A FUNCTIONALLY GRADED MATERIAL MODEL FOR THE TRANSMURAL STRESS DISTRIBUTION OF THE AORTIC VALVE LEAFLET

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Abstract

Heterogeneities in structure and stress within heart valve leaflets are of significant concern to their functional physiology, as they affect how the tissue constituents remodel in response to pathological and non-pathological (e.g. exercise, pregnancy) alterations in cardiac function. Indeed, valve interstitial cells (VICs) are known to synthesize and degrade leaflet extracellular matrix (ECM) components in a manner specific to their local micromechanical environment. Quantifying local variations in ECM structure and stress is thus necessary to understand homeostatic valve maintenance as well as to develop predictive models of disease progression and post-surgical outcomes. In the aortic valve (AV), transmural variations in stress have previously been investigated by modeling the leaflet as a composite of contiguous but mechanically distinct layers. Based on previous findings about the bonded nature of these layers (*Buchanan and Sacks, BMMB, 2014*), we developed a more generalized structural constitutive model by treating the leaflet as a functionally graded material (FGM), whose properties vary continuously over the thickness. We informed the FGM model using high-resolution morphological measurements, which demonstrated that the composition and fiber structure change gradually over the thickness of the AV leaflet. For validation, we fit the model against an extensive database of whole-leaflet and individual-layer mechanical responses. The FGM model predicted large stress variations both between and within the leaflet layers at end-diastole, with low-collagen regions bearing significant radial stress. These novel results suggest that the continually varying structure of the AV leaflet has an important purpose with regard to valve function and tissue homeostasis.

1. Introduction

While the aortic valve (AV) leaflets functionally resemble thin membranes, their composition and structure in actuality vary transmurally (Sacks et al., 1998; Stella and Sacks, 2007). Specifically, they are composed of three histologically distinct layers: the fibrosa, which consists primarily of a highly aligned collagen network; the spongiosa, which is made up mostly of glycosaminoglycans (GAGs); and the ventricularis, which contains both GAGs and collagen as well as a considerable amount of elastin (Gross and Kugel, 1931; Stella and Sacks, 2007; Vesely, 1998).

Due to their unique composition and structure, each leaflet layer is thought to have distinct mechanical properties and therefore contribute differently to the overall function of the AV leaflet. Specifically, the fibrosa layer dominates the leaflet's total mechanical response, while the ventricularis contributes secondarily to the response in the radial direction (Stella and Sacks, 2007; Vesely and Noseworthy, 1992). Though it has been speculated that the spongiosa facilitates shearing between the fibrosa and ventricularis layers (Mohri et al., 1972; Vesely and Boughner, 1989), more recent work indicates that the leaflet acts as a single bonded unit, with no detectable interlayer slippage (Buchanan and Sacks, 2014; Eckert et al., 2013; Stella and Sacks, 2007).

In light of these findings, there appears to be little benefit to developing discrete-layer AV models since such approaches imply that structural and mechanical properties, and therefore stress, are homogeneous within each layer. Discrete-layer models also assume that composition and structure change instantaneously between the layers, though the validity of this assumption has never been quantitatively evaluated (Stella and Sacks, 2007; Weinberg and Kaazempur Mofrad, 2007; Zhang et al., 2016). As an improved approach, we herein model the AV leaflet tissue as a functionally graded material (FGM), where various properties change gradually over the thickness. While this notion has been exploited in the past to bioengineer scaffolds and other medical materials (Erisken et al., 2008; Pompe et al., 2003), it has generally not been adopted in the study of native tissues, perhaps due to the high-resolution data required. Quantifying continuous transmural variations in structure and stress could have a substantial impact on our understanding of heart valve mechanobiology, since the deformation and biosynthetic behavior of valve interstitial cells (VICs) have been

shown to depend on local extracellular matrix (ECM) mechanical properties (Lee et al., 2015a; Pierlot et al., 2014; Pierlot et al., 2015). In the present study, we developed a FGM structural constitutive model for the AV leaflet to infer a more realistic transmural stress distribution under physiological deformation.

2. Methods

2.1. Theoretical framework

To account for the AV's transmural variations in structure and stress, we modeled the belly region of the leaflet as a FGM whose properties are homogeneous at any transmural location z_i but can vary along the direction of thickness $\mathbf{e}_z$. The belly region was specifically chosen since it represents a large portion of the valve (Billiar and Sacks, 2000a; Sacks et al., 1998; Sacks and Yoganathan, 2007). Also, though the present methods can be applied to other areas of the leaflet if the requisite information is available. Our approach was implemented in the context of the development of hyperelastic structural constitutive models for planar soft tissues, which partition the total mechanical response into individual tissue component contributions (Fan and Sacks, 2014; Kassab and Sacks, 2016; Lanir, 1983). For the AV, these constituents are the collagen fiber, elastin, and an isotropic “matrix” component, which combines the effects of GAGs, VICs, and water. Because elastin is only relevant in low-stress regimes (Vesely, 1998), we include its contribution in the matrix phase in the present study.

As in previous soft tissue models (Fan and Sacks, 2014; Fata et al., 2014; Zhang et al., 2016), the collagen phase was modeled as a collection of undulated linear elastic (with respect to their force-displacement relation) fibers with slack stretch λ_s , that are gradually recruited with strain. We model the effective slack stretch distribution using a recruitment function D , which is beta-distributed over $\lambda_s \in [1, \lambda_{ub}]$ with mean μ_D and standard deviation σ_D :

$$\begin{aligned}
D(\lambda_s) &= \begin{cases} \frac{y^{\alpha_D-1} (1-y)^{\beta_D-1}}{B(\alpha_D, \beta_D)(\lambda_{ub}-1)} & \forall y \in [0, 1] \\ 0 & \text{otherwise} \end{cases} \\
\text{for } y &= \frac{\lambda_s - 1}{\lambda_{ub} - 1}, \hat{\mu}_D = \frac{\mu_D - 1}{\lambda_{ub} - 1}, \hat{\sigma}_D = \frac{\sigma_D}{\lambda_{ub} - 1}, \\
\alpha_D &= \frac{-\hat{\mu}_D (\hat{\sigma}_D^2 + \hat{\mu}_D^2 - \hat{\mu}_D)}{\hat{\sigma}_D^2}, \text{ and } \beta_D = \frac{(\hat{\mu}_D - 1)(\hat{\sigma}_D^2 + \hat{\mu}_D^2 - \hat{\mu}_D)}{\hat{\sigma}_D^2},
\end{aligned} \tag{1}$$

where B is the Beta function. For an ensemble of fibers with a common direction $\mathbf{n}$, the second Piola-Kirchhoff stress is

$$\mathbf{S}_{\text{ens}}(\lambda) = \frac{\eta_c}{\lambda} \int_1^\lambda \frac{D(\lambda_s)}{\lambda_s} \left(\frac{\lambda}{\lambda_s} - 1 \right) d\lambda_s, \tag{2}$$

where η_c is the collagen elastic modulus and λ is the stretch along $\mathbf{n}$.

Within the AV leaflet, we assume that all ensembles are oriented orthogonally to $\mathbf{e}_z$, such that $\mathbf{n} \cdot \mathbf{e}_z = 0 \forall \mathbf{n}$. This assumption allows us to describe the 2D directional density of fibers at any z as a function of a single angle $\theta \in [-\pi/2, \pi/2]$, with $\mathbf{n} = [\cos\theta, \sin\theta, 0]^T$. Because heart valve collagen has a circumferential preferred direction (Billiar and Sacks, 2000b; Liao et al., 2007; Misfeld and Sievers, 2007; Sacks et al., 1998; Sacks and Yoganathan, 2007), we choose $\theta = 0$ to correspond to the circumferential axis and assume that the collagen orientation distribution function (ODF) $\Gamma(\theta)$ is beta-distributed over $[-\pi/2, \pi/2]$, with mean μ_Γ and standard deviation σ_Γ :

$$\begin{aligned}
\Gamma(\theta) &= \begin{cases} \frac{y^{\alpha_\Gamma-1} (1-y)^{\beta_\Gamma-1}}{\pi B(\alpha_\Gamma, \beta_\Gamma)} & \forall y \in [0, 1] \\ 0 & \text{otherwise} \end{cases} \\
\text{for } y &= \frac{\theta}{\pi} + \frac{1}{2}, \hat{\mu}_\Gamma = \frac{\mu_\Gamma}{\pi} + \frac{1}{2}, \hat{\sigma}_\Gamma = \frac{\sigma_\Gamma}{\pi}, \\
\alpha_\Gamma &= \frac{-\hat{\mu}_\Gamma (\hat{\sigma}_\Gamma^2 + \hat{\mu}_\Gamma^2 - \hat{\mu}_\Gamma)}{\hat{\sigma}_\Gamma^2}, \text{ and } \beta_\Gamma = \frac{(\hat{\mu}_\Gamma - 1)(\hat{\sigma}_\Gamma^2 + \hat{\mu}_\Gamma^2 - \hat{\mu}_\Gamma)}{\hat{\sigma}_\Gamma^2}.
\end{aligned} \tag{3}$$

The stress tensor for the collagen population is given by

$$\mathbf{S}_c(\mathbf{C}) = \eta_c \int_{-\pi/2}^{\pi/2} \frac{\Gamma(\theta)}{\lambda(\theta)} \left[\int_1^\lambda \frac{D(\lambda_s)}{\lambda_s} \left(\frac{\lambda(\theta)}{\lambda_s} - 1 \right) d\lambda_s \right] (\mathbf{n} \otimes \mathbf{n}) d\theta, \tag{4}$$

where $\mathbf{C} = \mathbf{F}^T \mathbf{F}$ is the right Cauchy-Green deformation tensor for a deformation gradient $\mathbf{F}$. Note that since ensemble stretch is direction-dependent, $\lambda = \lambda(\theta) = \sqrt{\mathbf{n}(\theta)^T \mathbf{C} \mathbf{n}(\theta)}$. In the special case of equibiaxial (EB) stretch, such that $\mathbf{C} = \text{diag}(\lambda^2, \lambda^2, \lambda_z^2)$ with $\lambda_z = \lambda^{-2}$ to ensure incompressibility, λ becomes angle-invariant and $\text{tr}(\mathbf{S}_c) = S_{\text{ens}}(\lambda)$.

We modeled the matrix phase as an incompressible neo-Hookean solid such that

$$\mathbf{S}_m(\mathbf{C}) = \mu_m (\mathbf{I} - \mathbf{C}_{33} \mathbf{C}^{-1}), \quad (5)$$

where μ_m is the matrix shear modulus (Fan and Sacks, 2014; Fata et al., 2014; Zhang et al., 2016). If transmural variations are ignored, the total tissue stress is simply

$$\mathbf{S}(\mathbf{C}) = \phi_c \mathbf{S}_c(\mathbf{C}) + (1 - \phi_c) \mathbf{S}_m(\mathbf{C}), \quad (6)$$

where ϕ_c is the collagen volume fraction. To model the AV leaflet as a FGM, we allow all *structural* features represented by the associated structural parameters ($\phi_c, \mu_D, \sigma_D, \lambda_{ub}, \mu_\Gamma, \sigma_\Gamma$) to vary with z , but assume the *intrinsic* fiber and the matrix mechanical properties (η_c, μ_m) remain constant throughout the tissue. Further, given the bonded nature of the leaflet tissue, we assume that tissue deformation is transmurally homogeneous (Buchanan and Sacks, 2014; Stella and Sacks, 2007; Zhang et al., 2016). The total tissue stress at any transmural location z is thus

$$\begin{aligned} \mathbf{S}(\mathbf{C}, z) = & \phi_c(z) \eta_c \int_{-\pi/2}^{\pi/2} \frac{\Gamma(\theta, z)}{\lambda(\theta)} \left[\int_1^\lambda \frac{D(\lambda_s, z)}{\lambda_s} \left(\frac{\lambda(\theta)}{\lambda_s} - 1 \right) d\lambda_s \right] (\mathbf{n} \otimes \mathbf{n}) d\theta \\ & + [1 - \phi_c(z)] \mu_m (\mathbf{I} - \mathbf{C}_{33} \mathbf{C}^{-1}) \end{aligned} \quad (7).$$

The associated average stress in any discrete thickness region $\zeta \in [z_1, z_2]$ given by

$$\bar{\mathbf{S}}_\zeta(\mathbf{C}) = \frac{1}{z_2 - z_1} \int_{z_1}^{z_2} \mathbf{S}(\mathbf{C}, z) dz \quad (8).$$

When parameters are estimated from the mechanical data alone, it is not possible to deduce continuous transmural variations in structure, as only the overall stress in a specimen can be measured during loading. In these cases, certain parameters can be approximated as constants over the thickness region of interest. When the recruitment function and ODF are approximated using a set of constant parameters $\Xi = \{\mu_D, \sigma_D, \lambda_{ub}, \mu_\Gamma, \sigma_\Gamma\}$, Eqn. 8 simplifies to

$$\begin{aligned}\bar{\mathbf{S}}_{\zeta}(\mathbf{C}) &= \frac{1}{z_2 - z_1} \int_{z_1}^{z_2} \phi_c(z) \mathbf{S}_c(\mathbf{C}|\Xi) + [1 - \phi_c(z)] \mathbf{S}_m(\mathbf{C}) dz \\ &= \phi_c^{\zeta} \mathbf{S}_c(\mathbf{C}|\Xi) + (1 - \phi_c^{\zeta}) \mathbf{S}_m(\mathbf{C})\end{aligned}\quad (9),$$

where ϕ_c^{ζ} is the (average) collagen fraction in ζ . The approximation resembles the form of Eqn. 6 and is useful particularly for validation purposes using data from intact leaflet or separated layer.

2.2. Quantitative transmural morphology

2.2.1. Histological analysis

The volume fraction of collagen is well known to vary between the AV's three layers (Stella and Sacks, 2007; Vesely, 1998); however the continuous transmural variation of ϕ_c has never been quantified. To obtain $\phi_c(z)$ experimentally, we excised square ($5 \times 5 \text{ mm}^2$) specimens from the belly regions of three porcine AV leaflets. 10- μm *en face* serial sections were cut through the entire thickness of each specimen and stained with Movat's pentachrome. Sections ($n=55$ per leaflet) were imaged using bright-field microscopy, which showed substantial transmural variation in the AV leaflet's composition (Fig. 1, top panel). The volume fractions occupied by collagen (ϕ_c), GAGs (ϕ_g), and elastin (ϕ_e) were then quantified along the normalized transmural coordinate $z \in [0,1]$, where $z=0$ is the surface facing the aorta and $z=1$ is the surface facing the left ventricle, using a color-based labeling method (A.1; Fig. 1, bottom panel). For consistency with conventional nomenclature, we defined boundary locations between the three layers from inflection points in $\phi_c(z)$, denoted $z_{F/S}$ and $z_{S/V}$.

2.2.2. Transmural CFA

In addition to tissue composition, fiber orientation also plays a significant role in determining the AV's mechanical behavior. To quantify transmural variations in collagen fiber architecture (CFA), a porcine AV leaflet was sectioned as in 2.2.1, and small-angle light scattering (SALS) data was collected from each section ($n=74$). We then estimated ODF parameters for each section by varying μ_r and σ_r to obtain a least-

squares fit of Eqn. 3 to each SALS intensity pattern (Billiar and Sacks, 2000b; Fata et al., 2014; Zhang et al., 2016). The resulting $\mu_r(z)$ and $\sigma_r(z)$ were smoothed for stress analysis using Taubin's algorithm (Taubin, 1995).

2.4. *Model validation*

2.4.1. *Mechanical data sources*

In order to examine the leaflet's angle-independent recruitment behavior, we gathered EB stretch data from four AV leaflet specimens (Fig. 2, Group 1), following established procedures for data collection and post-processing (Billiar and Sacks, 2000a; Zhang et al., 2015). Previous results have indicated that fibers in the fibrosa recruit sooner than in the ventricularis (Stella and Sacks, 2007). If recruitment within these two layers occurs in distinct stretch regimes (separated by $> 2\sigma_D$) such that the effective ranges of their recruitment functions overlap negligibly, then very high stresses (>1 MPa) would be necessary under EB stretch to significantly recruit ventricularis fibers, due to collagen's high stiffness following recruitment. Under this assumption, we thus loaded EB specimens sufficiently to observe a linear stress-strain curve (indicating full recruitment in the fibrosa) but not enough to engage the ventricularis, allowing us to consider the EB data essentially as a fibrosa-only response.

In addition, we utilized data previously reported by Stella and Sacks (2007) for six intact specimens as well as their individual ventricularis layers to examine the leaflet response under a variety of semi-physiological loading conditions and to investigate layer-wise differences in structure (Fig. 2, Group 2). For these specimens, we analyzed data from five protocols in which the membrane tensions along the circumferential and radial axes were controlled at ratios of 1:2, 3:4, 1:1, 4:3, and 2:1. Ventricularis data were referenced to the post-preconditioned unloaded state of their respective intact leaflet to allow for direct comparison.

2.4.2. *Parameter estimation*

All parameters (including those directly measured) were estimated by fitting our model to the biaxial mechanical data, which provided a means to obtain modulus and recruitment parameter values as well as to validate the ability to acquire orientation

information from mechanical data (Table 1). Because it is impossible to directly quantify stress variations within an individual test specimen, we fit the discrete form of the model (Eqn. 9), defining ζ appropriately for each specimen, and assumed that subsequent parameter results were representative of the tissue structure in the region of interest. The EB stretch dataset provided a good state from which to initially estimate elastic and recruitment parameters for the fibrosa, since its collagen network is fully recruited, orientation parameters can be ignored, and the ventricularis contribution is negligible (Zhang et al., 2016). Using a differential evolution algorithm (Storn and Price, 1997), we thus fit our EB data using the angle-invariant discrete model

$$\text{tr}(\bar{\mathbf{S}}_F) = \phi_c^F \mathbf{S}_{\text{ens}} + (1 - \phi_c^F) \text{tr}(\mathbf{S}_m), \quad (10)$$

where $F \equiv$ fibrosa and $\zeta_F \in [0, (z_{F/S} + z_{S/V})/2]$ includes the fibrosa and the top half of the spongiosa, $\bar{\mathbf{S}}_F$ is the average stress in ζ_F , and ϕ_c^F is the overall collagen fraction in ζ_F , prescribed from the image-based measurements (Fig. 2). In addition to moduli and recruitment parameters for the fibrosa, these results provided estimates for the ensemble Cauchy stress levels corresponding to full recruitment, defined as $T_{\text{ub}} = \lambda_{\text{ub}}^2 \mathbf{S}_{\text{ens}}(\lambda_{\text{ub}})$.

We determined the CFA parameters μ_r and σ_r as follows from the multi-protocol data. Because these specimens were not loaded to full collagen fiber recruitment, we defined λ_{ub} for each specimen using the average T_{ub} found in EB stretch data. The matrix modulus μ_m was also fixed at its average EB value. We first fit Eqn. 9 to the separated ventricularis data using the collagen fraction ϕ_c^V in $V \equiv$ ventricularis, with $\zeta_V \in [(z_{F/S} + z_{S/V})/2, 1]$, which yielded effective CFA and recruitment parameters for the ventricularis. Note that the fibrosa flattens from its initially wrinkled state radially following layer separation (Sacks et al., 1998; Stella and Sacks, 2007; Vesely and Lozon, 1993; Vesely and Noseworthy, 1992). As it is actually now known to be under substantial pre-stretch *in vivo* (Aggarwal et al., 2016), the fibrosa will be largely smooth in-vivo. Because this deformation is non-planar and thus cannot be precisely quantified through 2D fiducial marker tracking, we did not examine the response of individual fibrosa specimens. Instead, we solved for the effective fibrosa

response using $\bar{\mathbf{S}}_{\text{total}} = \phi_F \bar{\mathbf{S}}_F + \phi_V \bar{\mathbf{S}}_V$, where $\phi_F = (z_{F/S} + z_{S/V})/2$ and $\phi_V = 1 - \phi_F$ are fibrosa and ventricularis volume fractions (each including half the spongiosa). A Student's t-test was used to compare each fibrosa and ventricularis parameter, in order to identify potential transmural differences. For stress distribution analysis, each recruitment parameter was assumed to vary in a sigmoidal fashion through the spongiosa between its fibrosa and ventricularis values.

3. Results

3.1. Constituent volume fractions

Histological analysis revealed substantial transmural variations in the volume fractions of all constituents (Fig. 3). Consistent with previous studies (Stella and Sacks, 2007; Vesely, 1998), we found the fibrosa region to consist primarily of collagen, the spongiosa region to consist primarily of GAGs, and the ventricularis to hold most of the leaflet's elastin. Interestingly, we also found a spike in elastin content at the top of the fibrosa that lasted through about 5% of the thickness (Fig. 1 and 3b). The transmural collagen fraction of every specimen had two prominent peaks within the first and last thirds of the leaflet thickness, which became especially obvious after smoothing the averaged data (Fig. 3a). We defined approximate fibrosa/spongiosa and spongiosa/ventricularis transitions from the inflection points of ϕ_c at $z_{F/S} = 0.35$ and $z_{S/V} = 0.68$, which agree with previous findings (Stella and Sacks, 2007).

3.2. Collagen fiber orientation

The collagen ODF preferred fiber directions were observed to be close to zero throughout the leaflet (centered at $-3.77 \pm 4.82^\circ$). For all stress distribution analysis, we thus assumed a homogeneous circumferential preferred direction such that $\mu_T = 0^\circ$ everywhere. In contrast, the fiber splay was found to vary transmurally, increasing from $\sigma_T \approx 14^\circ$ in the fibrosa to about 21° in the ventricularis (Fig. 4a).

3.3. Parameter estimation from mechanical data

From $z_{F/S}$ and $z_{S/V}$, we defined the specimen intervals $\zeta_F \in [0, 0.515]$ and $\zeta_V \in [0.515, 1]$, assuming the spongiosa is equally divided during layer separation (Stella and Sacks, 2007), and determined $\phi_c^F = 0.508$ and $\phi_c^V = 0.172$ (Fig. 3a). The constitutive model was able to fit all EB stretch and multiprotocol data well ($R^2 > 0.9$; Fig. 5). Results from fitting EB stretch specimens suggested that on average, full recruitment occurs at $T_{ub} = 357 \pm 40$ kPa. Multiprotocol data was thus fit assuming $T_{ub} = 350$ kPa for simplicity. Statistical comparisons between the fibrosa and ventricularis parameters revealed significant structural differences between the two layers (Table 2). Specifically, fibers were found to be more tightly aligned in the fibrosa (smaller σ_F), consistent with our findings from the SALS data. In addition, the results indicate that fibers in the fibrosa recruit sooner (smaller μ_D and λ_{ub}) than in the ventricularis by stretch values $> 3\sigma_D$ on average, confirming that ventricularis fibers were not appreciably recruited in EB specimens. To arrive at a continuous transmural description, we assumed each recruitment parameter varied according to the shape of a logistic function (Fig. 4b).

3.4. Effective transmural stress distribution

To investigate how transmural variations in structure bring about transmural variations in stress *in vivo*, we evaluated the FGM model (Eqn. 7) using our final transmurally varying parameters (Fig. 3a and 4) at a circumferential and radial stretch of 1.1 and 1.7 respectively, which corresponds to an approximate end-diastolic state in the leaflet's belly region (Aggarwal et al., 2016). Our results show significant transmural variation in both the circumferential and radial components of the resulting Cauchy stress tensor (Fig. 6). Specifically, each of these quantities exhibited strong peaks within the fibrosa and ventricularis regions, indicating that these two layers dominate the intact response with little help from the spongiosa. Circumferential stress appeared to be concentrated in the fibrosa, while the ventricularis contributes primarily in the radial direction, consistent with previous studies (Stella and Sacks, 2007; Vesely and Noseworthy, 1992). The model also predicted significant transmural variations. For example, regions near the top and bottom of the leaflet are under much lower stresses

than the interior of their respective layers—a consequence of low collagen content near the boundaries.

4. Discussion

4.1. *Advantage over previous models*

In the present study, we developed a framework through which we could investigate transmural variations in effective AV leaflet tissue stress. Our approach is a logical extension of previous studies that have investigated local arterial wall and heart valve properties by treating the tissue as a composite of discrete layers (Gasser et al., 2006; Kroon and Holzapfel, 2008; Stella and Sacks, 2007; Weinberg and Kaazempur Mofrad, 2007; Yip and Simmons, 2011; Zhang et al., 2016). We note that the FGM model offers a significantly higher-resolution description of how different regions of the leaflet contribute to the overall structure and behavior of the intact valve.

4.2. *FGM model validation*

We extensively validated the FGM model using multiple types of biaxial mechanical data. In addition to predicting stress very well under a wide range of stretch states, the optimized model predicted layer-wise differences in fiber splay consistent with our CFA findings from SALS (Table 2). Direct quantification of more local transmural variations in the leaflet's mechanical properties is impossible due to the absence of experimental techniques that can back out local stress. In light of this, a structurally informed FGM model currently seems to be the best available method to deduce the overall transmural stress distribution in the AV leaflet.

4.3. *CFA in the intact leaflet*

In addition to providing a transmural description of stress, our results offer insights into how different regions of the leaflet sum together to determine the arrangement and architecture of fibers in the intact AV. One notable implication arising from our ODF results is that along different ensemble directions, the transmural distribution of collagen can vary drastically (Fig. 7a). At any angle θ_i , the collagen content is given by $\phi_c(z)\Gamma(\theta_i, z)$, which accounts for both transmural and directional

variations. Because σ_r generally increases with z , the ventricularis will contain a substantial share of the fibers at larger angles (e.g. $\theta = 30^\circ$), though these directions contain a small fraction of the total collagen. Along $\theta = 15^\circ$, the collagen content closely resembles the shape of the average transmural distribution, which is exactly $\phi_c(z)$. Since the recruitment parameters $\{\mu_D, \sigma_D, \lambda_{ub}\}$ also vary with z , the angular dependence of transmural collagen content implies that cumulative recruitment will also vary with angle, even though $D(\lambda_s, z)$ is assumed to be angle-invariant at all z (Fig. 7b). Our results suggest that in general, recruitment when $\lambda < 1.28$ occurs almost exclusively in the fibrosa, while fibers in the ventricularis only recruit at higher stretches. Moreover, the relative contribution of each layer is direction-dependent, with the ventricularis playing a greater role at larger angles.

4.4. Significance of results

We have quantified for the first time how mechanical properties vary through the thickness of the AV. An evaluation of the FGM model (Eqn. yielded smooth but variable distributions of stress across the leaflet (Fig. 6). Beyond demonstrating that there are large differences between the conventionally defined three layers, the stress distribution we inferred suggests that there is also considerable variation within each layer. From a biological perspective, this result could have significant implications, since VICs are known to deform *in vivo* and remodel their surrounding matrix in manners highly specific to the mechanical properties of their microenvironment (Lee et al., 2015a; Pierlot et al., 2014; Pierlot et al., 2015). Efforts to understand local valve tissue maintenance, growth, and adaptation to deviations in physiological function (e.g. pathology, pregnancy) may be limited if large regions within the leaflet are assumed to be transmurally homogeneous. The FGM modeling framework is a basis and a tool for making this common simplification unnecessary in future structural and mechanical investigations of the AV. More broadly, the same approach can be applied in studies of other functionally graded planar soft tissues, such as skin and blood vessel walls.

4.5. Limitations and future work

Aside from the significant transmural variations elucidated here, it is possible that heterogeneities within the leaflet plane also play a role in valve mechanics. Our histological images showed visible heterogeneity in the spatial distribution of constituents, especially in areas of transition between layers (Fig. 1). Moreover, fiber direction and splay are known to vary within heart valve leaflets, including the AV's (Pierlot et al., 2014; Sacks et al., 1998; Sacks and Yoganathan, 2007). While we have assumed these variations are negligible compared to those in the transmural direction, the mechanical effects of in-plane material and structural heterogeneities should be investigated in the future. We also only focused on the belly region of healthy AV leaflets. The transmural properties of various regions of the AV should thus be studied under a variety of physiological and pathophysiological conditions for comparison. Additionally, our recruitment parameter results could be further validated through direct quantification of local fiber undulation architecture (Lee et al., 2015b; O'Connell et al., 2008).

4.6. Conclusions

We have quantified substantial variations in constituent fractions, fiber alignment, and fiber crimp through the thickness of the AV leaflet. These data, together with estimated material properties, were used to define the parameters of a FGM model, which can be used to infer a continuous transmural distribution of stress for any state of 2D stretch in the leaflet. We have predicted that the AV experiences large transmural stress variations *in vivo*, with the fibrosa region bearing the most load overall, the spongiosa contributing very little to the total response, and the ventricularis playing a significant role radially. Our results also show intralayer structural and mechanical variations that could be important in the context of tissue remodeling. While not the final word, the present study has provided the first detailed description of local AV leaflet mechanics, and has shed light on the possible functional importance of the AV's continually varying transmural structure.

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A. Appendices

A.1. Color-based quantification of volume fractions from histological images

In pentachrome staining, collagen, GAGs, elastin, and cell nuclei have unique colors. To quantify the volume fraction occupied by each of these constituents in each section, the red-green-blue (RGB) color vector of each image pixel was compared to vectors that were characteristic of each relevant stain color as well as a neutral gray vector. Specifically, the angle γ_{ij} between a pixel's color vector $\mathbf{c}_i = [R_i, G_i, B_i]$ and each characteristic color vector $\mathbf{c}_j$ was computed via

$$\gamma_{ij} = \cos^{-1} \left(\frac{\mathbf{c}_i \cdot \mathbf{c}_j}{\|\mathbf{c}_i\| \|\mathbf{c}_j\|} \right) \quad (\text{A.1})$$

and the pixel was categorized according to its smallest γ_{ij} (Fig. 1, bottom panel). We chose the characteristic color vectors for collagen ($\mathbf{c}_c$), GAGs ($\mathbf{c}_g$), and elastin ($\mathbf{c}_e$) as

$$\begin{aligned} \mathbf{c}_c &= [255, 255, 200], \\ \mathbf{c}_g &= [200, 255, 255], \text{ and} \\ \mathbf{c}_e &= [255, 200, 255] \end{aligned} \quad (\text{A.2})$$

such that they were rotationally equidistant from the neutral color $\mathbf{c}_0 = [255, 255, 255]$. Note that because we only compared color vectors by angle, our categorization results were independent of the pixel brightness $\|\mathbf{c}_i\|$, and thus unaffected by variability in light intensity during imaging.

The histological results of all three leaflets were then averaged along the normalized transmural coordinate $z \in [0, 1]$, where $z = 0$ is the surface facing the aorta and $z = 1$ is the surface facing the left ventricle. Because the color of nuclei and elastin stains were largely indistinguishable, we utilized the fact that VICs are uniformly distributed through the leaflet and thus subtracted the lowest value from the nuclei/elastin data to arrive at the true elastin fraction $\phi_e(z)$ (Carruthers et al., 2012; Lee et al., 2015a). All average results were smoothed using Taubin's algorithm for visualization and stress distribution analysis (Taubin, 1995).

Tables

Table 1: Sources of parameter results

Parameter	Measured transmurally?	Estimated from EB stretch data?	Estimated from multiprotocol data?
ϕ_c	X	—	—
η_c^*	—	X	X
μ_D^*	—	X (F only)	X (F and V separately)
σ_D^*	—	X (F only)	X (F and V separately)
λ_{ub}^*	—	X (F only)	X (F and V separately)
μ_I	X	—	X (validation only)
σ_I	X	—	X (validation only)
μ_m	—	X	—

F: fibrosa, V: ventricularis. *Parameter results from multiple sources (excluding those obtained only for validation) were averaged when performing transmural stress distribution analysis.

Table 2: Summary of parameter estimates from mechanical data

Parameter	Fibrosa (F)	Ventricularis (V)	Comparison	p-value
η_c [MPa]*	62.8 ± 4.1	63.0 ± 6.4	—	0.492
μ_D^*	1.23 ± 0.01	1.34 ± 0.05	F < V	0.012
σ_D^*	0.022 ± 0.004	0.032 ± 0.003	—	0.053
λ_{ub}^*	1.28 ± 0.02	1.38 ± 0.05	F < V	0.027
μ_I [deg]	-1.1 ± 3.4	3.3 ± 4.3	—	0.276
σ_I [deg]	15.6 ± 1.0	17.9 ± 1.0	F < V	0.046
μ_m [kPa]	3.4 ± 0.8	—	—	—

All values are mean $\pm$ standard error. Comparisons yielding $p < 0.05$ were considered significant. *Fibrosa results are average of EB stretch and multiprotocol specimens.

Figure Captions

Fig. 1. (top) Representative histological sections from the fibrosa, spongiosa, and ventricularis, respectively. Collagen is stained yellow, GAGs are stained blue, and elastin and cell nuclei are stained purple. (bottom) Constituent labeling pipeline of a section representative of the fibrosa/spongiosa transition, showing a raw image of the section, the same image normalized by brightness to highlight variations in color, and a color map indicating how each pixel was categorized (cyan: collagen; bright yellow: GAGs; red: elastin/nuclei; dark blue: indiscernible).

Fig. 2. Overview of model parameter estimation pipeline, using EB stretch and multiprotocol biaxial mechanical data. Estimates of fibrosa parameters are obtained from both EB stretch and multiprotocol specimens, and then averaged to arrive at a final parameter set. Ventricularis parameters are obtained from multiprotocol data of separated ventricularis specimens.

Fig. 3. Average volume fractions ($N=3$ leaflets) of (a) collagen and (b) the matrix phase constituents for all sections along the thickness of the leaflet (black, $n=55$), overlaid by the same results after Taubin smoothing (red). Representative standard error bars are shown for a selection of equally spaced points. Boundaries between the leaflet's three traditional layers are defined by the inflection points of $\phi_c(z)$, from which the overall fibrosa and ventricularis specimen collagen fractions (ϕ_c^F and ϕ_c^V , respectively) were determined.

Fig. 4. (a) Fiber splay results for all sections (black, $n=74$), overlaid by the same results after Taubin smoothing (red). Splay oscillates locally but remains relatively constant within each half of the leaflet (average values for $\zeta_F=[0, 0.515]$ and $\zeta_V=[0.515, 1]$ shown in dotted red). (b) Mean recruitment stretch along the thickness of the leaflet, illustrating the assumed sigmoidal transition between the fibrosa and ventricularis values. Other recruitment parameters (σ_D, λ_{ub}) followed similar trends.

Fig. 5. Representative fits of the structural constitutive model to (a) EB stretch data and (b) multiprotocol data, showing excellent agreement ($R^2 > 0.9$ for every specimen). (c) Equibiaxial tension response for the specimen in (b), showing predicted contributions by the fibrosa ($\zeta_F=[0, 0.515]$) and ventricularis ($\zeta_V=[0.515, 1]$) halves of the leaflet. The fibrosa is almost solely responsible for bearing circumferential stress, but the two halves bear almost equal amounts of radial stress.

Fig. 6. Transmural distribution of Cauchy stress components for an approximate end-diastolic stretch state, showing significant interlayer and intralayer variations. Circumferential stress is concentrated in the fibrosa, while radial stress is more evenly divided between the two outer layers.

Fig. 7. (a) Collagen distribution along angles 0, 15, and 30° from the circumferential axis, accounting for both transmural and directional variations. The collection of fibers along $\theta = 15^\circ$ exhibit approximately the average transmural distribution of fibers within the leaflet (ϕ_c). (b) Cumulative fiber recruitment versus stretch along different directions, showing distinct contributions from the fibrosa (F) and ventricularis (V) layers. At larger angles, the ventricularis plays a more substantial role.

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REPRESENTATIVE IMAGES:

CONSTITUENT LABELING:

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Fig. 7