Marfan syndrome: Getting to the root of the problem
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Adapted from:
Circulating transforming growth factor-β as a prognostic biomarker in Marfan syndrome

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Abstract

**Background:** Patients with Marfan syndrome (MFS) are at risk for cardiovascular disease. Marfan associated mutations in the FBN1 gene lead to increased transforming growth factor-β (TGF-β) activation. The aim of this study was to investigate the role of plasma TGF-β as a biomarker for progressive aortic root dilation and dissection.

**Methods:** Plasma TGF-β level and aortic root diameter by means of echocardiography were assessed in 99 MFS patients. After 38 months of follow-up measurement of the aortic root was repeated and individual aortic root dilation curves were constructed. Clinical events were evaluated. The primary composite endpoint was defined as aortic dissection and prophylactic aortic root replacement.

**Results:** TGF-β levels were higher in MFS patients as compared to healthy controls (109 pg/ml versus 54 pg/ml, p<0.001). Higher plasma TGF-β levels correlated with larger aortic root dimensions (r=0.26, p=0.027), previous aortic root surgery (161 pg/ml versus 88 pg/ml, p=0.007) and faster aortic root dilation rate (r=0.42, p<0.001). During 38 months of follow-up, 17 events were observed (four type B dissections and 13 aortic root replacements). Patients with TGF-β levels above 140 pg/ml had a 6.5 times higher risk of experiencing the composite endpoint compared to patients with TGF-β levels below 140 pg/ml (95% CI: 2.1 to 20.1, p=0.001) with 65% sensitivity and 78% specificity.

**Conclusion:** Elevated TGF-β level in patients with Marfan syndrome is correlated with larger aortic root diameters and faster aortic root dilation rate. Level of plasma TGF-β predicts cardiovascular events and might serve as a prognostic biomarker in MFS.
Chapter 4: TGF-β as prognostic biomarker

Introduction

Marfan syndrome (MFS) is an autosomal connective tissue disorder caused by mutations in the FBN1 gene with high penetrance, but great clinical variability.\(^1,2\) Patients with MFS suffer from an increased risk of cardiovascular manifestations like (asymmetric) aortic root dilation, mitral valve prolapse, impaired biventricular function and aortic dissection, the latter being the main cause of premature death.\(^3-6\) However, it is hard to predict which MFS patient is prone for aortic dissection, since dissections may occur unexpectedly in non-dilated aortas and after prophylactic aortic root surgery.\(^7,8\)

Originally, it was thought that MFS was due to structural deficiency of fibrillin-1, leading to weakened microfibrils.\(^9\) This hypothesis provided a plausible explanation for aortic disease and eye lens dislocation, but other clinical features such as long bone overgrowth remained incompletely understood. Besides being a structural protein, fibrillin-1 normally binds to a large latent complex (LLC), which comprises the inactive form of transforming growth factor-β (TGF-β).\(^10\) Defective or deficient fibrillin-1, by FBN1 mutations, alters the matrix sequestration of the LLC resulting in activation and enhanced release of TGF-β into the extracellular environment.\(^10\) Free and active TGF-β is involved in many cellular processes, including growth inhibition, cell migration and extracellular matrix remodeling.\(^11\)

Studies with MFS mouse models have consistently demonstrated an increased TGF-β signaling.\(^12-14\) Treatment of these MFS mice with TGF-β neutralizing antibodies prevented the development of pathological changes in the aortic wall, progressive aortic root dilation,\(^20\) as well as attenuating of other clinical manifestations of MFS, including pulmonary alveolar septation,\(^15\) and myxomatous degeneration of the mitral valve.\(^14\) High circulating TGF-β is correlated with increased age and aortic diameters, and decreases upon losartan treatment in MFS mice.\(^16\) To our knowledge no clear correlation between circulating TGF-β and aortic diameter in humans with a classic MFS phenotype has been shown.

MFS is a highly variable disease in phenotype and age of onset of various manifestations, so a biomarker which predicts aortic disease activity and pathogenic events would be of tremendous value. The purpose of the current study was therefore to investigate whether increased circulating TGF-β levels at baseline can predict progressive aortic root dilation and aortic dissections.
**Methods**

**Patient population**

In this predefined sub-study of the COMPARE trial, we included 99 adult MFS patients from four academic centres in the Netherlands between 2008-2009 (Academic Medical Centre; Amsterdam Radboud University Nijmegen; Leiden University Medical Centre; University Medical Centre Groningen).\(^{17}\) In short, the COMPARE study is a multi-centre randomized clinical trial, investigating the effects of losartan on aortic dimensions. One of the secondary objectives is to determine whether TGF-β can be associated with different phenotypic expressions. Inclusion criteria include diagnosis of MFS according to the Ghent criteria\(^ {18}\) and age ≥ 18 years. Exclusion criteria are angiotensin converting enzyme (ACE) inhibitor usage and previous replacement of more than one part of the aorta. The trial was conducted with approval from institutional review boards in four participating academic hospitals in the Netherlands. Written informed consent was obtained from all participants.

**Baseline examination**

During the baseline visit, all patients were evaluated with extended clinical examination by two investigators (determination of MFS). History of cardiovascular surgery and use of cardiovascular drugs were obtained from patients’ medical records and questionnaires. For analysis of plasma TGF-β, venous serum blood was collected and measured in EDTA plasma samples. Total TGF-β1, i.e. latent and active TGF-β, was determined by means of ELISA using a commercially available kit (Bio-Rad, Richmond, Canada) following the manufacturer’s recommendations. In order to be able to measure the neo-epitope in active TGF-β, the latent TGF-β1 samples were first acid-activated before assaying. The TGF-β levels of MFS patients, measured in the same laboratory, were compared to TGF-β levels of 22 healthy controls. Healthy controls were recruited among the spouses and non-affected siblings of MFS patients.

**Follow-up**

Doppler echocardiography was performed at baseline and after every year of follow-up. Aortic root growth was calculated on basis of the aortic root diameter of the most recent echocardiogram minus the aortic root diameter of the baseline echocardiogram. Elective aortic root replacement was blinded for TGF-β levels and completely dependent on the judgement of treating cardiologist based on the 2010 ACC/AHA/AATS and ESC guidelines for the management of patients with Thoracic Aortic Disease.\(^ {19,20}\) Patients who had undergone aortic root replacement before the start of the study were excluded in this analysis. In cases with aortic root replacement during the study, the echocardiogram made before surgery was used if available. The follow-up period varied between two and five years, therefore the absolute growth in millimetre (mm) was divided by
time in months and multiplied by 12 to obtain a growth rate per year (mm/year). Data on events such as aortic dissection and elective aortic root replacement were taken from the databases of the participating hospitals and were evaluated after the two to five years of follow-up.

**Transthoracic echocardiography**

Transthoracic Doppler echocardiography was performed with a Vivid 7 (GE, Vingmed Ultrasound, Horton, Norway) ultrasound system by multiple echocardiographers. Aortic root diameters were measured in end-diastole at the level of the sinus of Valsalva. Aortic dimensions were measured using the leading edge to leading edge technique, consistent with the current American Society of Echocardiography guidelines. All echocardiographic images were acquired and recorded digitally, and analysed by a single observer (RF).

**Statistical analysis**

Data are presented as mean value ± standard error of the mean or as mean value (range) or as number of patients (percent) or as median and 95% confidence interval (CI), where appropriate. Comparisons between continuous variables were made by two-tailed Student t-tests. Linear regression analysis was used to identify determinants for progressive aortic dilation and clinical events. Receiver operator characteristic (ROC) analysis was performed to determine sensitivity and specificity of TGF-β in predicting clinical events. Kaplan-Meier analysis was used for survival comparison between patients with high (TGF-β > 140 pg/ml) and low TGF-β (TGF-β < 140 pg/ml). All statistical tests were two-sided and differences were considered statistically significant at p < 0.05. Data analysis was performed using the SPSS statistical package (19.0 for windows; SPSS Inc., Chicago, Illinois, USA). ROC analysis and figures were generated using GraphPad Prism (version 5.01 for Windows, GraphPad Software, San Diego California USA). The authors of this manuscript have certified that they comply with the Principles of Ethical Publishing in the International Journal of Cardiology.

**Results**

A total of 99 MFS patients (mean age: 36 years (range 18-62 years)), diagnosed according to the Ghent criteria of 1996, were enrolled, with a mean follow-up of 38 months (range 24–53 months). Thirty-one patients (31%) had a previous aortic root repair at baseline, with a mean age at first cardiovascular surgery of 31 years (range 12–56 years). In 84% of the MFS patients a FBN1 mutation was found. The MFS patients and healthy controls were comparable for age (36 years, SEM = 1 versus 34 years, SEM = 2, p = 0.27) and
gender (58% versus 55%, p= 0.8). The use of cardiovascular drug therapy and previous cardiovascular surgery was only observed in MFS patients. Baseline demographics of the study population are provided in Table 1.

Age and cardiovascular medical treatment
Baseline plasma TGF-β levels were significantly higher in patients with MFS compared to healthy controls (109 pg/ml, SEM = 12 pg/ml versus 54 pg/ml, SEM = 9 pg/ml, p = <0.001) (Figure 1A). In our cohort 78 patients used cardiovascular drug therapy, 74% of the patients were using β-blockers, three patients were on calcium channel blockers, one patient used a diuretic and one patient used flecainide (Table 1). Patients using β-blocker therapy did not have lower TGF-β levels compared to MFS patients without using cardiovascular medicine (109 pg/ml, SEM = 12 pg/ml versus 81 pg/ml, SEM = 15 pg/ml, p = 0.17) (Figure 1A). No significant difference in aortic root diameter was found between patients using β-blockers (43.8 mm, SEM = 0.6 mm) and patients without β-blocker treatment (41.6 mm, SEM = 1.1 mm, p = 0.09). Figure 1B shows no relation between age and level of plasma TGF-β in MFS patients (r² = 0.03, p = 0.07) nor in healthy controls (r² = 0.04, p = 0.35).

Aortic root dimensions, previous aortic root surgery and skeletal features
Within our MFS cohort 31% of the patients had undergone aortic root surgery before the start of the study. TGF-β levels were studied in the patient group without aortic repair and compared to the patients with their native aortic root. In patients without previous aortic root surgery, aortic root diameter was significantly correlated with circulating total TGF-β levels (n = 71, r = 0.26, p = 0.027) (Figure 2A). Furthermore, patients with an aortic root replacement before inclusion had significant higher circulating TGF-β levels in plasma compared to patients without prior cardiovascular surgery (164 pg/ml, SEM = 27 versus 88 pg/ml, SEM = 13, p = 0.005) (Figure 2B). No correlation was found between circulating TGF-β and body surface area (BSA) (r² < 0.001, p = 0.978). Moreover, no difference was demonstrated between TGF-β levels and patients with or without skeletal involvement (106 pg/ml, SEM = 14 versus 115 pg/ml, SEM = 25, p = 0.759), with or without a known FBN1 mutation (106 pg/ml, SEM = 13 versus 89 pg/ml, SEM = 28, p = 0.598) and between TGF-β levels and patients with or without ectopia lentis (101 pg/ml, SEM = 15 versus 116 pg/ml, SEM = 19, p = 0.539).

TGF-β levels and follow-up
Figure 3A shows a significant correlation between circulating TGF-β levels and aortic root dilation rate in patients with MFS after a mean follow-up of 3 years (n=67, r = 0.42, p < 0.001). Univariate analysis of gender, age, BSA and aortic root dimensions as predictors for aortic root dilation rate (mm/year) are described in Table 2. Besides plasma
levels of TGF-β no other significant predictor for aortic root dilation rate in our cohort was found. Correction for β-blocker usage revealed that TGF-β level was the sole independent parameter associated with aortic root dilation rate ($\beta = 0.45$, SEM $< 0.001$, $p < 0.001$).

During a mean period of 38 months follow-up 17 events were observed (four aortic type B dissections and 13 elective aortic root replacements indicated following the current guidelines), with a mean time to event of 28 months, SEM = 4 months. ROC curve analysis revealed an optimum cut-off value of 140 pg/ml for the composite clinical endpoint, with an area under the curve (AUC) of 0.71 (95% CI=0.58 to 0.84; $p = 0.006$), yielding a sensitivity of 65% (95% CI = 38% to 86%) and a specificity of 78% (95% CI = 68% to 86%) (Figure 3B). This cut-off value corresponded to the mean TGF-β level plus two times the standard deviation of our healthy controls (mean 54 pg/ml, standard deviation 41 pg/ml, cut-off value: 136 pg/ml). For detailed analysis of the effect of TGF-β plasma level, the total patient cohort was divided into two groups (Group 1: <140 pg/ml and Group 2: > 140 pg/ml). Kaplan-Meier survival analysis revealed that patients with a TGF-β level above 140 pg/ml had a significant higher probability of meeting the combined endpoint (log rank - test, $p = 0.006$) (Figure 3C), with a Hazard Ratio of 6.5 (95% CI: 2.1 to 20.1, $p = 0.001$).

Discussion

Our study is the first to demonstrate that TGF-β might serve as a prognostic biomarker, since a single determination of TGF-β level at baseline is an independent predictor for cardiovascular events in MFS. Patients with a TGF-β level above 140 pg/ml had a 6.5 times higher likelihood to reach the combined endpoint (aortic dissection and elective aortic root surgery). Moreover, we demonstrated a positive correlation between circulating TGF-β levels and aortic root diameters at baseline, as well as aortic root dilation rate after a mean follow-up of 38 months.

The importance of cardiovascular features in MFS was already emphasized by Murdoch in 1972, showing that lifespan in MFS patients was markedly shortened due to unpredictable cardiovascular events.23 Patients with MFS may develop an aortic dissection due to progressive dilation in the entire aorta,24 which can be predicted by both aortic diameter and aortic elasticity as independent parameters.6,25,26 Furthermore, the asymmetry of the aortic root might be of clinical importance in unexpected aortic root dissection.5 Increased clinical awareness and availability of a surgical technique for prophylactic aortic root replacement have greatly increased life expectancy by 30 years in the MFS population.27 However, patients with MFS are still at risk for aortic
dissection, predominantly in the distal aorta after successful prophylactic aortic root replacement.\(^4,28,29\)

Moreover, aortic dissection may also occur in patients without prior aortic dilation.\(^4,7,28,29\) In the last decades tremendous progress has been made in elucidating the common molecular mechanisms underlying the pathogenesis of MFS. However, despite increased \(FBN1\) mutation screening, clinical severity cannot be predicted yet, due to the unclear genotype-phenotype correlations. These observations highlight the need for an informative distinctive prognostic marker to monitor aortic disease in MFS.

Previous studies have shown that the aortic vessel of humans and mice with MFS had excessive TGF-\(\beta\) activation by studying phosphorylation of downstream signalling molecules, transcription factors SMAD 2/3.\(^{13,15,16,30}\) Haskett et al, demonstrated in MFS mice that disruption in the elastic lamina of non-aneurismal descending aortas due to biaxial testing is accompanied by an increasing TGF-\(\beta\) signaling and that TGF-\(\beta\) can regulate collagen organization.\(^{31}\) Furthermore, in a MFS mouse dissection model, it has

<table>
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<tr>
<th>Table 1. Baseline characteristics of the study population</th>
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<td><strong>Variables</strong></td>
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<tr>
<td>Age (years, range)</td>
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<tr>
<td>Male gender</td>
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<tr>
<td>BSA (m(^2))</td>
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<td>Aortic root dilatation</td>
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<td>absolute diameter (mm)</td>
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<td>mean z-score</td>
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<td>(FBN1) mutation *</td>
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<tr>
<td>Skeletal involvement</td>
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<tr>
<td>Dural ectasia</td>
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<tr>
<td>Cardiovascular surgery (%)</td>
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<tr>
<td>Aortic root replacement</td>
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<tr>
<td>David procedure</td>
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<tr>
<td>Bentall procedure</td>
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<tr>
<td>Mitral valve correction **</td>
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<tr>
<td>Distal aortic graft</td>
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<td>Cardiovascular medicine</td>
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<td>Calcium channel blockers</td>
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<tr>
<td>Flecainide</td>
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<td>Diuretics</td>
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</tbody>
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Data presented as number (percentage) or mean ± standard deviation.
Abbreviations: BSA = body surface area, \(FBN1\) = fibrillin 1 gene,
* 5 of 99 patients did not have \(FBN1\) diagnostics
** 1 of 3 patients also had had a Bentall procedure
been shown that serum TGF-β levels were significantly increased before a dissection occurred.\textsuperscript{32} These data suggest that pathological changes in the aortic wall, ultimately leading to dissection, might lead to dysregulation of TGF-β. This is consistent with the current model of MFS in which large latent complex fails to be sequestered by defective fibrilline-1, resulting in more bioavailable TGF-β, leading to chronic and excessive activation of the SMAD transcription factors.\textsuperscript{10,30,33} Along the line, TGF-β antagonism through TGF-β neutralizing antibodies or inhibition of the angiotensin II receptor type 1 (that stimulates TGF-β production) by losartan treatment prevented progressive aortic dilation\textsuperscript{15} and other clinical features in MFS mouse models. The therapeutic benefits of TGF-β antagonism, by losartan treatment, were confirmed in humans in a small MFS paediatric cohort.\textsuperscript{34} Plasma TGF-β levels might be increased in other diseased in other diseases such as myocardial infarction or hypertrophic cardiomyopathy is not very likely.

In the study of Matt et al, strong correlations were found between circulating TGF-β levels and aortic root diameters in MFS mice, however no correlations could be demonstrated in their human cohort.\textsuperscript{16} In our study, we did find a significant correlation between aortic root diameters and TGF-β levels, which is probably due to the clinically more severely affected MFS population as expressed in larger aortic root dimensions and z-scores. Furthermore, 26\% of the MFS population studied by Matt et al. used losartan treatment, with significantly lower TGF-β levels and another 7\% used ACE inhibitors.

**Figure 1.** (A) Mean and standard error of the mean of total TGF-β plasma levels in patients with Marfan syndrome (MFS) (black box), healthy controls (white box), MFS patients without cardiovascular (CV) drugs (dark grey box) and MFS patients with β-blocker use (light grey box). (B) Relation of plasma TGF-β levels and age in patients with Marfan syndrome (MFS) in black dots and healthy controls in white dots.
with a tendency towards lower TGF-β levels. ACE inhibitors prevent angiotensin II conversion leading to reduced signalling via both angiotensin receptors type 1 and 2, while losartan only inhibits the type 1 receptor. Both medications will give lower TGF-β production, however, the signalling via the type 2 receptor is beneficial for the vessel wall. For this reason we did not include MFS patients on ACE inhibitors in our cohort. In this highly variable population a clear correlation between TGF-β levels and aortic root diameters could not be found and no follow-up data was available. To our knowledge, the observed correlation in our study between TGF-β and progressive aortic root dilation rate - and even more remarkable - the predictive value of TGF-β levels on clinical events have not been reported before. Plasma TGF-β might thus serve as a prognostic biomarker in patients with MFS. Aortic root replacement in medical history showed higher plasma TGF-β levels compared to patients with their native aortic root. This may be caused by aortic stiffness alteration after prosthetic aortic root replacement or it may be due to permanent wall damage in other parts of the aorta.

Table 2. Univariate analysis for predictors for aortic root growth and clinical events

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<thead>
<tr>
<th>Baseline Characteristics</th>
<th>Aortic root growth</th>
<th>Clinical events</th>
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<tbody>
<tr>
<td></td>
<td>β</td>
<td>p</td>
</tr>
<tr>
<td>Gender</td>
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<tr>
<td>TGF-β level</td>
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<td>&lt;0.001</td>
</tr>
<tr>
<td>Aortic root diameter (mm) by echo</td>
<td>0.07</td>
<td>0.58</td>
</tr>
</tbody>
</table>

Abbreviations: echo: echocardiography

Figure 2. (A) Correlation between total plasma TGF-β levels and echocardiographic aortic root diameters in patients with Marfan syndrome. (B) Mean and standard of the mean of total TGF-β plasma levels in MFS patients without previous aortic root (AoR) surgery (black box) and MFS patients with previous AoR surgery (white box).
and aortic root diameters were not very strongly correlated, nevertheless TGF-β level is highly predictive for aortic events. This also suggests that high TGF-β levels are rather a consequence of severe wall damage than the cause of the aortic pathology. This implies that there might be an even more comprehensive pathway leading to the cardiovascular features of MFS. It has been suggested that inflammation plays a modifying role in the pathogenesis of MFS, and that inflammation might be a novel therapeutic target in MFS patients. More fundamental research to unravel new molecular pathways remains necessary to optimize treatment in MFS patients. In the meantime results of several multicentre, randomized, controlled clinical trials are awaited.

**Figure 3.** (A) Correlation between total plasma TGF-β levels and aortic root growth in mm/year measured by echocardiogram in patients with Marfan syndrome. (B) Area under curve (AUC) shows sensitivity and specificity of TGF-β as predictive value for the combined primary endpoint elective aortic root replacement and aortic dissection. (C) Kaplan-Meier curve shows percentage freedom of events of TGF-β < 140 pg/ml TGF-β > 140 pg/ml. Numbers of patients at risk of events are demonstrated below the figure.
Limitations

In this observational follow-up study, relatively small patient numbers were included and only four patients reached the clinical endpoint of aortic dissection. Therefore, the combined endpoint of aortic dissections with -physician driven- elective aortic root surgery was chosen.

Our study aimed to predict aortic root dilation rate and aortic events by a single baseline determination of plasma TGF-β level. An interesting point would have been the repetitive testing of TGF-β to garner precise information about the time dependency and stability of these values in each patient. A drawback for the implementation of TGF-β measurements as a prognostic tool for aortic damage may be the large variability in TGF-β measurements in different laboratories. Therefore, our data cannot be easily extrapolated to other centres, and normalized standards should be determined by each individual centre.

Asymmetrical shape of the aortic root in MFS can bias the echocardiographic measurements, other imaging modalities such as cardiac magnetic resonance imaging or computed tomography are better exam methods for aorta root measurements and follow-up.

Conclusion

Plasma TGF-β levels are correlated with aortic dimensions of adults with MFS. Our study is the first to demonstrate the prognostic value of TGF-β on aortic root dilation rate and clinical events, such as aortic dissection and elective aortic root surgery. Plasma TGF-β might therefore serve as a prognostic biomarker in MFS.

References

Chapter 4: TGF-β as prognostic biomarker


