BICUSPID AORTIC VALVE MORPHOTYPES SHOW DISTINCT MECHANISMS OF GENE EXPRESSION ALTERATION IN THE AORTA: RELEVANCE TO THE IDENTIFICATION OF POTENTIAL CIRCULATING BIOMARKERS

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OBJECTIVE

The pathogenesis of the aortopathy underlying the increased risk of aortic dilatation and dissection in bicuspid aortic valve (BAV) patients is currently poorly understood. Moreover, no available circulating biomarker, related to the risk or severity of the aortopathy, has been identified so far.

The Transforming Growth Factor-β (TGF-β) network is attracting an increasing attention, by virtue of its role in fibrosis, inflammation, cell proliferation and migration and extracellular matrix (ECM) remodeling and in light of its involvement in aortopathy syndromes, such as Loeys-Dietz and Marfan syndromes (**Fig. 1**).

In this study we aimed to gain more in-depth insights in the pathogenesis of aortopathy in BAV patients and to the identification of potential early biomarkers of diseas, by coupling a differential gene expression study in aortic samples with serum assay of the respective products.

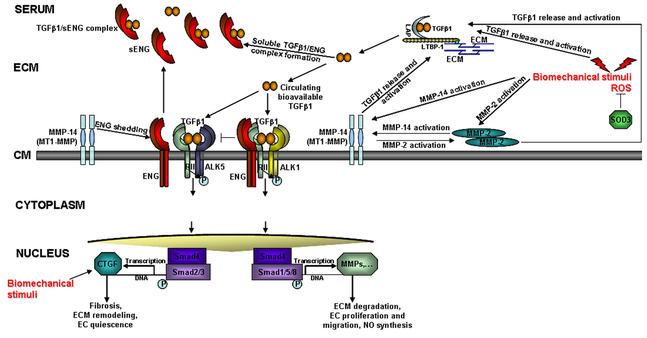


Figure 1: Interplays among the vascular remodeling players explored in the present study.
ECM stretching and proteolytic cleavage by various enzymes, including MMP-2 and MMP-14, can release the TGF-β1 homodimer from the latent complex. MMP-14 activates several MMPs, including MMP-2. MMPs can also be activated by biomechanical stimuli and ROS. SOD3 is a well-known extracellular ROS scavenger. In the canonical pathway, TGF-β1 binds to TGFβR2 that is auto-phosphorylated and phosphorylates TGFβR1 (ALK5), activating the TGF-β1, leading, among others, to the expression of pro-fibrotic CTGF. One of the known alternative pathways is mediated by TGFβR2/ALK1 and can ultimately lead to up-regulation of proteolytic enzymes. ENG is a regulatory co-receptor for TGF-β1: it can be cleaved by MMP-14, resulting in soluble ENG that can form inactive complexes with serum TGF-β1.

	Aorta donors	Serum donors	TAV	$BAV_{non\text{-}dil}$	BAV_{dil}
Male (%)	54.5	63.6	52.0	61.5	64.5
Age (years)	39.7±13	59.7±4.8*	69.7±7.8*†	60.9±15*‡	55.7±12*‡
Body surface area (m^2)	1.87±0.1	1.83±0.2	1.81±0.2	1.80±0.2	1.88±0.2
BAV morphotype (RL%/RN%)	-	-	-	56.4/43.6	67.7/32,2
Aortic diameter (cm)	3.0±0.6	N/A	3.6±0.6	3.7±0.5	5.0±0.5*†§
Left ventricle mass index (g/m^2)	N/A	N/A	152±43	143±37	125±44*§
Aortic valve area index (cm^2/m^2)	N/A	N/A	0.39±0.1	0.40±0.1	0.44±0.2
Hypertension (%)	22.7	18.2	82*†	69.2*†	58.1*†
Total Cholesterol (mg/dL)	N/A	173.2±6.4	178.0±10	176.8±12.0	172.8±7.6
Low-density-lipoproteins (mg/dL)	N/A	101.2±6.1	107.1±9.6	102.6±10.2	99.2±4.5
ARBs (%)	0	0	26*†	30.8*†	32.3*†
Statins (%)	0	18.2	44*	35.9	32.3
Aspirin (%)	0	0	26*†	20.5*†	16.1*†

Table 1. Clinical characteristics of the study groups.
Values are expressed as mean±SD, unless otherwise indicated. *=p<0.05 *vs.* aorta donors; †= p<0.05 *vs.* serum donors; ‡=p<0.05 *vs.* TAV; §=p<0.05 *vs.* $BAV_{non\text{-}dil}$. ARBs=angiotensin receptor blockers. N/A=not available, however BAV, aortic stenosis or aortopathy had been previously excluded.

METHODS

Patients: Aortic wall samples from 120 patients with aortic valve stenosis undergoing surgery, with or without associated aortopathy, and from 22 heart transplant donors with negative personal and familial history of BAV and/or aortopathy were included. Fifty patients had tricuspid aortic valve (TAV) stenosis with normal or mildly dilated ascending aorta (echocardiography-measured maximal aortic diameter ≤45mm), 39 (non-familial, non-syndromic) had a stenotic congenital BAV with normal or mildly dilated aorta (≤45mm, $BAV_{non\text{-}dil}$ group) and 31 had BAV stenosis with "ascending phenotype" aortic dilatation (>45mm, BAV_{dil} group). Clinical characteristics are summarized in **Table 1**.

Aortic wall and blood sample collection: At surgery, two aortic samples were retrieved from the so-called convexity (CVX: greater curvature) and the concavity (CCV: lesser curvature) regions respectively, 1–2 cm distal to the sino-tubular junction (from the ends of the transverse aortotomy in patients without dilatation). Samples were immediately stored in RNALater (Qiagen) at −80°C for subsequent RNA extraction. Blood samples were obtained preoperatively by venipuncture from all BAV and TAV patients as well as from 11 healthy volunteers in whom the presence of BAV or aortic diseases had been previously excluded.

RT-PCR and ELISA assays: We evaluated by RT-PCR gene expression variations of TGF-β1, connective tissue growth factor (CTGF), matrix-metalloproteinase 2 (MMP-2), MMP-14, endoglin (ENG) and superoxide dismutase 3 (SOD3) in the ascending aorta of TAV and BAV stenosis patients, and assessed by ELISA the serum concentration of the respective products.

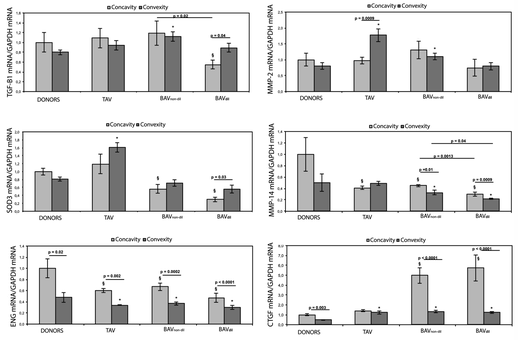


Figure 2: RT-PCR gene expression data of target molecules in thoracic aortic samples harvested from reference organ donors and from TAV, $BAV_{non\text{-}dil}$ and BAV_{dil} groups of patients.
*=p<0.05 *vs.* donor aortic CVX; §=p<0.05 *vs.* donor aortic CCV. The p values for significant differences in gene expression between two patient groups or between CVX and CCV within a group are shown as well.

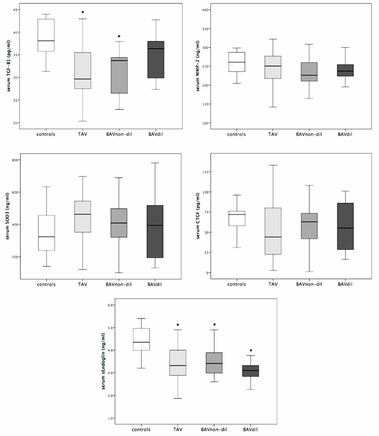
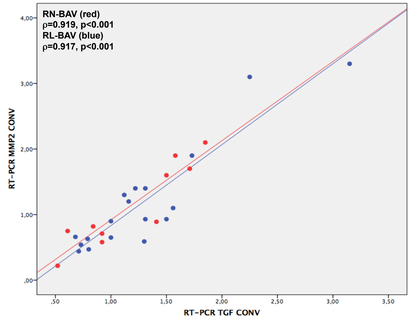


Figure 4: Box-plots of serum concentration of target molecules in healthy volunteers and in TAV, $BAV_{non\text{-}dil}$ and BAV_{dil} groups of patients.
*=p<0.05 *vs.* serum concentration in healthy subjects.



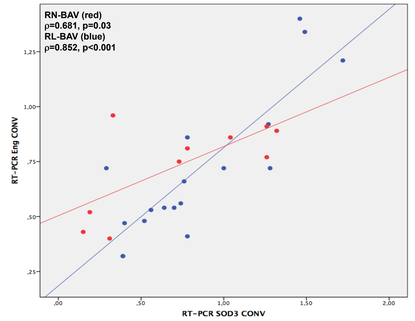


Figure 3: Scatter plots of Spearman correlations between mRNA expression level of target molecules in BAV patients divided in subgroups homogeneous for cusp fusion pattern of aortic valve (RL-BAV and RN-BAV).
Data refer to BAV aortic convexity. P<0.05 was considered statistically significant. N.S.: not significant.

Statistical analysis: Variables considered in the analysis included clinical features (**Table 1**), gene expression data and serum concentration of target molecules: each variable was preliminarily tested for normality of distribution and accordingly parametric (Student's t test or paired t test, Pearson correlations) or non-parametric methods (Mann-Whitney test or Wilcoxon test, Spearman correlations) were used for comparisons. Categorical variables were compared through the chi-square statistics with exact test. In the whole patient population and in the $BAV_{non\text{-}dil}$ and BAV_{dil} subgroups, correlations were assessed both between pairs of gene expression data and between the expression of each gene and the serum concentration of target proteins. Values of p<0.05 (two-tailed) were considered significant.

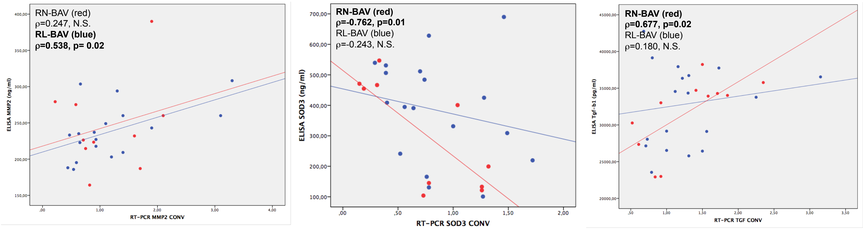


Figure 5: Scatter plots of Spearman correlations between mRNA expression level of MMP-2, SOD3 and TGF-β1 and serum concentration of respective products in BAV patients divided in subgroups homogeneous for cusp fusion pattern of aortic valve (RL-BAV and RN-BAV).
RT-PCR data refer to BAV aortic convexity. P<0.05 was considered statistically significant. N.S.: not significant.

RESULTS

RT-PCR data reveal that even in $BAV_{non\text{-}dil}$ stenosis patients, significant changes of gene expression *vs.* control aortas occur, including increased expression of TGF-β1, CTGF and MMP-2, decreased expression of MMP-14 and ENG and lack of the increased expression of SOD3 that was observed in TAV stenosis patients (**Fig. 2**). The expression levels of different genes showed morphotype-specific correlations, including MMP-2/TGF-β1 and SOD3/ENG expression in both RL- and RN-BAV patients (**Fig. 3**), and ENG/MMP-14 in RL-BAV and TGF-β1/MMP-14 in RN-BAV patients only (ρ=0.723 and 0.714, respectively, p=0.001).

TGF-β1 serum concentration significantly decreased in TAV and $BAV_{non\text{-}dil}$ patients *vs.* healthy subjects. ENG serum concentration decreased in all patients, more markedly in BAV_{dil} patients (**Fig. 4**).

RL-BAV patients and RN-BAV patients showed distinctive correlations between expression levels of some genes and serum concentration of the respective products: these included a significant correlation of both MMP-2 and TGF-β1 gene expression with serum concentration of MMP-2 in RL-BAV group, and with serum SOD3 concentration in RN-BAV (**Fig. 5**).

Finally, for what concerns the association between RT-PCR and/or ELISA data and medications, we highlighted a significantly lower TGF-β1 concentration in the serum of BAV-RL patients treated with Angiotensin receptor blockers (ARBs) (**Fig. 6**).

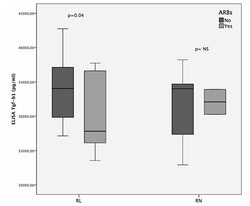


Figure 6: Box-plot of serum concentration of TGF-β1 in RL-BAV and RN-BAV patients treated with ARBs or untreated.

CONCLUSIONS

The results of this study suggest differences between valve morphotypes in the pathogenetic mechanisms underlying aortic wall maladaptive remodeling.

Different molecules could be tested as potential biomarkers in RL and RN types of BAV. Our results also highlight the importance of careful phenotypic stratification of patients in future trials testing the use of ARBs and other medications for prevention of aneurysm development in BAV patients.

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