Quantifying vascular damage by investigating stent-triggered mechanical and morphological alterations in coronary arteries

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ABSTRACT

Vascular damage develops with diverging severity during and after coronary stent implantation and is the prevailing stimulus for in-stent restenosis. Previous work has failed to link mechanical data obtained in a realistic in vivo or in vitro environment with data collected during imaging processes. We investigated whether specimens of porcine right coronary arteries soften when indented with a stent strut shaped structure, and if the softening results from damage mechanisms inside the fibrous collagen structure. To simulate the multiaxial loading scenario of a stented coronary artery, we developed the testing device ‘LAESIO’ that can measure differences in the stress-stretch behavior of the arterial wall before and after the indentation of a strut-like stamp. The testing protocol was optimized according to preliminary experiments, more specifically equilibrium and relaxation tests. After chemical fixation of the specimens and subsequent tissue clearing, we performed three-dimensional laser scanning confocal microscopy and second-harmonic generation imaging on the deformed specimens. We analyzed and correlated the mechanical response with structural parameters of high-affected tissue located next to the stamp indentation and low-affected tissue beyond the injured area. The results reveal that damage mechanisms, like tissue compression as well as softening, fiber dispersion, and the lesion extent, are direction-dependent, and the severity of them is linked to the strut orientation, indentation pressure, and position. The findings highlight the need for further investigations by applying the proposed methods to human coronary arteries. Additional data and insights might help to incorporate the observed damage mechanisms into material models for finite element analyses to perform more accurate simulations of stent-implantation.

1. Introduction

Despite the great efforts of scientists and manufacturers in recent years, in-stent restenosis (ISR) remains the most frequent and critical post-surgical event of percutaneous coronary intervention (PCI) cardiologists have to face [1,2]. During and after PCI including stent implantation, the coronary artery is exposed to a triaxial loading scenario in the supraphysiological range, i.e., above the hemodynamic loading. The expanding stent causes an increase in the diameter of the artery to an abnormally high value, i.e., the tissue gets stretched in the longitudinal and the circumferential directions. This mechanical stimulus and supraphysiological loadings provoke pathological processes in the form of cell migration and proliferation, resulting in neointimal thickening [3]. It is shown that the extent of ISR correlates with the severity of the stent-induced injury [4-9].

However, even newest generations of so-called drug eluting stents (DES) show similar fatality rates to uncoated bare metal stents (BMS) in the long-term (>5 years) of about 15–20% [1,2,10–12]. To lower the fatality rate of BMS and DES, scientists and manufacturers need to develop safer stents by significantly lowering the risk of damage caused by PCI. The actual moment this stent-induced damage occurs (also denoted as ‘vessel or vascular damage’) is difficult to determine. It must be assumed that vascular damage and subsequent neointimal hyperplasia start to develop as soon as the artery is exposed to supraphysiological loadings. The mechanical, as well as morphological alterations may become more severe and even irreversible with an increasing mechanical stimulus.

To prevent excessive vascular damage, the load applied on the artery must be reduced by optimizing the geometry or the material of the stent and the stent delivery system. Finite element analysis (FEA) has become a promising tool to fulfill this mission. Modern FEA is able to precisely simulate the interaction between all components of an expanding stent-delivery system as Geith et al. [13], Wiesent et al. [14], and He et al. [15] have shown. In addition, progressive constitutive models of arteries known in the literature [16,17] are meant to simulate the behavior of healthy or diseased arteries in a physiological or pathological state, but under the assumption that mechanical properties and the morphology remain constant. The equations of the latest constitutive material models often contain structure tensors in which experimentally validated structural parameters of different arterial layers are implemented. However, as soon as such a model is implemented in the FEA of PCI, a significant limitation is added: so far, acute-occurring biomechanical and morphological alterations that lead to vascular damage in the arterial tissue during the event of PCI are not considered in any material model.

Only a few studies are known which try to quantify the vascular damage inside arteries after PCI. Schwartz et al. [4] implanted stents in porcine arteries, prepared and analyzed histological sections with light microscopes, and evaluated the lesions with a stent injury score. Even though Schwartz et al. [4] created a revolu-
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tionary tool allowing damage classification by the degree of injury more than 25 years ago, the subjective evaluation of the respective stent injury score is still based on empirical data. Based on the mentioned injury score, Schwartz et al. [18] and Swier et al. [19] aimed to correlate the severity of the injury with the neointimal thickening. Nevertheless, the results presented in these studies cannot be correlated with the indentation pressure and the orientation of a stent strut, as well as the mechanical response of the tissue.

Pathological alterations in the tissue are only detectable if one compares the properties of an arterial wall before and after the iatrogenic event of PCI. To date, properties of coronary arteries can be quantified in three ways:

(i) One can observe the parthenogenesis of cardiovascular events, like neovascularization, neointimal formation, inflammation, endothelial dysfunction, apoptosis, thrombus formation, and angiogenesis, by detecting biochemical mechanisms, which is the focus of several biologically-oriented research efforts, cf. [20–25].

(ii) Mechanical alterations of the arterial wall can be determined by measuring the stress-stretch response of the tissue. Here, during standard tensile tests, specimens are stretched in one or two orthogonal directions, and their stress-stretch relationship is measured. The in vitro mechanical response of healthy and diseased arteries in a passive or active state to physiological and supraphysiological biaxial loadings have been substantially analyzed, as Chen and Kassab [26] summarized in their review article. However, experimental setups for conventional uni- and biaxial tensile tests are inadmissible to simulate the triaxial loading scenario of PCI. A precise in situ measurement of the indentation pressure, which interacts between the stent strut and the artery, is technically not feasible due to the tiny dimensions of the stents and the poor accessibility. Therefore, an experimental setup is needed to mimic the loading scenario of a stented artery under in vivo conditions and to enable the measurement of the prevailing stress-stretch relationship of the artery in parallel. However, to this point, it seems that no study has been published, which describes experiments with such a setup.

(iii) One can investigate the arterial morphology, which helps to explain the mechanical response. Technologies such as fluorescence microscopy with DAPI staining or antibody labeling and modern, high-quality multi-photon microscopy in combination with specific tissue clearing methods allow deep tissue imaging. Thus, a display of all constituents such as collagen, elastin, and vascular smooth muscle cells is feasible. Since the mechanical behavior of vascular tissue is highly dependent on fibrillar collagen type I, [27] q.v., in particular, second-harmonic generation (SHG) imaging has become an important tool for the investigation of structure-related alterations in the extracellular matrix [17, 28–32]. By analyzing the imaging data of SHG scans, constant values for structural parameters were obtained and incorporated into a constitutive model in order to capture the physiological and pathological mechanisms of healthy and diseased arterial tissues more precisely [17, 31]. However, to this day, structural parameters for injured tissue of stented arteries could not be provided. Nevertheless, we assume that most of these structural parameters, as well as the mechanical properties of the arterial tissue, will change due to PCI. Furthermore, it is very likely that the structural parameters of stented tissues vary with increasing indentation pressure exerted by the stent, and depend on the orientation of the stent struts, and also alter with increasing distance to the lesion.

For the proof of this hypothesis, new experimental and imaging methods are presented in this pilot study, which aims to observe the influence of PCI on the mechanical properties and morphology of porcine right coronary arteries (RCA). First, the theoretical background is explained to describe the in situ loading scenario of PCI and to impart the meaning of the structural parameters introduced by Holzapfel et al. [17]. Subsequently, a new experimental setup is presented that was built to mimic the in situ loading scenario during PCI with the real-time stress-stretch measurement as well as a well-proven testing procedure. This study also explains an imaging strategy, including adapted procedures for optical tissue clearing as well as three-dimensional (3D)-surface and SHG scans. Finally, the mechanical and structural data are presented, correlated, and key as well as further findings are interpreted.

2. Materials and Methods
2.1. Biomechanical Principles

2.1.1. In Situ Loading Scenario

In the following, we consider a planar and square segment from a homogeneous wall of a coronary artery, shown schematically in Fig. 1(a) and (b). Coronary arteries consist of three layers, intima, media, and adventitia, which are separated by transition layers around the membrana elastica interna and externa. In the following, the circumferential, longitudinal, and radial directions are denoted as $\theta$, $z$, and $r$, respectively. The physiological, i.e., hemodynamical or in situ loading of coronary arteries is the result of the interplay between the blood pressure, intraparietal active stresses, and the residual stresses inside the arterial tissue. For the sake of simplicity, only a static loading scenario was assumed in this study. Therefore, the dynamically changing blood pressure was replaced by a constant mean arterial pressure (MAP) $p_{\text{MAP}} \approx 100$ mm Hg $\approx 13.3$ kPa. The physiological values for stresses inside the tissue in the $\theta$- and $z$-direction can be approximated by using Laplace’s equation. Hence, corresponding physiological Laplace mean stresses in the $z$- and $\theta$-direction in an untreated, i.e. not stented artery are according to

$$
\sigma_{zz}^\text{MAP} = \frac{p_{\text{MAP}} L}{4\pi T} \quad \text{and} \quad \sigma_{\theta\theta}^\text{MAP} = \frac{p_{\text{MAP}} L}{2\pi T},
$$

where $L$ is the side length of the tissue segment and $T$ is the thickness of the arterial wall in the reference configuration, i.e., under physiological load. As Humphrey et al. [33] concluded in their review article, the projected pressure $p_{\text{MAP}}$ alone may not be sufficient to maintain a physiological stretch as the loading in the $z$-direction may remain constant for different pressures. Before further studies are carried out, we recommend that this problem should be investigated experimentally on porcine and/or human coronary arteries and the derivation of the stresses reconsidered according to prior and recent approaches [33–36].

If a stent strut of width $w$ and height $h$ indents with an indentation pressure $p_{\text{hat}}$, a lesion develops. The compression along the elevation profile of the cross-section of the lesion can be expressed as the nonlinear function

$$
\lambda_r(k) = \frac{t(k)}{T},
$$

where $t$ is the compressed wall thickness in the stented-configuration at the distance $k$ to the center of the strut indentation. The compression finds its smallest magnitude $\lambda_{\text{min}}$ directly under the stent at $k = 0$ and $t = t_{\text{min}}$, then the compression increases nonlinearly with an increasing $k$ and reaches its maximum $\lambda_{\text{max}}$ at $k_{\text{max}}$ and $t = t_{\text{L}}$, whereby the subscript L stands for ‘low-affected tissue’. Subsequently, we denote tissue beyond $k_{\text{max}}$ as ‘low-affected’, since the arterial wall gets only stretched in the $\theta$- and $z$-direction.
In vivo situation

Adventitia

Epicardial adipose tissue

Media

Intima

Fig. 1: Schematic illustration of the in situ loading scenario and fiber directions of a stented artery: (a) cross-section of a stented coronary artery labeled by a cylindrical coordinate system with two axes in the \( r \)- and \( z \)-direction and a third one in the \( \theta \)-direction; (b) planar and square segment of a stented artery with a damaged area surrounding the stent strut within the distance \( k \in [0, k_{\text{max}}] \), with a transformed rectangular Cartesian coordinate system. Here, the strut orientation angle is \( \phi = 90^\circ \). The springs symbolize the epicardial adipose tissue; (c) symmetric mean fiber directions \( \mathbf{m}_{i,1} \) and \( \mathbf{m}_{i,1} \) of the two fiber families of the low-affected tissue in the stented configuration, each making an angle \( \alpha_i \) with the \( \theta \)-direction \( \mathbf{e}_1 \), whereas \( \mathbf{m}_{i,1} \) denotes the unit out-of-plane vector; (d) symmetric mean fiber directions \( \mathbf{m}_{i,1} \) and \( \mathbf{m}_{i,1} \) of the high-affected tissue in the stented configuration next to the stent strut, with the angle \( \alpha_i \) and the unit out-of-plane vector \( \mathbf{m}_{i,1} \); \( \mathbf{e}_1, \mathbf{e}_2, \mathbf{e}_3 \) are the three unit basis vectors pointing in the \( \theta \)-, \( z \)-, and \( r \)-direction, respectively (see Fig. 2). The content of collagen fibril bundles enveloping SMC is rather low in the media of coronary arteries [37] and mainly oriented in the \( \theta \)-direction [35, 38]. According to [26], the adventitia can be divided into an inner adventitia (IA) and an exterior adventitia (EA). In the IA, the density of collagen bundles gets higher, and the fibers tend to align with the \( z \)-direction. The collagen content peaks in the EA with a rather high degree of fiber dispersion.

The mean fiber directions \( \mathbf{M}_i \) and \( \mathbf{M}_i \) for both symmetric fiber families of an untreated artery are located in the \((\mathbf{e}_1, \mathbf{e}_2)\)-plane. In this reference configuration, both mean fiber directions can be described through

\[
\mathbf{M}_i = \cos \alpha \mathbf{e}_1 + \sin \alpha \mathbf{e}_2 \quad \text{and} \quad \mathbf{M}_i = \cos \alpha \mathbf{e}_1 - \sin \alpha \mathbf{e}_2 ,
\]

where \( \alpha \in [-90^\circ, 90^\circ] \) is the mean fiber angle between the mean fiber directions \( \mathbf{M}_i \) and \( \mathbf{M}_i \) and the unit vector \( \mathbf{e}_i \) (see Fig. 1(c)). For the mathematical quantification of the fiber dispersion, Holzapfel et al. [17] introduced further the generalized structure tensors \( \mathbf{H}_i \) and \( \mathbf{H}_i \) as

\[
\mathbf{H}_i = \mathbf{A} \mathbf{I} + \mathbf{B} \mathbf{M}_i \otimes \mathbf{M}_i + (1 - 3 \mathbf{A} - \mathbf{B}) \mathbf{M}_i \otimes \mathbf{M}_i , \quad i = 4, 6 ,
\]

where \( \mathbf{M}_i \) is a unit out-of-plane vector given by \( \mathbf{e}_i \times \mathbf{e}_j \), \( \mathbf{I} \) is the identity tensor, and \( \mathbf{A} \) and \( \mathbf{B} \) are parameters according to

\[
\mathbf{A} = 2 \mathbf{c}_{\text{op}} \mathbf{F}_{\text{op}} \quad \text{and} \quad \mathbf{B} = 2 \mathbf{c}_{\text{op}} (1 - 2 \mathbf{c}_{\text{op}}) ,
\]
which are composed by the two scalar quantities $\kappa_p$ and $\kappa_{op}$ as

$$
\kappa_p = \frac{1}{2} - \frac{I_1(a)}{2I_0(a)},
$$

$$
\kappa_{op} = \frac{1}{2} - \frac{1}{8b} + \frac{1}{4} \sqrt{\frac{2}{zb}} \exp(-2b) \exp\left(\frac{2}{z\sqrt{b}} \text{erf}\left(\frac{2}{z\sqrt{b}}\right)\right),
$$

where $\text{erf}(\cdot)$ is the error function of $\cdot$, $a$ and $b$ are concentration parameters, and $I_0$ and $I_1$ are the modified Bessel functions of the first kind of order 0 and 1, respectively. If $\kappa_p$ increase and $\kappa_{op}$ decrease, the degree of the in- and out-of-plane dispersion becomes greater. In [17], the structural parameters are denoted by $a$, $\kappa_p$, and $\kappa_{op}$, which can be determined by SHG analysis, see, e.g., [31].

After PCI, the orientation of the respective stent strut is defined by the vector $\textbf{S}$ through

$$
\textbf{S} = \cos \phi \textbf{e}_1 + \sin \phi \textbf{e}_2,
$$

where $\phi$ is the stent orientation angle $\phi \in [-90^\circ, 90^\circ]$ is the angle between $\textbf{e}_1$ and $\textbf{S}$ in the $(\theta, z)$-plane, see Fig. 1(b). In the stented configuration, two cases must be considered:

(i) The low-affected tissue is located beyond a distance to the stent strut higher than $k_{\text{max}}$, as presented in Fig. 1(c). Therefore, $\textbf{M}_i$, $\textbf{M}_d$, $\alpha$ become $\textbf{m}_{\text{a}i}$, $\textbf{m}_{\text{a}d}$, $\textbf{m}_{\alpha i}$, $\alpha_i$.

(ii) The high-affected tissue is located next to the stent within $k < k_{\text{max}}$, as depicted in Fig. 1(d). Thus, the reference configuration transforms into $\textbf{m}_{\text{a}i}$, $\textbf{m}_{\text{a}d}$, $\textbf{m}_{\alpha i}$, $\alpha_i$.

We assume and aim to show within this study that the structural parameters $a$, $\kappa_p$, $\kappa_{op}$ of the low-affected tissue depend mainly on the indentation pressure $p_{\text{ind}}$. In the high-affected tissue, the structural parameters are expected to depend additionally on the stent strut orientation angle $\phi$. Finally, they are assumed to be dependent on the position, which means that they alter with the compression $\lambda_p(k)$. Either way, alterations of the structural parameters would influence the structure tensors $\textbf{H}_a$ and $\textbf{H}_d$, and, therefore, the material response of the stented tissue of coronary arteries.

### 2.2. In Vitro Simulation of Stent Implantation

#### 2.2.1. Experimental Setup LAESIO

For the simulation of the loading scenario of a stented coronary artery, we developed the LAESIO (Latin for lesion) testing device. As schematically depicted in Fig. 3(a), it consists of two main units: the biaxial extension unit (BIAX), which aims to simulate physiological stretching of the planar specimen in the $z$- and $\theta$-direction, and the triple-axis unit (TAU) which simulates the indentation pressure $p_{\text{ind}}$ between the stent strut and the arterial wall.

The BIAX setup is an arrangement of four linear stages LTM 60F-50 HSM (Owis, Staufen im Breisgau, Germany), two in $\theta$- and two in the $z$-direction, which can be controlled independently. The linear stages hold load cells KD40s (ME-Messsysteme, Henningsdorf, Germany) with a max. capacity of $\pm 5 \text{N}$ and an accuracy of 0.1%. These load cells are connected via extensions to clamps to which the specimens are attached using surgical sutures and hooks. The specimen and the clamps are submerged in a tissue bath. The fluid inside can be heated to 37°C with a coil, which is operated by the heating immersion circulator series ED (Julabo, Seelbach, Germany). To counteract evaporation during long-term tests, the infusion pump infusomat fmS (BBraun Melsungen, Melsungen, Germany) is connected to the bath. It keeps the fluid level above the specimen by injecting ideally 19 ml/h. For chemical fixing specimens under load, the phosphate-buffered saline (PBS) can be exchanged with formaldehyde (FA) solutions. A system consisting of an infusion set, two three-way stopcocks, and two bottles enables a fast exchange between PBS and FA. A syringe allows the user to drain the bath before exchanging the fluid or after testing. To monitor the tissue's true stress-stretch behavior, the four load cells measure the reaction forces of the arterial tissue, while a video extensometer (VE) underneath the bath tracks markers on the stained and illuminated adventitia. For 2D stretch measurements, the software Laser Speckle Extensometer 2.23.3.0 (Messphysik Materials Testing, Fürstenfeld, Austria) with a resolution of $\pm 0.15 \mu\text{m}$ and a sample rate of 20 Hz was used.

The TAU can be freely placed above the BIAX and the specimen. It is equipped with a straight stamp with its cross-section being identical to a square stent strut with a standard strut width of $w = 0.08 \text{ mm}$ of modern stents [39]. Artificial lesions are generated by indenting the strut into the arterial wall (see Fig. 3(b)). When the stent touches the specimen, the three-component force sensor (3CFS) K3D40 (ME Messsysteme) with a max. capacity of $\pm 2 \text{ N}$ and an accuracy of 0.05% is and connected to the strut, and it measures the reaction forces in all three directions. The position of the strut can be altered by three magnet coil motors of the product series PS01-23 (NTI AG LinMot & MagSpring, Spreitenbach, Switzerland). The stent strut in each direction is monitored by an absolute linear encoder MS01-1/D-SSI (LinMot) with a resolution of $\pm 0.001 \text{ mm}$.

The ideal choice of the strut shape is controversial. At this point, it must be noted that we decided not to consider the effect of curved strut segments or struts with round cross-sections in this early stage of our investigations. We used a straight strut segment with a total length $l$ of 3 mm, which is shorter than the side length $L$ of the specimen. This strut shape has the disadvantage that the strut ends induce stress singularities into the tissue of the floating specimen. However, preliminary tests on porcine aortas [40], and the obtained results, show that the compression underneath the strut is almost homogeneous along $l$. Therefore, we consider the following simplification for the calculation of the maximum strut indentation force $F_{\text{ind}}$, i.e.

$$
F_{\text{ind}} = p_{\text{ind}} l e l.
$$

The advantage of a shorter strut is that it is possible to investigate the tissue’s response next to the strut, and, therefore, if the tissue structure contains transition zones for fiber orientation and dispersion. Thus, the proposed strut design was beneficial for the first findings and insights during this early stage of our investigations.
but should be carefully reconsidered in future studies.

All actuators of the LAESIO testing device are controlled, and all sensors are monitored by the programmable logic controller (PLC) CX5130 (Beckhoff Automation, Verl, Germany). With the touch panel CP3911-0000 (Beckhoff Automation) and a control panel, the user was given the ability to change the testing protocol and parameters in real-time. For coding the machine software, SELMOmodeler (SELMO, Dobl, Austria) was used. The BIAx and TAU are encapsulated inside a sealed cabin with a fume hood to protect the user from direct contact with carcinogenic FA vapor (see Fig. 3(c)). A more detailed view of the interior of LAESIO is presented in Fig. 3(d).
2.2.2. Specimen Preparation and Storage

Two whole hearts of five-month-old domestic pigs were obtained from a local slaughterhouse. The RCA was used for our investigations because we found that more samples can be taken than from the main branches of the left coronary artery. Within a post-mortem interval of one hour, the RCA from where they emerge at the coronary ostium to the bifurcation of the right marginal artery was carefully excised and cut in tubes. To preserve all tubes from the main branches of the left coronary artery. Within a post-testing, the particular specimen was submerged in 37°C PBS (pH 7.4) and subsequently preconditioned. For preconditioning, every specimen was biaxially loaded with five cycles within the assumed physiological range, i.e., within the average hemodynamic loading at MAP. The absolute values of the tensile forces at MAP for the z- and θ-direction can be calculated according to

\[ f_z^{\text{MAP}} = \sigma_0^{\text{MAP}} A_z \]

\[ f_\theta^{\text{MAP}} = \sigma_0^{\text{MAP}} A_\theta, \]

where \( A_z = A_\theta \) is the out-of-plane cross-section of the specimen defined by the (r,θ)- and the (r,z)-plane, respectively. Substitution of Eq. (1) into Eq. (9) gives

\[ f_z^{\text{MAP}} = \frac{P_{\text{MAP}} L_z^2}{4\pi} \cdot \frac{\epsilon_0^{\text{MAP}}}{2\pi}, \]

where \( L \) was here replaced with \( L_0 \), which is the side length of the unloaded specimen. To measure \( L \) of the respective physiologically loaded specimen was technically not feasible. A fully automated force-controlled protocol ensured a linear loading and unloading behavior with a testing speed of \( v_{\text{BIAX}} = 1 \text{ mm/min} \) and with tensile forces within the physiological range \( f_z \in [f_{\text{MIN}} f_z^{\text{MAP}}] \) and \( f_\theta \in [f_{\text{MIN}} f_\theta^{\text{MAP}}] \). The force versus time curves have the shape of a triangular function with a constant ratio of \( f_z^{\text{MAP}} : f_\theta^{\text{MAP}} \) between both axes. The results of this study show that three preconditioning cycles are adequate for porcine RCA specimens (see Section 3.1).

### 2.2.2.4. Preliminary Tests

In order to investigate the mechanical response of coronary arteries to constant physiological (in situ) and supraphysiological (ia- trogenic) loadings caused by PCI over a specific time, two preliminary tests were designed and executed as follows.

(A) **Equilibrium Tests:** The in vitro mechanical response of the RCA tissue to physiological loadings can alter over time due to biochemical, thermodynamical, and mechanical processes inside the arterial tissue, e.g., vasoactivity [34] q.v., thawing, and preloading. Thus, this preliminary test was performed test on specimens SI and SII using a stretch-controlled protocol in order to determine the waiting time before the mechanical response becomes reproducible and an the specimen is ready for further testing. Five measurement series had to be carried out with a testing speed of \( v_{\text{BIAX}} = 1 \text{ mm/min} \), where every series stands for a sequence of five loading and unloading cycles. During every loading cycle, the testing device has stretched the specimen with a linearly increasing and subsequently decreasing stretch within the mentioned physiological range. The maximum stretch was reached when the load cells recognized forces greater or equal \( f_z^{\text{MAP}} \) and \( f_\theta^{\text{MAP}} \). In parallel, the force-stretch response of the tissue was recorded. The first series started at \( t = 0 \text{ min} \) and the last one at \( t = 180 \text{ min} \), with resting breaks of 30 min in between. Finally, the ideal equilibrium time \( t_{\text{eq}} \) was derived, which defines the interval between the experiment start and the moment where the stress-stretch response of the specimen was reproducible.

(B) **Relaxation Tests:** Quasi-static relaxation tests were carried out with a force-controlled protocol to determine the time range between the moment a stent is fully expanded and recolled inside the artery and the moment the viscoelastic tissue has adapted to the new loading scenario. Two specimens, S III and S IV, from two different RCA were stretched by the BIAx with a testing speed of \( v_{\text{BIAX}} = 1 \text{ mm/min} \) until the tensile forces at MAP \( f_z^{\text{MAP}} \) and \( f_\theta^{\text{MAP}} \) were reached. Afterwards, the linear stages stopped and kept their position. Finally, the strut of the TAU was subsequently indented with an indentation speed of \( v_{\text{IND}} = 1 \text{ mm/min} \) until an indentation force of \( F_{\text{IND}} = 2 \text{ N} \) was reached, which equals the maximum loading capacity of the 3CFS. The indentation force was kept constant, and the relaxation time \( t_{\text{RELAX}} \) measured after no change in the resulting tensile forces \( f_z \) and \( f_\theta \) could be registered anymore.
2.2.5. Indentation Tests

The proposed quasi-static indentation tests enable the documentation of alterations in the mechanical response of the RCA after they underwent supraphysiological loadings during stent implantation. Thus, after the mentioned equilibrium time $t_{equ}$ (see Fig. 4(a)) followed by the preconditioning process (see Fig. 4(b)), the measurement series I with five additional loading and unloading cycles were performed (see Fig. 4(c)). Every specimen was subsequently loaded and $F_{MAP}$ and $f_{\theta}$ kept constant. Finally, the strut was indented with a constant indentation pressure $P_{ind}$ (defined by the strut surface $A_{strut} = wL$ and the indentation force $F_{ind}$) for $t_{relax}$ minutes; (e) the second measurement cycle on the high-affected tissue is performed.

2.3. Structural Analysis

2.3.1. Optical Clearing

By finishing the measurement series II of Section 2.2.5, the strut was indented again, according to Fig. 4(d). The PBS inside the bath was replaced with a solution of 4% FA to chemically fixed under load. The tissue under force-controlled loading during a fixation time of $t_{fix} = 6$ h. Then, up to four specimens were stored at once inside the biopsy cassettes M508-Mircromesh (Simport Scientific, Saint-Mathieu-de-Beloeil, Canada) with four compartments and kept in 4% FA at room temperature. In every compartment, the specimen was embedded between two biopsy foam pads M476 (Simport Scientific) to prevent buckling.

An optical tissue clearing procedure described by Schriefl et al. [30] was utilized to increase the penetration depth during subsequent multi-photon microscopy without destroying the tissue. First, the biopsy cassettes with the specimens were exposed to ethanol solutions with concentrations of 50, 70, 95 and twice to 100% in steps lasting 45 min. Afterwards, the biopsy cassettes were submerged in 1:1 ethanol/benzyl alcohol, benzyl benzoate (BABB) solution for 4 h followed by an exposure to pure BABB of at least 12 h. Finally, each specimen was placed on a standard microscope slide between a washer, which formed a basin filled with BABB. The size of the washer was chosen in a way that its height was greater than the whole specimen. The washer with the specimen inside was sealed with a coverslip and nail polish to prevent the microscope lens.

2.3.2. Nonlinear Optical Imaging

To show if it is feasible to quantify alterations in the morphology of stented coronary arteries, 3D-surface and SHG scans were carried out on the specimens S V, VII, IX, XI, and XII of pig heart.
1, which were all chemically fixed in a loaded configuration. All scans were performed on a multi-photon SPM5 upright microscope (Leica Microsystems, Mannheim, Germany) at the Core Facility Bioimaging of the Biomedical Center, Ludwig-Maximilians-Universität München. An HC IRAPO L 25x/1.00W motCORR water immersion lens with a working distance of 2.6 mm was used. To observe potential alterations of the structural parameters \( k_{op}, k_{ip}, \) and \( \sigma \) of the artificially damaged and the control specimens on the micro-level and to define the geometrical extent of the lesion by defining \( k_{LZ} \) and \( \lambda_{LZ} \), two imaging processes were carried out:

(A) 3D-surface Scanning: The detection of elevation profiles of all lesions was achieved by performing 3D-surface scans on the specimens with the intima facing up in the \( r \)-direction (see Fig. 5). Confocal images were recorded in the reflection mode with an internal conventional photomultiplier tube by using solid-state laser excitation at 0.488 \( \mu \)m. The image pixel size was set to 0.5 \( \mu \)m. (B) Second-harmonic Generation Imaging: SHG analyses of the fibrillar collagen structure were carried out with pulsed InSight DSR laser (Spectra Physics, Mountain View, USA) and an excitation wavelength of 0.88 \( \mu \)m. An AT 420/40 emission filter with a BS 488LPRXR beam splitter was equipped to observe the collagen structure of the media and the adventitia layer. The intima was expected to be detached and removed during specimen preparation. Images were recorded with an external, non-descanned hybrid photodetector (HyD) in an 8-bit mode with an image pixel size of 0.1 \( \mu \)m, and frame accumulation of \( n = 3 \). For SHG scanning, specimens were physically quartered with the sections going through the center of the lesion, pointing in the \( \theta \)- and \( z \)-direction, as indicated by the blue and green planes in Fig. 5.

Figure 5: Sketch showing a quarter section of a specimen with a lesion pointing in the \( z \)-direction. Out-of-plane scans were taken in the \( (r,\theta) \)- and \( (r,z) \)-planes and in-plane \( (\theta,z) \) image stacks around the points \( P_L \) and \( P_H \). The normal vectors of the image stacks were parallel to the vectors \( Z_{Lz} \) and \( Z_{H} \), whereas \( Z_{H} \) is perpendicular to the tangent laying on the intima in \( P_H \) making the angle \( \epsilon \) with \( r \). \( k_{max} \) is the distance between the low-affected tissue and the strut indentation; \( t_L \) is wall thickness at \( P_L \); \( t_H \) is wall thickness at \( P_H \).

In the following, the \((\theta,z)\)-plane is denoted by ‘in-plane’ and the \((r,\theta)-\) and \((r,z)-\)plane are called ’out-of-plane’. A single-row mosaic of the in-plane image stacks had to be scanned with the first stack centered at point \( P_L(0, k_{max}, t_L) \) located in the low-affected tissue. It was not possible to obtain any information about the fiber dispersion from the tissue directly in the strut indentation due to severe compression and signal artefacts. Therefore, the location of the center of the last stack \( P_H(0, k_{max}/3, t_H) \) of the mosaic was chosen in a way that the images do not include the strut indentation itself. The distance between every image of the stacks was 5 \( \mu \)m.

Furthermore, out-of-plane scans had to be provided within the same range as the mosaic scan, with the normal vectors of the images being parallel to the long side of the lesion.

2.4. Data Analysis

2.4.1. Mechanical Data

In all testing scenarios, Cauchy stresses in the \( z \)- and \( \theta \)-direction were computed from mechanical data as follows:

\[
\sigma_{zz} = \frac{f_{z1} + f_{z2}}{2L_0 T_0} \lambda_z, \quad \sigma_{\theta\theta} = \frac{f_{\theta1} + f_{\theta2}}{2L_0 T_0} \lambda_\theta, \tag{11}
\]

where \( f_{z1}, f_{z2}, f_{\theta1}, f_{\theta2} \) represent the measured reaction forces at every load cell and \( \lambda_z = x_z/X_z \) and \( \lambda_\theta = x_\theta/X_\theta \) the stretches in the \( z \)- and \( \theta \)-direction, calculated with the marker distances from the reference \((X_z, X_\theta)\) and the deformed configuration \((x_z, x_\theta)\). We assume negligible shear deformations due to the orthotropic natural structure of healthy coronary arteries [46].

2.4.2. Structural Data

The 3D-surface scans were visualized with LAS X Life Science (Leica Microsystems). By virtually slicing the 3D-surface scans normal to the long side of the lesions, out-of-plane cross-sections of the lesion and the surrounding tissue were generated. With the help of these cross-sections, the values for \( \eta \) in the points \( P_L \) and \( P_H \) were measured. All SHG scans had to be preprocessed in Fiji (http://fiji.sc/Fiji, Ashburn, VA, USA). The normal vectors of the stacks in both points are denoted by \( Z_L \) and \( Z_H \). While \( Z_L \) is normal to the \((\theta,z)\)-plane, the high-affected tissue tends to bend due to the strut indentation. Therefore, all SHG images of the in-plane stack and the out-of-plane scans had to be rearranged to \( Z_L \) and tilted with the angle \( \epsilon \) around an axis going through \( P_H \) and being parallel to the \( z \)-direction (see Fig. 5). This was achieved by using the reslice and transform commands in Fiji.

The structural parameters \( k_{op}, k_{ip}, \sigma \) for native, low-affected, and high-affected tissues were obtained by following the workflow described by Schrieff et al. [30]. Briefly, the fiber directions for the media and adventitia were extracted from every in-plane SHG-image of the stacks and the out-of-plane images via a Fourier-based image analysis method with wedge filtering [29]. From the angular data set, the mean fiber angle was obtained with respect to \( \theta \). According to Eq. (6), the in-plane dispersion quantity \( k_{ip} \) of the media and adventitia was calculated. Due to the small thickness of the specimens, only one value of \( k_{ip} \) for both layers could be derived. All structural parameters were obtained and subsequently compared using MATLAB R2019a (The MathWorks, Natick, USA). Finally, the compression in the low- and high-affected tissue was calculated according to Eq. (2), i.e.

\[
\lambda_{Lz} = \frac{t_L}{T_0} \quad \text{and} \quad \lambda_{Hz} = \frac{t_H}{T_0}, \tag{12}
\]

where \( t_L \) and \( t_H \) is the wall thickness in the low- and high-affected area, respectively. The wall thickness of the unloaded specimen \( T_0 \) had to be used since it was technical not feasible to measure the respective thickness \( T \) of the physiologically loaded specimen.

3. Results

3.1. Mechanical Response

The curves in Fig. 6(a) show a representative preconditioning behavior of the RCA tissue loaded biaxially within the physiological range. In the \( z \)- and \( \theta \)-direction, the nonlinear stress-stretch relationship reveals no visible softening between the first and the third
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Fig. 6: Mechanical results taken from all three test scenarios: (a) representative preconditioning behavior of the RCA tissue of specimen S1 depicted as the Cauchy stress-stretch relationship during the first three physiological loading cycles; (b) representative material behavior of specimen SII reaching an equilibrium state after 30 min; (c) representative relaxation behavior, i.e., force vs. time relationship of specimen SIII loaded with an indentation force of $F_{\text{ind}} = 2\, \text{N}$; (d) comparison of the Cauchy stress-stretch behavior of specimens SV ($F_{\text{ind}} = 1\, \text{N}$) and S VII ($F_{\text{ind}} = 2\, \text{N}$) before and after strut indentation and with the long side of the strut pointing in the $z$-direction; (e) comparison of the Cauchy stress-stretch behavior of specimens SIX ($F_{\text{ind}} = 2\, \text{N}$) and SXI ($F_{\text{ind}} = 2\, \text{N}$) before and after strut indentation and with the strut pointing in the $\theta$-direction. Every curve in (b), (d) and (e) represents the mean of a measurement series of five loading, and unloading cycles.
loading and unloading cycles. Thus, porcine RCA display almost exclusively elastic behavior within the physiological range. For specimen S I, mean stresses of $\sigma^{\text{MAP}}_z = 18.6 \text{kPa}$, $\sigma^{\text{MAP}}_{\theta} = 38.1 \text{kPa}$, and stretches of $\lambda^{\text{MAP}}_z = 1.14$, $\lambda^{\text{MAP}}_{\theta} = 1.22$, and for S II $\sigma^{\text{MAP}}_z = 18.5 \text{kPa}$, $\sigma^{\text{MAP}}_{\theta} = 39.4 \text{kPa}$, and $\lambda^{\text{MAP}}_z = 1.14$, $\lambda^{\text{MAP}}_{\theta} = 1.25$ were obtained. Both graphs in Fig. 6 show that the hystereses are not pronounced, which is a sign of marginal viscoelasticity under physiological loading and the given testing rate.

Representative results of the equilibrium tests of S II are shown in Fig. 6(b). Especially in the $\theta$-direction, a significant drop of the Cauchy stress can be recognized after 30 min. Therefore, to secure an equilibrium of the mechanical response of the tissue, the equilibrium time was set to $t_{\text{eq}} = 45 \text{ min}$ for all subsequent indentation tests.

In addition, the curves in Fig. 6(c) present a representative relaxation behavior of the RCA during relaxation tests. When reaching the indentation force of $F_{\text{ind}} = 2 \text{ N}$, the biaxial response of the specimen peaked at $f_{\text{max}} = 0.71 \text{ N}$, $f_{\text{max}} = 1.40 \text{ N}$ for S III and $f_{\text{max}} = 0.95 \text{ N}$, $f_{\text{max}} = 1.41 \text{ N}$ for S IV, which results in supraphysiological Cauchy stresses of $\sigma^{\text{max}}_{z} = 101.0 \text{kPa}$, $\sigma^{\text{max}}_{\theta} = 203.7 \text{kPa}$ and $\sigma^{\text{max}}_{z} = 117.3 \text{kPa}$, $\sigma^{\text{max}}_{\theta} = 171.8 \text{kPa}$, respectively. While keeping the indentation force constant, the forces in the $\theta$- and $z$-direction dropped immediately, followed by a gradual decrease of approximately 14 and 4%, respectively. After about 30 min, almost no further decrease of the reaction forces was found. Based on these findings and by adding an extra buffer time, the timer for the strut indentation in the indentation tests was set to $t_{\text{eq}} = 45 \text{ min}$.

The representative Cauchy stress-stretch relationship of measurement series I of specimens S V, and S VII depicted in Fig. 6(d) exposes a very heterogeneous material response of vital porcine RCA tissue in both directions. Nevertheless, by comparing the data from measurement series I and II, i.e., data from healthy and injured tissue, softening of the tissue can be detected after the strut indentation. If the strut points were towards $z$, a longitudinal softening of $\Delta \lambda_z = 0.007$ (gain: 5.0%) after indenting with $F_{\text{ind}} = 1 \text{ N}$ and $\Delta \lambda_z = 0.014$ (gain: 11.5%) when indenting with $F_{\text{ind}} = 2 \text{ N}$, and a circumferential softening of $\Delta \lambda_{\theta} = 0.056$ (gain: 35.6%) after indenting with $F_{\text{ind}} = 1 \text{ N}$ and $\Delta \lambda_{\theta} = 0.129$ (gain: 51.8%) when indenting with $F_{\text{ind}} = 2 \text{ N}$ was measured.

If the strut points were towards $\theta$, a longitudinal softening of $\Delta \lambda_z = 0.017$ (gain: 12.5%) after indenting with $F_{\text{ind}} = 1 \text{ N}$ and $\Delta \lambda_z = 0.027$ (gain: 18.9%) when indenting with $F_{\text{ind}} = 2 \text{ N}$, and a circumferential softening of $\Delta \lambda_{\theta} = 0.057$ (gain: 25.8%) after indenting with $F_{\text{ind}} = 1 \text{ N}$ and $\Delta \lambda_{\theta} = 0.720$ (gain: 28.2%) when indenting with $F_{\text{ind}} = 2 \text{ N}$ was measured. This reveals a trend, which implies that in particular the circumferential softening increases remarkably with higher indentation pressures if the strut points were towards $z$. If the strut is turned towards $\theta$, the softening in the longitudinal direction seems to increase with the indentation pressure, while the increase of the circumferential softening is not as severe. An equal trend could be derived from the data of the specimens of pig heart 2. The test with the control specimen S XIII without strut indentation showed no difference in the material response. The Cauchy stresses and stretches ranged in intervals of $\sigma^{\text{max}}_{z} \in [16.5, 19.7] \text{kPa}$, $\sigma^{\text{max}}_{\theta} \in [40.6, 41.3] \text{kPa}$, $\lambda^{\text{max}}_z \in [1.12, 1.18]$, $\lambda^{\text{max}}_{\theta} \in [1.21, 1.30]$ when indenting with $F_{\text{ind}} = 1 \text{ N}$, and in $\sigma^{\text{max}}_{z} \in [17.8, 21.0] \text{kPa}$, $\sigma^{\text{max}}_{\theta} \in [42.8, 45.1] \text{kPa}$, $\lambda^{\text{max}}_z \in [1.14, 1.18]$, $\lambda^{\text{max}}_{\theta} \in [1.28, 1.38]$ when indenting with $F_{\text{ind}} = 2 \text{ N}$.

### 3.2. Structural Response

Elevation profiles of lesions generated with varying strut orientations and indentation pressures are presented in Fig. 7. In all four samples, the tissue bulges at the end of the indentation, while the compression in the area of interest, i.e., the center of the lesion and along the strut, is rather homogeneous. Every specimen got compressed more with increasing indentation pressure. However, differences in the extent and shape of the lesion can be detected between specimens with a strut orientation angle of $\phi = 0^\circ$ and $90^\circ$. Thus, the values for $k_{\text{max}} = [1.25, 1.00] \text{ mm}$ generated with $F_{\text{ind}} = [1, 2] \text{ N}$ and $\phi = 90^\circ$ were rather high, while the compression under the strut was, with $\Delta h_{\text{max}} = [0.11, 0.09]$, mostly moderate (see Fig. 7(a) and (b)). The sidewalls of the lesions protruded slightly with a small angle. The opposite can be observed when the strut is oriented in the $\theta$-direction with $\phi = 0^\circ$ (see Fig. 7(c) and (d)). The extent of the lesion is, with $k_{\text{max}} = [0.65, 0.60] \text{ mm}$, smaller, the compression under the strut, with $\Delta h_{\text{max}} = [0.09, 0.07]$, more severe, and the lesion exhibited steeper sidewalls next to the indentation. However, $k_{\text{max}}$ seems to decrease with increasing force in both cases (see Fig. 7(a) with (b), and (c) with (d)).

Detailed out-of-plane sections of the control specimen S XIII taken in the normal directions to the ($r,z$)- and ($r,\theta$)-plane, are presented in Figs. 8(a) and (b). Two layers are clearly distinguishable, the media and the adventitia, whereas the intima was not visible, and, therefore, considered as part of the media. In both sections, the adventitia is clearly divided into its sub-layers, the IA and EA, which take up two-thirds of the wall thickness. The quality of the signal is best with both out-of-plane sections in the EA, where thick and randomly dispersed bundles of wavy collagen fibers are present. However, in Figs. 8(a) and (b), the signal quality differs for the media and the IA. Thus, the fine collagen structure can be better obtained in the media if an out-of-plane section is generated parallel to the fibers in the ($r,\theta$)-plane (see Fig. 8(b)), while most of the thicker fiber bundles of the IA can be better distinguished in out-of-plane sections in the ($r,z$)-plane (see Fig. 8(a)).

Cross-sections of the left sides of the lesions of specimens S IX and S XI generated with $F_{\text{ind}} = 2 \text{ N}$ are depicted in Figs. 8(c) and (d). Again, the signal quality for the layers differs in both images. However, the angles of the sidewalls of the lesions are rather small if the strut is turned to the $z$-direction (see Figs. 8(c)), but the lesion is twice as wide in comparison to specimen S XI where the strut was oriented in the $\theta$-direction (see Fig. 8(d)). When comparing both sections, the media and the whole adventitia in Fig. 8(c) are getting gradually thinner over the entire lesion, while in Fig. 8(d) the two layers get only significantly compressed next to the lesion. Although the media, IA, and EA retain the reference ratio of their thicknesses even in the deepest point of the lesion if the strut points toward $z$, the media and IA get almost completely thinned out if the strut is oriented in the $\theta$-direction. In both cases, the fibers in the adventitia lose their waviness and align towards the lesion, as can be seen more severely in Fig. 8(c). In addition, the fiber density increases next to the lesion, especially in the indentation, and gaps can hardly be noticed. It is remarkable that no visible fiber rupture was detected, not even at the strut ends. Fibers next to the indentation are pushed aside, reoriented, and formed a ‘boat-like’ structure (see Fig. 8(e)). In the deepest point of the indentation, a consistently strong but blurred signal is dominating. Individual fibers and their orientation can no longer be determined.

Detailed in-plane sections of all layers and the respective intensity plot of the control specimen S XIII under physiological loading in the reference configuration are depicted in Fig. 9(a). The intensity plot illustrates the collagen fiber orientation and disper-
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Fig. 7: Half isometric 3D-surface scans of injured porcine RCA specimens: (a),(b) show scans of specimens SV and SVII generated with an indentation force of $F_{\text{ind}} = \{1, 2\}$ N, respectively, and the lesion points in the $z$-direction; (c),(d) show scans of specimens SIX and SXI generated with $F_{\text{ind}} = \{1, 2\}$ N, and the lesion points in the $\theta$-direction. All dimensions are in millimeter; $z_{\text{min}}$ = compression in the center of the lesion; $k_{\text{max}}$ = distance between the low-affected tissue and the strut indentation.

Fig. 8: Collection of SHG-images of porcine RCA tissue in the deformed configuration: (a),(b) are detailed out-of-plane sections of native tissue of specimen SXIII under physiological loading taken from the $(r,z)$- and $(r,\theta)$-plane, respectively. Only the media (M), the inner adventitia (IA), and the exterior adventitia (EA) are distinguishable; (c),(d) are out-of-plane images showing cross-sections of the lesions of specimens SVII and SXI in the $(r,z)$- and $(r,\theta)$-plane generated with an indentation force of $F_{\text{ind}} = 2$ N, with the strut pointing in the $z$- and $\theta$-direction, respectively; (e) in-plane section of SIX in the $(\theta,z)$-plane reveals the tendency of collagen fibers to take on a 'boat-shape' surrounding close to the indentation of the strut. The image plane was slightly tilted around $\theta$ so that the media can be seen on the left side and the IA on the right side. All dimensions are in mm.
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(a) Native, porcine RCA tissue

(b) Low-affected (at \( P_L \) in \( k = k_{\text{max}} \)) and high-affected (at \( P_H \) in \( k = k_{\text{max}} \)) tissues of specimens SVII and SXI in the reference configuration; (b) low-affected (at \( P_L \) in \( k = k_{\text{max}} \)) and high-affected (at \( P_H \) in \( k = k_{\text{max}} \)) tissues of specimens SVII and SXI in the deformed configuration treated with an indentation force of \( F_{\text{ind}} = 2 \text{ N} \), and the strut points in the \( z \)- and \( \theta \)-direction, respectively. All dimensions are in \( \mu \text{m} \). In the intensity plots, the abscissas refer to the fiber angle, where \( 0^\circ \) and \( \pm 90^\circ \) denote for the \( z \)- and \( \theta \)-direction, respectively. The preferred fiber directions are intensified with red areas and blue areas correspond to a low fiber density. \( \phi \) = strut orientation angle; \( k \in [0, k_{\text{max}}] \) = distance to lesion; \( P_L \) = point in the zone of low-affected tissue at distance \( k_{\text{max}} \) (see Fig. 5); \( P_H \) = point in the zone of high-affected tissue next to the lesion.

Fig. 9: Collection of in-plane SHG-images and intensity plots of the media (M), inner adventitia (IA), and exterior adventitia (EA) of porcine RCA tissue: (a) native, porcine RCA tissue of specimen SXIII in the reference configuration; (b) low-affected (at \( P_L \) in \( k = k_{\text{max}} \)) and high-affected (at \( P_H \) in \( k = k_{\text{max}} \)) tissue of specimens SVII and SXI in the deformed configuration treated with an indentation force of \( F_{\text{ind}} = 2 \text{ N} \), and the strut points in the \( z \)- and \( \theta \)-direction, respectively. All dimensions are in \( \mu \text{m} \). In the intensity plots, the abscissas refer to the fiber angle, where \( 0^\circ \) and \( \pm 90^\circ \) denote for the \( z \)- and \( \theta \)-direction, respectively. The preferred fiber directions are intensified with red areas and blue areas correspond to a low fiber density. \( \phi \) = strut orientation angle; \( k \in [0, k_{\text{max}}] \) = distance to lesion; \( P_L \) = point in the zone of low-affected tissue at distance \( k_{\text{max}} \) (see Fig. 5); \( P_H \) = point in the zone of high-affected tissue next to the lesion.

Discussion

In this pilot study, we quantified vascular damage by defining damage mechanisms inside the tissue by investigating mecha-
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Table 2
Mean values of the structural parameters $\alpha$, $\kappa_{ip}$ and $\kappa_{op}$, obtained from SHG images of the media and adventitia layers of the native specimen S XII in the reference configuration, and the injured specimens S V, VII, IX, XI in the stented configuration correlated with the indentation parameters, the resulting tissue softening, and compression.

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Indentation parameters</th>
<th>Softening</th>
<th>Compression</th>
<th>Layer</th>
<th>Structural parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$F_{ind}$/P$_{ind}$ (N/MPa)</td>
<td>$\phi$ (°)</td>
<td>$\Delta l_i/\Delta l_0$ (%)</td>
<td>$T_0$ (mm)</td>
<td>$t$ (mm)</td>
</tr>
<tr>
<td>S V</td>
<td>1.0/4.1</td>
<td>90 (long.)</td>
<td>4.96/35.63</td>
<td>1.25</td>
<td>0.85</td>
</tr>
<tr>
<td>High-aff.</td>
<td></td>
<td>90 (long.)</td>
<td>11.53/51.83</td>
<td>1.00</td>
<td>0.97</td>
</tr>
<tr>
<td>S VII</td>
<td>2.0/8.3</td>
<td>90 (long.)</td>
<td>12.54/25.84</td>
<td>0.65</td>
<td>0.66</td>
</tr>
<tr>
<td>High-aff.</td>
<td></td>
<td>0 (circ.)</td>
<td>18.90/28.17</td>
<td>0.60</td>
<td>0.94</td>
</tr>
<tr>
<td>S IX</td>
<td>1.0/4.1</td>
<td>0 (circ.)</td>
<td>18.90/28.17</td>
<td>0.60</td>
<td>0.94</td>
</tr>
<tr>
<td>High-aff.</td>
<td></td>
<td>0 (circ.)</td>
<td>18.90/28.17</td>
<td>0.60</td>
<td>0.94</td>
</tr>
</tbody>
</table>

$F_{ind}$ = indentation force; $P_{ind}$ = indentation pressure; $\phi$ = strut orientation angle with respect to $\theta$; $\Delta l_i/\Delta l_0$ = softening gain; $T_0$ = thickness of the unloaded specimen; $t$ = thickness of the specimen at point P$_i$ and P$_{op}$, respectively;
$\lambda_i$ = compression of the low- or high-affected tissue with $i = L, H$; $\alpha$ = angle between the mean fiber direction and the $\theta$-direction; $\kappa_p$, $\kappa_{ip}$, $\kappa_{op}$ = in- and out-of-plane dispersion quantities.

4.2. Damage Mechanisms

With the help of the proposed experimental indentation tests damage mechanisms such as tissue compression and softening, differences in the collagen fiber dispersion and lesion formation could be identified. These damage mechanisms depend significantly on (i) the contact pressure between the coronary artery and the indenting stent strut, (ii) the strut orientation, and (iii) the position, i.e., the distance between a point of interest inside the tissue and the center of the strut indentation.

4.2.1. Influence of Indentation Pressure

Higher indentation pressures seem to primarily increase the severity of damage mechanisms. Thus, higher pressures led to more softening in the $z$- and $\theta$-direction of all tested specimens, which could be a potential indicator for vascular damage. However, the percentage gain in the stretch due to softening was higher in the $\theta$-direction in all tests (see Figs. 6(d) and (e)). Furthermore, an indentation with a higher pressure led to a greater radial compression $\lambda_0$ of the tissue directly below the strut (see Figs. 7(a) and (b)). Again, $\lambda_0$ was more severe in the $\theta$-direction. On the contrary, the distance $k_{max}$ seemed to shrink to a higher indentation pressure.

4.2.2. Influence of Strut Orientation

Our findings demonstrate the importance of the strut orientation angle $\phi$ for the directional development of damage mechanisms. The out-of-plane section in Fig. 8(c) uncovers that a strut, which is oriented in the $z$-direction, cannot push fibers to the side

cal and morphological alterations. We have outlined that tissue of porcine coronary arteries softens when indented by a stamp which is shaped as a stent strut, but the severity is directional and changes with position, strut orientation and indentation pressure. It turns out that all of these mechanical phenomena are influenced by changes in the fibrillar collagen structure. We confirmed our hypothesis whereby the structural parameters $\alpha$, $\kappa_{ip}$, $\kappa_{op}$ introduced in [17] differ between native and injured tissues. The presented in vitro experimental methods combined with an advanced imaging strategy were highly efficacious for the quantification of strut-triggered mechanical and morphological alterations in porcine coronary arteries. We have demonstrated that the proposed experimental testing rig LAESIO has the potential to simulate the in situ loading scenario during PCI in an in vitro environment. Morphological analysis including tissue clearing and 3D-surface, as well as SHG scanning could be applied to deformed specimens. The determination of structural parameters for native, low-affected, and high-affected tissues following the approach proposed in [17] and their subsequent comparison turned out to be a potential tool to quantify changes in the fiber dispersion in the layers of coronary arteries. To our knowledge, this is the first study that provides information on how the mechanical behavior and structural parameters of porcine coronary arteries change due to an indenting stent strut. Despite a small number of tested specimens, we are able to identify key drivers for specific damage mechanisms after analyzing the experimental and imaging data.

4.1. Characteristics of Native Porcine RCA

After analyzing the results from experimental preconditioning and relaxation tests (compare with Figs. 6(a) and (b)), tissues of porcine RCA under physiological loading can be described as an anisotropic, nonlinear, and marginal viscoelastic material, which agrees with findings in the literature, see, e.g., [26]. Consistent with Wang et al. [47], the scanned media layers of untreated coronary arteries (Figs. 8(a),(b) and 9(a)) contained a fine collagen structure with a high degree of fiber orientation in the $\theta$-direction, with a small degree of dispersion indicated by rather small values for $\kappa_p$ (see Table 2). The results show that the adventitia can be subdivided into the IA and EA, which confirms the findings documented in [35, 38]. The thicker fibers in the IA tend to align more in the $\theta$-direction, while the wavy fiber bundles of the EA point mostly towards the $z$-direction. The degree of dispersion in the adventitia is significantly higher as in the media, as shown by larger values for $\kappa_{ip}$. 

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since they are aligned perpendicular to the long side of the strut. The affected fibers of the media and the IA resist against the indentation and get straightened and significantly compressed. This results in a severe softening of the tissue in the $\theta$-direction (see Fig. 6(d)). This trend coincides with our findings from preliminary tests with porcine descending aortas [40]. The degree of fiber dispersion, however, decreases with a higher indentation pressure, as shown by the smaller values for $k_{ip}$ (see Table 2). Both fiber families seem to align as the mean fiber angle $\alpha$ gets smaller. On the contrary, if oriented in the $\theta$-direction, the strut potentially squeezes through the collagen fibers of the media and IA as long as the strut orientation aligns with the orientation of the fibers. This might be the reason why the strut was able to indent deeper into the tissue. In addition, fewer fibers are affected by the indentation, causing less severe softening in the $\theta$-direction, but slightly more severe softening in the z-direction (see Fig. 6(d)), as the gap splits and, therefore, widens the tissue. The degree of fiber dispersion, however, is increasing, as $k_{ip}$ increases. In addition, both fiber families seem to split up more as $\alpha$ increases at higher indentation pressures.

4.2.3. Influence of Position

If the strut is oriented in the z-direction, the degree of dispersion in the high-affected tissue increases, as shown by higher values for $k_{ip}$. Even if the fibers get compressed, we assume them to get squeezed into each other in the $r$-direction and, due to the increased fiber density, pushed towards the strut ends, which leads to a greater dispersion; thus, also $\alpha$ increases. If the strut was oriented in the $\theta$-direction, $k_{ip}$ and $\alpha$ decrease in the high-affected tissue. We assume that this is caused by the local compression of the fibers in the $\theta$-direction. Through the increasing fiber density, the fibers have to align and the dispersion decreases.

4.3. Impact on Stented Arteries

To summarize these key findings, we want to transfer them to the scenario of a straight element of a stent strut that indents into the tissue of a porcine coronary artery, as depicted in Fig. 1(b). For a better understanding, we want to interpret the findings by consulting Fig. 10 together with Table 3. We now consider two cases of the stent strut indenting into the tissue with an indentation pressure beyond the physiological range:

Case (A) – strut points towards z ($\phi = 90^{\circ}$): A large number of collagen fibers in the media and IA get compressed along the entire strut length, but both layers keep their thickness ratio. In the EA, mainly fibers which are situated directly under the strut are affected. In addition, a flat but broad lesion starts to form. These broad lesions may provoke extensive cell proliferation and, therefore, pronounced in-stent restenoses. The fiber density increases inside the tissue, and the volume of the extra-cellular matrix shrinks next to the strut and beyond, as the indentation pressure is distributed over a large area. Thus, the tissue stretches which results in softening in the z- and in particular in the $\theta$-direction. We assume that the fibers in the media and IA get pushed into each other. Due to the increasing density, they give way to the neighboring fibers and cause an increase in the degree of the fiber dispersion and the mean fiber angle. Few affected fibers in the adventitia align along the strut due to stretching, i.e., the fiber dispersion decreases together with the mean fiber angle as the compression in the $\theta$-direction is only moderate.

Case (B) – strut points towards $\theta$ ($\phi = 0^{\circ}$): In comparison to case (A), the collagen fibers in the media and IA get compressed more and mainly in the immediate vicinity of the strut. The thick fiber bundles of the EA have to bear most of the load. The lesion is deeper, and its sidewalls are steep. This may cause only local restricted cell proliferation. Nevertheless, these narrow lesions located perpendicular to the flow direction could increase the risk of thrombus formation. The softening is now more severe in the z-direction since most of the fibers of the EA are affected. The softening in the $\theta$-direction is smaller if compared with case (A) as only a few fibers of the media and the IA get stretched under and next to the stent strut. The degree of the fiber dispersion in the adventitia increases as fibers get squeezed into each other, but decreases in the media as fibers align parallel to the stent strut. In both cases, the fibers directly under the strut might get severely squeezed or even destroyed, as in our tests, single fibers were not identifiable under the strut (see Fig. 8(e)).

4.4. Further Findings

The preconditioning behavior reveals that only three loading cycles are enough to reproduce the material behavior in the assumed physiological range. The stress-stretch relationship matches with the results of Wang et al. [47]. We found that a waiting time of 45 mins is adequate to reach a biochemical, thermodynamical, and mechanical equilibrium for porcine coronary arteries prior to tensile tests (see Fig. 6(b)). Only then it can be guaranteed that experimental data are reproducible. The activities of vital SMC in the tissue might be decisive for this phenomenon. We suppose that the waiting time is influenced by sample preparation and storage techniques. Humphrey [34] q.v. mentioned that this equilibrium also

![Fig. 10: Schematic sections of the fibrillar collagen structure of the media (M), inner adventitia (IA), and exterior adventitia (EA) as well as the development of the damage mechanism at increasing indentation pressure inside coronary arteries for the strut orientation angles $\phi = (0^{\circ}, 90^{\circ})$.](image)
depends on the imposed mechanical loads. Finally, we defined a relaxation time of at least 30 mins for the tissue of porcine RCA to overcome viscoelastic processes and creep if a strut indents with \( p_{\text{ind}} = 8.30 \text{ MPa} \) (compare with Fig. 6(c)). This rather long relaxation time is a sign of a very high amount of viscous material in the RCA tissue and might be caused by a high content of SMC in the medial layer [37].

LAESIO, the proposed testing device, was found to be suitable to realistically simulate the loading scenario of stented coronary arteries and to chemically fix the specimen under load. The testing protocol demonstrated to be robust and straightforward to perform.

The combination of 3D-surface as well as a set of in- and out-of-plane SHG scans enabled the determination of the lesion extent and the fibrillar collagen structure. The method proposed by Schriefl et al. [29, 30] could be adapted for analyzing alterations of the mean fiber angle and the fiber dispersion in low- and high-affected tissues of the medial and adventitial layers. The subsequent computer-assisted image preprocessing made it possible to derive structural parameters from deformed and injured tissue.

4.5. Limitations

In the biaxial extension tests, dynamic effects caused by the blood pressure and the rapid expansion of the stent were not considered and instead a static loading scenario was assumed (see Fig. 1(a)). Furthermore, we only investigated vascular injuries caused by straight strut struts. In the future, different stamp designs with curved struts should be added to the study. As it is technically not feasible to measure the indentation pressure of a strut in situ, the extremum of the loading range was taken from the FEA investigation of a Palmaz-Schatz stent [48], as a first reference. The boundary conditions between the myocardium and the adventitia were ignored during testing to enable stretch measurements. The number of samples in the subsequent tests should be significantly increased since tissues of coronary arteries tend to be heterogeneous and also seem to differ from pig to pig. The sample preparation, indentation tests, chemical fixation, tissue clearing, and the imaging process turned out to be very time consuming. Thus, a fixed and cleared specimen is ready after approx. 24 h, while the imaging takes up to 6 h. While performing SHG scans, we were confronted with poor signal quality in the media of some specimens, even after tuning the wave length and the resolution. Imaging issues caused by a weaker SHG signal in the media layer [37].

4.6. Future Aspects

At this stage, it is challenging to formulate recommendations for the improvement of constitutive and computational damage models, to simulate the presented damage mechanism with the finite element method. Within a continuum framework, an expansion of the model proposed in [17] seems to be feasible. Additional parameter sets need to be incorporated in such a model, which allow to fit the numerical results to an experimentally obtained function that describes the stress-stretch relationship in dependency of the radial compression \( \lambda_r \). For this purpose, whole specimens of healthy and diseased tissues have to be compressed homogeneously with defined varying pressures and the stress-stretch response in the \( z \) - and \( \theta \) -direction subsequently measured. Nevertheless, with this study we have shown that the material response and the injury development of stented coronary arteries seem to be influenced by the load-, direction-, and position-dependent damage mechanisms inside the tissue. Thus, the collagen structure adapts to the load, compresses, and fibers reorientate (see Fig. 8(e)), while the severity of the damage decreases with increasing distance to the stent strut. Therefore, a discrete modeling approach might be preferable to describe the dominant damage mechanism more accurately. Due to the lack of experimental data and for deeper insights into these pathological events, the proposed study should be pursued. Further experiments, including tests with healthy and diseased human tissues with extended test series including a wider variety of indentation pressures and strut orientation angles have to be carried out. By analyzing empirical data, Swier et al. [19] successfully linked the injury score of Schwartz et al. [4, 5] with the degree of neointimal growth. We suppose that the same injury score could be used to classify artificially created vascular injuries of the proposed experiments. In this way, cell growth could be correlated with mechanical and imaging data. Additionally, the contributions of other constituents to vascular damage formation such as elastin and proteoglycans should be investigated at the nano, micro, and macrolevels. Finally, the observation of stent-triggered alterations of the contractile or synthetic phenotype of endothelial cells, fibroblasts, and vascular smooth muscle cells as well as apoptosis and cell proliferation in active tissue is an important task to assign to see if the induced vascular damage is irreversible.

5. Conclusion

The main finding of this study is that PCI-triggered damage mechanisms such as tissue compression and softening, and changes in collagen fiber dispersion contribute to the formation of vascular damage. We demonstrated that the development of these damage mechanisms and the extent of lesions are directional, and the severity is linked to strut orientations, the indentation pressure, and the position. No fiber ruptures were discovered during our tests. To our knowledge, we present here the first approach that links multiaxial mechanical data of high-affected tissue with structural parameters obtained from three-dimensional scans of the collagen structure. The testing device LAESIO has shown to be suitable to simulate PCI in vitro and to quantify mechanical alterations. Our testing protocol was adapted to the findings of equilibrium and relaxation tests. Imaging methods like 3D-surface and SHG scans could be performed on deformed specimens.

This research should be considered as a foundation for further studies, which focus on the examination of vascular damage – the most potent stimulus for in-stent restenosis development. More data would be necessary to strengthen the statistical power of these findings and to enable a continuum or discrete framework for more accurate damage models used in finite element analyses to contribute further to the optimization process of stents.

Declaration of Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

We want to show our gratitude to S. Diezel from the Core Facility Bioimaging at the Biomedical Center, Ludwig-Maximilians-Universität München, for assisting the SHG-imaging process. A. Pukuluk and S. Sherifova from the Institute of Biomechanics, Graz University of Technology, for their advise in the image analysis,
and the members of the Regensburg Center of Biomedical Engineering for their helpful support, in particular F. Erzinger and F. Muehlenberg for assistance during the setup process of the first version of the testing rig.

This research was funded by the Bayerische Staatshochschule für Bildung, Bildung, Wissenschaft, and Arts within the project ‘OptiStent’ (AZ VIII.2-F1116.RE/17/3 and PIZ-225-18). Personnel funds, consumables, and imaging costs were subsidiary financed from the project ‘LAESIO’ (P 32713) of the Austrian Science Fund (FWF). Technical equipment was partly financed by the Regensburg Center of Biomedical Engineering (RCBE) and by private parties.

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References
Quantifying vascular damage by investigating stent-triggered mechanical and morphological alterations


