome patients [12].

As fibrillin-1 molecules are also found in the myocardium, primary cardiomyopathy may also develop in patients with Marfan syndrome. Asymptomatic mild biventricular enlargement and dysfunction, independent from age, gender and other cardiovascular manifestations, were observed in a remarkable portion of Marfan syndrome patients [16]. A further study has reported a mildly impaired systolic and diastolic left ventricular function in Marfan patients, not related to valvular disease [17]. These findings were consistent with a recently published meta-analysis, which described intrinsic cardiac impairment in Marfan syndrome [18].

1.1.1.1 Aortic aneurysm

Aneurysm is defined as a localized dilation of an artery with more than 50% of its normal diameter (*Figure 1*). Aneurysms tend to dilate asymptomatically over time, until they lead to life-threatening acute aortic events [19]. Most frequently, aortic aneurysms develop at the level of the sinus of Valsalva in patients with Marfan syndrome, but they can occur at other aortic levels as well [20]. The reasons behind the predominant aortic root involvement could be the high blood pressure prevailing there as well as the aortic root's different embryological origin in comparison to the other parts of the aorta [21].

The above described definition of aneurysm does not apply well for the aortic root and ascending aorta, as the chance of aortic dissection is significantly increased at aortic diameters well below the defined size. Based on the increase in the risk of dissection, an aorta between 4.0 and 4.4 cm is declared to be called dilated, and from 4.5 cm the term aneurysm is reasonable to be applied [22]. As body size influences aortic size, aortic diameters should be adjusted for age, body surface are (aortic size index) or height (aortic height index) to get more accurate information on the risk of dissection [23].

At histological level, aneurysm of the ascending aorta is mostly caused by (cystic) medial degeneration, characterized by smooth muscle cell dropout and elastic fiber degeneration, leading to a weakened aortic wall [24]. These alterations predispose aneurysms to dissection and rupture, both of which carry a high mortality rate. As 95% of aneurysms do not cause any symptoms before resulting in an acute aortic event, their detection can only be achieved by medical imaging in most of the cases, highlighting the significance of screening patients with suspected Marfan syndrome [25].

The gold standard tool for the assessment and follow-up of the aortic root and the proximal ascending aorta is 2D transthoracic echocardiography (TTE). Follow-up imaging is recommended annually, but in case of a larger aortic diameter or increased growth rate, more frequent screening needs to be applied. From the age of 18 years, vascular imaging of the thorax and abdomen should be carried out every 2-5 years with the use of computed tomography (CT) or magnetic resonance imaging (MRI) [14]. The American Heart Association/American College of Cardiology guideline on aortic diseases suggests an additional TTE evaluation of the aorta six months after the initial imaging diagnosis to assess aortic growth [22].

Figure 1 3D computed tomography reconstruction
of an aortic root aneurysm.
Image from the collection of the Department of
Cardiac Surgery, Heart and Vascular Center,
Semmelweis University

1.1.1.2 Aortic dissection

Aortic dissection is a tear in the inner layer of the aorta, which results in blood entering the aortic wall and separating its layers, potentially leading to life-threatening complications. The most common causes of death related to aortic dissection are pericardial tamponade leading to cardiogenic shock, aortic rupture, acute aortic valve insufficiency and acute myocardial infarction [9].

Several risk factors are associated with the development of aortic dissection, of which the most important ones are hypertension, smoking and connective tissue disorders such as Marfan syndrome. The presence of aortic aneurysm predisposes to dissection, which highlights the need for their surveillance and operative treatment when indicated. Further risk factors include older age, cocaine abuse, trauma, vascular inflammation and iatrogenic procedures [9].

Aortic dissection is characterized by the Stanford and the DeBakey classification systems. The Stanford system is more widely used in the clinical practice as it better aids the therapeutic decision making due to its simplicity. Stanford A dissections involve the ascending aorta and they necessitate an acute cardiac operation (*Figure 2*). In case of type B dissections, the ascending aorta is not affected and pharmaceutical therapy is the

treatment of choice in non-complicated cases. The DeBakey I and II type dissections correspond to the Stanford type A, in case of the latter the dissection is limited to the ascending aorta, while DeBakey I describes cases when both the ascending and descending parts of the aorta are involved. DeBakey type III equals to Stanford B, as the ascending aorta is not affected [9].

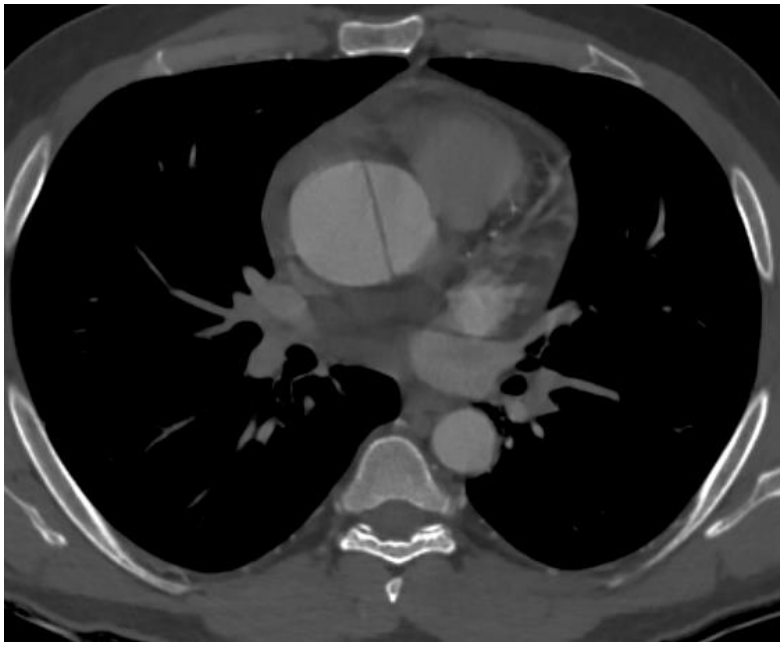

Figure 2 Computed tomography image of a type A aortic dissection.
Image from the collection of the Department of Cardiac Surgery, Heart and Vascular Center, Semmelweis University

Around two thirds of acute aortic dissections belong to the type A group. The average age at presentation in the general population is about 61 years [26]. Patients with Marfan syndrome suffer aortic dissection at a significantly younger age, and about 5% of all aortic dissections occur in people living with Marfan syndrome [26]. Without surgical intervention, the mortality of acute type A aortic dissection is 20% at 24 h, 30% at 48 h and 50% at two weeks after symptom onset [27]. The operation of an acute type A aortic dissection has a mortality rate of even 20-25%, while the mortality of a prophylactic aortic surgery, carried out for the prevention of dissection, is around 1-2% [9,10,26]. Furthermore, late overall mortality is also associated with acute operations [28]. Besides increased mortality, emergency aortic surgery is also associated with high morbidity and adverse long-term consequences. It has been reported that Marfan syndrome patients who underwent emergency operation due to acute type A aortic dissection were less likely to

have a valve-sparing procedure, presented more frequently with dissection and aneurysm formation of the distal aorta years after the initial surgery and had a poorer activity-related life quality compared to patients with an elective aortic operation [29]. Furthermore, trait anxiety level was significantly increased in Marfan patients who underwent acute life-saving aortic operation compared to the general population, however, patients with a prophylactic aortic root replacement did not have a higher anxiety trait than the general population [30]. These unfavorable outcomes emphasize the importance of the prevention of acute aortic dissection.

1.1.1.3 Arterial tortuosity

Arterial tortuosity describes the morphology of an artery with increased number or increased amplitude of curvatures and it has emerged as a promising predictor of cardiovascular manifestations in patients with Marfan syndrome [31]. Arterial tortuosity was mainly associated with aging, hypertension and atherosclerosis [32], but it has been recognized to be also a feature of genetic conditions associated with aortopathy, including Marfan syndrome and Loeys-Dietz syndrome (LDS) [33]. A rare autosomal recessive genetic condition, called arterial tortuosity syndrome is caused by biallelic mutations in the solute carrier family 2 member 10 gene (*SLC2A10*) and characterized by arterial tortuosity as the main feature [34].

Previous studies have shown that the aorta and the vertebral arteries have an increased tortuosity in Marfan syndrome and the degree of tortuosity correlates with the severity of aortic involvement [35,36]. However, the tortuosity of the examined vessels could be influenced by skeletal deformities, which are frequent clinical features of Marfan syndrome. Thus, investigating the tortuosity of arteries that are not affected by the skeletal abnormalities could provide a promising risk stratification tool for severe aortic involvement.

1.1.2 Musculoskeletal system

Musculoskeletal complications also reduce the life quality of Marfan syndrome patients, often requiring corrective surgery [37]. The most common manifestations are scoliosis, chest wall deformities such as pectus excavatum and pectus carinatum, pes planus, arachnodactyly and the characteristic thumb and wrist signs, dural ectasia, protrusio acetabuli and craniofacial features. The latter include malar hypoplasia, retrognathia,

enophthalmos, dolichocephaly and down-slanting palpebral fissures [38]. Dolichostenomelia, meaning disproportionally long extremities for the size of the trunk, presents in more than half of Marfan syndrome patients [12].

1.1.3 Ocular system

The burden of ophthalmic manifestations is also of high relevance in Marfan syndrome, as these patients carry an increased risk for ophthalmic diseases, need for ophthalmic surgery and medical treatment compared to the general population [39]. The most characteristic ocular finding is ectopia lentis, which occurs in approximately 60% of Marfan syndrome patients [12]. The other frequent ocular morbidity is myopia [12]. The incidence of retinal detachment and glaucoma are increased, and cataracts develop at earlier age in patients with Marfan syndrome [40].

1.2 Molecular background

1.2.1 Fibrillin-1 and microfibrils

Fibrillin-1 is a key glycoprotein in the extracellular matrix (ECM), as fibrillin-1 assembles into microfibrils, which are fundamental components of connective tissue. Through the formation of microfibrils, fibrillin-1 provides the base for elastic fiber assembly, thus being responsible for tissue elasticity. Microfibrils are especially abundant in elastic tissues like the aorta, lungs and skin [41]. Fibrillin-1 also plays a structural role without elastic fiber formation, as it is the case in the ciliary zonules of the eye [42,43].

Fibrillin-1 consists of 2871 amino acids and has a predicted molecular mass of 347 kDa [41]. The multidomain structure of fibrillin-1 is highly conserved, disulfide-rich and contains calcium-binding epidermal growth factor-like (cbEGF)/EGF domains, transforming growth factor-β-binding-like (TB) domains and hybrid domains with features of both TB and cbEGF domains [44]. A particularly important amino acid of the fibrillin-1 protein is cysteine (Cys), as it is responsible for disulfide bond formation, which is required for the appropriate structure and function of the protein [45]. There are 6 highly conserved cysteine residues in the EGF domain, creating 3 disulfide bonds in a characteristic way. Furthermore, cysteine is also a key component in the 8-cysteine containing modules, the so-called 8-Cys/TB domains [46].

Microfibrils are 10-12 nm sized structures and they form a beaded string with an average periodicity of about 55 nm [44]. Through the interaction with several other glycoproteins, such as latent transforming growth factor-β binding protein (LTBP), bone morphogenic protein (BMP), fibronectin and integrins, microfibrils play a crucial role in several structural and regulatory processes [47].

Considering its key function in the development of aortopathy and other manifestations of Marfan syndrome, it is important to discuss the regulatory role of microfibrils on the bioavailability of transforming growth factor-β (TGF-β). Microfibrils bind TGF-β and keep it in an inactive form, which is achieved through the association of microfibrils to LTBP. LTBPs are extracellular glycoproteins that share structural similarities with fibrillin-1, including the presence of TB and cbEGF domains [44]. LTBPs form the large latent complex (LLC) by binding the small latent complex (SLC) in the ECM, which consists of TGF-β and latency associated protein (LAP). LLC binds to microfibrils through the LTBP, thus a reduced level or abnormal structure of fibrillin-1 may result in an increased bioavailability of TGF-β [48].

1.2.2 The role of TGF-β

TGF-β is a growth factor with the effects of cell proliferation, migration, differentiation and survival regulation. An increased level of TGF-β results in alterations that could explain the characteristic features of Marfan syndrome, including the pathomechanism of aneurysm formation. The balance between matrix deposition and degradation is shifted towards degradation in aneurysm development, resulting in a weakened aortic wall. Dysregulation of TGF-β is a key component of this pathological vascular remodeling [49].

The link between elevated TGF-β signaling and manifestations of Marfan syndrome was first evidenced in 2003 by discovering the lung destruction reducing effect of TGF-β neutralizing antibodies in a Marfan emphysema mouse model [50]. In 2013, the potential role of TGF-β in the pathogenesis of aneurysm development was also described [51].

TGF-β exerts its effects through two different signaling pathways, the so-called canonical and noncanonical pathways. The canonical pathway is initiated by the binding of TGF-β to the heterotetrameric receptor complex of two type 1 receptors and two type 2 receptors. As a consequence of receptor activation, the receptor activated SMAD (R-

SMAD) is phosphorylated and binds to a common-mediator SMAD (co-SMAD; SMAD4), forming a complex that enters the nucleus and regulates the expression of target genes. The noncanonical way may also involve the mentioned SMAD proteins, however, it differs from the canonical one in many aspects. The key mediators of the noncanonical route are p38 mitogen-activated protein kinase (p38 MAPK), extracellular-signal regulated kinase 1 and 2 (ERK1/2), c-Jun N-terminal kinase (JNK) and the phosphoinositide 3-kinase (PI3K)/AKT pathways [52].

TGF-β increases the degree of collagen synthesis, leading to a reduced compliance of the aorta, which, together with a decreased elasticity due to elastic tissue impairment, contributes to aortic pathology. In addition to the reduced amount or abnormal structure of fibrillin-1, elastic tissue impairment is also caused by elastic fiber degradation due to the increased level of elastase and matrix metalloproteases (MMPs), as a result of elevated TGF-β levels [53]. An elevated level of MMPs in aneurysms has been extensively reported, with a particular significance of MMP2 and MMP9. These two MMPs belong to the group of gelatinases, which have the ability to degrade elastin and denaturated collagen [54]. Having an inhibitory effect on satellite cells required for muscle formation, increased TGF-β levels also result in a reduced muscle mass [48].

1.2.3 Genetics of Marfan syndrome

The *FBN1* gene is located on the long arm of chromosome 15 (15q21.1) and it consists of 65 exons. To date, more than 3500 mutations of the *FBN1* gene have been reported [55,56]. Around 25% of the disease-causing variants are *de novo* [12]. Most of the mutations are single nucleotid variants (SNVs), of which the most frequent ones are missense mutations [57].

Missense mutation is defined as a single base pair (bp) substitution leading to an amino acid change in that position [58]. SNVs also include nonsense and splice-site mutations [59]. In case of a nonsense mutation, a single nucleotid substitution introduces a premature termination codon (PTC) instead of an amino-acid-coding sense codon, which can result in nonsense-mediated mRNA decay (NMD) or in the premature termination of translation [60,61]. Splice-site mutations result in improper exon or intron recognition in the precursor mRNA by disrupting existing splice sites, creating new ones or activating cryptic ones, thereby leading to an abnormal transcript [62].

Frameshift mutations are caused by small insertions or deletions (indels) with a size of <50 bp not the multiple of three, resulting in the alteration of the reading frame of a coding gene, which can modify the translated protein or lead to NMD due to PTC [63].

Structural variants are genomic alterations involving at least 50 bp and can be categorized as deletions, duplications, insertions, inversions and translocations. One particular subtype of structural variants is copy number variation (CNV), mainly comprising deletions and duplications ≥50 bp [64]. CNVs have also been identified as disease-causing variants in patients with Marfan syndrome, emphasizing the need for their screening [65,66].

Mutations can be categorized into two distinct forms: haploinsufficient (HI) and dominant negative (DN) ones. HI variants of the *FBN1* gene lead to a reduced expression of the fibrillin-1 protein, meaning fibrillin-1 molecules with intact structure, but decreased quantity of microfibrils in the ECM [65]. Haploinsufficiency may be caused by the deletion of the entire allele/gene or by the deletion/mutation of gene regions (promoter, exon with start codon) that prevent transcription/translation (true haploinsufficiency). Furthermore, haploinsufficiency can also be caused by premature termination codon (introduced by nonsense or frameshift mutations or aberrant splicing) that lead to NMD (functional haploinsufficiency) [65,67]. On the other hand, DN mutations can alter the structure of the fibrillin-1 protein, resulting in an abnormal protein without reducing its quantity, thus both the normal and abnormal fibrillin-1 can be found in the ECM. ECM distraction may be caused by protein misfolding or dysfunction, hence by disturbed interactions with other ECM proteins [67]. Due to the especially important structural and functional role of the cysteine amino acid, DN mutations can be further divided based on cysteine involvement.

1.3 Genotype-phenotype correlations

Predicting disease severity based on the type of genetic variant could be a promising way to optimize care of Marfan syndrome patients, including the improvement of prophylactic surgery indications. However, to date only a few well established genotype-phenotype correlations have been described in Marfan syndrome. One of these is the association of mutations in exons 24-32, the so-called neonatal region, with a particularly severe form of the disease, termed early-onset or neonatal Marfan syndrome [68,69]. Diagnosis is

mostly made at or shortly after birth, and the affected patients are most likely to die within one month, barely surviving beyond 2 years. The most common cause of death is congestive heart failure secondary to valvular insufficiency [68]. However, patients carrying mutations in exons 24-32 have also been reported to present with atypically severe or classic Marfan syndrome. Patients with atypically severe Marfan syndrome develop aortic dissection or require aortic surgery at a remarkably younger age compared to classic Marfan syndrome patients [70]. A specific mutation of *FBN1*, p.Gly1013Arg, located in exon 24, has also been described in non-related individuals to lead to an atypically severe form of Marfan syndrome [70–72].

A generally accepted genotype-phenotype correlation is linked to the ocular system. Ectopia lentis, a characteristic feature of Marfan syndrome, occurs more frequently in patients with missense mutations, especially with cysteine involvement, compared to patients carrying PTC variants [73–75].

1.4 Diagnosis

1.4.1 Ghent nosology

Reflecting the growing amount of knowledge on connective tissue disorders as well as on molecular biology, the diagnostic criteria of Marfan syndrome has been evolving over the years. The first criteria for the diagnosis of Marfan syndrome was included in the Berlin nosology, which was created in 1986 and relied completely on clinical features [76]. Although still mainly based on clinical manifestations, the Ghent nosology published in 1996, aimed to improve the diagnosis and differential diagnosis of Marfan syndrome [77]. As the Ghent nosology still had several limitations, it needed to be further improved and today the diagnosis of Marfan syndrome can be established according to the Ghent nosology revised in 2010, which puts a special emphasis on aortic manifestations, ectopia lentis and the role of genetic testing [78]. A further important aspect of the diagnosis is the systemic score, which is acquired by scoring the presenting characteristic features of the disease.

The criteria required for the diagnosis is different in case of positive and negative family history. In the absence of affected family members, the diagnosis can be established by one of the following [78]:

A, aortic root dilation (Z-score ≥2) or dissection AND ectopia lentis

B, aortic root dilation (Z-score ≥2) or dissection AND disease-causing *FBN1* mutation

C, aortic root dilation (Z-score ≥2) or dissection AND a systemic score of ≥7

D, ectopia lentis and *FBN1* mutation with known aortic involvement

When the family history is positive for Marfan syndrome, the diagnosis can be established in the presence of one of the following: ectopia lentis, systemic score of ≥7 or aortic root dilation (Z-score ≥2 above 20 years of age and Z-score ≥3 below 20 years) or dissection.

Z-score expresses the deviation of a given measurement from a size- or age-specific population mean [79].

1.4.2 Genetic testing in Marfan syndrome

1.4.2.1 Benefits of genetic testing

Identifying the disease-causing genetic variation in patients with Marfan syndrome carries several benefits. First, it helps establishing the correct diagnosis and differentiates Marfan syndrome from other related connective tissue disorders that require a different therapeutic approach, thus patients can receive the appropriate care. Family members can undergo targeted screening of the known variant, which helps to confirm or exclude the diagnosis of Marfan syndrome. This approach is especially beneficial in childhood when the characteristic manifestations have not yet fully developed, making it difficult or rather impossible to establish the diagnosis [6], while patient care should be initiated as early as possible to successfully reduce the complications of the syndrome [14]. By excluding the disease, patients can avoid unnecessary follow-ups and it could also exert a positive effect on their quality of life due to the lack of uncertainty and also due to the finding that patients with Marfan syndrome have higher anxiety traits than the normal population [30]. Furthermore, the knowledge of a disease-causing variant could support family planning, and enables preimplantation diagnostics [80]. In addition, identifying the genetic variant could contribute to the investigation of genotype-phenotype correlations, through which a more personalized patient care could be achieved in the future [81].

1.4.2.2 Methods of genetic testing

Genetic testing can be carried out in various ways depending on the phenotype. When the clinical features are indicative for Marfan syndrome, single-gene testing for screening the *FBN1* gene could be an option, however, due to the remarkable overlap between Marfan syndrome and its related disorders, screening more genes seems to be a more helpful approach [82].

Genomics has been revolutionized by the technology of next-generation sequencing (NGS), also called massively parallel sequencing. Various NGS platforms are available and they are all able to sequence millions of small DNA fragments in parallel, thereby significantly reducing the time of genetic examinations compared to the previously gold-standard Sanger sequencing. Bioinformatics analysis delivers highly accurate data due to the increasing depth of sequencing (deep sequencing) [83]. NGS can be applied to sequence a specific panel of genes (targeted sequencing, TS), the coding parts of the genome (whole-exome sequencing, WES) and the entire genome (whole-genome sequencing, WGS).

TS enables the sequencing of the genes of interest with high read depth, while minimizing the chance of incidental findings and optimizing the interpretation of the detected variants, as only the disease-associated genes are examined. TS has a limitation in the detection of certain variants, especially CNVs. As new gene-disease associations may be discovered in the future, gene panels may need to undergo timely updates [66].

WES is able to detect genetic variants within the protein-coding sequences, which account only for about 1-2% of the whole genome, however, most of the known disease-causing variants can be found in these regions [84]. Applying WES enables the detection of novel gene-disease associations and due to its cost efficiency also to carry out trio analysis (sequencing both parents too), which latter helps to increase the power of analysis. During data analysis, *in silico* gene panels can be created, thereby reducing the chance of incidental findings and supporting the process of data interpretation. However, similarly to TS, WES has also incomplete coverage, thus hindering the detection of clinically relevant sequence variants [66,85].

A better coverage can be achieved by the use of WGS, which is also able to reliably detect CNVs and non-coding pathogenic variants, leading to a better diagnostic yield. Similarly to WES, *in silico* gene panels can be created to help data interpretation.

As the costs are continuously decreasing, the application of WGS may be more widespread in the future [86].

Several further methods can be used to detect CNVs, one of which is multiplex ligation-dependent probe amplification (MLPA), a cost-effective tool [87]. MLPA is a multiplex polymerase chain reaction (PCR) assay that uses several probes, which are specific for certain DNA sequences, thus enabling the evaluation of the relative copy number of each DNA sequence. At the end of the complex reactions, the height or area of PCR derived fluorescent peaks are measured, indicating the relative amount of target DNA sequence in the examined sample of DNA [88].

1.4.3 Differential diagnosis

Marfan syndrome is the most prevalent member of syndromic heritable thoracic aortic disease (HTAD), where systemic features of a genetically mediated condition present along with aortic involvement [20]. Patients with HTAD are at an increased risk for aortic aneurysm formation and acute aortic events, as abnormalities of the aorta lead to aortic wall weakness or abnormal hemodynamic profile [23].

The diagnosis of Marfan syndrome also requires the exclusion of features characteristic to related connective tissue disorders and other HTADs, the most important of which is LDS. There is a remarkable overlap between Marfan syndrome and LDS and other related disorders, sometimes making it difficult to make the correct diagnosis without genetic testing. However, due to their different severity and thus different therapeutic approach, establishing the correct diagnosis is crucial in order to provide the patients with the appropriate management.

1.4.3.1 Loeys-Dietz syndrome

Similarly to Marfan syndrome, LDS is a syndromic HTAD, inherited in an autosomal dominant manner. LDS is caused by mutations in the TGF-β signaling pathway and was first described in 2005 as an aggressive form of aortic disease with characteristic clinical features [89,90]. The typical phenotype is characterized by the triad of hypertelorism, bifid uvula and/or cleft palate, and cardiovascular features including generalized arterial tortuosity with widespread vascular aneurysm and dissection [91].

Based on the affected gene, LDS can be categorized into five groups. Mutations of the transforming growth factor-β receptor 1 *(TGFBR1)* and transforming growth

factor-β receptor 2 (*TGFBR2)* genes lead to LDS type 1 and type 2, respectively, and they represent the most severe form of the disease. LDS type 3 is associated with the SMAD family member 3 gene *(SMAD3*), while type 4 and type 5 are caused by variants in the transforming growth factor-β 2 (*TGFB2)* and transforming growth factor-β 3 (*TGFB3)* genes. Type 5 represents the mildest phenotype of LDS [92]. Furthermore, mutations of the SMAD family member 2 gene *(SMAD2)* have also been associated with the clinical characteristics of LDS [92], giving rise to the most recent type, LDS 6 (OMIM 619656). Importantly, LDS patients, especially the ones with mutations in the *TGFBR1* and *TGFBR2* genes, can present with a more severe cardiovascular phenotype than Marfan syndrome patients, experiencing aortic dissection at younger age and smaller diameters [93]. In addition, the rate of aortic dilation is about ten times faster than that observed in Marfan syndrome, as the aorta could even grow 1 cm in a year in severe LDS cases opposed to the 0.1 cm growth of Marfan aortas [94].

Taking the aggressive aortic features into account, prophylactic surgery is indicated in patients with LDS when the aortic diameter reaches 45 mm. Women with *TGFBR2* mutation, low body surface area and severe extra-aortic features are recommended to undergo a prophylactic operation from an aortic diameter of even 40 mm [95]. However, as LDS caused by mutations of the *SMAD2* and *TGFB3* genes is associated with a milder phenotype, it is reasonable to consider a prophylactic aortic surgery only when the aorta reaches 50 mm [20]. Given that the vascular manifestations are not limited to the aorta, patients with LDS need to undergo excessive vascular imaging at baseline and reasonably at least every two years to assess the status of the arterial system [22,96].

1.4.3.2 Further diseases for differential diagnosis

Several other diseases need to be considered in the differential diagnosis of Marfan syndrome (*Table 1*).

Table 1 The involved genes and main vascular manifestations of Marfan syndrome and related disorders. The listed disorders need to be considered in the differential diagnosis of Marfan syndrome.

	Gene	Main vascular features
Syndromic HTAD		
Marfan syndrome	*FBN1*	-Aortic aneurysm (mainly root) -Aortic dissection
Loeys-Dietz syndrome	*TGFBR1* *TGFBR2* *SMAD3* *TGFB2* *TGFB3* *SMAD2*	-Aneurysm and dissection throughout the arterial system -Arterial tortuosity -Aortic dissection at younger age and smaller diameter than in Marfan syndrome -Severity of vascular involvement depends on the affected gene
Vascular Ehlers-Danlos syndrome	*COL3A1*	-Dissection and rupture of medium sized arteries -Dissection and rupture of the aorta without preexisting dilation
Non-syndromic HTAD*		
	ACTA2	-Aortic dissection may occur without preexisting aneurysm -Prophylactic aortic surgery from 4.5 cm
	MYH11	Prophylactic aortic surgery from 4.5 cm
	MYLK	Prophylactic aortic surgery from 4.5 cm
	LOX	Prophylactic aortic surgery from 5 cm
	PRKG1	-Widespread vascular involvement -Prophylactic aortic surgery from 4.5 cm
Allelic disorders		
MASS phenotype	*FBN1*	Borderline and non-progressive aortic dilation
Marfan lipodystrophy	*FBN1*	Aortic dilation may present
Familial ectopia lentis	*FBN1*	No vascular involvement
Other genetic syndromes*		
Shprintzen-Goldberg syndrome	*SKI*	Rare and mild cardiovascular manifestations
Congenital contractural arachnodactyly	*FBN2*	Mildly dilated aorta in rare cases
Stickler syndrome	*COL2A1* *COL9A1* *COL9A2* *COL9A3* *COL11A1* *COL11A2*	No vascular involvement
Fragile X syndrome	*FMR1*	Aortic root dilation may present

ACTA2 - actin alpha 2, smooth muscle gene; *COL2A1* - collagen type II alpha 1 chain gene; *COL3A1* - collagen type III alpha 1 chain gene; *COL9A1* - collagen type IX alpha 1 chain gene; *COL9A2* - collagen type IX alpha 2 chain gene; *COL9A3* - collagen type IX alpha 3 chain gene; *COL11A1* - collagen type XI alpha 1 chain gene; *COL11A2* - collagen type XI alpha 2 chain gene; *FBN2* – fibrillin-2 gene; *FMR1* - fragile X messenger ribonucleoprotein 1 gene; HTAD - heritable thoracic aortic disease; *LOX* - lysyl oxidase gene; MASS phenotype - mitral valve prolapse, myopia, mild and non-progressive aortic dilation, nonspecific skin and skeletal manifestations; *MYH11* - myosin heavy chain 11 gene; *MYLK* - myosin light chain kinase gene; *PRKG1* - protein kinase cGMP-dependent 1 gene; *SKI* - SKI proto-oncogene gene
* Only some of the most common ones are listed here

Within the syndromic HTAD group, it is important to highlight vascular Ehlers-Danlos syndrome (vEDS). Vascular EDS is a rare disorder with an autosomal dominant inheritance, caused by mutations of the collagen type III alpha 1 chain gene *(COL3A1)*,

which encodes the chains of collagen type III. As collagen type III is a major component in the structure of arteries and hollow organs, patients with vEDS are at an increased risk of arterial and gastrointestinal rupture as well as rupture of the pregnant uterus [97]. Patient management is complicated by the fact that acute aortic events can occur at normal diameters in this patient population [98]. Furthermore, dissection and rupture affect medium sized arteries as well, even more frequently than the aorta [99].

Importantly, mutations in genes associated with syndromic HTAD, including *FBN1*, *TGFBR1, TGFBR2, SMAD3, TGFB2, TGFB3* and *COL3A1*, can also lead to aortopathies without meeting the criteria for the diagnosis of a syndromic disease. These conditions are termed non-syndromic HTAD [23]. Patients with non-syndromic HTAD suffer aortic dissection at a younger age than the general population, but usually not as young as Marfan syndrome patients. Some further genes that lead to non-syndromic HTAD are actin alpha 2, smooth muscle gene (*ACTA2*), myosin heavy chain 11 gene (*MYH11*), myosin light chain kinase gene (*MYLK*), lysyl oxidase gene (*LOX*), and protein kinase cGMP-dependent 1 gene (*PRKG1*) [100].

Further genetic syndromes also need to be taken into consideration in the differential diagnosis of Marfan syndrome, however, patients with these conditions rarely develop acute aortic events. These include familial ectopia lentis, MASS phenotype (mitral valve prolapse, myopia, mild and non-progressive aortic dilation, nonspecific skin and skeletal manifestations), Marfan lipodystrophy syndrome, Shprintzen-Goldberg syndrome, congenital contractural arachnodactyly, Stickler syndrome and fragile X syndrome. Familial ectopia lentis, the MASS phenotype and Marfan lipodystrophy are allelic disorders in the differential diagnosis of Marfan syndrome, meaning that they are also caused by mutations in the *FBN1* gene. The MASS phenotype is characterized by mitral valve prolapse, myopia, mild and non-progressive aortic dilation, nonspecific skin and skeletal manifestations overlapping with those of Marfan syndrome. Differentiating it from Marfan syndrome can be highly challenging in childhood without family involvement, thus it requires appropriate follow-up to monitor the state of the aorta [12].

1.5 Prevention of severe aortic manifestations

The currently available preventive options are blood pressure control and prophylactic aortic surgery. Prophylactic aortic surgery is carried out based on certain indications, of

which aortic diameter is an important factor. In patients with Marfan syndrome, a prophylactic aortic root replacement is indicated when the aortic diameter reaches 50 mm, which is smaller than the threshold in the general population. The threshold is further reduced to 45 mm when at least one of the following is present: positive family history for an acute aortic event, desire for pregnancy, growth rate greater than 3 mm/year or severe aortic regurgitation [95].

However, aortic dissection can occur at normal aortic diameters as well and not everyone with a dilated aorta suffers dissection [101]. Milleron *et al*. reported a low aortic dissection risk for Marfan syndrome patients who are treated according to the guidelines, however, three of the five type A dissections that occurred during the follow-up period, developed in aortas with a diameter smaller than 50 mm, one of which was even under 40 mm [102]. A study based on the GenTAC (National Registry of Genetically Triggered Thoracic Aortic Aneurysms and Cardiovascular Conditions) registry found a more frequent occurrence of aortic dissection in patients with Marfan syndrome compared to the findings of Milleron *et al*., however, consistently, some of the dissections developed at the size below the surgical threshold [103]. In a further study, 26% of Marfan syndrome patients suffered aortic dissection with an ascending aortic size below 45 mm [104]. Another study reported that 15% of type A dissections developed under 50 mm [105]. In a large mixed cohort including a small percentage of Marfan syndrome patients, about 28% of aortic dissections developed at an aortic size under 35 mm [106].

It is also important to note that operations carried out in younger age could lead to more reoperations and to an early initialization of life-long anticoagulation in case of mechanical valve implantation [107]. Thus, a prophylactic surgery should preferably be done as late as reasonably possible, but definitely before the occurrence of an acute aortic event. Importantly, the diameter threshold cannot be set unreasonably low, as it would mean that patients who would never experience an acute aortic event, would undergo a cardiac operation, which itself carries risks and has 1-2% mortality rate as mentioned above [108]. These indicate that patient selection, as well as the timing for a preventive operation need to be optimized. To achieve that it is required to identify predictors that could improve risk stratification.

2 Objectives

Given the high mortality rate of acute aortic dissection, and the findings that acute aortic events may occur even in the absence of substantial previous aortic dilation, the aim of the current work is to identify possible predictors of severe cardiovascular manifestations in patients with (suspected) Marfan syndrome to improve risk stratification and to optimize the indications for a prophylactic aortic operation. The possible predictive role of genotype-phenotype correlations and arterial tortuosity are investigated in this work.

Some work has already been published about the possible correlations of genetic background and aortic involvement severity in Marfan syndrome, however, as the results are inconclusive, further research is required to be carried out in this field. To enable the investigation of genotype-phenotype correlations, this thesis put a special emphasis on maximizing the mutation detection rate of genetic testing, including the demonstration of the importance of screening for structural genetic variants. In addition, due to the possible significant phenotypic overlap between aortopathies, further genes were also tested to reach the definite diagnosis.

Arterial tortuosity has been found to be a promising predictor of more severe aortic involvement in Marfan syndrome, however, as the published articles focus on vessels that may be influenced by the frequently encountered skeletal manifestations of the disease, we aimed to investigate the predictive role of the tortuosity of visceral arteries that are not affected by skeletal deformities. Arterial tortuosity and its development throughout the years is further presented through a case with several years of follow-up. The case of this patient also shows that peripheral arterial manifestation can develop in Marfan syndrome.

3 Methods

3.1 Genetic testing

3.1.1 The importance of copy number variation (CNV) detection

Prior to our main study on genetic testing in Marfan syndrome, we had demonstrated the relevance of CNVs as causative mutations of Marfan syndrome, which influenced the design of our study. An index patient with the clinical diagnosis of Marfan syndrome and her family members underwent genetic screening to confirm the diagnosis [109]. Ethical approval for this study was obtained from the Scientific and Research Ethical Committee of the Medical Research Council of Hungary (ETT-TUKEB, 12751-3/2017-EKU) and written informed consent was obtained from the patients.

NGS-based targeted gene panel test including all coding exons and flanking intronic regions of the *FBN1, TGFBR1, TGFBR2, SMAD3, TGFB2, TGFB3, ACTA2, COL3A1, MYH11* and SKI proto-oncogene *(SKI)* genes was carried out. MiSeq Personal Sequencer (Illumina, San Diego, CA, USA) was used to analyze amplicons [110]. As this technique is not suitable for the detection of deletions and duplications larger than 20 bp, 60x PE150 PCR-free WGS on a HiSeq X Ten platform (Illumina, San Diego, CA, USA) was performed subsequently [86]. Nexus Copy Number (BioDiscovery, El Segundo, CA, USA) software was used to analyze the WGS data for CNVs. To confirm the result, MLPA (P065/P066, MRC-Holland, Amsterdam, the Netherlands) [65] as well as standard PCR with a 407-bp amplicon spanning the deletion breakpoints followed by Sanger sequencing were applied.

3.1.2 Study design

Altogether 136 patients underwent genetic testing after informed genetic counselling and written consent (ETT TUKEB 12751-3/2017/EKU) [109] in our retrospective cross-sectional study. Phenotypic evaluation was carried out at the Marfan outpatient clinic at the Heart and Vascular Center of Semmelweis University in Budapest. All the involved patients are registered in the Hungarian Marfan Register, which is maintained by the Hungarian Marfan Foundation and includes more than 500 patients [111]. Patients were selected in the order of visiting the outpatient clinic. As Marfan syndrome shows a high phenotypic variability even between family members, we included first-degree relatives

who presented with Marfanoid features regardless of their systemic score. Among the 136 patients, 18 were first-degree relatives, therefore the investigated population covered 118 families.

3.1.3 First phase of genetic testing

3.1.3.1 Study population

The study consisted of two distinct phases. The inclusion criterion was the clinical diagnosis of Marfan syndrome in the first phase, resulting in the involvement of 57 patients. The clinical diagnosis was based on the revised Ghent nosology [78].

3.1.3.2 Single-gene analysis

Single-gene analysis was applied in the first phase of the study. As a first step, NGS with a Roche GS Junior platform was used to screen the *FBN1* gene [112]. After that, homopolymer regions were investigated with Sanger sequencing using ABI Prism 310 Genetic Analyser (Applied Biosystems). This technique was also used to confirm the detected disease-causing variants in both phases of the study.

3.1.4 Second phase of genetic testing

3.1.4.1 Study population

Patients with negative results from the first phase, as well as further 79, altogether 96 patients were enrolled in the second phase, where the inclusion criterion were the (suspected) clinical diagnosis of Marfan syndrome or Marfanoid habitus. Marfanoid habitus was defined as having a systemic score of at least 5 points.

3.1.4.2 Multi-gene panel analysis

An NGS-based multi-gene panel, involving the *ACTA2, COL3A1, FBN1,* potassium calcium-activated channel subfamily N member 1 *(KCNN1), MYH11, SMAD3, TGFB2, TGFBR1* and *TGFBR2* genes was applied in the second phase of the study. Mutations of these genes may lead to Marfan syndrome, LDS, vEDS or other HTADs. Altogether 96 samples were examined with this method. QIAseq targeted DNA custom panel (QIAGEN, USA) was used to prepare genomic DNA libraries, and the Illumina MiSeq platform (Illumina, San Diego, USA) was applied for the subsequent NGS. Annotation

of the Variant Call Format (VCF) files were carried out with the SnpEff software [113] and the ClinVar database [56]. Variants were classified with the VariantAnalyzer software, which was developed by the Budapest University of Technology and Economics.

3.1.5 Multiplex ligation-dependent probe amplification (MLPA)

As the applied sequencing methods are not or less capable to detect CNVs, and CNVs have been demonstrated to be important in the development of Mendelian disorders, including Marfan syndrome, we have used MLPA method (MRCHolland, Amsterdam, the Netherlands) to screen the *FBN1* and *TGFBR2* genes for CNVs in samples where no (likely) pathogenic mutations were identified after the above detailed sequencing steps [112].

3.1.6 Relevance of detected variants

Various databases were queried to interpret the pathogenicity of a detected variant. These databases included Varsome [114], Human Gene Mutation Database [55], Universal Mutation Database [115], dbSNP [116] and gnomADv2.1 non-Finnish population. American College of Medical Genetics and Genomics (ACMG) guidelines were followed for variant classification [117]. Pathogenic and likely pathogenic variants were considered disease-causing. Missense variants were classified as DN, while HI variants included nonsense, splice-site and frameshift mutations, as well as CNVs.

3.1.7 Investigations of genotype-phenotype correlations

The association between ascending aortic involvement, including dilation and dissection, and the type of disease-causing variant was investigated. Dilation was defined by the Z-score reaching at least 2 in patients above 20 years and 3 below 20 years of age [78]. Aortic involvement was compared between mutation positive and negative patients, between HI and DN mutations of the *FBN1* gene and between Marfan syndrome and LDS patients. Regarding the important role of cysteine in the structure of fibrillin-1, we have divided the DN mutations into variants that resulted in the elimination of a cysteine and DN variants that did not substitute this amino acid. The need for aortic surgery was also investigated in HI and DN variants, as well as the presence of well-known genotype-phenotype correlations in the patient cohort.

3.1.8 Statistical analysis

Two-sample t-test and chi-squared test were used for the comparison of certain groups, the results being considered significant at $p<0.05$. The general characteristics of the examined population were described by the mean and 95% confidence interval, while the systemic score was characterized by median with first and third interquartile ranges.

3.2 Arterial tortuosity

3.2.1 Patient selection

Patients with available helical CT angiography (CTA) from the Hungarian Marfan Register [111] were considered for the study [118]. Inclusion criteria were the diagnosis of Marfan syndrome and available arterial phase abdominal CTA images with appropriate quality to assess the tortuosity of the splenic and renal arteries. Marfan syndrome was diagnosed based on the revised Ghent nosology [78] and in almost half of the patients (likely) pathogenic variants were already identified in our ongoing genetic testing project. Patients were excluded in case of insufficient CTA image quality. Exclusions had to be made due to image noise, not appropriate coverage of the investigated arteries and the presence of pathological structures near the arteries that may influence the geometry of these vessels. After assessing the coverage of the analyzed visceral arteries by the CT slices and applying the exclusion criteria, of 114 Marfan syndrome patients, 37 were selected for further investigations. Control individuals matched for age and sex with a control-to-case ratio of 2:1, were selected from our clinical imaging database. Control subjects did not suffer from connective tissue disorders, otherwise the same exclusion criteria were applied for the selection of control individuals as presented above for Marfan syndrome patients. The study was approved by the Semmelweis University Regional and Institutional Committee of Science and Research Ethics (SE RKEB 72/2018), informed consent was waived due to the retrospective study design.

3.2.2 Severity groups

The following groups were used to classify Marfan syndrome patients based on aortic involvement severity:

Group A (n=5) - No aortic involvement requiring surgery at the time of CT scan: no aortic dissection, no significant aortic valve insufficiency and an ascending aortic diameter below 45 mm.

Group B (n=12) - Elective surgery carried out on the ascending aorta due to mild aortic involvement before the CT scan. Patients were required to meet the following criteria to be included in this group: ascending aortic diameter between 45-50 mm OR diameter at the level of the sinus of Valsalva between 45-48 mm with grade I-II aortic regurgitation AND aortic dilation rate of >2 mm/year OR positive family history for aortic dissection.

Group C (n=20) - Operation for annuloaortic ectasia with an ascending aortic diameter of >50 mm or >48 mm at the level of the sinus of Valsalva with grade III-IV aortic regurgitation before the CT scan; or type A aortic dissection confirmed by CT.

The aortic sizes were defined based on the European Society of Cardiology (ESC) and the European Association for Cardio-Thoracic Surgery (EACTS) guidelines [119].

3.2.3 CT angiography

Arterial phase CT images of the abdominal aorta, made with 256-slice CTA (Philips Brilliance iCT) at our institution were evaluated for tortuosity measurements. Control individuals with aortic CTA of the abdomen were selected from our clinical imaging database. The images were analyzed with a slice thickness of 1-2.5 mm and were reconstructed with traditional filtered back projection (FBP) or hybrid-type iterative reconstruction (IR). The amount of contrast agent and acquisition settings were based on local protocols.

3.2.4 Image segmentation and centerline export

Selected CT datasets were loaded to a dedicated workstation to generate centerlines for further assessment. Medis QAngio CT (v.3.1.0.1) was used for image analysis, which was carried out by a single reader blinded to patients' clinical data. Segmentation of the splenic and renal arteries were done manually by placing markers in the vessel lumen. The centerline of the splenic artery was extracted from the coelic trunk to the bifurcation at the hilus of the spleen, while the renal arteries were identified from their aortic origin to the renal hilus, selecting the largest branch with a diameter of at least 1.5 mm. The

segmented arteries were saved and the centerlines were exported with the Medis QAngio CT 3D Workbench (v 0.8) in a text format with the 3 dimensional vessel coordinates (*Figure 3*).

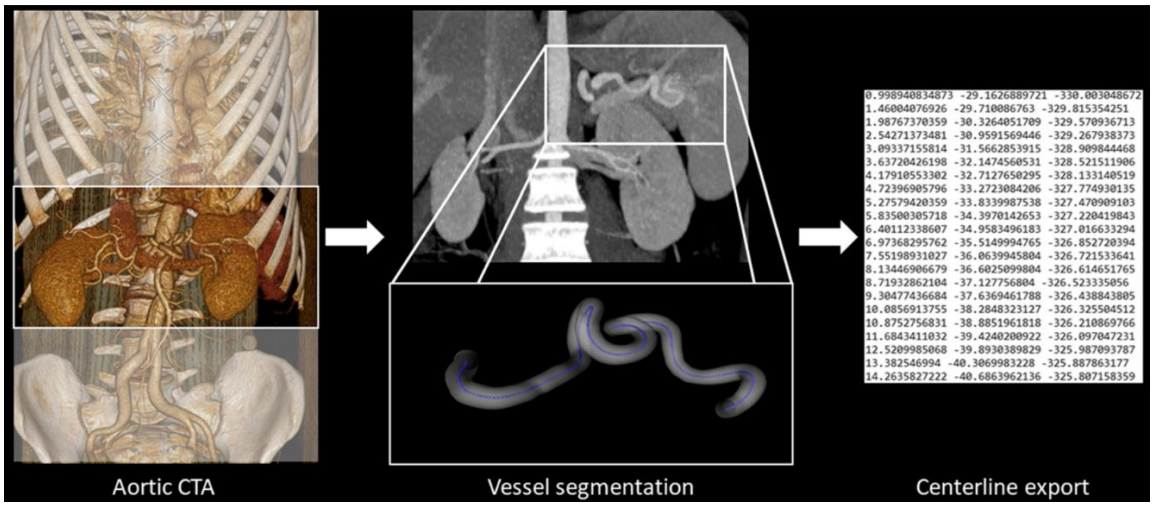

Figure 3 CT angiography images from Marfan syndrome patients and control subjects were used for the visceral arterial tortuosity study [118]. Manual segmentation of the vessel was carried out, followed by exporting the 3D coordinates of the centerlines for tortuosity metrics calculations.

3.2.5 Tortuosity metrics

Various tortuosity metrics were used to assess the geometric properties of the analyzed arteries (*Figure 4*). Distance metric (DM) gives us the ratio of the actual path length to the distance of the linear end points, with the downside of not being sensitive to the frequency of the curves. The inflection count metric (ICM) provides a solution for this issue, as it is calculated as the product of DM multiplied by the number of inflection points. DM and ICM are mainly able to detect high amplitude, low frequency curves. For the characterization of tight coils, sum of angles metric (SOAM) can be applied, which is mostly increased in the presence of high frequency curves. The algorithms to calculate these tortuosity metrics were implemented as described by Bullitt *et al*. [120], in JavaScript (Node.js) language as a server-side software plugin for the electronic version of the Hungarian Marfan Register. In addition to the above mentioned three metrics described by Bullitt *et al*. [120], a further metric, the ICM/SOAM metric is used to assess the contribution of amplitude and frequency to the tortuosity.

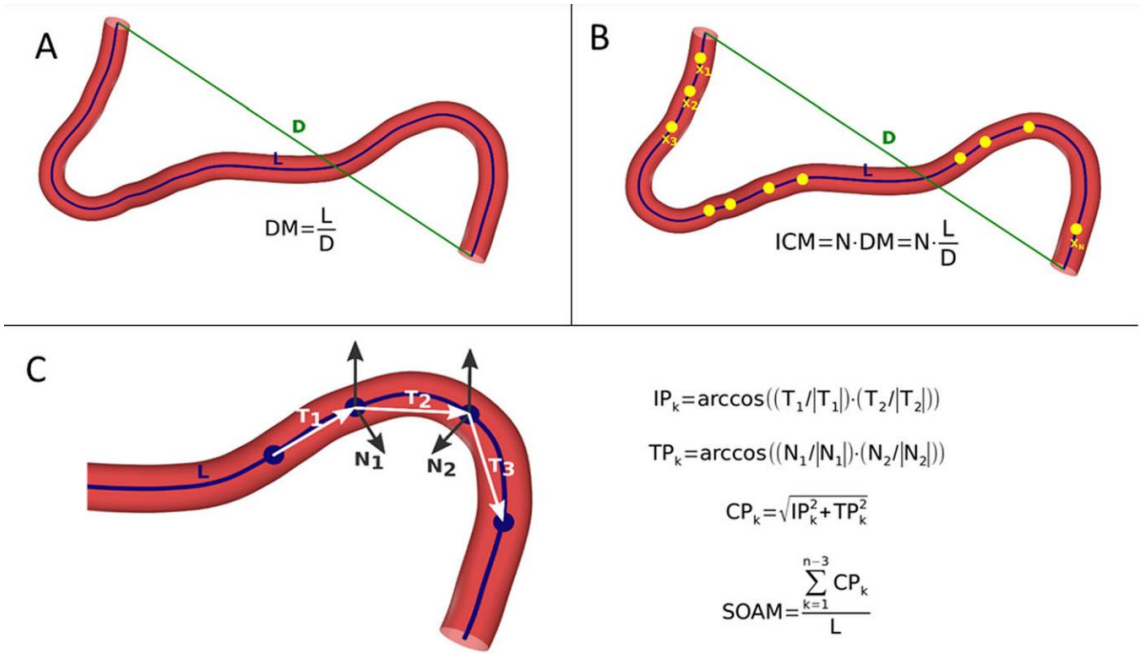

Figure 4 Schematic representation of the calculation of tortuosity metrics used in the visceral arterial tortuosity study [118]. **A,** Distance Metric (DM) gives us the ratio between the actual path length of the curve (L, blue centerline) and the linear distance between the end points of the curve (D, green straight line). **B,** Inflection count metric (ICM) is calculated by multiplying the DM by the number of inflection points (N), which are the points where the curve changes from convex to concave (indicated by yellow dots). **C,** Sum of angles metric (SOAM) is given by subdividing the arterial centerline into small segments (T1-T3 with white arrows) and summing the in-plane (IP_k) and torsional angles (TP_k) between these segments.

3.2.6 Statistical analysis

The R software environment was used for statistical analysis. Normality of the dataset was evaluated by Shapiro-Wilk test. Based on that, tortuosity metrics and most general characteristics parameters are reported as medians with interquartile ranges, further analyzed by non-parametric tests. According to that, Mann-Whitney U-test was used for the comparison of two groups, while Kruskal-Wallis test was applied to compare multiple groups. Pairwise Mann-Whitney U-test with Benjamin-Hochberg adjustment for multiple comparisons was the applied post-hoc test. Age is presented as mean with standard deviation and it was compared with ANOVA test between the different Marfan syndrome severity groups. Fisher's exact test was applied for the comparison of non-continuous variables.

3.2.7 Follow-up of tortuosity through a case

A patient with severe aortic involvement and bilateral internal mammary artery (IMA) aneurysms was followed-up with CT images, the tortuosity of the aorta and the internal

mammary arteries being assessed in addition to the monitoring of the aneurysms. The applied tortuosity metrics and evaluation method were the same as described above. CTA images were carried out in 2013, 2015, 2018, 2019 and 2020 [31]. This study was approved by the Semmelweis University Regional and Institutional Committee of Science and Research Ethics (SE RKEB 72/2018). The patient provided his written informed consent to participate in this study.

4 Results

4.1 Genetic testing

4.1.1 The importance of copy number variation (CNV) detection

The design of the genetic testing sequence in our study was influenced by a case of a Hungarian Marfan family [109]. Three members of this family had the clinical diagnosis of Marfan syndrome. The index patient, a 32-year-old female, had a systemic score of 9 points according to the revised Ghent nosology and had already undergone aortic root reconstruction surgery. The mother and the sister of the index case also presented with a systemic score of 9 points and conspicuous cardiovascular features. The father had some features characteristic to Marfan syndrome but did not fulfill the diagnostic criteria for the syndrome.

Screening the index patient with multi-gene panel has not revealed any disease-causing variants. As the applied gene panel is not always suitable for the detection of CNVs, a 60× PE150 PCR-free WGS, which has the ability of CNV detection, was carried out. No pathogenic SNVs or small indels were identified in the relevant HTAD genes. After analyzing the WGS data for CNVs, a 31,956-bp deletion of the *FBN1* gene (NM_000138.4:c.164+13846_442+1334del) was revealed *(Figure 5)*. The deletion has been confirmed with MLPA and PCR followed by Sanger sequencing. The detected deletion of coding exons 2-4 is predicted to lead to a frameshift and a premature termination codon [NP_000129.3:p.(Pro56Cysfs*3)], thus, possibly resulting in functional haploinsufficiency through NMD.

As a next step, the patient's first degree relatives were screened for the detected *FBN1* mutation by MLPA and Sanger sequencing, and the variant was identified in the patient's mother and sister, but not in the father.

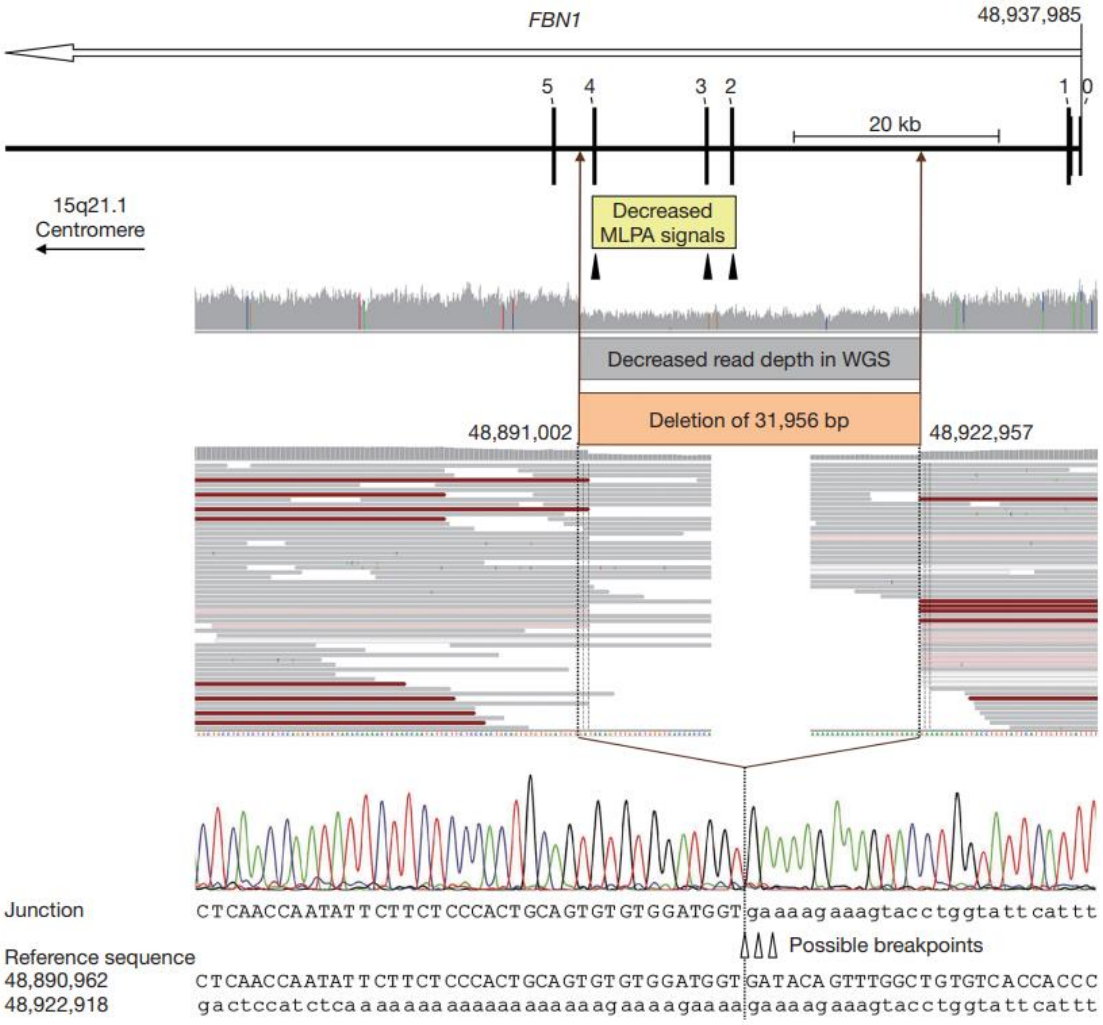

Figure 5 Schematic representation of the detected *FBN1* deletion involving exons 2-4, and an overview of the corresponding multiplex ligation-dependent probe amplification (MLPA), whole-genome sequencing (WGS) and Sanger sequencing results from our study that emphasizes the relevance of copy number variation screening [109]. The open arrow on the top of the image indicates the direction of transcription. Below that the bars and their numbering represent the exons (the 0 non-coding exon and the exons 1-5 are shown). The yellow box indicates the region with decreased normalized MLPA signals, and filled triangles show the positions of the 3 MLPA probes in exons 2-4. Integrative Genomics Viewer (IGV) is used to display WGS data. Colored bars indicate positions with ≥20% non-reference alleles. Gray bars indicate the aligned reads, red bars show read pairs with larger insert size than expected (due to the deletion), while reads with low quality are indicated by white and pale red bars. The gray and the brown box demonstrate the deleted region of the genomic DNA. Open triangles indicate the three possible break and rejoining positions. The dotted lines mark the most 3' possible breakpoints in *FBN1* transcription direction. The GRCh37 human genome reference is used.

4.1.2 First phase of genetic testing

4.1.2.1 Study population

The first phase of genetic screening involved 57 patients with the clinical diagnosis of Marfan syndrome who underwent a single-gene analysis of *FBN1* [121]. Of these patients

19 (33%) were men and 38 (67%) were women, and the average age at the time of genetic screening was 33 (30–37) years. Based on the revised Ghent nosology, the median systemic score was 8 points (ranging from 7 to 10) for this patient population.

4.1.2.2 Identified genetic variants

The mutation detection rate was 60%, as 34 (likely) pathogenic variants of *FBN1* were identified with the means of NGS and Sanger sequencing. As expected from literature data, the most frequent mutations were missense ones with 17 of them having been identified (50%). Besides that, 8 nonsense (23%), 5 frameshift (15%) and 4 splice-site (12%) variants have been detected.

As the above described case has demonstrated, CNVs can also be the disease-causing variants for Marfan syndrome, thus it is recommended to screen for them in case of negative results of SNV and small indels testing. Accordingly, we applied MLPA method to screen the *FBN1* gene for CNVs in 19 of the 21 negative cases, which resulted in the detection of large deletions in 3 patients. Two of these CNVs were the ones presented above and they served as positive controls. The third CNV involved the deletion of exons 1 and 2.

4.1.3 Second phase of genetic testing

4.1.3.1 Study population

In the second phase of the genetic study, a multi-gene panel was applied to screen the 17 patients with negative results after the first-phase of genetic testing, as well as 79 newly enrolled patients with the clinical diagnosis of Marfan syndrome or Marfanoid habitus. [121]. The examined cohort included 52 (54%) men and 44 (46%) women, the average age at the time of genetic screening was 35 (21–49) years. The median systemic score was 8 points (ranging from 7 to 9) for this patient population.

4.1.3.2 Identified genetic variants

Altogether 30 pathogenic variants have been identified, 27 of which (90%) have affected the *FBN1* gene. The *FBN1* variants comprised 3 missense (11%), 7 nonsense (26%), 7 frameshift (26%) and 10 splice-site (37%) mutations. One pathogenic nonsense mutation was detected in the *TGFBR2* (3.3%) gene, while 2 pathogenic frameshift mutations affected the *TGFB2* (6.7%) gene, both of which are associated with LDS. Furthermore,

13 likely pathogenic variants were identified in *FBN1*, of which 10 were missense mutations (76.9%) and 3 were in-frame deletions (23.1%). In addition, the LDS-associated *TGFBR1* was affected by 2, and the *SMAD3* by another 2 likely pathogenic missense variants. Patients with identified disease-causing variant were 36 (33-40) years old, and had a median systemic score of 8 (ranging from 7 to 9).

Variants of unknown significance (VUS) were also detected in 8 individuals, 3 affecting the *FBN1* (37.5%), 2 the *MYH11* (25%), 2 the *ACTA2* (25%) and 1 the *KCNN* (12.5%) gene. Clinical relevance of these detected VUS could be established in the future, thus they require timely reconsideration.

The detected variants and their distribution among the examined genes are presented in *Figure 6*.

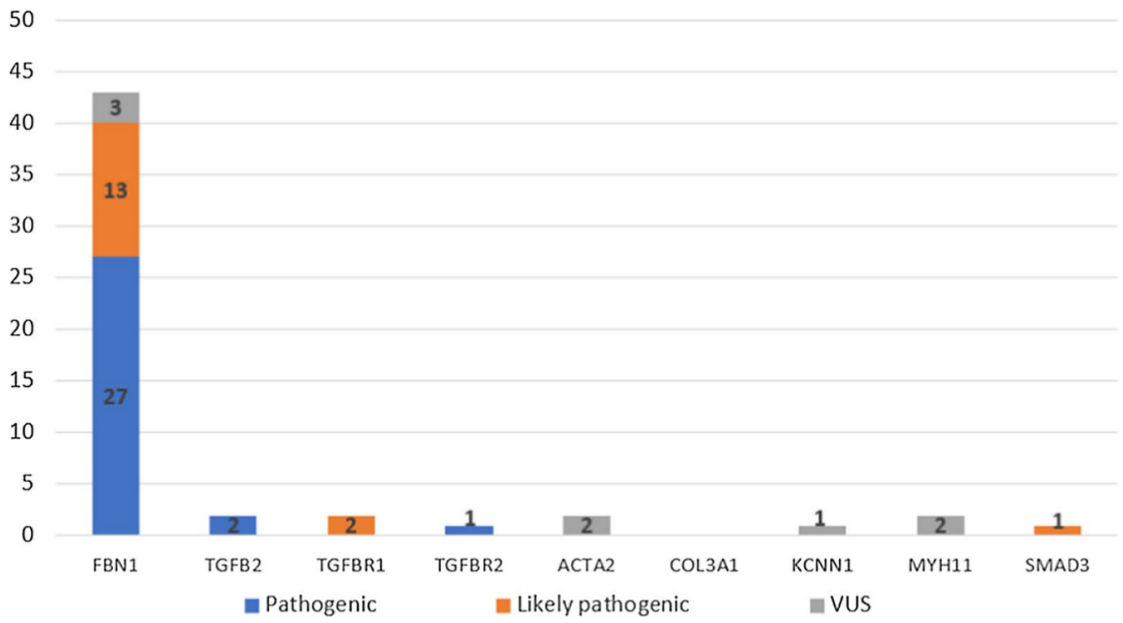

Figure 6 The pathogenic, likely pathogenic mutations and variants of unknown significance (VUS) detected by the multi-gene panel in our genotype-phenotype correlations study are shown [121].

In case of negative results in the second set of patients, MLPA was carried out to examine the presence of CNV. Out of 30 measurements, one CNV was identified resulting in the deletion of exons 3-4 of the *FBN1* gene.

4.1.4 Overall results

The summary of the steps of genetic testing and the overall outcomes are shown in *Figure 7*.

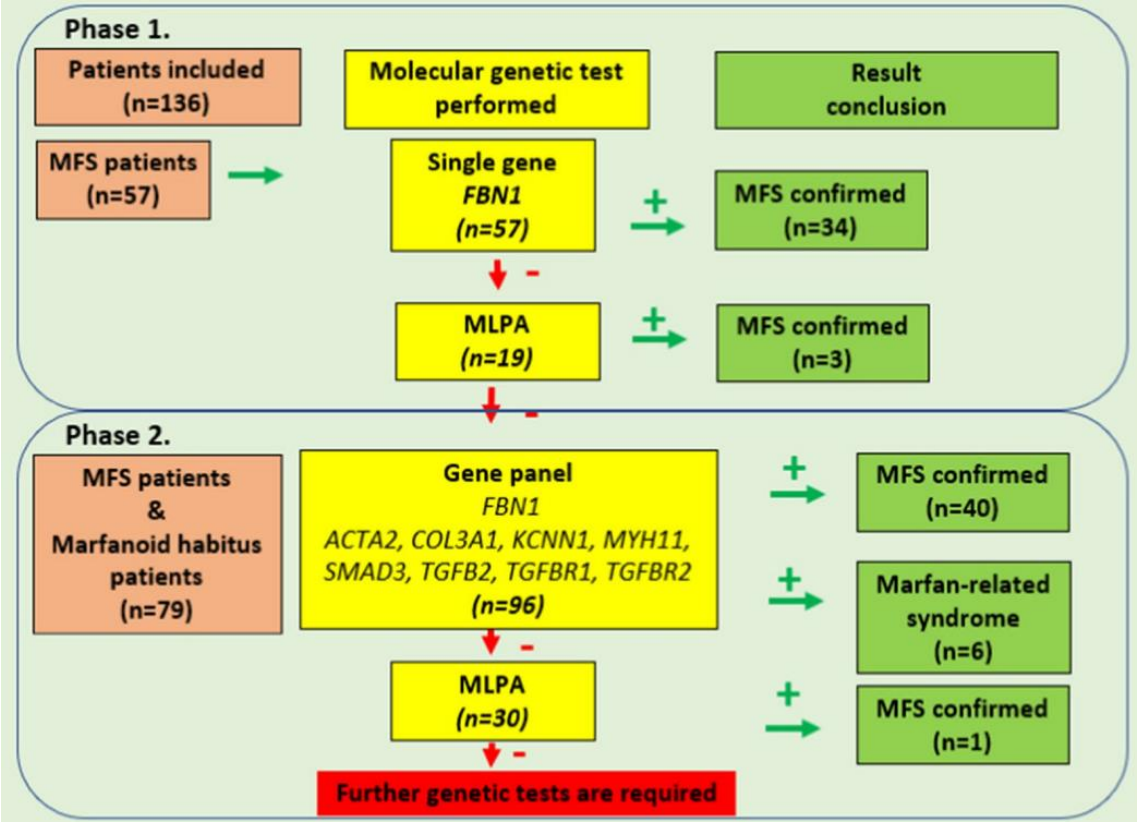

Figure 7 The two phases of genetic testing steps with the outcomes from our genotype-phenotype correlations study are demonstrated [121]. The *FBN1* gene was screened for clinically diagnosed Marfan syndrome (MFS) patients during Phase 1. In Phase 2, a multi-gene panel was applied to test the negative samples from Phase 1, as well as new patients with Marfanoid habitus, meaning a systemic score of at least 5 points, and clinically diagnosed Marfan syndrome. In case of negative results, multiplex ligation-dependent probe amplification (MLPA) was carried out to screen for copy number variations in both phases. The diagnosis of Marfan syndrome was confirmed in 78 cases, while 6 patients were diagnosed with Loeys-Dietz syndrome, which is a Marfan-related syndrome.

4.1.4.1 General characteristics of patients

The general characteristics and the systemic score of patients with and without a detected genetic variant are shown in *Table 2*. It is clearly demonstrated that apart from the body mass index (BMI), which was greater in case of an identified mutation (p=0.042), patients did not show any relevant difference in the two groups.

4.1.4.2 Variant detection rate

At the end of the genetic testing, 65% of the first set of patients (37/57) had a detected disease-causing *FBN1* variant, while mutation identification rate of the *FBN1* was 52% (41/79) in the second set of patients.

Altogether, the detection rate of *FBN1* mutations appeared to be 57% (78/136), while the overall variant detection was 62% (84/136).

4.1.4.3 Diagnoses

The genetic testing confirmed the diagnosis of Marfan syndrome in 78 individuals, while the diagnosis of LDS was established in 6 patients.

Table 2 General characteristics of the examined cohort with and without a detected disease-causing variant. Significant difference is marked with an asterisk (*) [121].

	Studied population (n = 136)	**Positive for mutation** (n = 84)	**Not positive for mutation** (n = 52)	***p* value** (positive vs. not positive)
Male (%)	46	42	54	0.166
Age (years)	35 (33–38)	37 (34–40)	33 (28–37)	0.113
Anthropometric (measured)				
Height (cm)	183.7 (181.8–185.7)	183.4 (180.9–185.8)	184.4 (181.2–187.6)	0.604
Lower segment (cm)	96.1 (94.6–97.5)	96.3 (94.4–98.1)	95.8 (93.3–98.3)	0.748
Arm span (cm)	188.1 (186.0–190.3)	188.4 (185.6–191.1)	187.8 (184.2–191.3)	0.786
Foot size	42.8 (42.3–43.4)	42.8 (42.2–43.5)	42.9 (42.0–43.8)	0.956
Weight (kg)	70.3 (67.3–73.3)	72.0 (68.3–75.7)	67.5 (62.3–72.7)	0.153
Anthropometric (calculated)				
Body Mass Index (BMI; kg/m^2)	20.7 (20.0–21.4)	21.3 (20.4–22.1)	19.7 (18.5–21.0)	0.042*
Body surface area (m^2)	1.88 (1.84–1.93)	1.91 (1.85–1.96)	1.85 (1.77–1.93)	0.230
Upper segment–lower segment ratio (USLS)	0.92 (0.90–0.94)	0.91 (0.88–0.93)	0.93 (0.90–0.97)	0.218
Arm span-height ratio (ASHR)	1.024 (1.018–1.030)	1.027 (1.019–1.036)	1.018 (1.008–1.028)	0.158
Systemic score	8 (7–9)	8 (7–9)	8 (7–9)	0.249

4.1.5 Genotype-phenotype correlations

As a next step, we analyzed the associations between genetic variants and ascending aortic involvement (dissection and/or dilation) with the aim of identifying possible predictors of more severe cardiovascular manifestations by comparing the mutation types of the *FBN1* gene.

4.1.5.1 Aortic involvement in dominant negative and haploinsufficient variants

When comparing the aortic involvement between DN (n=30) and HI (n=48) variants of the *FBN1* gene, we have revealed a tendency of the HI group leading to aortic dissection and/or dilation more frequently than DN mutations (90% and 73%, respectively, p=0.061).

4.1.5.2 Subdividing the dominant negative group based on cysteine involvement

As the amino acid cysteine is a key structural element of the fibrillin-1 protein through disulfide bond formation, we have created a group where the mutation eliminates a cysteine (DN Cys) and a further one where cysteine is not substituted (DN non-Cys).

DN Cys (n=18) mutations led to aortic involvement significantly more frequently, than DN non-Cys (n=12) variants (89% and 50%, respectively, p=0.018). Given that HI mutations were also associated with frequent aortic involvement, and we aimed to create a classification that could be useful in the clinical practice, we have grouped the DN Cys and HI mutations together and compared this combined group to the DN non-Cys variants. It was found that DN non-Cys variants were less frequently associated with aortic dissection and/or dilation than the combined group of HI and DN Cys ($p<0.001$) *(Figure 8a).*

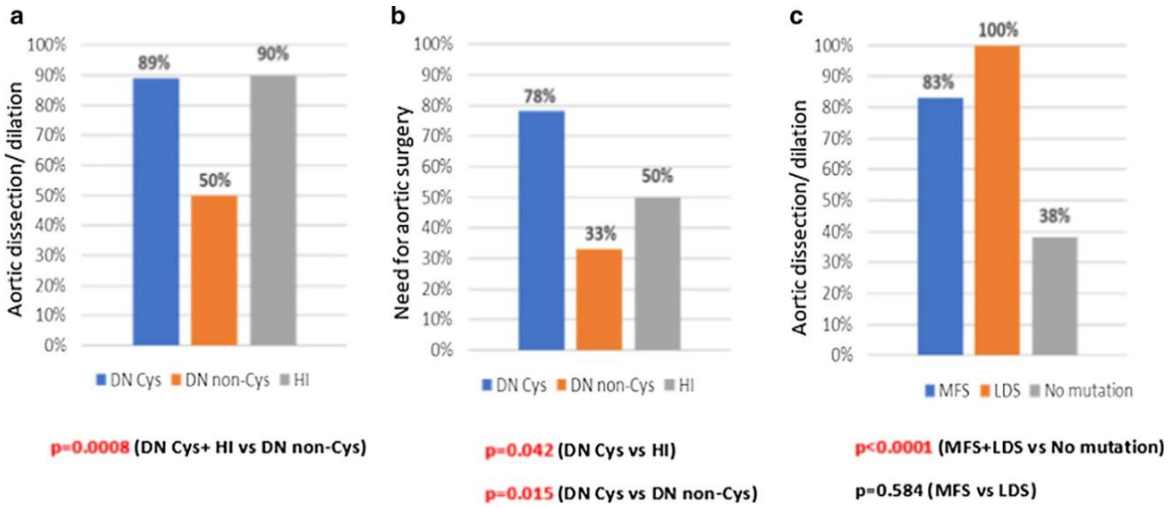

Figure 8 Ascending aortic involvement comparisons from our genotype-phenotype correlations study [121]. **a,** The combined group of haploinsufficient (HI; n=48) and dominant negative mutation eliminating a cysteine (DN Cys; n=18) variants led to aortic dissection/dilation more frequently than DN mutations without cysteine elimination (DN non-Cys; n=12). **b,** Aortic surgery was required more frequently in DN Cys variants than in the other two mutation types. **c,** Patients with identified disease-causing variants (n=84) presented with a significantly higher aortic involvement rate than individuals with no detected mutation (n=52). No difference was observed between Marfan syndrome (MFS; n=78) and Loeys-Dietz syndrome (LDS; n=6) patients in terms of aortic involvement.

We also examined the need for aortic surgery among the three mutation types. Patients with DN Cys variants required aortic surgery significantly more frequently than patients with HI (78% vs 50%, p=0.042) and DN non-Cys variants (78% vs 33%, p=0.015) *(Figure 8b)*.

Importantly, no significant age difference was present among the mutation types at the time of the surgery and at the time of the last follow-up. The mean age at the time of surgery was 36 (28-44) years for the DN Cys, 32 (15-48) years for the DN non-Cys and 35 (31-38) years for the HI groups (DN Cys vs DN non-Cys p=0.605; DN non-Cys vs HI p=0.524; DN Cys vs HI p=0.757). The mean age at the last follow-up was 43 (36-51) years for the DN Cys, 34 (28-40) years for the DN non-Cys and 38 (34-41) years for the HI groups (DN Cys vs DN non-Cys p=0.074; DN Cys vs HI p=0.151; DN non-Cys vs HI p=0.382).

4.1.5.3 Aortic involvement by diagnosis

The frequency of aortic involvement was also analyzed among patients with the diagnosis of Marfan syndrome, LDS and patients where no disease-causing variant was identified.

Of Marfan syndrome patients, 83% (65/78) presented with aortic dissection and/or dilation, while this was 100% (6/6) in case of LDS (p=0.584) *(Figure 8c). Table 3* shows the general characteristics, as well as the systemic score of Marfan syndrome and LDS patients. The systemic score in LDS appeared to be significantly lower than in Marfan syndrome (p=0.013), and patients with LDS tended to be younger than patients with Marfan syndrome (p=0.057) at the last follow-up. However, despite the lower systemic score and younger age, patients with LDS presented with a particularly severe cardiovascular phenotype, as all of them had a dilated aorta, and two of them had already undergone a prophylactic aortic root surgery.

Significantly less people without a detected mutation developed aortic involvement (38%, 20/52) than patients with an identified disease-causing variant (p<0.001) *(Figure 8c).*

Table 3 General characteristics of patients with the diagnosis of Marfan syndrome (MFS) and Loeys-Dietz syndrome (LDS). Significant differences are marked with an asterisk (*) [121].

	MFS (n = 78)	**LDS (n = 6)**	***p* value**
Male (%)	41	50	0.667
Age (years)	37.5 (34.4–40.6)	26.7 (19.8–33.5)	0.057
Anthropometric (measured)			
Height (cm)	183.5 (180.9–186.0)	182.2 (170.2–194.2)	0.777
Lower segment (cm)	96.7 (94.8–98.5)	91.6 (82.2–101.0)	0.153
Arm span (cm)	189.0 (186.1–191.8)	181.5 (168.2–194.8)	0.138
Foot size	42.9 (42.2–43.6)	42.6 (37.9–47.3)	0.854
Weight (kg)	72.7 (68.9–76.6)	63.3 (48.8–77.9)	0.164
Anthropometric (calculated)			
Body mass index (BMI; kg/m^2)	21.49 (20.59–22.39)	18.94 (15.97–21.93)	0.106
Body surface area (m^2)	1.92 (1.86–1.98)	1.78 (1.53–2.03)	0.209
Upper segment–lower segment ratio (USLS)	0.90 (0.88–0.92)	1.01 (0.84–1.18)	0.020*
Arm span-height ratio (ASHR)	1.030 (1.021–1.039)	0.997 (0.980–1.012)	0.025*
Systemic score	8 (7–9)	6.5 (6–7)	0.013*

4.1.5.4 Already established genotype-phenotype correlations

The neonatal region comprising exons 24-32 of the *FBN1* gene has been recognized to be associated with the most severe form of the disease. In our cohort, 8 disease-causing variants were found in this region of *FBN1*, and only one of them led to severe cardiovascular manifestation, as the patient required prophylactic aortic surgery at the age of 17 years. Six of the remaining 7 cases also developed aortic dilation, but without

reaching the surgical threshold. Frequency of aortic involvement did not differ between patients with mutations in the neonatal regions (7/8) and patients with mutations in other regions (58/70) (p=0.739).

In consistence with the literature, DN Cys mutations led to ectopia lentis more frequently than the HI and DN non-Cys variants (p=0.011 and p=0.008, respectively).

4.2 Arterial tortuosity

4.2.1 Study population

The general characteristics (including risk factors for atherosclerosis) of the patient cohort divided into 3 different severity groups based on aortic involvement, as well as the general characteristics of the control group are presented in *Table 4* and *Table 5* [118]. Apart from hypertension being more frequent in Group B compared to the other groups, the severity groups of Marfan syndrome patients did not differ from each other. No difference could be observed in terms of atherosclerosis risk factors between Marfan syndrome and control individuals: hypertension (p=0.832), hyperlipidemia (p=0.478), smoking (p=0.413), diabetes (p=0.663) and history of coronary artery disease (p=1.000).

Table 4 General characteristics of Marfan syndrome patients according to their severity groups. Significant differences are marked with an asterisk (*). Based on *Agg et al. Orphanet J Rare Dis. 2020 Apr 15;15(1):91* [118]. SSc- Systemic score

Groups	A (*n* = 5)	B (*n* = 12)	C (*n* = 20)	All (*n* = 37)	p value
Age at CT (years)	32.4 ± 2.6	37.5 ± 6.6	42.3 ± 14.3	39.4 ± 11.6	0.187
Male	2	8	12	22	0.657
Involvement of cardiovascular system	No intervention required	Mild involvement required intervention	Severe cardiovascular involvement	Involvement of varying degree	
Anthropometric (measured)					
Height (cm)	184.0 [180.0-185.0]	194.0 [182.0-199.2]	187.5 [181.2-195.8]	186.0 [181.5-197.5]	0.351
Arm span (cm)	183.0 [182.0-187.0]	191.0 [189.0-210.0]	186.0 [184.2-204.5]	189.0 [183.0-204.0]	0.263
Lower segment (cm)	90.0 [90.0-99.0]	95.0 [94.0-104.0]	98.0 [93.2-99.8]	97.0 [91.8-103.0]	0.445
Foot size (cm)	28.0 [25.3-28.0]	29.0 [26.7-30.2]	27.7 [26.7-29.7]	28.0 [26.7-30.0]	0.663
Weight (kg)	70.0 [60.0-72.0]	74.0 [63.8-93.5]	82.0 [72.8-96.5]	75.0 [65.0-93.0]	0.242
Anthropometric (calculated)					
Arm span to height ratio	1.01 [1.01-1.02]	1.02 [1.00-1.04]	1.02 [1.01-1.07]	1.02 [1.01-1.05]	0.586
Upper segment to lower segment ratio	1.00 [0.82-1.06]	0.92 [0.84-0.96]	0.90 [0.86-0.97]	0.92 [0.85-1.01]	0.680
Body Mass Index (BMI; kg/m^2)	20.5 [18.5-21.3]	20.6 [19.6-24.1]	23.0 [21.4-26.0]	21.5 [19.6-25.2]	0.128
Body surface area (m^2) - Mosteller	1.90 [1.73-1.92]	2.01 [1.81-2.27]	2.08 [1.92-2.26]	1.97 [1.81-2.26]	0.263
Ghent nosology					
Positive family history (%)	80.0	58.3	35.0	48.6	0.153
Systemic score (SSc)*	5.0 [2.0-7.0]	8.0 [6.5-9.0]	8.0 [5.8-9.0]	8.0 [5.0-9.0]	0.176
SSc < 7 pts. (%)	60.0	25.0	30.0	32.4	0.477
SSc 7-10 pts. (%)	40.0	58.3	65.0	59.5	0.591
SSc > 10 pts. (%)	0.0	16.7	5.0	8.1	0.707
Risk factors for atherosclerosis					
Hypertension (%)	0.0	66.7	25.0	35.1	0.013* Group B p=0.03*
Hyperlipidemia (%)	0.0	8.3	15.0	10.8	1
Smoking (%)	0.0	25.0	5.0	10.8	0.171
Diabetes (%)	0.0	0.0	5.0	2.7	1
History of coronary artery disease (%)	0.0	0.0	5.0	2.7	1

Table 5 General characteristics of the control group [118].

	Control group (*n* = 74)
Age at CT (years)	39.7 ± 11.5
Male	44
Risk factors for atherosclerosis	
Hypertension (%)	32.4
Hyperlipidemia (%)	6.8
Smoking (%)	18.9
Diabetes (%)	5.4
History of coronary artery disease (%)	2.7

4.2.2 Tortuosity of the visceral arteries in Marfan syndrome compared to controls

The distance metric (DM) of the splenic and the right and the left renal arteries was revealed to be greater in patients with Marfan syndrome compared to controls (2.44 [1.92-2.80] vs. 1.75 [1.57-2.18] p<0.001; 1.16 [1.10-1.28] vs. 1.11 [1.07-1.15] p=0.011; 1.40 [1.29-1.70] vs. 1.13 [1.09-1.23] p<0.001 respectively) *(Figure 9A).*

Inflection count metric (ICM) of the splenic artery was significantly increased in comparison to the control individuals (31.43 [22.75-42.39] vs. 26.02 [17.86- 34.30] p=0.026), the right renal artery only tended to have a larger ICM (14.95 [10.65-18.53] vs. 12.03 [9.26- 15.17] p=0.056), and no difference in ICM could be observed in case of the left renal artery (9.26 [7.13-13.27] vs. 9.73 [7.63-12.72] p=0.841) *(Figure 9B).*

The right and left renal arteries of Marfan syndrome patients had a significantly lower sum of angles metric (SOAM) than the control group (0.55 [0.45-0.65] vs. 0.62 [0.53-0.71] p=0.024; 0.43 [0.38-0.53] vs. 0.55 [0.49-0.64] p<0.001, respectively). No significant difference could be observed in SOAM in case of the splenic artery (0.45 [0.35-0.52] vs. 0.48 [0.39-0.58] p=0.116) *(Figure 9C).*

We further investigated the tortuosity between the two groups by analyzing the ICM/SOAM parameter. The splenic, the right and the left renal arteries all showed a significantly increased ICM/SOAM in Marfan syndrome patients compared to control individuals (73.35 [62.26-93.63] vs. 50.91 [43.19-65.62] p<0.001; 26.52 [20.69-30.24] vs. 19.95 [16.47-22.95] p<0.001; 22.81 [18.64-30.96] vs. 18.38 [15.29-21.46] p<0.001) *(Figure 9D).*

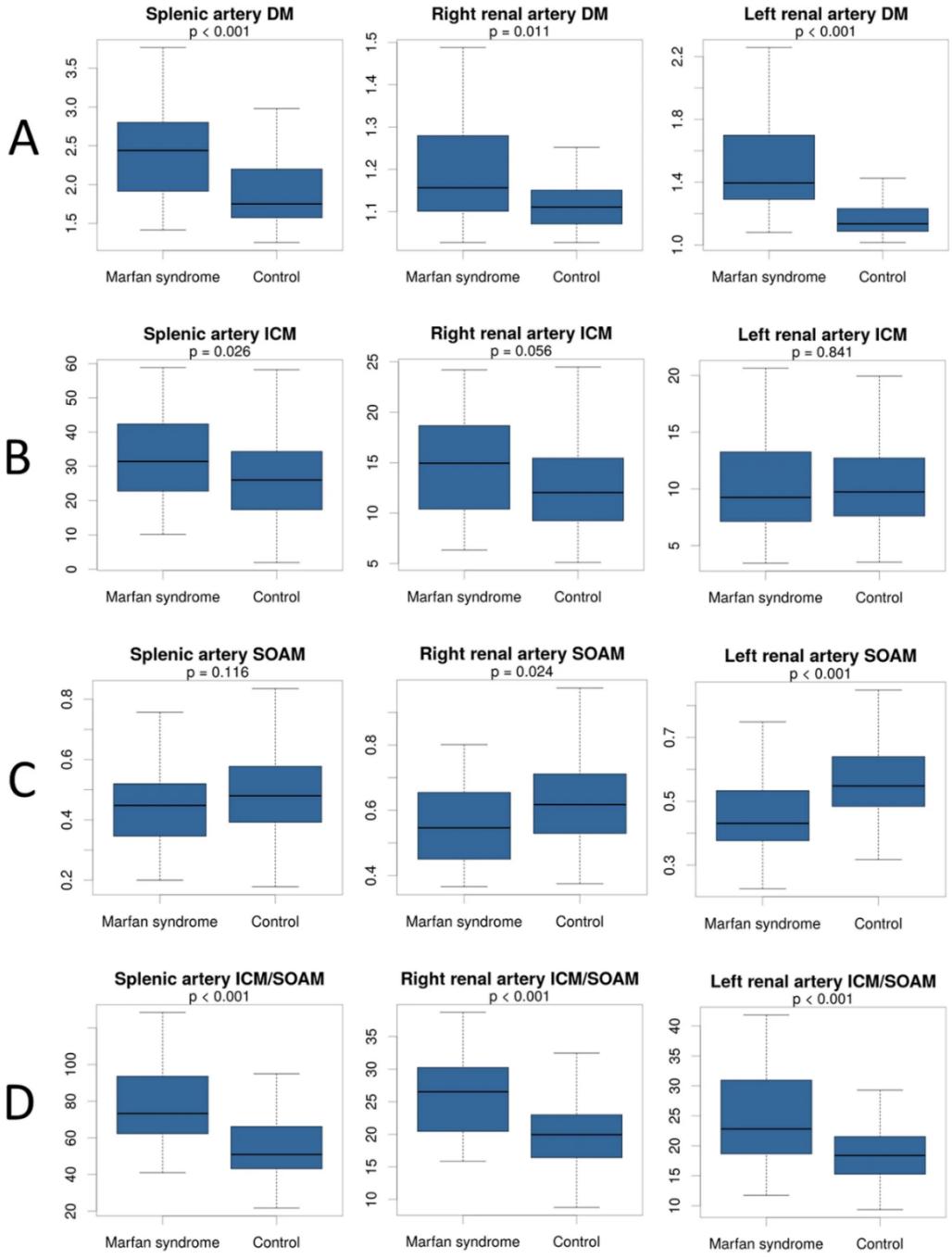

Figure 9 Comparison of the tortuosity metrics of the splenic artery and the right and left renal arteries between Marfan syndrome patients and control subjects in our visceral arterial tortuosity study [118]. **A,** Distance metric (DM) was significantly increased in Marfan syndrome patients in all three arteries. **B,** Inflection count metric (ICM) showed a similar tendency as DM. **C,** Sum of angles metric (SOAM) was higher in control individuals. **D,** ICM/SOAM was significantly higher in case of Marfan syndrome compared to controls.

4.2.3 Tortuosity of the visceral arteries in the severity groups of Marfan syndrome

The DM showed a significant difference between the severity groups in case of the right and left renal arteries (Kruskal-Wallis p=0.045 and 0.049, respectively). Marfan

syndrome patients without severe aortic involvement (Group A) compared to Marfan syndrome patients who underwent aortic surgery due to various indications (Group B and C) had significantly lower DM in case of the right (p=0.039 and p=0.039) and the left (p=0.041 and p=0.041) renal arteries *(Figure 10A)*. In terms of the other tortuosity parameters, the difference was significant only when comparing the left renal ICM/SOAM of Group A and Group B (Kruskal-Wallis p=0.040; p=0.023) *(Figure 10B)*.

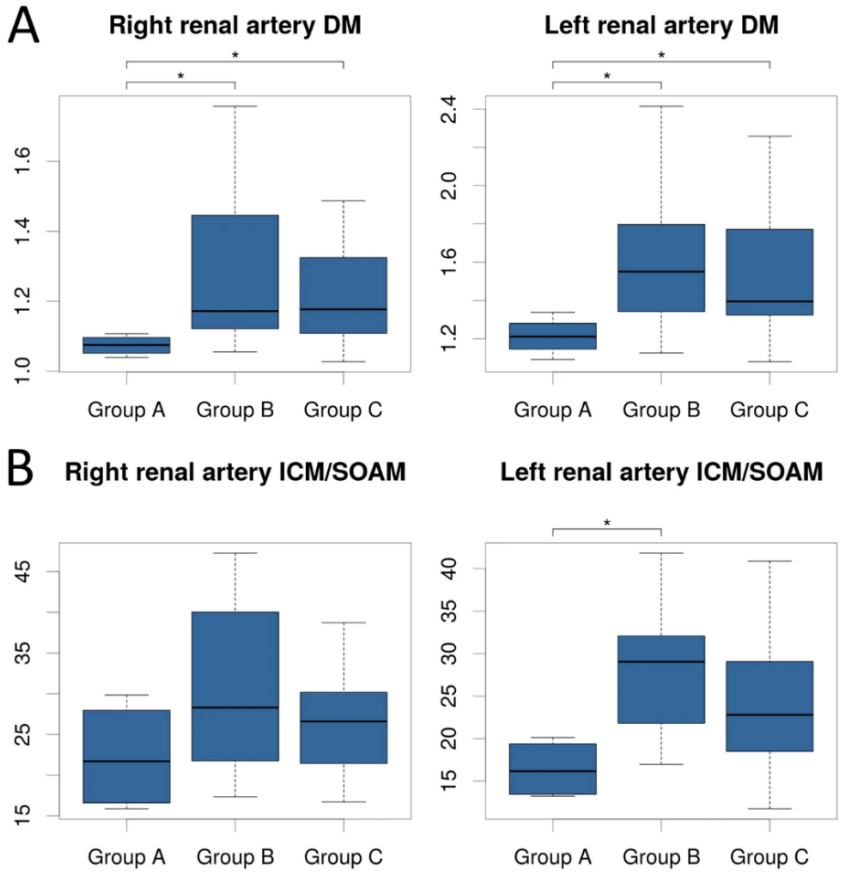

Figure 10 Comparison of the tortuosity of the right and left renal arteries between the severity groups of Marfan syndrome patients in our visceral arterial tortuosity study [118]. Group A- patients without aortic involvement. Group B- patients with prior elective surgery on the ascending aorta. Group C- patients operated on for annuloaortic ectasia or type A aortic dissection. Patients who underwent aortic surgery (Group B and C) had significantly increased distance metric (DM) (**A**) and inflection count metric/sum of angles metric (ICM/SOAM) (**B**) parameters of both renal arteries compared to Marfan syndrome patients without aortic involvement (Group A).
Asterisk (*): p<0.05.

4.2.4 Follow-up of tortuosity through a case

A 41-year-old Marfan syndrome patient, who had previously undergone aortic surgery, presented with a saccular pearl-string-like aneurysm on the RIMA and a single aneurysm

on the LIMA [31]. We have opted for follow-up and blood pressure control instead of intervention of these aneurysms due to the lack of guidelines, the tortuosity of the affected vessels, the asymptomatic state of the patient and a previous cardiac surgery. Because of the regular follow-ups with CT scan, we could analyze the tortuosity of the internal mammary arteries and the aorta and its changes throughout the years.

We evaluated the DM, ICM, SOAM and ICM/SOAM tortuosity parameters. The changes of these parameters are demonstrated in *Figure 11*. As the aneurysms progressed, DM demonstrated an increase in case of the RIMA and the LIMA, which shows a progression of the tortuosity of these vessels. The thoracic aorta showed an overall increase in the ICM, SOAM and ICM/SOAM parameters, indicating a progression of tortuosity with increasing amplitude and frequency of the curves, dominated by the rise in amplitude as suggested by ICM/SOAM.

It is important to note that this patient had severe aortic involvement as aortic root surgery and later on aortic arch replacement needed to be carried out. Furthermore, peripheral aneurysms can rarely be observed in Marfan syndrome patients, and this finding together with a progressive tortuosity could indicate that the blood vessels are severely affected, thus putting the patient in risk for vascular events.

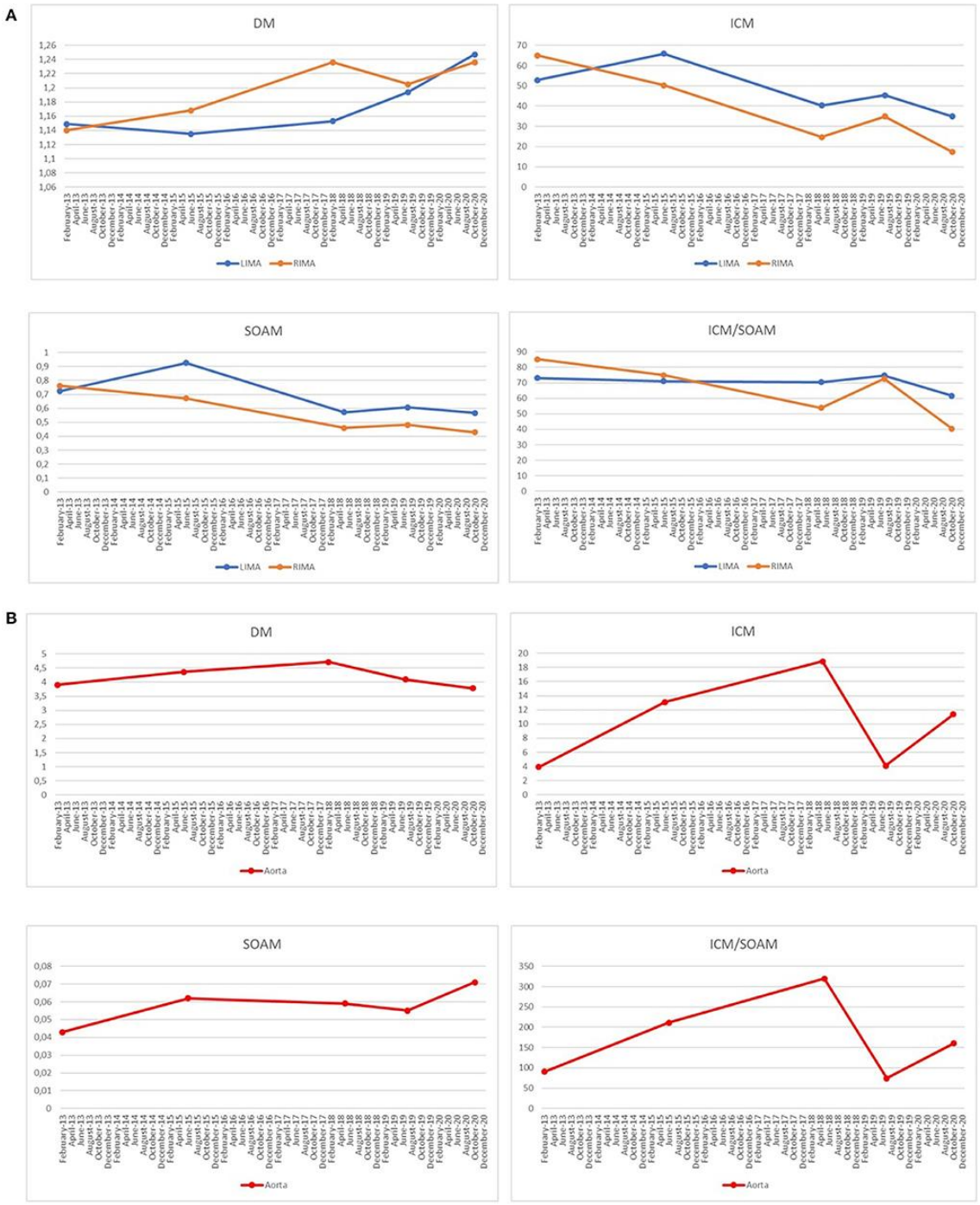

Figure 11 Changes of vessel tortuosity from our case presentation [31]. **A,** Distance metric (DM) has been increasing over the years in case of the left internal mammary artery (LIMA) and right internal mammary artery (RIMA), indicating a progression in tortuosity. The other tortuosity parameters are less conclusive as they all show a decreasing tendency. **B,** The thoracic aorta is shown to have an increasing tortuosity with increasing amplitude and frequency of the curves indicated by the overall rise of the ICM, SOAM and ICM/SOAM parameters.

5 Discussion

This thesis identified potential predictors of more severe aortic involvement by the means of genotype-phenotype correlations and arterial tortuosity in patients with Marfan syndrome. These findings could contribute to an improved risk stratification and thereby to an optimization of indication and timing of a prophylactic aortic surgery. Furthermore, this thesis established a possible genetic testing sequence for patients with the clinical diagnosis of Marfan syndrome and Marfanoid habitus.

5.1 Genetic testing of Marfan syndrome patients

In order to study and to make use of genotype-phenotype correlations, it is important to optimize the mutation detection in patients with Marfan syndrome. Our genetic testing strategy has evolved with time, hence there were two distinct phases in our screening design [121]. First, we only screened the *FBN1* gene with NGS in patients with clinically diagnosed Marfan syndrome. The applied technology is not able to detect CNVs, which are large deletions and duplications (>50 bp), accounting for about 10% of Mendelian disorders, a category that also involves Marfan syndrome [109]. The above presented case has also confirmed the significance of CNV screening of the *FBN1* gene in case of negative results after SNV and small indels testing [109]. Thus, we have started to routinely use MLPA in case of negative results after NGS. Due to the remarkable overlap between the symptoms of Marfan syndrome and its related disorders, mainly LDS [81], and the different therapeutic approach required in the various aortopathies, we have decided to use a multi-gene panel as a first tier genetic testing tool and we also started to screen patients with Marfanoid habitus in order to identify patients with related disorders.

There are various approaches for genetic testing of Marfan syndrome patients in the literature yielding in a wide range of detection rates. The overall rate of identifying a disease-causing variant was 62% in our study. Baetens *et al*. screened clinically diagnosed Marfan syndrome patients with a single-gene analysis by NGS followed by MLPA and they reached a 92% success rate in mutation detection [112]. On the other hand, Arnaud and colleagues managed to get a detection rate of only 56% after screening the *FBN1* gene in patients with suspected Marfan syndrome [122]. In case of multi-gene aortopathy panels, the success rate of disease-causing variant identification has also been reported to

be as low as 10.3% [123]. Similarly, Lerner-Ellis *et al*. reached a positive result only in 19% of tested individuals with the suspicion of Marfan syndrome, LDS and other aortopathies, however, as the authors state, patient selection was not carried out by experts in the field, which could have contributed to the low success rate [124].

In our patient cohort, the general characteristics and the systemic score, apart from their BMI, did not show any difference between patients with identified disease-causing variants and individuals without detected variants, indicating that the lower mutation identification rate was not likely to be caused by inappropriate patient selection. One contributing factor to the lower success rate in variant detection could be the causative role of deep intronic variants, that have already been shown to result in Marfan syndrome, but could not be investigated in our study [125]. A further explanation could be that the disease-causing variant may be found in another gene not present in the applied multi-gene panel, possibly in genes the mutations of which lead to Marfanoid features without severe aortic involvement, as individuals without an identified mutation had a smaller rate of aortic involvement than patients with a detected variant.

As around 5% of the identified mutations in our cohort were CNVs, we have also highlighted the relevance of CNV screening in case of negative results after screening for SNVs and small indels. Negative cases after a 15-gene panel also underwent MLPA testing in a study by Yang *et al*., resulting in the detection of 5 large deletions in the *FBN1* gene, hence finding a CNV in approximately 8% of the cases that were negative with panel assay [126]. The importance of CNV screening in syndromic aortopathies was also highlighted by Takeda *et al*., as they identified CNVs in around 47% of patients without a detected disease-causing variant after undergoing NGS-based genetic testing [127].

The mutation detection rate could be improved by the use of WES and WGS. WES is used to sequence the coding regions of all genes, thus can examine all the genes associated with the disease and could also reveal novel gene-disease associations. However, similarly to targeted sequencing, WES has an incomplete coverage of the exome, thereby may miss clinically relevant variants [66,85]. Due to its beneficial properties, WES has been suggested to be used as a first-tier screening method in general in all cases without a clear differential diagnosis [128]. On the other hand, WGS has the most continuous coverage and the ability to detect variants throughout the genome,

including non-exonic variants and CNVs [66]. Its routine use is currently limited by its high costs.

5.2 Prognostic role of the involved gene

The importance of screening multiple genes in patients with a suspicion of Marfan syndrome is clearly demonstrated by the finding that 7% of our patient cohort carried a (likely) pathogenic variant in LDS-associated genes, thereby establishing the diagnosis of LDS. These LDS patients tended to be younger and had a significantly lower systemic score than our Marfan syndrome cohort, despite of which all of them presented with aortic involvement, two of them already having undergone a prophylactic aortic surgery. This data emphasizes the need for the screening of patients with Marfanoid features but without reaching the required 7 points for the systemic involvement according to the revised Ghent nosology. Furthermore, patients with LDS can also fulfill the diagnostic criteria of Marfan syndrome, stressing the need for multi-gene panels. An example of that is the work from Nistri *et al*., where they presented a patient who fulfilled the diagnostic criteria of Marfan syndrome, but a mutation in the *TGFB2* gene was later identified, altering the diagnosis to LDS [129]. Furthermore, in a study from Franken and colleagues, 4 *TGFBR1*, 4 *TGFBR2*, 1 *TGFB2*, 2 *SMAD3* and 1 *MYH11* mutations were identified in patients, who fulfilled the revised Ghent criteria but did not have an *FBN1* mutation [81]. There are some distinctive features between Marfan syndrome and LDS that could aid the differential diagnosis, however, they do not present in all cases and there is emerging data that some of the specific features may develop in the other syndrome as well. Ectopia lentis is a unique feature of Marfan syndrome, discriminating it from LDS, however, ectopia lentis was identified in a patient with genetically confirmed LDS without any variants in the *FBN1* gene [130]. Our research group has also described the case of a genetically confirmed Marfan syndrome patient who developed peripheral aneurysms, which is rather a characteristic feature of LDS [31]. These findings underline the significance of genetic testing involving multiple genes, not only in patients with Marfanoid features, but in clinically diagnosed Marfan syndrome patients as well.

The importance of differentiating between Marfan syndrome and LDS is due to their substantially different management approach necessitated by the more aggressive course of LDS. LDS patients are recommended to undergo a prophylactic aortic surgery

at smaller diameters and to have a follow-up with more extended imaging examinations [93].

5.3 Genotype-phenotype correlations

We investigated the potential usefulness of genetic background in the prediction of aortic manifestations in Marfan syndrome with the aim of improving risk stratification and thereby to optimize the indications and timing of a prophylactic aortic surgery [121]. In our cohort, we have identified mutation types with more frequent and more severe ascending aortic involvement. DN variants without cysteine substitution represented a group with less frequent aortic dissection and/or dilation compared to the combined group of HI and DN Cys mutations. We have grouped HI and DN Cys variants together as they both frequently led to aortic involvement and we intended to create a classification system which could be easily implemented in the clinical practice. However, DN Cys variants may result in more severe aortic manifestations than HI ones, as patients with a DN Cys variant had to undergo aortic surgery more frequently than individuals in the HI group, despite showing no age difference at the time of surgery and at the last follow-up. As cysteine is a particularly important amino acid in the structure of fibrillin-1 due to its disulfide bond forming role, mutations that affect cysteine disrupt fibrillin-1 domain conformation and multimerization, thereby impairing its function and making it more vulnerable to proteolysis [131].

Several articles have been published about genotype-phenotype correlations in Marfan syndrome, however, apart from a few well-established associations, the results have been conflicting, necessitating further studies. The reasons behind the inconclusive results could be the small sample sizes given Marfan syndrome is a rare disorder, differences in study design, the role of possible genetic modifiers [132] and differences between the investigated individuals in factors that influence the status of the aorta, such as blood pressure.

A few studies have been published where no difference could be observed in terms of aortic involvement among the mutation types. Loeys and colleagues compared the occurrence of major cardiovascular manifestations between Marfan syndrome patients with missense mutations substituting a cysteine amino acid (23/27) and patients with PTC variants (29/33), and they revealed no difference. The need for aortic surgery was not

discussed in this work [133]. Comparable findings were reported by Arbustini *et al*. when they studied the same mutation types as in the paper from Loeys and colleagues [134]. Similarly, when comparing PTC variants and missense mutations with cysteine substitution, Comeglio *et al*. revealed no difference in cardiovascular involvement [135]. These results are consistent with our finding that DN Cys and HI mutations lead to cardiovascular manifestations with comparable frequencies.

The importance of treating missense mutations that eliminate a cysteine amino acid separately was also indicated in a large study involving 1013 probands with identified *FBN1* mutation, which showed that variants with cysteine elimination had a higher probability of ascending aortic dilation than mutations creating a cysteine. No significant difference could be observed between other mutation types in terms of cardiovascular severity [74]. Consistently, PTC and missense mutations led to cardiovascular involvement with similar probability in a pediatric cohort [136].

Schrijve and colleagues evaluated the genotype-phenotype correlations in Marfan syndrome patients with PTC variants and mutations with cysteine substitution. Aortic dissection occurred more frequently in the PTC group, and it was the dominant indication for aortic surgery. On the other hand, the most common indication for aortic operation was a dilated aorta in the cysteine substitution group [73]. Aortic dilation and/or dissection developed more frequently in PTC mutations than in cysteine substitutions, however, it did not reach a statistical significance in a paper by Rommel *et al*. Aortic dissection was only reported in case of these two mutation types, being more common in the PTC group. Interestingly, aortic involvement in patients with missense mutations without cysteine involvement was comparable to PTC and cysteine substitution variants, but without aortic dissection being reported [137]. A further study has revealed missense mutations without cysteine involvement to be more deleterious in terms of cardiovascular manifestations than missense mutations with cysteine involvement. A significantly higher probability of cardiovascular involvement was reported in PTC or splice variants in comparison to missense ones [138]. The more severe cardiovascular effect of missense mutations without cysteine involvement is inconsistent with our findings, as well as with most of the published data in the literature. The above mentioned work from Faivre *et al*. with 1013 included patients described a tendency for more frequent ascending aortic dilation in case of missense mutations with cysteine involvement compared to other

missense variants [74]. A further publication has revealed that DN variants with cysteine substitution tended to be associated with aortic involvement more frequently than other DN variants. The study has also found that patients with HI mutations experienced aortic dissection more often and at an earlier age compared to DN patients, however, the difference was statistically not significant. Cox regression analysis did not reveal any significant difference in the risk of aortic dissection, prophylactic aortic surgery and death among the mutation types [139].

Several papers have been published that found significant differences in cardiovascular manifestations of Marfan syndrome patients among the various mutation types. Baudhuin *et al*. analyzed the occurrence of aortic dissection and prophylactic aortic surgery in 179 Marfan syndrome patients. Most of the variants in patients with aortic event belonged to truncating or splicing categories, the frequency of missense mutations was only about 20%. In patients without an aortic event, truncating or splicing variants accounted for only approximately 40% of variants. Furthermore, aortic event occurred at a younger age in the group of truncating or splicing variants compared to missense ones. Missense mutations were not further divided based on cysteine involvement [140]. Similar findings were observed in the work of Becerra-Muñoz *et al*. Patients with a truncating variant (frameshift or nonsense) had an aortic event significantly more frequently than patients with a missense mutation (57.1% vs 13.6%). The age at the occurrence of the aortic event did not differ significantly between the two groups [75]. In the paper from Franken and colleagues, the development of severe aortic phenotype was analyzed in a prospective design among HI and DN variants. Altogether 357 patients were involved and followed-up for a mean duration of about 8 years. HI variants were found to be more deleterious than DN ones, as patients with HI variants had 2.5-fold increased risk for cardiovascular death, 2.4-fold increased risk for the combined endpoint of aortic dissection and cardiovascular death and a 1.6-fold increased risk for any cardiovascular event in comparison to Marfan syndrome patients carrying a DN variant [81]. In a further work from Franken *et al*., aortic diameter, the dilation rate of the main aortic segments and the clinical endpoints of dissection and death were analyzed in a large cohort of almost 300 Marfan syndrome patients with HI or DN variants. There was no difference in terms of age and body surface area among the two mutation types. The HI group had a significantly larger aortic root at baseline compared to the DN group, however, they

showed no difference in terms of aortic dissection and aortic surgery. Dilation of aortic root and ascending aorta was also more pronounced in the HI group after a mean follow-up time of around 5 years. In addition, the risk for the combined endpoint of dissection and death was 3.3-fold increased in case of HI mutations compared to DN ones. In case of individual endpoints of cardiovascular death, aortic dissection or aortic surgery, no difference could be observed [141]. A further group of mutations associated with severe aortic involvement defined by the need for aortic root replacement, type A aortic dissection and related death, have been identified in the work of Takeda *et al*. When HI and DN variants were compared, the HI group was associated with a higher risk for the listed aortic events. However, a subgroup of DN, the DN-CD group, comprising variants affecting or creating cysteine residues and in-frame deletion variants in exons 25–36 and 43–49, led to a lower cumulative event-free survival than the other missense mutations (DN-nonCD group), resulting in a 6.3-fold higher risk. Both the DN-CD and HI groups had a significantly higher risk of severe aortic events than the DN-nonCD group. No significant difference could be observed between the DN-CD and HI groups, however, the DN-CD variants tended to be associated with more severe aortic involvement than the HI ones. Furthermore, the aortic root was larger in the combined group of HI and DN-CD compared to the DN-nonCD one [142]. Mutations with cysteine involvement were also found to lead to more severe aortic phenotype in a pediatric cohort. Compared to missense mutations without affecting a cysteine amino acid, mutations with cysteine involvement were associated with a higher prevalence of the dilation of the sinus of Valsalva and tricuspid valve prolapse. Cardiovascular manifestations did not differ significantly between patients with missense/frameshift variants and with splicing mutations. When missense/frameshift variants were compared with nonsense ones, no significant difference could be observed either. Interestingly, pulmonary artery dilation was described to have earlier onset in patients with missense variants compared to patients with nonsense/frameshift ones [143]. In the study of Xu *et al*, frameshift and nonsense mutations were significantly more common in patients with aortic dissection compared to aortic aneurysm, while missense variants more frequently appeared in patients with aneurysm compared to dissection. In this work, effect of genotype differences was investigated also at the histological level. Frameshift and nonsense variants were found to result in fewer elastic fibers and fewer and more disorganized smooth muscle cells in

the aortic wall in comparison to the effect of missense variants. It is important to stress that dominant negative mutations were not divided based on cysteine involvement in this study [144]. Arnaud and colleagues reported genotype-phenotype correlations in their work including more than 1500 genetically confirmed Marfan syndrome patients. According to their findings, PTC mutations were associated with a higher risk of aortic dissection and aortic surgery as well as with a larger mean aortic root diameter than in-frame mutations. In-frame variants with cysteine elimination led to a severe cardiovascular phenotype with more aortic dissection or surgery. Thus, similarly to our findings, PTC and in-frame variants with cysteine elimination made up the high risk group for severe aortic involvement. Interestingly, in-frame variants introducing a cysteine amino acid were associated with a mild phenotype, and in-frame variants not involving cysteine carried an intermediate risk for aortic events [131].

Based on our findings and the discussed literature, we propose an aortic management strategy for Marfan syndrome patients according to their variant type. This suggestion is demonstrated in *Figure 12*. Briefly, mutations should be categorized into haploinsufficient, dominant negative with cysteine elimination and dominant negative without cysteine elimination groups. We recommend that HI and DN Cys variants should be treated similarly as high-risk variants and thus a more frequent follow-up and an earlier prophylactic surgery may be considered in case of patients with these variants. DN non-Cys variants should be treated as lower risk for aortic involvement, thus the current guidelines should be applied in terms of follow-up and prophylactic surgery.

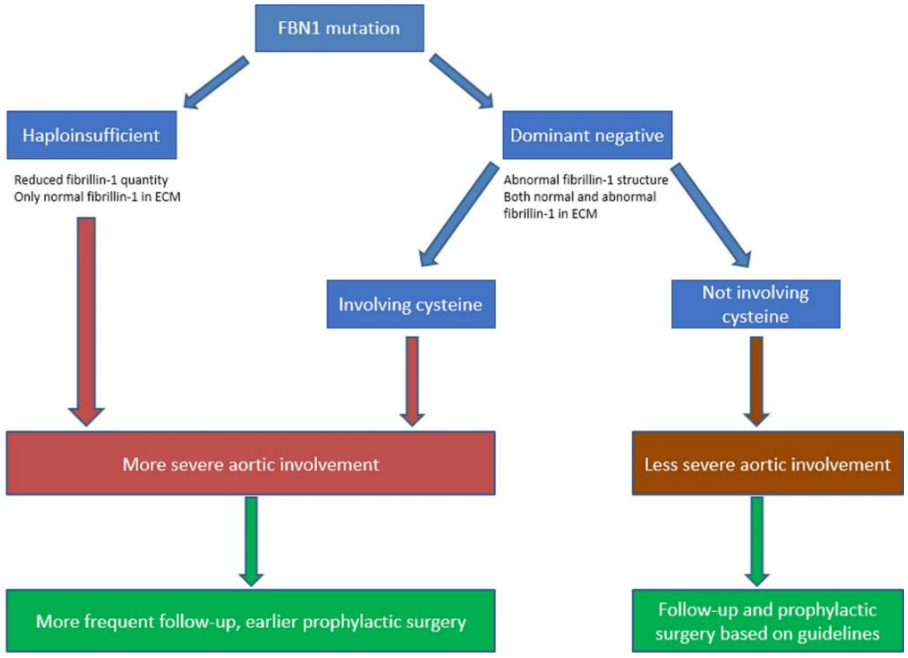

Figure 12 Proposed management of aortic manifestations of Marfan syndrome patients based on the type of disease-causing genetic variant. As, according to our findings and data from the literature, haploinsufficient (HI) and dominant negative mutation eliminating a cysteine (DN Cys) are associated with a higher risk for aortic involvement than dominant negative mutation not eliminating a cysteine (DN non-Cys), patients carrying HI or DN Cys variants should be followed-up more frequently and an earlier prophylactic aortic surgery should be considered than in case of DN non-Cys mutations. Blue boxes represent the genetic variant type, color red shows a more severe, while the brown color indicates a less severe aortic involvement. Green boxes represent the proposed management approach [145].

5.4 Arterial tortuosity

According to the literature and our results, arterial tortuosity also has the potential to contribute to the improvement of risk stratification for severe aortic involvement, and therefore to the prevention of acute aortic events in Marfan syndrome patients. We have shown that patients with Marfan syndrome present with increased tortuosity of the splenic and both renal arteries compared to controls, furthermore, more tortuous renal arteries were associated with more severe aortic phenotype, making tortuosity of the renal arteries a potential predictor of serious cardiovascular manifestations [118].

Based on the hypothesis that arterial tortuosity represents vascular fragility, S. Morris and colleagues investigated the tortuosity of vertebral arteries in patients with connective tissue disorders and analyzed its association with the severity of cardiovascular features. They developed a magnetic resonance angiography (MRA) index for vertebral artery tortuosity (Vertebral Tortuosity Index=VTI) and applied it for 90 patients with Marfan syndrome, LDS, EDS or nonspecific connective tissue disorders and

30 control individuals. More than half of the patients had the diagnosis of Marfan syndrome. It was found that Marfan syndrome and LDS patients had an increased VTI compared to controls. Importantly, a higher VTI was associated with more severely dilated aortic root, a more frequent need for cardiac surgery and a younger age at dissection, cardiac surgery and death [35]. The Aortic Tortuosity Index (ATI) was established to further assess the tortuosity in a 3 year-long follow-up study involving more than 200 patients with Marfan syndrome. The authors revealed that ATI correlated with age, aortic root diameter and the rate of aortic volume expansion. Patients with higher ATI had a significantly higher probability of meeting the combined endpoint of prophylactic aortic surgery, aortic dissection and death. Furthermore, ATI was the only predictor for type B aortic dissection [36].

These two studies analyzed the tortuosity of vessels that run in close proximity to skeletal structures, thereby the properties of these vessels could be highly influenced by the skeletal manifestations of Marfan syndrome. As an example, pectus excavatum, which develops in about two thirds of Marfan syndrome patients [146], could have a relevant impact on the geometry of the aorta. This limitation of the above mentioned studies led us to examine the tortuosity of visceral arteries that are not influenced by the skeletal features of the syndrome.

Furthermore, we used additional metrics that can provide a more complex characterization of vessel tortuosity. These metrics have shown that the tortuosity of the investigated arteries was dominated by higher amplitude and lower frequency curves [147].

In our case presentation, we followed the evolvement of arterial tortuosity for years in a Marfan patient with severe cardiovascular manifestations [31]. Parallel to the progression of LIMA and RIMA aneurysms, the tortuosity of the LIMA, RIMA and the aorta has been increasing, thereby showing an association between peripheral aneurysmal growth and the degree of tortuosity. As the patient had undergone a Bentall surgery prior to acquiring the data for tortuosity analysis, the relationship between the degree of tortuosity and aortic aneurysmal growth could not be assessed [31].

The predictive role of arterial tortuosity was assessed in other aortopathies as well. Chu *et al*. investigated carotid arterial tortuosity in 54 LDS patients and calculated the Carotid Artery Tortuosity Index (CATI). Higher CATI was found to be associated with

the need for aortic root replacement, making it a promising predictor of more severe aortic involvement [148]. Arterial tortuosity was revealed to be a marker of more severe cardiovascular involvement in a subset of vEDS patients. An increased height-adjusted VTI was found to be associated with an increased rate of cardiovascular events in patients with high-risk genetic variants who were under 40 years [149].

The pathological background for the development of arterial tortuosity is not completely understood yet. As a possible explanation, the role of oxidative stress has been hypothesized to be a driver for the manifestations of arterial tortuosity syndrome [150]. Another hypothesis outlines the abnormal lengthening of the arteries in a fixed space as the main cause of tortuosity development. This lengthening may be due to a maladaptation to axial stress, with the aim of reducing stress along the vessel. It is also possible that the abnormal vessel wall in genetic conditions experiences less axial tension which can lead to increased tortuosity [33]. The increased level of TGF-β has also been postulated as the cause of tortuous arteries [36].

5.5 Further potential predictors of severe aortic involvement

Further factors have also been proposed as possible predictors of severe aortic manifestations [145]. These can be grouped into biomarkers that are measured in blood and biomarkers assessed by medical imaging. The former group involves TGF-β, the predictive role of which has been extensively studied. As TGF-β is an important factor in the development of many features of Marfan syndrome, its elevated level could be expected to be associated with a more severe phenotype [48]. Some papers report no correlation between disease manifestation and TGF-β levels [151,152], but others found that elevated TGF-β levels were associated with more severe aortic involvement and thus, could have a prognostic role [51,153]. Another promising molecular predictor is homocysteine, which, when is in excess amount, can damage the fibrillin-1 protein [154]. Consistently, elevated homocysteine level was associated with severe cardiovascular involvement in Marfan syndrome patients [155,156]. MMPs play an important role in the development of cardiovascular Marfan syndrome features, as they lead to elastic fiber degradation [53]. Accordingly, up-regulation of the *MMP3* gene in peripheral blood mononuclear cells and the plasma levels of the soluble form of the extracellular MMP inducer (EMMPRIN) were associated with aortic manifestation severity [153,157].

Apart from arterial tortuosity, other factors assessed by medical imaging could also provide help in the prediction of severe aortic involvement. One of these factors is aortic stiffness, mainly characterized by distensibility and pulse wave velocity. It has been shown that Marfan syndrome patients without advanced aortic manifestations have stiffening throughout the entire aorta, thus impaired biomechanics could indicate a diseased aorta, serving as a potential early marker for aortic involvement [158]. Follow-up studies demonstrated that impaired biomechanics of the aorta are related to the rate of dilation and aortic events, making them promising predictors of severe aortic manifestations [159–161]. An emerging new field is the assessment of hemodynamic and biomechanical properties of the aorta with the aim of four dimensional flow cardiac magnetic resonance imaging, which has already provided some interesting insights into the potential associations between various measurable parameters and the degree of aortic involvement in Marfan syndrome [162–165].

Figure 13 shows a list of the potential predictors of aortic involvement severity in Marfan syndrome patients, while *Figure 14* demonstrates the possible mechanisms and connection points of these predictors [145].

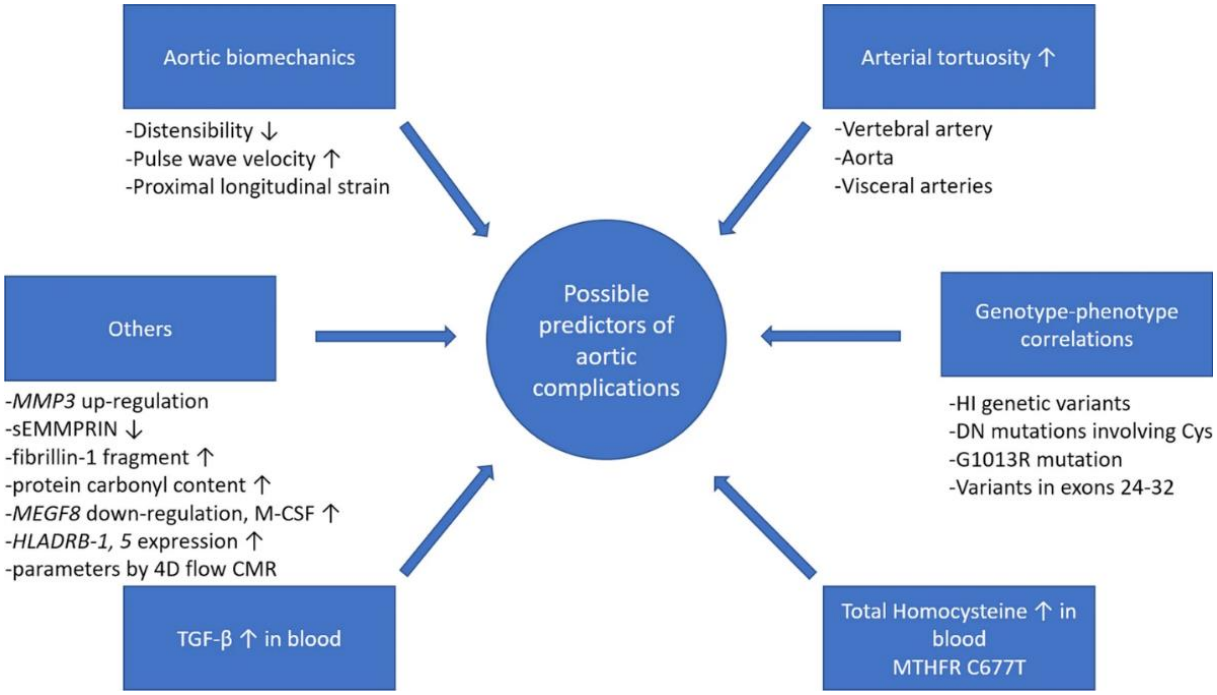

Figure 13 The possible predictors of aortic complications reported to date are demonstrated in this figure. These include biomarkers measured in blood like transforming growth factor-β (TGF-β) and homocysteine, radiological biomarkers such as aortic biomechanics and arterial tortuosity, genotype-phenotype correlations and further potential predictors [145].

CMR - cardiac magnetic resonance; Cys - cysteine; DN - dominant negative; G1013R mutation - a mutation resulting in the amino acid change of glycine to arginine at the 1013th position; HI - haploinsufficient; *HLADRB-1* - major histocompatibility complex, class II, DR beta 1 gene; M-CSF - macrophage colony-stimulating factor; *MEGF8* - Multiple EGF Like Domains 8 gene; *MMP3* - matrix metalloprotease 3 gene; MTHFR - methylenetetrahidrofolat-reductase; sEMMPRIN - soluable form of extracellular MMP inducer; Variants in exons 24-32 - variants affecting the *FBN1* gene at the region of exons 24-32, the so-called neonatal region

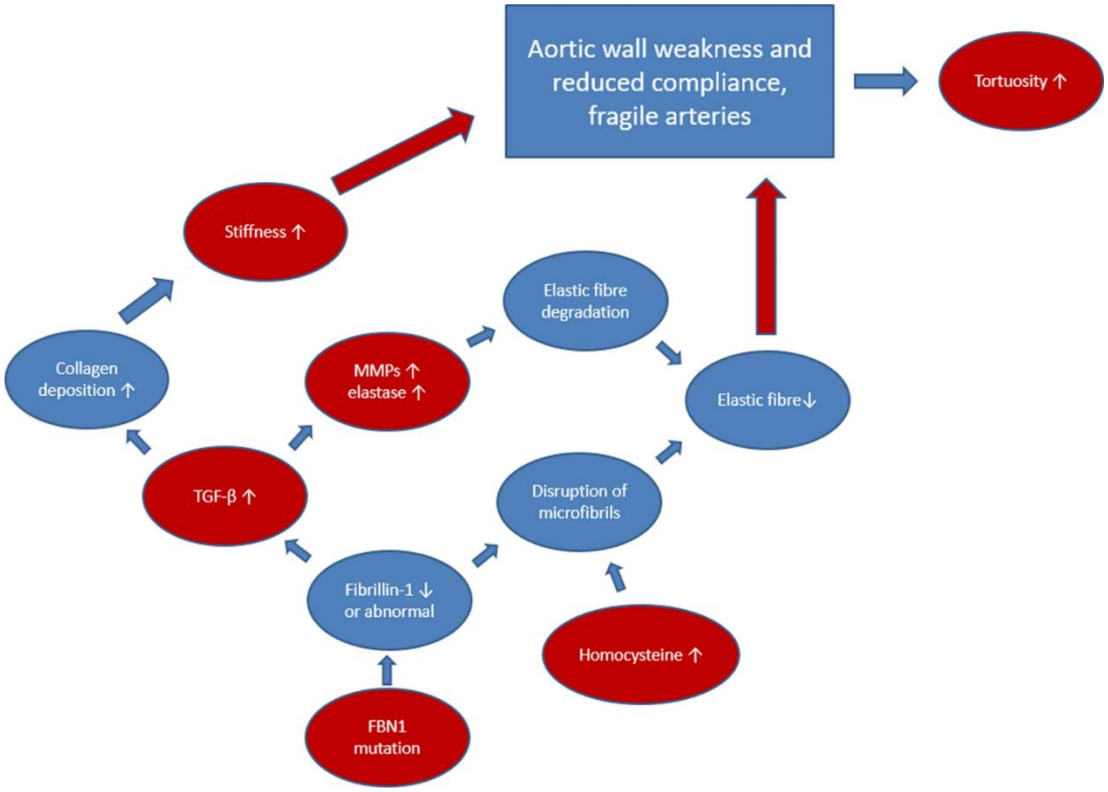

Figure 14 Pathomechanism of aortic wall alterations in Marfan syndrome, as well as the connection points and relationships of the potential predictors. The red figures represent potential predictors of severe aortic involvement, while the blue ones illustrate other relevant aspects in the pathomechanism of aortic manifestations. The red arrows show the final causal steps in the development of aortic involvement, while the blue arrows demonstrate the connection between the pathological processes leading to aortic complications [145]. *FBN1* - fibrillin-1 gene; MMP - matrix metalloprotease; TGF-β - transforming growth factor-β

5.6 Limitations

The presented studies have some limitations. Relatively small sample size and retrospective design are limitations for both the genetic and the tortuosity investigations. However, the number of included patients is comparable to other existing studies in these fields and we were able to demonstrate significant findings with the included cohorts. It is important to stress that Marfan syndrome is a rare disorder, making it difficult to reach large sample sizes.

As we applied targeted screening, we could only screen a limited number of genes and we could not sequence deep/non-canonical regions, thereby potentially missing the diagnoses in some cases. However, we screened the most frequently encountered genes in HTAD and we could demonstrate the relevance of using a multi-gene panel in Marfan syndrome patients and patients with Marfanoid habitus.

The diagnosis of Marfan syndrome was not genetically confirmed in all patients included in the tortuosity study. As discussed above, patients who fulfill the currently applied diagnostic criteria of Marfan syndrome may still suffer from a related disorder of Marfan syndrome, thus we cannot exclude the possibility that some of the patients had another diagnosis other than Marfan syndrome. However, as all included individuals met the diagnostic criteria of Marfan syndrome and the other genetic disorders are rare compared to Marfan syndrome, this limitation is not likely to invalidate our results.

Large prospective studies are required to validate our findings to enable them to be applied in the care of patients with Marfan syndrome.

6 Conclusions

The examined markers were shown to be promising in the prediction of more severe aortic involvement in patients with Marfan syndrome.

We first demonstrated that dominant negative mutations with cysteine elimination and haploinsufficient variants of the *FBN1* gene may lead to more severe aortic manifestations than dominant negative mutations without cysteine amino acid substitution. Furthermore, DN Cys variants may even be more deleterious than HI ones, as they required aortic surgery more frequently.

The other potential predictor is visceral arterial tortuosity, which we have demonstrated to be increased in case of Marfan syndrome patients compared to control subjects. Increased tortuosity of both renal arteries was associated with more severe aortic involvement, thereby offering a promising predictor.

Based on these findings and the reported results in the literature, Marfan syndrome patients with DN Cys and HI variants and Marfan syndrome patients with increased renal arterial tortuosity may need to undergo more frequent follow-up and a prophylactic surgery at smaller aortic diameters than patients who belong to the lower risk groups.

Furthermore, we conclude that a multi-gene panel should be applied for the genetic testing of patients with Marfan syndrome, with subsequent CNV screening in negative cases. We also emphasize the need for testing patients with Marfanoid habitus not meeting the clinical diagnosis of Marfan syndrome, in order to identify LDS patients, who are likely to present with severe aortic involvement.

7 Summary

Marfan syndrome belongs to the syndromic heritable thoracic aortic diseases and it is caused by mutations of the *FBN1* gene. Aortic aneurysm and dissection are the main causes of morbidity and mortality of the syndrome. A prophylactic aortic surgery to prevent acute aortic events is mainly indicated based on aortic diameters. However, aortic dissection may occur by normal or mildly dilated aortas, and on the other hand, patients with dilated aorta may never experience acute aortic events. Furthermore, a prophylactic surgery has lower mortality and short- and long-term postoperative morbidity rate than the operation of an acute aortic dissection. Thus, predictors of severe aortic involvement in Marfan syndrome patients need to be identified to optimize the indications and timing of prophylactic aortic operation.

We assessed the role of genotype-phenotype correlations and arterial tortuosity in the prediction of more severe aortic involvement in patients with Marfan syndrome. Genetic testing of patients with Marfan syndrome and Marfanoid habitus was carried out with single-gene-, gene-panel- and copy number variation (CNV) analyses.

We could differentiate between *FBN1* variant types based on their aortic involvement severity. Haploinsufficient (HI) variants and dominant negative mutations with cysteine elimination (DN Cys) led to more severe aortic involvement than dominant negative mutations without the elimination of cysteine (DN non-Cys). Furthermore, DN Cys variants appeared more deleterious than HI ones. We also demonstrated the relevance of CNV screening and the use of a multi-gene panel in this patient population.

We evaluated the visceral arterial tortuosity of Marfan syndrome patients with different aortic involvement severity and control individuals. CT angiography images were analyzed with dedicated software tools and various tortuosity metrics. The renal arteries and the splenic artery were examined as their geometry is not influenced by the common skeletal abnormalities of the syndrome. All three vessels showed increased tortuosity in Marfan syndrome compared to the general population and more tortuous renal arteries were associated with more severe aortic phenotype.

In conclusion, the type of genetic variant and the degree of renal artery tortuosity could be predictors of severe aortic involvement, thereby could potentially be used for optimizing the indication and timing of prophylactic aortic surgery in Marfan syndrome patients.

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9 List of own publications

9.1 Own publications involved in the current thesis

IF=20.423

1. **Stengl R**, Bors A, Ágg B, Pólos M, Matyas G, Molnár MJ, Fekete B, Csabán D, Andrikovics H, Merkely B, Radovits T, Szabolcs Z, Benke K. Optimising the mutation screening strategy in Marfan syndrome and identifying genotypes with more severe aortic involvement. Orphanet J Rare Dis. 2020 Oct 15;15(1):290. doi: 10.1186/s13023-020-01569-4. **IF=4.123 (2020)**

2. **Stengl R**, Ágg B, Pólos M, Mátyás G, Szabó G, Merkely B, Radovits T, Szabolcs Z, Benke K. Potential predictors of severe cardiovascular involvement in Marfan syndrome: the emphasized role of genotype-phenotype correlations in improving risk stratification-a literature review. Orphanet J Rare Dis. 2021 May 31;16(1):245. doi: 10.1186/s13023-021-01882-6. **IF=4.302 (2021)**

3. **Stengl R**, Ágg B, Szilveszter B, Benke K, Daradics N, Ruskó B, Vattay B, Merkely B, Pólos M, Szabolcs Z. Case Report: Morphological characterization and long-term observation of bilateral sequential internal mammary artery aneurysms in a patient with confirmed FBN1 mutation. Front Cardiovasc Med. 2021 Jun 16;8:697591. doi: 10.3389/fcvm.2021.697591. **IF=5.848 (2021)**

4. Benke K, Ágg B, Meienberg J, Kopps AM, Fattorini N, **Stengl R**, Daradics N, Pólos M, Bors A, Radovits T, Merkely B, De Backer J, Szabolcs Z, Mátyás G. Hungarian Marfan family with large *FBN1* deletion calls attention to copy number variation detection in the current NGS era. J Thorac Dis. 2018 Apr;10(4):2456-2460. doi: 10.21037/jtd.2018.04.40. **IF=2.027 (2018)**

5. Ágg B, Szilveszter B, Daradics N, Benke K, **Stengl R**, Kolossváry M, Pólos M, Radovits T, Ferdinandy P, Merkely B, Maurovich-Horvat P, Szabolcs Z. Increased visceral arterial tortuosity in Marfan syndrome. Orphanet J Rare Dis. 2020 Apr 15;15(1):91. doi: 10.1186/s13023-020-01369-w. **IF=4.123 (2020)**

9.2 Own publications not involved in the current thesis

IF=25.802

1. Pólos M, Benke K, Ágg B, **Stengl R**, Szabó A, Nagy Á, Ruskó B, Hedberg J, Radovits T, Susánszky É, Merkely B, Székely A, Szabolcs Z. Psychological factors affecting Marfan syndrome patients with or without cardiac surgery. Ann Palliat Med. 2020 Sep;9(5):3007-3017. doi: 10.21037/apm-20-546. Epub 2020 Aug 19. **IF=2.595 (2020)**

2. Pólos M, **Stengl R**, Şulea CM, Benke K, Bartha E, Ágg B, Koppányi Á, Hartyánszky I, Székely A, Németh E, Kovács A, Merkely B, Szabolcs Z. Stratégiai szemléletváltás a Marfan-szindrómás betegeken végzett aortagyök-rekonstrukciókban [Changing strategies in aortic root reconstruction in Marfan syndrome]. Orv Hetil. 2021 May 2;162(18):696-704. Hungarian. doi: 10.1556/650.2021.32080. **IF=0.707 (2021)**

3. Veres G, Benke K, **Stengl R**, Bai Y, Stark KA, Sayour AA, Radovits T, Loganathan S, Korkmaz-Icöz S, Karck M, Szabó G. Aspirin Reduces Ischemia-Reperfusion Injury Induced Endothelial Cell Damage of Arterial Grafts in a Rodent Model. Antioxidants (Basel). 2022 Jan 18;11(2):177. doi: 10.3390/antiox11020177. **IF=7.0 (2022)**

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5. Veres G, Benke K, **Stengl R**, Weber P, Marina E, Szabó G, Karck M. Long-Term Outcomes Stratified by Age in Patients with a Mechanical versus Biological Mitral Valve Replacement. J Cardiovasc Dev Dis. 2022 Oct 6;9(10):339. doi: 10.3390/jcdd9100339. **IF=2.4 (2022)**

6. Șulea CM, Mártonfalvi Z, Csányi C, Haluszka D, Pólos M, Ágg B, **Stengl R**, Benke K, Szabolcs Z, Kellermayer MSZ. Nanoscale Structural Comparison of Fibrillin-1 Microfibrils Isolated from Marfan and Non-Marfan Syndrome Human

Aorta. Int J Mol Sci. 2023 Apr 20;24(8):7561. doi: 10.3390/ijms24087561. **IF=5.6***

7. Benke K, **Stengl R**[#], Stark KA, Bai Y, Radovits T, Loganathan S, Korkmaz-Icöz S, Csonka M, Karck M, Szabó G and Veres G (2024) Zinc-aspirin preconditioning reduces endothelial damage of arterial grafts in a rodent model of revascularization. Front. Cardiovasc. Med. 10:1288128. doi: 10.3389/fcvm.2023.1288128 **IF=3.6***

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