Quantification of the passive and active biaxial mechanical behaviour and microstructural organization of rat thoracic ducts

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Mechanical loading conditions are likely to play a key role in passive and active (contractile) behaviour of lymphatic vessels. The development of a microstructurally motivated model of lymphatic tissue is necessary for quantification of mechanically mediated maladaptive remodelling in the lymphatic vasculature. Towards this end, we performed cylindrical biaxial testing of Sprague–Dawley rat thoracic ducts ($n = 6$) and constitutive modelling to characterize their mechanical behaviour. Spontaneous contraction was quantified at transmural pressures of 3, 6 and 9 cmH₂O. Cyclic inflation in calcium-free saline was performed at fixed axial stretches between 1.30 and 1.60, while recording pressure, outer diameter and axial force. A microstructurally motivated four-fibre family constitutive model originally proposed by Holzapfel et al. (Holzapfel et al. 2000 J. Elast. 61, 1–48. (doi:10.1023/A:1010835316564)) was used to quantify the passive mechanical response, and the model of Rachev and Hayashi was used to quantify the active (contractile) mechanical response. The average error between data and theory was $8.9 \pm 0.8\%$ for passive data and $6.6 \pm 2.6\%$ and $6.8 \pm 3.4\%$ for the systolic and basal conditions, respectively, for active data. Multi-photon microscopy was performed to quantify vessel wall thickness ($32.2 \pm 1.60$ μm) and elastin and collagen organization for three loading conditions. Elastin exhibited structural ‘fibre families’ oriented nearly circumferentially and axially. Sample-to-sample variation was observed in collagen fibre distributions, which were often non-axisymmetric, suggesting material asymmetry. In closure, this paper presents a microstructurally motivated model that accurately captures the biaxial active and passive mechanical behaviour in lymphatics and offers potential for future research to identify parameters contributing to mechanically mediated disease development.

1. Introduction

The lymphatic system is a robust network of vessels that plays a critical role in many functions including immune cell trafficking, transport of lipids from the gut to the bloodstream and tissue fluid balance [1]. Each of these functions is directly dependent on the ability of lymphatic vessels to spontaneously and rhythmically contract to transport fluid against an unfavourable pressure gradient, a function which, if impaired, leads to lymphoedema [2]. Lymphatic vessels consist primarily of collagen, elastin and lymphatic muscle cells. Vessel contractility is dependent on the ability of the muscle cells to synchronously contract in order to manipulate the geometry of the extracellular matrix and increase the transmural pressure, thereby expelling fluid across a one-way valve. The mechanical environment probably contributes significantly to the overall contractile function of these vessels, leading some to study the relationship between...
intrinsic contractile function and mechanical cues such as magnitude and direction of flow, inlet and outlet pressure, or pressure gradient [3–7]. Also directly related to contractile function is the ability of the vessel to withstand appropriately in response to mechanical loading, motivating studies that characterize the active and passive mechanical behaviour of lymphatic vessels [8–11]. Confocal microscopy has also been reported to characterize the vessel microstructure and geometry, which dictate the mechanical responses of the vessels [9,12].

Lymphatic vessels exhibit a nonlinear mechanical response, are nearly elastic, heterogeneous and anisotropic, and experience large deformation under normal physiological loading [13]. Several important lumped parameter models have been proposed that quantify lymph propulsion through the lymphatic vasculature [14–19]. These models have deepened our understanding of lymphatic biomechanics; however, most models to date either neglect the nonlinear, anisotropic behaviour of the tissue or employ empirical pressure–diameter relations. There is a pressing need for a predictive microstructurally motivated mathematical model that captures the key characteristics of the mechanical response, namely the material nonlinearity and anisotropy, in the framework of finite elasticity. Such a model would represent a key step in exploiting important biomechanical frameworks for modelling lymph transport and mechanically mediated growth and remodelling of lymphatics; such models hold the potential to advance our understanding of lymphatic function (or dysfunction) and disease progression.

The purpose of this paper is to develop a mathematical constitutive model for the passive (i.e. no contribution from contraction of muscle cells) and active (contractile) mechanical behaviour of the rat thoracic duct. We performed cylindrical biaxial testing to quantify the passive mechanical behaviour and spontaneous contractile response of rat lymphatic vessels, nonlinear regression to identify best-fit material parameters for a four-fibre family constitutive model [20] and active contractile model [21], and multi-photon microscopy to quantify vessel thickness and elastin and collagen organization. The fitted material parameters for the proposed passive and active constitutive models captured well the tissue mechanical response. Microscopy revealed structural ‘fibre families’ of elastin oriented nearly circumferentially and axially. Collagen fibre distributions varied significantly from sample to sample, often exhibiting non-axisymmetric organization, suggesting material asymmetry—a characteristic not captured in the passive constitutive model. Taken together, the reported constitutive models provide a platform for accurately quantifying the mechanical response of the rat thoracic duct, which may be incorporated into models for lymph transport and growth and remodelling, to better understand lymphatic function and disease progression.

2. Material and methods

2.1. Vessel isolation

Male Sprague–Dawley rats (300–350 g) were obtained from Jackson Laboratories (Bar Harbor, ME, USA) and kept on a 12 L:12 D cycle in a temperature-controlled room at 22°C. The animals were housed and cared for according to the guidelines set forth by the National Institutes of Health (NIH) for the care and use of experimental animals. All experimental procedures were reviewed and approved by the Georgia Tech Institutional Animal Care and Use Committee (IACUC).

Rats were anaesthetized with a combination of diazepam (2.5 mg kg \(^{-1}\) body weight) and droperidol/fentanyl (0.3 mg kg \(^{-1}\)) via intramuscular injection. After the animals reached a surgical plane, the chest cavity was opened and the thoracic duct was visualized immediately post-mortem by removing surrounding tissue and organs. Care was taken to excise the vessel from the same location each time; however, priority was given to identifying a location that was without valves or branches. Vessels were carefully excised and placed in physiological saline solution (PSS) consisting of the following constituents (in mM) at room temperature: 145.0 NaCl, 4.7 KCl, 1.17 MgSO\(_4\), 2.0 NaH\(_2\)PO\(_4\), 5.0 dextrose, 2.0 sodium pyruvate, 0.02 EDTA and 3.0 MOPS (pH = 7.4 at 37°C). Vessels were mounted on opposing cannulae in a bath of PSS and carefully cleaned of all perivascular tissue. All chemicals were obtained from Sigma-Aldrich (St Louis, MO, USA).

2.2. Biomechanical testing

Thoracic ducts containing a single lymphangion (n = 6), excluding valves, were mounted on opposing glass cannulae using silk sutures (figure 1) within a custom computer-controlled biaxial testing device [22] in PSS in a temperature-controlled incubator at 37°C. The unloaded length was at least 10 times the unloaded radius and data were collected near the midpoint between mounting sutures to sufficiently attenuate end effects during testing and isolate the mechanical response of a central region of the vessel. Vessels were visualized using an inverted microscope (2.5× magnification) and digital camera (Allied Vision Technologies, Marlin F-033B) while controlling the transmural pressure and axial length and recording the outer diameter and axial force measurements. Inlet and outlet pressure were measured using inline pressure transducers (Honeywell FPG 060-E418-11), and transmural pressure was calculated as the average of the inlet and outlet pressure. Outer diameter was recorded using an edge detection subroutine in LaVision, axial length was controlled using linear actuators (Newport Precision LTA series) connected to the cannulae, and axial force measurements were measured using a 50 g load cell (Delta Metrics 99-2636-050G). Vessels were pressurized to just above 0 cmH\(_2\)O in order to prevent collapse, and the unloaded length and diameter of each vessel was determined using a custom program in LaVision. Vessels were then axially stretched to 30% beyond the unloaded length (\(\lambda = 1.3\)), pressurized to 3 cmH\(_2\)O and given 30–60 min to equilibrate to 37°C in order to establish contractility.

Prior to data collection for this study, preliminary experiments were performed on five vessels to establish the experimental testing protocol. Axial force versus transmural pressure and transmural pressure versus diameter behaviour demonstrated little variation for axial stretch values below 1.3; thus, the lower limit of axial stretch was chosen as 1.3. For axial stretches above 1.6, transmural pressure versus diameter behaviour became nearly linear, suggesting that collagen fibres had possibly become maximally engaged. After this transition to linear behaviour, no differences were observed in transmural pressure versus diameter behaviour other than a reduction in diameter and increased slope. Thus, the upper limit of axial stretch for the testing protocol was chosen as 1.6. Also, from these preliminary experiments, vessels exhibited contractile amplitudes more than 25% of unloaded diameter at 3 cmH\(_2\)O
and axial stretches of 1.30; thus, vessels that exhibited a contractile amplitude of less than 25% of the unloaded diameter were not considered representative (e.g. due to damage during isolation and mounting) and excluded from analysis. Note that the maintenance of basal tone was not evaluated as an exclusion criterion.

Once contractility was established, contractile function was assessed at transmural pressures of 3, 6 and 9 cmH2O (noting that contraction-induced changes in transmural pressure were neglected). At each transmural pressure, the vessel was given a period of 5–10 min for equilibration. The same procedure was repeated for axial stretches of λ = 1.4 and 1.5. Temporal changes in end diastolic and systolic diameters were observed following changes in loading conditions; thus, data for analysis were taken only after the minimum and maximum diameters reached steady-state conditions.

After contractile function was assessed at fixed pressure, the PSS in the bath and the system was replaced with Ca2+-free PSS, which contained all of the same constituents as PSS with the exception of replacing CaCl2 with 3.0 mM EDTA to inhibit vessel contractility and quantify the ‘passive’ mechanical response. Vessels were preconditioned by ramping the transmural pressure from 0 to 15 cmH2O at axial stretch values of λ = 1.30, 1.35, 1.40, 1.45, 1.50, 1.55 and 1.60. Note that an axial stretch value of λ = 1.6 was excluded from passive testing in two samples because transmural pressure versus diameter behaviour transitioned to a linear shape at a stretch of 1.55, thus precluding the need to test at higher values of axial stretch. Following preconditioning, vessels were subjected to three loading cycles of transmural pressure values from 0 to 15 cmH2O at axial stretches identical to those during preconditioning. Plots depicting transmural pressure versus diameter and axial force versus pressure were generated during preconditioning. Plots depicting transmural pressure versus diameter and axial force versus pressure were generated for all fixed length conditions. Axial force versus axial length data were generated for conditions of fixed transmural pressure. Axial force data were retrieved for values of transmural pressure at intervals of 1 cmH2O for each value of axial stretch, resulting in up to 16 curves each with six data points.

2.3. Multi-photon imaging
Following mechanical testing, the device was placed under a LSM 710 META inverted confocal microscope (Zeiss) to visualize the microstructure across the entire wall of live, unfixed lymphatic vessels [23]. The vessels were imaged using a 40×/1.3NA immersion objective (Zeiss). Vessels were maintained in Ca2+-free PSS during testing. The META module was set to a bandpass filter of 380–420 nm and excited at 800 nm in order to detect backward-scattering second harmonic generation signal from collagen. Elastin emissions were detected by setting the META module to a 500–550 nm bandpass configuration and using 488 nm excitation [24].

z-stacks were collected under three loading conditions: a low-load condition (p = 3 cmH2O, λ = 1.3), a medium-load condition (p = 9 cmH2O, λ = 1.45) and a high-load condition (p = 20 cmH2O, λ = 1.6 or maximum during passive testing). Orthogonal views of these z-stacks were then used to calculate vessel thickness in a loaded configuration. Unloaded vessel thickness was calculated from these values for each of the three conditions, assuming incompressibility, and the unloaded thicknesses used in modelling were calculated as the average of the unloaded thickness values determined from each of the three loading conditions.

2.4. Quantification of collagen organization
The angular distribution of collagen fibres was quantified for each optical slice of the z-stacks using a custom Matlab program that uses a fast Fourier series algorithm as described previously [25]. Using a fast Fourier transform, a power spectrum was generated in order to provide a histogram of the frequency intensities between −90° and 90° at 4° increments (reported as ‘normalized intensity’). Note that an angle of 0° corresponds to an orientation in the axial direction. The mean fibre angle distribution was determined by taking an average of each bin across all slices in a z-stack.

2.5. Constitutive modelling
We modelled the lymphatic vessels as an incompressible, thin-walled cylinder in the framework of finite elasticity such that the Cauchy stress can be defined by equation (2.1),

\[ \mathbf{T} = -p \mathbf{I} + 2 \mathbf{F}^T \frac{\partial W}{\partial \mathbf{F}} \]

where \( T \) is the Cauchy stress, \( p \) is the Lagrange multiplier that enforces incompressibility, \( F \) is the deformation gradient, \( W \) is the strain energy function and \( C \) is the right Cauchy–Green strain tensors. The components of the deformation gradient and the right Cauchy–Green strain tensor for inflation and extension of an axisymmetric thin-walled tube are

\[ F = \text{diag}(\lambda_1, \lambda_2, \lambda_3) \quad \text{and} \quad C = \text{diag}(k_{xx}^2, k_{yy}^2, k_{zz}^2), \]

where \( \lambda_1 = h/H, \lambda_2 = r_m/R_m \) and \( \lambda_3 = L/L \), where \( h, r_m \) and \( L \) and \( H, R_m \) and \( L \) are the loaded and unloaded thickness, midwall radius and axial length, respectively.

Conservation of mass for the incompressible solid vessel wall requires that \( \text{det}[F] = \lambda_1 \lambda_2 \lambda_3 = 1 \), which leads to the constraint,

\[ h(r_m + h) \lambda^2 = H(R_m + H) L. \]

Equilibrium requires that

\[ T_{\theta \theta} = \frac{p r_m}{h} = T_{\theta \theta,\text{act}} = -C_{rr} \frac{\partial W}{\partial C_{rr}} + C_{\theta \theta} \frac{\partial W}{\partial C_{\theta \theta}}, \]

and

\[ T_{z z} = \frac{f_z}{h(2r_m + h)} = -C_{rr} \frac{\partial W}{\partial C_{rr}} + \frac{C_{\theta \theta} \partial W}{\partial C_{\theta \theta}}, \]

where \( f_z \) is the axial force, \( P \) is the transmural pressure, \( T_{\theta \theta,\text{act}} \) and \( T_{z z,\text{act}} \) are the mean circumferential and axial components of the Cauchy stress, respectively, \( T_{\theta \theta,\text{act}} \) is a constitutive equation that describes the stress generated from ‘active’ muscle cell contraction, \( C_{\theta \theta} (j = r, \theta, z) \) are the in-plane components of \( C \), and \( W = C W \) is the strain energy density function that describes the ‘passive’ mechanical response. Note that \( f_z = f_{z0} + P r_m^2 \), where \( f_{z0} \) is the force measured by the load cell during testing, and the second term accounts for the end cap pressure which is due to the load exerted on the cannula in the axial direction by the luminal pressure. Given that the average unloaded thickness-to-radius ratio is 0.134, a two-dimensional framework was used such that stresses in the radial direction were assumed to be negligible in comparison with circumferential and axial stress.

We considered the four-fibre family strain energy density function of Baek et al. [26], which was adapted from the original function described by Holzapfel et al. [20]; namely,

\[ W = b (c - 3) + \sum_{l=1,2,3,4}^4 \frac{b_l}{2} \exp(\frac{b_l}{2}((\lambda^l)^2 - 1)^2) - 1, \]

where \( b, b_l \) and \( b_4 \) are material parameters, \( c = tr(C) = C_{rr} + C_{\theta \theta} + C_{zz} \) is the first invariant of \( C \), \( (\lambda^l)^2 = C_{\theta \theta} \sin^2(\alpha^l) + 2C_{rr} \sin(\alpha^l) \cos(\alpha^l) + C_{zz} \cos^2(\alpha^l) \) is the stretch of the \( l \)-th fibre family and \( \alpha^l \) is the angle between the axial and fibre directions. For inflation and extension tests (given appropriate material symmetry), \( C_{\theta \theta} = 0 \), so that \( (\lambda^l)^2 = C_{\theta \theta} \sin^2(\alpha^l) + C_{rr} \cos^2(\alpha^l) \). We assume that fibre families 1 and 2 correspond to fibres oriented in the circumferential and axial directions, respectively, and are therefore prescribed as opposed to calculated. We also assume material symmetry, such that \( \alpha^2 = -\alpha^1 = \alpha \) and \( b_1 = b_2 \) and \( b_3 = b_4 \). Thus, equation (2.6) contains seven material parameters \( b, b_1, b_2 \) and \( b_3 \) with \( k = 1, 2, 3 \) and one structural parameter (\( \alpha \)).
We employed the model of Rachev & Hayashi [21] for the active stress due to muscle cell contraction; namely,
\[
\frac{\tau_{\text{act}}}{\text{max}} = T_{\text{act}} \lambda_0 \left(1 - \left(\frac{\lambda_0}{\lambda_m - \lambda_0}\right)^2 \right),
\]
where \( T_{\text{act}} \) is a parameter dependent on the activation of the muscle cells, \( \lambda_m \) is the stretch at which the muscle contraction is maximum and \( \lambda_0 \) is the stretch at which muscle-induced force generation ceases. Due to both tonic and rhythmic contraction of lymphatic muscle cells, the parameter \( T_{\text{act}} \) was calculated for conditions describing the basal tone as well as the systolic contraction of the vessel, resulting in separate curves depicting the pressure values of \( \frac{\tau_{\text{act}}}{\text{max}} \) during active testing. These parameters were prescribed in order as 5% less than the minimum circumferential stretch achieved during passive testing plus 5%. The parameter \( \lambda_0 \) was imposed on the unloaded length was 3.57 mm, respectively (table 1); the thickness-to-radius ratio of 13% in the unloaded state in which some contractile tone is maintained as opposed to a mechanically passive state in which muscle cells generate no force.

\( T_{\text{bas}} \) was calculated as a function of transmural pressure to support the observation of a myogenic response. Constant values of \( T_{\text{bas}} \) were determined for each loading condition, resulting in three values of \( T_{\text{bas}} \) and three values of transmural pressure. A linear regression was performed on the data to determine values for parameters \( a \) and \( b \) following the form \( T_{\text{bas}} = f(P) = aP + b \), thus characterizing \( T_{\text{bas}} \) (and consequently \( T_{\text{mat}} \)) as a function of transmural pressure. In some cases, regression yielded a nearly vertical line due to almost identical maximum radii in the diastolic condition, and therefore one point was excluded from the regression so as to ensure that calculated parameters indicated a positive linear relationship between \( T_{\text{bas}} \) and transmural pressure. Following linear regression, the Matlab subroutine lsqnonlin was used to calculate \( T_{\text{act}} \) for a range of transmural pressure values for the purpose of plotting theoretical curves. A minimum of \( -b/a \) was imposed on the solver to prevent predictions of negative active stress.

2.6. Parameter estimation

The passive mechanical parameters and structural parameter were determined using the Matlab nonlinear regression function lsqnonlin and minimizing the error function
\[
e = \frac{1}{2} \sum_{i=1}^{n} \left( \frac{P_{\text{model}}^i - P_{\text{exp}}^i}{P_{\text{exp}}^i} \right)^2 + \sum_{i=1}^{n} \left( \frac{f_{\text{model}}^i - f_{\text{exp}}^i}{f_{\text{exp}}^i} \right)^2,
\]
where \( n \) equals the number of data points acquired during biaxial testing, \( P_{\text{model}}^i \) and \( f_{\text{model}}^i \) are the model simulation of transmural pressure and axial force values for data point \( n \), determined from equations (2.4) and (2.5), with \( T_{\text{act}} = 0 \) (and, thus, \( T_{\text{bas}} = 0 \)), \( P_{\text{exp}}^i \) and \( f_{\text{exp}}^i \) are the measured transmural pressure and axial force for data point \( n \), \( P_{\text{exp}} \) and \( f_{\text{exp}} \) are the average transmural pressure and axial force values over all data points, respectively.

After the passive parameters were determined via regression with the passive data, the values of \( T_{\text{bas}} \) and \( T_{\text{act}} \) were determined by minimizing equation (2.7) to the maximal contraction occurring during systolic contraction (\( T_{\text{act}} = T_{\text{sys}}^\text{max} \)) and minimum contraction occurring during diastolic relaxation (\( T_{\text{act}} = T_{\text{bas}}^\text{min} \)) at transmural pressure values of \( P = 3, 6 \) and 9 cmH2O. The parameter \( \lambda_0 \) was prescribed as the maximum circumferential stretch achieved during passive testing plus 5%. The parameter \( \lambda_0 \) was prescribed as 5% less than the minimum circumferential stretch achieved during active testing. These parameters were prescribed in order to capture the entire working range of active contraction. All parameter values are reported for individual samples as well as in the form of the mean ± s.e.m.

2.7 Statistical analysis

To evaluate significant differences in contractile amplitude as a function of mechanical loading, a two-tailed Student’s t-test was performed between all individual groups assuming equal variance between groups. Evaluations were performed between groups at varying axial stretches and fixed transmural pressure as well as varying transmural pressure and fixed axial stretches. Significance was considered as \( p < 0.01 \).

3. Results

3.1. Passive and active mechanical response

Average unloaded thickness was 32.2 ± 1.60 \( \mu \text{m} \), the average unloaded length was 3.57 ± 0.17 mm, and the average unloaded radius was 240 ± 10.9 \( \mu \text{m} \), respectively (table 1); thus, the thickness-to-radius ratio of 13% in the unloaded thickness supports the thin wall assumption and the diameter-to-length ratio suggests that end effects may be neglected within a central region of the vessel (figure 1). Loaded geometry was used to determine unloaded thickness from the incompressibility assumption. Standard errors associated with the calculation of unloaded thicknesses, based on measurements from the low-, medium- and high-loading scenarios, averaged 2.67 \( \mu \text{m} \), which is 8% of the mean unloaded thickness, and suggests that incompressibility may be a reasonable assumption. For a single value of axial stretch, the amplitude of spontaneous contractions decreased (i.e. the vessel becomes more like a conduit) as transmural pressure increased (figure 2). Pressure-induced amplitude reduction was significant between all values of transmural pressure at a fixed axial stretch of 1.3, but not 1.4 or 1.5. Similarly, contractile amplitude was markedly diminished upon increasing axial stretch at fixed values of transmural pressure (figure 2). Axial stretch-induced amplitude reduction was significant between all values of axial stretch at fixed transmural pressure values of 3 and 6 cmH2O. Differences were observed only between 1.3 and 1.5 at a transmural pressure of 9 cmH2O. Contractile frequency, however, was not diminished (data not shown).

Passive mechanical behaviour was observed and showed a stiffening response with increasing axial stretch, typical of soft tissues from the vasculature (figure 3). Axial force measurements had little variation at the lower values of axial stretch but generally increased to maximum force values ranging from 4 to 15 mN at the highest stretch values. Qualitatively, isobaric axial force versus axial stretch curves consistently intersected at axial stretch values between 1.3 and 1.4; the intersection of isobaric axial force–stretch curves is a characteristic response observed in blood vessels and often ascribed as the \( \text{in vivo} \) axial stretch [23,27]. We did not measure the \( \text{in vivo} \) axial stretch of the rat thoracic ducts during excision; however, an axial stretch of 1.4 seems reasonable based on our qualitative observations.

3.2. Material parameter calculation

Nonlinear regression yielded material parameters that provided a good fit of the passive mechanical testing data
Table 1. Measurements and calculations of loaded and unloaded geometry for individual rat thoracic ducts. Vessels were mounted on a biaxial testing device and oriented in a traction-free condition. Unloaded length and outer radius were determined by inspection using a custom LABVIEW program. Loaded thicknesses from three loading conditions were determined using confocal microscopy. The loading conditions consisted of low loading ($p = 3\ \text{cmH}_2\text{O}$, $l = 1.3$), mid-range loading ($p = 9\ \text{cmH}_2\text{O}$, $l = 1.45$) and high loading ($p = 20\ \text{cmH}_2\text{O}$, $l = 1.6$ or highest stretch value during passive testing) corresponding to $h_{\text{low}}$, $h_{\text{mid}}$ and $h_{\text{high}}$, respectively. Unloaded thickness ($H$) was calculated by applying the known loaded and unloaded geometry to the incompressibility assumption and averaging the unloaded thickness calculations from the low-, mid- and high-loading conditions. s.e.m. for each calculation of the average unloaded thickness is also reported.

<table>
<thead>
<tr>
<th></th>
<th>$L$ (mm)</th>
<th>$R_o$ (µm)</th>
<th>$h_{\text{low}}$ (µm)</th>
<th>$h_{\text{mid}}$ (µm)</th>
<th>$h_{\text{high}}$ (µm)</th>
<th>$H_{\text{ave}}$ (µm)</th>
<th>s.e.m. (µm)</th>
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<tbody>
<tr>
<td>1</td>
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<td>16.0</td>
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<tr>
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<tr>
<td>5</td>
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<td>260</td>
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</tr>
<tr>
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<td>280</td>
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<td>16.0</td>
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<tr>
<td></td>
<td>average</td>
<td>3.57</td>
<td>240</td>
<td>19.2</td>
<td>16.9</td>
<td>15.2</td>
<td>32.2</td>
</tr>
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|    | s.e.m.   | 0.17       | 10.9                   | 0.78                  | 0.63                   | 0.69                  | 1.60        | 0.45
In general, the majority of the error in the passive fits appeared to occur either in the toe region of the plots where the collagen fibres become engaged or in the higher values of axial stretch in which collagen fibres appear to be constantly engaged.

Regression yielded material parameters that provided a good fit of the contractile testing data (table 3 and figure 5). Average errors were calculated using equation (2.8), and the values associated with the active parameters $T_{\text{act}}^{\text{max}}$ and $T_{\text{act}}^{\text{bas}}$ were $6.6 \pm 2.6\%$ and $6.8 \pm 3.4\%$, respectively. The parameter $a$ provides physical insight into the dependence of the basal parameter on transmural pressure. Calculation of this parameter showed relatively consistent basal tone in all samples with the exception of specimen 5, which had very little dependence on transmural pressure. Although a myogenic response was absent from this specimen, we believe this to be an indication of the variability in lymphatic behaviour rather than an outlier in the dataset, as the contractility for that specimen met the inclusion criteria defined in the methods. Note also that, for specimen 3, a single datum point was excluded from the error calculation in the basal condition due to a skewed error calculation. The inaccuracy was affected by the large slope of the basal curve (thus a very large change in transmural pressure is required for modest changes in diameter). Eliminating this datum point from the error calculation had little effect on the theoretical transmural pressure versus diameter curve for specimen 3.

### 3.3. Collagen and elastin organization

A single outer layer of collagen and a single inner layer of elastin were consistently identified in all samples using confocal microscopy (figure 6). Similar observation of the layered structure of lymphatic vessels was reported by Arkill et al. [12]. Qualitative observations of the fluorescence spectrum of elastin suggested the presence of elastin fibre families primarily oriented at two distinct angles that were near axial and circumferential directions. Collagen fibres showed a more undulated morphology at lower loading conditions and appeared to recruit fibres more gradually throughout the loading process than elastin. Despite consistency in the elastin fibre orientations, significant differences in collagen fibre distributions were observed across samples,

![Figure 3](http://rsif.royalsocietypublishing.org/)

**Figure 3.** Passive transmural pressure versus diameter (**a**) and axial force versus transmural pressure (**b**) response of specimen 4 for a range of axial stretch values. Lowest and highest values of axial stretch are denoted by arrows. Note that increased axial stretch induces an increase in axial force and a stiffening response that occurs at progressively lower diameter values. (**c**) Isobaric axial force versus axial length curves for specimen 4. The intersection point of the curves has previously been used to estimate the in vivo axial stretch of arteries. (**d**) Transmural pressure versus outer diameter data for six thoracic ducts at an axial stretch of 1.3. Note the large variability in geometry between samples. The maximum diameter for the largest vessel is approximately 50% larger than that of the smallest vessel (approx. 800 \(\mu\)m compared with approx. 530 \(\mu\)m). The specimen denoted in red corresponds to the vessel represented in (**a–c**).
Table 2. Passive material parameter calculations for rat thoracic ducts using a four-fibre constitutive model. Data are reported as the mean ± s.e.m. The family of parameters $b_i^1$ exhibits a much higher degree of variability than the family of parameters $b_i^2$. The structural parameter exhibits consistency, and all parameters yield error values that suggest reasonable fits of the experimental data.

<table>
<thead>
<tr>
<th></th>
<th>$b$ (dyne cm$^{-2}$)</th>
<th>$b_1^1$ (dyne cm$^{-2}$)</th>
<th>$b_2^1$ (dyne cm$^{-2}$)</th>
<th>$b_3^1$ (dyne cm$^{-2}$)</th>
<th>$b_4^1$ (dyne cm$^{-2}$)</th>
<th>$b_1^2$ (dyne cm$^{-2}$)</th>
<th>$b_2^2$ (dyne cm$^{-2}$)</th>
<th>$b_3^2$ (dyne cm$^{-2}$)</th>
<th>$\alpha$ (°)</th>
<th>error (%)</th>
</tr>
</thead>
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4. Discussion

Lymphatics play a key role in a diverse set of physiological functions, including tissue fluid balance, lipid absorption and immune cell trafficking. As is the case for other soft tissues, mechanically mediated biological mechanisms probably play a central role in lymphatic function (or dysfunction) and disease progression. Experimental studies have shown that lymphatic contractility is altered following changes in transmural pressure [10,28] and changes in axial pressure gradients [3,5], and clinical data have shown that lymphoedema leads to dramatic changes in lymphatic vessel geometry (e.g. thickness, radius, lymphatic muscle hyperproliferation) [29]. While it is well known that tissues remodel in response to changes in their local mechanical environment, altered loading conditions such as those found in lymphoedema have yet to be directly linked. To study mechanically mediated remodelling of lymphatics, accurate quantification of the local mechanical environment of lymphatics is required; however, a critical gap remains in quantifying the biaxial mechanical response of lymphatic tissue and modelling the mechanical behaviour in a framework that allows quantification of the local mechanical environment for relevant applied loads and vessel geometries. Such a model represents a critical step in the investigation of mechanically mediated dysfunction and disease progression.

To that end, we have identified material parameters, combining the models from Baek et al. [26] and Rachev & Hayashi [21], to accurately describe the mechanical behaviour of rat thoracic ducts to passive cylindrical biaxial testing and spontaneous contraction.

4.1. ‘Microstructurally motivated’ passive constitutive model

The functional forms of the constitutive equations were motivated by the tissue microstructure; however, microscopy revealed both consistencies and inconsistencies between the microstructure captured in the constitutive model and that observed in the tissue. First, although elastin is often modelled as an isotropic material [25,30–32], qualitative observations of elastin orientation suggested nearly circumferentially and nearly axially oriented fibres. Second, although we did not quantify their organization, there are nearly circumferentially oriented smooth muscle cells. Third, we observed collagen fibres oriented at off-axis angles. Fourth, in addition to these elastin structural components, collagen structural components, and smooth muscle cells, an amorphous, presumably isotropic mechanically significant solid, probably containing proteoglycans, glycosaminoglycans, water and other extracellular matrix constituents, exists in which these structural components are embedded. Thus, the proposed four-fibre family model (equation (2.6)) captures many of the key salient features of the observed microstructure. Namely, the axial fibre family...
parameters by the summation of the basal and systolic contributions. Contributions to the behaviour was calculated from the combination of passive plus active stresses and provides a good fit of the active data. Theoretical simulation of transmural pressure versus circumferential stretch behaviour for specimen 2 at an axial stretch value of \( \lambda \) = 1.3. Theoretical simulation of transmural pressure versus circumferential stretch behaviour was calculated from the combination of passive plus active stress and provides a good fit of the active data. Contributions to the behaviour from the basal (dashed line) and systolic (dotted line) parameters are denoted on the graph. Note that the maximal systolic condition is determined by the summation of the basal and systolic contributions.

captures the contribution from the axially oriented elastin structural element (and perhaps the contribution from axial collagen components), the circumferential fibre family captures the combined role of passive smooth muscle and the circumferentially oriented elastin structural element (and perhaps the circumferential collagen components), the fibre families at \( \pm \alpha \) capture the combined contribution of collagen fibres oriented off-axis, and the isotropic term captures the contribution from the amorphous solid.

There are, however, several important inconsistencies between our microstructurally motivated constitutive model and the observed microstructure. For several vessels, the collagen fibre distributions were asymmetric, often with a single peak oriented off-axis (figure 7), suggesting material asymmetry. To accurately identify material parameters for an asymmetric material requires one to quantify both the shear strains and the torsional load during fixed length pressurization; more rigorously, one should also perform torque-twist tests on the tissue [33]. Although quantifying shear strains can be done via tracking of markers or particle image velocimetry, the measurement of applied torque is technically challenging and has not been reported for tissues of this calibre.

Without this torsion boundary condition, proper analysis to identify material parameters for a proposed asymmetric constitutive model cannot be performed. Indeed, other asymmetries are also apparent; namely, the elastin fibres and smooth muscle cells are not oriented at exactly 0° and 90°. Note that material asymmetries exist in most arteries, yet rarely are torsional loads quantified (particularly in small-calibre vessels) and rarely are these material asymmetries incorporated into the functional form of the constitutive model, perhaps because the nominal values and biological significance of these shear strains and stresses are thought to be of secondary importance compared with the normal strains and stresses. Thus, although our model is motivated by microstructure, we do not use measured microstructure as an input to the constitutive model. Despite the technical challenges required for such an effort, the results herein motivate the need for incorporating micro-structural measurements into the constitutive model [25] and quantifying relevant boundary conditions and deformations so that such modelling efforts may be performed.

It is worth noting that the passive model contains limitations in the ability to recapitulate the experimental data. Difficulties were consistently encountered in capturing the highly nonlinear pressure versus diameter response at lower axial stretch values (figure 5) because of the sharp strain-stiffening transitions. Similarly, the axial force versus transmural pressure simulations in some cases produced negative force values at lower axial stretch and higher transmural pressure values (figure 4b) because experimental force values approached zero in such cases. These difficulties could present potential problems in simulating lymphatic functional behaviour, especially in attempting to predict mechanically mediated lymphatic failure; thus, future investigations should aim to improve upon the current model in order to better capture the highly dynamic nature of these vessels. However, given the reasonably low error associated with the passive fits, the theoretical model was considered to be sufficient for characterizing the behaviour of lymphatic vessels in this context.

4.2. Sample-to-sample variations and material heterogeneity

We observed significant variations in mechanical response (figure 3) and material parameters (tables 2 and 3), geometry (table 1) and microstructural organization (figure 7) across samples; such variability is not uncommon in the lymphatic
circulation [13,34,35]. Loaded thicknesses in rat thoracic ducts reported in the literature are consistent with the present data [9,13]; geometric and functional parameters generally have large standard errors, suggesting large sample-to-sample variations. Similarly, others have used confocal microscopy to quantify angle orientation in bovine mesenteric lymphangions and report similar variability to that reported herein [12]. Geometric variability in human thoracic ducts as a function of longitudinal position has been reported [34] and we (data not shown) and others have reported heterogeneities in the microstructural organization along the length of a single lymphangion [9].

Such heterogeneities and sample-to-sample differences in geometry, microstructure and mechanical behaviour render quantifying a unified constitutive model for the rat thoracic duct challenging. Indeed, it further motivates the need for a constitutive model that links microstructural measurements to the tissue-level mechanical response. The goal of this paper was to isolate the mechanical behaviour of a central, nearly homogeneous region of a lymphangion, quantify the deformation and boundary conditions for this central region, identify an appropriate constitutive model for this homogeneous region that captures well the tissue-level mechanical response, and quantify the microstructure of the same region. We submit that an accurate constitutive equation that links the microstructural architecture to the tissue-level biomechanical behaviour for a homogeneous region is a required first step before the material heterogeneities can be accurately modelled. Nevertheless, recognizing that the lymphatic circulation contains large sample-to-sample variability is an important characteristic to report. Our field still lacks a reasonable explanation as to why, with two seemingly similar breast cancer patients, one patient progresses to secondary lymphoedema following mastectomy and the other does not. Of course, there are many factors at play in disease progression; however, ‘normal’ variations in the geometry, structure, and mechanical and functional behaviour may be one important factor.

4.3. Sample-to-sample variation in passive material parameters

Passive parameters obtained via nonlinear regression spanned multiple orders of magnitude (table 2); such variations may be due to several factors. First, given that we observed significant sample-to-sample variations in the mechanical response, geometry and microstructural organization, it is perhaps not surprising that there were also large variations in material parameters obtained via nonlinear regression. Second, identification of material parameters for exponential constitutive models via regression to biaxial mechanical testing data often results in large deviations across samples, in some cases spanning two to three orders of magnitude in murine tissue and 10–15 orders of magnitude in human tissue [23,36,37], even when sample-to-sample variations in mechanical behaviour are low; non-uniqueness may be a factor [20,38]. This observation highlights the limitation that, although microstructural observations motivated the functional form of the constitutive equation, the resulting model remains a phenomenological description of the mechanical behaviour of the tissue. For example, the material parameter $b$, which is the modulus in the term motivated by the presence of an
amorphous material in which fibres are embedded, varies from 0.00018 to 661.6 dynes cm$^{-2}$; it may be unreasonable to suggest that the modulus of the amorphous solid varies by six orders of magnitude across samples. A target of future work should be to explore parameter estimation techniques that employ more defined parameter constraints [39]. Note that we were unable to identify models with fewer material parameters (e.g. two- or three-fibre family models) that provided an adequate fit to data. All parameters were restricted to be positive; however, as our goal was to identify a model that provided the best fit to data, no other restrictions were placed on their value. We also surveyed a broad range of initial guesses during regression to ensure that a global minimum was identified. Restricting the values of material parameters in order to ascribe a more physical meaning and microstructural motivation to each may be an opportunity for future work [40].

Figure 7. Frequency intensity plots for low- (blue), mid- (green) and high- (red) loading conditions as a function of orientation angle for six rat thoracic ducts (a–f for specimens 1–6, respectively). An angle of 0° denotes an orientation in the axial direction. Primary angle orientations were easily identified by a large peak in frequency intensity. Peaks associated with secondary angles were more difficult to interpret. Data suggest that material asymmetry exists in all specimens. Note that the highest normalized intensity values occur in the high-loading conditions of every specimen, indicating increased alignment of collagen fibres as loading conditions are increased.
4.4. Modelling spontaneous contraction and basal tone

Lymphatic muscle cells have characteristics in common with both smooth muscle cells, which exhibit tonic contraction, and cardiac muscle cells, which exhibit rapid and spontaneous contraction [41,42]; both characteristics are observed here and are captured in the active model. The contraction results suggest that the maximum contraction is captured well by the functional form of Rachev and Hayashi, but that the basal tone that occurs during the resting phase exhibited a pressure (or strain) dependence beyond that described in the strain-dependent response in equation (2.7): modelling a linear dependence between the basal parameter and transmural pressure provided a reasonable fit to data. In general, material parameters should not be prescribed as a function of applied loads (e.g. pressure), but rather as a function of an appropriate strain measure; this is a limitation of the current active model. However, with the data reported herein, at fixed length (i.e. fixed axial strain), the diastolic diameter (and circumferential strain) remained nearly constant with increasing pressure, presumably due to a myogenic response from the muscle cells; such singularities prohibit relating basal tone to strain using the original model in equation (2.7) because the model cannot produce the highly nonlinear behaviour necessary to recapitulate maintenance of load-dependent tone without prescribing unrealistic values for the parameters $\lambda_{\delta \varepsilon}$ and $\lambda_{\delta \varepsilon}$. Davis et al. [43] reported myogenic responses in rat lymphatic vessels that are time-dependent following a step-change in transmural pressure and reported [3] a decrease in maximum diastolic diameter following an increase in afterload in the rat mesentery. Gasheva et al. [44] have reported increases in end diastolic diameter of rat thoracic ducts due to pumping-induced production of nitric oxide. Further, these data suggest that contraction amplitude (figure 2) and frequency (not shown) are highly dependent on the axial stretch; however, the current active model does not consider such axial dependencies. Developing a new functional form for the active stress as a function of appropriate circumferential and axial strain measures and material constants (and performing a broader range of theoretically motivated experiments to quantify these material constants for lymphatic vessels) should be a target of future work.

Taken together, these findings provide a framework for future investigations targeting lymphatic growth and remodelling as they relate to the progression of diseases such as lymphoedema. Given that an important role of the lymphatic system is the transport of fluid from tissues to the venous system, many functional parameters that have been quantified in the literature relate directly to the ability of the vessels to transport fluid (e.g. contractile amplitude and contractile frequency). It is unknown how these functional parameters may be affected by the internal forces experienced in these vessels, and it therefore remains necessary to quantify these forces. It is well known that mechanical factors such as transmural pressure, stretch and flow direct tissue growth and remodelling [30,40,45–47], and quantification of stresses in lymphatics will provide a foundation for simulating growth and remodelling in an effort to quantify the effect of mechanically mediated geometrical changes on common functional parameters describing contractile behaviour. This proposed model is the first to successfully simulate both the active and passive behaviour of a lymphatic vessel using a microstructurally motivated model and provides potential for long-term predictions of mechanically mediated maladaptive remodelling as well as identification of primary parameters contributing to the development of diseases relating to the lymphatic vasculature.

5. Conclusion

In closure, this paper presents experimental data and a computational model to accurately quantify the active and passive mechanical behaviour of rat thoracic ducts and highlight the significant variations in mechanical response, geometry and microstructural organization across rat thoracic duct samples. This study has several limitations: the model does not directly input microstructural measurements into the constitutive model, nor does it capture the observed material asymmetry or heterogeneity; there are large deviations in calculated material parameters across samples; the basal tone was correlated to pressure (an applied load) rather than an appropriate strain measure; and the contractile response neglects axial stretch dependencies. There is a pressing need to develop a microstructural model that accurately captures the mechanical response of lymphatics, including material heterogeneities and asymmetry and multi-faceted contractile function. Such a model can be incorporated into computational frameworks for lymph transport function (or dysfunction) and tissue growth and remodelling in health and disease. This paper provides an important first step towards this end.

Authors’ contributions. A.W.C. carried out mechanical testing and confocal imaging, developed the mathematical framework, analysed mechanical testing data, participated in the design of the study and drafted the manuscript; Z.V.N. carried out excision of lymphatic vessels, assisted with analysis of mechanical testing data and participated in the design of the study; R.S. assisted with confocal imaging and analysed confocal data; J.B.D. and R.L.G. conceived, designed and coordinated the study and helped draft the manuscript. All authors gave final approval for publication.

Competing interests. We declare we have no competing interests.

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