

ORIGINAL RESEARCH

Dysregulation of Endothelial Nitric Oxide Synthase Does Not Depend on Hemodynamic Alterations in Bicuspid Aortic Valve Aortopathy

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BACKGROUND: Bicuspid aortic valves (BAVs) predispose to ascending aortic aneurysm. Turbulent blood flow and genetic factors have been proposed as underlying mechanisms. Endothelial nitric oxide synthase (eNOS) has been implicated in BAV aortopathy, and its expression is regulated by wall shear stress. We hypothesized that if turbulent flow induces aneurysm formation in patients with a BAV, regional differences in eNOS expression would be observed in BAVs.

METHODS AND RESULTS: Ascending aortic specimens were harvested intraoperatively from 48 patients with tricuspid aortic valve (19 dilated, 29 nondilated) and 38 with BAV (28 dilated, 10 nondilated) undergoing cardiac surgery. eNOS mRNA and protein concentration were analyzed at the convex and concave aortic wall. In nondilated aortas, eNOS mRNA and protein concentration were decreased in BAV compared with tricuspid aortic valve (all $P < 0.05$). eNOS expression was increased in association with dilation in BAV aortas ($P = 0.03$), but not in tricuspid aortic valve aortas ($P = 0.63$). There were no regional differences in eNOS mRNA or protein concentration in BAV aortas (all $P > 0.05$). However, eNOS expression was increased at the concave wall (versus convexity) in tricuspid aortic valve dilated aortas (all $P < 0.05$).

CONCLUSIONS: Dysregulated eNOS occurs independent of dilation in BAV aortas, suggesting a potential role for aberrantly regulated eNOS expression in the development of BAV-associated aneurysms. The absence of regional variations of eNOS expression suggests that eNOS dysregulation in BAV aortas is the result of underlying genetic factors associated with BAV disease, rather than changes stimulated by hemodynamic alterations. These findings provide insight into the underlying mechanisms of aortic dilation in patients with a BAV.

Key Words: aortic valve ■ ascending aortic aneurysm ■ bicuspid aortic valve ■ endothelial nitric oxide synthase ■ hemodynamics

The bicuspid aortic valve (BAV) is the most common congenital cardiovascular malformation, with a prevalence of 0.5% to 2%.^{1–3} Of those afflicted, ≈50% to 60% will develop an aortic aneurysm during their lifetime.^{1,2,4–6} Compared with patients with a tricuspid aortic valve (TAV), BAV is associated with larger aortic root dimensions and a higher progression rate of dilation, suggesting that the pathogenesis of thoracic aortic dilatation associated with BAV is different.^{7,8} Turbulent flow as a result of the asymmetric

opening of the valve in BAV has been postulated as an essential determinant for the development of aortic dilatation.^{5,9–11}

The high incidence of dilatation in asymptomatic patients with BAV and the progression of aortic dilation after aortic valve replacement^{12,13} has been linked to intrinsic changes within the aortic wall, offering an alternate theory for the development of BAV-associated thoracic aortic dilatation. Compared with patients with a TAV, medial changes such as thinning and

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CLINICAL PERSPECTIVE

What Is New?

- Herein, we report changes in the mRNA expression and protein concentration of endothelial nitric oxide synthase in the ascending aorta of patients with a bicuspid aortic valve (BAV) compared with patients with a tricuspid aortic valve.
- The mRNA and protein levels of endothelial nitric oxide synthase in the aorta are not impacted by hemodynamic alterations associated with BAVs.
- The mRNA expression of endothelial nitric oxide synthase is increased in the concave wall of the aorta in patients with a tricuspid aortic valve.

What Are the Clinical Implications?

- The bicuspid aortic valve is the most common cardiovascular malformation, and it is associated with a strong predilection for ascending aortic aneurysm.
- Although hemodynamic alterations are associated with the BAV, our findings suggest that ascending aortic dilatation in patients with a BAV occurs independently of turbulent flow patterns.
- Our discovery of regionally variable eNOS expression in tricuspid aortic valve aortas prompts further research into the role of molecular biologic patterns in aortic remodeling.

Nonstandard Abbreviations and Acronyms

BAV	bicuspid aortic valve
eNOS	endothelial nitric oxide synthase
HMBS	hydroxymethylbilane synthase
R-L	right and left coronary cusp fusion
TAV	tricuspid aortic valve
VSMC	vascular smooth muscle cells

fragmentation of elastic lamellae, loose attachments of vascular smooth muscle cells to their surrounding lamellae and precocious vascular smooth muscle cell apoptosis and senescence have been found in BAV aortas.^{14–20} These changes to the aortic wall indicate an inherent structural defect that is not observed in TAV aortas and may therefore be the result of genetic influences associated with BAV.

There is evidence to suggest that endothelial nitric oxide (NO) synthase (eNOS) may be a potential contributor to BAV development. Using animal models, Lee and colleagues²¹ observed BAV morphology

in 5 of 12 eNOS knockout mice. Furthermore, studies of human aortopathy showed that eNOS expression is altered in the thoracic aortic wall of BAV patients compared with patients with a TAV.^{22–24} It is unclear, however, whether these changes in the eNOS system are genetically determined or if they are the consequence of turbulent flow and altered wall stress.

Magnetic resonance imaging in patients with a BAV has clearly indicated that blood flow patterns and subsequent aortic wall shear stress are altered in patients with a BAV compared with a TAV.^{9,25} Furthermore, aortic wall shear stress in patients with a BAV appears to depend also on the cusp fusion morphology, with the most common fusion pattern (right and left coronary cusp fusion [R-L]) displaying increased shear stress in the convex wall of the proximal and mid-ascending aorta.^{26,27} However, whether this change in wall shear stress is the primary mechanism of aortic dilatation in patients with a BAV remains unknown.²⁸ To determine if eNOS expression is influenced by turbulent flow in BAV aortas, we analyzed the mRNA expression and protein levels of eNOS at various regions within BAV aortas.

METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request. This prospective study was conducted in accordance with the Declaration of Helsinki and was approved by the locally appointed Ethics Committee (Ethikkommission bei der Ärztekammer des Saarlandes, No. 205/10). All patients gave written informed consent.

Samples

We collected samples of ascending aortic tissue from 86 patients undergoing aortic valve reconstruction or replacement, with or without ascending aortic replacement. The samples were extracted at the convexity and concavity of the ascending aorta, 5 to 10 mm cranial to the sinotubular junction and from the midascending aorta (Figure 1). Transesophageal echocardiography was used to characterize aortic dimensions, and aortic valve morphology was determined intraoperatively. Tissue samples were immediately snap frozen in liquid nitrogen and stored at -80°C . If sinus or tubular ascending aortic diameter were >40 mm, the ascending aorta was considered dilated. BAV was present in 38 patients, and TAV was present in 48 patients. The distribution of valve morphology and aortic dilatation of the patients are summarized in Table 1. Further clinical characteristics are reported in Table 2.

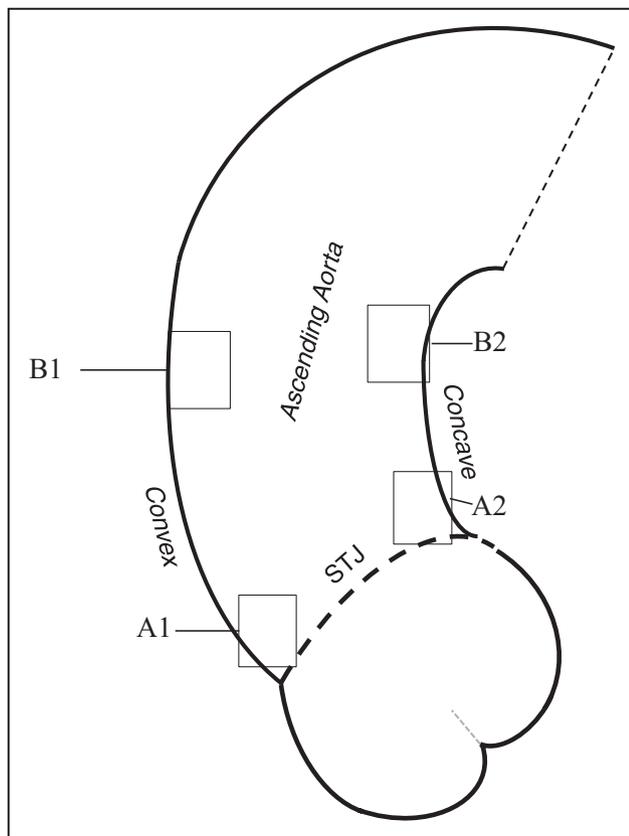


Figure 1. Schematic representation of the human aorta, with tissue sampling sites indicated.

To assess for endothelial nitric oxide synthase (eNOS) mRNA expression and protein concentration in the ascending aorta of patients with a bicuspid aortic valve, tissue samples were harvested from the convex and concave walls of the aorta, adjacent to the sinotubular junction (STJ) and at the level of the mid-ascending aorta. Samples from patients with a tricuspid aortic valve were used as comparison. Sample sites are indicated by rectangular boxes: samples of convex wall (A1) and concave wall (A2) adjacent to the STJ and samples of convex (B1) and concave wall (B2) from the mid-ascending aorta were harvested. This schematic representation indicates a bicuspid aortic valve, with a partially fused commissure (gray dotted line).

RNA Isolation and Complementary DNA Synthesis

RNA isolation was performed with the mirVana PARIS Kit (Ambion, Austin, TX). Frozen tissue samples were homogenized using an Ultra Turrax T8 homogenizer (Ika, Staufen, Germany) and an ultrasonic processor UP100H (Hielscher, Teltow, Germany). Further isolation was performed according to the manufacturer's recommendations. DNase digestion and RNA cleanup were done with the RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA quantity and quality were determined with an Infinite 200 NanoQuant (Tecan, Mannedorf, Switzerland). RNA integrity was confirmed with an Agilent 2100 Bioanalyzer and the Agilent 6000 Nano Kit (Agilent Technologies, Santa

Table 1. Patient Characteristics Based on Valve Morphology

	Tricuspid (n = 48)	Bicuspid (n = 38)
Age, y	63.9±13.6	54.7±15.1
Sex, male, %	68.8	92.1
Aortic valve pathology, n (%)		
Stenosis	13 (27.1)	29 (76.3)
Insufficiency	27 (56.3)	3 (7.9)
Combined	8 (16.7)	6 (15.8)
Comorbidities, n (%)		
Hypertension	29 (60.4)	12 (31.6)
Diabetes mellitus	2 (4.2)	0
Hyperlipidemia	7 (14.6)	7 (18.4)
Coronary artery disease	14 (29.2)	2 (5.3)
Medication, n (%)		
β-Blocker	20 (41.7)	17 (44.7)
Angiotensin-converting enzyme inhibitor	19 (39.6)	14 (36.8)
Angiotensin 1-receptor antagonist	7 (14.6)	5 (13.2)
Diuretic	14 (29.2)	13 (34.2)
Insulin	3 (6.3)	0
Calcium channel blocker	10 (20.8)	3 (7.9)
Statin	15 (31.3)	7 (18.4)
Aldosterone antagonist	6 (12.5)	1 (2.6)

Clara, CA). Reverse transcription was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's recommendations. Quantitative real-time polymerase chain reaction was performed with TaqMan Gene Expression Assays (Applied Biosystems) nitric oxide synthase 3 (Hs01574659_m1) on 96-well TaqMan Plates (Applied Biosystems). The plates were run in a StepOnePlus Real Time PCR System (Applied Biosystems). Each well contained 20 ng of complementary DNA and had a total reaction volume of 10 mL, consisting of 5 mL complementary DNA diluted with diethylpyrocarbonate-treated water and 5 mL TaqMan Gene Expression MasterMix (Applied Biosystems). All samples were run as triplicates. The gene expression levels of nitric oxide synthase 3 in patients with a BAV were determined relative to the expression levels in samples from patients with TAV without aortic dilatation using eukaryotic transcription initiation factor 2B, subunit 1, ets-related transcription factor 1, and hydroxymethylbilane synthase as internal control genes. These genes have been identified as reference genes for expression analyses on aortic tissue from patients with different valve morphologies in previous studies.²³ The expression level of nondilated TAV samples was set as 1. For comparisons between convex and concave regions of the aorta, relative expression of

Table 2. Patient Characteristics Based on Aortic Dimensions

	TAV Normal	TAV Dilated	BAV Normal	BAV Dilated
	n = 29	n = 19	n = 10	n = 28
Age	66.7±7.2	59.7±5.4	59.4±4.7	52.9±8.3
Sex (M/F)	18/11	16/3	08/2	26/1
Maximal aortic diameter (cm)				
Mean	3.2±0.8	5.3±1.1	3.6±0.24	4.7±0.48
Standard error	±0.8	±1.1	±0.24	±0.48
Range	2.4–3.9	4.2–8.1	3.2–3.9	4.0–5.3
Ejection fraction	54.4±2.4	63.2±2.4	56.7±5.8	58.5±2.2
Gradient over aortic valve				
Mean, mm Hg	21.2±3.9	5.0±0.6	13.6±5.1	10.0±2.0
Peak, mm Hg	35.0±5.7	9.5±1.1	24.6±8.3	16.8±3.1
LVOT diameter, cm	2.34±0.1	2.7±0.1	2.5±0.1	3.0±0.1

Values are presented as mean±SE. BAV indicates bicuspid aortic valve; LVOT, left ventricular outflow tract; and TAV, tricuspid aortic valve.

nitric oxide synthase 3 normalized to the reference genes was compared within each patient, and mean differences were compared within groups. Relative quantification values for nitric oxide synthase 3 were determined with the 2^{-DDCT} method.

Protein Quantification

Samples were lysed by mechanical force and the protein portion was isolated by using Protein Quant Sample Lysis Kit (Life Technologies, Rockville, MD). To determine the protein content within the lysate, Micro BCA Protein Assay (Thermo Scientific, Waltham, MA) was performed. The tissue lysates were then stored at -80°C for further examination at a later date. Protein expression of eNOS was ascertained by western blot analysis. The samples were diluted with Roti Load 1 (Carl Roth GmbH, Karlsruhe, Germany) and loaded onto polyacrylamide Bis-Tris 4 to 12% gels (Life Technologies). After separation of the protein fractions by electrophoresis, the samples were blotted onto Roti-PVDF transfer membranes (Carl Roth GmbH) and blocked with phosphate-buffered saline/milk powder 5% for 1 hour at room temperature. The membranes were then incubated with primary antibodies against eNOS (Anti-eNOS/NOS Type III Mouse, 1:1.000; BD Transduction Laboratories, Franklin Lakes, NJ) and β -actin (β -Actin 8H10D10 Mouse mAB, 1:200.000; Cell Signaling Technology, Danvers, MA) at 4°C overnight. After washing the membranes with phosphate-buffered saline +/- Tween20, an incubation with a secondary antibody (Peroxidase-conjugated AffiniPure Goat Anti-Mouse IgG, 1:1.000; Jackson ImmunoResearch, West Grove, PA) was executed for 1 hour at room temperature. Chemiluminescent staining (Pierce ECL Western Blotting Substrate, Thermo Scientific) was used for detection of the crucial protein bands. All

eNOS bands were evaluated as a relative quantity subject to the respective β -Actin bands within the same line. For comparisons between convex and concave regions of the aorta, relative eNOS concentration (eNOS/ β -actin) was compared between the convexity and the concavity within each patient, and mean differences were compared within groups.

Statistical Analysis

Data analysis was performed using SPSS Statistics 19 software (IBM, Ehningen, Germany). We analyzed eNOS expression in the ascending aortic wall and the aortic diameter by the Pearson product moment correlation coefficient or the Spearman correlation when data were not normally distributed. All data sets were tested for normality by the D'Agostino and Pearson omnibus test. In the case of normally distributed distributions, comparisons were made by the Student *t*-test. Nonparametric distributions were compared by the Whitney *U*-test.

RESULTS

Endothelial Nitric Oxide Synthase Expression Is Altered in the Aorta From Patients With a Bicuspid Aortic Valve Compared With Patients With a Tricuspid Aortic Valve

Ascending aortic tissue was harvested from a total of 86 patients. Aortic samples were characterized into four groups according to valve morphology and ascending aortic diameter: TAV-nondilated ($n = 29$), TAV-dilated ($n = 19$), BAV-nondilated ($n = 10$) and BAV-dilated ($n = 28$). Clinical information for all patients is presented in Tables 1 and 2.

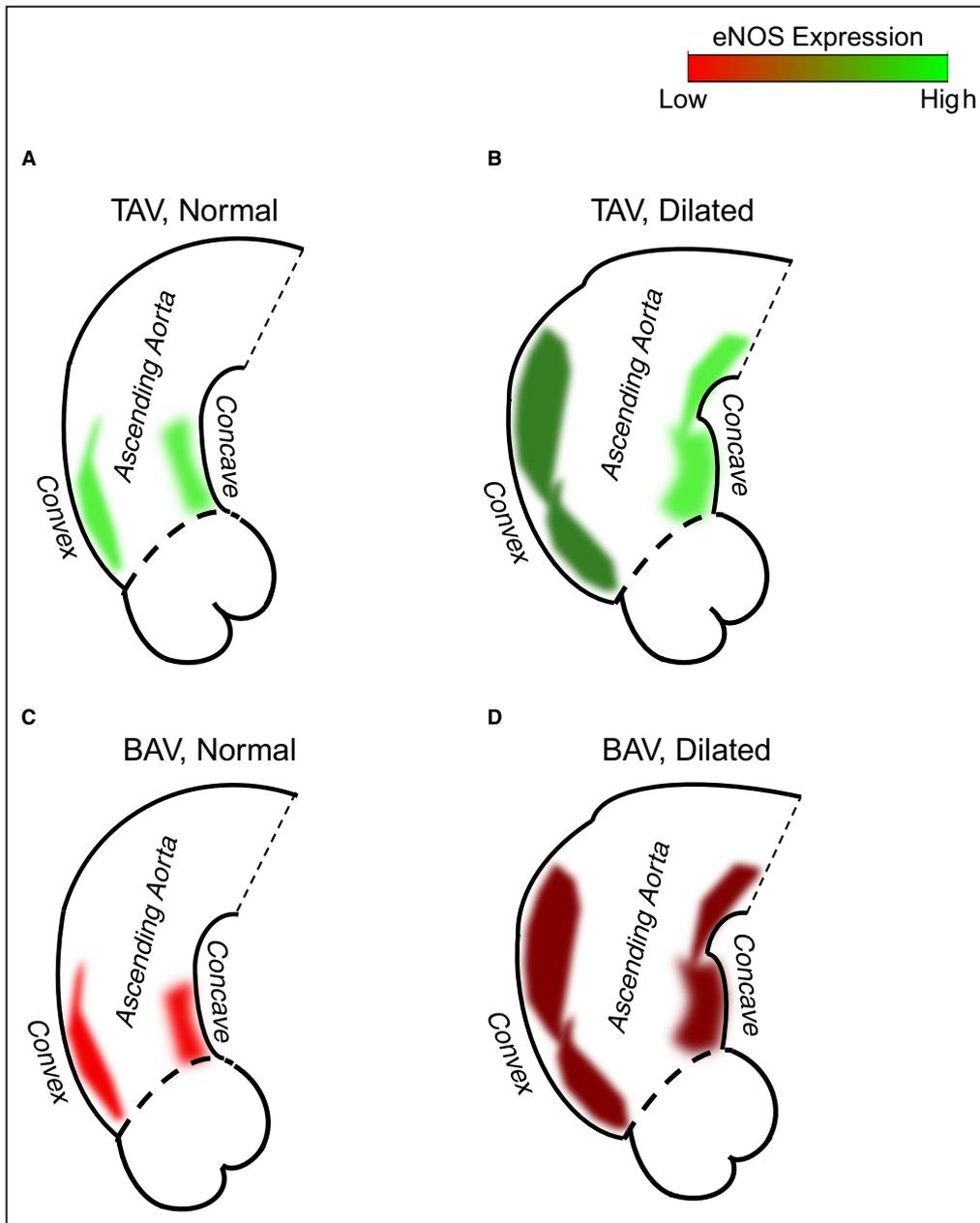


Figure 2. Schematic summary of endothelial nitric oxide synthase (eNOS) expression in normal and dilated aortas associated with tricuspid and bicuspid aortic valves.

A, Schematic representation of a normal (nondilated) aorta associated with a tricuspid aortic valve (TAV). eNOS expression is relatively high (green shading) in both the convex and concave walls of the aorta. **B,** Schematic representation of a dilated aorta associated with a TAV. eNOS expression is relatively high in the concave wall, with decreased expression (darker green) in the convex wall of the aorta. **C,** Schematic representation of a nondilated aorta associated with a bicuspid aortic valve (BAV). eNOS expression is relatively low (red shading) in both the convex and concave walls. **D,** Schematic representation of a dilated aorta associated with a BAV. eNOS expression is relatively lower than in nondilated BAV aortas (darker red shading) and similar to that of dilated TAV aortas in the convex wall (dark shading). Within the BAV dilated aorta, eNOS is equally expressed between concave and convex walls.

Previous studies have shown that eNOS is aberrantly expressed in BAV-associated aortas compared with TAV-associated aortas.²³ To confirm a role for aberrant eNOS expression in BAV aortopathy

in our study population, we assessed for the gene and protein expression of eNOS in normal and dilated aortas from patients with a TAV or with a BAV. In the absence of pathological dilation, eNOS mRNA

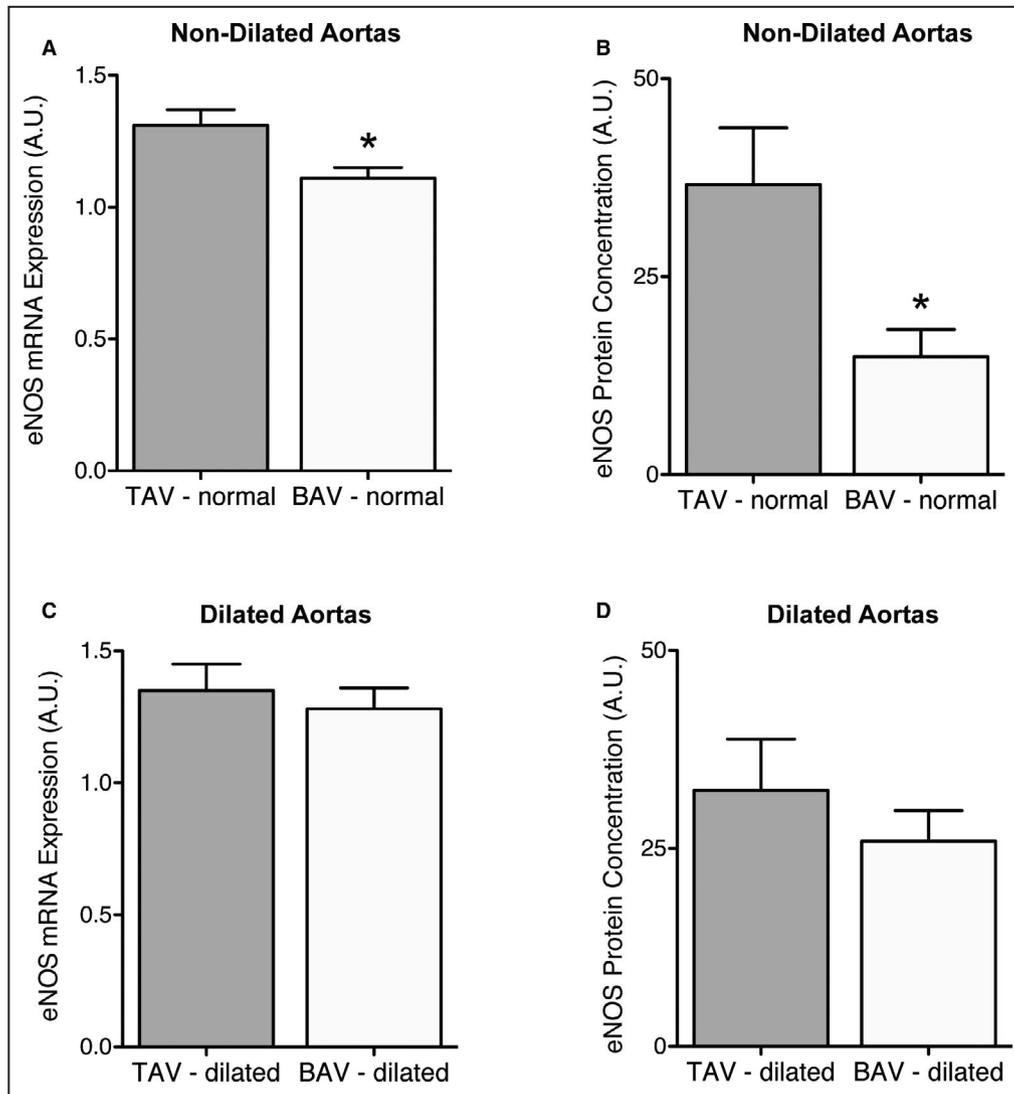


Figure 3. Endothelial nitric oxide synthase (eNOS) expression is altered in bicuspid aortic valve (BAV) nondilated aortas compared with tricuspid aortic valve (TAV) nondilated aortas.

A, Graph depicting the eNOS mRNA expression in nondilated (normal) aortas from patients with a TAV or a BAV. **B**, Graph and corresponding western blot image depicting the eNOS protein expression in normal aortas from patients with a TAV and patients with a BAV. eNOS protein expression values are normalized to beta-actin protein expression. **C**, Graph depicting the eNOS mRNA expression in dilated aortas from patients with a TAV or BAV. **D**, Graph and corresponding western blot image depicting the eNOS protein expression in dilated aortas from patients with a TAV and patients with a BAV. eNOS protein expression values are normalized to beta-actin protein expression. * = significance at $P < 0.05$; AU indicates arbitrary units. Data are presented as mean (bars) \pm SEM (error bars).

expression was significantly decreased in the aortas of patients with a BAV (1.11 ± 0.04) compared with those with a TAV (1.31 ± 0.06 , $P = 0.02$; Figure 2A, 2C, and 3A). Furthermore, eNOS protein concentration was decreased by 2.5-fold in BAV nondilated aortas compared with TAV nondilated aortas ($P = 0.02$; Figure 3B). This finding indicates a potential role for aberrantly regulated eNOS expression in the development of aneurysms, specific to those associated with BAVs. On the other hand, when comparing

dilated aortas from patients with a BAV and patients with a TAV, we found that there was no significant difference in eNOS mRNA expression between TAV (1.35 ± 0.10) and BAV aortas (1.28 ± 0.08 ; $P = 0.90$; Figure 2B, 2D, and 3C). Similarly, there was no significant difference in protein concentration between TAV and BAV dilated aortas ($P = 0.13$). This further implies that aberrant eNOS expression in BAV aortas may precede aneurysm development and is therefore a feature of BAV aortopathy.

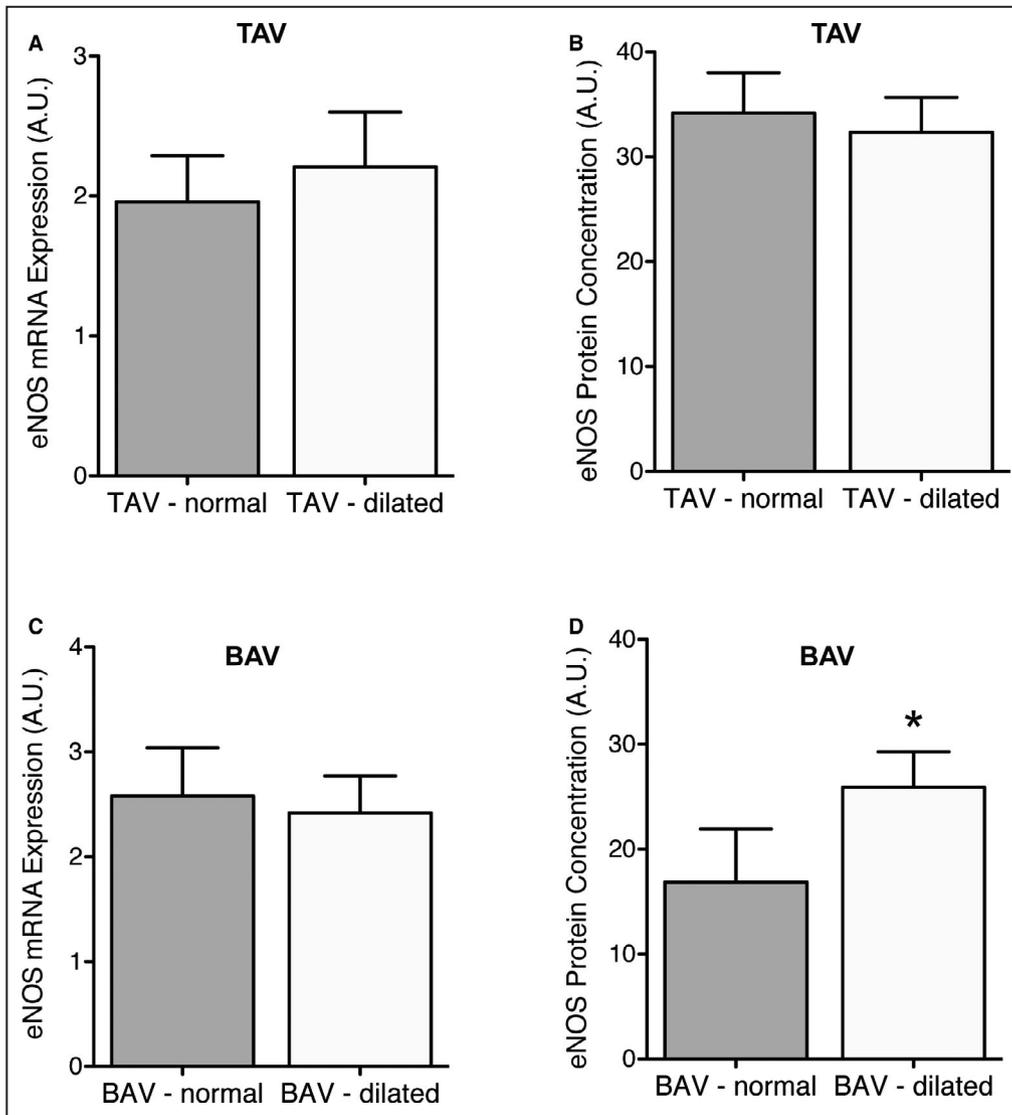


Figure 4. Endothelial nitric oxide synthase (eNOS) expression in the aorta is altered in response to dilation in patients with a bicuspid aortic valve (BAV) but not patients with a tricuspid aortic valve (TAV).

A, Graph depicting the eNOS mRNA expression in patients with a TAV with nondilated (normal) and dilated aortas. **B,** Graph and corresponding western blot image depicting the eNOS protein expression in normal and dilated aortas from patients with a TAV. eNOS protein expression values are normalized to beta-actin protein expression. **C,** Graph depicting the eNOS mRNA expression in normal and dilated aortas from patients with a BAV. **D,** Graph and corresponding western blot image depicting the eNOS protein expression in normal and dilated aortas from patients with a BAV. eNOS protein expression values are normalized to beta-actin protein expression. * = significance at $P < 0.05$; AU indicates arbitrary units. Data are presented as mean (bars) \pm standard error of the mean (error bars).

eNOS Expression Alterations Are Exacerbated in Response to Dilation in Patients With a BAV But Not in Patients With a TAV

As eNOS expression is altered in BAV nondilated aortas, we next sought to determine whether this finding is exacerbated in the presence of dilation. In patients with a TAV, we found no significant changes

between nondilated and dilated aortas in either mRNA expression ($P = 0.63$; Figure 4A) or protein concentration ($P = 0.73$; Figure 2A, 2B, and 4B) of eNOS. Remarkably, we found similar results for gene expression in patients with a BAV, as eNOS mRNA expression did not change in the presence of dilation ($P = 0.79$; Figure 4C). This was also the case when comparing patients with similar BAV configurations, in that there was no difference in eNOS expression

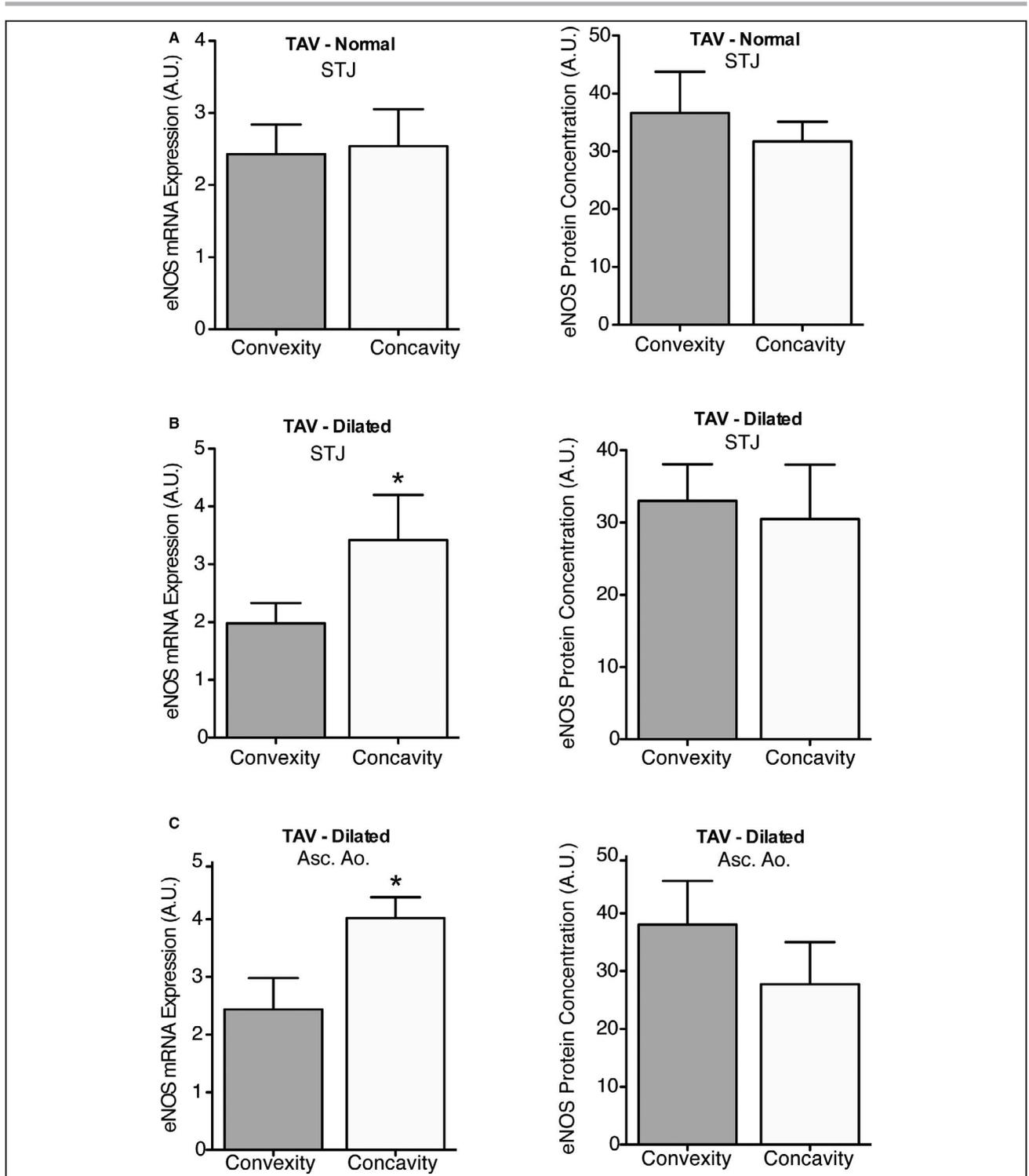


Figure 5. Endothelial nitric oxide synthase (eNOS) expression is altered at the concavity of the aortic wall compared with the convexity in dilated tricuspid aortic valve (TAV) aortas.

Graphs depicting the endothelial nitric oxide synthase (eNOS) mRNA expression (left) and protein expression (right) in nondilated (normal) aortas (A) and dilated aortas (B) from patients with a TAV. eNOS expression is compared between the convex and the concave wall of the aorta at the level of the sinotubular junction (STJ). C, Graphs depicting the eNOS mRNA expression (left) and protein expression (right) in dilated aortas from patients with a TAV. eNOS expression is compared between the convex and the concave wall of the aorta at the level of the mid-ascending aorta (Asc. Ao.). * = significance at $P < 0.05$; AU indicates arbitrary units. Data are presented as mean (bars) ± standard error of the mean (error bars).

between nondilated and dilated aortas from patients with an R-L BAV ($P=0.55$) or from patients with a right and noncoronary cusp fusion BAV ($P=0.61$). However, there was a significant increase in eNOS protein expression in dilated aortas compared with nondilated aortas in the presence of a BAV (25.92 ± 3.35 versus 16.87 ± 5.07 , respectively; $P=0.04$; Figure 2C, 2D, and 4D). This increased concentration was maintained when evaluating patients with an R-L BAV ($P=0.04$), and a trend for increased eNOS concentration was observed in dilated aortas in patients with a right and noncoronary cusp fusion BAV ($P=0.08$).

There Are No Regional Differences in eNOS Expression in BAV-Associated Aortas

To test the hypothesis that altered blood flow patterns associated with a BAV-induced regional differences in aortic wall remodeling and consequent dilation, we assessed for eNOS expression at various regions of the aortic wall in both patients with a TAV and patients with a BAV. In patients with a TAV and a nondilated aorta, we found no significant difference in either eNOS mRNA expression or protein concentration between the convexity (outer curvature) and the concavity (inner curvature) of the aortic wall at the level of the sinotubular junction ($P=0.92$ and $P=0.52$, respectively; Figures 2A, 5A). Interestingly however, in dilated aortas from patients with a TAV, there was a significantly increased expression of eNOS mRNA in the concavity of the proximal aorta adjacent to the sinotubular junction compared with the convex wall ($P=0.002$; Figures 2B, 5B). Similarly, eNOS mRNA was significantly increased at the concavity compared with the convexity of the mid-ascending aorta (convexity, 2.44 ± 0.54 versus concavity, 4.02 ± 0.36 ; $P=0.003$; Figure 5C) in TAV-associated dilated aortas. However, no significant changes in protein concentration were observed in TAV dilated aortas between the convexity and concavity at either the sinotubular junction ($P> 0.99$; Figure 5C) or the mid-ascending aorta ($P=0.7$; Figure 5D).

Although changes to blood flow and wall shear stress in the presence of a BAV have been well described,²⁷ we did not observe any regional difference in eNOS expression in BAV-associated dilated aortas. Namely, there were no changes in eNOS mRNA expression or protein concentration between the convexity and concavity at the sinotubular junction of dilated BAV aortas ($P=0.71$ and $P=0.57$, respectively; Figures 2C, 6A) or at the mid-ascending aorta of BAV-associated dilated aortas ($P=0.7$ and $P=0.51$, respectively; Figures 2D, 6B). Finally, we assessed for differences in eNOS expression between patients with a BAV with different cusp fusion morphologies. At the

level of the sinotubular junction, there was no significant difference in eNOS expression at either the convexity ($P=0.44$) or the concavity ($P=0.47$) when comparing patients with an R-L BAV and patients with a right and noncoronary sinus BAV (Figure 6C). Furthermore, there was no significant difference in eNOS expression at the level of the mid-ascending aorta between patients with a BAV with an R-L and a right and noncoronary cusp fusion BAV (convexity, $P=0.65$, concavity, $P=0.46$; Figure 6C).

DISCUSSION

BAV is associated with dilation of the ascending aorta, though the underlying mechanisms are incompletely understood. Although valve-related hemodynamics may play a role, there is evidence suggesting that genetic abnormalities underlie both the development of the BAV and the associated aortic degeneration. In this study, we showed that alterations in eNOS expression and concentration occur independent of pathological dilation of the BAV aorta. Furthermore, eNOS expression remains unchanged throughout the BAV aortic wall, including at regions that are susceptible to changes in shear stress. These findings emphasize a genetic component as the underlying cause of aortic dilation in patients with a BAV.

In support of the hemodynamics theory for aortic dilation in patients with a BAV, several studies have provided evidence for regional differences in the degree of pathological remodeling in BAV aortas. For instance, the expression of matrix metalloproteinases²⁹ and senescence markers²⁰ were increased in regions susceptible to increased wall shear stress in BAV aortas. However, these parameters were not regionally assessed in the aorta of patients with a TAV, so whether these findings are BAV specific remains unknown. Furthermore, regional differences were observed in extracellular matrix protein expression in BAV aortas, but similar findings were observed in the aorta of patients with Marfan syndrome with a TAV,³⁰ suggesting that these findings may not be exclusive to BAV aortas. These results led us to question whether hemodynamic alterations underlie aortic dilation in patients with a BAV or whether they simply exacerbate genetically influenced aortic remodeling.

Although dysregulated eNOS has been implicated in BAV aortopathy, the specific role of eNOS in aortic dilation is unclear. This could be because most groups have studied only mRNA expression or protein concentration of eNOS in the aorta, which yielded inconsistent findings. For example, studies by Henn et al²³ and Kotlarczyk et al³¹ showed increased eNOS mRNA expression and activation in BAV aortas compared with TAV aortas. Conversely, studies

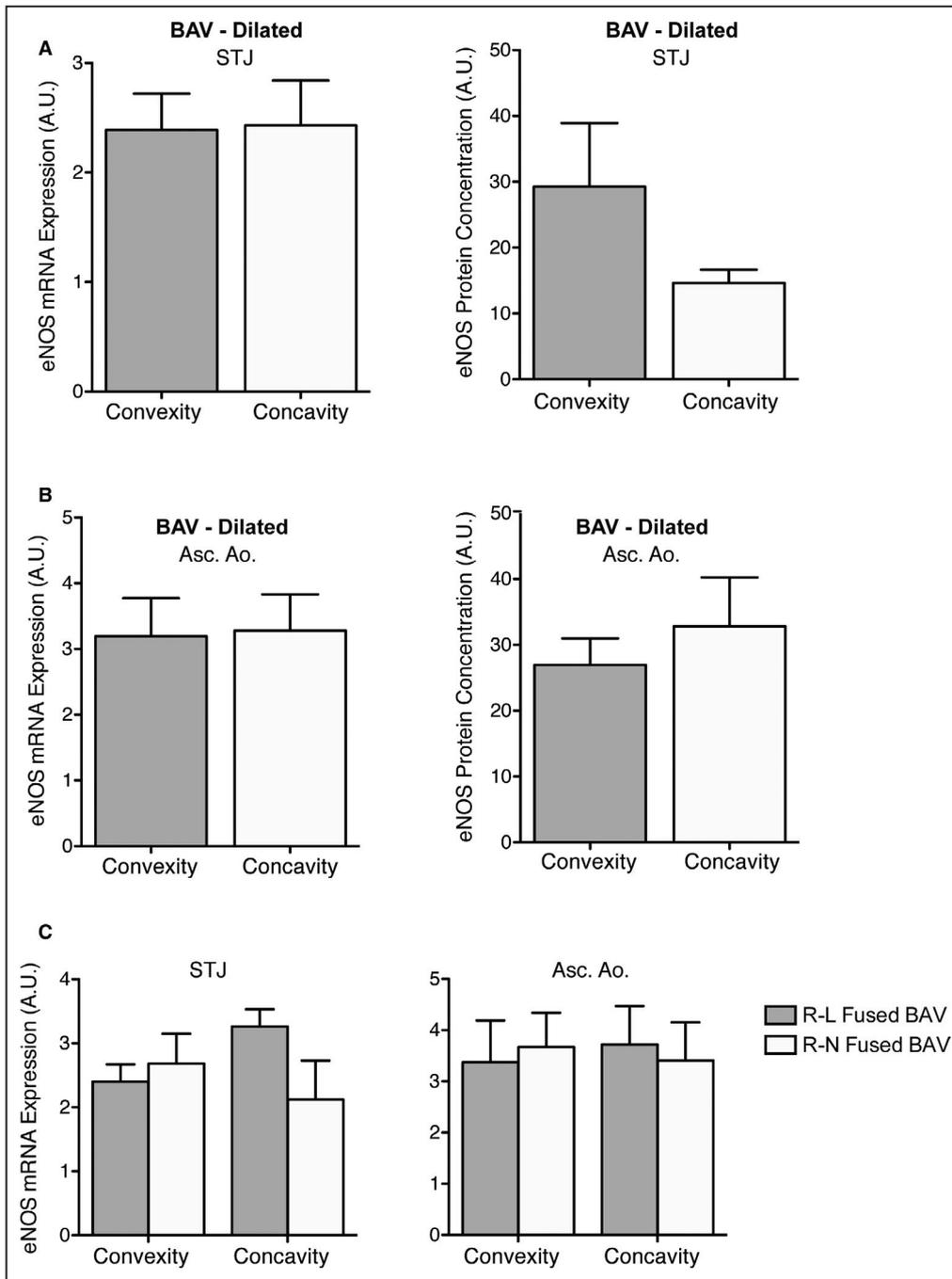


Figure 6. There are no regional differences in endothelial nitric oxide synthase (eNOS) expression in the aortic wall in dilated bicuspid aortic valve (BAV) aortas.

A-B, Graphs depicting the eNOS mRNA expression (left) and protein expression (right) in dilated aortas from patients with a BAV. eNOS expression is compared between the convex and the concave wall of the aorta at the level of **(A)** the sinotubular junction (STJ) and **(B)** the mid-ascending aorta. **C,** Graphs depicting eNOS mRNA expression between BAV aortas with right and left coronary cusp (R-L) fusions and right noncoronary cusp (R-N) fusions at the convexity and the concavity from the sinotubular junction (STJ) and the mid-ascending aorta (Asc. Ao.) in BAV aortas. * = significance at $P < 0.05$; AU indicates arbitrary units. Data are presented as mean (bars) \pm SEM (error bars).

by Mohamed et al²⁴ and Aicher et al²² showed a decrease in eNOS protein concentrations in dilated BAV aortas compared with dilated TAV aortas. In light of these inconsistent findings, we chose to study both

the mRNA expression and the protein concentration of eNOS in the aorta.

In this study, we observed a significant decrease in both eNOS mRNA expression and protein concentration

in nondilated BAV aortas compared with normal TAV aortas. This implies that dysregulation of eNOS occurs independent of pathological dilation in BAV aortas. Reduced eNOS expression may have implications in aortic degeneration, as reduced eNOS was shown in association with increased matrix metalloproteinase 2 and decreased collagen content in buckled arteries.³² Interestingly, this difference in eNOS concentration between patient groups disappears in the case of aortic dilation, as eNOS protein levels increase in dilated BAV aortas compared with nondilated BAV aortas. This could indicate a secondary role for aortic remodeling in further influencing eNOS regulation in BAV aortas. This finding is inconsistent with the results by Aicher et al,²² which showed that eNOS levels are negatively correlated with aortic diameter in patients with a BAV. However, there were only 5 patients with a BAV with a dilated aorta included in this study, and 80% of these patients had eNOS levels above the trendline in the correlation analysis, effectively implying increased eNOS levels in the case of dilation. Taken together, our results reveal evidence for a relationship between eNOS dysregulation and the initiation of aortic dilation in patients with a BAV that may be further influenced by aortic remodeling.

Studies have shown that eNOS expression is regulated by wall shear stress.²⁸ Therefore, if aortic dilation in patients with a BAV is primarily attributable to altered hemodynamics, we would expect the most pronounced changes in eNOS expression to occur at regions of altered shear stress. Although prior studies have shown regional differences in wall shear stress in the aorta of patients with a BAV,^{9,25} which is further influenced by BAV fusion type,^{26,27} we did not detect any corresponding regional differences in eNOS mRNA expression or protein concentration in BAV aortas, independent of cusp fusion pattern. These results are in contrast to findings in other diseases impacting the aorta, including atherosclerosis, where eNOS mRNA expression and protein concentration are reduced in atherosclerotic-susceptible regions of increased wall shear stress in mouse and human arteries.^{33,34} Therefore, it is plausible that dysregulated eNOS expression in BAV aortas occurs independent of hemodynamic alterations, and is likely attributable to underlying genetic factors that override flow-induced expression patterns.

As expected, we did not detect any regional differences in eNOS expression in normal TAV aortas. Interestingly however, eNOS mRNA expression was significantly decreased at the convexity of TAV dilated aortas. There is limited data on hemodynamic alterations associated with dilated TAV aortas, but an abnormal helical flow pattern was observed in a group of patients with a TAV with a dilated ascending aorta.¹⁰ Although this helical flow pattern does not appear to preferentially direct flow toward the convexity of the aorta, it provides

evidence for aneurysm-associated hemodynamic alterations that are not dependent on the presence of a BAV. In fact, helical flow patterns in the ascending aorta may be linked to pressure changes across the left ventricular outflow tract, which occur when the geometry between the left ventricular outflow tract and the ascending aorta is distorted.³⁵ This angular change between the left ventricular outflow tract and the aorta can occur with diseases of the aortic valve,³⁶ but also as a result of ascending aortic elongation, which occurs in response to aging.³⁷ Interestingly, a recent study showed that aortic elongation is associated with an increased prevalence of aortic adverse events,³⁸ most likely linked to the observation that aortic dilatation concurs with elongation. Further work is needed to define clearly if the mechanisms of aortic elongation are identical to those of aortic dilatation, and if aortic elongation contributes to the regional differences in eNOS transcript expression observed in TAV-associated aneurysms.

In summary, the current work confirms the involvement of dysregulated eNOS in BAV-associated aortopathy. However, since eNOS expression is not regionally variable in BAV aortas, it is likely that dysregulated eNOS in BAV aortopathy is the result of genetic factors associated with the disease, rather than changes stimulated by hemodynamic alterations. Furthermore, the interesting finding of regionally variable eNOS transcript expression in TAV-associated dilated aortas prompts further research into the possible relationship between ascending aortic elongation, associated hemodynamic alterations, and aortic wall remodeling, regardless of disease background.

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Disclosures

None.

REFERENCES

1. Fedak PW, Verma S, David TE, Leask RL, Weisel RD, Butany J. Clinical and pathophysiological implications of a bicuspid aortic valve. *Circulation*. 2002;106:900–904.

2. Siu SC, Silversides CK. Bicuspid aortic valve disease. *J Am Coll Cardiol*. 2010;55:2789–2800.
3. Basso C, Boschello M, Perrone C, Mecenero A, Cera A, Bicego D, Thiene G, De Dominicis E. An echocardiographic survey of primary school children for bicuspid aortic valve. *Am J Cardiol*. 2004;93:661–663.
4. Carro A, Teixido-Tura G, Evangelista A. Aortic dilatation in bicuspid aortic valve disease. *Rev Esp Cardiol (Engl Ed)*. 2012;65:977–981.
5. Girdauskas E, Borger MA, Secknus MA, Girdauskas G, Kuntze T. Is aortopathy in bicuspid aortic valve disease a congenital defect or a result of abnormal hemodynamics? A critical reappraisal of a one-sided argument. *Europ J Cardio-Thoracic Surg*. 2011;39:809–814.
6. Michelena HI, Desjardins VA, Avierinos JF, Russo A, Nkomo VT, Sundt TM, Pellikka PA, Tajik AJ, Enriquez-Sarano M. Natural history of asymptomatic patients with normally functioning or minimally dysfunctional bicuspid aortic valve in the community. *Circulation*. 2008;117:2776–2784.
7. Larson EW, Edwards WD. Risk factors for aortic dissection: a necropsy study of 161 cases. *Am J Cardiol*. 1984;53:849–855.
8. Tadros TM, Klein MD, Shapira OM. Ascending aortic dilatation associated with bicuspid aortic valve: pathophysiology, molecular biology, and clinical implications. *Circulation*. 2009;119:880–890.
9. Barker AJ, Markl M, Burk J, Lorenz R, Bock J, Bauer S, Schulz-Menger J, von Knobelsdorff-Brenkenhoff F. Bicuspid aortic valve is associated with altered wall shear stress in the ascending aorta. *Circ Cardiovasc Imaging*. 2012;5:457–466.
10. Hope MD, Hope TA, Meadows AK, Ordovas KG, Urbani TH, Alley MT, Higgins CB. Bicuspid aortic valve: four-dimensional MR evaluation of ascending aortic systolic flow patterns. *Radiology*. 2010;255:53–61.
11. Robicsek F. Aortic media in bicuspid valve disease. *Ann Thoracic Surg*. 2003;76(1):337–338.
12. Andrus BW, O'Rourke DJ, Dacey LJ, Palac RT. Stability of ascending aortic dilatation following aortic valve replacement. *Circulation*. 2003;108(9):2951–2959.
13. Borger MA, Preston M, Ivanov J, Fedak PW, Davierwala P, Armstrong S, David TE. Should the ascending aorta be replaced more frequently in patients with bicuspid aortic valve disease? *J Thoracic Cardio Surg*. 2004;128:677–683.
14. Bartram U, Molin DG, Wisse LJ, Mohamad A, Sanford LP, Doetschman T, Speer CP, Poelmann RE, Gittenberger-de Groot AC. Double-outlet right ventricle and overriding tricuspid valve reflect disturbances of looping, myocardialization, endocardial cushion differentiation, and apoptosis in TGF-beta(2)-knockout mice. *Circulation*. 2001;103:2745–2752.
15. Bauer M, Pasic M, Meyer R, Goetze N, Bauer U, Siniawski H, Hetzer R. Morphometric analysis of aortic media in patients with bicuspid and tricuspid aortic valve. *Ann Thorac Surg*. 2002;74:58–62.
16. de Sa M, Moshkovitz Y, Butany J, David TE. Histologic abnormalities of the ascending aorta and pulmonary trunk in patients with bicuspid aortic valve disease: clinical relevance to the Ross procedure. *J Thoracic Cardio Surg*. 1999;118:588–594.
17. Mohamed SA, Misfeld M, Hanke T, Charitos EI, Bullerdiek J, Belge G, Kuehnell W, Sievers HH. Inhibition of caspase-3 differentially affects vascular smooth muscle cell apoptosis in the concave versus convex aortic sites in ascending aneurysms with a bicuspid aortic valve. *Ann Anat*. 2010;192:145–150.
18. Nataatmadja M, West M, West J, Summers K, Walker P, Nagata M, Watanabe T. Abnormal extracellular matrix protein transport associated with increased apoptosis of vascular smooth muscle cells in Marfan syndrome and bicuspid aortic valve thoracic aortic aneurysm. *Circulation*. 2003;108(9):3291–334.
19. Razavi HM, Hamilton JA, Feng Q. Modulation of apoptosis by nitric oxide: implications in myocardial ischemia and heart failure. *Pharmacol Ther*. 2005;106:147–162.
20. Balint B, Yin H, Nong Z, Arpino JM, O'Neil C, Rogers SR, Randhawa VK, Fox SA, Chevalier J, Lee JJ, et al. Seno-destructive smooth muscle cells in the ascending aorta of patients with bicuspid aortic valve disease. *EBioMedicine*. 2019;43:54–66.
21. Lee TC, Zhao YD, Courtman DW, Stewart DJ. Abnormal aortic valve development in mice lacking endothelial nitric oxide synthase. *Circulation*. 2000;101:2345–2348.
22. Aicher D, Urbich C, Zeiher A, Dimmeler S, Schafers HJ. Endothelial nitric oxide synthase in bicuspid aortic valve disease. *Ann Thoracic Surg*. 2007;83:1290–1294.
23. Henn D, Perttunen H, Gauer S, Schmied W, Porras C, Such M, Schafers HJ. GATA5 and endothelial nitric oxide synthase expression in the ascending aorta is related to aortic size and valve morphology. *Ann Thoracic Surg*. 2014;97:2019–2025.
24. Mohamed SA, Radtke A, Saraei R, Bullerdiek J, Sorani H, Nimzyk R, Karluss A, Sievers HH, Belge G. Locally different endothelial nitric oxide synthase protein levels in ascending aortic aneurysms of bicuspid and tricuspid aortic valve. *Cardiol Res Pract*. 2012;2012:165957.
25. Barker AJ, Lanning C, Shandas R. Quantification of hemodynamic wall shear stress in patients with bicuspid aortic valve using phase-contrast MRI. *Ann Biomed Eng*. 2010;38:788–800.
26. Mahadevia R, Barker AJ, Schnell S, Entezari P, Kansal P, Fedak PW, Malaisrie SC, McCarthy P, Collins J, Carr J, et al. Bicuspid aortic cusp fusion morphology alters aortic three-dimensional outflow patterns, wall shear stress, and expression of aortopathy. *Circulation*. 2014;129:673–682.
27. Rodriguez-Palomares JF, Dux-Santoy L, Guala A, Kale R, Maldonado G, Teixido-Tura G, Galian L, Huguet M, Valente F, et al. Aortic flow patterns and wall shear stress maps by 4D-flow cardiovascular magnetic resonance in the assessment of aortic dilatation in bicuspid aortic valve disease. *J Cardiovasc Magn Reson*. 2018;20:28.
28. Ranjan V, Xiao Z, Diamond SL. Constitutive NOS expression in cultured endothelial cells is elevated by fluid shear stress. *Am J Physiol*. 1995;269:H550–H555.
29. Guzzardi DG, Barker AJ, van Ooij P, Malaisrie SC, Puthumana JJ, Belke DD, Mewhort HE, Svystonyuk DA, Kang S, Verma S, et al. Valve-related hemodynamics mediate human bicuspid aortopathy: Insights from wall shear stress mapping. *J Am Coll Cardiol*. 2015;66:892–900.
30. Della Corte A, De Santo LS, Montagnani S, Quarto C, Romano G, Amarelli C, Scardone M, De Feo M, Cotrufo M, Caianiello G. Spatial patterns of matrix protein expression in dilated ascending aorta with aortic regurgitation: congenital bicuspid valve versus Marfan's syndrome. *J Heart Valve Dis*. 2006;15:20–27;discussion 27.
31. Kotlarczyk MP, Billaud M, Green BR, Hill JC, Shiva S, Kelley EE, Phillippi JA, Gleason TG. Regional disruptions in endothelial nitric oxide pathway associated with bicuspid aortic valve. *Ann Thoracic Surg*. 2016;102:1274–1281.
32. Xiao Y, Liu Q, Han HC. Buckling reduces eNOS production and stimulates extracellular matrix remodeling in arteries in organ culture. *Ann Biomed Eng*. 2016;44:2840–2850.
33. Won D, Zhu SN, Chen M, Teichert AM, Fish JE, Matouk CC, Bonert M, Ojha M, Marsden PA, Cybulsky MI. Relative reduction of endothelial nitric-oxide synthase expression and transcription in atherosclerosis-prone regions of the mouse aorta and in an in vitro model of disturbed flow. *Am J Pathol*. 2007;171:1691–1704.
34. Oemar BS, Tschudi MR, Godoy N, Brovkovich V, Malinski T, Luscher TF. Reduced endothelial nitric oxide synthase expression and production in human atherosclerosis. *Circulation*. 1998;97:2494–2498.
35. Allen BD, Choudhury L, Barker AJ, van Ooij P, Collins JD, Bonow RO, Carr JC, Markl M. Three-dimensional haemodynamics in patients with obstructive and non-obstructive hypertrophic cardiomyopathy assessed by cardiac magnetic resonance. *Eur Heart J Cardiovasc Imaging*. 2015;16:29–36.
36. Aboulhosn J, Child JS. Left ventricular outflow obstruction: subaortic stenosis, bicuspid aortic valve, supraaortic stenosis, and coarctation of the aorta. *Circulation*. 2006;114:2412–2422.
37. Adriaans BP, Heuts S, Gerretsen S, Cheriex EC, Vos R, Natour E, Maessen JG, Sardari Nia P, Crijns H, et al. Aortic elongation part I: the normal aortic ageing process. *Heart*. 2018;104:1772–1777.
38. Wu J, Zafar MA, Li Y, Saeyeldin A, Huang Y, Zhao R, Qiu J, Tanweer M, Abdelbaky M, Gryaznov A, et al. Ascending aortic length and risk of aortic adverse events: the neglected dimension. *J Am Coll Cardiol*. 2019;74:1883–1894.