Damage and failure in the carotid artery: a mechanistic approach

Lauren Beatty Priddy

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DAMAGE AND FAILURE IN THE CAROTID ARTERY: A MECHANISTIC APPROACH

By

Lauren Beatty Priddy

A Thesis
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Master of Science
in Biomedical Engineering
in the Department of Agricultural and Biological Engineering

Mississippi State, Mississippi

August 2010
DAMAGE AND FAILURE IN THE CAROTID ARTERY: A MECHANISTIC APPROACH

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Blunt carotid artery injury (BCAI), resulting primarily from automobile accidents, is a major contributor to the high mortality and morbidity rates associated with carotid artery dissection. More work is needed to characterize carotid artery injury mechanisms, quantify stages of damage, and elucidate failure modalities as a result of this type of injury. The present study examines the structure and mechanics of the carotid artery in the circumferential and axial directions by employing uniaxial tensile testing, high speed videography, interruption testing, scanning electron microscopy (SEM), histological analysis, real-time environmental SEM assessment, and atomic force microscopy (AFM). Results are as follows: (i) the carotid artery exhibits anisotropic, viscoelastic behavior; (ii) intimal failure precedes ultimate tissue failure, and the layers in order of increasing strength are intima, adventitia, and media; (iii) tissue damage accumulates as strain level increases, and failure occurs as a result of void nucleation, void growth, and void coalescence.
DEDICATION

This research is dedicated to my parents, Hamp and Kim Beatty. Thank you for the love, encouragement, support, and inspiration you have provided me. For your constant sacrifice and generosity, I am truly grateful. You instilled in me the belief that through hard work, anything is possible. Thank you for being my heroes.

To Matthew, you are my rock, the smartest person I know. Thank you for having the answers to all my questions. Going through this process with you made it that much more enjoyable. I am so proud to call you my husband.

To all my friends and family, thank you for molding me into the person I am today. I appreciate each and every one of you.
ACKNOWLEDGEMENTS

I would like to acknowledge the students, faculty, and staff and Mississippi State University who had a hand in contributing to the completion of this work. Dr. Jun Liao, my major professor, deserves special thanks for his sacrifice of time and assistance. I am grateful to all the members of my graduate committee, Dr. Lakiesha Williams, Dr. Mark Horstemeyer, and Dr. Allen Crow, for their direction and help with my project. I appreciate the unending efforts from Amanda Lawrence and Bill Monroe at the Electron Microscope Center. Thanks also to Jean-Luc Bouvard at the Center for Advanced Vehicular Systems, Jeff Koon with Dynamic Imaging, and Sophia Hohlbauch with Asylum Research. Finally, I am grateful for the help of my fellow students, particularly Joe Chen, Scott Tran, John Clemmer, and Abel Lowry, for their dedication.
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CHAPTER I
MOTIVATION

Although the incidence of blunt carotid artery injury (BCAI) is low, mortality rates (40%) and correlated chronic neurological morbidity rates (40-80%) are very high [1-5]. BCAI causing carotid artery dissection results primarily from automobile accidents. The effects of dissection include headaches and neck pains, Horner’s syndrome, cranial nerve damage, transient ischemic attack, stroke, and death [6].

A thorough investigation into the biomechanics of the nondiseased carotid artery is an important step in understanding the progression of damage of the vessel, even to failure, that often accompanies traumatic events. Few studies have examined the mechanical behavior of the individual arterial layers, and this remains a limiting factor particularly in the understanding of dissection failure. A detailed description of the macroscale mechanical properties is facilitated through various multiscale experimentation techniques to delineate the layer-specific material behavior. The data herein serves as a basis for future constitutive modeling efforts and contributes to the development of the virtual human model at Mississippi State University.

Research Objectives

The main objectives of this study were (i) to examine the anisotropic, viscoelastic nature of the carotid artery, and (ii) to determine damage mechanisms and failure
modalities of the carotid artery. The best method of predicting material behavior during damage progression is a systematic approach to understanding how damage accumulates and how the altered microstructure influences the tissue’s mechanical response. The increasing ultrastructural damage of the carotid artery during loading can be characterized by analyses of (i) the uncrimping and breakage of elastin and collagen fibers, and (ii) the stretching of smooth muscle cells. Since each arterial layer (intima, media, and adventitia) has distinct morphological, structural, and mechanical properties, characterizing each layer in terms of damage progression is critical to understanding the failure mechanics of the artery as a whole.

Uniaxial tensile interruption tests on artery strips oriented both longitudinally and circumferentially were performed to various strain levels. Immediately after testing, with strain held constant, tissues were fixed to preserve their morphological state. For failure testing, a high speed camera was utilized to distinguish between intimal failure and ultimate failure of the tissue. Scanning electron microscopy (SEM) and histological analysis were applied to observe morphological alterations of collagen and elastin fibers and smooth muscle cells. An in situ tensile testing coupled with environmental SEM was utilized to examine arterial microstructure at various strain levels. This novel procedure is beneficial because of its capability to capture instantaneous changes without traditional dehydration and metallic coating methods. Image analysis was performed to assess initiation and propagation of voids within the intimal layer revealed by environmental SEM. For microscale tissue characterization of the multilayered carotid artery, atomic force microscopy (AFM) was utilized to obtain force maps through the wall thickness, which elucidated the mechanical properties of each layer.
Overview

The present report is organized in a traditional style, with the subsequent chapters as follows: *Introduction, Methods, Results, Discussion,* and *Conclusion.* *Introduction* includes background and previous research on (i) arterial microstructure and physiology, (ii) the artery’s major mechanical roles related to structure and function, and (iii) the causality and mechanics of arterial dissection. *Methods* provides a detailed explanation of the various experiments that were performed. Following the same order as the previous chapter, *Results* gives findings from each experimental technique. *Discussion* contains a much more descriptive explanation of the results and suggests theories as to the bases of the findings. Also mentioned are problems encountered during experimentation, limitations of the study, and related future efforts. *Conclusion* provides a synopsis of the aforementioned text.
CHAPTER II
INTRODUCTION

Arterial Microstructure

The arterial system functions to transport blood from the heart to capillaries throughout the body. The pulmonic portion of the arterial system consists of low-pressure vessels that move deoxygenated blood from the heart to the lungs, whereas the (much larger) systemic portion of the arterial system contains high-pressure vessels that carry oxygenated blood from the heart to the rest of the body [7]. The largest systemic vessel is the aorta, which branches into the brachiocephalic artery and subsequently the right common carotid artery, the vessel of interest in this study (Figure 1). Note that the portions of the left and right common carotids in the neck are anatomically identical.
Arterial microstructure varies with location, age, disease, and species. In general, arteries are classified as either elastic or muscular. Elastic arteries (i.e. aorta, main pulmonary artery, common carotids, and common iliacs) are larger in diameter and located closer to the heart. Muscular arteries (i.e. coronaries, cerebrals, femorals, and renals) are smaller in diameter and located closer to the arterioles. Transitional arteries (i.e. external carotids) are intermediary in location and exhibit traits of both the elastic and muscular arteries [7]. All arteries are composed of three layers: tunica intima, tunica media, and tunica adventitia (Figure 2), each with different physiological and mechanical characteristics.
The intima, which is the innermost layer, is made of a single layer of endothelial cells and a basal lamina (basement membrane) beneath. Endothelial cells are primarily flat and elongated along the direction of blood flow, as they are influenced by changes in stress and strain caused by blood pressure and altered blood flow. Endothelial cells are surrounded by tight occluding junctions that regulate transport processes through the endothelium, and in-plane gap junctions that provide cell-cell communication via ion transport. The basal lamina just beneath comprises type-IV collagen, proteoglycans, and the adhesion molecules laminin and fibronectin. Although it functions somewhat as structural support, it serves mainly as the meshwork on which endothelial cells can attach and grow [7].

The internal elastic lamina, which connects the intima and media (middle layer), is a layer of fenestrated elastin that provides for transport of water and nutrients across the wall and for communication between cells. The media contains smooth muscle cells
(SMCs) embedded in a network of elastin and collagen fibers (primarily types I, III, and V), along with a ground substance of proteoglycans. Vascular smooth muscle cells are spindle-shaped and covered by a basement-type membrane. SMCs communicate through gap junctions and transmit force through surrounding collagen fibrils. The media contains 10 μm-thick repeating units (40-70 in a thick elastic artery) of concentrically-oriented SMCs covered by thin elastin sheets similar to the internal elastic lamina [8]. These units, along with the collagen fibers between the elastin sheets, are collectively named the musculo-elastic fascicle and represent the basic structural and functional unit of the media [9, 10] (Figure 3). The outermost sheet, the external elastic lamina, separates the media and adventitia.

Figure 3. Representation of the musculo-elastic fascicles found in elastic arteries such as the carotid. C and L define the circumferential and longitudinal orientations, respectively. Note the highly organized elastin fibers (E) and smooth muscle cells (Ce), as well as the collagen fibril matrix (M) and collagen fiber bundles (F). Adapted from [10].
The adventitia, or outer layer, is composed mainly of type I collagen in a dense scaffold, surrounded by fibroblasts, elastin, nerves, and the vasa vasorum.

Figure 4. Detailed schematic of the heterogeneous layers of the healthy artery. From [11].

**Arterial Physiology**

The endothelium functions as a smooth, nonthrombogenic barrier between the vessel wall and the blood, while allowing the passage of substances to and from the bloodstream. Moreover, the endothelium serves many important biological functions, as it controls coagulative processes; synthesizes vasoactive substances, growth factors, and connective tissue; and alters plasma lipoproteins for transport into the arterial wall. Shear stress from the blood flow causes endothelial cells to lengthen and orient themselves in
the direction of flow. Thus, although a small percentage of the wall thickness, the endothelium has a primary role in (local) vascular mechanics [7].

The media is the thickest of the three arterial layers. Smooth muscle cells therein make up anywhere from 25-60% of the dry weight of the artery wall [12]. Similar to skeletal muscle, smooth muscle generates its maximum force at a unique length. This unique length cannot be achieved without a considerable amount of passive tension in smooth muscle before activation [13], an important factor to consider in constitutive modeling. SMCs are oriented nearly circumferentially [8], which allows them to function in the contraction of the vessel wall, thereby controlling the dilation of the vessel [12]. At the molecular scale, the amount of intracellular calcium determines contraction of smooth muscle. Intracellular calcium levels range from 100 nM at basal state to 600-800 nM during complete contraction [13].

The adventitia is only approximately 10-30% of the arterial wall thickness [7, 14]; however, it serves as a protective covering and provides partial structural support, as it limits overstretching of the vessel. Fibroblasts serve a major function in the maintenance of the adventitia through the synthesis of type I collagen [7].

**Material Symmetry**

Results on material symmetry from previous literature are conflicting. For example, experiments have been performed on intact arteries; therefore, the descriptions of the tissue’s material symmetry are based on global response, instead of the local response of individual constituents. The earliest known study that examined material symmetry of the artery wall excised from a living animal was on the canine aorta and
carotid artery [15]. Pressurization, axial extension, and torsion were performed, and the authors determined that planes of symmetry coincided nearly with the circumferential, radial, and axial directions. In other words, the global tissue response was cylindrically orthotropic. These conclusions have been assumed in the majority of later studies [16-18].

In another case, average axial and circumferential stresses at equal strain levels were measured for active and passive canine carotid arteries [19]. The results show that the carotid artery is stiffer in the circumferential direction than in the axial direction (with respect to an unloaded reference configuration). Findings from a subsequent study on rabbit carotids confirm these conclusions [17]. In contrast, others indicate that bovine carotid arteries are stiffer axially than circumferentially [16]. The conflicting reports could be attributed to the difference in species studied. Nonetheless, further exploration into material symmetry of the carotid artery, including its multi-layered material properties, should be performed to clarify this issue.

As shown by histology, the orientation of various arterial wall components along different axes indicates that the artery is locally anisotropic [7]. As stated before, medial smooth muscle cells and the thin elastin sheets that surround them have been shown to be oriented primarily circumferentially. More specifically, a previous study found a correlation between orientation and interluminal pressure. It was determined that the elastic laminae are oriented axially at the in vivo length and circumferentially at ≥60mmHg [20]. This realignment due to force occurs equally through the cross-section, and is possibly due to the residual stress present in the wall [7].
An earlier study determined that the average orientation of collagen fibers in the media and adventitia is in the circumferential direction [21]. However, more recently it was found that medial collagen is oriented at approximately ±10° with respect to the circumferential direction, and adventitial type I collagen fibers are oriented at nearly ±45° between the axial and circumferential directions [22].

**Multilayered Heterogeneity**

Due to the varying constituents and their organization in each of the three arterial layers, the mechanical properties are different from layer to layer. However, most previous studies consider material behavior within each layer to be homogeneous [23]. The normal intima, made of a thin basal lamina and a single layer of endothelial cells, contributes little to the mechanical strength of the artery [24]. However, previous research shows that in aged arteries, particularly in one study on the aged coronary artery, the intima has increasing thickness and strength [25]. The media is the thickest of the three layers and contributes most of the mechanical strength and structural integrity under physiological conditions [26]. Due to the large amount of smooth muscle cells therein, the media is thought to contribute significantly to the viscoelastic response of arteries [27]. The adventitia, composed of fibroblasts in a collagen scaffold, is also a load-bearing layer of the vessel. These observations were first made in 1880 [28], but few subsequent studies have further examined arterial heterogeneity. The next known study that involved separation of arterial layers employed uniaxial tensile testing of canine aortic media and adventitia in the axial and circumferential directions [29]. Following, another group stretched coronary artery intima uniaxially [30]. More recently, uniaxial
tensile testing was performed on each of the individual arterial layers, which were found to exhibit significant mechanical heterogeneity; also, each layer showed a nonlinear, inelastic, anisotropic behavior under large deformations [25].

**Incompressibility**

Besides connective fibers and cells, approximately 70-80% of the wet weight of the artery is water (mainly extracellular). Therefore, the artery can be regarded as a “mixture-composite,” with the primary solid constituents collagen, elastin, and smooth muscle cells, and the fluid part comprising extracellular water. For most experimental and theoretical applications on mechanical behavior, the artery wall is assumed a homogenous solid. Arteries are not truly incompressible, but due to the movement of water across the wall as a result of load, arteries deform nearly isochorically under various loading conditions [31, 32]. Another method of characterizing a material as incompressible is taken from linear elasticity theory, where a material is assumed incompressible if the bulk modulus is much greater than the shear modulus. In one such study, an experimentally derived bulk modulus (K) was compared to shear modulus (G) using a previously determined Young’s modulus value [33], and the ratio of K to G was found to be on the order of $10^3$, thereby rationalizing the assumption of incompressibility for arteries [34].

**Residual Stress**

Residual stress in excised, unloaded arteries is a critical factor in defining the appropriate reference configuration(s) for constitutive modeling of the material, as first
shown by two groups independently in the 1980s [17, 18]. Intact, excised, unloaded arteries open as a result of a longitudinal cut; the ring, once cut, tends to minimize its stored strain energy, thereby relieving the residual stress and transitioning into the stress-free configuration. This opening effect suggests the transmural stresses within the intact, unloaded arterial ring: the inner wall is in compression, and the outer wall is in tension. Histology confirms this phenomenon [35] by showing a greater degree of undulation of the internal elastic lamina in the intact, unloaded artery as compared to the opened artery. Furthermore, elastin was found to contribute to a large portion of the residual strain in the healthy artery wall [36].

**Passive Mechanical Response of Arteries**

In the passive (noncontractile) state, which is of interest in this study, arteries are viscoelastic, as shown by exhibiting the following: (i) hysteresis, (ii) stress relaxation under constant deformation, and (iii) creep under constant load. However, unlike most other viscoelastic materials, arteries have been shown to behave relatively independent of strain rate at low ranges [37-39]. Furthermore, viscoelastic effects of large (elastic) arteries such as the carotid can be assumed relatively negligible due to the lower smooth muscle cell content as compared to muscular arteries [22], and even more so in vivo due to cyclic loading [40]. It follows that material descriptions of large arteries are not limited to the viscoelastic realm but can also utilize hyperelastic formulations, as was the case in a recent work on human common carotid arteries [41].

After multiple cycles of cyclic loading in the physiological range (preconditioning), the mechanical response of the artery becomes repeatable. In testing
protocols that include preconditioning, the artery has been described as pseudoelastic [39]. Hence preconditioning of arteries is necessary to ensure the subsequent mechanical response of the tissue is accurate.

Cyclic pressure coupled with axial stretch of arteries has been previously characterized, and results show that an increase in axial stretch leads to circumferential stiffening of the vessel [7]. On the other hand, axial mechanical response was nearly identical in three separate tests when the diameter was held at different constant strain levels [42]. Furthermore, many previous studies have shown that the axial force needed to maintain constant axial strain during increased inflation (i) increases at axial strains greater than those in vivo, (ii) decreases at axial strains less than those in vivo, and (iii) is nearly constant at axial strains close to those in vivo. One major benefit resulting from this behavior is that the artery does no axial work during cyclic inflations at the in vivo length, thereby conserving energy [7].

**Contributions of Arterial Constituents**

Since collagen is more highly crimped in the unloaded state, elastin carries the majority of the mechanical force in the low load (physiological) regime. Once uncrimped, collagen dominates the stiffer region of the stress-strain curves at high stresses [43]. Previous investigators found that the percentage of collagen fibers recruited (bearing load) at physiological pressures was below 10% [44-46]. Smooth muscle cells are mainly responsible for the viscoelastic mechanical response of both passive and active arteries [27, 47].
As determined by histomorphometry, the area fractions of collagen and elastin as functions of total cross-sectional area of the rat carotid artery wall were 0.203 and 0.306, respectively [48]. Furthermore, a study on various canine arteries determined that the average wall composition of the carotid artery as a percentage of total dry weight was 50.7% collagen and 20.1% elastin, and as a percentage of wet weight was 71.1% water [49] (Table 1). Smooth muscle cells make up approximately 25-60% of the total wall dry weight [12]. Similarly, volume fractions of the primary constituents of abdominal aortas were as follows: 54.8% collagen, 22.7% elastin, and 22.6% smooth muscle cells [50].

Table 1. Average percentage of primary constituents of various canine arteries. From [49].

<table>
<thead>
<tr>
<th>Artery</th>
<th>% H₂O</th>
<th>% Collagen</th>
<th>% Elastin</th>
<th>C:E ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abdominal aorta</td>
<td>70.4 ± 0.4</td>
<td>45.5 ± 1.7</td>
<td>30.1 ± 1.7</td>
<td>1.58 ± 0.15</td>
</tr>
<tr>
<td>Carotid</td>
<td>71.1 ± 0.1</td>
<td>50.7 ± 2.1</td>
<td>20.1 ± 1.0</td>
<td>2.55 ± 0.13</td>
</tr>
<tr>
<td>Coronary</td>
<td>63.2 ± 1.0</td>
<td>47.9 ± 2.6</td>
<td>15.6 ± 0.7</td>
<td>3.12 ± 0.21</td>
</tr>
<tr>
<td>Femoral</td>
<td>68.0 ± 0.3</td>
<td>44.5 ± 1.4</td>
<td>24.5 ± 1.6</td>
<td>1.89 ± 0.14</td>
</tr>
<tr>
<td>Mesentery</td>
<td>70.8 ± 0.5</td>
<td>38.1 ± 1.7</td>
<td>26.5 ± 1.7</td>
<td>1.51 ± 0.15</td>
</tr>
<tr>
<td>Renal</td>
<td>70.4 ± 0.7</td>
<td>42.6 ± 1.6</td>
<td>18.7 ± 1.8</td>
<td>2.46 ± 0.27</td>
</tr>
</tbody>
</table>

% H₂O is per wet weight, whereas protein is per dry weight. C:E, collagen to elastin ratio.

These proportions of arterial wall constituents are important in modeling efforts whereby the overall strain energy function (SEF) can be separated into (i) isotropic (representing elastin) and (ii) anisotropic, or cylindrically orthotropic (representing collagen) SEF. Regardless of the linearly elastic material behavior of elastin, the arrangement and interaction of elastin sheets with one another result in elastin behaving more nonlinearly as a whole unit in the artery [51].
Deformation

Besides the residual stresses present in unloaded arteries, many arteries are held under a relatively large amount of axial stretch in vivo, and this stretch remains approximately constant throughout the cardiac cycle [52]. Previous studies have shown that excision of the carotid artery results in the following reduction in length: 57%, 51%, and 61% in canines, 60% and 70% in rabbits, and 69% in rats [18, 19, 39, 53-55]. Similarly, axial retractive forces, or the forces required to return a vessel to its in situ length, in canine carotid arteries were approximately 0.36 N via experimental measurement [56]. In addition, canine carotid arteries have been found to undergo further longitudinal strains in vivo ranging from 20-60% caused by cervical extension [57, 58]. However, extensions caused by blood flow are quite small [7].

Circumferentially, the canine carotid artery has been reported to be stretched 57% at 100 mmHg (within physiological range) in vivo [18]; another group reported a similar stretch of 59% [19].

Typical pressure values in arteries during systole and diastole are 120 mmHg and 80 mmHg, respectively. Changes in carotid artery diameter throughout the cardiac cycle range only from 6-10% [57]. Additionally, radial pulsation (systolic-diastolic radius divided by mean radius) of the human carotid artery was found to be ±0.5% [59, 60]. Although arteries experience very low radial strains, the coupling of these radial strains with relatively large (finite) longitudinal and circumferential deformations results in the need to assess the nonlinear material behavior in the large strain regime. Furthermore, besides the physiological range of strains already discussed, even larger strains can be
Arterial dissection is a tear in the inner layers of the artery wall; it can, however, extend into the media, and rarely, continue into the adventitia. Failure of one or more layers without catastrophic tissue failure is referred to as subfailure. Dissection alters the normal path of blood flow and allows for the formation of an intramural hematoma, which can have effects from minor wall narrowing to total occlusion of the vessel (Figure 5).

Figure 5. (A) Intimal dissection resulting in stenosis and development of a blind pouch, which can lead to thrombus formation. (B) Subadventitial dissection resulting in some amount of luminal obstruction. Also, the artery may dilate slightly, or bulge enough to create a pseudoaneurysm. In both A and B, a false lumen is formed. Arrows indicate direction of blood flow. From emedicine.medscape.com.
The initial tear can propagate both circumferentially and longitudinally [61], redirecting a portion of the blood flow path and forming a “false lumen,” called as such because the blood flows between artery layers. If the dissection extends into the outermost portion of the wall, the bulging of the wall can lead to (i) a pseudoaneurysm (as opposed to a true aneurysm, in which all three layers of the wall form the bulge) [62]; (ii) secondary tear formation, and/or (iii) complete rupture of the vessel, which is often lethal [63].

Dissections take place in many arterial types, including the aorta [64], coronary artery [65], and carotid artery [6]. In most cases of carotid artery dissection, the extracranial portion of the artery is affected [6], probably due to the higher rate of injury reported for cervical portions of the vessel [66].

Causes

Dissection of the artery may occur as a result of trauma, arteriopathy, or abnormal geometry of the vessel. Trauma cases can be divided into two major groups: (i) acute trauma and (ii) delayed trauma [62]. Acute trauma, also known as blunt carotid artery injury (BCAI), occurs when the head-neck complex is subjected to hyperextension, lateral rotation, or interaction with blunt objects. BCAI causing carotid artery dissection results primarily from automobile accidents, with one study finding the occurrence 45% over a 9-year study period [67, 68]. BCAI also results from other situations causing sudden, often unexpected head/neck injuries such as sports games [69] and other physical exercise [70]; violent vomiting [71], coughing, or nose blowing [72]; and physical abuse [73]. Delayed trauma can be caused by the head/neck complex remaining in an unnatural
forced position for long periods of time, as it can be in certain surgery procedures of the head [74, 75].

Several arteriopathies, most of which are connective tissue disorders, can contribute to the onset of carotid artery dissection. For example, fibromuscular dysplasia is a condition that involves smooth muscle cell hyperplasia, fibrous tissue proliferation, and elastin fiber damage [76]. Other conditions found to contribute to the initiation of arterial dissection include Ehlers-Danlos Syndrome Type IV (abnormal type III collagen), type I collagen point mutation [77], Marfan’s Syndrome (fibrillin deficiency), and α₁-antitrypsin deficiency [78, 79]. Previous work suggests structural arteriopathies that alter the native structure of collagen lower the strength of the artery, thereby reducing the force necessary for dissection propagation [61]. In the case of those that degrade elastin, the artery wall becomes stiffer and is likely to dissect at lower strains [80]. More specifically, Marfan’s Syndrome was found to have a correlation with increased frequency of aortic hypertension [81].

Chronic systematic hypertension is the most prevalent factor leading to aortic dissection and has been found in 62–78% of patients with aortic dissection [63]. Additionally, atherosclerosis, aneurysms, and coarctation of the aorta are contributing factors to the risk of aortic dissection [63, 82]. (For a synopsis on the mechanics of atherosclerosis, see Appendix A.)

**Prevalence and Effects**

The overall incidence of carotid artery dissection as found from two independent studies was 2.6 and 2.9 cases per 100,000 people [83, 84]. The effects of dissection
include headaches and neck pains, Horner’s syndrome, cranial nerve damage, transient ischemic attack, and stroke [6]. Horner’s syndrome, caused by disruption of the sympathetic nerves within the artery wall, has been found ipsilaterally to the affected carotid in up to half of the dissection cases [85, 86]. Previous studies have shown the occurrence of cranial nerve compromise to be 2-16% [85, 87-89]; damage to multiple cranial nerves has also been reported [90]. The more life-threatening result of carotid artery dissection that typically happens subsequently to the previously mentioned effects is acute ischemia distal to the tear, which has been found in approximately 80% of cases [85, 86]. A brief ischemia may result in a transient ischemic attack; however, a more prolonged ischemia may lead to partial blockage of the retinal artery [91, 92], optic neuropathy [93], or complete stroke [94, 95].

While the incidence of BCAI is low, mortality rates (40%) and correlated chronic neurological morbidity rates (40-80%) are very high [1-5]. One review determined that of the carotid artery dissection cases, 50% had normal neurological function, 21% had mild pathologies, 25% moderate to severe pathologies, and 4% died [72]. Similar findings reported the mortality and severe morbidity rates of patients with carotid artery dissections at approximately >30% [85, 96-98].

Carotid artery dissections were the primary cause in 2.5% of first-time strokes from data of 1200 stroke patients [99] and have been estimated to cause up to 20% of strokes in patients under 30 years of age [100]. The combinatory effect of a dissection and stroke is particularly grim. Of 30 cases of first-time stroke and dissection, 23% died within one week of the stroke, and 48% had chronic disability [99]. Although children 18 years and younger account for less than 5% of the extracranial dissection cases, the
carotid artery is the most commonly affected vessel; however, neurologic outcome of children after diagnosis is very encouraging [101]. Recurrent dissection has been found to occur in up to 4% of carotid artery dissection cases [102, 103].

**Diagnosis**

Elapsed time after a dissection is a critical factor in the diagnosis of carotid artery dissection. From a report on 80 patients with carotid artery dissection, 88% of the 42 strokes happened within the first week after dissection [104]. However, apparent effects of a dissection are often delayed for weeks or even months. Without treatment, acute aortic dissections resulted in a 90% mortality rate within weeks; nonetheless, the majority of deaths were less than 48 hours after the dissection event [105]. Intimal tearing without ultimate failure of the vessel is the most common type of dissection that leads to postponed symptomatology [2, 5, 106-108] due to the vessel maintaining sufficient mechanical integrity to withstand most physiologic loading. A comparison of two studies found that a more aggressive screening technique yielded more reported cases of blunt cerebrovascular injuries including BCAI [109, 110]. Other studies had similar results when screening for asymptomatic lesions [68, 106, 111], revealing that blunt cerebrovascular injuries have likely been underdiagnosed in the past.

The most commonly recognized tool for evaluating bodily injury as a result of trauma is the Abbreviated Injury Scale (AIS) [112]. Various AIS scales were developed by the Association for the Advancement of Automotive Medicine (AAAM) in 1971 and have been updated periodically. A more recent study developed a grading scale specifically for BCAIs with the levels defined as follows: grade I was vessel irregularity
or dissection with <25% stenosis; grade II included thrombus formation, raised intimal flap, and dissection or intramural hematoma with ≥25% stenosis; grade III involved pseudoaneurysm; grade IV was vessel occlusion; and grade V included complete vessel transection [68]. The study found an increase in risk of stroke as injury grade increased. Thus, the grading scale is a starting point by which more accurate prognoses and therapeutics can take place; nonetheless, damage criteria, especially those based on microscale injury mechanisms, must be more readily studied to improve upon current diagnostic techniques.

Mechanics

Even with the obvious life-threatening consequences of carotid artery dissection, few experimental works on the mechanics of this phenomenon exist. Dissection of porcine aortic media was examined in three studies [61, 113, 114], and dissection of human aortic media was performed more recently [115]. A previous work on circumferential tensile testing of rat carotid arteries reported intimal subfailures but did not quantify the vessel mechanics [116]. Overall, only two known groups to date present a mechanics based approach of carotid artery dissection [117, 118]. One utilized a guillotine drop technique from various heights onto inflated, intact carotid vessels to obtain high strain rate (≤7.3 s⁻¹) impact “pinching” of the artery and developed an associated finite element model [118]. The other work implemented longitudinal quasi-static testing (displacement rate of 1.0 mm s⁻¹) of common carotid artery strips to failure and determined that all subfailures took place in the intima [117]. Despite the varying
methodologies, both groups found that intimal failure preceded ultimate failure [117, 118].

*Damage*

Since so few studies of this nature exist, more work is needed to characterize carotid artery injury mechanisms and quantify stages of damage as a result of this type of injury. The previously mentioned drop technique study labeled post-impact specimens as either damaged or undamaged by inspection with the unaided eye; arterial microstructure was not examined [118]. Histological analysis from one study on peeling tests of the abdominal aortic media cites damage as disruption and tearing primarily between the elastic laminae; however, all samples were peeled to the same extent. Mechanical data is labeled to include a zone denoting damage, but no explanation of how this portion was selected is given [115]. No known studies on the varying degrees of microstructural damage that inevitably can result from carotid artery dissection exist to date.
CHAPTER III
METHODS

Sample Preparation

Porcine carotid arteries were obtained from a local abattoir (Sansing Meat Services, Maben, MS) within ten minutes of slaughter of the animals. Excess connective tissue and fat were trimmed off the outside of the vessels. Specimens were stored in 0.01M phosphate buffered saline (PBS) (Sigma-Aldrich, St. Louis, MO) at -20°C until use; storage in frozen conditions does not considerably affect artery mechanics [119], and other groups have employed this method [55]. For each carotid artery specimen, a longitudinal cut was performed, and the artery was opened to expose the intimal layer. Dogbone-shaped samples oriented longitudinally and circumferentially were procured via a custom-designed steel dogbone die (Figure 6) (Center for Advanced Vehicular Systems, Mississippi State University). Samples were shaped as such to ensure that failure occurred near the middle of the gauge length. Approximate sample size was 10 mm, 5 mm, and 1.5 mm for gauge length, width, and thickness, respectively (Figure 7); dimensions were measured with digital calipers and recorded.
Figure 6. Schematic of custom-designed dogbone die (Center for Advanced Vehicular Systems, Mississippi State University) used to cut artery samples.

Figure 7. Artery sample with intima exposed showing length and width after procurement with dogbone die.
Mach-1™ Micromechanical Testing System

The Mach-1™ Micromechanical Testing System (Biomomentum, Inc., Canada) was utilized for mechanical testing of arterial tissues. Before each test, calibration of the Mach-1™ machine was performed to ensure accurate data measurements.

Stainless steel teeth grips for securing dogbone tensile specimens were attached to the base and top of the Mach-1™. The 10 kg load cell was used for each tensile test; the linear actuator caused displacement of the top rod upwards while the bottom rod remained stationary, thereby applying tension. Immediately prior to testing, samples were placed with the ends between the grips and were slightly slack in the reference (zero-stress) state. The grip-to-grip distance was initialized to the gauge length of the specimen. Herein, all strain specifications in the protocol were calculated based on the initial gauge length of the sample. Displacement controlled loading velocity throughout testing was 10 mm min⁻¹. These criteria were input into the Mach-1™ software.

Each sample was kept in PBS until testing to avoid dehydration. To maintain the hydrated state of the tissue during testing, PBS was misted intermittently on the sample.

Failure Testing

To begin testing, samples (n = 7 for each direction) were subjected to 10 cycles of preconditioning [39] to 10% tensile strain. Samples were then loaded to 70% and 100% strain in the circumferential and axial directions, respectively, and subsequently unloaded to obtain hysteresis curves. The strain levels dependent on sample orientation were chosen based on a strain level within the subfailure region. Samples were then progressively loaded to failure (Figure 8). All samples failed within the gauge length.
Figure 8. Sample of porcine carotid artery during tension to failure testing. (a) Mach-1™ tensile setup, and (b) specimen immediately after failure.

Subfailure Testing

Two samples of the same dimensions as used for failure testing were used in subfailure testing. The samples were oriented along each major axis and stained with 0.1% Picrosirius red stain for 8 min, washed shortly with water, placed in PBS, and stored at 4°C for 1 hr. The water and PBS washes were repeated, and the samples were kept at 4°C until testing (≤ 24 hrs). The short duration of staining allowed only the outer layers (intima and adventitia) to stain red; the stain did not penetrate into the media, so the media remained its native (white) color.

The most useful methodology to examine intimal subfailures relative to ultimate failure is to open the vessel to expose the intima and subject the tissue to longitudinal failure testing [120]. The current study employs similar techniques but extends the procedure to include analysis of both longitudinally and circumferentially oriented samples. Therefore, 10 cycles of preconditioning to 10% tensile strain were performed,
and samples were subsequently distracted to failure. During loading to failure, high resolution digital images of the intima and underlying media exposed by intimal damage were captured at $\geq 60$ fps (MotionScope M-5 Color Camera, Redlake Inc., San Diego, CA).

** Interruption Testing**

Similarly to the failure testing protocol, 10 cycles of preconditioning to 10% tensile strain were done. Interruption tensile tests were performed to the following strain levels: 25%, 40%, 50%, and 100% (circumferentially oriented samples); and 45%, 60%, 70%, and 100% (longitudinally oriented samples). Strain levels were chosen based on examination of the average failure curves from both orientations. The greater degree of stiffness (and associated shorter toe region) of the tissue in the circumferential direction warranted lower strain levels of interest than those in the longitudinal direction. However, to have a basis for comparison between the two groups, the highest strain level chosen was the same (100%) for both.

Immediately following each interruption test, a small plastic bag filled with 10% neutral buffered formalin (NBF) was placed around the sample, and if necessary, additional NBF was siphoned into the bag so that the sample was completely submerged. Samples were fixed in NBF for $\geq 2$ hrs.

** Scanning Electron Microscopy**

From each fixed sample, two sections were cut within the gauge length ($n = 4$ for each direction) to examine both the intimal and adventitial sides. Six 10-minute washes
in distilled water followed by secondary fixation for 2 hrs in osmium tetrox ide were performed. The six 10-minute washes in distilled water were repeated; then dehydration with 35%, 50%, 70%, 95%, and 100% ethanol for 10-15 min took place, using two washes at each concentration. Critical point drying (Polaron E-300) was completed, and sections were mounted to stubs using carbon tape with either the intima or adventitia facing up. Au-Pd sputter coating was performed, and samples were stored in a desiccating cabinet until viewing. The Zeiss Evo® 50 and JEOL JSM-6500F were used to capture images of the intimal and adventitial surfaces of porcine carotid artery ultrastructure.

**Histology**

The fixed samples from interruption testing were also used for histological analysis. Three small cross-sectional slices approximately 1.5 × 1.5 mm (side view) were obtained from the gauge length of fixed circumferential and longitudinal samples (n = 4 for each direction) (Figure 9). Samples were embedded in paraffin, sectioned to a thickness of 6 μm, and subjected to Masson’s trichrome (TRI) and Verhoeff’s-Van Gieson (VVG) staining (Appendix B).
Figure 9. Schematic of cutting technique for samples fixed in 10% NBF prepared for histological staining.

For the samples stained with Masson’s trichrome, collagen was stained blue, smooth muscle cell cytoplasm and erythrocytes were stained red, and cell nuclei were stained blue-black. With regards to the VVG stain, the Verhoeff’s component was specific for elastin, while the Van Gieson component was specific for collagen. Elastin and cell nuclei were stained purple to black, collagen was stained bright red, smooth muscle cells appeared pink, and other tissue elements were colored yellow.

Using optical microscopy, each sample (n = 3 for each orientation and strain level) stained for both Masson’s trichrome and Verhoeff’s was imaged at 10x to view ultrastructural changes as a function of strain level.

Environmental Scanning Electron Microscopy: Real-time Observation of Tissue Damage

Dogbone specimens oriented circumferentially and longitudinally with dimensions 10 mm, 5 mm, and 1.5 mm for gauge length, width, and thickness,
respectively, were kept frozen at -20°C or on ice until testing. A small square piece of black tape (~0.5 × 0.5 mm) was placed near the center of the gauge length to serve as a marker. The ends of the sample were sandwiched between stainless steel teeth grips on the in situ electromechanical actuation, 1000 lb Tensile Substage for SEM (Ernest F. Fullam, Inc.) located within the Zeiss VP Evo® 50 SEM chamber.

Displacement controlled loading velocity throughout testing was 10 mm min\(^{-1}\). Samples were subjected to 10 cycles of preconditioning to 10% tensile strain. Subsequent loading in tension to various strain levels (0%, 25%, 40%, and 50% for circumferential; 0%, 45%, and 60% for longitudinal) was observed to capture the propagation of damage (crack growth, void nucleation, etc.) in the tissue.

While displacement occurred, the electron beam was turned off to decrease exposure time of the sample. Once a desired strain level was reached, the beam was turned on, and images were captured in approximately the same location. To slow the dehydration damage process, the following measures were taken: (i) a degraded vacuum was used in variable pressure (VP) mode, (ii) the chamber underwent multiple purge cycles with water vapor, and (iii) a sponge soaked in PBS was kept below the sample and remained in contact with the sample throughout testing. Before failure of the tissue, image capture time at each strain level was \(\leq 4\) min.

**Microstructural Analysis**

ImageAnalyzer v.2.2-0 software (Center for Advanced Vehicular Systems, Mississippi State University) was used for microstructural analysis of histological and environmental SEM images obtained from samples subjected to sequential levels of
strain as previously mentioned. Damage (i.e. cracks, voids) was quantified by means of various parameters, which, combined, provided a measurement tool for damage progression as a function of strain level.

The goal of thresholding each image was for the software to recognize voids and cracks produced by deformation. This involved setting the threshold values (i) near 0 for Evo SEM image analysis to identify voids colored black, and (ii) near 255 for histological image analysis to distinguish voids colored white. Minimum void size was set to 5 μm².

The parameters obtained for each image during analysis included the following: object count, object area fraction, number density, mean object area, and mean object nearest neighbor distance (nnd). Object count is the number of voids present in the image, and object area fraction is the ratio of total void area to total image area. Number density equals the object count divided by the total image area. Mean object area represents the average area of voids, and mean object nnd is a measure of the average distance between neighboring voids. Total image areas were approximately 1.20 × 10⁵ μm² and 8.00 × 10⁵ μm² for environmental SEM and histology, respectively.

Statistical analysis was performed using One Way Analysis of Variances (ANOVA). The differences in each parameter based on strain level were considered statistically significant when \( p \) is less than 0.05. The Holm-Sidak test was used for post hoc pair-wise comparisons versus the control group (SigmaStat 3.0, SPSS Inc., Chicago, IL).
**Atomic Force Microscopy**

Atomic force microscopy (AFM) was performed on the cross section of a normal human carotid artery using the MFP-3D-BIO AFM (Asylum Research) with a Nikon TE-2000U for imaging (Figure 10). The system was located in an acoustic isolation hood on a vibration isolation table. Samples were maintained at -80°C or in 1% dimethyl sulfoxide (DMSO) in PBS on dry ice until testing. A section of approximately 0.5 mm thickness was mounted onto a glass slide using 5 Minute® Epoxy (Devcon) with the cross section facing upwards.

Figure 10. AFM system including the MFP-3D-BIO AFM (Asylum Research) and Nikon TE-2000U for imaging.
The basic AFM setup includes a cantilever with a sharp tip at one end that scans the surface of a specimen. The tip is kept in close proximity to the sample surface, and the interaction force between the tip and the sample induces a deflection of the cantilever according to Hooke’s law. Light from a laser is reflected off the cantilever and collected by a position sensitive detector, thereby measuring deflection of the cantilever.

Using topview optics, the laser was aligned on top of the mechanically driven cantilever, and the cantilever was positioned over the area of interest (Figure 11). Electric force microscopy (EFM), a non-contact technique often used on soft biological samples, was employed on the artery sample. During EFM, the tip oscillated at its resonance frequency above the sample surface while a direct current (DC) bias was applied between the tip and the sample. The electric field induced a force gradient between the tip and the sample that resulted in a shift in the cantilever’s resonance frequency. The change in resonance led to a change in phase lag between the drive and response of the cantilever. Locations of phase shift indicated disturbances in the electric field between the tip and the sample, which corresponded to local variations in conductivity.

For each of the three arterial layers, force maps with individual force-displacement curves from $16 \times 16$ points over a $90 \times 90 \mu m$ total area were obtained.
Figure 11. Selection of area for force mapping. Red dot indicates tip of cantilever, and teal box designates force map area (90 × 90 μm).
CHAPTER IV

RESULTS

Data Processing

Output data from the Mach-1™ is in the form of time, displacement, and force. Engineering strain was calculated by dividing the displacement by original gauge length in the loading direction. Engineering stress was obtained by dividing force by initial cross sectional area. As previously stated, the artery is assumed incompressible, so engineering stress and strain were converted to true stress and strain via the following:

\[ \sigma_{true} = \sigma_{eng} (1 + \varepsilon_{eng}) \]  

(1)

\[ \varepsilon_{true} = \ln(1 + \varepsilon_{eng}) \]  

(2)

Hysteresis

Loading and unloading curves of both circumferential and longitudinal samples exhibit marked differences, showing the viscoelastic response of the carotid artery (Figure 12).
Figure 12. Representative hysteresis curves in the circumferential and longitudinal directions. The varying strain levels to which samples were stretched were chosen based on the extensibility of samples oriented in the two directions.

Hysteresis data was analyzed by calculating the area under the loading curve ($A_l$) and the area under the unloading curve ($A_u$). The area between the curves (hysteresis) was compared based on orientation of samples. Average hysteresis, or energy dissipation per unit volume, was $98.45 \pm 16.30 \text{ J m}^{-3}$ and $26.20 \pm 36.37 \text{ J m}^{-3}$ for circumferential and longitudinal samples, respectively ($n = 4$ for each) (Figure 13).
Figure 13. Energy dissipation per unit volume (hysteresis) from tensile testing of arterial specimens oriented circumferentially and longitudinally (n=4 for each orientation). Error bars indicate standard deviation.

Hysteresis was also quantified by normalizing the enclosed area of the loading and unloading curves to the area under the loading curve [121] via the following formula:

\[
A = \left(\frac{A_f - A_u}{A_f}\right) \times 100
\]  

Percentage of hysteresis from circumferential samples (59.18 ± 4.413%) was more than double that from longitudinal samples (22.74 ± 18.31%) (n = 4 for each) (Figure 14).
Figure 14. Hysteresis as a percentage of total area under loading curve from tensile testing of arterial specimens oriented circumferentially and longitudinally (n=4 for each orientation). Error bars indicate standard deviation.

**Failure Testing**

Stress-strain curves obtained from testing along each orientation indicate a heel (transition region) separating two distinct regimes: (i) the toe region and (ii) the linear region (Figure 15). As previously determined, the toe region represents the mechanical response of elastin, as the collagen fibers are crimped in this low load state. The transition region is the point at which collagen fibers are gradually recruited; they become the primary load-bearing constituent at the start of the linear region and continue to accumulate increasingly greater stresses until failure.
Failure testing in tension of arterial specimens oriented circumferentially and longitudinally (n = 5 for each orientation). Error bars indicate standard deviation.

Failure stress and failure strain were averaged based on direction (n = 6 for each) (Table 2), as an indicator of the strength of the tissue and its anisotropic mechanical behavior. Failure stress and associated strain were 2852.40 ± 557.93 kPa and 68.45 ± 8.29%, respectively, in the circumferential direction, and 1074.88 ± 366.80 kPa and 83.55 ± 5.60%, respectively, in the longitudinal direction.
Table 2. Failure stress and strain of circumferential and longitudinal samples (n = 6 for each).

<table>
<thead>
<tr>
<th>Orientation</th>
<th>Failure stress (kPa)</th>
<th>Failure strain (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Circumferential</td>
<td>2852.40 ± 557.93</td>
<td>68.45 ± 8.29</td>
</tr>
<tr>
<td>Longitudinal</td>
<td>1074.88 ± 366.80</td>
<td>83.55 ± 5.60</td>
</tr>
</tbody>
</table>

Extensibility was defined by the strain at which the extension of the linear portion of the stress-strain curve crosses the x-axis. Circumferential and longitudinal extensibilities were 46.71 ± 8.414% and 57.78 ± 5.481%, respectively. Maximum tensile modulus was calculated by determining the slope of the linear region of the stress-strain curve. Maximum tensile modulus was 12.90 ± 3.134 MPa for circumferential samples and 3.900 ± 1.827 MPa for longitudinal samples (Table 3).

Table 3. Extensibility and maximum tensile modulus based on sample orientation (n = 5 for each).

<table>
<thead>
<tr>
<th>Orientation</th>
<th>Extensibility (%)</th>
<th>Maximum tensile modulus (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Circumferential</td>
<td>46.71 ± 8.414</td>
<td>12.90 ± 3.134</td>
</tr>
<tr>
<td>Longitudinal</td>
<td>57.78 ± 5.481</td>
<td>3.900 ± 1.827</td>
</tr>
</tbody>
</table>

Subfailure Testing

The true stress-true strain data was synchronized with the high speed images from failure testing of stained samples. Subfailure was defined as a compromise of the structural integrity penetrating part of the transmural depth (i.e. visible crack, tear).
Ultimate failure was defined as the last point on the stress-strain curve at which the vessel was able to maintain a considerable amount of stress compared to the maximum tensile stress.

The sequential images and corresponding table reveal that intimal failure occurred before ultimate failure of the tissue (Figures 16 and 17, Tables 4 and 5). This is evidenced by (i) delamination of the intima (stained red) and media (unstained) and (ii) propagation of tears in the intima while the media remains intact. Interestingly, the stress-strain curves do not indicate any abnormalities at the point of initial crack formation in the intima (Figure 18). Note also the increase in stress and strain between intimal failure and ultimate failure.

![Image sequence obtained from strain to failure tension testing of circumferential sample. Intima was stained with Picro-sirius red, while media remained native yellow color. Arrow indicates location of crack initiation.](image-url)
Table 4. Data in the form of time, strain, and stress for the image sequence in Figure 13. Note that the stress and strain follow the same trend as shown for multiple circumferential samples in failure testing.

<table>
<thead>
<tr>
<th>Image</th>
<th>Time after intimal failure (s)</th>
<th>True strain (%)</th>
<th>True stress (kPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>0.00</td>
<td>72.65</td>
<td>3838.04</td>
</tr>
<tr>
<td>b</td>
<td>2.60</td>
<td>74.16</td>
<td>4157.22</td>
</tr>
<tr>
<td>c</td>
<td>4.90</td>
<td>75.48</td>
<td>4164.71</td>
</tr>
<tr>
<td>d</td>
<td>5.33</td>
<td>75.73</td>
<td>3447.96</td>
</tr>
<tr>
<td>e</td>
<td>5.65</td>
<td>75.91</td>
<td>2664.85</td>
</tr>
<tr>
<td>f</td>
<td>6.22</td>
<td>76.23</td>
<td>796.45</td>
</tr>
</tbody>
</table>

Figure 17. Image sequence obtained from strain to failure tension testing of longitudinal sample. Intima was stained with Picro-sirius red, while media remained native yellow color. Arrows indicate locations of crack initiations.

Table 5. Data in the form of time, strain, and stress for the image sequences in Figure 14. Note that the stress and strain follow the same trend as shown for multiple longitudinal samples in failure testing.

<table>
<thead>
<tr>
<th>Image</th>
<th>Time after intimal failure (s)</th>
<th>True strain (%)</th>
<th>True stress (kPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>0.00</td>
<td>80.94</td>
<td>1264.03</td>
</tr>
<tr>
<td>b</td>
<td>3.05</td>
<td>82.76</td>
<td>1393.79</td>
</tr>
<tr>
<td>c</td>
<td>6.17</td>
<td>84.57</td>
<td>1523.59</td>
</tr>
<tr>
<td>d</td>
<td>9.25</td>
<td>86.34</td>
<td>1562.53</td>
</tr>
<tr>
<td>e</td>
<td>10.46</td>
<td>87.03</td>
<td>294.90</td>
</tr>
<tr>
<td>f</td>
<td>10.47</td>
<td>87.03</td>
<td>241.86</td>
</tr>
</tbody>
</table>
Figure 18. Stress-strain curves of circumferential and longitudinal samples, indicating points along the failure region of the curves associated with high speed images.

Subfailure stress and strain were normalized to ultimate failure stress and strain. Circumferentially, intimal subfailure initiated at 87.88% of the stress and 94.29% of the strain at ultimate failure. Longitudinal subfailure began at 81.29% of the stress and 93.10% of the strain at ultimate failure (Tables 6 and 7).
Table 6. Subfailure strain and ultimate failure strain based on sample orientation.

<table>
<thead>
<tr>
<th>Orientation</th>
<th>Subfailure strain (%)</th>
<th>Ultimate failure strain (%)</th>
<th>Subfailure strain normalized to ultimate failure strain (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Circumferential</td>
<td>71.31</td>
<td>75.63</td>
<td>94.29</td>
</tr>
<tr>
<td>Longitudinal</td>
<td>80.94</td>
<td>86.94</td>
<td>93.10</td>
</tr>
</tbody>
</table>

Table 7. Subfailure stress and ultimate failure stress based on sample orientation.

<table>
<thead>
<tr>
<th>Orientation</th>
<th>Subfailure stress (MPa)</th>
<th>Ultimate failure stress (MPa)</th>
<th>Subfailure stress normalized to ultimate failure stress (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Circumferential</td>
<td>3.595</td>
<td>4.091</td>
<td>87.88</td>
</tr>
<tr>
<td>Longitudinal</td>
<td>1.264</td>
<td>1.555</td>
<td>81.29</td>
</tr>
</tbody>
</table>

Scanning Electron Microscopy

As can be seen from sequential SEM images of increasing strain level, the percentage of fibers (mostly collagen) aligned in the loading direction appears to increase as strain level increases (Figures 19-22). The micrographs of the adventitial surface appear much more irregular than those of the intimal surface, which is to be expected due to the composite makeup of the adventitia. The formation of microcracks is visible in the higher strain samples, particularly the adventitial side of the circumferential sample at 100% strain (Figure 19d). As a whole, the circumferential samples seem to accumulate more damage than the longitudinal samples.
Figure 19. SEM of porcine carotid artery adventitia after uniaxial tensile loading in the circumferential direction to (a) 25% strain at 330x, (b) 40% strain at 330x, (c) 50% strain at 550x, and (d) 100% strain at 482x with arrows showing microcracks. Line indicates loading direction.
Figure 20. SEM of porcine carotid artery adventitia after uniaxial tensile loading in the longitudinal direction to (a) 45% strain at 600x, (b) 60% strain at 500x, (c) 70% strain at 550x, and (d) 100% strain at 400x. Line indicates loading direction.

In the intimal image sequences (Figures 21 and 22), the fibers at the lower strain levels show a marked helical arrangement. At higher strains these fibers tend to straighten (i) at approximately 45° to the loading direction for circumferentially oriented samples, and (ii) in the direction of loading for longitudinally oriented samples. Void formation is also present in the intimal samples at higher strains.
Figure 21. SEM of porcine carotid artery intima after uniaxial tensile loading in the circumferential direction to (a) 25% strain at 430x, (b) 40% strain at 400x, (c) 50% strain at 450x, and (d) 100% strain at 443x. Vertical line indicates loading direction.
Figure 22. SEM of porcine carotid artery intima after uniaxial tensile loading in the longitudinal direction to (a) 45% strain at 430x, (b) 60% strain at 370x, (c) 70% strain at 430x, and (d) 100% strain at 450x. Vertical line indicates loading direction.

In the case of one sample, an intimal tear was preserved before complete intimal failure occurred, and as a result, the leading edge of the crack and the medial layer beneath are exposed. Delaminated elastin laminae in the media are revealed (Figure 23a, b), as well as frayed elastin fibers at higher magnifications (Figure 23c, d). Observation of the microstructure of the media further suggests that the (medial) failure mechanism is delamination of the elastic laminae.
Figure 23. SEM of porcine carotid artery intima after uniaxial tensile loading in the circumferential direction. Intimal tear with exposed media beneath the dissection point shown at (a) 103x, (b) 345x, and (c) 939x. Note particularly (d) damaged elastin fibers in the media at 863x. Vertical line indicates loading direction.

Histology

In general, sequential strain level images from samples stained with Masson’s trichrome and Verhoeff’s-Van Gieson illustrate a straightening of musculo-elastic fascicles, composed of smooth muscle cells, thin elastin sheets, and collagen fiber bundles, as compared to their undulated nature in the control (unloaded) images (Figures 24-26, 28-29). Due to the primary orientation of the musculo-elastic fascicles
(circumferentially), this phenomenon is more apparent in the longitudinally loaded samples (Figures 26 and 29) in which musculo-elastic fascicles align along (rather than into) the plane of sectioning. Sequential strain level images demonstrate an increase in damage (i.e. voids, microcracks) with increased strain. (Note that all images are oriented with the adventitia in the upper portion. Due to the thickness of the artery wall, the intima is not shown.)

Figure 24. (a) Masson’s trichrome and (b) Verhoeff’s-Van Gieson staining of porcine carotid artery cross-section control (unloaded) samples. 10x magnification.
Figure 25. Masson’s trichrome staining of porcine carotid artery cross-section after uniaxial tensile loading in the circumferential direction to (a) 25% strain, (b) 40% strain, (c) 50% strain, and (d) 100% strain. 10x magnification. Arrows indicate voids. Note sample orientation with circumferential (C), longitudinal (L), and radial (R) directions.
Figure 26. Masson’s trichrome staining of porcine carotid artery cross-section after uniaxial tensile loading in the longitudinal direction to (a) 45% strain, (b) 60% strain, (c) 70% strain, and (d) 100% strain. 10x magnification. Arrows indicate voids. Note sample orientation with circumferential (C), longitudinal (L), and radial (R) directions.

Microstructural analysis of the samples stained with Masson’s trichrome shows (with the exception of one strain level) that as strain level increases: (i) void count remains lower than its value at the first strain level (25%), and (ii) mean void area increases (Tables 8 and 9).

In samples of both orientations, void area fraction at strain levels below 100% is relatively low compared to the nearly doubled void area fractions at 100% strain. Additionally, void area fraction decreases between (i) 25% and 40% in the
circumferential samples, and (ii) 45% and 70% in the longitudinal samples. For the circumferential samples, many of the differences in each parameter based on strain level were found to be significantly different using post hoc pair-wise comparisons versus the control when p is less than 0.05. However, none of these differences were found significantly different among the parameters measured for longitudinal samples.

Table 8. Image analysis of voids as a function of strain level from circumferential samples stained with Masson’s trichrome (n=3). *Indicates p<0.05 versus control.

<table>
<thead>
<tr>
<th>Tensile strain</th>
<th>Control</th>
<th>25%</th>
<th>40%</th>
<th>50%</th>
<th>100%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Object count</td>
<td>1782 ± 181</td>
<td>3703 ± 714*</td>
<td>3050 ± 979*</td>
<td>3540 ± 542*</td>
<td>4690 ± 666*</td>
</tr>
<tr>
<td>Object area fraction (1x10^{-2})</td>
<td>2.87 ± 0.557</td>
<td>7.86 ± 2.21</td>
<td>6.84 ± 2.80</td>
<td>8.72 ± 4.21*</td>
<td>12.5 ± 4.37*</td>
</tr>
<tr>
<td>Number density (μm^{-2}) (1x10^{-5})</td>
<td>2.24 ± 0.228</td>
<td>4.73 ± 1.03*</td>
<td>3.83 ± 1.23*</td>
<td>4.44 ± 0.681*</td>
<td>5.89 ± 0.836*</td>
</tr>
<tr>
<td>Mean object area (μm^2)</td>
<td>12.8 ± 1.31</td>
<td>16.5 ± 1.05</td>
<td>17.5 ± 3.97</td>
<td>19.0 ± 6.04</td>
<td>22.2 ± 11.4</td>
</tr>
<tr>
<td>Mean object nd (μm)</td>
<td>11.2 ± 0.473</td>
<td>8.47 ± 0.741*</td>
<td>9.49 ± 1.29*</td>
<td>8.74 ± 0.481*</td>
<td>7.83 ± 0.571*</td>
</tr>
</tbody>
</table>

Table 9. Image analysis of voids as a function of strain level from longitudinal samples stained with Masson’s trichrome (n=3).

<table>
<thead>
<tr>
<th>Tensile strain</th>
<th>Control</th>
<th>45%</th>
<th>60%</th>
<th>70%</th>
<th>100%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Object count</td>
<td>1782 ± 181</td>
<td>5665 ± 2962</td>
<td>3714 ± 1247</td>
<td>3347 ± 1490</td>
<td>4676 ± 2098</td>
</tr>
<tr>
<td>Object area fraction (1x10^{-2})</td>
<td>2.87 ± 0.557</td>
<td>11.3 ± 7.54</td>
<td>8.22 ± 3.78</td>
<td>6.89 ± 4.23</td>
<td>17.2 ± 11.4</td>
</tr>
<tr>
<td>Number density (μm^{-2}) (1x10^{-5})</td>
<td>2.24 ± 0.228</td>
<td>7.11 ± 3.72</td>
<td>4.66 ± 1.57</td>
<td>4.20 ± 1.87</td>
<td>5.87 ± 2.63</td>
</tr>
<tr>
<td>Mean object area (μm^2)</td>
<td>12.8 ± 1.31</td>
<td>14.4 ± 5.06</td>
<td>17.0 ± 2.80</td>
<td>15.7 ± 3.14</td>
<td>26.7 ± 9.55</td>
</tr>
<tr>
<td>Mean object nd (μm)</td>
<td>11.2 ± 0.473</td>
<td>7.62 ± 1.92</td>
<td>8.42 ± 1.28</td>
<td>9.14 ± 1.80</td>
<td>8.01 ± 2.10</td>
</tr>
</tbody>
</table>

Upon examination of voids at higher magnifications, particularly of the longitudinally oriented images in which the collagen fibers and smooth muscle cells are
aligned horizontally, it was found that voids form primarily between neighboring elastin laminae. This is evidenced by the separation of collagen fibrils (blue) located between layers of elastin laminae – made of elastin and smooth muscle cells (red) (Figure 27).

Figure 27. Representative void formation in a longitudinal sample from uniaxial tensile loading to 100% strain at (a) 10x, (b) 20x, and (c) 40x. Note that the separation occurs between collagen fibrils (blue) within the musculo-elastic fascicle.

Observation of samples stained with Verhoeff’s-Van Gieson reveals similar results as those stained with Masson’s trichrome. A trend of increased damage in the form of voids is apparent as strain level increases (Figures 28 and 29).
Figure 28. Verhoeff’s-Van Gieson staining of porcine carotid artery cross-section after uniaxial tensile loading in the circumferential direction to (a) 25% strain, (b) 40% strain, (c) 50% strain, and (d) 100% strain. 10x magnification. Arrows indicate voids. Note sample orientation with circumferential (C), longitudinal (L), and radial (R) directions.
Figure 29. Verhoeff’s-Van Gieson staining of porcine carotid artery cross-section after uniaxial tensile loading in the longitudinal direction to (a) 45% strain, (b) 60% strain, (c) 70% strain, and (d) 100% strain. 10x magnification. Arrows indicate voids. Note sample orientation with circumferential (C), longitudinal (L), and radial (R) directions.

Quantification of the voids based on strain in the circumferentially oriented samples shows that as strain increases: (i) void count, void area fraction, void density, and mean void area (with the exception of one strain level) increase, and (ii) average nearest neighbor distance decreases (Table 10). Microstructural analysis of the longitudinally oriented samples indicates similar but less obvious development of damage as strain increases (Table 11). Similarly to the samples stained with Masson’s trichrome, more of the differences in parameters (compared to control) measured for the
circumferential samples were found significantly different than those from the longitudinal samples (p<0.05).

Table 10. Image analysis of voids as a function of strain level from circumferential samples stained with Verhoeff’s-Van Gieson (n=3). *Indicates p<0.05 versus control.

<table>
<thead>
<tr>
<th>Tensile strain</th>
<th>Control</th>
<th>25%</th>
<th>40%</th>
<th>50%</th>
<th>100%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Object count</td>
<td>1397 ± 161</td>
<td>1386 ± 536</td>
<td>1964 ± 550</td>
<td>2815 ± 293*</td>
<td>3358 ± 282*</td>
</tr>
<tr>
<td>Object area fraction (1x10⁻²)</td>
<td>2.86 ± 1.49</td>
<td>3.13 ± 0.578</td>
<td>5.10 ± 1.24</td>
<td>6.86 ± 0.645*</td>
<td>9.22 ± 2.00*</td>
</tr>
<tr>
<td>Number density (μm⁻²) (1x10⁻⁵)</td>
<td>1.75 ± 0.202</td>
<td>1.74 ± 0.673</td>
<td>2.47 ± 0.691</td>
<td>3.53 ± 0.367*</td>
<td>4.21 ± 0.354*</td>
</tr>
<tr>
<td>Mean object area (μm²)</td>
<td>16.0 ± 7.15</td>
<td>19.5 ± 7.28</td>
<td>21.3 ± 5.43</td>
<td>19.7 ± 3.72</td>
<td>22.0 ± 4.89</td>
</tr>
<tr>
<td>Mean object nnd (μm)</td>
<td>12.1 ± 0.281</td>
<td>11.2 ± 1.09</td>
<td>10.7 ± 0.929*</td>
<td>9.40 ± 0.382*</td>
<td>9.04 ± 0.316*</td>
</tr>
</tbody>
</table>

Table 11. Image analysis of voids as a function of strain level from longitudinal samples stained with Verhoeff’s-Van Gieson (n=3). *Indicates p<0.05 versus control.

<table>
<thead>
<tr>
<th>Tensile strain</th>
<th>Control</th>
<th>45%</th>
<th>60%</th>
<th>70%</th>
<th>100%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Object count</td>
<td>1397 ± 161</td>
<td>1736 ± 261</td>
<td>1515 ± 1091</td>
<td>3670 ± 1366</td>
<td>3706 ± 2181</td>
</tr>
<tr>
<td>Object area fraction (1x10⁻²)</td>
<td>2.86 ± 1.49</td>
<td>2.61 ± 0.950</td>
<td>2.31 ± 1.51</td>
<td>9.58 ± 4.49*</td>
<td>8.26 ± 4.16*</td>
</tr>
<tr>
<td>Number density (μm⁻²) (1x10⁻⁵)</td>
<td>1.75 ± 0.202</td>
<td>2.18 ± 0.328</td>
<td>1.90 ± 1.37</td>
<td>4.61 ± 1.71</td>
<td>4.65 ± 2.74</td>
</tr>
<tr>
<td>Mean object area (μm²)</td>
<td>16.0 ± 7.15</td>
<td>11.8 ± 3.23</td>
<td>12.4 ± 1.40</td>
<td>20.3 ± 2.13</td>
<td>19.3 ± 4.71</td>
</tr>
<tr>
<td>Mean object nnd (μm)</td>
<td>12.1 ± 0.281</td>
<td>10.9 ± 0.490</td>
<td>11.9 ± 3.21</td>
<td>8.26 ± 0.484</td>
<td>9.31 ± 2.49</td>
</tr>
</tbody>
</table>

Environmental Scanning Electron Microscopy: Real-time Observation of Tissue Damage

Evo SEM images of carotid artery intima acquired from failure testing of dogbone specimens also indicate a trend of increasing damage as strain level increases (Figures 30 and 31). Quantification of voids via image analysis confirms this phenomenon (Tables
12 and 13). In the cases of both circumferentially and longitudinally oriented samples, as strain level increases: (i) number of voids (and correspondingly, void density) decrease, (ii) area fraction of voids increases, (iii) mean area of voids increases, and (iv) nearest neighbor distance between voids increases.

Figure 30. Evo SEM of porcine carotid artery intima after uniaxial tensile loading to failure in the circumferential direction (a) unloaded after 10% preconditioning at 500x, (b) to 25% strain at 700x, (c) to 40% strain at 700x, and (d) to 50% strain (global failure) at 700x. Horizontal line indicates loading direction. Arrows indicate voids.
Table 12. Image analysis of voids as a function of strain level from Evo SEM coupled with uniaxial tensile loading to failure in the circumferential direction.

<table>
<thead>
<tr>
<th>Tensile strain</th>
<th>25%</th>
<th>40%</th>
<th>50%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Object count</td>
<td>205</td>
<td>139</td>
<td>126</td>
</tr>
<tr>
<td>Object area fraction (1x10⁻²)</td>
<td>2.46</td>
<td>2.54</td>
<td>3.36</td>
</tr>
<tr>
<td>Number density (μm⁻²) (1x10⁻³)</td>
<td>1.92</td>
<td>1.20</td>
<td>1.08</td>
</tr>
<tr>
<td>Mean object area (μm²)</td>
<td>12.83</td>
<td>21.24</td>
<td>30.99</td>
</tr>
<tr>
<td>Mean object nnd (μm)</td>
<td>10.35</td>
<td>11.94</td>
<td>11.99</td>
</tr>
</tbody>
</table>

Figure 31. Evo SEM of porcine carotid artery intima after uniaxial tensile loading to failure in the longitudinal direction (a) unloaded after 10% preconditioning at 500x, (b) to 45% strain at 700x, and (c) to 60% strain (global failure) at 700x. Horizontal line indicates loading direction.
Table 13. Image analysis of voids as a function of strain level from Evo SEM coupled with uniaxial tensile loading to failure in the longitudinal direction (n=2).

<table>
<thead>
<tr>
<th>Tensile strain</th>
<th>45%</th>
<th>60%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Object count</td>
<td>27.50 ± 14.85</td>
<td>12.00 ± 14.14</td>
</tr>
<tr>
<td>Object area fraction (1x10⁻²)</td>
<td>11.61 ± 1.86</td>
<td>14.45 ± 2.04</td>
</tr>
<tr>
<td>Number density (µm⁻²) (1x10⁻⁴)</td>
<td>1.69 ± 0.147</td>
<td>1.12 ± 1.32</td>
</tr>
<tr>
<td>Mean object area (µm²)</td>
<td>693.6 ± 170.2</td>
<td>4565 ± 5561</td>
</tr>
<tr>
<td>Mean object nnd (µm)</td>
<td>28.08 ± 2.81</td>
<td>100.5 ± 88.57</td>
</tr>
</tbody>
</table>

Atomic Force Microscopy

The force-displacement data from seven selected points on the force maps from the intima, media, and adventitia (Figure 32) were input into the Hertz model to determine elastic moduli (E) of each layer (Figure 33) using Equation 4. F is applied force, υ is Poisson’s ratio of the artery, δ is the deflection of the artery, and α is the tip cone half-angle.

\[
E = \frac{\pi}{2} \frac{F(1-\nu^2)}{\delta^2 \tan \alpha}
\]  

(4)

Due to large variation, the highest and lowest elastic modulus values were not included in statistical analysis. Results from AFM force mapping show that the material stiffness varies from layer to layer, with an increase in stiffness from the intima to the adventitia to the media.
Figure 32. Representative automated elasticity map from scan of media. Points were chosen for further analysis of elastic modulus values.

Figure 33. Representative force-displacement curves with calculated elastic modulus values from scan of media.
Average elastic moduli values were $23.992 \pm 24.028$ kPa, $3086.0 \pm 2149.4$ kPa, and $876.98 \pm 1205.0$ kPa for the intima, media, and adventitia, respectively (n = 5 for each) (Table 14).

Table 14. Elastic modulus from AFM for each arterial layer.

<table>
<thead>
<tr>
<th>Layer</th>
<th>Elastic modulus (kPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intima</td>
<td>$23.992 \pm 24.028$</td>
</tr>
<tr>
<td>Media</td>
<td>$3086.0 \pm 2149.4$</td>
</tr>
<tr>
<td>Adventitia</td>
<td>$876.98 \pm 1205.0$</td>
</tr>
</tbody>
</table>
Choice of Tension Testing

The methodology of uniaxial tensile testing on carotid artery samples oriented axially and circumferentially provides a good basis for revealing many mechanical attributes of the tissue. Although some would argue that more complicated experimentation is necessary to thoroughly characterize the tissue, biaxial tests, including planar biaxial and extension-inflation tests, have negligible advantage over separate uniaxial tests along different orientations because neither testing procedure is able to capture the (ideal) three-dimensional response of an anisotropic material such as artery [25].

Additionally, ultimate tensile stress indicates a tissue’s maximum resistance to fracture (dissection), which is a significant concern especially in blunt carotid artery injury cases. Previous work concluded that the primary local traumatic injury mechanism of the carotid artery is tension in the vessel secondary to hyperextension of the neck [122]. Other research groups suggest the main injury mechanism is blunt object interaction with the neck [123, 124]. In either case, the carotid artery is subjected to tension (on at least one side). Therefore, the current study employed tension experiments to examine damage accumulation and failure mechanisms in the carotid artery.
Reference State

As has been previously mentioned, arteries retract considerably in length upon excision, so knowing the amount of retraction is important when examining stress-strain data from tests on the excised artery. Due to the nature of the tissue procurement process in the present study, however, measurement of the in vivo length of porcine carotid arteries was not possible. Regardless of the amount of preload artery samples are subjected to, the values of subfailure stress and strain normalized to ultimate failure stress and strain are expected to be repeatable [117]. It is believed that preload amount affects only the length of the toe region. Nonetheless, the zero-stress state (as in an excised, opened artery) is a good reference state, both mechanically and morphologically, to which various loading scenarios can be applied. In addition, a detailed description of loading conditions, such as that included in this study, is vital for contribution to future constitutive models.

Rational for Porcine Carotid Artery as Suitable Surrogate

The porcine common carotid artery average inner diameter ranges from 5 – 6.1 mm with a wall thickness of 1.2 ± 0.4 mm. Similarly, the human carotid artery has an outer diameter of 8.9 ± 2.0 mm and a thickness of 1.1 ± 0.4 mm [117, 125]. In terms of mechanical properties, longitudinal elastic modulus of human carotid arteries was reported as 4.1±1.6 MPa [117], further supporting the present results and the assumption that the porcine carotid artery is an appropriate model for the human carotid artery.
**Hysteresis**

Data obtained from uniaxial tension load-unload cycles shows the expected viscoelastic response of soft tissue, with a difference in loading and unloading curves. Hysteresis was calculated based on sample orientation. Average hysteresis in the circumferential direction was much higher than the corresponding axial value, despite the fact that circumferential samples were loaded to only 70% strain, while axial samples were loaded to 100% strain. The same trend was observed when comparing the hysteresis normalized to the area under the loading curve; the percentage was greater circumferentially than axially. A previous group who used similar analysis techniques on separated layers of human iliac arteries found that all tissue layers oriented along the circumferential direction had larger hysteresis percentages than those along the axial direction [121]. Although overall hysteresis values were less than those reported in the present study, this could be due to differences in ending strain level, as the previous study did not report strain (or stress) levels to which the tissues were distracted.

**Material Symmetry**

Upon examination of stress-strain results from uniaxial tensile testing of carotid artery samples, a marked anisotropic behavior is apparent. Furthermore, failure stress and strain based on sample orientation indicate that the artery is much stiffer circumferentially than axially. The circumferentially oriented samples withstand greater stresses but at significantly lower strains to failure, whereas the axial samples accumulate much lower stresses but deform to a greater degree of stretch. High strain rate tension experiments (80-100 s\(^{-1}\)) on human aortas found likewise that ultimate stress was 41%
higher in the circumferential direction than in the longitudinal direction [126]. As previously stated, axial retractive forces from a report on canine carotid arteries were 0.36 N on average [56]. When comparing to data from the present study, 0.36 N corresponds to about 40% strain from axial extension (n = 6) of porcine specimens. Considering this and the prior findings that carotid artery removal results in about a 51-70% reduction in length of the vessel [18, 19, 39, 53-55], it is believed that the greater extensibility of axial samples as compared to that of circumferential samples can be attributed in part to the large amount of axial stretch under which the artery is held in its native state.

As would be expected, the stiffer behavior of the artery in the circumferential direction is further delineated through a comparison of maximum tensile moduli values. The maximum tensile modulus of circumferential samples (12.90 ± 3.134 MPa) is more than four times that of longitudinal samples (3.900 ± 1.827 MPa) and supports previous findings that the artery is stiffer circumferentially [17, 19].

**Microstructural Contributions to Material Symmetry**

The highly organized microstructure of arteries, particularly that of the media, contributes significantly to the mechanical response under loading. The orientations of various components such as smooth muscle cells, elastin, and collagen vary throughout transmural depth.

Smooth muscle cells are oriented nearly circumferentially [8]. Collagen fibers in the media are oriented about 10° up and down with respect to the circumferential direction, whereas those in the adventitia have approximately a ±40° orientation.
compared to the circumferential direction [22]. Undoubtedly, the preferential organization of collagen fibers and smooth muscle cells about the circumferential axis in the media, the most mechanically relevant layer, contributes to the stiffer macroscopic mechanical response in the circumferential direction in contrast to the greater extensibility of the longitudinal direction. The results obtained from previous studies in which the media was stiffer circumferentially than axially [25, 121] parallel the present findings. More specifically, collagen fibers are the primary contributors to the anisotropic nature of the artery [27]. Circumferentially oriented tensile testing transmits load primarily along the long axis of collagen fibers, which have a stiff mechanical response. As expected, the stiffer mechanical response of circumferential samples correlates with a higher ultimate tensile strength as compared to longitudinal samples. On the other hand, loading in the longitudinal direction distracts the tissue transverse to the collagen fiber orientation, causing the load to be carried mainly in the collagen fibril interconnections. The ability, even tendency, of the fibrils to separate is believed to contribute to the higher strain to failure seen in longitudinally distracted specimens.

Collagen is responsible for carrying load beyond the low stress toe region of the stress-strain curve; the elastic modulus of collagen in tension has been previously reported as approximately 200 MPa [24, 59], indicating a very high tensile strength. The cohesive strength force per width ratio of collagen type IV of the eye lens was found to be about 200 mN mm$^{-1}$ [127], and this same type collagen is beneath endothelial cells in the intima. Also, from direct tension tests of the human aortic media, the cohesive strength opposing dissection was 140.1 kPa [115]. Due to the high tensile strength of collagen as compared to its cohesive strength, it is believed that the failure mechanism of
the artery involves the separation of the circumferentially oriented collagen fibrils from each other and/or from the surrounding elastic laminae, which are also circumferentially oriented at pressures $\geq 60\text{mmHg}$ [20], by breakage of the fibrillar crosslinks (Figure 27). This mode of failure is thought to occur since the cohesive strength that binds fibrils together (longitudinally) is much lower than the tensile strength along the direction of the collagen fibrils (circumferentially). The reorientation of the elastic laminae from the axial to the circumferential direction as internal pressure increases provides additional structural alignment in the circumferential direction and contributes to the greater strength of the tissue in the circumferential direction.

The earliest known studies to cite this phenomenon as mode of dissection propagation suggested that tears typically spread between adjacent elastic laminae [113, 128]. More specifically, the interconnections between collagen fibrils and elastic laminae resist the propagation of a dissection, while dissection occurs between collagen fibrils [113]. Other previous findings on tear propagation in porcine thoracic aortas concluded that failure occurs via the separation of smooth muscle cells and elastin, which forms a void within the elastic laminae, and this phenomenon occurs at a critical strain value [61]. An additional work found damaged elastin layers through the use of histology and proposed that the failure of these layers results in void formation and the aforementioned microdamage within the media [129].

Also, it has been previously postulated that during peeling tests of the aortic media, collagen fiber tearing occurs via slippage of collagen fibrils [62]. A more recent study on bidirectional peeling tests of aortic media determined that circumferential dissections spread primarily between elastic laminae, while axial dissections propagated
from one elastin layer to adjacent elastin layers [115]. Furthermore, the authors cited that
the layered organization of the media results in the tendency of the laminae to peel during
failure; note also that in all tests conducted, a dissection within an elastic lamina did not
occur. These results collectively support the aforementioned theory that \textit{damage
propagates due to the separation of components of the musculo-elastic fascicle,
specifically the collagen fibers located between the elastin sheets}. Overall, the structure
and organization of various material constituents in the artery wall play critical roles in
the anisotropic mechanical properties of the artery.

\textbf{Subfailure Testing}

High speed imaging of the failure tests allowed for investigation into (i) failure
(dissection) of the intima versus overall failure and (ii) modes of dissection failure of the
carotid artery.

\textit{Intimal Failure versus Ultimate Failure}

In both orientations, intimal failure was apparent first, and ultimate failure
occurred approximately 6.25 s and 10.5 s after intimal crack initiation for the
circumferential and longitudinal samples, respectively (Tables 4 and 5). Subfailure stress
and strain normalized to ultimate failure stress and strain parallel the results of similar
testing on porcine thoracic aortas obtained from young (6 month old) pigs [117]. It is
believed that during intimal failure, damage accumulates in the media, which
subsequently causes failure of the media.
A study that employed inflation and axial extension on porcine coronary arteries (whole, intima-media portion, and adventitia portion) found that stiffness decreased from intima-media to whole to adventitia at physiological ranges of stretch [130]. Another previous work determined that the intima is the least extensible of the three layers [131], so it stands to reason that initial damage/failure in the artery wall would occur in the intima (it fails at lower stresses due to its lack of compliance). Subfailure of the intima is thought to be a product of localized tension, which can be attributed to blunt trauma, or global deformation secondary to blunt trauma [67].

As revealed by the image sequences, the adventitia is the final layer to fail. Similar work showed that the adventitia has a high tensile strength, and as applied force increases, the adventitia begins to carry the majority of the load [121]. Interestingly, a related study on tensile testing of individual layers determined that the failure strains of all three layers were nearly the same, whereas the average ultimate tensile stress of the adventitial samples was about three times that of the intimal and medial samples [25]. The ability of the adventitia to withstand greater stresses to failure may be explained by its greater degree of compliancy than the other layers; because of these characteristics, intimal failure will likely always precede ultimate (adventitial) failure [118]. A previous work on tensile testing of the internal carotid artery in the longitudinal direction showed that intimal failure preceded ultimate failure [120]. A later study by the same group utilized two high speed cameras positioned on opposite sides of strip porcine thoracic aortas and human carotid artery samples to capture failure of the intima and adventitia relative to ultimate tissue failure [117]. Results showed that the adventitia remained intact until ultimate failure during all tests. On the other hand, failure of the intima
without ultimate vessel failure occurred in 93% of samples, and all subfailures occurred in the intima. Similarly to the current study, resultant stress-strain curves indicate no apparent changes at the point of intimal damage initiation. This subfailure is believed to contribute to the delayed symptomatology often associated with carotid artery dissections due to the artery’s ability to maintain the majority of its mechanical functionality even after subfailure [117]. This phenomenon is evidenced by an increase in stress and strain between intimal failure and ultimate failure (also found in the current study). Although on the macroscale the artery may appear mechanically stable, the effects of an intimal tear can decrease blood flow to the head or neck enough to cause an acute ischemic attack. Acute ischemic attacks have been reported in approximately 80% of carotid artery dissection cases [85, 86]. Nonetheless, delayed onset of symptoms is thought to be the primary contributor to high morbidity and mortality associated with blunt carotid artery injury, and early diagnosis is the key to minimizing the potential effects of an artery dissection [117]. Still, however, the level of damage the artery can undergo without triggering life-threatening neurological events remains unknown.

*Modes of Dissection Failure*

Detailed information on failure modalities was also delineated from the high speed images of stained samples, as well as from observing failure in nonstained samples during uniaxial tensile tests in the circumferential and axial directions. In each case, a single crack initiated along the edge of the sample. The initiation point of the crack could be due to sample preparation, whereby collagen fibers at the sample edge were inevitably cut; the possible resultant retraction may be sufficient to weaken the tissue at the edges.
Regardless of orientation of the stained sample, the crack subsequently propagated in the axial direction (Figures 16 and 17). Similar findings from uniaxial tensile testing of an excised human iliac intima found that the intimal tear spread axially to failure [121]. However, one study cited that aortic dissection propagates both axially and circumferentially [61].

Intimal damage exposes thrombogenic collagen (type IV) located just beneath endothelial cells in the basal lamina, encouraging the aggregation of platelets, which can lead to partial or total thrombosis and stroke. If the dissection is such that the intimal flap is raised, the subsequent distribution of blood beneath the intima can form a thrombus and cause the flap to extend further into the lumen, thereby occluding the vessel to a greater extent [68].

Related to the aforementioned conclusion of collagen fibril debonding as a mechanism for arterial failure, a recent study on human cornea (collagen type IV) delineated a possible cascade of events that precedes tissue failure. Tissue damage is likely coupled with collagen laminae slippage; this slippage may be the result of a decrease in interlamellar and/or interfibrillar cohesive strength, which could be caused by enzymatic degradation [132].

Also interesting to note is that in the longitudinal sample, ultimate failure occurred at a site above the initial crack site, and the crack that caused ultimate failure propagated very quickly compared to the initial crack (Figure 17). A previous study verified that physiological pressures were sufficient to cause the propagation of intimal tears in porcine thoracic aortas [61]. Results also showed that tear depth at the leading edge was greater than that at the non-propagating edge, with a 2% difference in depth
enough to affect the direction of propagation. This finding indicates that the initial dissection event could be the primary factor in the development of a false lumen [61] and other morbid conditions.

**Interruption Testing**

The purpose of interruption testing at various strain levels was to examine the irreversible changes to the microstructure incurred during tension testing and expectedly, in trauma scenarios. The strain levels to which samples were distracted were determined based on examination of the average failure curves from both orientations. Due to the greater degree of stiffness (and associated shorter toe region) of the tissue in the circumferential direction, strain levels chosen were, as a whole, less than those in the axial direction. However, to have a basis for comparison between the two groups, the highest strain level chosen was the same (100%) for both.

Fixation of the sample immediately after testing was completed (and while the respective strain level was held constant) was crucial to ensure that stress relaxation of each sample was minimized. Because the fixation process is not instantaneous, a small amount of stress relaxation likely occurred. Nonetheless, it is believed that the stress relaxation that took place during the present methodology – submersion of the sample within a 5 sec window of test completion – is negligible with regards to altering the microstructural damage in the artery samples.
Scanning Electron Microscopy

The longitudinally oriented intima at low strain appears to have a more highly organized helical arrangement as compared to the circumferentially oriented intima. This greater degree of crimping is thought to contribute to the larger elongation to failure of samples in the longitudinal direction.

Observation of the microstructure of the media further substantiates the aforementioned hypothesis that medial failure occurs due to delamination of the elastic laminae. One previous study performed partial peeling tests of human abdominal aortic media, fixing samples after peeling to the middle of their length occurred. Results revealed damage to the media in the form of separated elastic laminae and the smooth muscle cells and elastin therein [115]. Furthermore, it is believed that during loading, the interlaminar collagen fibrils viscously slide past each other, dissipating load, and eventually breaking their crosslinks.

At a certain level of deformation beyond physiologic range, the adventitia morphs into a rigid “jacket-like” cylinder that prevents the smooth muscle in the media from acute overstretching [7, 121, 133], thereby serving as a protective mechanism to minimize global deformation. From the observation of adventitial micrographs in the present study, the circumferential samples appear to accumulate a greater degree of damage than the longitudinal samples. This is not surprising because the mechanical data at corresponding strain levels indicates that circumferentially oriented samples fail at lower strains than the more pliable longitudinally oriented samples.
Histology

Masson’s trichrome stain was used to distinguish between collagen fibers and smooth muscle. Verhoeff’s-Van Gieson (VVG) stain was often used to reveal the shrinking, thinning, tearing, and/or loss of elastin fibers due to damaged or diseased vasculature. The combination of the Verhoeff’s stain and Van Gieson stain provides structural information that would not be seen with either single component.

For the samples stained with Masson’s trichrome (TRI), it is quite possible that the decrease in void area fraction between 25% and 40% in the circumferential samples, and 45% and 70% in the longitudinal samples can be attributed to these strains corresponding to the strains to which the artery is loaded physiologically. As previously mentioned, the artery is subjected to relatively large amounts of physiological deformation, approximately 57-59% circumferentially and up to 70% longitudinally [18, 19, 39, 53-55]. Cervical extension and lateral rotation can cause further longitudinal deformation from 20-60% beyond physiological range [57, 58]. The initial decrease in void area fraction, coupled with its subsequent doubled increase at 100% strain, suggests an acceptable level of strain the artery can withstand without incurring significant amounts of microdamage, specifically 40% circumferentially and 70% longitudinally. Interestingly, these strains correspond approximately to each heel, or transition region, of the failure curves, respectively (Figure 15).

The decrease in number of voids as strain increases signifies void coalescence. Correspondingly, the average area of the voids increases with strain, indicating that growth of the voids contributes to failure. Voids coalescence may occur as a result of natural coalescence, a joining of voids due to an increase in void size, and/or via a void
sheet mechanism, whereby voids join through the extension of a thin sheet between them. Regardless of the type of coalescence mechanisms at work, larger (but fewer) voids are present with increased strain.

The more clearly defined trends in damage progression from analysis of samples stained with Verhoeff’s-Van Gieson (VVG) can be explained by the difference in staining techniques. The three samples from which sections for both types of histological staining were obtained were cut from a single dogbone sample distracted to a certain strain level. Therefore, although the TRI stained samples do not show trends of increased damage as distinctly as those stained with VVG, it is believed that this can be attributed solely to variations in the detection capabilities of the stains.

Overall, both types of staining reveal an increase in mean void area. However, unlike the samples stained with TRI in which the number of voids remains lower than the initial void number, samples stained with VVG show an increase in number of voids. The increase in void count as strain level increases suggests that another mechanism of failure that occurs is void nucleation. These results are viewed not as conflicting, but as a means to elucidate multiple mechanisms that contribute to damage progression and failure in the tissue. Therefore, it is believed that failure in the carotid artery is due to void nucleation, void growth, and void coalescence.

**Environmental Scanning Electron Microscopy: Real-time Observation of Tissue Damage**

Similarly to the findings from histological analysis, a decrease in number of voids coupled with an increase in mean area of the voids further substantiate the theory that
both void coalescence and void growth contribute to damage accumulation and failure in the carotid artery.

At present, no known work coupling tensile testing with environmental SEM on artery exists. Due to the novelty of the procedure, a few minor complications were encountered throughout its execution. These are discussed below.

The transfer of energy from the electron beam to the specimen is mainly in the form of heat. Due to the low heat conductivity of biomaterials such as artery, the beam can cause damage to the sample in the form of drying. The drying caused by the beam is also a function of scan size (magnification) and accelerating voltage [134]. Each of these factors was optimized to allow for the maintenance of tissue structural integrity for as long as possible.

The samples tested in the Evo SEM failed at lower strains (and presumably lower stresses) than those from testing in the Mach-1™. Both setups employed identical preconditioning procedures and the same constant displacement rate throughout. Thus, the discrepancy is believed to be due primarily to the dehydration of the samples caused by the charging in the SEM, as compared to the samples in the Mach-1™ that were kept moist by spraying with PBS during testing.

Hydration of samples in the SEM chamber was difficult, mainly because the vacuum pumping process draws PBS out of the sponge and distributes it throughout the chamber. As a result, the sponge can only hold a small amount of PBS. This minimum fluid capacity affects the degree to which the sample can maintain hydration because the sponge is the sample’s primary means of remaining moist. Although the sample stays
hydrated for a longer period of time than without the PBS-filled sponge, the allowable exposure time of a sample in the SEM chamber is still relatively short.

Although Evo SEM experiments included testing of fresh porcine carotid artery samples, no endothelial cells were visible on the intimal surface. The lack of endothelial cells suggests that cell death occurred as a result of sample storage on ice. Previous studies have shown that storage time and preservation solution affect the amount of injury that endothelial cells experience [135, 136]. More specifically, one work determined that endothelial cell injury developed after 12 hours of frozen storage in normal saline solution [135].

Since artery specimens were held at constant strain during imaging intervals, even for a short period of time, it is known that some stress relaxation occurred. Due to the nature of the testing procedure, this could not be avoided. However, the amount of stress relaxation that occurs during quasi-static loading is assumed insignificant.

Furthermore, the electromechanical stage load cell is 1000 lb, which is more than 45 times the strength of the Mach-1™ load cell (10 kg). The maximum force that the artery can withstand is approximately 1 kg. This load is so small that it would be difficult to distinguish from noise data generated by the electromechanical stage, so mechanical data from the Evo SEM procedure is not reported.

**Atomic Force Microscopy**

The results from the AFM multilayer force mapping indicate that of the three layers, the intima is least stiff, followed by the adventitia, and then the media (Table 8). These findings support the high speed imaging results that show intimal failure before
ultimate failure. Because much of the previous research efforts have been focused on the artery as a single material, measuring the mechanical properties of the individual artery layers is very beneficial for assisting in the development of accurate multilayered material models.

Bending tests have been the primary means of assessing the modulus of each layer. During bending tests of artery strips, one side of the tissue is in compression, while the other side is in tension. Due to this fact, the elastic moduli can be measured from only two parts of the cross section. Therefore, the intima and media are assumed as one unit and the adventitia as another. The use of these data for subsequent modeling efforts may be a reasonable delineation, however, because the intima, made of a single layer of endothelial cells, is relatively thin and contributes little mechanically to the overall vessel. As a result, the following data on the intima-media portion can be assumed to represent primarily the mechanical response of the media. From one previous bending study on rat aortas, the elastic modulus of the inner intima-media layer, which consists of endothelial and smooth muscle cells and elastin sheets, was three to four times larger than that of the outer adventitia layer which is made of collagen and small amounts of fibroblasts and elastin [26]. Another study on porcine thoracic aorta found that intimal-medial elastic modulus was $43.2 \pm 15.8$ kPa, while adventitial elastic modulus was $4.70 \pm 1.72$ kPa [137]. The lower modulus values, as compared with the current study, could be attributed to the previous procedures involving tests in stress-strain regimes at or below physiologic range.

From uniaxial tension to failure testing, maximum tensile moduli were 12.90 MPa for circumferential samples and 3.900 MPa for longitudinal samples. These values are
even greater than the elastic moduli obtained from AFM testing. These discrepancies could be attributed to the varying methodologies used, such as stress state (tension versus compression) and length scale (macroscale versus microscale). Also, note the lower standard deviation values from tension testing as compared to AFM testing. As would be expected, obtaining force maps from areas approximately $5 \times 5 \mu m$ would induce a larger degree of error due to the highly complex microstructure of the artery. It should be recognized, however, that from AFM force mapping, the average elastic modulus for the media (3.086 MPa) is on the order of the maximum tensile modulus determined from tension testing. This is not surprising because as previously mentioned, the media is the most mechanically significant of the three arterial layers.

The high standard deviation from elastic modulus of the adventitia could be due in part to the structural heterogeneity of the layer as compared to the more organized, regular arrangement of collagen fibers and smooth muscle cells in the media. From another study, tension tests on the separated layers of human coronary arteries resulted in large stress-strain variation among adventitial samples [25].

**Limitations**

*Need for Examination of Active Arterial Response*

Correlating mechanical properties to biological response (i.e. growth, remodeling, and disease) of each arterial layer is a significant challenge. Although the experiments in the present study characterize the passive artery under various mechanical scenarios, it is important to realize that the quantity, arrangement, and mechanical state of major structural components can vary with time as the components are produced, remodeled,
and degraded [47]. For example, previous works have found that axial stretch is a critical factor in vascular remodeling [138, 139]. As is the case in other fiber-dominated soft tissues, results from previous studies on arteries indicate that collagen synthesis increases in response to loading [48, 140, 141]. Additionally, the effects of wall shear stress were not explored, though it is known that shear stress leads to adaptations of vessel geometry, structure, and composition [142-144].

Microstructural Analysis of Histology

Some of the voids in the histological images recognized by the image analysis software may be present under physiological conditions. Hence, these voids would not be a result of damage to the tissue and therefore, should be distinguished from the voids that are a product of arterial injury. Future work will involve reexamining the size of voids believed to indicate damage and altering the corresponding thresholding property by increasing minimum void size.

Sample Number for Subfailure and Evo SEM Tests

The small number of samples tested with the present high speed image capture modality is a limiting factor of the extent to which the hypotheses presented herein are credible. It should be noted that experimentation resources readily accessible do not include a high speed camera with resolution of the caliber as the one used in this study (the camera used in this procedure was borrowed from an industrial associate). Additionally, the Evo SEM study is preliminary; the procedure must be repeated to validate the present results. Nonetheless, the developed methodologies and results
obtained serve as a baseline for measuring carotid artery damage and failure mechanisms which future work will hopefully extend.

*Age-dependent Variations*

The slight variation in mechanical response among uniaxial tests within the groups of axially and circumferentially oriented samples can likely be attributed to age differences. Research shows that in aged arteries, the thickness and strength of the intima increases [25]. Also, collagen cross-linking (which could result in a stiffer behavior) through advanced glycation end products tends to increase with age [145, 146]. Additionally, previous work has shown that the adventitia carries an increasing amount of load in aged human arteries [133].

*Future Work*

An improved, more cost-effective alternative to previously developed injury risk scales for the assessment of traumatic injury is the development of computational models that characterize the human body in more detail and are able to more accurately predict the risk of injury as a result of various types of trauma. A comprehensive tissue characterization utilizing multiple experimental approaches, such as the one described herein, provides structural information for a more extensive, detailed computational model. For example, the reported elasticity map of the trilayered arterial structure will assist in future efforts to establish a constitutive model composed of the individual layers. Nonetheless, the level of damage the artery can accumulate without triggering life-threatening neurological events remains unknown. In the future, important parameters
representing various structural and functional properties, such as collagen and elastin fiber tortuosity and orientation, and endothelial cell and smooth muscle cell disruption, can be developed from results such as those in the present study and incorporated into a viscoelastic, anisotropic constitutive model.
CHAPTER VI

CONCLUSION

The carotid artery exhibits anisotropic, viscoelastic behavior as determined by uniaxial tensile loading-unloading and loading to failure in the longitudinal and circumferential directions. The artery is less extensible circumferentially than longitudinally, which is not surprising due to the lower amounts of strain to which the artery is held physiologically in the circumferential as compared to the longitudinal direction. On the macroscale, high speed videography reveals that intimal failure precedes ultimate (adventitial) failure of the tissue. Atomic force microscopy elucidates likewise results based on elastic moduli of the individual arterial layers; the layers in order of increasing stiffness are intima, adventitia, and media. Medial collagen is the primary structural component that contributes to the anisotropy of the tissue, as collagen is aligned circumferentially. As deformation in the carotid artery increases, collagen fibers elongate, and eventually debond, forming voids between adjacent elastic laminae in the musculo-elastic fascicles. Microstructural analysis of sequential strain level images from histology and environmental SEM coupled with tensile testing reveal that damage accumulates as strain level increases. Trends of void area fraction suggest tolerable levels of strain the artery can withstand before a significant amount of damage develops, specifically 40% and 70% in the circumferential and longitudinal directions,
respectively. Failure occurs as a result of void nucleation, void growth, and void coalescence. The novelty in the present study is the use of multiple imaging methodologies to quantify and explain the damage progression that is believed to occur in carotid artery dissection. Furthermore, the current work delineates the mechanics of the carotid artery at multiple length scales, thereby contributing to future constitutive modeling efforts.
REFERENCES


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APPENDIX A

ATHEROSCLEROSIS OVERVIEW
Cardiovascular disease, including heart disease and stroke, is the leading cause of death in the western world, and it is expected to be the number one cause of death worldwide in the twenty-first century [7, 147]. Approximately 60% of myocardial infarctions are caused by the rupture of a vulnerable plaque [148]. Many victims of myocardial infarction with no previously visible symptoms die before they reach a hospital; this is the case in about 95% of sudden cardiac arrest victims [149]. Furthermore, many of the total number of myocardial infarction victims are not aware of their huge health threat until the attack occurs.

Atherosclerotic plaques form in the intima and inner media layers of the artery wall and consist of a lipid-rich necrotic core and calcium deposits covered by a fibrous cap [150]. Plaques become vulnerable when the necrotic core is large and the fibrotic cap is thin (<65 μm) [151]. As a plaque increases in size, it causes hardening and narrowing of the arteries and restricts blood flow, sometimes completely, often leading to a myocardial infarction. Atherosclerotic plaque rupture is thought to be affected by multiple factors, including but not limited to: mechanical forces, plaque geometry, vessel remodeling, blood composition, chemical environment, and luminal surface inflammation [7, 152]. Moreover, many studies have revealed that mechanical forces play a major role in the progression of plaque and the rupture process [149, 151-154]. Baldewsing et al. found through the use of intravascular ultrasound elastography that elastic modulus (of the lipid and the cap) is a very critical variable in quantifying the vulnerability of a plaque, but Poisson’s ratio is not related to rupture risk [153]. Trivedi et al. performed an in vivo study by MRI coupled with computational analysis, and determined that symptomatic plaques have greater average shear stresses than asymptomatic plaques
Additionally, fluid-structure interactions (FSI) play a large role on the mechanical stresses in the walls of severely stenosed arteries [149]. FSI in conjunction with MRI computations have been studied extensively by Tang et al. and have shown an improvement in the prediction accuracy of plaque vulnerability and rupture [149, 152]. As extensive as the studies by Tang and his colleagues are, however, they do not characterize vulnerability as it relates to multilayered vessel structure, anisotropic properties, or viscoelastic properties.

Due to the multitude of factors that contribute to atherosclerotic plaque rupture, a basis to quantify plaque vulnerability could greatly enhance clinical diagnosis. Zheng et al. compiled a list of significant variables related to plaque geometry and mechanical forces that combine to determine an overall biomechanical marker, which offers a more exact prediction of rupture than any single factor [151]. Nonetheless, accurate computational models are needed to determine the mechanical effects of plaque on the arterial wall, predict subsequent abnormal blood flow patterns, and determine the rupture risk of a plaque.

Previous data has shown that diseased arteries are stiffer than healthy arteries [155-157]. Furthermore, a decrease in stretchiness of the arterial wall leads to accelerated progression of atherosclerosis [158, 159]. Giannattasio et al. additionally revealed that a plaque affects the mechanical properties not only at the plaque site but also along the proximal arterial wall. In addition, the hardening of arteries restricts blood flow, which can raise blood pressure and further increase rupture risk of a plaque. In conclusion, a precise measure of the mechanical properties of the carotid artery with
atherosclerotic plaque would be greatly beneficial towards the development of a better diagnostic tool for the prediction of cardiovascular disease.
APPENDIX B

HISTOLOGICAL STAINING PROTOCOLS
MASSON’S TRICHSROME METHOD FOR CONNECTIVE TISSUE

FIXATION: Bouin’s Fixative or 10% Neutral Buffered Formalin.

TECHNIQUE: Paraffin. Cut at 6 micrometers.

SOLUTIONS:

1. Bouin’s Fixative
2. Weigert’s Iron Hematoxylin Solutions A&B
3. Biebrich Scarlet – Acid Fuchsin Solution
4. Phosphotungstic – Phosphomolybdic Acid
5. Aniline Blue Masson’s Trichrome
6. Acetic Acid 1% Aqueous

STAINING PROCEDURE:

1. Deparaffinize and hydrate to distilled water.
2. Mordant in Bouin’s Fixative for 1 hour at 56°C.
3. Cool and wash in running water until yellow color disappears.
4. Rinse in distilled water.
5. Place in Weigert’s Iron Hematoxylin Working Solution for 10 minutes.
6. Wash in running water for 10 minutes.
7. Rinse in distilled water.
8. Place in Biebrich Scarlet – Acid Fuchsin for 2 minutes. Save solution.
9. Rinse in distilled water.
10. Place in Phosphotungstic – Phosphomolybdic Acid for 10 to 15 minutes.
11. Place in Aniline Blue Solution for 5 minutes. Save solution.
12. Rinse in distilled water.
13. Place in Acetic Acid 1% Aqueous for 3 to 5 minutes. Discard solution.
14. Dehydrate in 95% Alcohol, Absolute Alcohol, and clear in Xylene, 2 changes each.
15. Mount with Poly Mount or any other acceptable mounting medium.

RESULTS:

Nuclei……………………………………………………………………………….Black
Cytoplasm, Keratin Muscle Fibers, Intercellular Fibers……….Red
Collagen……………………………………………………………………………….Blue

*Note: To insure proper mordanting, Bouin’s Fixative should be preheated to 58 – 60°C.
VERHOEFF’S STAIN FOR ELASTIC TISSUE

FIXATION:  Any fixative may be used.
TECHNIQUE:  Paraffin.  Cut at 6 micrometers.
SOLUTIONS:

1. Hematoxylin 5% Alcoholic.........Verhoeff’s Solution A
2. Ferric Chloride 10% Aqueous........Verhoeff’s Solution B
3. Lugol’s Iodine Working Solution.....Verhoeff’s Solution C

**Mix in order, just before use and filter:

Solution A..............................20 mL
Solution B.................................8 mL
Solution C.................................8 mL

4. Ferric Chloride 2% Aqueous
5. Sodium Thiosulfate 5% Aqueous
6. Van Gieson Solution

STAINING PROCEDURE:

1. Deparaffinize and hydrate to water.
2. Rinse in running tap water – 3 minutes.
5. Differentiate in Ferric Chloride 2% Aqueous, a few dips at a time, until tissue begins to appear gray.
6. Rinse in tap water to remove excess chloride. Check under microscope for correct differentiation. Elastic Fibers should be clear, sharp black, and surrounding elements colorless. The differentiation is very important. The section should be differentiated to the fine fibers. Fine fibers can be found around small blood vessels and in skin near the epidermis. The slides should be slightly under differentiated to offset the Van Gieson’s reaction which follows. The Picric Acid in Van Gieson’s will remove some of the stain from the elastic fibers. If fine fibers are not visible, the slide has been overdifferentiated. Repeat process from step 2.
7. Treat in Sodium Thiosulfate 5% Aqueous for 1 minute.
8. Wash well in tap water – 5 minutes. Recheck under microscope.
9. Counterstain in Van Gieson Solution. Timing differs with type of specimen and size. Consult pathologist before staining. Do not dehydrate in alcohol, because this will remove the Basic Fuchsin part of the Van Gieson Solution and the slides will take on a purple cast.
11. Mount with Poly Mount or any other acceptable mounting medium.
RESULTS: 
Elastic Fibers..............................................Black
Collagen.......................................................Red
Muscle, Cornified Epithelium.......................Yellow
Nuclei.......................................................Blue to Black