Featured Article

Elastin integrity in bicuspid valve-associated aortopathy is associated with altered biomechanical properties and influenced by age

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Background: Aortic wall remodelling in bicuspid aortic valve (BAV) patients is heterogeneous and characterized by elastin fiber breakdown alongside impaired biomechanics. However, the relationship between aortic histopathological changes and biomechanics are incompletely understood. We clarify the influence of elastin fiber integrity on ex vivo aortic wall mechanical properties in BAV patients, and explore the influence of patient age.

Methods: Aortic tissue samples (N=66) from 19 BAV patients undergoing prophylactic ascending aortic resection surgery were analyzed. Semi-quantitative histopathological analysis was conducted to assess elastin fiber integrity including elastin content and elastic fiber fragmentation. *Ex vivo* biaxial mechanical testing generated stress-strain curves from which physiological [low-strain tangential modulus (LTM), transition zone onset stress (TZo)] and supraphysiological [transition zone end stress (TZe) and high-strain tangential modulus (HTM)] mechanical properties were obtained. Relationships between histopathology and mechanical properties were determined using a linear mixed effect model. BAV patients were subdivided according to 'younger' and 'older' age groups (i.e., 51–60 and 61–70 years old, respectively).

Results: No statistically significant differences in elastin content were observed between younger and older BAV patients. Older patients showed greater elastin fiber fragmentation compared to their younger cohort (74% versus 61%). Elastin fiber histopathology was associated with differences in physiological mechanical properties: elastin fragmentation corresponded with lower LTM (P=0.005) and TZo (P=0.044) in younger BAV patients and higher LTM (P=0.049) and TZo (P=0.001) in older BAV patients. Histopathology changes were significantly associated with supraphysiological mechanical properties only in older BAV patients: decreased elastin integrity was associated with increased TZe (P=0.049) and HTM (P<0.001).

Conclusions: Elastin histopathologic changes in BAV aortopathy correspond with differences in mechanical properties and this relationship is influenced by patient age. These novel findings provide additional mechanistic insights into aortic wall remodeling and support a more nuanced stratification of BAV patients by age.

Keywords: Bicuspid aortic valve (BAV); bicuspid; aortopathy; aneurysm; biomechanics

Submitted Jan 05, 2022. Accepted for publication Jun 07, 2022.
doi: 10.21037/acs-2022-bav-17
View this article at: https://dx.doi.org/10.21037/acs-2022-bav-17

Introduction

Bicuspid aortic valve (BAV) disease is the most common congenital heart defect and affects one in every 50 persons (1). Progressive weakening of the ascending aorta in these patients (i.e., aortopathy), places them at an increased risk of aortic dissection or rupture (2). The mechanisms underlying this aortopathy are unclear. Abnormal histopathology within the BAV aortic wall is a well-accepted macroscopic manifestation of microscopic extracellular matrix changes mediated by cellular proteins including matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs) (1,3). Our group and others previously showed that valve-mediated hemodynamics influence the expression of aortic remodeling in BAV-associated aortopathy (4-10), and that regional differences in hemodynamics predict both the regional expression of histopathological disease within these aortas as well as degree of elastin fiber derangement—a key mediator of aortic wall mechanical properties (8,9,11).

In a small subset of patients, we found that greater elastin dysfunction corresponded to increased stiffness, a mechanical metric that has been previously linked to aortic disease (9). However, this was a limited pilot study. The influence of histopathological derangement of elastin fibers and their consequences on aortic wall biomechanics in BAV-associated aortopathy remain unclear. Furthermore, the influence of age in histopathological and biomechanical studies of BAV patients are poorly defined.

Given that aortic diameter alone is an inadequate criterion to guide surgical management of these patients (12-14), understanding the mechanical properties of the BAV aorta will help further clarify how to best address this cardiovascular pathology.

The present study sought to compare the relationship of elastin fiber integrity with *ex vivo* mechanical properties representing both physiological and supraphysiological mechanical loading of ascending aortic tissue from patients with BAV aortopathy in the 6th and 7th decades of life (51–70 years).

Methods

Patients

Nineteen patients undergoing elective prophylactic ascending aortic resection at the Foothills Medical Centre (Calgary, Alberta, Canada) and Northwestern Memorial Hospital (Chicago, IL, USA) between April 2016 and January 2020 were enrolled in this study. All BAV patients were greater than 18 years of age and had no prior history of aortic surgery, dissection, rupture, or connective tissue disorder. This study was conducted with the approval of the of the Conjoint Faculties Research Ethics Board at the University of Calgary (REB14-0084) and the Northwestern University Institutional review board (STU00204434) with written informed consent obtained from all participants. Demographic data such as age, sex, aneurysm size, and history of hypertension was also collected. The degree of stenosis and regurgitation were determined through continuous-wave Doppler ultrasound and graded based on clinical guidelines (15).

Data collection

Tissue samples were collected as permitted by the extent of the ascending aorta resection. The resected tissue was subdivided into circumferential regions: anterior, greater curvature, posterior, and lesser curvature. These regions were further divided into *ex vivo* mechanical testing specimens (12 mm × 12 mm square) with the circumferential and axial orientations noted. The histology samples were collected from the *ex vivo* specimens after mechanical testing. Specimens were flash frozen in VWR tissue freezing compound and stored at −80 ℃ until testing. When the circumferential region was too small for mechanical specimen to be obtained, the specimen was excluded from the study.

The mechanical testing of the tissue was performed as previously described (16). Specimens were subject to planar biaxial testing (ElectroForce System, TA Instruments, Springfield MO, with two 22N load cells). Specimens

were pre-loaded to 0.05N then subjected to a series of displacement-controlled testing protocols (1:1, 1:0.5, 0.5:1; where '1' indicates a maximum of 60% displacement based on the specimen's edge length). Tissue deformation was tracked through five dots placed in the central region of the sample; force was recorded by the testing device in the two directions. Mechanical behaviour was determined from the deformation gradient under the assumption of tissue homogeneity and incompressibility. Measures were taken in the testing set up and post-processing to ensure shear stress was low enough to be considered negligible. Cauchy stress and stretch were determined via the deformation gradient from the local dot tracking, the measured forces, and the dimensions of the specimens for each protocol. For better comparison across specimens, each of the resulting stress-strain relationships from the same specimen were fitted to surface representing the mechanical behaviour of that specimen (16). From this surface, the true equi-stretch (1:1 stretch ratio) mechanical behaviour was obtained. Four mechanical parameters were determined from the equi-stretch behaviour. A tangential modulus, similar to a stiffness measure, was determined for each linear region of the behaviour. A low-strain tangential modulus (LTM) was determined from the initial linear region. If a second linear region was present after the transition zone, a high-strain tangential modulus (HTM) was calculated. For specimens displaying a non-linear transition region, the onset and end stresses (TZo and TZe, respectively) were recorded. For the present study, LTM and TZo were defined as metrics of physiological loading conditions, while TZe and HTM were those of supraphysiological loading conditions.

For histological analysis, tissue was formalin-fixed and paraffin-embedded prior to 5 µm sectioning. Musto-Movat pentachrome staining was performed, and entire tissue sections digitized with Aperio ImageScope digital scanning (Leica Biosystems, Aperio ImageScope, Version 12.4.3.5008) for colorimetric analysis of the aortic media elastin. Each specimen was also graded by a pathologist for level of elastin fragmentation in accordance with the consensus statement for evaluation of non-inflammatory aortopathy from the Society for Cardiovascular Pathology (17).

Statistical analysis

A custom statistical analysis Python software pipeline (Python Software Foundation, https://www.python.org/) was developed using the SciPy, Pingouin, and Statsmodel packages (18-21). Descriptive statistics were presented as mean ± standard deviation. The significance threshold was set to 0.05 for all tests. A Shapiro-Wilk test was implemented to assess the normality of each continuous variable. For non-normally distributed data, a Box-Cox transformation was applied to ensure normality. To assess the equivalence of variance among the mechanical properties and elastin fragmentation a Levene test was performed. To determine statistically significant relationships among the mechanical properties and the elastin quantities, a linear mixed effect model was used with inter-patient variability set as a random effect. The normality and heteroscedasticity of the residuals of the models were assessed through the Shapiro-Wilk and the White test respectively. P values are presented along with the Marginal Coefficient of Determination which is the ratio of variance explained by the explanatory variable as comparted to the total variance in the data. The graphs presented in this study were created using the Plotnine package. Relationships between the mechanical properties and elastin were examined with respect to the total population and in age categories based on decades (i.e., 51–60 and 61–70 years old). Mechanical and elastin properties were also correlated with diameter size via a Spearman correlation due to the non-linearity of the diameter data even after a Box-Cox transformation

Results

Patient characteristics

A total of 66 aortic tissue samples were collected from 19 patients: 10 patients were categorized as the younger population group (i.e., 51–60 years at the time of aortic resection) and nine were categorized as the older population group (i.e., 61–70 years). Patient demographics and preoperative characteristics are summarized in *Table 1*. There were significant differences in mid ascending aneurysm size between the younger and older population (P=0.034) with the younger age group having a larger diameter, however there was no significant difference between the age groups for the Sinus of Valsalva. There were also no significant differences between mechanical properties or elastin properties and diameter size within each age group.

Age group differences

A comparison between each elastin and mechanical property

Table 1 Patient characteristics

Clinical characteristics	Age group 51–60 years (N=10 patients)	Age group 61–70 years (N=9 patients)
Age (years) ± SD	56±2	64±3
Female, n [%]	1 [10]	0 [0]
BAV classification, n [%]		
Type 1, RN	2 [20]	3 [33]
Type 1, RL	5 [50]	5 [55]
Type 2, RL/RN	2 [20]	0 [0]
Other	1 [10]	1 [11]
Aortic valve function, n [%]		
AS: none/mild/moderate-severe	2 [20]/1 [10]/7 [70]	1 [11]/3 [33]/5 [55]
AR: none/mild/moderate-severe	2 [20]/3 [30]/5 [50]	4 [44]/3 [33]/2 [22]
Ascending aorta diameter (cm), mean ± SD		
Sinus of Valsalva	4.1±0.5	4.6±0.4
Mid ascending aorta	5.1±0.2	4.7±0.4
Cardiovascular risk factors, n [%]		
Hypertension	2 [20]	2 [22]
Diabetes	0 [0]	1 [11]
Surgical procedure: aortic valve, n [%]		
Repair	0 [0]	0 [0]
Replacement	9 [90]	9 [100]
None	1 [10]	0 [0]
Surgical procedure: ascending aorta, n [%]		
Ascending aorta replacement	7 [70]	4 [44]
Root replacement	0 [0]	0 [0]
Hemi-arch	3 [30]	5 [56]

Data presented as mean ± SD or number [%]. Comparison between age groups (Student *t*-test) indicated with P values. SD, standard deviation; BAV, bicuspid aortic valve; RN, right-noncoronary cusp fusion; RL, right-left coronary cusp fusion; AS, aortic stenosis; AR, aortic regurgitation.

between the two age groups can be found in *Table 2*. While all the mechanical properties were greater for the older age group, no statistically significant differences between the older and younger BAV patients were appreciated. Similarly, no statistically significant difference in elastin content was noted between age groups, although semi-quantitative scoring of elastin fragmentation demonstrated that a greater proportion of the older BAV patients exhibited mild or moderate disease compared to their younger counterparts (*Table 2*).

Elastin and physiological mechanics

No statistically significant relationships between the elastin content in all BAV patients and LTM and TZo were noted, but significant differences emerged upon the stratification by young and older age groups (*Figure 1*). In the younger BAV population, LTM and elastin content were positively correlated such that greater elastin content corresponded with higher LTM (*Figure 1A*; P=0.032, marginal R^2=0.694). In contrast, this correlation was negative for the older BAV

Table 2 *Ex vivo* biaxial mechanical testing properties of BAV aortic wall tissue

Aortic wall tissue characteristic	Age group 51–60 years (N=36 samples)	Age group 61–70 years (N=30 samples)	P value
Histopathology			
Elastin content (%), mean ± SD	35±6	33±6	0.780
Elastin fragmentation score, n [%]			
Absent	14 [39]	7 [22]	–
Mild	19 [53]	19 [61]	–
Moderate	3 [8]	4 [13]	–
Circumferential biomechanics (kPa), mean ± SD			
LTM	429±105	447±137	0.670
TZo	31±9	40±20	0.193
TZe	93±31	106±37	0.420
HTM	1,261±734	1,604±898	0.390
Axial biomechanics (kPa), mean ± SD			
LTM	342±112	356±108	0.520
TZo	24±8	34±16	0.075
TZe	67±28	82±29	0.120
HTM	956±781	1,378±790	0.058

Data presented as mean ± SD or number [%] for aortic wall tissue samples. Comparison between age groups (Student *t*-test) indicated with P values. SD, standard deviation; LTM, low strain modulus; TZo, transition zone onset stress; TZe, transition zone end stress; HTM, high strain modulus.

population where greater elastin content corresponded with a lower LTM (*Figure 1B*; P=0.037, marginal R^2=0.95). Both populations also demonstrated significant associations between circumferential LTM and elastin fragmentation; younger BAV patients exhibited lower LTM where fragmentation was present compared to those without fragmentation (*Figure 1C*; P=0.005, absent to mild), whereas older BAV patients demonstrated lower LTM in samples without fragmentation (*Figure 1D*; P=0.049, absent to mild). For the axial direction, only the older population showed a significant relationship between LTM and elastin content; LTM was significantly higher when elastin fragmentation was present (P=0.047, absent to mild). Circumferential TZo was associated with elastin fragmentation in both BAV age groups. Younger BAV patients demonstrated lower TZo alongside increased elastin fragmentation (*Figure 1E*; P=0.044, absent to mild), while older BAV patients exhibited increased TZo with increased fragmentation (*Figure 1F*; P=0.001, absent to mild; P=0.049, absent to moderate).

Elastin and supraphysiological mechanics

Statistically significant relationships between measures of elastin integrity and supraphysiological mechanical properties were only appreciated in the older BAV patients (*Figure 2*). Elastin content inversely correlated with circumferential TZe (*Figure 2B*, P=0.049, marginal R^2=0.98), and worsened elastin fragmentation corresponded with increased circumferential HTM (*Figure 2D*, P<0.001 absent to mild, P=0.001 absent to moderate). No statistically significant associations were observed between histopathology and axial supraphysiological mechanical properties.

Discussion

BAV aortopathy is characterized by elastin fiber degeneration within the aortic wall, as well as aberrant proximal aortic biomechanics (3,22,23). However, the relationship

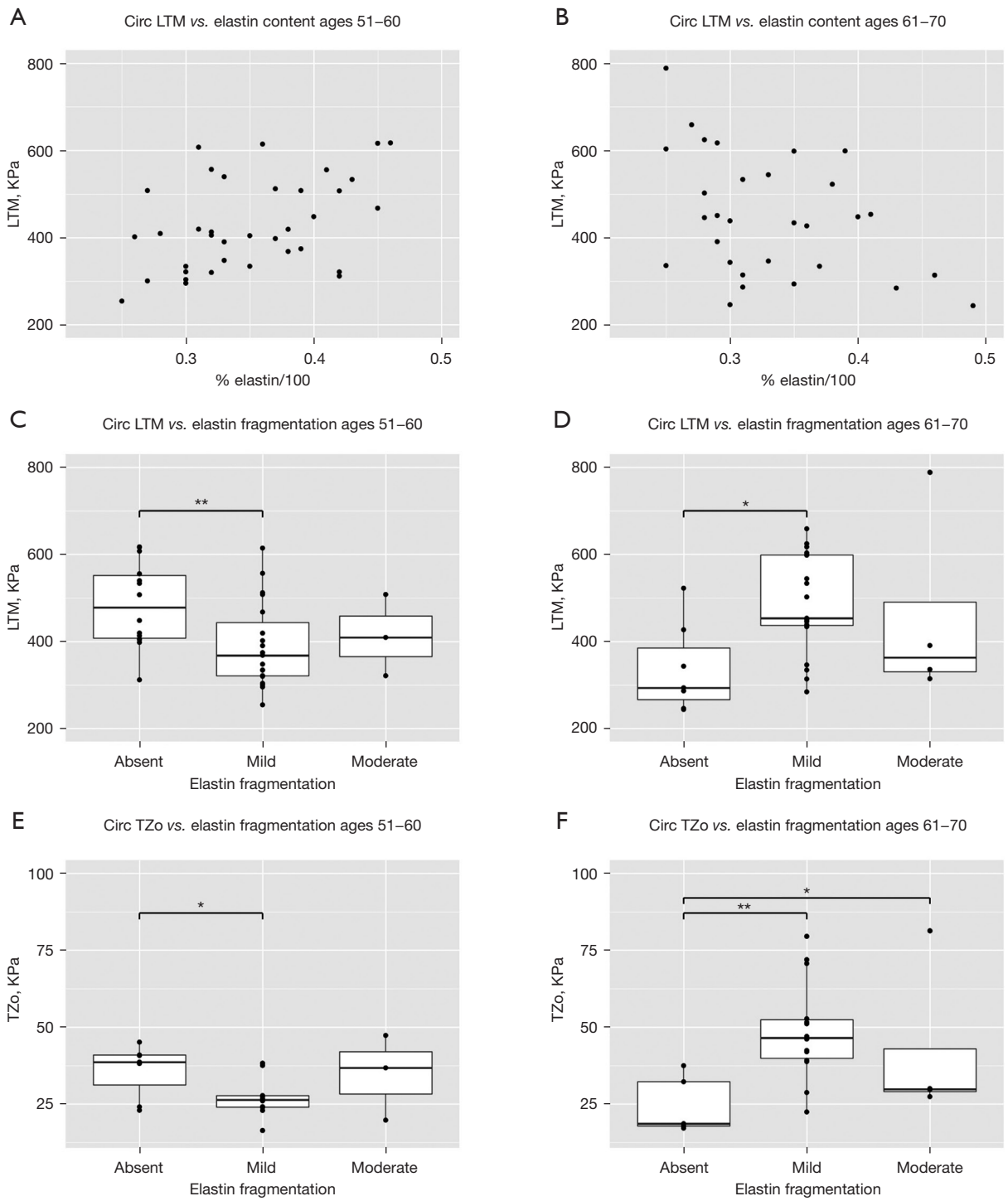

Figure 1 Elastin integrity and *ex vivo* circumferential physiological mechanical properties. Scatter plots of elastin content and LTM for ages 51–60 years (A) and 61–70 years (B). Elastin fragmentation semi-quantitative score and LTM (C,D) or TZo (E,F). Horizontal lines indicated mean values. Dots denote aortic tissue samples. LTM, low-strain tangential moduli; TZo, transition zone onset stress. *, P<0.05; **, P<0.01.

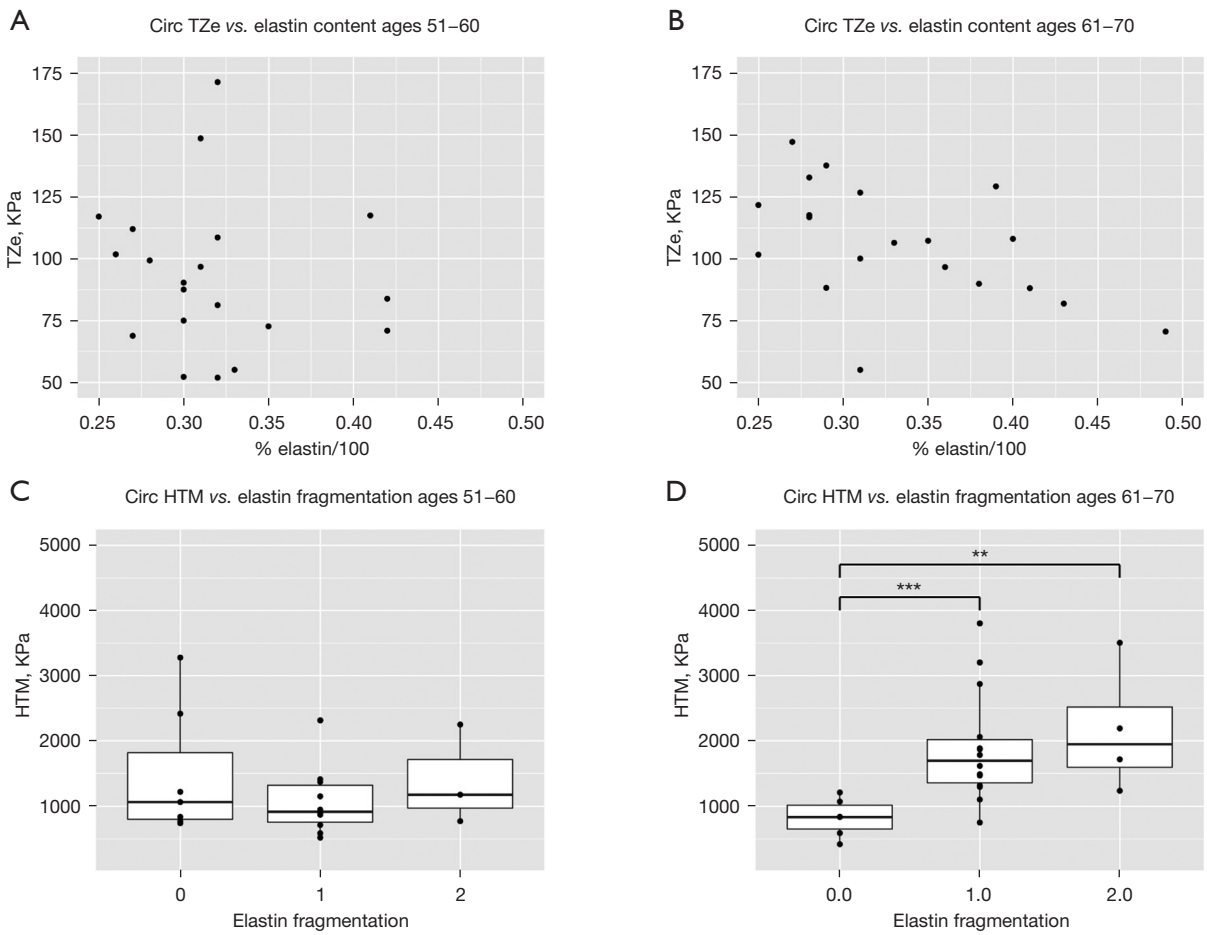

Figure 2 Elastin integrity and *ex vivo* circumferential supraphysiological mechanical properties. Scatter plots of elastin content and TZe for ages 51–60 years (A) and 61–70 years (B). Elastin fragmentation semi-quantitative score and HTM (C,D). Horizontal lines indicated mean values. Dots denote aortic tissue samples. TZe, transition zone end stress; HTM, high-strain tangential moduli. **, P<0.01; ***, P<0.001.

between the two, especially with respect to age remain incompletely defined. Most tissue studies group all BAV patients together despite studies documenting the need for more nuanced stratification of this heterogeneous patient population. Using 66 tissue samples from 19 BAV patients, we compared the relationship between elastin fiber integrity and *ex vivo* biomechanical properties from patients undergoing ascending aortic surgery. We report that: (I) abnormal elastin histopathology corresponds with altered biomechanics in the proximal BAV aorta, and; (II) this relationship differs by age (i.e., 51–60 years old versus 61–70 years old) and with respect to physiological and supraphysiological mechanical properties.

Heterogeneous aortic wall remodeling is well-documented in the BAV aorta (5-10). Compared to tricuspid aortic valve (TAV), histopathological studies support a unique remodeling process in the BAV aortic wall where heterogeneous expression of disease corresponds with both the cusp fusion pattern as well as valve-mediated hemodynamics (4,6-9,11). In the present study, we similarly found variable elastin fragmentation in the BAV aortic wall and build on previous findings by documenting differences in elastin integrity between BAV patients in their sixth and seventh decades of life (i.e., "younger" and "older" patients, respectively). These changes in elastin are in turn associated with differences in *ex vivo* aortic wall mechanical properties. These properties are primarily dictated by elastin and collagen fibers with the former governing mechanical behaviour in a low strain region (characterized by LTM) prior to collagen recruitment (TZo), and facilitates large

deformations under physiological loading conditions. In contrast, collagen bears higher loads at supraphysiological conditions and is only engaged when a critical threshold of strain is reached (24). This results in a non-linear transition region leading to fully-engaged collagen fibers (TZe) and a second linear region that is predominantly collagen-mediated (HTM).

In the present study with respect to physiological material properties, we report that decreased elastin fiber integrity in the younger group of BAV patients was associated with a decrease in both LTM and TZo, suggestive of decreased stiffness and a lower threshold for collagen fiber recruitment for mechanical loading, respectively. By contrast, decreased elastin fiber integrity was associated with an increased LTM and TZo in the older BAV patients, consistent with increased aortic wall stiffness. Furthermore, compromised elastin integrity in older BAV patients also corresponded with supraphysiological material properties consistent with a higher threshold for complete collagen fiber engagement (i.e., elevated TZe) alongside increased stiffening (i.e., increased HTM). These divergent findings between younger and older BAV patients could potentially be attributed to the aging effects with the biomechanics of the older patients being affected by both disease progression and aging. Given the natural history of arterial aging, elastin gradually breaks down without compensatory replacement, and collagen fibers become the predominant mechanical bearing unit of the aortic wall (23,25). The result is an overall stiffening of arteries that is itself an independent predictor of aortic dilatation and the need for aortic surgery (26-28), as well as a key driver of aortopathy pathogenesis (29-32). Our data suggest that the mechanical properties of the older patients follow this aging effect with a stiffening associated with elastin breakdown occurring in both the low-strain (LTM) and high-strain regions (HTM). Interestingly, in the younger group, elastin fiber breaks down more readily and leads to aortic wall compliance. Increased compliance has been previously associated with aortic histopathologic alterations and disease progression but has yet to be associated with any aging effects (33). Aortopathy has been considered a form of accelerated vascular aging as characterized by elastin degradation, reduction in smooth muscle cell number and contractile capacity as well as increased collagen content (34,35). However, our study suggests that the effect on the resulting biomechanics may be more complex with the interplay between aortic disease and aging. Given that the BAV aortic wall exhibits considerable regional heterogeneity in enzymatic activity and histopathology (5-10), both of which are influenced by valve-mediated hemodynamics that is itself influenced by patient age (36), our data provides a direct interrogation of the elastin integrity and mechanical properties, and build on previous work by showing that these relationships in the BAV aorta are further influenced by age, and warrant more nuanced stratification of this heterogeneous patient population.

Several limitations for this work exist. First, although a substantial number of tissue samples were analyzed, they were derived from only 19 separate patients. Future studies already underway will include a greater number of patients from a variety of age groups. Second, no comparison of the present data to TAV aortic wall in either health or disease was carried out. Future interrogations using these controls will be important to clarify whether the relationship between elastin and mechanical properties observed in this study are similar in non-BAV aortic wall. Third, given the important role of collagen in governing aortic wall biomechanics and the dynamic behaviour of the aorta, these data were limited to only elastin. Combined elastin and collagen histopathology analysis with mechanical testing is warranted. Lastly, the mechanical testing employed in this paper is limited by its *ex vivo* approach, and the interactions between the natural stresses of tissue *in vivo* as well as the living cells within the aortic wall are not accounted for in our analysis. Further validation with *in vivo* aortic mechanics is needed.

In summary, elastin disruption in the BAV aorta is associated with *ex vivo* mechanical behaviour of aortic tissue, and this relationship differs based on BAV patient age and with respect to physiological and supraphysiological mechanical properties. These novel data further our understanding of the influence of elastin fiber integrity on aortic biomechanics and support age-related considerations for future studies of BAV patients. Moreover, this work encourages nuanced assessment of BAV patients as a heterogeneous patient population. Further study is warranted to delineate how these mechanical properties can best direct the surgical management of BAV aortopathy.

Acknowledgments

Funding: This work was supported by National Institutes of Health grant (NIH HL133504 AJB, ESDM, PWMF), NSERC Discovery Programme (ESDM), an Alberta

Innovates Health Solutions MD-PhD Studentship (DGG), and by the Melman Bicuspid Aortic Valve Program, Bluhm Cardiovascular Institute (PWMF).

Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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Cite this article as: Nightingale M, Guzzardi DG, Barker AJ, Malaisrie SC, McCarthy PM, Markl M, Di Martino ES, Fedak PWM. Elastin integrity in bicuspid valve-associated aortopathy is associated with altered biomechanical properties and influenced by age. Ann Cardiothorac Surg 2022;11(4):426-435. doi: 10.21037/acs-2022-bav-17

