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A comprehensive study of layer-specific morphological changes in the microstructure of carotid arteries under uniaxial load

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Abstract The load bearing properties of large blood vessels are principally conferred by collagen and elastin networks and their microstructural organization plays an important role in the outcomes of various arterial pathologies. In particular, these fibrous networks are able to rearrange and reorient spatially during mechanical deformations. In this study, we investigate for the first time whether these well-known morphological rearrangements are the same across the whole thickness of blood vessels, and subsequently if the underlying mechanisms that govern these rearrangements can be predicted using affine kinematics. To this aim, we submitted rabbit carotid samples to uniaxial load in three distinct deformation directions, while recording live images of the 3D microstructure using multiphoton microscopy. Our results show that the observed realignment of collagen and elastin in the media layer, along with elastin of the adventitia layer, remained limited to small angles that can be predicted by affine kinematics. We show also that collagen bundles of fibers in the adventitia layer behaved in significantly different fashion. They showed a remarkable capacity to realign in the direction of the load,

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whatever the loading direction. Measured reorientation angles of the fibers were significantly higher than affine predictions. This remarkable property of collagen bundles in the adventitia was never observed before, it shows that the medium surrounding collagen in the adventitia undergoes complex deformations challenging traditional hyperelastic models based on mixture theories.

Keywords Carotid artery · microstructure · adventitia · media · uniaxial load

1 Introduction

Cardiovascular disorders are a significant public health issue affecting ageing populations globally and causing considerable public health expenses (31% of total mortality in 2012 - source: World Health Organization). In numerous cases, the disorder involves significant changes in the vascular mechanical properties, generating extensive studies about arterial biomechanics and mechanobiology. In this respect, a common approach to vascular biomechanics consists in submitting samples of arterial tissue to mechanical bench tests in order to characterize their macroscopic mechanical properties. Existing investigations [4,37,14] consisted in applying a tensile loading on flat samples of arterial material, revealing in particular the material's ability to undergo large strains and a characteristic stiffening occurring above a given tensile stress. In order to capture this complex mechanical behavior and a potential anisotropy of the response, uniaxial tensile tests have been performed independently in the axial and circumferential directions [32,8], and separately to the different layers of the composite structure of the arterial wall [22,46]. In parallel to this macroscopic characterization of the mechanical properties, the microstructure of the vascular wall has been extensively analyzed by different microscopy techniques. In particular scanning electron microscopy allowed a morphological analysis of the arterial microstructure at the micron scale [20,55,12,35]. *As for dynamic 2nd harmonic microscopy, it enabled simultaneous image acquisition and optical analysis of collagen fiber orientation, by means of the collagen's optical property of second harmonic generation in the presence of*

23 an intense laser beam coupled to a polarizer [43,48,26]. Confocal microscopy and
24 multiphoton microscopy enabled live imaging with independent emissions signals
25 corresponding to elastin and collagen [50] providing a 3D point of view on morpho-
26 logical characteristics of the vascular wall, and allowing the evaluation of volume
27 fractions of the different components of the microstructure [31]. These techniques
28 revealed the morphology of each concentric layer (intima, media, adventitia). Con-
29 cerning the intima, it is composed of endothelial cells, oriented longitudinally [37].
30 As for the media, it is composed principally of circumferentially oriented smooth
31 muscle cells and collagen fibers embedded in an elastin network [56,10,53,13,16].
32 Finally, the adventitia is composed of thick collagen bundles and of a net of heli-
33 cally oriented elastic fibers [6]. Recently, a special attention has been dedicated to
34 further characterize collagen and elastin fibers, for instance by measuring the wavi-
35 ness of adventitial collagen [36], fiber segment length and the radially-connecting
36 fiber density in the media [49,29]. Also, several studies started investigating the
37 link between the arterial tissue's macroscopic mechanical response and the associ-
38 ated rearrangements of its microstructure, by coupling mechanical testing with live
39 microscopy. The latter studies confirmed the load-bearing properties of collagen
40 fibers and revealed a progressive morphological rearrangement under load, namely
41 decrimping and reorientation of the collagen fibers in the direction of the load, as
42 well as the subsequent stiffening of the material's response [48,45,18,54]. Those
43 tests consisted in the application of uniaxial tension on flat samples [52,21,38],
44 biaxial tension on flat samples [39,23,47,27,28,7], or tension-inflation on cylindri-
45 cal samples [19,58,17]. The observed morphological rearrangements concerned in
46 particular bundle waviness and orientation [6,40,28,54].

47 As a conclusion, extensive characterizations of both the mechanical layer-
48 specific anisotropic behavior of the arterial wall, and the load-free microstructure
49 morphology exist. Moreover, studies aiming at the characterization of load-induced
50 microstructure rearrangements also exist and come up with advanced insights into
51 the coupling between macro-mechanical response and tissue microstructure rear-

52 rangements. However neither the underlying microscopic mechanisms governing
53 the load-induced microstructure rearrangements nor the inter-layer differences in
54 the rearrangements of the collagen and elastin networks have been characterized.
55 This paper is devoted to bridge these gaps in knowledge, by answering the fol-
56 lowing questions: are the well-known morphological changes of the vascular tissue
57 under load (uncrimping, realignment) dependent on the direction of the load, on
58 the vascular layer, and on the constituent under consideration? Are these mor-
59 phological changes governed exclusively by the rule of affine transformations [5,
60 25]?

61 **2 Materials and methods**

62 **2.1 Sample preparation**

63 Seven carotid arteries (Fig. 1(a)) were harvested from healthy male New Zealand
64 White rabbits, weighing 3 kg approximately. Excisions were realized at the Veteri-
65 nary Campus of the Université de Lyon (VetAgro Sup, Marcy l'Étoile, FR). Rab-
66 bit cadavers, previously sacrificed under compliance with the NIH Guide for Care
67 and Use of Laboratory Animals, were kindly provided by Centre Lago (Vonnas,
68 FR). The length of each carotid was measured *in vivo* and *ex vivo*, i.e. immedi-
69 ately after harvesting (Table 1 - columns 2 and 3), in order to evaluate the *in vivo*
70 pre-stretch condition (computed as the ratio of the *ex vivo* length to the *in vivo*
71 length, Table 1 - column 4). The arteries were immediately frozen at -20 °C until
72 the day of the experimental tests and unfrozen in a bath of phosphate-buffered
73 saline (10x PBS, pH 7.1) at ambient temperature (24 °C). 10 mm long cylindrical
74 portions were excised from the arteries and longitudinally cut open, with a result-
75 ing width of approximately 5 mm. For each artery, a 0.5 mm long ring (Fig. 1(b))
76 was also extracted for optical measurement of the arterial thickness (Table 1 - last
77 column). This resulted in a cross-sectional area of $0.5 \pm 0.1 \text{ mm}^2$. The rectangular
78 strips were cut into dogbone shapes [21] aligned along the three following in-plane

79 directions: circumferential, longitudinal, and an intermediate direction making a
80 45° angle with respect to the longitudinal direction (see Fig. 2(a) for a sketch of
81 the sample preparation). In the following, this intermediate direction will be re-
82 ferred to as the diagonal direction. Twelve samples (four in each orientation group)
83 were dedicated to mechanical testing coupled to multiphoton microscopy, while 6
84 additional samples (two in each orientation group) were dedicated to mechanical
85 testing alone.

86 2.2 Tensile test

87 A screw-driven high precision tensile machine (Deben[®] Microtest tensile/compression
88 stage) was used for uniaxial tensile tests, with a 150 N capacity load cell (Fig. 1(c)).
89 The load cell signal provided a 0.01 N precision with satisfactory stability. The
90 two heads of the tensile device moved in opposite directions, while the force and
91 the displacement were recorded. Particular care was taken to ensure that the proto-
92 col for sample fixation limited the risk of inducing any pre-strain or pre-stress
93 in the sample. Each sample underwent quasi static triangular preconditioning.
94 According to the choice made by Hill et al. [21], a displacement-speed control was
95 applied a rate of $0.5 \text{ mm}\cdot\text{min}^{-1}$, corresponding to a relative elongation speed of
96 0.2 min^{-1} , with fixed target tensile force equal to 1N. During the mechanical test,
97 the sample was continuously immersed in PBS at a constant ambient tempera-
98 ture of 24 °C. After preconditioning, four measures of the sample width in the
99 unloaded configuration were recorded using a caliper and averaged [21]. An image
100 of the harvested ring of the artery was taken using a macro optic objective (Nikon
101 D7200[®] optical camera equipped with Nikon AF-S VR Micro-Nikkor[®] optical
102 105 mm f/2.8G IF-ED lens). The caliper in 0.5 mm opening position was placed
103 next to the sample ring in order to calibrate the pixel size and subsequently measure
104 the thickness of the arterial wall. The measures of the sample width and thick-
105 ness allowed the computation of the reference cross-sectional area A_0 (under zero
106 load, after preconditioning) and of the first Piola-Kirchhoff (engineering) stress

107 [21] $\sigma_{PK} = F/A_0$, where F is the displacement-dependent measure of the tensile
 108 force. The stretch was computed from the actual and reference inter-clamp length
 109 of the strip (respectively l and l_0) as: $\lambda = l/l_0$. The reference length of the strip
 110 was defined as the interclamp length of the strip after preconditioning.

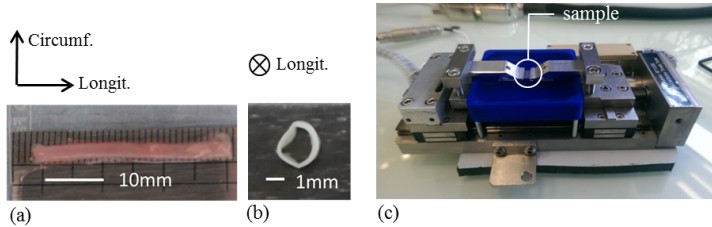


Fig. 1 (a) Excised carotid artery from a New Zealand White rabbit; (b) Cross-sectional ring of the artery for optical thickness measurement; (c) Tensile machine.

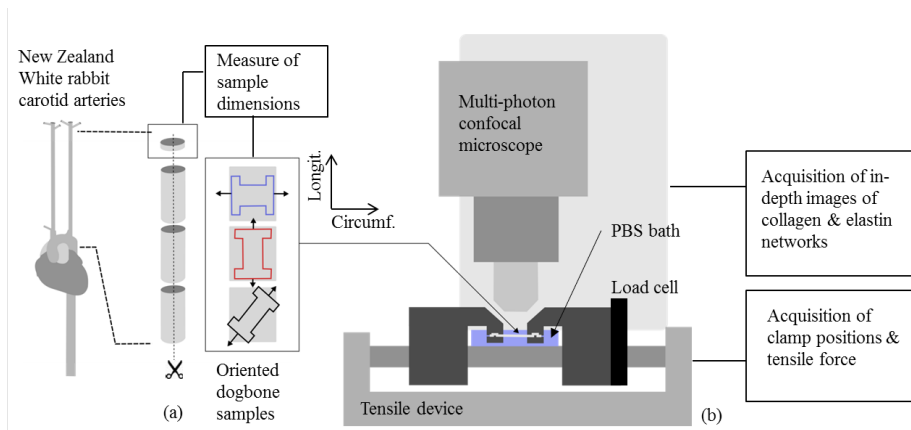


Fig. 2 (a) Schematic representation of the vascular tissue samples, prepared from cut-open cylindrical portions of New Zealand White rabbit carotid arteries; (b) Schematic representation of the experimental setup composed of a tensile machine coupled to a multiphoton microscope.

111 2.3 Multiphoton Microscopy

112 A multiphoton microscope (NIKON, A1R MP PLUS) of the IVTV platform (En-
 113 gineering and Ageing of Living Tissues Platform, ANR-10-EQPX-06-01) was used

114 to image the collagen and elastin networks of the samples (Fig. 2(b)). Setting the
115 excitation wavelength to 870 nm [21] allowed collecting autofluorescence and col-
116 lagen second harmonic generation (SHG) signals through 500-550 nm and 400-492
117 nm band-pass filters respectively [25], without any staining or fixation. The imag-
118 ing resolution was set to 0.5 μm in all directions [21,40] with a 512 μm^2 imaging
119 window. This setting resulted in stacks of images characterized by 60 to 90 μm
120 thicknesses, depending on the quality of the signal at in-depth focal position. In
121 order to achieve an optimal compromise between image quality and acquisition
122 time, the scan speed was set to 0.25 frames per second, with two-frame averaging.
123 This setting resulted in acquisition times of approximately 20 to 30 minutes for a
124 given elongation configuration. Each arterial tissue sample underwent two imag-
125 ing sequences: one sequence with the adventitial side facing the objective of the
126 microscope after five preconditioning cycles [21]; and the other sequence with the
127 intimal side facing the objective after two additional stabilizing preconditioning
128 cycles. Images were taken in the unloaded configuration and in three gradually
129 loaded configurations, respectively at 0.2 N, 0.5 N, and 0.8 N. Before imaging, a
130 period of 10 to 15 minutes was observed in order to stabilize the tissue after initial
131 material creep. Creep dynamics were previously analyzed and it was shown that 10
132 minutes were sufficient to avoid an important creeping. It was also observed by live
133 imaging that possible further creep did not modify noticeably the microstructure
134 morphology.

135 2.4 Image analysis

The image stacks representing collagen and elastin were analyzed in terms of spa-
tial orientation of the fibrous networks. Previous studies have investigated the
transmural angle (radial direction) of the fibers and showed that it is negligible in
comparison to the in-plane angle [38,36,40]. They have also showed that the most
relevant morphological changes in the microstructure occur in the circumferential
and longitudinal directions. Subsequently, we focused in the following on the anal-

ysis of the in-plane angles of the fibrous networks. Prior to the analysis of relative angle densities, the image stacks were pre-processed so that they could represent the true tissue morphology on two-dimensional images while enabling liable angle density extraction. To this aim, the stack portion representing the adventitial layer of the vascular wall was subdivided into three equally thick sublayers (20-30 μm) and each sublayer was projected orthogonally onto a single image using a maximum intensity projection algorithm. The choice of three sublayers allowed to achieve an optimum between image pixel density and fiber trans-mural continuity (due to non-zero transmural angles which could cause bias in the angle density analysis). Pre-processing of the media stack consisted in a projection in the thickness of one representative lamella (5-10 μm), after verification of the equivalent morphologies of the different lamellae. The resulting set of 2D images used for the analysis of the fiber angle density consisted in three images of the adventitial collagen, three images of the adventitial elastin, one image of the medial collagen and one image of the medial elastin (Fig. 3(a)). The relative angle densities of the fibrous networks seen on each of these images were analyzed by combining wedge-shape integration of the Fourier power spectrum [2,42,41] (Fig. 3(b)) and a custom method for the extraction of local density maxima with their associated dispersions (Fig. 3(c)). Local density maxima were determined by a standard peak detection algorithm. The associated dispersions were evaluated by first determining an arbitrary density threshold that represented a cumulative percentile of total fiber angles extracted from the image, and secondly by reading out angle values corresponding to this threshold. Explicitly, let θ represent a local orientation angle, and $d(\theta)$ the angle density of the analyzed image; the angle density threshold p is the angle density below which the fraction $\alpha_p = 0.8$ of the total fibers is counted, i.e. the solution of the following equation:

$$\alpha_p = \frac{\int_0^\pi \min(d(\theta), p) d\theta}{\int_0^\pi d(\theta) d\theta} = 0.8. \quad (1)$$

136 We evaluated corresponding angles about each detected local maximum in order
137 to extract related relative dispersions (Fig. 3(c)).

138 2.5 Predicting the amplitude of fiber rotation by affine reorientation

The assumption of affine reorientation considers that the fibers are continuously embedded in their surrounding matrix and are therefore constrained to the same deformation gradient as the matrix [3,5,25]. For a uniaxial tensile loading, the fiber orientations θ can be computed as a function of the initial angle θ_0 and deformation stretch λ (Fig. 3(d)) by:

$$\theta = \arctan(\lambda \tan \theta_0). \quad (2)$$

139 The angle values predicted by Equation (2) are compared to the experimentally
140 measured angles in order to analyze to which extent this affine reorientation can
141 predict true fiber network reorientations. In particular, the adventitial collagen
142 bundles showed, in the load-free state, a dense crimped configuration for which it
143 was difficult to extract global orientations of the bundles using traditional image
144 processing techniques (Fourier analysis, local gradients, ...). In order to avoid a
145 subsequent imprecision of the analysis, **a specific method was designed to isolate**
146 **the global fiber rotation mechanism from the uncrimping mechanism, and to test**
147 **the hypothesis of affine kinematics only on the global reorientation of the fibers. To**
148 **this aim**, we considered a deformation scenario which starts in the diagonally de-
149 formed state and reaches the circumferentially and longitudinally deformed states
150 in two sequenced steps: diagonal unloading followed by circumferential or longi-
151 tudinal loading. **Accordingly, only deformed configurations with straight collagen**
152 **bundles needed to be processed for fiber angle analysis.** Prediction errors were com-
153 puted by dividing the difference between experimental and predicted fiber angles
154 by the experimental fiber angle.

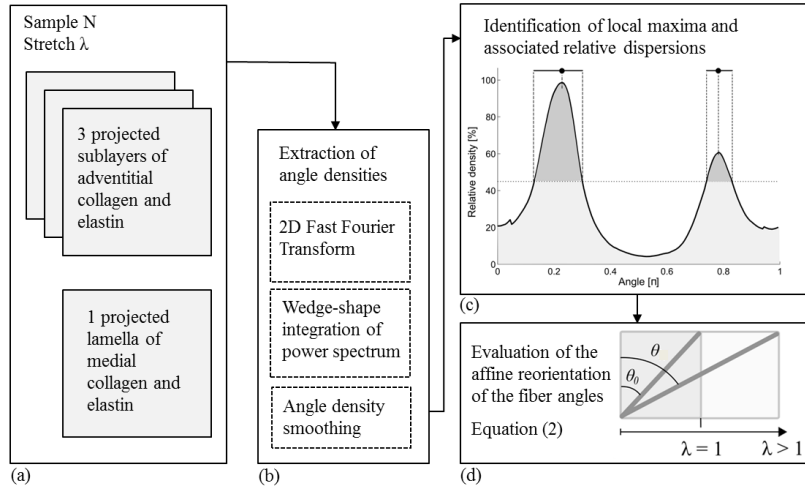


Fig. 3 Summary of the image analysis method. (a) Partial z-projections of adventitial and medial microstructure image stacks; (b) Fast Fourier 2D analysis of microstructure images; (c) Local maxima detection and associated relative dispersions evaluation on smoothed fiber angles density function; (d) Analytical computation of affine fiber reorientation.

155 3 Results

Specimen	<i>Ex vivo</i> length [mm]	<i>In vivo</i> length [mm]	<i>In vivo</i> stretch [-]	Thickness [mm]
1	4.3	7.1	1.6	0.18
2	4.1	6.9	1.6	0.17
3	4.3	7.0	1.6	0.19
4	4.2	6.9	1.6	0.16
5	4.3	7.5	1.7	0.18
6	4.0	6.5	1.6	0.18
7	3.9	6.6	1.7	0.19

Table 1 Geometrical measurements of the harvested carotid arteries.

156 3.1 Macromechanical response of the arterial tissue

157 Engineering stress evaluated against stretch revealed a macroscopic anisotropy of
 158 the material's mechanical response, as shown by (i) the characteristic stiffening
 159 of the samples starting at different stretch values depending on the direction of
 160 the load (Fig. 4(a)); (ii) the estimated elastic moduli in the final loading regime

161 (Figure 4(b)-middle), and (iii) the stretch values when reaching 0.5 MPa (Figure
162 4(b)-right). In particular, the mean estimated elastic moduli in the final loading
163 regime was 50% higher in the circumferential direction than in the longitudinal
164 direction; and 20% higher in the circumferential direction than in the diagonal
165 direction. Accordingly, the mean stretch when reaching 0.5 MPa was 20% lower
166 in the circumferential direction than in the longitudinal direction; and 15% lower
167 than in the diagonal direction. Concerning the estimated elastic moduli in the
168 initial loading regime (Figure 4(b)-left), the comparison of mean values showed
169 lower differences between the sample families as their standard deviation amounts
170 to 8%. Comparing the measured *in vivo* stretches to the longitudinal mechanical
171 response (Table 1), the carotid were subjected *in vivo* to a longitudinal stress of
172 about 0.1 MPa, in the soft region of the curve (Fig. 4 - red curves).

173 3.2 Microstructure morphological rearrangements under uniaxial load

174 Fibrous networks of the arterial microstructure revealed varying morphologies de-
175 pending on the biological constituent (elastin, collagen), on the considered arterial
176 layer (adventitia, media), and on the direction of the applied uniaxial load (Fig.
177 5). Fig. 5(a) shows the microstructure of the unloaded arterial tissue, and Fig.
178 5(b)(c)(d) show the microstructure under the three considered loading conditions,
179 which corresponded to the same uniaxial force of 0.8 N. For each constituent of
180 the imaged microstructure, we provide on Fig. 6 a polar representation of fiber
181 orientations (local maxima of angle density with associated dispersions, see section
182 2.4 for more details) with the relative elongation λ and the fiber angle θ as polar
183 coordinates. The angles $\pi/2$, $3\pi/4$, and π represent the circumferential, diagonal,
184 and longitudinal directions respectively.

185 Adventitial collagen was organized in thick bundles showing a crimped mor-
186 phology in the load-free state (Fig. 5(a) - upper left). The analysis of in-plane
187 fiber angles (Fig. 6(a)) suggested a preferred direction close to the longitudinal
188 direction, with an important dispersion ($\pm\pi/6$) due to bundle direction variabil-

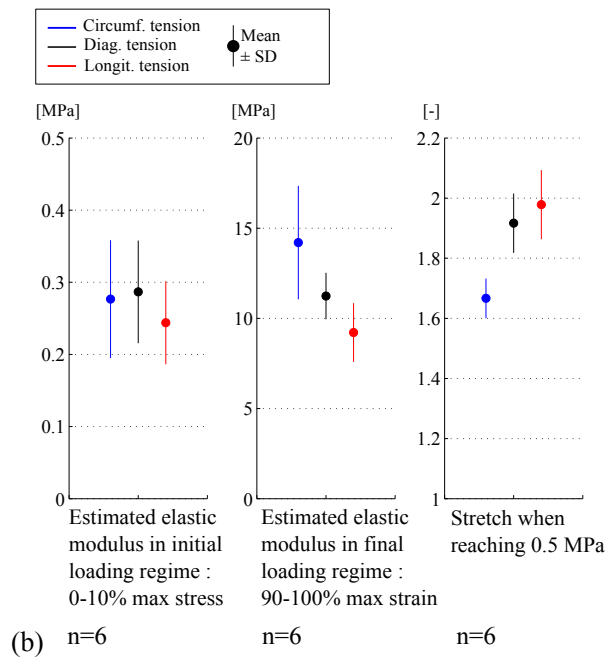
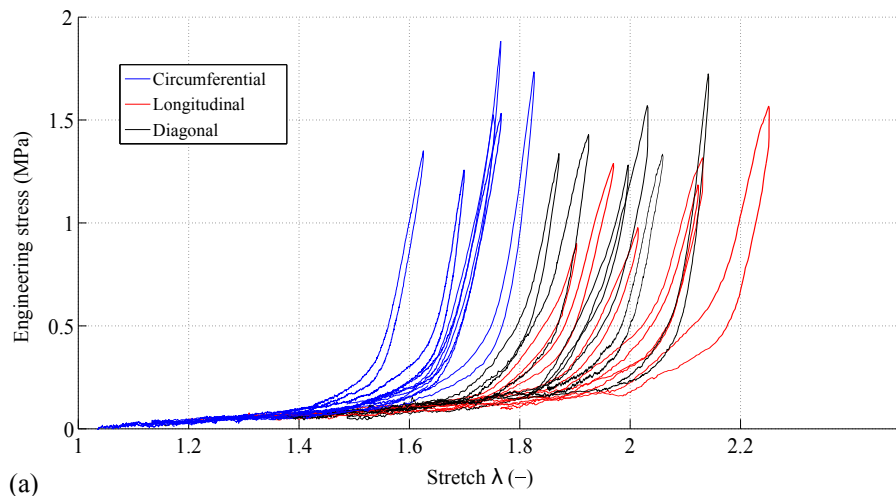


Fig. 4 (a) Macroscopic mechanical response: engineering stress PK v.s. stretch plotted for the three loading directions (blue: circumferential, black: diagonal, red: longitudinal); (b) Statistical representations of the stiffness before (left) and after (middle) collagen engagement, and stretch when reaching 0.5 MPa (right); n stands for the number of investigated samples

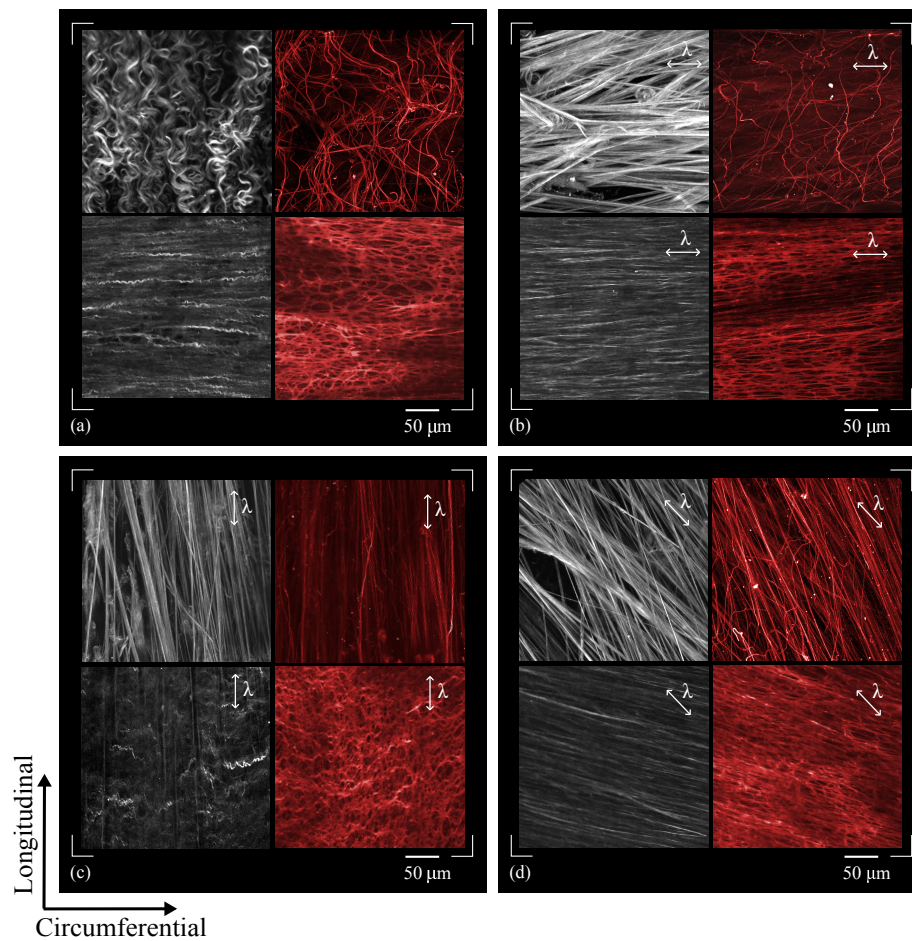


Fig. 5 Arterial microstructure imaged under multiphoton microscope. (a) Load-free state, (b) circumferential load ($F = 0.8 \text{ N}$, $\lambda = 1.7$), (c) longitudinal load ($F = 0.8 \text{ N}$, $\lambda = 2.1$), and (d) diagonal load ($F = 0.8 \text{ N}$, $\lambda = 1.9$). The upper row of each image represents the adventitia; the lower row represents the media. Collagen network is represented in grey (left), elastin network is represented in red (right).

189 ity and to the influence of crimping, which limited the liability of interpretation.
 190 These collagen bundles underwent an uncrimping process and all appeared to align
 191 close to the direction of the load, whether its direction was circumferential (Fig.
 192 5(b) - upper left), longitudinal (Fig. 5(c) - upper left) or diagonal (Fig. 5(d) -
 193 upper left). In the three deformed states, in-plane fiber orientations (Fig. 6(a))
 194 showed identically limited dispersions ($\pm\pi/12$) around their respective local angle
 195 maximum.

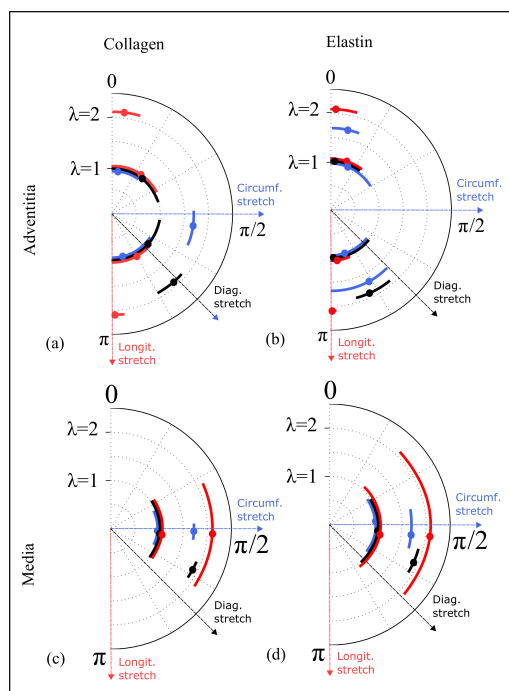


Fig. 6 Orientations of fiber networks represented with local angle density maxima and associated dispersions, for each constituent, layer, and load direction, under 0 and 0.8 N tensile load: (a) and (e) correspond to adventitial collagen, (b) and (f) to adventitial elastin, (c) and (g) to medial collagen, and (d) and (h) to medial elastin. The blue, black, and red colors correspond to the circumferential ($\lambda = 1.7$), diagonal ($\lambda = 1.9$), and longitudinal ($\lambda = 2.1$) directions respectively. Represented network orientations correspond to the mechanical deformations of samples 1-3, tested respectively in the circumferential, diagonal and longitudinal directions.

196 Adventitial elastin took the form of a low-density meshwork, composed of thin
 197 fiber segments showing a preferred longitudinal direction with variously oriented
 198 transverse segments (Fig. 5(a) - upper right). The analysis of in-plane fiber an-
 199 gles at rest (Fig. 6(b)) confirmed the longitudinal orientation, with an important
 200 dispersion ($\pm\pi/6$) due to the variously oriented transverse fiber segments. Under
 201 load, the longitudinally oriented fiber segments underwent limited morphological
 202 changes: when the loading direction was circumferential (Fig. 5(b) - upper right),
 203 the global orientation of the meshwork did not change significantly (a maximum
 204 of $\pi/6$ rotation - Fig. 6(b)), although a spreading of the meshwork was noticeable
 205 (compare Fig. 5(a) - upper right and 5(b) - upper right). Almost unchanged disper-
 206 sions around local density maxima (Fig. 6(b)) confirmed this limited morphological

207 change. When the load was applied longitudinally (Fig. 5(c) - upper right), trans-
208 verse segments aligned in the direction of the load, while the fibrous meshwork
209 remained oriented in the longitudinal direction, which led to an unchanged angle
210 density (Fig. 6(b)). When the load was applied in the diagonal direction (Fig.
211 5(d) - upper right), the meshwork underwent a limited reorientation towards the
212 loading direction (a maximum rotation of $\pi/6$ and angle dispersions reduced by a
213 factor 2 - Fig. 6(b)), with transverse segments aligning with the principal segments
214 orientations.

215 Medial collagen did not agglomerate into variously oriented bundles as in the
216 adventitia, but took the form of thin parallel fibers distinctively oriented in the
217 circumferential direction (Fig. 5(a) - lower left) and showed a higher crimping fre-
218 quency. Its morphology underwent limited changes under uniaxial load: when the
219 loading direction was circumferential (Fig. 5(b) - lower left), the fibers uncrimped
220 and remained aligned in the circumferential direction (Fig. 6(c)). When the load-
221 ing direction was diagonal (Fig. 5(d) - lower left), the fibers uncrimped - with a
222 decrease of the dispersion of local angles by a factor 2 (Fig. 6(c)) - and underwent
223 a limited realignment in the loading direction - below $\pi/6$ rotation for the density
224 maximum of local angles (Fig. 6(c)). When the load was longitudinal (Fig. 5(c)
225 - lower left), i.e. perpendicular to the network direction, the fibers did not align
226 in the loading direction and their crimping increased, as shown by the angular
227 dispersion increased by a factor 2 (Fig. 6(c)).

228 The medial elastin morphology was different from the one of adventitial elastin:
229 at rest (Fig. 5(a) - lower right), it took the form of a dense, structured meshwork
230 preferentially oriented in the circumferential direction. Under load, it underwent
231 limited morphology changes, namely partial alignment of transverse fiber segments
232 in the loading direction, when the loading direction was circumferential (Fig. 5(b)
233 - lower right) or longitudinal (Fig. 5(c) - lower right); the maxima of angle density
234 remained unchanged, while the angular density dispersion decreased by a factor

235 2 (Fig. 6(d)); and a limited global reorientation when the loading direction was
236 diagonal, with rotation of the angle density maximum below $\pi/6$.

237 3.3 Prediction of reorientations using an affine model

238 Table 2 presents the experimental values of local angle density maxima with the
239 upper and lower angle bounds corresponding to the evaluated dispersions, together
240 with the theoretical angles computed by applying the affine reorientation (Equa-
241 tion (2)) to the local angle maxima. The represented results in Table 2 refer to the
242 testing of samples 1 to 3, respectively in the circumferential, diagonal and longitu-
243 dinal directions. Fig. 7 presents a statistical synthesis of fiber angles under uniaxial
244 load, together with the theoretical angles computed by applying the affine reori-
245 entation (Equation (2)) to the local angle maxima. The represented results refer
246 to the four sample groups tested in the three aforementioned directions (samples
247 1 to 12). Results show that the affine model predicted well the reorientation of the
248 medial microstructure (collagen and elastin) as well as of the adventitial elastin,
249 with prediction errors ranging from 1 to 7% (see Table 2 and compare black and
250 white symbols in Fig. 7) and a limited variability of fiber orientation between the
251 analogous samples (Fig. 7). Concerning adventitial collagen, for which we illus-
252 trated the ability to reorient along the different tested loading directions (Fig. 5,
253 Fig. 6), the affine model largely under-predicted the fiber reorientation, as proven
254 by the high prediction error (12 to 25%, see Table 2), observed independently on
255 the analogous samples (Fig. 7, upper left).

256 4 Discussion

257 Our results represent an original insight into the morphological changes that occur
258 in the arterial tissue microstructure under variously oriented uniaxial loads. The
259 experiments were performed on rabbit carotid arteries and focused on collagen
260 and elastin networks of the media and of the adventitia. Our results suggest that

Group 1 : samples 1-3		(a) Initial	(b) Final 1	(c) Final 2
		$\lambda_{diag} = 1.9 \pm 0.1$	$\lambda_{circ} = 1.7 \pm 0.05$	$\lambda_{long} = 2.1 \pm 0.2$
Collagen - adv.	Experimental [π]	$0.76^{+0.07}_{-0.02}$	$0.55^{+0.07}_{-0.06}$	$0.97^{+0.03}_{-0.05}$
	Affine prediction [π]	-	0.69	0.85
	Prediction error [π]	-	25%	12%
Elastin - adv.	Experimental [π]	$0.86^{+0.04}_{-0.09}$	$0.83^{+0.12}_{-0.13}$	$0.98^{+0.02}_{-0.01}$
	Affine prediction [π]	-	0.89	0.97
	Prediction error	-	7%	1%
Collagen - med.	Experimental [π]	$0.61^{+0.04}_{-0.03}$	$0.51^{+0.02}_{-0.02}$	$0.52^{+0.16}_{-0.17}$
	Affine prediction [π]	-	0.52	0.54
	Prediction error	-	2%	4%
Elastin - med.	Experimental [π]	$0.60^{+0.07}_{-0.06}$	$0.53^{+0.03}_{-0.08}$	$0.53^{+0.22}_{-0.21}$
	Affine prediction [π]	-	0.50	0.51
	Prediction error	-	6%	4%

Table 2 Evaluation of the predictive accuracy of affine reorientation. Fiber angles are provided for initial and final states of two deformation scenarii (Samples 1-3) : (a) to (b) and (a) to (c)

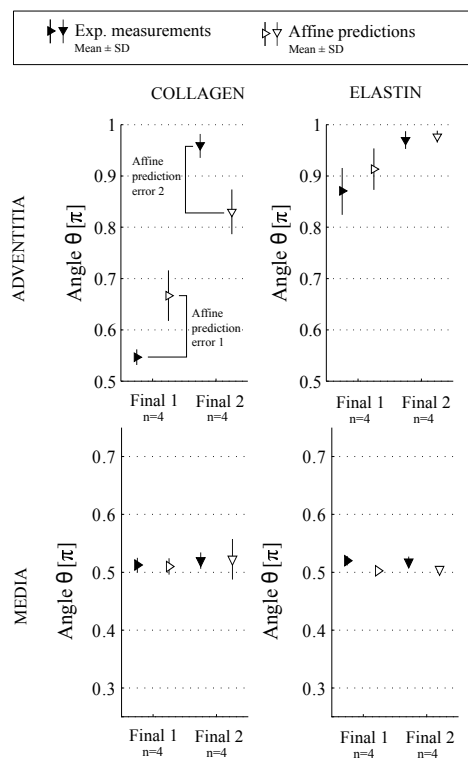


Fig. 7 Synthesis of the predictive accuracy analysis of affine reorientation. Final network orientations (Final state 1 and Final state 2) are averaged on $n=4$ samples under analogous deformation. Affine prediction errors in the case of adventitial collagen reorientation amount to 0.12π and 0.13π when reaching final states 1 and 2 respectively, corresponding to 22% and 14% error.

261 the arterial tissue is **more compliant** in the longitudinal direction, as seen by a
 262 delayed stiffening phase. **This result is consistent with previous studies performed**
 263 **by pressurizing and axially elongating dog carotids [34,11], rat and rabbit carotids**

[11] and by applying uniaxial tension on flat samples of human ascending aortas [8]. Conversely, this anisotropy has been shown contrary (stiffer tissue in the longitudinal direction) for human coronary arteries [33] and dog coronary arteries [34]. Indeed, no consensus has been gained up to now as regards the anisotropy of arteries, evidencing the complex interplay of load bearing contributions between the media and the adventitia. Recent results [22] show clearly that the media is stiffer in the circumferential direction, while the adventitia is stiffer in the longitudinal direction. These results are in good agreement with the orientation of the fiber networks in each layer, but do not permit to draw any conclusion on the overall anisotropy of the tissue. In order to further understand the origin of this complex anisotropy, the distribution of load bearing properties between the media and adventitia should be further characterized along with the study of microstructure rearrangements.

Concerning the arterial microstructure in the load-free state, our results illustrate the layer-specific morphologies of the collagen and elastin networks which are similar to previous investigations [31,36]. In the deformed configuration however, our results bring a novel comprehensive understanding of morphological changes occurring within the arterial microstructure. Under uniaxial tensile loadings, affine kinematics [3,5] explain the reorientations of adventitial elastin, medial elastin, and medial collagen. This kinematically limited reorientation corresponds to a tight embedding of the fibers in their surrounding matrix, which could correspond to near-equivalent shear moduli of the fibers and of their surrounding matrix, or to the existence of molecular links which hinder further rotation of the inclusions within the matrix. Besides, these fibrous networks are unable to fully align in any of the presently applied uniaxial loading directions. Conversely, the adventitial collagen showed a remarkable potential to reorient in any of the applied loading directions (circumferential, longitudinal, diagonal). This reorientation is characterized by significant rotation amplitudes, that remain underestimated by affine reorientations. *Although the protocols employed in the literature vary both*

293 in terms of characterization techniques (focusing on fiber decrimping [30] or on
294 global fiber reorientation [24]) and in terms of applied mechanical loadings (uni-
295 axial, equi-biaxial [30]), our result is consistent with previous studies investigating
296 collagen fiber kinematics in other tissues like porcine aortic leaflets [3], bovine
297 pericardium [3], tissue constructs [5] or human liver capsule [24] and suggests that
298 other mechanisms are activated in the reorientation of adventitial collagen under
299 load.

300 Several limitations stem from the differences existing between our testing pro-
301 tocol and the real *in vivo* conditions. First of all, we should comment on the fact
302 that we have considered temperature conditions lower than *in vivo* conditions.
303 Zemanek et al. [57] studied the influence of the testing temperature on the me-
304 chanical response of the arterial wall and showed that samples are stiffer at ambient
305 temperature than at *in vivo* temperature. There is no consensus on the impact of
306 sample freezing on the change in the mechanical response of the sample [57, 1, 51, 9].
307 However in the present study, test temperature and preservation conditions were
308 identical for all experiments, and allowed to perform an inter sample comparison
309 of the stiffness change during loading or among diverse load directions. Also, to
310 our best knowledge, there is no study on the influence of the temperature on the
311 fiber rotation. Secondly, we have applied a quasi-static elongation speed, which
312 cannot render the dynamic conditions of the pulsatile hemodynamic load seen in
313 large vessels. However, given the small variations in the arterial pressure and the
314 preferred helical orientation of the adventitial collagen bundles, the orientations of
315 the latter are believed not to vary significantly during a cardiac cycle. The choice
316 of a quasi-static loading was made so as to ensure that the mechanical response
317 was independent of the strain rate, as well as to limit the relaxation effects that
318 would impair repeatability of the results. We should also comment on the choice
319 of directions and ranges of load that go beyond the physiological load directions
320 and ranges. While tension-inflation or equi-biaxial protocols allow the simulation
321 of near physiological loadings, the present protocol was applied to reach our objec-

322 tive of comparing inter-layer and inter-constituent changes of the fiber networks
323 within the microstructure subjected to diversified loading scenarii. Besides, the
324 high stretches reached successively during preconditioning and imaging may have
325 induced damage in the tissue, enhancing non-affine fiber rotation. However it is
326 assumed that in this case, the increase of fiber rotation would have affected all
327 imaged fibrous networks, not only adventitial collagen. Accordingly, we did not
328 observe any sign of damage within the imaged tissue, on any fibrous network of
329 the arterial wall.

330 Furthermore, it should be mentioned that what we call arterial microstructure
331 in this work is incomplete from the biological point of view, as our multipho-
332 ton microscopy provided only images of elastin and collagen fibers, with a spatial
333 resolution of the order of 1 μm . The analysis could be complemented by the char-
334 acterization of the deformations and reorientations of non-cellular components
335 under varying levels of smooth muscle tones, as the latter mechanism is known to
336 play an active role in arterial adaptive plasticity during the in vivo pressure cycle.
337 At a lower scale, our analysis could also be complemented by the description of
338 collagen fibrils rearrangements within bundles during decrimping and during post-
339 decrimping stretching. Moreover, the observed morphology changes during uniaxial
340 tension in carotid samples should be compared to morphology changes occurring
341 in identically deformed carotids from other species, with different smooth mus-
342 cle, collagen and elastin content. To the authors' knowledge, identical deformation
343 protocol has not been yet applied to samples of other arteries or other species
344 carotids. However, and to engage the discussion, the recent studies of Keyes et al.
345 [27] on mouse aortas, showed that adventitial collagen could undergo high ampli-
346 tude rotations under pressurization (up to $\pi/3$), while medial networks kept their
347 orientation unchanged. On the contrary, the results of Chen et al. [7], performed
348 on porcine coronary arteries, illustrated under flat biaxial tension the adventitial
349 collagen's decrimping mechanism but a limited rotation potential. Nevertheless,
350 our analysis was intended as a first step, focusing on the major load-bearing com-

ponents of the arterial wall. Furthermore, a nano-scale imaging resolution could investigate the influence of nanoscale aldehyde cross-links on the observed morphology rearrangements. Such an analysis would give biological foundations to the observed reorientations, explaining why several fibers can encounter large rotations whilst others are restricted to small rotations.

Finally, the level of representativeness of the microscopy images with associated angle distributions of collagenous and elastic networks should be commented. In particular concerning automated angles extraction, the Fast Fourier Transform algorithm applied on adventitial collagen at rest (high density crimped bundles) provided information about local angles but did not allow the extraction of global collagen bundle orientation. In order to capture full morphological rearrangements of adventitial collagen under load, a specific method should be developed so as to analyze collagen orientation at different scales: crimping scale (10 μm) and global bundle scale (100 μm). With the objective of comparing true reorientations with affine predictions, we here overcame this image processing issue by comparing only deformed configurations, for which the orientations at crimping scale and at global bundle scale are identical.

5 Conclusion and outlooks

We have shown that arterial microstructure presents inter-layer differences between collagen network and elastin network morphologies. These inter-layer differences concern in particular the ability to rearrange under variously oriented uniaxial loads. We have shown that even though the fibers of the vascular microstructure undergo the same macroscopic deformation, they rearrange in their own specific way, and differently from one vascular layer to the other. This finding brings additional insight for current multi-scale model formulations **which take into account specific kinematics of arterial collagen and its relation to the surrounding matrix [44,15]**. It also confirms the need to consider the arterial wall as a composite structure composed of different mechanically relevant layers with different mechanical

379 properties [22]. Our results also suggest the need to further investigate the un-
380 derlying mechanisms that govern adventitial collagen rearrangement, in particular
381 uncrimping and post-uncrimping fiber rotation. Given that the affine reorientation
382 underestimates real fiber rotation of adventitial collagen, it appears interesting
383 to propose a micromechanical origin to collagen fiber rotation. In particular, we
384 hypothesize that the macroscopic strain concentrated in a highly heterogeneous
385 microstructure induces a heterogeneous field of displacement gradients, itself gen-
386 erating microscopic spin that can be important if the shear moduli of the fibers
387 and of the matrix are significantly different. These spins may induce material ro-
388 tation within the structure which superimposes with affine reorientation. Future
389 multi-scale models could test this hypothesis by accounting for the existence of
390 local spin vectors, promoting fiber rotations.

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