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Regional Structural and Biomechanical Alterations of the Ovine Main Pulmonary Artery During Postnatal Growth

The engineering foundation for novel approaches for the repair of congenital defects that involve the main pulmonary artery (PA) must rest on an understanding of changes in the structure-function relationship that occur during postnatal maturation. In the present study, we quantified the postnatal growth patterns in structural and biomechanical behavior in the ovine PA in the juvenile and adult stages. The biaxial mechanical properties and collagen and elastin fiber architecture were studied in four regions of the PA wall, with the collagen recruitment of the medial region analyzed using a custom biaxial mechanical-multiphoton microscopy system. Circumferential residual strain was also quantified at the sinotubular junction and bifurcation locations, which delimit the PA. The PA wall demonstrated significant mechanical anisotropy, except in the posterior region where it was nearly isotropic. Overall, we observed only moderate changes in regional mechanical properties with growth. We did observe that the medial and lateral locations experience a moderate increase in anisotropy. There was an average of about 24% circumferential residual stain present at the luminal surface in the juvenile stage that decreased to 16% in the adult stage with a significant decrease at the bifurcation, implying that the PA wall remodels toward the bifurcation with growth. There were no measurable changes in collagen and elastin content of the tunica media with growth. On average, the collagen fiber recruited more rapidly with strain in the adult compared to the juvenile. Interestingly, the PA thickness remained constant with growth. When this fact is combined with the observed stable overall mechanical behavior and increase in vessel diameter with growth, a simple Laplace Law wall stress estimate suggests an increase in effective PA wall stress with postnatal maturation. This observation is contrary to the accepted theory of maintenance of homeostatic stress levels in the regulation of vascular function and suggests alternative mechanisms regulate postnatal somatic growth. Understanding the underlying mechanisms, incorporating important structural features during growth, will help to improve our understanding of congenital defects of the PA and lay the basis for functional duplication in their repair and replacement. [DOI: 10.1115/1.4023389]

Introduction

The pulmonary trunk lies between the pulmonary valve and the bifurcation (BFN) point into the right and left pulmonary arteries and carries oxygen-deficient blood from the right ventricle toward the lungs. It has been estimated that worldwide 600,000 babies are born annually with significant congenital heart disease [1], wherein normal growth and hemodynamic function of the pulmonary arteries and aorta become disrupted either due to structural heart defects and/or genetic disorders [1–5]. Congenital heart and related vascular defects cause increased flow and pulmonary pressure leading to unfavorable vascular remodeling that results in pulmonary arterial hypertension [1,2,6]. In children, pulmonary arterial hypertension is a severe condition that leads to substantial remodeling of the extracellular matrix in the proximal pulmonary arteries [7].

Congenital abnormalities of these arteries often necessitate surgical repair or the use of autologous tissue and synthetic biomaterials as vascular grafts [8–10]. The patency of the synthetic conduit replacements is limited, often requiring further surgical reinterventions due to lack of adaptation to the normal growth of the child and/or functional failure of the graft [11]. The autologous conduit replacements are limited in supply and may not adjust to the different flow environment of the graft site. The optimal vascular replacement should be able to accommodate somatic growth and closely mimic the structure, function, and physiologic environment of native vessels. In recent years, there has been a growing interest in the development of a living autologous tissue graft that could address the critical need for growing substitutes in the repair of congenital cardiovascular defects [12–15].

While the biomechanical changes of postnatal growth in the aorta have been previously studied [16–20], there has not been any study on the postnatal growth behavior of the main pulmonary artery. Knowledge of the biomechanical and structural properties of the arterial wall and adaptations during the postnatal growth period are essential to understanding the alteration due to a disease state such as hypertension and aging [21–23]. For example, Lammers et al. [7] have delineated the prominent role of elastin in the alteration of pulmonary artery mechanics in hypertensive calves. However, they point out that in order to fully realize the nature of this disease phenomenon, the elastin structure of both the normal PA and that of the hypertensive arterial wall has to be

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Fig. 1 (a) Anterior view of the ascending aorta (AA) and pulmonary trunk (PT) with medial aspect outlined (dashed square), showing the sinotubular junction (STJ) and bifurcation (BFN), which define the main pulmonary artery (PA). (b) Locations of biaxial testing samples excised from the PA's anterior (A), medial (M), posterior (P), and lateral (L) walls. Also shown are the estimated change in the PA circumference with growth (circumferential growth stretch, λ_0) from Ref. [26], highlighting regions of large and small growth deformations, used to guide the specimen selection locations. (c) Sample unloaded ring (top) and stress-free (bottom) states shown for circumferential luminal and abluminal residual strain measurements at the STJ and BFN.

quantified. Moreover, a detailed knowledge of native PA biomechanical function during normal postnatal growth is a prerequisite to establishing functional requirements for a replacement PA in congenital disorders. Based on these considerations, in the present study we characterized the regional biomechanical properties of the PA wall during postnatal growth in an ovine model at two time points. We quantified the regional biaxial mechanical behavior, residual strain, and microstructural characteristics of collagen and elastin. Moreover, we quantified the collagen recruitment behavior as a function of age using a biaxial device under multiphoton microscopy.

Methods

Anatomical Definitions and Specimen Preparation. In this study, the PA is defined as the region of the pulmonary trunk bounded proximally by the pulmonary valve sinotubular junction (STJ) and distally by the BFN (Fig. 1(a)). Note that the pulmonary trunk is physically connected to the ascending aorta through connective tissue on its medial aspect (Fig. 1(a)), and the auricle of the left atrium lies on its lateral aspect proximal to the BFN. As in our related studies [24,25], the Dorset ovine animal model was used for having a similar cardiovascular system to that of humans and a relatively fast growth rate that would allow a feasible timeline. It was revealed in our previous geometric studies [25] that postnatal changes in the PA wall geometry, although noteworthy, were not dramatic. Henceforth, two age groups of 4–5 months and 10–12 months old, representing the juvenile and adult stages, were considered sufficient to represent the alterations of the biomechanical behavior during postnatal growth. The age of the animal was verified by the size of its pulmonary trunk. For each age group twenty five fresh Dorset sheep pulmonary trunk specimens were obtained from a local slaughterhouse. Specimens were transported in phosphate-buffered saline (PBS) on ice for about 45 min before being either directly tested or stored in the -80°C freezer for a few days before testing.

Biaxial Mechanical Testing. Based on the estimated surface growth patterns [26], we chose four regions to describe the biaxial mechanical behavior of the PA wall around its circumference (Fig. 1(b)). A detailed description of the biaxial testing device has been previously presented [27]. For the present study, arterial wall samples ($n = 10$ per age group) from each region were cut into squares of 10–15 mm per side. Thickness measurements were taken at three locations using a micrometer Starrett® Model 1010

thickness gauge (The L. S. Starrett Company, Rochester, NY) and averaged. Four small cut portions of polypropylene suture were affixed to the middle region of the specimen in a square pattern using a small amount of cyanoacrylate glue to serve as fiducial markers. Specimens were submerged in a room temperature PBS bath and mounted onto the biaxial device with the circumferential and longitudinal directions aligned with the device's axes. The marker positions in the free-floating (no-load) reference states were saved before preconditioning, after preconditioning, and after full protocol testing. Each specimen was taken to maximum equibiaxial stress of 35 kPa, which approximately represented the peak systolic stress. The loading duration was set to 10 s resulting in strain rates of 3–4%/s and 5–6.5%/s, in circumferential and longitudinal directions, respectively. The equibiaxial stress protocol results are presented for each region. Additionally, each medial wall sample was tested under the estimated biaxial physiological stress ratio of 35:17.5 to compare with the collagen recruitment measurement study outlined below. After testing, samples were placed in freshly prepared 2% paraformaldehyde solution and refrigerated at 4°C overnight for later structural quantification.

Residual Strain Measurements. The residual strain measurements were performed right after the tissue arrived at the lab from the slaughterhouse. One ring from the STJ and one ring from the BFN regions (Figs. 1(b) and 1(c)) of the fresh PA ($n = 10$ per age group) were excised. To facilitate transmural strain measurements, a large number of black water-insoluble ink dots were placed uniformly along the thickness of the ring with a fine brush (Fig. 1(c)). Next, to allow for tissue reequilibration and inhibit protease activity, the ring was placed in PBS-PMSF solution at 37°C for 20 min. Thereafter, the ring was placed in a tank containing PBS and papaverine HCl to relax the smooth muscle cells, with the temperature maintained at 37°C by circulating the tank solution. The rings were attached point-wise with a high viscosity glue to a small insulated wire piece (1 mm in diameter) attached to a stable base to keep the ring in one location and the edges in the right orientation towards the camera. After a few minutes, a lateral cut was made on the ring, and a custom-written LabVIEW program (National Instruments Corporation, Austin, TX) was run to acquire an image every 2 min for 30 min. The ImageJ software (National Institute of Health Image³) was used to trace the luminal and abluminal surface boundaries. The luminal and abluminal

³See <http://rsb.info.nih.gov/ij/>

circumferential residual Green's strain $E_{rs\theta}$ was then determined using standard methods [28].

Multiphoton Microscopy (MPM). The Olympus FV1000 MPM system consisted of a mode-locked Ti: Sapphire laser (Chameleon Vision, Coherent, Santa Clara, CA) and an Olympus upright optical microscope. The excitation wavelength was set to 830 nm, with the emission wavelengths received by two filters: The emission wavelength was set to 400 ± 100 nm for the second harmonic generation (SHG) signal of collagen, and the second channel was set at 525 ± 50 nm for the elastin two-photon excitation autofluorescence (TPEF). Time-lapse images were acquired using FluoView software (Olympus). Using a 25X water-immersion objective, XY scans ($508 \times 508 \mu\text{m}$; $0.497 \mu\text{m}/\text{pixel}$ resolution) were performed at $2 \mu\text{sec}/\text{pixel}$. Depending on the section quality, either an image stack or a single XY scan was acquired of each slide from the sectioned tissue samples.

Structural Quantification in the Unloaded Configuration. The following approach was applied to the biaxial test specimens ($n = 5$ per location per age group). For visualization of the arterial layer structure, one circumferential strip was cut from each biaxial testing sample for transverse sectioning. The remaining specimen was left for en-face sectioning to quantify the collagen and elastin fiber content and/or orientation as outlined in the following paragraph. The fixed samples were paraffin-embedded, the strip samples were each cut transversely into $5 \mu\text{m}$ thick sections for histological staining, and en-face sectioning was performed at $50 \mu\text{m}$ slice thickness for use in multiphoton imaging. Verhoeff-Van Gieson stain for elastin was applied to strip sections for identification of arterial layer structures. Different arterial layers were visually identified and their thicknesses manually measured using ImageJ software.

The MPM technique, as explained above, was used to obtain the collagen and elastin structure of three sections of tunica media of each biaxial specimen with one section proximal to intima, one in the central region, and one proximal to the adventitia in the unloaded state. For each section, the elastin and collagen channels of MPM images were separated (Fig. 2(a)) and summed—inten-

sity projection of MPM image stack into the X1-X2 plane of the arterial wall was obtained using ImageJ, giving a 2D projection from the 3D stacks. The relative elastin and collagen content of each image was quantified using the ImageJ color threshold tool. Due to the presence of the collagen fiber crimp structure, an overall collagen fiber axis could not be accurately determined in the unloaded state (see below for the loaded state, where we could clearly identify fiber axes). Therefore, only elastin fiber orientation was determined in the stress-free state using the methods described in [31] (Fig. 2(b)). Both mean elastin fiber orientation and normalized orientation index (NOI), as a degree of orientation dispersion from the mean fiber alignment, were computed.

Measurement of Collagen Recruitment. To both visualize and quantify the collagen fiber network behavior in three dimensions under real-time deformations, biaxial stretching tests were performed in the Olympus FV1000 MPM system described above. A miniaturized biaxial deformation system was custom developed to fit the space constraints of the MPM system (Fig. 3). The miniaturized biaxial stretching system consisted of both an imaging and motion control system, with four miniature hybrid linear actuators (Haydon-Kerk, CT) that were controlled with a customized LabVIEW program. For the imaging system, strain was calculated real-time with a customized LabVIEW program and a firewire camera (Sony Inc., Tokyo, Japan) equipped with a 55 mm telecentric lens (CBC Inc., New York). The customized LabVIEW software allowed the user to control displacement while continuously displaying the real-time strain.

The 10×10 mm specimens were dissected from the medial aspect of the juvenile ($n = 5$) and adult ($n = 5$) ovine PA wall (Fig. 1(b)). To expose the tunica media, the tunica intima was gently peeled off using micro forceps and micro scissors. Most of the loose connective tissue of tunica adventitia in the center of the specimen was clipped, and four small cut portions of polypropylene suture were attached as fiducial markers to the center of the abluminal surface of the specimen. Two silk suture loops of equal length were attached to each side of the specimen with four stainless steel hooks, and the specimen was placed in the stretcher system's PBS bath at room temperature. The stainless steel hooks

Fig. 2 (a) Collagen (red) and elastin (green) of a proximal section of tunica media shown on left with each fiber population individually displayed in middle. (b) A depiction of orientation analysis of elastin fibers is demonstrated through superimposed regional fiber orientation vectors (white arrows) with corresponding normalized fiber orientation distribution graph.

Fig. 3 The miniature biaxial stretching device combined with MPM system. Specimen's reflection in mirror captured by camera underneath the device stage (bottom).

attached to each side of the specimen were coupled to the linear actuator through suture lines that looped over carriage mounts. These carriages were free to rotate to allow for equal tension in the suture lines, and the specimen mounted onto the device such that X_1 and X_2 axes of the device corresponded with circumferential and longitudinal directions of the sample (Fig. 3). The fiducial markers were visualized with a mirror angled at 45 deg, which was mounted below the miniaturized biaxial stretcher system with a transparent cast acrylic base (Fig. 3). The specimens were taken through three equibiaxial stretch cycles of preconditioning at the rate of 0.5 mm/s, then taken up to 65% to 70% equibiaxial stretch to insure complete recruitment of the collagen fibers.

The excitation wavelength was chosen at 830 nm at about 11% to 13% laser transmissivity with the same aforementioned SHG and TPEF filter wavelengths. The sampling speed was set to 2 μ s/pixel with a 2 line Kalman filter, and the scanning had an incremental z-step of 0.6 μ m. SHG and TPEF images of collagen and elastin fibers were acquired in the stress-free post-preconditioned state and at each stretch level. At every 10% increase in stretch level, the tissue extension was held fixed during the approximate 30 min of image acquisition. The maximum imaging depth reached ranged from about 70 μ m to about 150 μ m from the luminal side. Image stacks were imported into the Imaris software (Bitplane, Switzerland) for 3D visualization and quantitative analysis of 3D collagen fiber trajectories. Due to the relatively high density of the collagen fiber packing, the typical number of fibers that were accurately traceable was ~ 25 .

Tortuosity was defined as the ratio of fiber-path (fiber arc) length to the chord length, with the fiber arc length measured in 3D reconstructed images by using the semi-automated Filament

function in Imaris. Fibers were manually selected, and the fiber tracing was performed using the fast marching algorithm, which is a generalization of the 2D method described in Ref. [29]. The chord length was determined based on the best fit line to the 3D fiber trace, rather than the end-to-end length, so that the chord length was not dependent upon the choice of the end points [30]. An important advantage of using the 3D renderings is that tracing of the longer fibers is more accurate than with 2D methods since portions of fibers that travel out of the plane, or that overlap, may be included. The 2D elastin fiber orientations were quantified in both fully loaded and in post-preconditioned unloaded states, and collagen fiber orientation was quantified in the former state. First, superimposed image stacks of collagen and elastin were obtained as previously mentioned. The previously implemented algorithm [31] was then applied to quantify the orientation distribution of each fiber type.

Statistical Analysis. For the analysis of biaxial mechanical properties, a mixed factorial analysis of variance (ANOVA) was performed for the between-subjects variables of age and within-subject variables of location and direction (SPSS software, IBM Inc., New York). The effect size r was then calculated to determine whether the difference between data was substantive (the threshold for a large effect is defined to be above 0.5). The circumferential residual strain data was analyzed using the paired and independent t-test accordingly. A one-way ANOVA was performed (SPSS software) to evaluate spatial variability of structural measurements. Levene's test for homogeneity of error variance was subsequently performed to determine the appropriate post hoc analysis. The multiple post hoc comparisons were made using the Bonferroni correction when error variances were similar, and the Games-Howell post hoc adjustment was made when homogeneity of variance was violated. The independent t-test was applied (SPSS software) to assess the changes with growth in each location. In the case of collagen recruitment measurements, the paired and independent t-test was used (SPSS software) to assess the within group parameter of deformation level and in-between group variable of growth. In all the above analysis, the statistical significance was considered to have been reached for $p < 0.05$. All values are reported in terms of mean $\pm$ standard error.

Results

Mechanical Responses. The biaxial mechanical responses for each protocol were stable and became repeatable after the first two to three preconditioning cycles. As expected, the biaxial loading paths were nonlinear but without a prominent toe region similar to other arterial tissues [32] (Fig. 4). The circumferential direction was consistently stiffer than the longitudinal direction in all regions except the posterior wall, where the biaxial behavior was nearly isotropic in both age groups (Fig. 4). The compliance (defined as the deformation at the peak stress) in the circumferential direction was not significantly heterogeneous. The longitudinal compliance of the lateral wall considerably increased with growth ($p < 0.01$, $r = 0.57$, Fig. 4). In the juvenile stage, the anterior and medial regions exhibited a similarly larger degree of anisotropy than the posterior wall ($p < 0.02$, Fig. 5(a)) based on the comparison of their maximum longitudinal-to-circumferential stretch ratio. However, the posterior and lateral walls were not significantly different in their biaxial behavior in the juvenile group (Fig. 5(a)). The anisotropy of the medial wall increased substantially during the postnatal growth period ($p < 0.006$, $r = 0.64$) while it was regularly maintained in the anterior and posterior regions (Fig. 5(a)).

For the residual strain measurements, we found that the abluminal circumferential residual strain was consistently larger compared to the luminal surface ($p < 0.001$, Fig. 5(b)). In the juvenile stage, the respective luminal and abluminal residual strains of -0.23 ± 0.02 and 0.39 ± 0.05 at the STJ decreased

Fig. 4 Results from the equibiaxial stress protocol for each of four regions displayed for juvenile (circles) and adult (triangles) groups. Responses demonstrated mild nonlinearity and were circumferentially stiffer than longitudinally in all regions except the posterior wall, which was nearly isotropic in both age groups. The longitudinal compliance (maximum measured deformation) of lateral wall increased significantly with growth.

Fig. 5 (a) Ratio of maximum longitudinal to circumferential stretch of equi-biaxial stress protocol in four regions of PA with growth. Here, the anisotropy of the medial wall increased substantially during postnatal growth while it was maintained in the other regions. (b) Circumferential residual strains at the luminal and abluminal surfaces in juvenile and adult ovine PA. Larger residual strain values were measured on abluminal surface than luminal surface in both age groups; BFN residual strain decreased considerably over time while it was maintained at STJ.

to -0.18 ± 0.02 and 0.35 ± 0.03 , respectively, at the BFN (Fig. 5(b)). However, this distal decrease in residual strain was not significant in the juvenile group. While the STJ residual strain was relatively preserved with growth, it did significantly decrease at the BFN from juvenile to adult stage (Fig. 5(b)). This decline in amount of residual strain at the BFN with growth occurred both at the luminal surface, decreasing from -0.18 ± 0.02 to -0.10 ± 0.02 , and at the abluminal surface, from 0.35 ± 0.03 to 0.19 ± 0.02 ($p < 0.02$, $r = 0.5$). This finding was further confirmed by the adult group's considerably smaller residual strain at the BFN compared to the STJ, with residual strains of -0.22 ± 0.02

and 0.30 ± 0.05 at luminal and abluminal surfaces, respectively ($p < 0.01$).

Physical Dimensions and Layer Structure. Collagen and elastin were closely associated with each other and formed laminae in the tunica intima and media (Fig. 6). Elastin exhibited two forms: a dense network structure and a thin sheetlike structure. The elastin compact network arrangement gradually became sparse distally, moving from the intima towards the adventitia (Fig. 6). In the tunica adventitia, collagen formed thick undulated

Fig. 6 MPM image set showing different layer structures of PA. The top image is a montage of a transverse image sections of PA anterior wall with each layer labeled. MPM images of representative en-face sections of intimal, medial, and adventitial layers (bottom) displaying collagen (red) and elastin (green) structure and content in each layer: Collagen was relatively thin and had fine crimp structure in media and formed into thicker fiber bundles in adventitia; relative elastin content of media ($62 \pm 1\%$) was significantly larger than collagen while collagen was dominant fiber in adventitia ($63 \pm 2\%$).

Fig. 7 (a) Thicknesses (in mm) of excised specimens of adult and juvenile PA. Anterior and posterior samples were consistently thicker than the medial and lateral samples. (b) Relative thickness of each arterial layer in transverse section of PA wall: media is substantially thicker than the intima and adventitia.

bundles running mostly in the circumferential direction with elastin dispersed in between in the form of randomly oriented fiber strands (Fig. 6). Uniform or very distinct internal and external elastic lamellae were not present in the ovine PA wall; rather, they mainly appeared as sparse perforated narrow sheets.

The spatial variations of the PA wall thickness were significant, with the anterior and posterior locations having similarly larger thicknesses (1.6 ± 0.08 mm) compared to medial and lateral positions (1.14 ± 0.05 mm, $p < 0.006$) (Fig. 7(a)). The regional thickness change with growth was not significant ($p > 0.06$). The regional variation of arterial layer structure was negligible in both age groups. The relative thickness of each layer did not significantly change with growth. The PA wall mostly consisted of the tunica media, occupying $81 \pm 0.8\%$ of the wall thickness, and

substantially smaller wall volume was comprised of tunica adventitia and intima, taking up $11 \pm 0.7\%$ and $7.3 \pm 0.4\%$ of the wall thickness, respectively (Fig. 7(b)). There was not a significant alteration in the relative collagen and elastin content of the tunica media spatially and with growth. The collagen area fraction was measured to be $38 \pm 1\%$ (Elastin: $62 \pm 1\%$) in the tunica media and $63 \pm 2\%$ (Elastin: $37 \pm 2\%$) in the tunica adventitia.

Determination of the fiber orientation for the elastin fibers in multiphoton images was successful (Fig. 2(b)). Interestingly, there was no significant transmural variation in elastin fiber orientation distribution based on the mean fiber orientation and NOI. The elastin fiber structure was considerably heterogeneous and was nonuniformly altered with growth. The mean elastin fiber orientation and the NOI of the anterior and posterior walls did not change

Fig. 8 Regional variations in mean elastin fiber orientation and NOI from the PA wall with growth. (a) Elastin alignment in medial wall did not change significantly with growth, whereas the anterior and medial wall elastin fiber alignment was significantly closer to circumferential direction than posterior wall. (b) NOI of lateral wall was less than other regions in juvenile stage. In lateral wall, significant increase in circumferential mean orientation of elastin fibers with growth (from 23 ± 6 deg to 1 ± 7 deg) was associated with a significant drop in NOI from $60 \pm 3\%$ to $46 \pm 4\%$. Overall, only the lateral region demonstrated statistically significant growth changes in both mean fiber direction and NOI.

with growth (Fig. 8). The mean fiber orientation values indicated that elastin fibers were considerably more circumferentially aligned on the anterior aspect of the PA wall, 0 ± 5 deg, compared to the posterior aspect, 30 ± 8 deg ($p < 0.005$, Fig. 8(a)). However, according to the NOI quantities, the degree of fiber alignment of anterior wall, $38 \pm 3\%$, was not significantly different from that of the posterior wall, $44 \pm 3\%$ (Fig. 8(b)).

The increase in circumferential alignment of medial wall elastin fibers with growth did not prove to be statistically significant ($p = 0.07$, Fig. 8). The degree of elastin fiber dispersion in the medial wall ($49 \pm 2\%$) was relatively less than the anterior wall ($p = 0.02$, Fig. 8(b)). A significant change in the elastin fiber structure with growth was measured in the lateral wall, where elastin fibers became considerably more circumferentially aligned, with the mean fiber orientation changing from 23 ± 6 to 1 ± 7 deg ($p = 0.006$, Fig. 8(a)). The NOI of elastin fibers in the lateral wall decreased from $60 \pm 3\%$ to $46 \pm 4\%$ ($p = 0.01$, Fig. 8(b)) revealing an increase in degree of fiber scattering. The change in elastin fiber orientation of the lateral wall with growth resulted in a more uniform degree of fiber scattering and more heterogeneous mean fiber alignment in the PA wall. Overall, the mean fiber orientation appeared to be a clearer indicator than the NOI of the spatial variation and growth changes in the biaxial mechanical properties of the PA wall.

Real-Time Biaxial Stretching MPM. The average out-of-plane fiber angle was measured to be 8.2 ± 0.8 and 11 ± 1 deg in juvenile and adult groups, respectively (no significant difference between the two groups). It was verified that the elastin orientation distribution was maintained during equibiaxial deformation based on the comparison of both the mean fiber orientation and NOI of unloaded and maximally deformed states in both age groups (Fig. 9). The mean elastin and collagen fiber orientations were similar and nearly circumferential (1 ± 2 deg) within the depth reached from the luminal side in intact medial wall specimens (Fig. 9). This result confirms our visual observations of close collagen and elastin fiber alignment in the unloaded sections of tunica media in all regions of the PA wall. The collagen fiber tortuosity did not significantly change between the stress-free and 10% stretch in both age groups ($p > 0.4$). Therefore, the tortuosity values of fibers in both these configurations were combined to calculate the mean fiber tortuosity of the stress-free PA wall.

The collagen fibers had a more tortuous trajectory in the adult medial wall specimens, 1.31 ± 0.01 , than the juvenile group,

1.23 ± 0.01 ($p = 0.006$, Fig. 10(a)) in the stress-free states. The tortuosity was calculated to be 1.04 ± 0.01 for juvenile specimens and 1.02 ± 0.002 for adult samples at their respective applied maximum equibiaxial deformations (Fig. 10(a)). Henceforth, the critical fiber recruitment tortuosity, beyond which a fiber was considered straight, was defined to be 1.04. The decrease in fiber tortuosity with respect to applied deformation correlated well with the increase in fiber recruitment. The fiber recruitment started after $\lambda = 1.20$ in adult specimens and after $\lambda = 1.10$ in the juvenile group (Fig. 10(b)). At $\lambda = 1.30$, less than 10% of collagen fibers were recruited on average in both age groups. The difference between recruitment behavior of the two age groups did not become significant until after $\lambda = 1.4$ ($p < 0.004$, Fig. 10(b)), where $28 \pm 2\%$ and $44 \pm 4\%$ of collagen fibers were recruited in juvenile and adult specimens, respectively. Additionally, the rate of recruitment with respect to deformation was ~ 1.4 times faster at this deformation level in the adult stage compared to the juvenile group.

Discussion

Mechanical Behavior. The regional mechanical behavior of the aorta and its postnatal growth adaptations has been investigated in several previous studies [16,18,20,33–35]. While there have been a few studies of pulmonary artery mechanics [36–39], to date there have not been any other rigorous investigation of the regional pulmonary arterial mechanical behavior as a function of growth. We determined that the PA wall was circumferentially stiffer in both age groups implying a more dominant circumferential fiber orientation. Previous studies of the PA biaxial behavior in other animals report similar directional properties [37]. The PA wall exhibited a maximum of about 40% circumferential and 40–70% longitudinal strains under equibiaxial stress. The large circumferential and longitudinal extensibility of the PA wall is of great significance since during cardiac systole about one-third of the heart's stroke volume can be stored in the PA alone leading to significant attenuation of blood flow pulsatility in the rest of the pulmonary system [40]. Longitudinal compliance, particularly in the juvenile group, was substantially more spatially variable than the circumferential behavior among the four regions studied. The heterogeneity in the longitudinal stiffness resulted in nearly isotropic behavior on the posterior wall and a substantial degree of anisotropy in the anterior wall.

The biaxial behavior of the medial and lateral walls underwent changes with growth, while that of the anterior and posterior walls

Fig. 9 The mean collagen (red) and elastin (green) fiber orientation histograms at peak physiological stretch in the juvenile and adult medial PA wall quantified in the MPM-biaxial-deformation experiments. Also shown for the elastin are the histogram results at low stretch, which were similar to the peak stretch results, supporting the assumption that equi-biaxial stretch induced no change in fiber alignment.

was maintained. The circumferential growth deformation patterns reported by Fata et al. [26] were also found to be substantially altered in the same medial and lateral regions. Although circumferential growth seems to have been restricted in the lateral location, the circumferential stiffness was not affected here, yet the longitudinal compliance considerably increased with growth. The medial region exhibited a significantly larger degree of anisotropy in the adult group with larger longitudinal compliance and, to a lesser degree, a larger circumferential stiffness, noting that neither of these directional changes was individually statistically significant.

Collectively, these findings support the assumption made by Fata et al. [26] that the computed *in vivo* growth deformation patterns (Fig. 1(b)) were due to changes in the vessel surface geometry and not from modifications in the mechanical behavior with growth. We note further that the ascending aorta and auricle of the left atrium increasingly constrained the growth in the circumferential dimension of the medial and lateral regions, respectively, during postnatal maturation as revealed in our previous study [26] (Fig. 1(b)). Thus, the alteration in mechanical behavior of medial and lateral locations may be due to the changes in the local surface geometry in these regions. Further studies are

Fig. 10 Growth adaptations in collagen fiber (a) tortuosity and (b) recruitment behavior of medial aspect of PA wall measured at different equibiaxial deformation levels. Overall, the adult group demonstrated greater initial tortuosity and recruitment rate compared to the juvenile state. (c) Biaxial stress-stretch behavior of medial aspect of adult PA wall near physiological biaxial stress levels demonstrated near equibiaxial deformation.

needed to determine the underlying mechanisms, such as possible alteration in longitudinal stresses, causing the alteration in mechanical properties of these regions.

Residual Strain Patterns. It is well established that the vascular system contains residual strain in the no-load state. It has been demonstrated that this mechanism generates nonuniform prestress transmurally in the vessel wall, thus, reducing the concentration of the circumferential stress at the inner wall at normal physiological pressure [41–43]. The residual strain patterns along the PA wall have been described in previous studies [44–46], but the alteration in this pattern with growth has not been previously reported. The measured circumferential residual strains during the postnatal maturation period were considerably heterogeneous and became more nonuniform with growth. The abluminal E_{RS0} was substantially larger than that of the luminal surface. Although the transmural distribution of E_{RS0} was not quantified, this observation is an indication that the neutral bending axis is located proximally (near the luminal surface), implying that the medial layer is stiffer; hence, more mechanically significant than the adventitial layer [28]. Since the change in the abluminal residual strain between the two age groups was consistent with that of the luminal surface, the position of the neutral bending axis is probably relatively maintained with growth. There was an average of about 18% circumferential residual strain present at the BFN on the luminal surface in the juvenile stage that fell to approximately 10% in the adult stage. The residual strain was relatively maintained at the STJ while it significantly decreased at the BFN, implying that the PA wall remodels toward BFN while it maintains its structure near the right ventricular outflow region. As revealed in the previous studies [25,26], the PA tapers distally with growth. This decrease in diameter at BFN and drop in hoop stress in that region may be a correlate to the decrease in residual strain.

Wall Geometry and Structure. The anterior and posterior walls were much thicker than the medial and lateral locations, suggesting the existence of larger wall tension in these regions (assuming homogeneity of mean stress). Yet, there were no measured increases in wall thickness and no significant changes in its spatial variation with growth. Given the measured 1.4 fold increase in diameter, no detectable changes in thickness, and the absence of any measured change in blood pressure, a simple Laplace law estimate suggests that the hoop stress in the PA wall rises by about 40% with growth.

The tunica media is considered to be the most mechanically significant arterial layer as measured to occupy the major part of the PA wall. However, despite the inferred rise in the hoop stress, the thickness of this layer was maintained with growth. Therefore, the change in size of the tunica media is not part of an adaptive response to change in wall stress during postnatal growth. The relative thickness of this arterial layer was also maintained in the four designated regions at a given growth stage. The quantified elastin content of the tunica media was significantly larger than its collagen content indicating that elastin is the more dominant structural protein in the PA wall. The absence of a toe region in the stress-strain loading path indicates that elastin response dominated the mechanical response, particularly in the low-load zone.

One goal of this study was to correlate changes in collagen and elastin organization with the alterations in mechanical behavior during postnatal maturation. Our results demonstrated that the elastin fiber network had a distinct alignment with a moderate degree of dispersion, implying that mechanical behavior of the PA elastin network is direction-dependent as demonstrated in a few recent studies of aortic elastin [47,48]. The heterogeneity in the mechanical anisotropy of the PA wall (Fig. 4) was mostly comparable with the spatial variation in the elastin mean orientation (Fig. 9(a)). For example, the elastin fibers in the medial and anterior walls were more circumferentially aligned than the posterior

wall correlating with the substantially larger anisotropy of former regions compared to the latter, where the organization of the elastin fiber network was for the most part maintained with growth. A significant alteration in elastin organization was measured on the lateral wall, where the mean fiber alignment became nearly circumferential while the fiber orientation dispersion increased as revealed by the drop in the NOI. Thus, the increase in longitudinal compliance of the lateral wall is explained by the change in the mean fiber orientation towards the circumferential direction.

Collagen Recruitment Behavior. The gradual collagen fiber recruitment has been postulated to be the underlying cause for the nonlinear stiffening of the arterial wall with increasing stress [49]. Hill [50] combined a uniaxial loading device with the MPM system to directly measure the 3D collagen fiber paths and evaluate the collagen recruitment. However, uniaxial testing does not sufficiently replicate the biaxial nature of cardiovascular tissues' in situ boundary conditions. While related approaches in arterial mechanics utilizing biaxial deformations have recently appeared in the literature [51,52] and been used in arterial modeling [53,54], no study known to the authors have focused on the collagen recruitment behavior nor with growth.

Measurement of 3D fiber orientation demonstrated that the medial collagen fibers lie nearly in the plane of the wall. This supports the selection of a 2D form of distribution rather than use of the 3D conical splay orientation distribution of the fibers in the mechanical modeling of the vessel wall [55,56]. It was verified that the equibiaxial deformation did not cause noticeable change in the orientation distribution of elastin fibers, meaning that fiber rotation with deformation was minimal; thus, collagen recruitment was independent of fiber orientation. The measured circumferential and longitudinal stretch values of adult medial PA wall samples near peak physiological biaxial stress levels were measured to be 1.37 ± 0.03 and 1.41 ± 0.03 , respectively (Fig. 10(c)). Thus, the applied equibiaxial deformations approximately replicated the physiological biaxial deformation experienced by the PA wall under the *in vivo* condition.

The collagen fibers had significantly more tortuous trajectories in the adult group compared to the juvenile stage (Fig. 10(a)). However, this feature did not lead to a slower overall collagen fiber engagement with deformation. On the contrary, while having a slightly slower recruitment rate (with respect to deformation) below 30% stretch level, the collagen fibers were recruited at a significantly faster rate in the adult specimens with the difference becoming significant starting at $\lambda = 1.4$, where only 30% to 40% of fibers were recruited (Fig. 10(b)). However, comparison of this data with the biaxial stress-stretch responses reveals that this stretch level to be near or beyond the maximum physiological deformation level of the PA wall, especially in the circumferential direction (Figs. 10). The alteration in the collagen recruitment behavior with growth suggests a modification in the collagen structural properties.

In this study, we only considered the medial collagen fibers in the proximity of luminal surface of the arterial wall. The visual inspection of the collagen fiber crimp structure throughout the stress-free PA wall revealed a small increase in the crimp period from intima towards the adventitia (Figs. 2 and 6), implying that the collagen engagement occurs earlier (with respect to deformation) proximal to tunica intima under planar biaxial loading. Therefore, the contribution of collagen fibers is more substantial more proximally in the medial layer. A more thorough investigation of the collagen recruitment behavior throughout the arterial wall, especially in the *in-situ* cylindrical configuration, is necessary. However, due to the depth limitation of the MPM, similar direct measurements of the whole PA wall are not currently feasible. The larger average tortuosity of the collagen fibers in the stress-free adult specimens could be due to the smaller compressive circumferential residual strains in the adult PA wall compared to the juvenile stage. Thus, in the unloaded *in-situ*

configuration, the collagen fibers probably have a more similar level of undulation between the two age groups.

Summary. In the present study, we quantified the postnatal growth patterns in the structural and biomechanical behavior of the ovine PA. The PA wall demonstrated significant mechanical anisotropy, except in the posterior region where it was nearly isotropic, and overall moderate changes in regional mechanical properties with growth. Structurally, there were no measurable changes in collagen and elastin content of the tunica media with growth, with the collagen fibers recruiting more rapidly with strain in the adult compared to the juvenile. Perhaps our most interesting finding was that we did not find evidence that the stress was maintained in the PA wall during postnatal growth. Rather, our results suggest that there is an average of a 40% increase in hoop stress over the growth period. Therefore, unlike the arterial wall remodeling due to hypertension [57–59], the increase in elastin and collagen mass is not a mechanistic response to the change in wall stress during the postnatal growth period. Ongoing studies in our lab are focusing on structurally based constitutive models of the PA collagen and elastin fiber network in order to gain insight into the role of the modification in collagen recruitment with growth and mechanical significance of collagen within normal physiological loading.

It has been suggested that the structure of an artery adapts to changes in shape during perinatal and postnatal maturation. The driving mechanism is the maintenance of internal stresses levels and/or the equilibrium mechanical tension per elastin lamellar unit [42,60,61]. Yet, this was not the case here, although additional studies are clearly warranted to support this finding. This observation suggests an alternative mechanism that regulates postnatal somatic growth. Understanding these underlying mechanisms, through developing constitutive and finite models that incorporate the quantified structural features of normal growth, will help to improve our understanding of congenital defects of the PA and lay the basis for functional duplication in their repair and replacement.

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