

Possible extracardiac predictors of aortic dissection in Marfan syndrome

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Abstract

Aim: According to previous studies, aortic diameter alone seems to be insufficient to predict the event of aortic dissection in Marfan syndrome (MFS). In determining the optimal timing of preventive aortic root replacement (ARR) aortic growth rate is of importance, as well as family history, however, none of them appear to be decisive. Thus, the aim of this study was to search for possible predictors of aortic dissection in MFS.

Methods: A Marfan Biobank consisting of 79 MFS patients was established. 39 MFS patients who underwent ARR were assigned into three groups based on the indication for surgery (dissection, annuloaortic ectasia and prophylactic surgery). Transforming growth factor- β (TGF- β) serum levels were measured by ELISA, relative expression of c-Fos, matrix metalloproteinase 3 and 9 (MMP-3 and -9) were assessed by RT-PCR. Clinical parameters, including anthropometric variables and the original Ghent criteria were also analysed.

Results: Among dissection patients TGF- β serum level was elevated (43.78 ± 6.52 vs. 31.64 ± 4.99 ng/l, $p < 0.0001$), MMP-3 was up-regulated ($\text{Ln}2^a = 1.87$, $p = 0.06$), arm span to height ratio (ASHR) was increased (1.053 vs. 1.014 ; $p = 0.027$ in patients older than 20 years) and striae atrophicae were more common (92% vs. 41% $p = 0.027$) compared to the annuloaortic ectasia group.

Conclusions: We found four easily measurable parameters (ASHR, striae atrophicae, TGF- β serum level, MMP-3) that may help to predict the risk of aortic dissection in MFS. Based on these findings a new benign-malignant classification of MFS is also proposed, which could be taken into account when determining the timing of prophylactic ARR.

Keywords

Marfan syndrome, Aortic dissection, Predictors

Introduction

Marfan syndrome is an autosomal dominantly inherited genetic disorder affecting the body's connective tissue. The prevalence of the syndrome is 1:3000-1:5000 [1]. Among others it involves the cardiovascular system, eyes and the skeletal system. Most patients with Marfan syndrome were shown to have a mutation in the fibrillin-1 (FBN1) gene [1, 2]. The mutation causes decreased Large Latent Complex (LLC) sequestering ability of the FBN1 protein, which in turn leads to elevated tissue and serum concentrations of Transforming Growth Factor- β (TGF- β). This affects the expression of many proteins in the connective tissue through both the canonical, Smad mediated signal transduction and non-canonical pathways including MAP kinases (ERK1/2, JNK1, p38) with AP-1 transcription factor (c-Fos/c-Jun complex) as a downstream mediator [3]. High tissue and plasma activity of matrix metalloproteinases (MMPs) – especially gelatinases (MMP-2 and 9) and MMP-3 – are prominent [4, 5]. These molecular alterations entail the fragmentation of the connective tissue fibres, and results in an inelastic and tear-prone connective tissue. Due to this inelasticity the wall of the aorta is not able to resist the force arising from blood pressure, leading to the formation of aortic pathologies typical to the syndrome [6].

According to Détaint and colleagues by the age of 60 in nearly 100 percent of the individuals with Marfan syndrome, varying degrees of aortic root dilatation will have been developed and three quarters of the patients would have undergone aortic root replacement (ARR), as the indication criteria for surgery are fulfilled: either increased aortic diameter to critical level, symptomatic aortic valve insufficiency or Stanford type „A” dissection [7]. In most of the Marfan cases, aortic dissection presents an acute life-threatening condition, and is to be treated by surgery on an emergency basis. Consequently, prophylactic ARR should be given priority in Marfan syndrome patients' care in order to prevent aortic dissection.

Optimal timing of these properly planned operations with less potential of complications has

decisive importance with regard to the life expectancy and quality of life of patients with Marfan syndrome [8]. However, cardiac surgical interventions performed unreasonably early (with small aortic diameters) in young adulthood or childhood may lead to early and late complications in some cases leading to reoperation or redo surgery [9, 10]. Moreover, in cases with mechanical valve implantation lifelong anti-coagulation is needed [11]. These factors can significantly worsen the quality of life of the asymptomatic young patients. In addition, numerous studies have shown that aortic dissection can also develop in individuals with smaller aortic diameters compared to those dimensions, which are included in the current surgical indication criteria [6, 12]. These data suggest that aortic diameter alone is not an appropriate indicator of aortic dissection.

To optimise the timing of the preventive surgical intervention, it would be necessary to create a model that could precisely determine the risk and also the probable time of aortic dissection for each individual. Hence we established a systematic Marfan Biobank with relatively large collection of samples, in order to gain a better overview of the molecular pathways and pathomechanism underlying the diverse cardiovascular manifestations of the syndrome. With the use of the data from the biobank, in this article we present four possible extracardiac predictors of aortic dissection in Marfan syndrome and we propose a new benign-malignant classification indicating the probable cardiovascular outcome of the syndrome.

Materials and methods

Patients

Selection of the patients was based on the data from the Hungarian Marfan Registry (HMR) set up and maintained by the staff of the Cardiac Surgery Department at Semmelweis University. Clinical data of 221 Hungarian patients with Marfan syndrome were collected in the HMR between 1988 and 2011. In each patient enrolled in the HMR, diagnosis of the syndrome was established according to the original Ghent nosology [13]. However in 2011, when patients were selected for the current study, the diagnosis was verified in every case with the use of the revised (2010) Ghent criteria [14].

Establishment of the Marfan Biobank

63 patients from the HMR were chosen randomly. First and second degree relatives of these 63 patients, who were diagnosed with Marfan syndrome and had a record in HMR, were also assigned to this group (another 16 patients). Thus a total of 79 patients were enrolled in the Marfan Biobank. Between October 2011 and April 2012 clinical data and biological samples (serum, blood, genomic DNA, cDNA from PBMC) from 37 women (47%) and 42 men (53%) with Marfan syndrome were collected in the biobank. At the time of the establishment of the biobank the patients' average age was 33.2 ± 13.5 years.

Forty out of the 79 patients in the biobank underwent cardiac surgery (primary type of the surgery were ARR in 39 cases and mitral valve replacement in 1 case). Among the 39 patients with ARR surgical indication was aortic dissection in 12 cases (Group 1; no distinction between the 9 acute and 3 chronic cases was applied in this study), annuloaortic ectasia in 12 cases (Group 2) and prophylactic surgery in 15 cases (Group 3). Annuloaortic ectasia was defined as the presence of dilated aortic annulus accompanied by severe (grade III-IV), symptomatic aortic insufficiency. In contrast, ARRs performed on asymptomatic patients, who had an aortic diameter over 40 mm – measured at the level of the sinus of Valsalva – or an aortic growth rate exceeding 5 mm/year were classified as prophylactic surgery (Table 1).

It should be also underlined, that while aortic dissection and annuloaortic ectasia are well-defined, distinct manifestations of Marfan syndrome, patients in the prophylactic group could have developed either aortic dissection or annuloaortic ectasia without the preventive ARR. Therefore prophylactic group should be treated as a mixed, intermediate population.

Sample collection was conducted 6.7 ± 5.1 years after the surgery (Table 1). The samples required for the biobank were collected and handled according to international protocol at the Cardiac Surgery Department of Semmelweis University. The samples were processed in 2 hours following the collection. Oral mucosa samples were collected from 5 subjects under 14 and their extracted genomic DNA was stored.

Control group

TGF β 1 ELISA

For the measurement of serum active TGF- β 1 levels a solid phase ELISA was used, which was designed to measure biologically active TGF- β 1 (R&D Systems). 0.25 ml Serum samples were acidified to a pH of 2–3 with 50 μ l 1N HCL for 10 minutes to activate latent TGF- β , and then re-neutralized to pH 7–8 with 50 μ l 1.2N NaOH/0.5 M HEPES. Results were expressed in ng/ml.

cDNA synthesis and real-time PCR assay

PBMC was isolated from 5 ml of the anticoagulated blood samples (containing EDTA) of the 76 adult patients. Total RNA from PBMC cells content was isolated with Quick RNA microprep kit (Zymo Research). RNA concentration was determined using NanoDrop spectrophotometer (NanoDrop). Reverse transcription was performed in 20 μ l target volume using 5 μ g whole RNS, 75 pmol random hexamer primer, 10 mM dNTP (Invitrogen), 20 U M-MuLV Reverse Transcriptase enzyme (MBI Fermentas) and 1x-es buffer (MBI Fermentas). The reaction mix was incubated for 2 hours at 42°C. The reverse transcriptase reaction solution was diluted three-fold with nuclease-free water. For the real-time PCR assay, 1 μ l diluted cDNS (approximately 15 ng RNA-equivalent) and

1 x SYBR Green Master Mixet (Applied Biosystems) were used. Primers were designed using Primer Express Software (Applied Biosystems). Primer sequences are detailed in Supplementary Data. Real-time PCR was performed in 20 µl target volume using 1 µl cDNA, 1 pmol, gene-specific Forward and Reverse primer and 1 x SYBR Green PCR Master mix. All real-time PCR were performed using the MX3000 Real-time PCR (Stratagen) system with the following settings: 40 cycles at 95°C, denaturing process for 15 seconds, annealing at 60 °C, chain elongation and detection for 60 seconds. For each gene, relative expression was normalized using the human *GAPDH* gene as standard.

ΔC_t values were calculated as $C_t(\text{target gene}) - C_t(\text{GAPDH endogenous control gene})$ in the same sample, while α value was defined as $\Delta C_t(\text{control sample}) - \Delta C_t(\text{target sample})$. This value was used to calculate expression fold value ($\ln 2^\alpha$) by the equation. Significantly up- or downregulated if the $\ln$ ratio of the α value is >1 or <-1 , respectively, with $p < 0.05$. The ELISA and real-time assays were validated.

Clinical variables

In the 3 specified subgroups we assessed sex ratio, family history, age, anthropometric parameters, presence of major and minor criteria in the original Ghent nosology whereas the systemic score was calculated on the basis of the revised Ghent nosology from 2010 [13, 14].

We defined positive family history as the presence of at least one first- or second-degree relative with the diagnosis of Marfan-syndrome. In cases where the above condition was not met we considered the family history negative (sporadic cases).

Measurement (height, lower segment - LS, arm span - AS, weight) and calculation (upper segment - US, upper segment to lower segment ratio - USLS, arm span to height ratio - ASHR) of anthropometric values were carried out according to the instructions of Ghent nosology [13].

Length parameters and weight were expressed in centimetres and kilograms respectively. Shoe sizes were assessed in the units of continental European system. We used Mosteller's formula to calculate body surface area (BSA) and the well-known formula for body mass index (BMI).

Along with the collection of biological samples the presence of major and minor Ghent criteria – queried from HMR database – were verified with the use of collected cardiological, ophthalmological, orthopaedic and dermatological evidences and inspection.

Obviously, criteria representing aortic pathologies – which the grouping was based on – were not assessed. Criteria with poor sensitivity and sporadic incidence in the studied population were also omitted from our investigation (dilatation of the main pulmonary artery, calcification of the mitral annulus, acetabular protrusion of any degree, down-slating palpebral fissures, malar hypoplasia, abnormally flat cornea, hypoplastic iris or hypoplastic ciliary muscle causing decreased miosis, apical blebs, lumbosacral dural ectasia, recurrent or incisional herniae).

Since the closure of the epiphyseal plates takes place around the age of 20, and before that age there are dynamical changes in weight, we also specified each anthropometric parameter and derived Ghent criteria in a separate table for patients older than 20 years.

Statistical methods

As the prophylactic group is an intermediate, mixed population (see above), it should not be considered as a third group for ANOVA test. Thus, in case of continuous parameters with normal distribution two-sample t-test was performed. Non-normally distributed variables were compared with the use of Mann–Whitney *U* test. In case of dichotomic parameters – with regards to the small sample size – Fisher's exact test were calculated. The internationally accepted probability ($p < 0.05$) was chosen for significance level. Statistical calculations were performed with Statistica 11 software.

Results

Molecular biological parameters

TGF- β serum level was significantly higher (see values below) in all three groups established according to the surgical indication than in the control (25.7 ± 4.1 ng/l) group ($p < 0.0001$ for all three groups).

TGF- β level measured in the dissection (43.78 ± 6.52 ng/l) group was significantly higher, than that measured in the annuloaortic ectasia (31.64 ± 4.99 ng/l) and prophylactic (34.86 ± 5.60 ng/l) groups ($p < 0.0001$ and $p = 0.001$). There was no difference in TGF- β values between the annuloaortic ectasia and prophylactic groups.

The MMP-3 gene showed significant up-regulation in dissection patients with Marfan syndrome in comparison with control patients ($\text{Ln}2^a = 2.22$, $p = 0.05$). There was no change in the MMP-3 gene expression level of the prophylactic and annuloaortic ectasia groups when compared to the control group ($\text{Ln}2^a = -0.05$, $p = 0.08$ and $\text{Ln}2^a = 0.31$, $p = 0.09$ respectively).

The MMP-3 gene showed up-regulation in dissection patients when compared to the prophylactic and annuloaortic ectasia individuals; in the comparison between dissection vs. prophylactic groups the difference was significant ($\text{Ln}2^a = 1.87$, $p = 0.06$ and $\text{Ln}2^a = 2.25$, $p = 0.05$). There was no difference in the MMP-3 gene expression level between prophylactic and annuloaortic ectasia patients ($\text{Ln}2^a = 0.23$, $p = 0.07$).

MMP-9 gene showed significant up-regulation in dissection patients compared to the control patients ($\text{Ln}2^a = 1.85$, $p = 0.04$). MMP-9 gene showed up-regulation in annuloaortic ectasia patients compared to the control patients, however, this change was insignificant ($\text{Ln}2^a = 1.74$, $p = 0.07$). In prophylactic patients there was no difference in the MMP-9 gene expression level ($\text{Ln}2^a = 0.8$, $p = 0.05$) compared to the control group.

The MMP-9 gene showed significant up-regulation in dissection patients compared to prophylactic patients ($\text{Ln}2^{\alpha}=1.05$, $p=0.05$). Compared to the annuloaortic ectasia patients there was no significant difference in the MMP-9 gene expression level of dissection patients with Marfan syndrome ($\text{Ln}2^{\alpha}=0.11$, $p=0.03$). Similarly, there was no significant difference in the MMP-9 gene expression level between annuloaortic ectasia and prophylactic patients ($\text{Ln}2^{\alpha}=0.94$, $p=0.05$).

In the expression of the c-FOS gene there was no difference between the dissection, annuloaortic ectasia and prophylactic groups compared to the control group ($\text{Ln}2^{\alpha}=-0.38$, $p=0.09$; $\text{Ln}2^{\alpha}=0.03$, $p=0.08$; $\text{Ln}2^{\alpha}=-0.27$, $p=0.12$). Investigating the subgroups established according to the surgical indication there was no difference between them in the c-FOS gene expression (dissection vs. annuloaortic ectasia: $\text{Ln}2^{\alpha}=-0.34$, $p=0.08$; dissection vs. prophylactic: $\text{Ln}2^{\alpha}=-0.08$, $p=0.08$; $\text{Ln}2^{\alpha}=-0.23$, $p=0.09$) (Figure 2).

Clinical variables

Our findings from the analysis of clinical parameters are illustrated in Tables 2 and 3.

These results highlight the fact that presence of two criteria from the Ghent nosology, namely increased ASHR and striae atrophicae, were more common among dissection patients than in the annuloaortic ectasia group (58% vs. 17% $p=0.089$, and 92% vs. 41% $p=0.027$). The difference was significant in the case of striae. There was as well a significant difference in the occurrence of ASHR when ignoring the data of patients under 20 and focusing on the prevalence of ASHR only in older patients (64% vs. 9% $p = 0.031$).

Comparing dissection and annuloaortic ectasia groups the same tendency was true for ASHR as a continuous parameter (without age filter: 1.046 vs. 1.018 $p=0.098$; for patients older than 20: 1.053 vs. 1.014 $p = 0.027$). It must be stated that the probability of the difference in the lower segment values was near to the significance level when analysing the above mentioned two groups (dissection 93.6 cm vs. annuloaortic ectasia 101.9 cm; $p = 0.059$) However, comparison of patients

over 20 clearly suggests that we should exclude the possibility of a real significant difference (94.9 cm vs. 102.2 cm $p = 0.108$).

Body height, lower segment and shoe size (given in European units) were significantly smaller in the dissection group compared to the prophylactic group (178.6 cm vs. 189.6 cm $p = 0.021$; 93.6 cm vs. 100.7 cm $p = 0.027$; 42.3 vs. 45.0 $p = 0.039$ respectively). These differences – with the exception of body height – were not significant for patients over 20 ($p = 0.042$; $p = 0.058$; $p = 0.074$), even though probability remained close to the significance level. (Figure 3)

Although, increased ASHR proved to be significantly more common in the dissection group than in the prophylactic group (64% vs. 20% $p = 0.043$), when applying the age filter the difference was not significant ($p = 0.057$). When analysing continuous ASHR we could not observe a significant difference either without age filter or in groups of patients over 20 ($p = 0.293$ and $p = 0.523$). Myopia over 3 dioptries was more common among prophylactic patients compared to dissection patients (47% vs. 8% $p = 0.043$).

When comparing the results of annuloaortic ectasia and prophylactic groups only the systemic score showed significant difference (7 vs. 8 points $p = 0.040$) (Figure 4).

We found no significant differences in any other comparison of the groups.

Discussion

The key issue in the treatment of patients with Marfan syndrome is the prevention of the life-threatening aortic dissection for which - according to the current state of knowledge - the only solution seems to be aortic root replacement. The increasing risks of acute aortic dissection caused by progressive aortic dilatation can only be diminished by preventive cardiac surgical procedures

performed at small aortic diameters at the early stages of the disease [15]. Furthermore, the short and long-term survival rates for patients having undergone prophylactic surgery, as well as the rates of complications and subsequent reoperations are far more favourable than for those undergoing ARR procedures in emergency situation [8; 16]. In the past two decades ARR surgery tends to be performed at smaller and smaller aortic diameter (in 1991 at >6 cm [17]; in 1994 at >5.5 cm [16]; in 2005 at >4.5 cm [12]; nowadays certain experts recommend it at even >3 cm [18]). Thus, because of the progressive nature of aortic dilatation, patients are much younger nowadays when surgical intervention is performed.

However, ARR performed in a young patient – as any surgical treatment – may have considerable risks. After the ARR procedure, several life-threatening complications may occur even among patients having no cardiac symptoms prior to the operation [9-11].

It has also been pointed out that in case of Marfan syndrome, aortic dissection may occur at smaller aortic diameters (even smaller than those contained in the surgical indication criteria) [6, 12]. Thus, considering literature data presented above, aortic diameter considered critical, alone does not seem to be sufficient and satisfactory to predict the formation of aortic dissection.

In order to rule out these limitations several attempts have been made. To exclude the strong variations in body size typical to Marfan syndrome patients, Aalberts et al. specified *aortic diameters* normalized to body surface area and patient age, instead of using the absolute value [19]. In addition, patients' family history of aortic dissection as well as *aortic growth rate* is taken into account by most of the protocols [19, 20]. Nevertheless, besides these factors other clinical and molecular markers would be necessary to predict the trends describing the progression and direction of the aortic pathology.

Being aware of the prognostic parameters (both clinical and molecular biological) of aortic dissection, preventive surgical intervention – which usually exposes Marfan syndrome patients to considerable risks and potential complications – should only be carried out if there is a real danger of aortic dissection. Optimal timing for ARR procedure could be also calculated more precisely, which would likely improve the life expectancy and quality of life of patients with Marfan syndrome.

As Meijboom et al. showed in their research, the growth rate of the aortic dilatation is quite variable among Marfan syndrome patients [12]. At early stages, when the aortic growth rate is slow („slow grower”) the disease is asymptomatic. However, during the decades, patients develop annuloaortic ectasia, which leads to aortic valve insufficiency. In this case, the risk of dissection is lower. In contrast, rapid growth of the aortic root („fast grower”) can be characterized by an increased risk of aortic dissection.

Based on the above data, patients with Marfan syndrome should be assigned into two groups: *malignant* (with an increased risk of dissection) and *benign* (with a reduced risk of dissection). However, instead of the growth rate alone as Meijboom et al suggested, we hypothesize that the main difference between the two groups lies in the presence (or absence) of certain, yet unmapped, pathological alterations at the molecular and tissue level which can cause aortic dissection even in case of aortic diameters close to normal [6, 12].

We also assume that these molecular alterations, if present in a patient, can also influence the pattern of the extracardiac manifestations of the syndrome. The aim of our study was to identify these molecular indicators and extracardiac manifestations with the use of clinical and molecular biological data from the Marfan Biobank.

The highly significant difference in the TGF- β serum level between the dissection and annuloaortic ectasia groups indicates that increased TGF- β levels not only play an important role in the development of the symptoms, but also the rate of the increase may correlate with the risk of dissection.

Furthermore, increased MMP-3 expression in the PBMC cells of the patients with dissection compared to the annuloaortic ectasia group, apparently contributes to the development of the severe pathologic changes leading to aortic dissection. Since in terms of c-FOS expression no distinction could be identified between these two groups, it seems unlikely that the detrimental effects of increased TGF- β level are resulting from the activation of the ERK1/2, JNK1, p38 mediated non-canonical pathways and the consequent up-regulation of the AP-1 transcription factor. Thus, it is more likely that Smad mediated canonical or Rho-like GTPase and PI3K/Akt mediated non-canonical pathways are responsible for the signal transduction causing the up-regulation of MMP-3 and destruction of the aortic wall [3].

Results from an earlier study show that MMP-3 is also responsible for the activation of latent TGF- β ; therefore, at this central point of the signalling pathway a positive feedback cycle should be considered [21] (Figure 5).

It should be also stated that sample collection happened 7.1 ± 5.1 years after the event of aortic dissection, thus it can be ruled out that the above mentioned molecular alterations (elevated TGF- β levels, up-regulated MMP-3) is caused by the dissection itself. (see Table 1)

Our findings also indicate that certain groups of patients with Marfan syndrome can be characterized by yet unidentified phenotypic correlations. The development of aortic dissection is significantly more often associated with increased ASHR levels and atrophic striae compared to annuloaortic ectasia. The prevalence of increased ASHR in the group of dissection patients has also

shown a significant difference when comparing to the prophylactic group.

Though there is limited data available on the molecular mechanisms causing atrophic striae, based on previous publications and on our gene expression results, we assume that the increased MMP-3 and MMP-9 activity in dissection patients is responsible for the degradation of the connective tissue fibres [4, 5, 22]. This destruction process may affect the dermis and the subcutaneous layer of the skin, as well as the aortic wall. Therefore MMP-3 and MMP-9 can be considered as common mediators in the pathogenesis of both aortic dissection and atrophic striae.

It is also known that the tissue and serum levels of TGF- β , which increase the expression of the above enzymes, are elevated in patients with Marfan syndrome [23]. Our results suggest that this difference is more pronounced in dissection patients. In addition, TGF- β also contributes to bone development [24], which might explain the frequent constellation of ASHR, elevated TGF- β levels and aortic dissection.

Without undergoing a preventive ARR, patients in the prophylactic group would likely develop either annuloaortic ectasia or aortic dissection. Therefore, this subgroup should be considered quite inhomogeneous and, thus according to our expectation, this group is characterized by phenotypic signs and parameters falling between the values of the malignant and benign groups. Our expectation proved to be true in case of the ASHR and TGF- β serum level, since these parameters showed a value, which ranged just between the values of the other two groups. Contrary to our presumptions, when comparing the prophylactic group to the two other groups (annuloaortic ectasia, dissection) we found significant phenotypic differences (MMP-9 expression, body height, lower segment, foot size, myopia over 3 dioptries, systemic values). Differences in anthropometric parameters did not disappear completely even if we excluded patients under 20. The fact that number of male patients in the dissection group is higher than in the prophylactic group (33.3% vs.

73% $p=0.057$) can serve as an explanation for the differences in body shape; however, for the other remarkable differences (MMP-9 expression, ocular manifestation, systemic values) no such trivial explanation was found.

When analysing the MMP-9 expression of the prophylactic group compared to the dissection patients we found a considerable increase in the MMP-9 expression. Although this difference was also present between the annuloaortic ectasia and prophylactic groups, but the probability did not reach significant level. These findings suggest, that the increased MMP-9 expression is responsible for the aortic dilatation, rather than for the destruction of the aortic wall which leads to dissection. Therefore, the MMP-9 expression – at least according to our present findings – is not suitable to make a clear distinction between the hypothetical malignant and benign types of Marfan syndrome.

Ocular manifestations and increased systemic score in the prophylactic group are prominent and easily recognizable features that together may facilitate early diagnosis of Marfan syndrome, and thus, programmed prophylactic surgery can be performed in these patients. This hypothetical reasoning could provide a possible explanation for our unexpected findings in connection with ocular manifestation and high systemic score observed in the prophylactic group. However, additional investigations and further refinement of the malignant-benign distinction are needed to elucidate the background of these surprising results.

Conclusions

Based on our findings we conclude that our hypothetical dignity groups (benign vs. malignant) are distinct not only from the cardiovascular perspective, but also in terms of phenotypic signs related to other organs (ASHR, striae), which can be caused by molecular alterations demonstrated above (TGF- β , MMP-3). Thus, a total of four – two molecular and two clinical – factors which might predict aortic dissection were found: elevated TGF- β serum level (1), increased expression of

MMP-3 gene in peripheral blood mononuclear cells (2), increased arm span to height ratio (3) and the presence of striae atrophicae (4). Differences in the extracardiac clinical parameters can be detected quite easily by inspection (striae) or using a tape measure (ASHR), while molecular factors could be assessed by relatively inexpensive laboratory tests (ELISA, RT-PCR).

Similar phenotypic features of the skeletal system or the skin - which may predict the cardiovascular pathologies - have not been described in the international literature of Marfan syndrome so far. Likewise, there are also few studies in which the connection between phenotypic differences is analysed [25].

Limitations, future objectives

Our current findings are not sufficient to define how many of these four predictive factors are necessary to assign a Marfan syndrome patient to the benign or the malign group; neither can we specify the optimal timing of prophylactic ARR procedure in the malign or benign group.

In order to prove our hypothesis regarding to the classification of patients with Marfan syndrome into benign and malignant groups, we consider it necessary to repeat the examinations involving a larger number of patients, as well as to carry out further serum level measurements, genetic and gene expression tests. Investigation of molecular pathways underlying certain phenotypic relationships would provide further evidence for the existence of the above mentioned benign-malignant groups and would support their characterization. Summarizing the clinical and molecular biological results in a multivariate model could facilitate the elaboration of a personalized score that would help to choose the optimal timing of the prophylactic ARR procedure.

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Table 1. Demographic data of patients in the Marfan Biobank

	1.Dissection	2. Annuloaortic ectasia	3. Prophylactic ARR	Operated	Not operated	Biobank	Control
Patients	12	12	15	40	39	79	
Male	4	9	11	24	18	42	
Female	8	3	4	16	21	37	
Under 14	0	0	0	0	5	5	
Under 20	1	1	0	2	13	15	
Mean age	44.6 ± 14.2	38.1 ± 9.2	35.6 ± 6.5	38.6 ± 10.9	27.7 ± 13.7	33.2 ± 13.5	
Operation to sample collection interval (yrs)	7.1 ± 5.1	6.4 ± 6.3	6.7 ± 4.2	6.7 ± 5.1	NA	NA	NA

Table 2. Clinical data without age filter

	1. Aortic dissection	2. Annuloaortic ectasia	3. Prophylactic	p value		
				1 vs 2	1 vs 3	2 vs 3
Patients	12	12	15			
Male	4	9	11	0.100	0.057	1.000
Anthropometric (measured)						
Height	178.6 ± 10.3	186.1 ± 11.7	189.6 ± 12.5	0.106	0.021	0.473
Lower segment (cm)	93.6 ± 9.5	101.9 ± 10.9	100.7 ± 6.3	0.059	0.027	0.727
Arm span (cm)	186.9 ± 13.8	189.6 ± 15.2	196.2 ± 11.9	0.657	0.072	0.216
Foot size	42.3 ± 2.6	44.0 ± 3.1	45.0 ± 3.7	0.148	0.039	0.463
Weight (kg)	74.9 ± 14.8	75 ± 17.0	80.7 ± 19.9	0.990	0.413	0.441
Anthropometric (calculated)						
Upper segment (cm)	85.0 ± 7.8	84.2 ± 9.9	88.9 ± 8.7	0.839	0.240	0.209
Body Mass Index (BMI; kg/m ²)	23.4 ± 3.8	21.45 ± 3.6	22.3 ± 4.5	0.204	0.496	0.597
Body surface area (m ²)	1.921 ± 0.227	1.961 ± 0.277	2.050 ± 0.300	0.705	0.230	0.435
Upper segment - Lower segment ratio (USLS)	0.918 ± 0.132	0.838 ± 0.147	0.883 ± 0.084	0.173	0.408	0.325
Arm span - Height ratio (ASHR)	1.046 ± 0.042	1.018 ± 0.039	1.036 ± 0.041	0.098	0.523	0.255
Ghent nosology (%)						
Mitral valve prolapse	58%	75%	73%	0.667	0.683	1.000
Involvement of ascending aorta	8%	0%	13%	1.000	1.000	0.487
Pectus carinatum	33%	67%	60%	0.220	0.252	1.000
Pectus excavatum requiring surgery	17%	8%	0%	1.000	0.188	0.444
Reduced upper to lower segment ratio	33%	50%	20%	0.680	0.662	0.217
Increased arm span to height ratio	58%	17%	20%	0.089	0.057	1.000
Wrist sign	83%	83%	87%	1.000	1.000	1.000
Thumb sign	83%	83%	93%	1.000	0.569	0.569
Scoliosis of > 20° or spondylolisthesis	83%	58%	80%	0.371	1.000	0.398
Severe scoliosis	58%	41%	40%	0.684	0.449	1.000
Reduced extension at the elbows	17%	33%	7%	1.000	0.569	1.000
Medial displacement of the medial malleolus causing pes planus	33%	33%	47%	1.000	0.696	0.696
Heel deformity	8%	16%	13%	1.000	1.000	1.000
Pectus excavatum of moderate severity	25%	33%	40%	1.000	0.683	0.236
Asymetric chest	33%	33%	60%	1.000	0.252	0.252
Joint hypermobility	75%	42%	47%	0.214	0.239	1.000
Highly arched palate with crowding of teeth	58%	42%	67%	0.684	0.706	0.258
Facial appearance	50%	16%	40%	0.400	0.707	0.683
Dolichocephaly	25%	17%	27%	1.000	1.000	0.662
Enophthalmos	8%	8%	20%	1.000	0.605	0.605
Retrognathia	42%	8%	27%	0.155	0.683	0.342
Ectopia lentis	25%	16%	33%	1.000	0.696	0.408
Myopia over 3 diopter	8%	16%	47%	1.000	0.043	0.217
Increased axial length of globe	0%	8%	0%	1.000	1.000	0.444
Spontaneous pneumothorax	8%	8%	0%	1.000	0.444	0.444
Striae atrophicae (stretch marks)	92%	41%	67%	0.027	0.182	0.258
Systemic score	7 [5-8]	7 [5.5-8]	8 [8-9]	0.860	0.088	0.040
Positive family history (%)	50%	50%	67%	0.827	0.381	0.381

Table 3. Anthropometric data of patients over 20 years

	1. Aortic dissection	2. Annuloaortic ectasia	3. Prophylactic	p value		
				1 vs 2	1 vs 3	2 vs 3
Patients	11	11	15			
Male	4	8	11	0.198	0.109	1.000
Anthropometric (measured)						
Height	179.9 ± 9.6	187.2 ± 11.64	189.6 ± 12.5	0.122	0.042	0.633
Lower segment (cm)	94.9 ± 8.7	102.2 ± 11.4	100.7 ± 6.3	0.108	0.058	0.682
Arm span (cm)	189.4 ± 11.4	190.1 ± 15.8	196.2 ± 11.9	0.903	0.154	0.272
Foot size	42.6 ± 2.3	44.0 ± 3.3	45.0 ± 3.7	0.272	0.074	0.483
Weight (kg)	77.1 ± 13.3	77.4 ± 15.5	80.7 ± 19.9	0.954	0.611	0.660
Anthropometric (calculated)						
Upper segment (cm)	85.0 ± 8.2	85.1 ± 9.9	88.9 ± 8.7	0.982	0.261	0.313
Body Mass Index (BMI; kg/m ²)	23.8 ± 3.7	21.96 ± 3.32	22.3 ± 4.5	0.224	0.365	0.830
Body surface area (m2)	1.957 ± 0.199	2.001 ± 0.252	2.050 ± 0.300	0.659	0.382	0.662
Upper segment - Lower segment ratio (USLS)	0.904 ± 0.128	0.846 ± 0.151	0.883 ± 0.084	0.340	0.621	0.425
Arm span - Height ratio (ASHR)	1.053 ± 0.037	1.014 ± 0.39	1.036 ± 0.041	0.027	0.293	0.185
Ghent nosology (%)						
Reduced upper to lower segment ratio	36%	45%	20%	0.335	0.407	0.218
Increased arm span - height ratio	64%	9%	20%	0.031	0.042	0.613

Figure legends

Figure 1. TGF- β serum levels in the aortic dissection, annuloaortic ectasia and prophylactic groups and in the control patients expressed in ng/ml.

Figure 2. $\text{Ln}2^{\Delta\alpha}$ values describing the relative expression of the MMP-3 (a), MMP-9 (b) and c-FOS (c) genes. ΔCt is $\text{Ct}(\text{target gene}) - \text{Ct}(\text{GAPDH endogenous control gene})$ in the same sample, α value is $\Delta\text{Ct}(\text{control sample}) - \Delta\text{Ct}(\text{target sample})$. This value is used to calculate expression fold value by the equation (expression fold value $\text{Ln} 2^{\Delta\alpha}$). Significantly up- or downregulated if the Ln ratio of the α value is >1 or <-1 , respectively, with $p < 0.0$.

C/D – control group vs. aortic dissection group

C/A – control group vs. annuloaortic ectasia group

C/P – control group vs. prophylactic group

A/D – annuloaortic ectasia group vs. aortic dissection group

P/D – prophylactic group vs. aortic dissection group

P/A – prophylactic group vs. annuloaortic ectasia group

Figure 3. Arm span to height ratio (ASHR) values in the annuloaortic ectasia and aortic dissection groups with and without age filter (patients over the age of 20 years).

Figure 4. Systemic score according to the revised Ghent nosology (Loeys et al, 2010) in the annuloaortic ectasia and prophylactic groups.

Figure 5. This figure depicts a hypothetical positive feedback cycle in the homeostasis of connective tissue fibres. As shown in the picture fibrillin-1 mutation leads to elevated serum and tissue Transforming Growth Factor- β levels, as the ability of fibrillin-1 protein to sequester latent TGF- β decreases. Through intracellular signal transduction active TGF- β up-regulates matrix

metalloproteinases (MMP-3 and MMP-9). Increased MMP activity in the connective tissue in turn leads to the integrin dependent activation of latent TGF- β which results in further increase of the active TGF- β tissue level. During the growth of children with Marfan syndrome elevated TGF- β levels may cause increased arm span to height ratio (ASHR) through the proliferation and early differentiation of osteoprogenitor cells. Both aortic dissection and striae (stretch marks) may be the consequence of increased tissue activity of MMPs. This positive feedback cycle could provide a link between aortic dissection and the four extracardiac predictors (TGF- β serum level, MMP-3 expression, ASHR and striae atrophicae) described in this article.

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Author contributions

Name	Contribution
Bence Ágg	Study design, collection of samples, analysis and interpretation of clinical data, writing sections of the manuscript
Kálmán Benke	Collection of samples, analysis and interpretation of clinical data, writing sections of the manuscript
Krisztina Marosi	Collection (ELISA, RT-PCR measurements), analysis and interpretation of molecular biological data
Ferenc Tarr	Manuscript revision, professional advices
László Daróczi	Collection, analysis and interpretation of clinical data
Zsolt B. Nagy	Collection (ELISA, RT-PCR measurements), analysis and interpretation of molecular biological data, writing sections of the manuscript
Béla Merkely	Head of the department, institutional background, manuscript revision
Zoltán Szabolcs	Head of the research group, study design, manuscript revision

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Abbreviations

ARR – Aortic root replacement
ASHR – Arm span to height ratio
BMI – Body mass index
BSA – Body surface area
cDNA – Complementary DNA
c-Fos – Cellular oncogene Fos (FBJ murine osteosarcoma viral oncogene homolog)
DNA – Deoxyribonucleic acid
ELISA – Enzyme linked immunosorbent assay
FBN1 – fibrillin-1
HMR – Hungarian Marfan Registry
LLC – Large latent complex
MAD – Mothers against decapentaplegic
MAP kinase – Mitogen-activated protein kinase
MMP – Matrix metalloproteinase
PBMC – Peripheral blood mononuclear cell
RT-PCR – Real time – polymerase chain reaction
Sma – Small mutant *Caenorhabditis elegans* gene
Smad – Sma + MAD
TGF- β – Transforming growth factor- β