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The TGFβ system and *TIMP1* and *3* genotypes in Turner syndrome—Relation with aortic congenital malformations

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Abstract

Objective: Cardiovascular complications and congenital malformations are known traits in Turner syndrome (TS), which increases mortality. Women with TS have varying phenotype and cardiovascular risks. A biomarker assessing the risk for cardiovascular complications could potentially reduce mortality in high-risk TS and reduce screening in TS participants with low cardiovascular risk.

Design, Patients, Participants and Measurements: As part of a study initiated in 2002, 87 TS participants and 64 controls were invited to magnetic resonance imaging of the aorta, anthropometry, and biochemical markers. TS participants were re-examined thrice lastly in 2016. The focus of this paper is the additional measurements of transforming growth factor beta (TGFβ), matrix metalloproteinase (MMP's), tissue inhibitor of matrix metalloproteinase (TIMP), peripheral blood DNA and their associations with TS and the cardiovascular risk and congenital heart disease.

Results: TS participants had lower TGFβ1 and TGFβ2 values compared to controls. snp11547635 heterozygosity was not associated with any biomarkers but was associated with increased risk of aortic regurgitation. TIMP4 and TGFβ1 were correlated with the aortic diameter at several measuring positions. During follow-up, the antihypertensive treatment decreased the descending aortic diameter and increased TGFβ1 and TGFβ2 levels in TS.

Conclusion: TGFβ and TIMP's are altered in TS and may play a role in the development of coarctation and dilated aorta. snp11547635 heterozygosity was not found to impact biochemical markers. Further studies should investigate these biomarkers to further unravel the pathogenesis of the increased cardiovascular risk in TS participants.

KEYWORDS

antihypertensive treatment, cardiovascular risk, snp11547635, TGFβ, tissue inhibitor of matrix metalloproteinase, Turner syndrome

1 | INTRODUCTION

Turner syndrome (TS) associates with an increased prevalence of congenital left-sided heart disease along with an increased incidence and mortality from aortic dissection.[1] Aortic dilation is a key risk marker for aortic dissection in TS with the risk especially increased with coexisting 45,X karyotype, left-sided congenital heart disease such as bicuspid aortic valves and aortic coarctation, hypertension and pregnancy.[2–6] Nevertheless, risk stratification is fraught in TS because not all aortic dissections are predicted by these risk factors, and an increased understanding of the underlying disease mechanisms may help identify new and improved markers of the risk of aortic dissection.

The aetiology of congenital heart disease in TS remains an enigma, but a genetic origin is hypothesized, and recent studies have implicated a relation to the expression of the tissue inhibitor of matrix metalloproteinase 1 (*TIMP1*) and *TIMP3* genes and changed methylation patterns, having complementary effects.[7,8] It seems that having being haploinsufficient for the *TIMP1* gene (the gene is situated on the X chromosome and females with 45,X would, therefore, only have one gene), and simultaneously having a certain single-nucleotide polymorphism (SNP) (snp11547635) in the *TIMP3* gene (on chromosome 22), increases the risk of developing bicuspid valves.[7] How the changed expression and methylation of these TIMPs could affect the development of bicuspid aortic valves and perhaps coarctation of the aorta is not known.[9] When it comes to the causative mechanisms of progressive aortic disease, transforming growth factor beta (TGFβ) cytokines are implicated in aortic disease of Marfan syndrome, Loyes–Dietz syndrome and familial thoracal aortic aneurism, with TGFβ upregulations and TGFβ receptor defects.[10] In the TGFβ superfamily, primarily the TGFβ1 isoform associates with changes of the extracellular matrix within the arterial wall leading to reduced arterial elasticity and increased dilation. Matrix metalloproteinases (MMPs) activate TGFβ, and increased MMP2, MMP3 and MMP9 are regarded as essential for reduced arterial elasticity. Other regulatory mechanisms of the production and activation of TGFβ1 remain unclear, but wall shear stress and angiotensin II are coupled to TGFβ activation.[11,12] The TGFβ2 isoform has a prominent role in the fetus, but probably holds a secondary role in progressive aortic dilation and a third isoform, TGFβ3, does not seem to be associated with aortic disease to any noticeable extent.[13]

This study hypothesized that circulating cytokines, karyotype effect and gene polymorphisms are perturbed in TS and especially so in the subset with bicuspid valves, aortic coarctation and/or progressive aortic disease. To that end, these parameters were assessed in a 10-year follow-up study of aortic disease, including diagnosis of congenital heart disease along with prospective registration of aortic diameter, blood pressure and left ventricular function.[14–17]

2 | METHODS

2.1 | Study design and recruitment

This study was carried out as a part of a prospective observational study,[14–17] consisting of a baseline visit followed by two repeat visits (first follow-up visit 2.4 years [1.4–3.5 years], second follow-up visit 2.4 years [2.1–2.7 years]). Participants with TS verified by karyotyping ($N = 102$) were included consecutively through advertisements in the National Society of Turner Contact Groups in Denmark and the local outpatient clinic. Exclusion criteria were malignant disease, clinically significant liver disease and mechanical heart valves. Healthy age-matched female controls ($N = 65$) were also recruited. Participants eligible for this study included 87 with TS and 65 controls, which has been previously described.[14]

2.2 | Circulating biomarkers

Plasma lipids and triglycerides were measured using an automated commercially available system (Aeroset; Abbott Diagnostics). The biomarkers, sMMP (MMP2, -3 and -9), TIMP (TIMP1, -2, -3, -4) and TGFβ (TGFβ1, -2 and -3) were measured by Multiplex assays (RnD Systems) according to the manufacturer's instructions. Samples were measured in duplicates and analysed using the BioPlex Manager 4.1 software (BioRad). There was a systematic difference in the levels of TGFβs at the three visits, and we did, therefore, not compare absolute levels between study visits but transformed measurements at each visit to standard deviation scores (SDS), which were then subsequently compared.

2.3 | Anthropometry, treatment and clinical biomarkers

Weight, height, and body mass index (BMI) were collected. Blood tests were performed after an overnight fast. A medical history was taken regarding medications, including antihypertensive treatment (AHT) and allowing categorisation into (i) participants receiving AHT throughout the study, (ii) participants starting AHT during the study (between baseline and final visits) and (iii) participants never receiving AHT during the study. Aortic valve morphology and aortic coarctation were determined using echocardiography and magnetic resonance imaging (MRI),[14,16–18] and aortic diameter was measured in a standardized manner using MRI at nine standardized positions as described previously.[14] Aortic coarctation was defined as concentric narrowing of the aortic lumen with a posterior shelf-like structure; aberrant right subclavian artery: origin of the right subclavian artery distally to the left subclavian artery and bovine aortic arch: common aortic origin of the innominate and left subclavian arteries.[15]

2.4 | Second-sex chromosome status and SNP determination

X and Y chromosome information from karyotypes was used to assess the presence of any secondary sex chromosome and the estimated *TIMP1* copy number was calculated (TIMP1 effect). We based the effect on karyotypes and estimated the *TIMP1* copy number based on the percentage of cells with a second X chromosome. A 45,X karyotype was scored with a TIMP1 effect of 1 and a 46,XX karyotype in controls was scored with a TIMP1 effect of 2. In cases of mosaicism, the TIMP1 effect ranged between 1 and 2 depending on the degree of mosaicism measured in blood DNA; a 45,X/46,XX karyotype with a mosaicism degree of 50% would equal a TIMP1 effect of 1.5.

DNA was isolated from peripheral blood with a standard protocol. *TIMP3* SNP analysis was performed using 10 ng of DNA with primer sets for four SNPs (rs9862, rs11547635, rs149161075, rs369072080) using a genotyping protocol (Thermofisher), performed on a Viia 7 Realtime polymerase chain reaction system (Thermofisher). Carriers of the putative risk allele, rs11547635, were determined.

2.5 | Statistical methods

All statistical computations were performed using STATA 16.1. Continuous variables were expressed as medians with ranges (median [range]) or mean ± standard deviations [mean ± SD]). Distributions were compared using Student's independent *t* test, or Wilcoxon signed-rank test. $p < .05$ was considered statistically significant. We used the Spearman correlation coefficient in TS and controls combined to determine the relationship between the TIMP1 effect and the TGFβs, the MMPs and TIMPs, since these data were nonparametric. Linear regression was used to correlate TGFβ, MMPs and TIMPs to aortic diameter. Analysis of variance tests were used to compare between groups. We used SDS to compare TGFβ levels between visits. Bonferroni correction was performed when analysing the SNP data and only results that remained significant are shown. In all other formal analyses, we made no formal correction for multiple testing because of the explanatory nature of the correlation analyses.

3 | RESULTS

In the current study, 87 women (aged 38 ± 11 years, range: 18–62 years) with TS verified by karyotyping (45,X: $n = 47$ (54%); other karyotypes (mosaics, isochromosomes): $n = 40$ (46%)) were included. Sixty-four healthy women taking no daily medication (oral contraceptives accepted), were recruited through advertising, served as controls, and examined at baseline. All 87 TS women were examined at baseline, second visit (follow-up time mean ± SD) and final visit (follow-up time mean ± SD). At baseline, TS participants and controls had similar ages, while anthropometrics, as expected, were significantly different (Table 1). Among TS, 25/87 (29%) had bicuspid aortic valve (BAV), 40/87 (46%) had elongated transverse aortic arch (ETA) and 11/87 (13%) had aortic coarctation, whereas none were affected among the controls. Among TS 37/87 (43%) did not receive AHT during the follow, 20/87 (23%) started AHT during the study and 23/87 (26%) never received AHT, 7/87 had unknown AHT status. No TS participants stopped taking AHT during the follow-up period. Primary AHT was Angiotensin II antagonist (35%), angiotensin-converting enzyme-inhibitors (26%) and β1-inhibitors (24%). Calcium antagonist (18%) and thiazides (29%) were used as primary and secondary AHT, spironolactone was only used as secondary AHT in 18%. TS participants who started AHT during the follow-up period had a significant reduction in blood pressure [systolic: −14 mmHg, 95% confidence interval, CI (−3, −25); diastolic: −9 mmHg 95% CI (−2, −17)], and had significant reduction of the diameter in aortic position 8 [−0.53 mm 95% CI (−0.83, −0.23)] (Figure 1). The reduction in blood

TABLE 1 Anthropometrics and descriptive indices [means ± standard deviation, *n* (%)] in Turner syndrome and healthy female controls at baseline.

	Controls	Turner syndrome
Participants (*n*)	65	87
Age (years)	40 ± 12	38 ± 10
Karyotype [45,X/non-45,X, *n* (%)]		47/40
		54%/46%
Body surface area	1.75 ± 0.2	1.49 ± 0.2*
Height (cm)	167 ± 6.3	147 ± 7.2*
Weight (kg)	68.5 ± 11.8	57.6 ± 12.4*
BMI (kg/m^2)	23.4 ± 3.8	26.6 ± 5.6*
Bicuspid aortic valve	0/67	25/87 = 29%
Elongated transverse aortic arch	0/67	40/87 = 46%
Coarctation of aortae	0/67	11/87 = 13%
Total cholesterol	5.03 ± 0.8	5.15 ± 0.9
LDL cholesterol	2.90 ± 0.8	2.78 ± 0.8
HDL cholesterol	1.67 ± 0.3	1.94 ± 0.6*
Oestrogen replacement therapy (baseline/follow-up)		
Ongoing [*n* (%)]	-	74 (85%) / 54 (79%)
Duration (years)	-	16 ± 10 / 22 ± 10
Growth hormone treatment (previous)		23 (26%)
Duration	-	5.3 ± 3.0

Abbreviation: BMI, body mass index; HDL, high density lipoprotein; LDL, low density lipoprotein.

*$p < .01$ using Student's independent *t* test comparing women with Turner syndrome with controls.

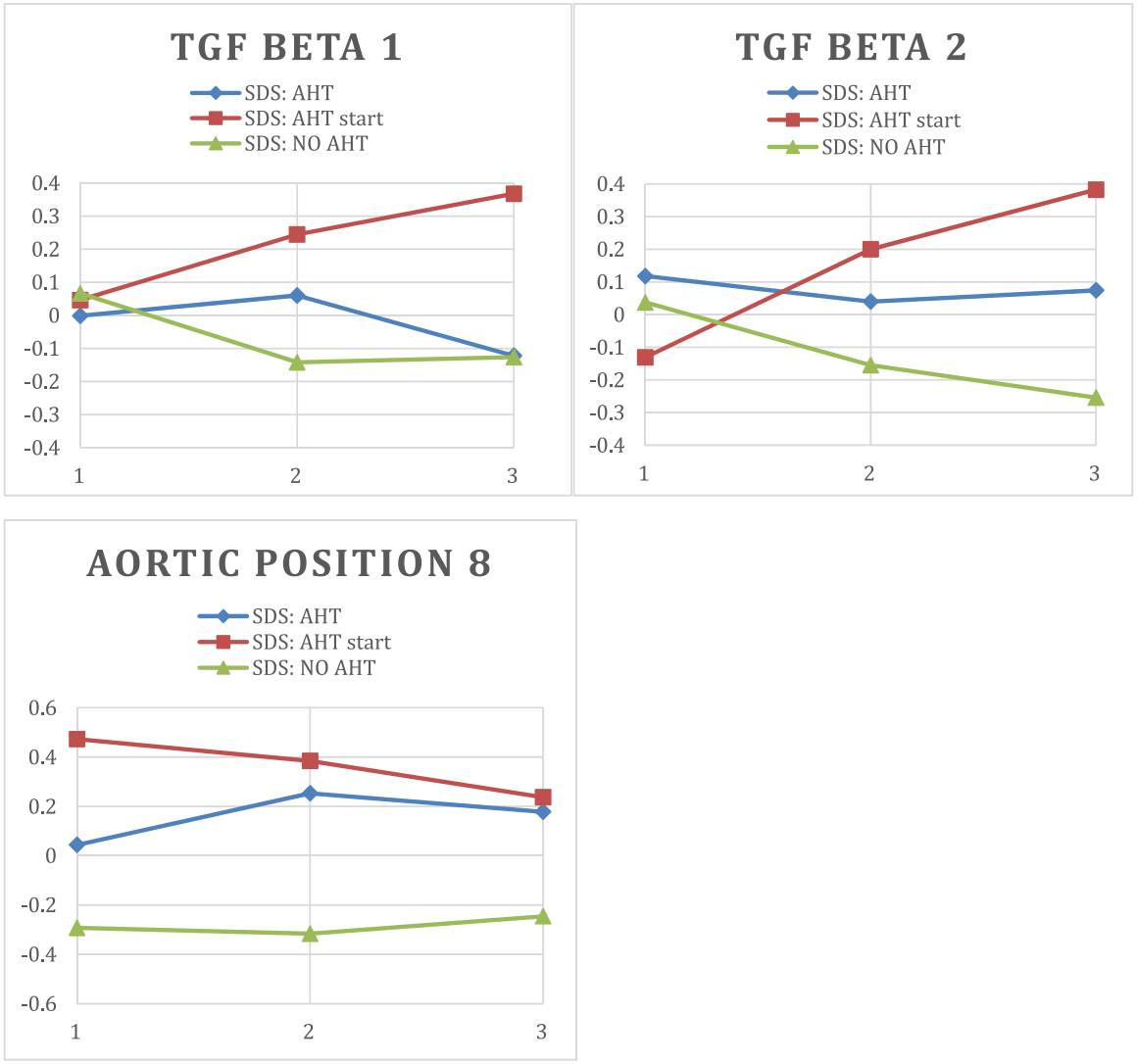

FIGURE 1 Standard deviation scores (SDS) of transforming growth factor beta 1 (TGFβ1) and TGFβ2 and aortic diameter in women with Turner syndrome (TS) from visits 1, 2 and 3, stratified between TS receiving antihypertensive treatment (AHT) throughout the study, TS starting AHT during follow-up and TS participants never receiving AHT. Changes, compared to the two other groups (AHT and no AHT) were significantly different for TGFβ1 ($p < .05$), for TGFβ2 ($p = .001$) and for aortic diameter (position 8) ($p = .033$).

pressure was significant in those who started AHT during the study and compared to those who received AHT throughout the study ($p < .03$) and to TS participants who never received AHT ($p = .04$) (Figure 1). TS participants who started AHT had a significant rise in TGFβ1 SDS ($p < .05$) and TGFβ2 SDS ($p < .01$) over time, compared to both those who never received AHT and those who received AHT throughout the study (Figure 1). There was no difference in biomarkers between the different types of AHT.

At baseline, TS participants had significantly lower TGFβ1 SDS ($p < .001$) and TGFβ2 SDS ($p < .001$) compared to controls (Figure 2), but no significant differences were evident in TGFβ3, MMP2, -3 and -9, or TIMP1, -2, -3 and -4 between TS and controls (Table 2). Having TS and being heterozygote for snp11547635 did not significantly affect TGFβ1, -2 and -3, MMP2, -3 and -9 or TIMP1–4. However, heterozygosity for snp11547635 TS significantly increased the risk of aortic regurgitation ($p < .001$), but not aortic stenosis or mitral valve regurgitation. The *TIMP1* effect was significantly associated with TGFβ1 and TGFβ2 SDS ($p < .001$), but not with MMP2, -3, -9 or TIMP14 (Table 3). TIMP4 was significantly associated with the aortic diameter at position 3 (r_s: 0.28, $p = .01$), position 5 (r_s: 0.27, $p = .02$), position 6 (r_s: 0.36, $p = .0014$) (Figure 3) in TS participants, at all three visits. As previously shown, TS participants had significantly smaller aortic size at the distal transverse arch (position 5) and aortic isthmus (position 6) ($p < .001$), whereas the rest of the aortic positions remained equal compared to controls. TGFβ1 was positively correlated with aortic size 6 at the first visit ($\beta = 2043$ 95% CI (875–3211 $p < .001$) in cases and controls combined. Changes in aortic diameter across the study were not associated with any biomarkers. TS participants with BAV and ETA had no altered biomarkers compared to TS without. TS participants with aortic

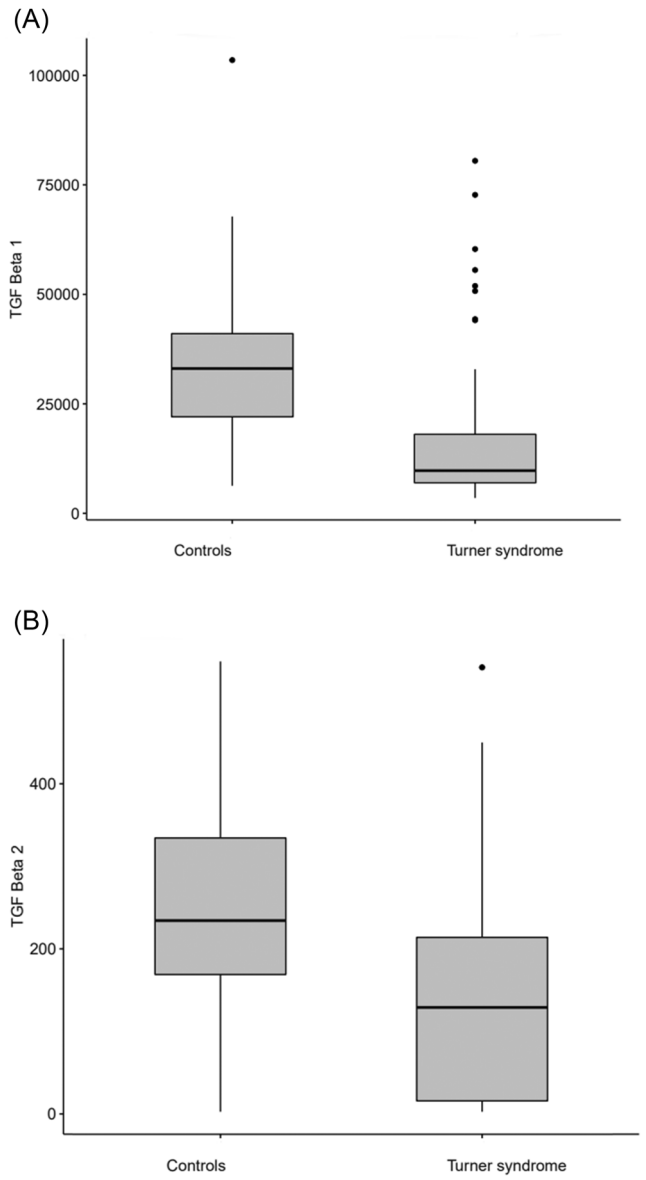

FIGURE 2 (A) Transforming growth factor beta 1 (TGFβ1), (B) and TGFβ2 in Turner syndrome ($n = 87$) and healthy age-matched controls ($n = 65$) at baseline. One outlier of TGFβ2 = 2099 has been removed for clarity. Wilcoxon rank-sum different $p < .0001$.

TABLE 2 THE TGFβ system [median (range)] in Turner syndrome ($n = 87$) and healthy age-matched controls ($n = 65$) at baseline.

	Controls	Turner syndrome
TGFβ system		
MMP2	171,737	172,870
	(116,799–244,539)	(103,664–274,669)
MMP3	8093	8349
	(2937–23,654)	(3270–171,477)
MMP9	390,404	408,015
	(136,723–1,191,300)	(135,220–1,796,200)
TGFβ1	33,073	9746
	(6295–103,484)	(3493–80,490)**
TGFβ2	241	129
	(3–2100)	(3–541)**
TGFβ3	64	64
	(7–8296)	(7–669)
TIMP1	146,604	148,166
	(4508–212,694)	(4549–253,246)
TIMP2	98,691	101,593
	(753–124,492)	(583–142,858)
TIMP3	9392	9047
	(117–41,271)	(116–53,348)
TIMP4	1296	1298
	(36–2364)	(57–4284)

Abbreviations: MMP, matrix metalloproteinase; TGFβ, transforming growth factor beta; TIMP, tissue inhibitor of matrix metalloproteinase.
**$p < .0001$ using Wilcoxon rank-sum test to compare women with Turner syndrome with controls.

coarctation had higher TGFβ1 and MMP9. TS participants with mitral and aortic regurgitation had 9.6% ($p = .02$) and 12.2% ($p = .01$) higher levels of MMP2 at the last visit, the results, though similar, were not significant at the other visits.

4 | DISCUSSION

The present study examines aortic disease, congenital and acquired, risk factors for aortic dissection and possible circulating biomarkers. We hypothesized that members of TGFβ family of cytokines and related peptides could either function as biomarkers for the development of disease, such as aortic dilation, or inform us concerning the pathophysiology of these conditions. It remains an enigma why some females with TS and a 45,X karyotype develop both coarctation of the aorta and bicuspid aortic valves, while others with the same karyotype, and thus presumably the same genotype, have a seemingly normal heart and branching arteries.

We show that TGFβ1 and -2 were significantly lower among females with TS and that the *TIMP1* effect was associated with TGFβ1 and -2, respectively. All the measured MMPs and TIMPs were similar among TS and controls. Only one previous study has studied the TGFβ system and TS participants and found higher TGFβ1 values and lower TGFβ2 values, conflicting somewhat with the present results, yet both demonstrate that the TGFβ system is perturbed in TS participants.[19]

The circulating level of TIMP4 correlated with several aortic diameters both in the ascending aorta and at the distal transverse aortic arch and aortic isthmus, at each of the three visits in TS participants. However, no significant association was seen in controls.

TABLE 3 r_s in Turner syndrome and controls combined to determine the relationship between the TIMP1 effect and the TGFβs, the metalloproteases as well as the TIMPs.

TIMP1 effect	MMP2	MMP3	MMP9	TGFβ1	TGFβ2	TGFβ3	TIMP1	TIMP2	TIMP3	TIMP4
r_s	0.02	−0.06	−0.05	0.55	0.43	0.07	-0.04	−0.05	−0.01	0.04
p Value	.8	.5	.5	<.001	<.001	.5	.6	.6	.9	.7

Note: For details see text.

Abbreviations: MMP, matrix metalloproteinase; r_s, Spearman correlation coefficient; TGFβ, transforming growth factor beta; TIMP, tissue inhibitor of matrix metalloproteinase.

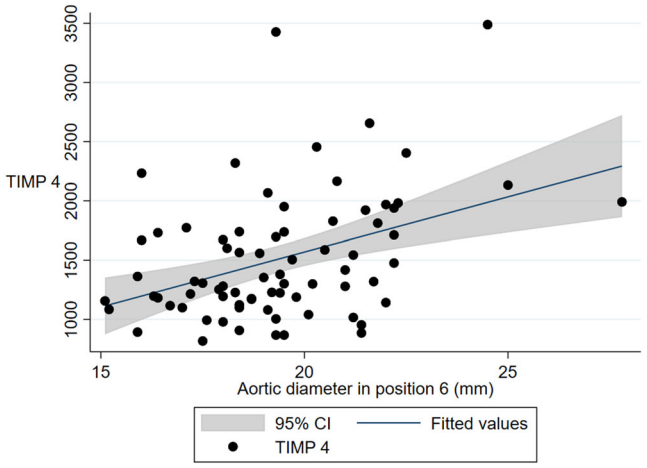

FIGURE 3 Linear regression between TIMP4 and diameter of aortic position 6 in TS participants. *β* coefficient = 92, 95% CI (44–142). CI, confidence interval; TIMP, tissue inhibitor of matrix metalloproteinase; TS, Turner syndrome.

Previously we and others have shown that this position, where coarctation of the aorta normally is present, is smaller than among controls.[15,20] TGFβ1 was positively correlated with aortic position 6 using linear regression and significantly lower in TS participants indicating that it could be a future biomarker for the reduced diameter. The findings regarding reduced diameter in aortic position 6 and the rates of ETA, BAV and coarctation of the aorta have all been found in previous studies at similar rates.[15,20] ETA and BAV were not associated with changes in biomarkers. The positive correlation between diameter aortic position 6 with TIMP4 and TGFβ1 is new and indicates that lower values of TGFβ1 or TIMP4 could somehow be associated with the diameter of the aorta at position 6, whether it be mechanistically or just an incidental association.

We hypothesized that snp11547635 heterozygosity might be related to reduce circulating TIMP levels, but we did not find this relation; however, snp11547635 heterozygosity was associated with aortic regurgitation but not aortic stenosis or mitral valve regurgitation. Previous studies have found decreased TIMPs in participants with aortic regurgitation.[21] This lack of association between TIMP levels, aortic stenosis, mitral valve regurgitation and snp11547635 heterozygosity in the present study could, however, be due to a small sample.

Twenty participants started AHT during the follow-up period. Blood pressure decreased significantly and concomitantly we observed a significant increase in TGFβ1 and TGFβ2 compared to all other TS participants. These results do not match previous mouse studies that have shown that losartan (angiotensin-II antagonist) is an antagonist to TGFβ1 and is able to reduce the level of TGFβ1.[22] This study showed no difference in TGFβ1 if participants received losartan. To our knowledge, there are no human studies that have accessed the effect of AHT on the TGFβ system. The TS participants who started AHT during follow-up had a slight reduction of aortic diameter at position 8 (descending aorta); however, future studies with larger sample size are needed to confirm whether this reduction is real or just an incidental finding. Indeed, it may be so that the descending aorta is especially sensitive to blood pressure reductions.

This study must, of course, be viewed as an explorative study aiming at finding new biomarkers and relating such biomarkers to the genetic makeup of TS patients. As such, a larger sample size would have been preferable. Also, the fact that karyotyping was performed in blood and not in any of the target tissues, such as the heart, the large branching arteries and the endothelium, should be considered as a drawback, as mosaicism cannot be excluded in 45,X patients.

We conclude that TS participants have reduced TGFβ1 and TGFβ2 compared to controls. The *TIMP1* effect was associated with TGFβ1 and TGFβ2. snp11547635 heterozygosity did not impact TGFβ's, MMP's or TIMP's but was associated with an increased risk of aortic regurgitation. TIMP4 and TGFβ1 are measurably associated with the diameter of the aorta in several positions and potentially associated with coarctation of the aorta. TGFβ1 and -2 levels increase when blood pressure is reduced in TS participants. TS participants who continue AHT or with no indication for AHT have stable TGFβ1 and TGFβ2 levels. AHT treatment may reduce the diameter of the descending aorta in TS participants.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

Informed consent was obtained from each participant, and the study protocol adhered to the 1975 Declaration of Helsinki as reflected in a priori approval by Aarhus County Ethical Scientific Committee (Denmark) (# 20010248). The study is also reported to Clinicaltrials.gov (NCT01678274).

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