Oscillatory wall strain reduction precedes arterial intimal hyperplasia in a murine model

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Oscillatory wall strain reduction precedes arterial intimal hyperplasia in a murine model

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By

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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AAA</td>
<td>Abdominal aortic aneurysm</td>
</tr>
<tr>
<td>ANG II</td>
<td>Angiotensin II</td>
</tr>
<tr>
<td>apoE</td>
<td>Apolipoprotein-E</td>
</tr>
<tr>
<td>CAD</td>
<td>Coronary artery disease</td>
</tr>
<tr>
<td>CT</td>
<td>Computed Tomography</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EEL</td>
<td>External elastic lamina</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
</tr>
<tr>
<td>HA</td>
<td>Hyaluronic acid</td>
</tr>
<tr>
<td>HDM</td>
<td>High density mapping</td>
</tr>
<tr>
<td>IEL</td>
<td>Internal elastic lamina</td>
</tr>
<tr>
<td>IH</td>
<td>Intimal hyperplasia</td>
</tr>
<tr>
<td>LCCA</td>
<td>Left common carotid artery</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>OCT</td>
<td>Optical coherence tomography</td>
</tr>
<tr>
<td>PAD</td>
<td>Peripheral artery disease</td>
</tr>
<tr>
<td>RCCA</td>
<td>Right common carotid artery</td>
</tr>
<tr>
<td>SMC</td>
<td>Smooth muscle cell</td>
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Abstract

Cardiovascular diseases (CVD) remain the most common cause of death in the United States. Additionally, peripheral artery disease affects thousands of people each year. A major underlying cause of these diseases is the occlusion of the coronary or peripheral arteries due to arteriosclerosis. To overcome this, a number of vascular interventions have been developed including angioplasty, stenting, endarterectomies and bypass grafts. Although all of these methods are capable of restoring blood flow to the distal organ after occlusion, they are all plagued by unacceptably high restenosis rates. While the biological reactions that occur as a result of each of these methods differ, the initiating factor of both the primary atherosclerosis and subsequent failure of vascular interventions appears to be intimal hyperplasia (IH).

Intimal hyperplasia is most simply defined as the expansion of multiple layers of cells internally to the internal elastic lamina of the blood vessel. This excessive cellular growth leads to arterial stenosis, plaque formation and inflammatory reactions. Despite extensive research the underlying factors that cause IH remain unclear. A quantity of research to date has implicated endothelial cell mechanosensation as the mechanism by which IH is initiated with evidence positively correlating wall shear stress with IH. Others, however, have demonstrated that changes in the stresses applied to the wall in vitro can modulate IH independent of hemodynamic shear stress. Thus, relations between wall tensile stress and IH in vivo may shed light on the underlying mechanisms of IH. Since noninvasive measurement of wall tensile stress in vivo is difficult, it is most feasible to measure oscillatory wall strain which is intimately related to wall tensile stress through the mechanical properties of the arterial wall. In this dissertation, we hypothesize that reductions in oscillatory wall strain precede the formation of intimal hyperplasia in a murine model.

To test our hypothesis, we first developed a novel, high spatial and temporal resolution method to measure oscillatory wall strains in the murine common carotid artery. We validated this method both in vitro using an arterial phantom and in vivo using a murine model of abdominal aortic aneurysms. To assess relationships between strain and IH, we applied our strain measurement technique to a recently developed mouse model of IH. In this model, a suture is used to create a focal stenosis and reduce flow through the common carotid artery by 85%; resulting in proximal IH formation. Using this approach, we identified a relationship between oscillatory strain reductions and IH. Subsequent analysis demonstrated that early reductions in mechanical strain just 4 days after focal stenosis creation correlate with IH formation nearly 1 month later.

Since IH is not expected to form by day 4 in this model, we went on to assess changes in gross vascular morphology at day 4. We discovered that, although strains are significantly reduced by day 4, no significant IH can be observed, suggesting that changes in wall structure are resulting in strain reductions. At day 4 post-op, we observed cellular proliferation and leukocyte recruitment to the wall without intimal hyperplasia. These studies suggest that early reductions in mechanical strain may be an important predictor of IH formation. Clinically, this relation could be important for the development of novel techniques for predicting IH formation before it becomes hemodynamically significant.
1 Overview

1.1 Introduction

The 2013 update of the American Heart Association’s heart disease and stroke statistics indicates that 32.3% of deaths in the United States list cardiovascular disease (CVD) as the underlying cause and that more than 50% of death certificates mention CVD as a contributing factor to death.\(^1\) Although the death rates per 100,000 Americans due to CVD have declined from 864.9 in 1999 to 243.9 in 2009, the prevalence of CVD is expected to rise from 35.6% to 40.8% by the year 2030.\(^1\) These statistics indicate a real clinical need to improve our understanding and treatment of CVD and its causes.

Coronary artery disease (CAD) and peripheral artery disease (PAD) both contribute significantly to mortality and morbidity in the United States, each affecting more than 14 million Americans.\(^1\) CAD is estimated to have been responsible for over 1.2 million hospital stays in 2004 and was determined the most expensive condition treated in hospitals and caused more than 50% of cardiovascular events.\(^1\) Similarly, PAD caused 14,000 deaths in 2009 and also contributes to CVD prevalence.\(^2\)

Although a number of evidence based interventions have been applied to treat CAD and PAD, these interventions fail to address the underlying causes of the diseases.\(^3\) Present day treatments for CAD and PAD include angioplasty, stents, endarterectomies, and bypass grafts. Although these treatments have decreased the mortality rates due to arterial occlusive diseases, all continue to suffer from unacceptably high failure rates which in some cases exceed 50%.\(^2,4,5\) While the biologic adaptation underlying the failure of each of these treatments differs subtly, the underlying cause of both the primary arterial occlusive disease and the subsequent treatments appears to be intimal hyperplasia (IH).\(^6-11\)
Intimal hyperplasia is characterized by the expansion of multiple layers of cells expressing alpha smooth muscle actin (αSMA) internally to the internal elastic lamina of a blood vessel (Figure 1-1).\textsuperscript{3,12} This expansion often results in the appearance and aggregation of foam cells, accumulation of cholesterol and the eventual formation of atherosclerotic plaques.\textsuperscript{3,12,13} In the healthy adult artery, smooth muscle cells (SMCs) remain outside the internal elastic lamina and maintain a homeostatic level of growth to maintain vessel size and structure with a very low rate of both proliferation and death.\textsuperscript{14-18} In the pathological state, however, SMCs can be seen to migrate into the intimal compartment of the vessel. This proliferative and migratory response is the primary underlying mechanism that initiates arteriosclerosis, arterial occlusion and the eventual end organ ischemia associated with CAD, PAD and virtually all contemporary treatments of the disease.\textsuperscript{3,6-11,14}

\textbf{Figure 1-1: Staining of vessels with intimal hyperplastic lesions.} A) Masson’s Trichrome stain of a mouse carotid artery with intimal hyperplasia. B) Masson’s Trichrome stain with overlaid green tracings of the internal and external elastic laminae. C) Close-up of region shown in B. D) Dual stain of Ki-67 (red) and alpha smooth muscle actin (green) showing cells expressing alpha smooth actin within the internal elastic lamina and cellular proliferation at the edge of the vessel lumen.
Although a number of hypotheses have been made as to why IH occurs, a long line of evidence suggests that cellular sensing of the mechanical environment that the artery is exposed to provides the cues that initiate IH. Low wall shear stress,\textsuperscript{19} high wall tensile stress \textsuperscript{20} and the combination of both factors\textsuperscript{21} may play a role in the formation of IH in the vessel wall. Others suggest that wall strain, which is closely related to wall stress, may play a more direct role in IH formation.\textsuperscript{22} Although a number of researchers have implicated wall shear stress as the primary factor affecting IH formation,\textsuperscript{23-26} shear-based explanations alone fail to predict many aspects of IH.\textsuperscript{27,28} A leading hypothesis is that the arterial wall actively maintains a homeostatic level of wall stress.\textsuperscript{29-31} This theory is supported by the observation that the number of lamellar units in any given artery is proportional to blood vessel diameter.\textsuperscript{32} By maintaining this relationship, the vascular wall keeps the mechanical tension per lamellar unit within an amazingly narrow physiological range, even across numerous mammalian species.\textsuperscript{32} Since wall stress and strain are intimately related through the mechanical properties of the vessel wall, others have suggested that wall strain, not stress, is the underlying state sensed by the cell.\textsuperscript{31} The sheer volume of literature relating the mechanical environment to IH suggests that \textit{in vivo} assessment of arterial mechanics may be useful for the characterization, diagnosis and prevention of IH and arterial occlusive diseases.

Given that wall stress is difficult to measure accurately \textit{in vivo}, analysis of wall strain represents a more promising mechanical property to assess in living models. \textit{In vivo} wall strain has been assessed using a number of methodologies which are both invasive and non-invasive including MRI,\textsuperscript{34,35} ultrasound\textsuperscript{36} and high speed light imaging based methods\textsuperscript{37}. Herein we
develop a novel method to assess cyclic arterial strain in a murine model and determine the relationship between cyclic wall strain and IH.

1.2 Overall goal and hypothesis

The overall goal of this study is to determine the relationship between cyclic wall strain and IH in a small animal model of arterial occlusive disease. We hypothesize that reductions in oscillatory wall strain precede the formation of intimal hyperplasia in a murine model (Figure 1-2). The expected outcome of this dissertation is to provide compelling evidence to support the above hypothesis and to enable future research to establish if mechanical strain can be used to predict IH formation in the mouse or in larger animals.

To systematically test our hypothesis, this dissertation was divided into three specific aims and 5 objectives. Herein, we first aim to develop and validate a novel, high spatial and temporal resolution method for measuring cyclic wall strains in the murine model. We then aim to demonstrate that a relationship exists between cyclic strain and IH. Finally, we aim to determine the detailed temporal changes in both cyclic strain and IH and demonstrate that changes in cyclic strain precede IH formation in this model.
Figure 1-2: Overall hypothesis of this dissertation. We hypothesize that, using a murine model of IH, we can demonstrate that reductions in cyclic circumferential wall strain temporally precede the formation of IH in the corresponding region of the vessel.

1.3 Specific Aim I: Develop and apply a noninvasive, ultrasound-based method for measuring mechanical strain in the murine vasculature

Recent developments in high frequency ultrasound technology have considerably improved both the spatial and temporal imaging resolution of ultrasonography systems, allowing researchers to accurately measure luminal diameter in vivo in mice. Simultaneously, improvements in speckle tracking technology in ultrasound imaging has allowed for objective quantification of both myocardial strains and strain rates in small animal models. In this Aim, we propose that the combination of high frequency ultrasound and speckle tracking algorithms can be used to quantify and assess mechanical strains on the murine arterial wall in vivo.
1.3.1 **Objective 1A: Validate an ultrasound-based strain measurement technique**

A number of measurement techniques have been applied to measuring strains on the arterial wall both *in vitro* and *in vivo*. Given that we eventually aim to apply our strain measurement technique to an *in vivo* murine model, the technique must be both noninvasive and have a large enough spatial resolution to resolve changes in diameter of vessels on the micron scale. High frequency ultrasound meets both of these requirements. In this objective, we used telecentric optics to accurately and dynamically measure the diameter of an *in vitro* murine artery phantom. Simultaneously, we measured strains using our novel ultrasound-based method and demonstrated that we produce similar results with both techniques.

1.3.2 **Objective 1B: Apply the ultrasound based method *in vivo* in a murine model of vascular disease**

Although IH remains the overall focus of this study, the development of a non-invasive, high resolution strain measurement method for *in vivo* studies would be beneficial to the study of a number of vascular diseases. In this objective, we chose to apply our novel ultrasound technique to a murine model of abdominal aortic aneurysm (AAA) formation. In this objective, we hypothesized that high-frequency ultrasound scan biomicroscopy combined with speckle-tracking can be used to define the role of oscillatory wall strain in AAA formation in defined aortic anatomic regions in the Angiotensin II apolipoprotein-E deficient mouse model of AAAs. The results of this objective demonstrate the utility of our novel ultrasound method and showed that it can be effectively applied to mouse models of arterial disease.

1.4 **Specific Aim II: Evaluate if a relationship exists between oscillatory wall strain and intimal hyperplasia formation**

In Specific Aim I, we validated the measurement accuracy and precision of our ultrasound strain measurement technique and demonstrated that it can be effectively applied to a living...
model of arterial disease. In Aim II, we apply this method to a novel model of arterial intimal hyperplasia and examine how dynamic mechanical strain relates to IH.

In a previous study in our collaborating lab, we have demonstrated a novel, technically simple, model of IH formation in the mouse. In this model, a focal stenosis is created on the murine carotid artery. In the resulting publication detailing this method, we showed that immediately after creation of the focal stenosis, wall shear stress and cyclic wall strain were significantly reduced in the artery proximal to the stenosis. In objective 2, we combine this novel model with our ultrasound-based strain measurement method to better understand the relationship between cyclic strain and IH formation in the mouse.

1.4.1 Objective 2: Determine what relationship exists between mechanical strain and pathological wall remodeling

In order to assess the relationship between IH and cyclic strain, in objective 2 we used the ultrasound strain measurement technique validated in Aim I to assess cyclic strains in the region proximal to the stenosis pre-operatively and at day 27 after focal stenosis. We then collected arterial tissues for histological analysis at day 28. In this objective, we demonstrate that when we create a focal stenosis in our mouse model, we induce the formation of IH and the complete occlusion of ~30% of arteries by 28 days post-surgery. In this objective, we discovered that there was a relationship between cyclic strains measured at day 27 and IH at day 28. The existence of this relationship suggests that changes in mechanical strain may play an underlying role in the processes that eventually result in IH formation.

1.5 Specific Aim III: Determine if changes in oscillatory wall strain precede formation of intimal hyperplasia in vivo

In Specific Aim II, we provide evidence that a relationship exists between acute changes in mechanical strain and IH formation at the ~1 month time point in our murine model. In the final
objective of this dissertation, we seek to more clearly define this temporal relationship and to
delineate exactly when both cyclic strain and IH formation occur in this model

1.5.1 Objective 3A: Determine the temporal progression of wall strain in the
data stenosis model

In the objective 3A experiment, we use data collected from the animals in Objective 2 to
aves changes in strain at just 4 days post-op. Regional reductions in oscillatory wall strain
were quantified at day 4 and compared to histological outcome at day 28. Relationships
between early changes in mechanical strain and eventual IH

1.5.2 Objective 3B: Determine the temporal progression of intimal
hyperplasia in the focal stenosis model

In objective 3B, we will use and additional in vivo study in our mouse model of intimal
hyperplasia. In this study, IH was assessed just 4 days after creation of the focal stenosis to both
validate that strain reductions occur and to determine if morphological changes or IH are
visible at day 4.
2 Background

2.1 Introduction

2.1.1 The cardiovascular system
The cardiovascular system serves as the primary nutrient and waste transport system in the body. It is responsible for carrying oxygen, nutrients and hormones to the body’s tissues and removing carbon dioxide and other wastes. The cardiovascular system is composed of two primary components: the heart and the blood vessels. The heart is an electromechanical pump which continually beats throughout a person’s life. With each beat, the heart creates pressure gradients which in turn force blood into the vascular system.\(^45\)

The vascular system is a system of tubular blood vessels which transfer blood to and from the body’s tissue with each heartbeat. Although often simplified and considered a system of pipes that transports blood, the vasculature is a complex, living and changing system that varies considerably in the body in different anatomic locations. Blood vessels are composed of three different layers, each of which functions to maintain vascular homeostasis.

2.1.2 Vascular structure
The outermost layer of the blood vessel is the adventitial layer. This layer primarily functions to reinforce the vessel and to anchor it to surrounding tissues. The adventitia is composed mostly of interwoven collagen fibers and is infiltrated with fibroblasts.\(^46\) The high collagen content of the adventitia plays a role in preventing vascular rupture at high pressures.\(^47\) Additionally, in larger blood vessels, the adventitia can contain a network of smaller blood vessels (the vasa vasorum) which supply nutrients to the vessel wall, which is too distant to obtain nourishment from the vessel lumen.\(^45\) The vasa vasorum is seen primarily in larger mammals and is not found in small mammals such as mice.\(^46\)
Just inside of the adventitial layer is the external elastic lamina, which is a fenestrated, circumferentially oriented sheet of elastin. Throughout the medial layer of the blood vessel, these elastic laminae occur alternately in the radial direction with layers of vascular smooth muscle cells (SMCs) and proteoglycan rich extracellular matrix (ECM). Thin fibers of elastin intersect the SMC layers connecting the lamina and forming a continuous three dimensional network throughout the vessel. Although the number of lamina in the arteries of a given animal may vary greatly throughout the vascular system, the average tension per lamellar unit falls into a surprisingly narrow physiological range, suggesting that lamellar tension is physiologically maintained across vessels of different sizes and even across many mammalian species. This concept is further supported by studies demonstrating that removal of SMC function in the adult artery does not reduce the static mechanical properties of the vessel. This suggests that the mechanical properties of the vessel are mainly due to the collagen and elastin fibers in the media and are not due to SMC muscle tone.

On the inside of the innermost elastic lamina, or internal elastic lamina, is the intimal layer of the blood vessel. In the healthy artery, the intimal layer is typically formed of a monolayer of endothelial cells which produce and attach to a basal lamina that is supported by the internal elastic lamina. In some larger animals, including humans, the healthy intimal layer also contains some SMCs in addition to the underlying basal lamina. Although the intimal layer does not contribute significantly to vascular mechanics, it does play an important role in maintaining vascular homeostasis. The intimal layer forms a barrier between the blood and the underlying basal lamina. Denudation of the endothelial layer results in local protein adsorption from the blood stream and can eventually result in thrombosis. In addition to its barrier
function, the intimal layer also plays an important role in mechanosensation of flow through the blood vessel and provides molecular cues that control SMC tone in the medial layer.\textsuperscript{58-61}

The layers of blood vessels can be clearly observed using histological staining techniques and biomicroscopy. Masson’s trichrome stain differentiates the layers of the arterial wall clearly by staining the elastic lamina bright red, the vascular smooth muscle cells red, the collagenous fibers in the adventitia blue and the nuclei black. Figure 2-1 shows a Masson’s Trichrome stain of the healthy murine carotid artery. Although all blood vessels are composed of adventitial, medial and intimal layers, the size and structure of these three layers vary considerably throughout the vascular system. The vascular system can be broadly divided into 3 separate types of blood vessels: arteries, capillaries and veins. Arteries are the first conduits in which blood travels as it exits the heart. Arteries are typically large, muscular vessels which are conditioned to withstand the high, pulsatile pressures created by the beating heart. The largest artery, the aorta, is the first vessel which comes off of the heart. As blood travels through the vasculature from the heart, it is branched into arteries and arterioles, which eventually branch into capillaries. Capillaries are typically formed of only a monolayer of endothelial cells and allow for nutrient transport to the surrounding tissues. After leaving the capillaries, blood flows into the venules and then into the larger veins before returning to the heart. Given that blood pressures and flow rates through the different types of blood vessels differ considerably throughout the vascular system, the relative compositions of the three different vascular layers also vary by vessel type.
Figure 2-1: Structure of the healthy artery. (A) Schematic of the different layers of the blood vessel. (B) Masson's trichrome stain of a full carotid arterial cross-section in a healthy adult mouse carotid artery. (C) A high magnification image of the arterial wall showing the internal (black arrow) and external (green arrow) elastic laminae. (D) Immunohistochemical stain of a full mouse arterial section stained for endothelial lining (CD-31, red), smooth muscle cells (myosin heavy chain, green), and nuclei (Hoechst 33342, blue) L represents the vessel lumen, M represents the medial layer and A represents the adventitial layer. (E) 100x magnification image of vessel wall from D.
2.1.3 Mechanical forces on the vessel wall

In addition to having a structure targeted at withstanding mechanical forces in their respective locations in the body, many blood vessels also actively react to changes in blood pressure and flow to maintain homeostasis. In particular, blood vessels have been shown to sense and react to two different forces: hemodynamic luminal shear stress and wall tensile stress. Figure 2-2 shows the major forces applied to the blood vessel.

Hemodynamic shear stress is the shear force applied to the endothelial cells in the vessel lumen by the flow of blood through the vessel and can be computed using Equation (2-A). The majority of research assumes that the blood has a constant viscosity and therefore can be considered as a Newtonian fluid.\(^{62-69}\)

\[
\tau(u) = \mu \frac{du}{dy} \tag{2-A}
\]

Using advanced mechanical modelling techniques, the non-Newtonian properties of blood can be included in shear stress analysis. These techniques demonstrate that Newtonian assumptions can grossly underestimate average wall shear stress on the wall and overestimate the maximum shear stress.\(^{70}\) Despite this evidence, limitations in data available in most in vivo studies force most researchers to continue using Newtonian fluid assumptions.

Vascular endothelial cells are equipped to sense and respond to the luminal shear forces applied to them. A number of cell surface receptors, ion channels and the cell cytoskeleton have been shown to specialize in mechanotransduction of wall shear.\(^{71}\) Under normal physiological conditions, steady laminar blood flow generates, on average, a shear stress of approximately 15 dynes/cm\(^2\).\(^{72}\) When sensing this physiological level of shear, endothelial cells release nitric oxide
and other chemical mediators which maintain medial SMCs in a quiescent state and promote vasodilation of the vessel wall.73

Similarly to hemodynamic shear stress, tensile stress in the vessel wall has also been shown to be maintained homeostatically in the healthy artery.74 Tensile stress is most simply defined as the force per unit cross-sectional area acting on a given surface. In three dimensions, stress is represented as a 2nd order tensor with 6 independent stress components representing the force per unit area on the sides of a volume element. In the arterial wall, this tensor is typically defined in cylindrical coordinates with 3 normal components and 3 shear components. The normal components of arterial stress are circumferential (σθθ), longitudinal (σll), and radial (σrr) stress and the shear components are σrθ, σlθ, σlr. (Normal stresses are shown in Figure 2-2) Under a given mechanical stress, the blood vessel will deform until the forces within the vessel reach equilibrium.

Mechanical strain is a measure of the distortion of the vascular wall. Although there are a number of mathematical definitions of strain,75 the most commonly used definition in the literature for blood vessels is the Green (Lagrangian) strain tensor. For this reason this definition will be used throughout the rest of this dissertation. Like wall stress, strain is also a 2nd order tensor with 6 independent components. Strains in the vessel wall due to the internal blood pressure are limited to the strains in the normal directions: radial strain, longitudinal strain and circumferential strains. Although others have assessed radial76 and longitudinal77 strains, experimental data77 shows that circumferential strains in blood vessels are larger than the other strain components. For this reason, we focus solely on circumferential strains for the remainder of this dissertation. In Green’s definition of strain, the deformation of a body is
determined as the change from a reference configuration to a final configuration using the starting configuration as the reference point. Optimally, the reference configuration for strain is defined as the point where the body is under no stress. Defining this configuration in the artery is complicated by the fact that arteries under no external forces still having residual stresses internally.\textsuperscript{78} This is further complicated \textit{in vivo} where the external forces (i.e. blood pressure) are never zero. Therefore, in this dissertation, we will measure oscillatory wall strain. This strain is defined using the vessel at diastole as a reference configuration and the vessel at systole as the final configuration. Although this definition provides no information about the absolute strain state of the vessel wall, it does measure how much strain is being applied to the vessel wall cyclically with each cardiac cycle and is easily measured \textit{in vivo}.

Since wall stress and wall strain are intimately related through the mechanical properties of the vessel wall, wall strain has consequently been implicated as a mediator of vessel homeostasis in addition to wall stress.\textsuperscript{33, 79} \textit{In vitro} studies have demonstrated that both vascular SMCs and vascular endothelial cells respond to changes in cyclic stress/strain independently of wall shear stress.\textsuperscript{80} This suggests that shear stress and strain mechanosensation pathways independently contribute to changes in wall composition seen during neointima formation. Together with shear stress mechanotransduction mechanisms, the vascular wall is able to maintain a homeostatic level of internal pressure and luminal flow.
Chapter 2: Background

2.2 Vascular disease

In this dissertation, we aim to unravel relations between vascular mechanics and intimal hyperplasia. In order to fully describe the importance of these relations, we must first examine the known relationships between vascular mechanics, IH and vascular disease currently described in the literature. In the following sections we will discuss these relationships.

2.2.1 Relation between intimal hyperplasia and vascular disease

Despite extensive research and funding, cardiovascular diseases remain the number one cause of death in first world countries.¹ Although a number of pathologies underlie cardiovascular diseases, many pathologies begin with occlusion of the arterial lumen via arteriosclerosis. Pathologies affected by arteriosclerosis include myocardial infarction, stroke,
and many others. Arteriosclerosis is characterized by the progression from intimal thickening, to inflammatory response, and finally to the formation of fatty streaks and atherosclerotic plaques.\textsuperscript{3} One of the major factors that initiates the formation of atherosclerotic plaques is IH,\textsuperscript{3} and a number of researchers have demonstrated that mechanical forces underlie the propagation of intimal hyperplastic lesions.\textsuperscript{14, 26, 81-85}

Intimal hyperplasia (IH) can be defined as the proliferation of cells in multiple layers internally to the internal elastic lamina. Further, IH cells are observed to express αSMA either permanently or temporarily.\textsuperscript{3} Others add that the term IH should be reserved for tissues that are both proliferative and without common orientation or clear organization. Cells in IH tend to be spread out with abundant matrix and few, if any, collagen or elastin fibers.\textsuperscript{13} Although IH is typically considered to be a completely pathological response, there are some cases in which IH occurs during healthy physiology. The most commonly referenced cases of this non-pathological IH are the closing of the ductus arteriosus and the closure of umbilical arteries.\textsuperscript{86} Others also report a benign form of IH in the healthy artery. This benign IH has a similar appearance to that of pathological IH but does not significantly reduce lumen area or flow.\textsuperscript{53, 54} Given that the underlying causes of IH and the relation between pathological and benign IH remain unclear, the connection between IH and disease is an important field of study with widespread impact on vascular disease.

\textit{In vivo}, IH is a precursor and the primary underlying cause for a number of vascular diseases and the subsequent failure of many vascular surgical interventions. Atherosclerosis, the stiffening and stenosis of the artery due to plaque formation, has been shown to occur in regions of preexisting intimal hyperplasia.\textsuperscript{87} Atherosclerotic plaques can go on to cause luminal
narrowing, and eventually the total blockage of the arterial lumen. Blockage of blood flow results in distal organ ischemia, and eventually cell death. This IH-initiated pathway has been shown to contribute to a number of diseases including stroke, coronary artery disease, peripheral artery disease, and restenosis after a number of clinical interventions. The following sections of this dissertation will delineate the current literature that relates mechanical forces to biological outcome in intimal hyperplasia.

2.2.2 Relation between Wall shear stress and vascular disease

Blood flow through the healthy artery in vivo is typically laminar and maintains a time-averaged shear stress on the vascular endothelial cells of 10-15 dynes/cm² (1-1.5 Pascals). Regions of low wall shear stress and oscillatory flow have been positively correlated with IH in vitro and in vivo, while high levels of laminar blood flow (shear stress) results in decreased IH formation. Additionally, increases in blood flow in vessels with preexisting IH can induce regression of IH. Although high blood flow is typically considered atheroprotective, extremely high shear stresses have also been associated with IH formation.

Given that, in the arterial circulation, shear stress constantly changes as the heart cycles between diastole and systole, it is necessary to clearly define what “low shear stress” means. Typically, low shear stress in the arteries refers to shear stress with a time average magnitude of less than about 10 dynes/cm² but can refer to larger or smaller values depending on the arterial conditions or species. A number of in vitro studies have demonstrated that endothelial cells can sense and respond to changes in hemodynamic shear stress. Of the many biological changes caused by changes in hemodynamic shear stress, the most commonly cited is the nitric oxide (NO)/endothelial nitric oxide synthase (eNOS) pathway. In vitro, it has
been demonstrated that increases in applied shear stress results in increases in NO production in arterial endothelial cells.\textsuperscript{104} NO has also been shown to prevent vasoconstriction of vessels in the microcirculation suggesting that it both causes vasodilation and reduces the effects of vasoconstrictors.\textsuperscript{105} Colotti \textit{et al.} demonstrated that treatment of IH-prone vessels with nitric oxide (NO) reduces IH production in an \textit{ex vivo} model.\textsuperscript{106} Further, \textit{in vivo} studies have demonstrated that local release of NO can reduce intimal hyperplasia in both the IH-prone artery and the stented artery.\textsuperscript{107, 108}

Although extensive research has demonstrated the importance of the NO/eNOS pathway in effecting vasodilation after changes in wall shear stress, the mechanism by which the endothelial cells actually sense shear stress still remains unclear.\textsuperscript{109} One hypothesis to date involves mechanosensation of fluid shear via torque applied to the endothelial glycocalyx. The glycocalyx is a carbohydrate rich layer of molecules that lines the outside of the cell membrane.\textsuperscript{45} A number of recent studies suggest that endothelial sensation of torque on the glycocalyx results in changes in cell membrane permeability which therefore results in release of NO molecules.\textsuperscript{109-111}

\textbf{2.2.3 Relation between wall tensile stress and strain and vascular disease}

In addition to the wall shear stress applied to endothelial cells, the vessel wall is also cyclically exposed to mechanical strain and stress in both the circumferential and longitudinal directions with each cardiac cycle.\textsuperscript{112} Although mechanical strain is fundamentally different from mechanical stress, the close relationship between the two parameters makes it difficult to separate their effects.
Although this stress is applied to the endothelial cells of the intima, the majority of the stress is supported by the SMCs of the medial layer. Although the exact mechanisms of stress mechanotransduction remain unknown, a plethora of research demonstrates that stress and/or strain mechanotransduction plays a role in vascular disease. Evidence shows that increases in intraluminal pulse pressure (and therefore stress and strain) result in compositional changes in the blood vessel wall. Additionally, excessive mechanical stretch can result in SMC hypertrophy, localized inflammatory reactions and degradation of wall structural elastin and collagen. All of these changes could contribute to changes in wall structural properties.

Reductions in mechanical stretch also yield changes in SMC phenotype. One study demonstrated that reducing circumferential strains applied to arteries in vitro resulted in SMC proliferation and increased production in inflammatory cytokines. These correlations suggest that vascular cells sense changes in both shear stress and tensile stress (and/or strain) and react to changes in their mechanical environment.

Some research suggests that the arterial wall actively maintains a homeostatic level of wall stress. This theory is supported by the observation that the number of lamellar units in any given artery is proportional to blood vessel diameter across a number of mammalian species. By maintaining this relationship, the vascular wall keeps the mechanical tension per lamellar unit within an amazingly narrow physiological range.

Compared to the well-established effect of shear stress, the impact of wall strain on the development of intimal hyperplasia is much less defined, and thus the focus of the current proposal. From a mechanical standpoint, the artery can be considered a composite structure with three mechanically unique layers. Given that the intima in the healthy artery is typically

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only one cell layer thick, it is generally considered to not contribute to healthy arterial wall mechanics.\textsuperscript{119, 120} Previous studies have examined the different contributions of the arterial wall layers to wall mechanics in both the healthy\textsuperscript{120} and diseased arteries.\textsuperscript{121} These studies demonstrated that the material properties vary significantly amongst the three layers\textsuperscript{120} and that the mechanical properties of the intimal layer can contribute significantly to mechanical properties after pathological neointima formation has occurred.\textsuperscript{121}

Smooth muscle cells are the most prominent cell type in the blood vessel and therefore are the cells that are affected most by cyclic strains. A number of studies have demonstrated relationships between oscillatory strains and biological changes in vascular SMCs. In vitro studies have demonstrated that oscillatory strains result in increases in SMC synthesis of collagen\textsuperscript{122-124} and elastin as well as glycosaminoglycans (GAGs) such as versican and hyaluronic acid.\textsuperscript{14, 80, 125} Further, studies have shown that the magnitude of ECM and GAG synthesis by SMCs are dependent upon strain magnitude, strain frequency and time in culture.\textsuperscript{80} Recent ex vivo studies have proposed that mechanical strain works independently of the wall shear stress to cause intimal hyperplasia.\textsuperscript{126, 127} Cyclic strain has been shown to cause structural and compositional changes in both the endothelial and smooth muscle cells of vein grafts.\textsuperscript{128, 129} Evidence in vivo suggests that wall strain correlates positively with intimal thickening.\textsuperscript{73, 130} In the cell culture setting, mechanical stretch activates several pathways capable of regulating smooth muscle cell phenotype,\textsuperscript{131, 132} but biologic information in the in vivo setting is limited. This dearth of mechanistic knowledge into links between strain and intimal hyperplasia is largely the result of limited capabilities in the measurement of strain in the intact animal.
2.3 Techniques for measuring vascular mechanics in vivo

Although a large quantity of research has been completed to characterize in vitro cellular reactions to mechanical stresses and strains, true clinical relevance is best realized through in vivo research in animal models and in the clinic. A major hurdle that must be overcome is the need to accurately and consistently measure mechanical stresses and strains in vivo. Optimum measurement of mechanical stresses and strains in vivo would include non-invasive approaches that have limited effects on animal physiology. The problems associated with in vivo studies are exacerbated in small animal models of vascular disease, such as the mouse, because of the small size of rodent vasculature. In this section, we will discuss different methods that are used to measure mechanics in vivo in both animal models and humans.

2.3.1 Measurement of wall shear stress

Wall shear stress measurements in vivo are typically made using either MRI or ultrasound-based of blood flow through the blood vessel. Ultrasound approaches use either transit time of an ultrasound pulse through the vessel to determine bulk blood flow velocities\textsuperscript{133} or use the Doppler effect to determine local average blood velocities\textsuperscript{134}. Others utilize phase-contrast MRI-based measurements to estimate wall shear stress from flow rates.\textsuperscript{135} Of these two methods, ultrasonography provides both higher spatial and temporal resolutions.\textsuperscript{136}

The simplest approach to measuring flow velocities using ultrasonography is the use of transit-time flowmeters to measure bulk flow through the blood vessel. Transit-time flowmeters are limited in that they require that the vessel be exposed in order to measure the flow through the vessel. From bulk flow measurements, it is possible to estimate shear stress at the vessel wall using equation (2-B) below where $\tau$ is shear stress, $\mu$ is the dynamic velocity of blood, $Q$ is the volumetric flow rate, and $d$ is the vessel’s diameter:

\begin{equation}
\tau = \frac{6 \mu Q}{d^2}
\end{equation}
This formula assumes that the blood has a fully developed, laminar and parabolic flow profile. Additionally it assumes that the blood has constant dynamic viscosity (typically ~3.3 cP) and that the vessel has a constant, circular cross-section. Although these assumptions may be accurate enough to calculate time average shear stresses in a healthy blood vessel, they vastly underestimate the shear stresses due to pulsatile flow and non-laminar flow profiles.

Continuous wave Doppler ultrasound has been used extensively to quantify changes in blood flow in humans for over 30 years. This approach, based on the Doppler Effect, continuously transmits and receives sound waves through the blood vessel wall. The frequency shift of the sound waves received by the transducer compared to those sent are related to the velocity of blood flow through the vessel. The primary limitation of this approach is that it provides no information about where in the ultrasound field of view the measured flow is occurring. Additionally, it is not possible to simultaneously measure continuous-wave ultrasound data and collect ultrasound images, making it even more difficult to interpret flow measurements.

In order to overcome some of the issues associated with continuous-wave Doppler ultrasonography, pulsed-wave Doppler ultrasonography was developed. Pulsed wave Doppler ultrasonography differs from continuous-wave ultrasonography only in that brief pulses of ultrasound wave are emitted. Based on the timing of these pulsed waves and the known speed of sound in tissue, pulsed-wave Doppler made it possible to determine the location of flow velocities. This finding also allows for the use of duplex ultrasound where Doppler velocity

\[
\tau = \frac{32\mu \cdot Q}{\pi \cdot d^3} \quad (2-B)
\]
flows and B-mode ultrasound images are simultaneously collected. Even with pulsed-wave Doppler ultrasonography, the method by which shear stress is calculated is limited by a number of assumptions. With both pulsed-wave Doppler, wall shear stress is typically computed based on the maximum velocity ($V_{\text{max}}$):

$$\tau = \frac{4 \cdot \mu \cdot V_{\text{max}}}{d}$$

(2-C)

This formula once again makes the same assumptions as that used for transit-time flowmeters. As a result, it is prone to substantial errors that may cause it to underestimate differences in regional shear stress.\textsuperscript{140} Despite these limitations, a number of clinical studies utilize Doppler ultrasound shear stress calculations for both research and diagnosis.\textsuperscript{141-143}

2.3.2 Measurement of wall tensile stress

Unlike hemodynamic shear stress, tensile stress in the vascular wall cannot be measured based on blood flow. Rather, the most relevant physical method of estimating tensile stress in the vascular wall is through use of pressure measurements. The most simplistic approach to estimating wall stresses is the use of Laplace’s law and assuming the artery is a thin walled cylindrical pressure vessel with constant thickness and homogeneous mechanical properties.\textsuperscript{36, 144}

To better estimate wall stresses, it is often necessary to constitutively model the blood vessel.\textsuperscript{78, 145} Although constitutive modelling is not a direct method of measuring wall stresses \textit{in vivo}, it can be made to be patient specific by combining 3D imaging (such as MRI or CT scans) with blood pressure measurements. Combining these two measurements into one computer model can provide data about mechanical stresses for a specific artery.\textsuperscript{146, 147} Given that
computational modelling is time consuming, and requires that a number of assumptions be made about arterial mechanics, it may not be the best in vivo measure of wall mechanics.

2.3.3 Measurement of wall strain

Wall tensile stress is directly related to tensile strain in the vessel wall through the mechanical properties of the arterial wall. Given that a number of studies have demonstrated that the mechanical properties of the arterial wall can be changed in the diseased state, it is reasonable to assume that there will be changes in in vivo wall stresses and strains due to changes in wall mechanics. Relative to measuring wall tensile stresses in vivo, measurement of wall strains is comparatively easy. In order to measure wall strains, it is generally necessary to define a zero strain state from which strains will be measured. Ideally, the zero strain state would be defined with the vessel under zero pressure. Since it is not possible to measure the arterial diameter under zero pressure in vivo, the zero strain state for most in vivo studies is typically defined as the state of the vessel at diastole.

A number of approaches have been used to measure wall strains in vivo. In humans, MRI, and both external and intravascular ultrasound have all been used to estimate oscillatory wall strains. MRI has been used in humans to measure circumferential strains in both the carotid artery and the descending thoracic aorta. Others have reported using MRI to measure carotid strains in the mouse, but EKG gating must be used to overcome temporal resolution limits.

Ultrasound has been used in humans to assess mechanical strains in a number of studies. In the majority of these studies, speckle tracking algorithms are applied to longitudinal B-mode images of arteries and displacements in each direction are computed. From these displacements,
average strains can be determined. Use of longitudinal B-mode views provides robust data for accurate calculation of average strains but does not allow for regional strain measurement. Others have used external ultrasound to measure cross-sectional diameters in humans and estimate strains based on diameter changes. Use of cross-sectional ultrasound images allows for the measurement of circumferential strains in a specific arterial region but is hindered by changes in probe angle.

In rodents, measurement of vascular strains is more difficult due to the small size and fast heart rates of mice and rats. These conditions require methods with significantly higher spatial and temporal resolutions then is necessary in humans. Goergen et al. reported using MRI to measure aortic strains in the mouse with sufficient spatial resolution and using EKG gating to overcome temporal resolution limits. Others have reported using longitudinal ultrasound images to measure strains in the mouse. Newer ultrasound systems used primarily in the cardiac literature now report spatial and temporal resolutions that are sufficient to accurately image cardiac cycles in the mouse. As discussed in section

2.4 Summary
A plethora of studies have demonstrated that vascular cells respond to changes in their mechanical environment and that these changes in mechanical environment can induce pathological changes in the vessel wall. Although the majority of previous work focuses on endothelial mechanosensation of wall shear stress, evidence suggests that changes in wall shear stress alone do not fully explain the phenomenon of neointimal formation. Herein, we focus on the relationship between cyclic deformation of the arterial wall and the eventual intimal hyperplastic outcome in a murine model. By defining
a relationship between noninvasively determined oscillatory wall strains and intimal
hyperplasia, we will develop a better understanding of how changes in arterial mechanical
environments relate to vascular disease.
3  **Objective 1A: Validation of ultrasound strain measurement**

3.1  **Introduction**

Ultrasound has emerged as a tool to enhance understanding of vascular diseases in both the clinical and research setting. One of the most commonly employed models is the murine model, due to its well-defined genome and relatively low cost. Recently, high-frequency ultrasound imaging systems (e.g. Vevo 2100, FUJIFILM Visualsonics, Toronto, ON, Canada) have been developed to noninvasively micro-image mice in vivo, and such approaches can reliably detect and measure the mouse aortic diameter in vivo. At the same time, speckle-tracking technology in ultrasound imaging has emerged as a quantitative, objective technique to accurately evaluate myocardial function and dynamics. Speckle tracking functions by tracing the displacement of speckles during the cardiac cycle to measure strain and strain rate, and it has been used in the assessment of mouse models of cardiac dysfunction. In this chapter, we aim to validate a novel system for measuring wall strains noninvasively using an in vitro phantom model.

A number of previous studies have employed in vitro phantoms of the artery to test novel ultrasonic measurement methods for both flow and a number of vascular disease analyses. Although these different phantoms vary in complexity, the simplest of them are composed of a piece of tubing suspended in a hydrogel solution. A pressure gradient or cyclic flow waveform is created within the tube which can be used to evaluate the ability of ultrasound to measure flow or other properties within the vascular phantom. More complex phantoms include highly sophisticated pulsatile flow pumps and harvested blood vessels with real blood flow through the lumen. Since the goal of this objective is to demonstrate that ultrasound strain measurements using our novel approach have similar results to those found using other
methods, we will use an extremely simple silicone tube model of a blood vessel with precisely defined dimensions.

To provide a benchmark measurement for the strains measured using ultrasound, we will employ a high speed camera system with telecentric lenses to simultaneously measure the strain of the tube. Telecentric lenses are widely used in machine vision to measure the dimensions of products during manufacturing. In a typical camera lens system, the magnification of the system is modified significantly due to changes in focus setting and distance of the lens from the object. This property of traditional camera lenses can distort images recorded with a camera causing images to inaccurately represent the size of objects in the camera field. One approach to overcoming lens distortion is to use telecentric lens systems. Telecentric lenses create nearly orthographic projections of objects being imaged in the image plane. This means that objects that are in focus and those that are out of focus will still be projected at their actual size. Telecentric optics creates a distinct advantage in optical measurement methods because they minimize measurement error due to the perspective distortion. In conjunction with telecentric lenses, we used high density mapping (HDM), an optics-based measurement approach that we previously developed to determine regional displacements in a number of biological tissues. In this objective, we will first demonstrate the accuracy of HDM by comparing HDM strain measurements with sonomicrometry, then validate our ultrasound-based method against HDM.

3.2 Materials and methods
  3.2.1 Validation of High Density Mapping against sonomicrometry
Sonomicrometry has long been considered a gold standard for distance measurement in medical displacement and strain analyses. To test the ability of HDM to measure strains, two
sonomicrometry crystals were attached to a 2 inch piece of ¼ inch I.D. Penrose tubing (Penrose drain, Cardinal Health, McGaw Park, IL). This tubing was attached to a syringe pump and oscillatory pressure gradients were created in the closed system to simulate oscillatory wall strains. Displacements were simultaneously measured via two 2mm sonomicrometry crystals sutured to the tubing surface (Triton, San Diego, CA) and a high speed camera (Fastcam 1280pci, Photron USA, San Diego, CA) connected to a telecentric lens (486mm precision telecentric lens, Computer Optics Inc., Hudson, NH). Displacements from the optics system were computed using HDM\textsuperscript{181} and oscillatory wall strains were computed using both methods. Average strain magnitudes across several cycles were calculated. Simultaneously recorded strains from the cycles were compared using a paired t-test. A sample image used for HDM analysis is shown in Figure 3-1.

![Figure 3-1: Comparison of sonomicrometry and HDM strains. Two sonomicrometry (sono) crystals were sutured to the wall of Penrose tubing. HDM speckle tracking was used to simultaneously determine strains.](image_url)
3.2.2 Development of an in vitro model of pulsatile pressure

Given that ¼ inch Penrose tubing is much larger than the size of blood vessels analyzed in the remainder of this dissertation, ultrasound-based measurement of strain was validated using a small diameter silicone tube. Given that the smallest available sonomicrometry crystals are larger than our tubing phantom, validation of ultrasonography was completed using HDM and not sonomicrometry. In order to complete this validation, we first aimed to create an in vitro model of arterial pulsatile pressure. Using similar methodology to previous ultrasound vascular phantoms, a small diameter (1.473 mm I.D.; 1.956 mm O.D., SMI Manufacturing, Saginaw, MI) silicone tube was securely attached to two 1/8 inch barbed connectors. One side of the tubing was connected to a 60 mL syringe (Figure 3-2). The tubing was cleared of air bubbles and the system was closed using a luer valve. The tubing was speckled using a 50:50 mix of silicon carbide microparticles (Silicon carbide 400 grinding compound, Alfa Aesar, Ward Hill, MA) and reflective paint (Scotchlite Reflective Ink 8017, 3M, St. Paul, MN) to create a unique light intensity distribution across the tubing surface. The tubing was secured to a custom optics rig and covered with optically clear ultrasound gel (Aquasonic Clear, Parker labs, Fairfield, NJ). A large glass coverslip was pulled back against the ultrasound gel and bubbles were removed using cotton swabs yielding a flat, undistorted view of the tubing. A telecentric lens and camera were then attached to the optics rig and the camera axis was aligned perpendicular to the coverslip.
Figure 3-2: Ultrasound validation experimental setup. A telecentric lens attached to a high speed video camera was used to make diameter measurements on a murine blood vessel-sized tube in vitro. Simultaneously, strains were measured using the proposed ultrasound measurement system. The resulting circumferential strains were compared between the two measurement methods.

The 60 mL syringe was then connected to a syringe pump and controlled via a computer running pump system software (New Era pump systems, Farmingdale, NY). Cyclic strains were applied to the tubing wall at flow rates of 6, 8, 10, 12, 14 and 16 mL/min for periods of 1, 1.5, 2, 2.5 and 3 seconds. While running each protocol, both ultrasound and video data were simultaneously recorded as described below.
Figure 3-3: Close-up of ultrasound validation tubing setup. A large glass coverslip is used to create an optically flat surface behind which resides the ultrasound gel. This allows for clear, undistorted imaging of the tubing wall which has been pre-speckled to create a large light intensity distribution.

3.2.3 Ultrasound data acquisition and analysis

A 700 MHz ultrasound probe (MS700) and FUJIFILM Visualsonics Vevo2100 ultrasound system were used to record luminal wall strains within the tube. The ultrasound transducer was placed in the ultrasound gel and the tube lumen was visualized and brought into focus. Several consecutive cycles were recorded at a frame rate of 300 Hz. Cyclic mechanical strains as measured at the lumen of the vessel using Green’s definition were calculated and reported. Given that strains measured via ultrasound are 1 dimensional only, the stretch ratio was computed and green’s strain was calculated via equation (3-A). Cumulative Green’s strains were computed for each cycle and averaged for at least 5 cycles in each dataset.
3.2.4 Video data acquisition and analysis

A digital camera (EOS 7D, Canon USA, Melville, NY) was attached to the telecentric lens and the tubing was brought into focus. Videos were recorded at maximum resolution and 30 frames per second simultaneously with the ultrasound system. High Density Mapping, a speckle tracking program previously developed in our lab along with custom MatLab programs was used to calculate strains on the outside of the vessel using Green’s definition. Briefly, HDM uses a phase correlation algorithm in 2 dimensions to efficiently determine displacements of regions of an image. As seen in Figure 3-4, small 16 by 16 pixel size sub-regions are compared between two sequential images and combined via phase correlation in the Fourier domain. After subsequent application of an inverse Fourier transform, it is possible to determine the pixel level displacement of the region. Using the sub-pixel algorithm first described by Foroosh et al., it is then possible to determine the displacement of the region with an accuracy of 0.09 pixels and a precision of 0.02 pixels. This process is iterated across all sub-regions (overlapping by 8 pixels) and images in a dataset to produce displacement fields. From these displacement fields, the displacement gradient tensor is determined and cumulative green’s strain is computed (Figure 3-5). For this study, only the normal plane strain in the circumferential direction is reported.
Chapter 3: Objective 1A

3.2.5 Theoretical relationships
Since commercially available tubing in the size range targeted in this study was limited, the tubing used herein had a considerably thick wall compared to its radius. For this reason, thin-walled tubing mechanical assumptions could not be used. To better understand the expected relationship between strains measured on the inside wall of the tubing (ultrasound) and strains measured on the outside (HDM), the theoretical relationship was determined mathematically.

3.2.6 Statistics
Statistical analyses were executed using Sigmaplot 11 (Systat Software, San Jose, CA). The resulting ultrasound and image strains for each pump condition were compared using a Pearson product moment correlation. Statistical differences between sonomicrometry and HDM strains were determined using a paired t-test. Statistical powers are reported for all negative statistical results.
3.3 Results

3.3.1 Comparison of sonomicrometry and optical strain measurements

Mechanical strains generated on Penrose tubing wall by a syringe pump were

simultaneously recorded via a high speed video camera and sonomicrometer. Strains computed

using HDM (from the high speed video) and sonomicrometer were averaged across 7 cycles.

Data are presented as mean ± std and statistical comparisons and a paired t-test yielded no

significant difference between the two methods (p=0.39, Power: 0.05). The resultant strain

values are presented in Figure 3-6.

![Figure 3-6: Simulated wall strains in Penrose tubing](image)

Data show mean oscillatory wall strain as computed via sonomicrometry and high density mapping for 7 simultaneously recorded cycles. No significant difference between the two groups was seen using a paired t-test.

3.3.2 Comparison of optical and ultrasound strain measurements

Although strain measurements were recorded at 4 different flow rates for 5 different

amounts of time, upon analysis, usable strain recordings were only found using HDM for the 8
combinations that produced the largest strains. Thus, data reported below is for flow rates of 10, 12, 14 and 16 mL per minutes and flow times of 2.5 and 3 seconds only. For the selected 8 combinations, clear strain curves were computed using both HDM and US. Sample regions of interest and curves for each method in Figure 3-7: Sample regions of interest and strain curves.

Peak strains for corresponding recordings from both HDM and ultrasound were averaged across at least 5 cycles and plotted with ultrasound strain on the x-axis and HDM strain on the y-axis (Figure 3-8).

**Figure 3-7: Sample regions of interest and strain curves.** A) Ultrasound region of interest showing tubing lumen with selected ultrasound tracing and resulting strain curve. B) HDM images with region of interest (red) sub-image size (green square) and sub-image shift (blue square).
Figure 3-8: Comparison of wall strains recorded with ultrasound and HDM. Note that strains measured with ultrasound were measured at the tubing lumen while strains measured with HDM were measured on the outer wall. Line indicates the linear regression of the data.

As seen in Figure 3-8, strains measured via HDM were significantly lower than those measured with ultrasound. Despite this difference, comparison of the data from the two methods yielded a Pearson’s product moment correlation coefficient of 0.83. The indicated linear regression of the data resulted in a slope of 0.23 with the linear fit equation (3-B):

\[
E_{\text{HDM}} = 0.23E_{\text{US}} - 0.002
\]  

(3-B)

3.3.3 Theoretical relationships between lumen and outside strains

The tubing used in this study has a thickness that is almost 25% of its inner radius. As a result, it must be considered a thick-walled pressure vessel and its change in thickness cannot be ignored. Since pressures were not measured in this study, the most relevant relationship to our study is the relation of strain at the outer wall (experimentally measured using HDM) to luminal strain (experimentally measured by ultrasound). Since Green’s strain was implemented
for all calculations, Equation (3-A), was used to calculate strain from stretch ratios with E representing Green’s strain and λ representing the stretch ratio.

To determine the relationships between Green’s strain for the inner and outer walls, the wall cross-sectional area (and therefore volume) were assumed to be constant. This assumption yielded the relationship in Equation (3-C) where \( r_o \) and \( r_i \) respectively are the outer and inner radius before stretching and \( R_o \) and \( R_i \) are the outer and inner radii after distension:

\[
\frac{r_o^2 - r_i^2}{r_o^2} = \frac{R_o^2 - R_i^2}{R_o^2} \tag{3-C}
\]

Given that the stretch ratio (\( \lambda \)) is the ratio of the radius after distension to the radius before distension, the following relation between inner and outer strains can be determined. A complete derivation of Equation (3-D) is described in Appendix C:

\[
E_o = E_i \cdot \frac{r_i^2}{r_o^2} \tag{3-D}
\]

Using this ratio, a theoretical strain relationship curve can be determined and compared to the actual data. This curve is shown in Figure 3-9 alongside the experimental data. The resultant theoretical relationship indicated that the strain measured via HDM is theoretically expected to be 0.57 times the strain measured with ultrasound.
3.4 Discussion

In this chapter, we aimed to validate our ultrasonography-based approach to measuring small diameter vascular strains against another method of measuring strains. In order to accomplish this objective, a simple *in vitro* ultrasound phantom of a blood vessel was constructed and placed in a system designed specifically to allow for simultaneous comparison of strains measured with two different methods. Due to our expertise and publication history in the field of optics-based strain measurements, we chose to use our high density mapping software to measure regional displacements on the outside of the tubing wall. To enhance this approach, telecentric optics were employed to minimize perspective distortion.

To demonstrate the capabilities of our HDM-based strain measurement approach, HDM strains were directly compared to those measured via sonomicrometry, a method often used as the gold standard for displacement measurements. Using ¼ inch diameter Penrose tubing, we demonstrated that no difference in oscillatory wall strain values was determined between
sonomicrometry and HDM. Due to the large size (2mm diameter) of available sonomicrometry crystals, we were unable to use sonomicrometry in a direct comparison with our ultrasound-based strain measurement approach. We therefore directly compared HDM with ultrasonography.

The tubing strains that could be produced by our system were primarily limited by the amount of force that could be generated by our syringe pump and the high stiffness of small diameter tubing that was commercially available at the time of this study. As a result of these system limitations, the mechanical strains experimentally measured with both HDM and ultrasound in this chapter were extremely low (<2.5%) and therefore much smaller than those expected in vivo, where reported values are 20-25%.

Despite these limitations, the results presented in Figure 3-8 demonstrate a clear positive correlation between the data measured using the two methods with a Pearson’s correlation coefficient of 0.83. Although the data do correlate, the relationship observed was definitely not 1:1; with strains measured by HDM considerably less than those measured by ultrasound. In order to better understand the relationship we should expect between these two measurements, the theoretical relationship was determined by assuming constant tubing volume. By deriving and applying Equation (3-D), we demonstrated that the theoretical strains measured via HDM at the tubing wall are expected to be smaller than those measured by ultrasound. This theoretical finding supports the experimental findings found in this chapter.

The second factor we suspect may have affected our measurements in this chapter is the lower limit of the spatial resolutions of both the ultrasound and optics based measurement systems. Since the methods used by the ultrasound system to measure displacements are
proprietary, it is not possible for us to determine the spatial resolution of the system. Using an image of a scale with known length, the imaged pixel size for the HDM measurements reported in this chapter is 28.6 μm. The region of interest that was used for HDM analysis was 64 pixels (1830 μm) tall. Given that the reported theoretical displacement accuracy of HDM is 0.09 pixels\(^1\) (2.57μm in this study)\(^,\) the smallest strain that can be accurately determined in this study is 0.14%. This strain level was calculated by dividing the smallest pixel displacement that can be measured by the 64 pixel region height. Since the strains measured via HDM in this chapter are very close to or below this value, the reported HDM measurements are likely not very accurate due to the system’s resolution limits. To overcome these limitations, other methods of measuring strain should be used in the future to validate our ultrasound-based measurement approach. One method that has been used increasingly in medical imaging recently is optical coherence tomography (OCT).\(^2\), \(^3\) OCT uses the same principles as ultrasound imaging, but uses the optical reflective properties of tissues rather than the sonic impedance to determine the tissue. Given that the wavelength of light waves are significantly smaller than that of high frequency sound waves, the resolution of OCT can be as high as 1 μm and some systems have reported temporal resolutions greater than 1 kHz.\(^4\) Future studies should utilize a higher resolution state-of-the-art, such as OCT, to more accurately validate ultrasound-based strains.

Despite the limitations in the system used herein, the strains measured between the two methods correlate well and are on the same order of magnitude as the theoretical values. An improved testing system with the capability to produce larger tubing strains would likely yield an even better correlation between the two methods. Given that strain measurements made
using ultrasound used in the remainder of this dissertation will not be compared between measurement methods, the limited correlation demonstrated in this chapter shows that our ultrasound-based approach can provide usable measurements even when near its resolution limits. In the next chapter of this dissertation, we will apply our ultrasound system to in vivo measurement of arterial strains.
4  **Objective 1B: In vivo testing of ultrasound vascular strain methodology**


4.1 **Introduction**

In Objective 1A (Chapter 3) of this dissertation, we aimed to demonstrate that ultrasound strain measurements can be used to measure strain, as validated with optical measurement techniques, in a small analog of a blood vessel. For Objective 1B, we aimed to test this system on a live, *in vivo* model of vascular disease. For this study, we chose to analyze strains in a murine model of abdominal aortic aneurysm (AAA). The mouse model was chosen primarily because it is extensively used in the study of the mammalian vasculature and the diseases that affect it.

4.1.1 **Aortic aneurysms**

Despite knowledge insights over the last decades, the molecular mechanisms of aortic aneurysmal disease remain obscure. Beyond some known clinical risk factors, biomechanical forces contribute to abdominal aortic aneurysm (AAA) development, progression, and rupture.\(^{190-192}\) The composition and mechanical properties of AAAs are different from those of normal aortas,\(^{193}\) with AAAs being stiffer and less distensible than non-aneurysmal aortas.

4.1.2 **Murine models of aortic aneurysms**

Murine models have emerged as a tool to enhance understanding of aneurysms,\(^{169,170}\) though biomechanical studies have been limited in these models. One of the most commonly employed models involves subcutaneous delivery of angiotensin II (Ang II) into apolipoprotein
E-deficient (apoE/-) mice via an implanted osmotic pump.\textsuperscript{169, 194-197} Despite widespread clinical value, the use of longitudinal imaging in these aneurysm models remains scarce and under-utilized because the small size of the animals make the quantification and characterization of AAA progression difficult and time-consuming. Current methods for measuring aneurysm biomechanics and progression are simplistic in that they assess only the gross appearance of the aorta,\textsuperscript{198} the maximum diameter of the aorta in situ,\textsuperscript{199} or the luminal diameter \textit{ex vivo}.\textsuperscript{200} To date, a few \textit{in vivo} imaging studies have been done on small animal AAA models, but they also only measured aortic diameters.\textsuperscript{38, 201-203} Recently, high-frequency ultrasound imaging systems (e.g. Visualsonics Vevo 2100, Visualsonics, Toronto, ON, Canada) have been developed to noninvasively micro-image mice \textit{in vivo}, and such approaches can reliably detect and measure the luminal diameter of AAA in apoE/- mice infused with Ang II.\textsuperscript{38, 39} At the same time, speckle-tracking technology in ultrasound imaging has emerged as a quantitative, objective technique to accurately evaluate myocardial function and dynamics. Speckle tracking functions by tracing the displacement of speckles during the cardiac cycle to measure strain and strain rate,\textsuperscript{40-43} and it has been used in the assessment of mouse models of cardiac dysfunction.\textsuperscript{173}

\subsection{4.2 Materials and methods}

\subsubsection{4.2.1 Mouse model}

Male apoE deficient mice (B6.129P2-Apoetm1Unc/J) were purchased from the Jackson Laboratory (Bar Harbor, ME), maintained on a 12-hour light-dark cycle, and received water and assigned chow \textit{ad libitum}. All animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee and complied with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 85-23, Revised 1996).
Chapter 4: Objective 1B

At 16 weeks of age, osmotic mini-pumps (Alzet Scientific Products, Model 1002, Cupertino, CA) loaded with Ang II (500-1,000 ng/min/kg) were sterilely implanted in the dorsal subcutaneous tissues under 1-2% isoflurane inhalant anesthesia with oxygen at 1 L/min, and wounds closed with absorbable sutures.

4.2.2 Dietary perturbations

All animals received a 10 kcal% fat standard chow diet after weaning (D12450B; Research Diets Inc., New Brunswick, NJ). The normal chow group was maintained on this diet throughout the course of the experiment. To study the effect of diet induced obesity on aortic wall elasticity, animals designated as high fat were pre-fed with a 60 kcal% fat western diet (D12492; Research Diets Inc.) 6 weeks prior to Ang II infusion and maintained on this diet during the study. Table 4-A summarizes the experimental groups and the specific diets over time.

Table 4-A: Experimental groups for Objective 1A

<table>
<thead>
<tr>
<th>Diet</th>
<th>Age at Implant</th>
<th>Ultrasound acquisition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Chow (10 kcal% fat)</td>
<td>16 Weeks</td>
<td>Preop, Day 3 (n=8) Preop, Day 14, Day 28 (n=10)</td>
</tr>
<tr>
<td>High Fat (60 kcal% fat)</td>
<td>16 Weeks</td>
<td>Preop, Day 3 (n=6) Preop, Day 14, Day 28 (n=10)</td>
</tr>
</tbody>
</table>

4.2.3 Study design

High resolution ultrasonography was performed using a Vevo 2100 Imaging System with MS700 (30-70 MHz) and MS550D (22-55 MHz) linear array transducers (Visualsonics). For short term study groups, data was collected before implantation (pre-op) and at day 3 after
implantation, while for long term groups, data was collected preoperatively, at day 14, and at day 28 after implantation. Mice were anesthetized using 1-2% isoflurane inhalant anesthesia with oxygen at 1 L/min and animals were placed in supine position on a moveable, heated stage maintained at 37°C. Hair was removed from the abdomen and thorax using a depilatory cream. Warmed ultrasound transmission gel was applied to the ventral surface of the animal and the probe was positioned on a fixed stand perpendicular to the stage. After adequate visualization of the aortic segments of interest, one hundred frame (at 240-270 frames per second) B-mode cine image sequences were then recorded over an average of 2-3 cardiac cycles. Since aortic pathologies associated with Ang II infusion frequently occur at defined locations along the aorta (arch and suprarenal > infra-renal and thoracic), six different anatomic segments were chosen to allow for comparisons of mechanical strains at these locations (Figure 4-1).
Figure 4-1: Segments of interest analyzed in the thoracic and abdominal aorta. Celiac trunk (CT), superior mesenteric artery (SMA) and renal arteries (RA). The thoracic region = cross-section 5mm proximal to the CT; the supraceliac region = cross-section 1mm proximal to the CT; the paravisceral region = cross-section between the CT and SMA; and the juxtarenal region = cross-section 1mm distal to the SMA.

Ultrasound cine loops collected during the imaging procedure were analyzed to calculate circumferential strains. Cine loops were loaded into Vechostrain 2100 Advanced Cardiac Analysis Software (Visualsonics) and analyzed using the manufacturer’s cardiac speckle tracking algorithm. A closed spline of 48 points was manually drawn around the edge of the vessel cross-section in the ultrasound image. Accuracy of speckle tracking throughout the cine loop was verified visually. If tracking did not pass visual inspection, splines were redrawn until adequate tracking was achieved. The frame-to-frame displacements of each of the 48 points throughout the cine loop were the output for strain analysis. Additionally, representative frames from the ultrasound images were saved to allow determination of vessel
regions that did not have enough speckle contrast for adequate analysis (regions that correspond to renal artery branching or echogenic shadow artifacts) (Figure 4-2).

![Sample ultrasound image of an aorta](image)

Figure 4-2: Sample ultrasound image of an aorta. Orange line indicates manually drawn spline. Green traces show the path of the tracking points (yellow) throughout several cardiac cycles. The dashed red line indicates a region that has poor contrast (likely due to a branching vessel) and therefore poor tracking. These regions were dropped from the analyses to improve accuracy. Yellow scale bar is 1-mm in length.

### 4.2.4 Strain calculation

Displacements from the Vevostrain software were then downloaded into a custom MatLab (Mathworks, Natick, MA) program and analyzed to determine circumferential strain from displacements. Strains were determined at each of the 48 points around the vessel. Circumferential strains were calculated using the definition of the Cauchy strain tensor for cylindrical coordinates Equation (4-A).\(^{75}\)

\[
\varepsilon_{\theta\theta} = \frac{1}{r} \left( \frac{\partial u_\theta}{\partial \theta} + u_r \right) \tag{4-A}
\]

Radii were measured by determining the distance from each point to the centroid of the points around the vessel. It was assumed that the local radius around each measured point was constant at any particular time point. The strains at each point around the vessel were averaged.
to give a mean incremental strain from frame to frame. The analysis points were then compared with images from the ultrasound machine. Regions that appeared dark and which had no speckle contrast were then manually dropped from the analysis. An example of these regions is shown with a dashed red line in Figure 4-2. After excluding such un-interpretable regions, average strain curves were plotted, and peaks and troughs were located and manually verified using a custom MatLab program. The maximum circumferential strain value was calculated as the average difference between consecutive peaks to troughs throughout each cine loop. Resulting means were then averaged in each experimental group at each aortic location and reported as mean ± SEM. In addition to calculating strains, vessel cross-sectional areas were determined from scaled ultrasound images. These areas were then used to determine if vessel diameters changed throughout the study.

4.2.5 Statistics
Statistical analyses were executed using Sigmaplot 11 (Systat Software, San Jose, CA). Comparisons between two groups were completed using an unpaired two-tailed Student’s t-test. Comparisons between 3 or more experimental groups and between time points were made using one-way ANOVA with Tukey post-hoc analyses. All data is reported as mean ± SEM.

4.3 Results
4.3.1 Effects of diet on aortic strain
The normal chow and high fat mice gained weight as expected based on vendor supplied data (Jackson Laboratory, Bar Harbor, ME). To confirm the ability to measure strain in the mouse aorta, pre-op baseline data for mice in the four study groups were analyzed. Data from all normal chow diet mice with all high fat diet mice were compared first (Figure 4-3). The results demonstrate a consistency of measurements in the same aortic location with animals
consuming the same diet. Additionally, our strain values were similar (maximum strains ~19-20%) to those reported by Goergen et al. using MRI imaging.\textsuperscript{35} It also shows no statistically significant differences between normal chow and high fat diet mice at each segment along the aorta preoperatively. However, differences in mechanical strain along the aorta in the normal chow and high fat groups were noted. The major trend seen here is that in both the normal chow and high fat groups, the ascending aorta shows statistically significant higher strain values than the other anatomical locations (P < .05). Additionally, the lowest strain values were observed in the arch segment (P < .05).
Figure 4-3: Comparison of baseline preoperative strain magnitudes. Comparisons are made across 6 aortic locations between normal chow and high fat diet mice. Statistical statements are noted in the inset table. No statistically significant differences were found between mice of different diets in any aortic location, though differentials were noted among segments as depicted. All statistical statements indicate that $P < .05$; all groups contain 15 or more animals. *Indicates statistical difference from high-fat ascending aorta; #indicates statistical difference from normal chow ascending aorta; and @indicates statistical difference from high-fat aortic arch.

4.3.2 Short term impact of Angiotensin-II treatment on mechanical strain

Using the short-term arm of the study, the acute effects of the Ang II treatment (1000ng/kg/min) on mechanical strain in normal chow and high fat mice (Figure 4-4, A) were isolated. There is a consistent trend toward decreased strains in all aortic locations after just 3 days of Ang II treatment in the normal chow group. Reductions in the supraceliac, thoracic, and ascending aortic locations were statistically significant ($P < .05$). None of these animals showed changes in aortic diameter at day 3 post-Ang II infusion.
Similar results were found in the short-term study for the mice fed on a high fat diet (Figure 4-4, B). In this group, there was a similar trend toward decreased strain at the day 3 time point, with statistically significant decreases occurring in the supraceliac segment. Unlike the normal chow group, however, two animals in this group formed aortic aneurysms (at least 1.5 times greater diameter than initial measurement; paravisceral and supraceliac regions) by day 3 post-op, while one animal died of aortic arch rupture prior to day 3.

4.3.3 **Long term effects of angiotensin II treatment on mechanical strain**

Long-term changes in strain were analyzed for both the normal chow and high fat groups receiving Ang II (500ng/kg/min). In both groups there was a progressive trend towards decreased mechanical strains from pre-op to day 14 to 28 strains (Figure 4-4, C and D). The largest reduction of strain was observed in the para-visceral, and the supra-celiac segments in high fat diet mice (Figure 4-4, D). The majority of these reductions in strain corresponded directly with aneurysm formation. It should be noted that both the normal chow and high fat groups contained animals with and without aneurysm development by day 28.
Figure 4-4: Short and long term effects of Ang II on normal and high fat animals. (A) Short-term effects of Ang II treatment on animals fed on normal chow diets. (B) Short-term effects of Ang II treatment on animals consuming only high fat diets since week 10 of age. (C) Long-term effects of Ang II treatment on normal chow animals. (D) Long-term effects of Ang II on high fat animals. * Indicates P<.05.

4.3.4 Relative effects of different factors on mechanical strain
The design of this study enabled the isolation of the effects of Ang II treatment from those of aneurysm formation. The percent change in strain from pre-op strain due to Ang II treatment alone, aneurysm formation at day 14, and aneurysm formation at day 28 for normal chow and high fat animals was determined (Figure 4-5). These data suggest that both the Ang II treatment and the actual formation of aneurysm play a role in reducing strain in the aorta. The results in Figure 4-5 also suggest that aneurysmal degeneration and physical remodeling of the wall may play a large role in decreasing circumferential strains. Mural thrombus was a
common finding in the Ang II mice. The thrombus likely contributed to the overall decrease in strain of the aneurismal aortic wall. However, the current study does not have enough power to tease out the component contributions of mural thrombus vs other factors on the decreased strain found in aneurismal segments.

Overall in this study, there were clear differences in aneurysm formation between the normal chow and high fat diet groups. At day 3 in the short term arm of our study, 0% (n=8) of normal chow animals had formed aneurysms. High fat animals at day 3, however, demonstrated an aneurysm formation rate of 50% (n=6). One of these high fat animals died of aortic arch aneurysm and rupture prior to day 3. In the long term arm, similar rates of aneurysm formation between normal chow and high fat animals was observed. At week 4 there was an aneurysm rate of 70% (including animals which died of aneurysmal arch rupture by week 4). In contrast an aneurysm formation rate of 88% was seen in high fat animals. We were not, however, able to distinguish a difference in pre-Ang II strain measurements in animals that subsequently develop aneurysms vs no aneurysms (no predictive capability).
Figure 4-5: Effects of Ang II and aneurismal degeneration on aortic wall strain. Data are reported as percent change in strain from pre-operative strain. The Ang II group contains only animals that did not have aneurysms at Day 3 from the early study. Day 14 and Day 28 aneurysmal degeneration groups only contain data from animals that had aneurysmal expansion (increase in size of 150% or more) detected via ultrasound scans. Data for normal chow and high fat groups are shown in (A) and (B), respectively.

4.4 Discussion

AAAs lead to over 15,000 deaths per year in the United States alone, and some even estimate the annual death rate from AAA to be as high as 30,000. Most are asymptomatic until catastrophic rupture, and ruptured AAAs continue to have high morbidity and mortality.204 Hence, there is an undeniable need to fully understand the pathophysiology of AAAs and to improve patient selection for expansion and rupture prediction and prevention.

The exact pathogenesis of AAA is complex and still unknown, but clinical risk factors are known which include smoking, male gender, age, family history, previous vascular disease,
hypertension, and hypercholesterolemia. Biologically aortic wall inflammation and oxidative stress appear to hold a role in AAA formation. The immune system is also believed to be mechanistically involved in the development of AAAs, along with a reduction in the density of smooth muscle cells, elastin and collagen fibers, which are degraded by proteolytic enzymes (mostly matrix metalloproteinases). The aortic wall structurally depends on elastin and collagen, but in aneurysm disease medial elastin degradation (elastolysis) occurs, causing dilatation of the aorta.

Biomechanical forces stand as a pivotal factor in AAA development and rupture. Peak AAA wall stress may be superior to maximum diameter in differentiating rupture risk. Additionally, due to unevenly distributed wall stresses, overall AAA wall stress does not directly depend on maximum diameter.

Ultrasound serves as a useful tool not only clinically for infrarenal aortic aneurysm surveillance, but also experimentally for quantification of aortic morphology and biomechanics. Contemporary high-frequency ultrasound represents a significant improvement over prior techniques in evaluating the aorta in murine AAA models. The approach is relatively non-invasive imaging, can be performed longitudinally, allows for examination of aneurysm prone/protected regions, and in combination with analytical approaches such as speckle tracking for strain analyses it provides more information than previous murine AAA imaging studies that only measured maximum aortic diameter. The primary downside is the expense of the device and technical expertise needed to acquire image data, though it has been shown that high-frequency ultrasound can be performed with relatively small intra-individual and inter-individual variance. Other groups have used in vivo pulse wave velocity in non-aneurysm

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studies to show that apoE-/− mice infused with Ang II have significantly increased vessel stiffness compared with controls after 30 days. However, this report did not provide localized stiffness data. Other related imaging modalities are in development for in vivo murine studies—for example quantification of murine aortic cyclic strain and motion using 3D MR angiography in relation to its implications for AAA growth.

In the current experiment high frequency ultrasound was used to assess aortic wall strain in apoE-/− mice at selected time points during Ang II-induced aortic aneurysm development. Wall strain correlated with the pathological events that occurred in different segments of the aorta. Baseline wall strain was measured in selected segments along the aorta, revealing a rather high strain in the ascending aorta, while the aortic arch manifested the lowest basic strain. As Ang II was given to our mice, the ascending segment did not manifest aneurysmal dilatation. However, as expected, the arch responded dramatically by developing aneurysms, some of which ruptured within the very early stage (3 days). Cao et al. described the phenomenon of early development of aneurysm with subsequent rupture, ranging at 25% of all mice. In their series, 44% presented with arch aneurysms. The prevalence of early development of aortic aneurysm correlates with age. Also, it is assumed to represent a genetic predilection of some of the apoE-/− mice, presenting with aortic arch structural aberration, which yields a stiffer vessel wall. The later phase of Ang II infusion (as assessed on days 14 through 28) was characterized by a significant reduction of wall strain in the supra-renal segment, namely the para-visceral, and supra-celiac regions. It is not surprising, therefore, that those segments are most affected by aneurysm formation in this model. Notably, the aneurysmal dilatation by itself leads to an increased vessel diameter, which further decreases
wall strain locally. The data collected via longitudinal non-invasive assessments emphasize the significance of anatomical localization of regional circumferential wall strain characteristics as a predictor for potential future events.

Dietary manipulations of murine models have emerged as a useful experimental tool, and such manipulations are known to impact factors key to development of arterial aneurysms. Obesity has been shown to increase peri-aortic adipose tissue inflammation leading to enhanced incidence of AAA in the Ang II murine model. We sought to characterize the effect of these changes on the aortic wall elasticity and association with enhanced aneurysm formation. Under the conditions of our experiments we found no differences between normal chow mice and high fat mice in any region along the aorta preoperatively. This suggests that the hyperlipidemic state associated with short term high fat feeding alone does not significantly alter the structural components of the aortic wall, but rather enhances the inflammatory and remodeling response associated with Ang II treatment. There was no significant difference between atherosclerotic plaque deposition on the aortic wall observed at autopsy between the normal chow and high fat animals. However, longer term high fat feeding as well as more advanced age may lead to enhanced atherosclerotic changes that can result in loss of aortic elasticity and increased risk for aneurysmal degeneration, but this remains to be characterized in future investigations.

Potential parallel applications for high frequency ultrasound are numerous. Our methods could be used to quantify circumferential strain in other murine models of disease and after interventions (e.g. statins, anti-hypertensive therapies, anti-inflammatory agents, etc.). Furthermore, most AAAs are asymmetric. It has been shown that asymmetry/shape has a substantial influence on the distribution of wall stress within the aneurysm, and our strategies...
could be expanded to interrogate the effect of asymmetric circumferential strain on AAA formation and rupture. Clinically there are significant shortfalls with current AAA management pathways, as the fundamental strategy of using size imaging as a stand-alone to diagnose and provide prognostic information regarding AAA does not provide complete data to identify which AAAs are most likely to continue to increase in size and be at risk for rupture. A more complete ability to predict AAA progression may allow improved AAA management.

A limitation of this study is that blood pressure was not closely measured in the mice. However, it has been reported previously that blood pressure does not affect Ang II related AAA development, and it is likely that for the range of blood pressures for the experimental animals the impact on strain is minimal. Systemic blood pressure is necessary for calculation of stiffness index and other relevant hemodynamic parameters. We assumed that at day 3 no animals would develop gross aneurismatic changes, and that any difference in strain measured would be from the effect of Ang II alone rather than contributions from structural changes from an enlarging aneurysm/thrombus. The combination of high fat feeding and high Ang II dose led to unexpected aneurysm development at day 3. It is possible that by reducing the dose of Ang II we could decrease the rate of early aneurysm formation and better determine the effects of the Ang II treatment alone. Finally, due to the resolution limitations of current ultrasound technology, we were unable to accurately differentiate between the intra-luminal and extra-luminal aortic wall. For these reasons, we only calculated and reported the circumferential strains (not radial strain) on the intra-luminal wall, which was clearly visible in the ultrasound images.
In conclusion, the combination of high-frequency ultrasound imaging and speckle tracking allows the quantification of the changes in circumferential strain of the aorta during AAA development in apoE-/− mice infused with Ang II. The combination of these techniques demonstrated that the aorta significantly stiffens (has decreased strain) shortly after the infusion of Ang II into apoE-/− mice and after aneurysm formation in these mice. Measurements at later time points revealed progressive decrease in strain in the supra-renal aorta, which is the segment most likely to be involved in aneurysm formation. The delineation of the biomechanical factors and natural history of AAA development in animal models of the disease in combination with our continuously improving armamentarium of biologic assays will provide insights into the factors associated with initiation and propagation of AAAs in humans.

4.5 Conclusions

The methodologies used to measure strains in this chapter represent a novel and useful approach to noninvasively determine wall strains in the murine model. In this chapter, we demonstrated that there are baseline differences in strain amongst aortic regions with the greatest arterial strain in the ascending aorta region. Additionally, we found that mechanical strains in several regions were reduced very soon after the induction of aneurysms in the apoE-/− mouse model infused with Ang II. These reductions in strain demonstrate that our method can measure small enough strain changes to determine changes in a murine model with small sample sizes and that this method is sensitive enough to determine changes in aortic strains due to disease conditions. This method enables research that noninvasively examines changes in vascular strains in a variety of biological systems. In the following chapters of this dissertation, this method is applied to a novel model of vascular intimal hyperplasia.
5 Objectives 2: Determination of the relationship between circumferential wall strain and intimal hyperplasia

Portions of this section are taken from The Journal of Vascular Surgery, In Press, John T Favreau, Chengwei Liu, Peng Yu, Ming Tao, Christine Mauro, Glenn R. Gaudette, C. Keith Ozaki, “Acute reductions in mechanical wall strain precede the formation of intimal hyperplasia in a murine model of arterial occlusive disease”, Copyright 2013, with permission from Elsevier (See Appendix A).

5.1 Introduction

In the first aim of this dissertation, we validated a novel method for measuring mechanical strains on the murine arterial wall using high frequency ultrasound. Having demonstrated the accuracy and precision of our method compared to another measurement technique, and applied our method to an in vivo model, our next objective is to determine what relationship exists between cyclic circumferential wall strain and intimal hyperplasia (IH).

In this chapter, we hypothesize that there is a direct positive relationship between reduced cyclic arterial wall strain and the formation of IH. To test this hypothesis, we combined a validated murine model of IH\textsuperscript{37} with our method for measuring arterial wall strains in vivo\textsuperscript{44} to unravel the relationship between wall strain and neointima formation.

5.2 Materials and methods

5.2.1 Study design

All animal experimental protocols used in this study complied with the Guide for the Care and Use of Laboratory Animals\textsuperscript{214} and were approved by our local Institutional Animal Care and Use Committee. Male C57BL/6J mice (n=10) were purchased from the Jackson Laboratory (Bar Harbor, ME) and maintained on a normal chow diet.
5.2.2 **Mouse model**

At eight weeks of age, general anesthesia was induced with 2% isoflurane gas in an induction chamber and then maintained via a nose cone with 1.0-1.5% isoflurane gas. A focal stenosis was created in the left distal common carotid artery (LCCA) as described previously.\(^\text{37}\) Briefly, the LCCA was exposed and visualized via a midline incision using a surgical microscope (OPMI-MD, Carl Zeiss, Germany). A focal stenosis was created approximately 1mm proximal to the common carotid bifurcation by tying a 9-0 nylon suture around both the artery and a blunt 35 gauge needle (external diameter of 0.14 mm, item No. NF 35 BL; World Precision Instruments, Inc., Sarasota, FL). After removal of the needle, the vessel diameter was reduced by ~78%. Our previous studies have shown this diameter reduction will produce a reduction in luminal flow of ~85%.\(^\text{37}\) After creation of the focal stenosis, the surgical incision was closed and mice were allowed to recover.

5.2.3 **Ultrasound data acquisition**

Ultrasound cine loops of both the LCCA and right common carotid artery (RCCA) were recorded preoperatively and on post-op day 27. For ultrasound acquisition, mice were anesthetized using 1-2% isoflurane and body temperature was maintained on a moveable platform heated to 37 °C. A chemical depilatory was used to remove hair from the neck and pre-warmed Aquasonic 100H ultrasound transmission gel (Parker Laboratories, Inc., Fairfield, NJ) was applied. Using a Visualsonics Vevo 2100 Ultrasound Imaging System with MS700 50 MHz linear array transducer (Visualsonics Inc., Toronto, ON, Canada), cine loops of cross-sections of both the RCCA and LCCA were recorded. For the LCCA, loops were recorded 1mm, 2mm and 3mm proximal to the focal stenosis and at similar locations for the RCCA (Figure 5-1). Cine loops for all time points and locations were recorded at 432 frames/s with 500 consecutive...
frames acquired. Efforts were made to maintain depth of anesthesia with a heart rate of at least 475 beats/minute and a respiratory rate of 75-100 breaths/minute during all data collection.

Figure 5-1: Objective 2 study design. A focal stenosis (green arrow) was made in the left common carotid artery 1mm proximal to the carotid bifurcation. Mechanical (via ultrasound) and histological parameters were assessed 1mm, 2mm, and 3mm proximal to the focal stenosis on the left side, and in corresponding locations on the contralateral side (blue lines).

5.2.4 Strain calculations
Ultrasound cine loops were loaded into VevoVasc software (Version 3.6.2.0, Visualsonics Inc.) and vascular walls were traced using the automatic edge detection tool. Movement of the carotid artery wall was then tracked through at least 5 cardiac cycles and vessel area was recorded over time. From the resulting area change curves, ideal vessel diameters were computed (assuming a circular cross-section) and circumferential wall strain curves (using Green’s definition) were calculated across the 5 cardiac cycles. Average magnitude of cyclic wall strain from diastole to systole was calculated for each animal, time point and location.
Additionally, mean diastolic and systolic cross-sectional areas were computed and reported for each time point.

**5.2.5 Tissue Harvest and Histology**

On post-op day 28, mice were again anesthetized using 1-2% isoflurane anesthesia. The chest cavity was opened and the left ventricle of the heart was punctured with a 25 gauge cannula and blood was drained through an incision in the inferior vena cava. Lactated ringer’s solution was perfused under physiological pressure (100 mmHg) for 2-3 minutes then replaced with 10% buffered formalin for 5 minutes. The LCCA and RCCA were collected and fixed in the same formalin solution overnight.

Tissue specimens were then dehydrated, cleared through several changes of ethanol and xylene and embedded in paraffin. Tissue cross-sections (6-μm thick) were collected throughout the tissue. Using the carotid bifurcation as a reference for the RCCA and the focal stenosis as a reference for the LCCA, tissue cross-sections were collected 1mm, 2mm and 3mm proximal to the focal stenosis and at corresponding locations on the right (the same locations at which wall strain was assessed using ultrasound). At each location, Masson’s trichrome stain was employed to visualize arterial structures via a microscope with high resolution camera (Axio A1 microscope with vision 4.7 software; Carl Zeiss). The lumen dimensions, internal elastic lamina (IEL) length and external elastic lamina (EEL) length were identified and traced. Assuming a circular cross-section *in vivo*, the areas and radii of the lumen, IEL and EEL were determined. From these values, mean intimal thickness, wall thickness, and intima:media thickness ratio were calculated.
5.2.6 **Statistics**

Data are presented as mean and standard error of the mean. All statistical analyses were completed in Sigmaplot version 11 (Systat Software Inc., San Jose, CA, USA). Statistical comparisons amongst spatial regions for strain, histological measurements and diameters were performed using a one way ANOVA followed by a Tukey post-hoc test. Differences between pre-op and post-op day 28 timepoints and between contralateral control and experimental artery were determined using a paired t-test (paired by animal). Statistical powers are reported for all negative statistical results. P<0.05 was considered significant for all tests.

5.3 **Results**

All animals survived surgery to tissue harvest. Three of the 10 mice had occluded LCCAs on harvest and their morphology data were not included in any of the analyses. Histology was available on all of the remaining animals. Due to technical difficulties related to visualizing the relatively low flow artery and edema, some day 27 strain data was unusable and thus is missing. The baseline data for these animals was included in the strain analyses. Table 5-A summarizes the data available and used for the following analyses.

<table>
<thead>
<tr>
<th>Timepoint</th>
<th>2mm Strain (# of Regions)</th>
<th>3mm Strain (# of Regions)</th>
<th>4mm Strain (# of Regions)</th>
<th>Histology (# of animals)</th>
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Table 5-A: Sample sizes used for strain and histological analyses
5.3.1 **Wall strain analysis**

Wall strains at baseline (pre-op) were consistent in all regions of both the left and right common carotid artery with no significant differences (Figure 5-2; p=0.56; Power: 0.22). At day 27, strains were reduced compared to pre-op measurements (p<0.001). Strains on the contralateral artery remained unchanged compared to pre-op strains at day 27 (p=0.719; Power: 0.05). No regional differences in strain were seen amongst either the stenosis or contralateral locations at any time point.

![Figure 5-2: Regional differences in strain.](image)

*Figure 5-2: Regional differences in strain.* Wall strains were assessed on the left (stenosed) and right (unaltered) common carotid artery in the regions shown in Figure 5-1. No differences amongst spatial locations were found on either side at any time point. (n=8 in all groups) *indicates P<.05 compared to pre-op values (tested via paired t-test).

5.3.2 **Histological analysis of wall adaptations**

Histological analysis of tissues collected at day 28 was completed in all animals with a patent LCCA (n=7). Intimal thickness and wall thickness were computed at 2, 3 and 4 mm
proximal to the carotid bifurcation (regions which correspond to those where ultrasound data was acquired). IH occurred near the focal stenosis of the LCCA, but none was observed in the contralateral RCCA (Figure 5-3A). No regional differences were found in any calculated parameter amongst the three spatial regions. Mean wall thickness of the LCCA tended to be higher than that of RCCA, with a significantly 56% thicker wall 3 mm proximal to the bifurcation (P =0.012) (Figure 5-3B).

Figure 5-3: Morphological analysis of day-28 carotid arteries. (A) The intimal thickness of LCCA was significantly higher than that of RCCA at the same location. (B) LCCA tends to have higher wall thickness than its counterpart RCCA. (C) Representative histology for each of the three regions on the LCCA and RCCA. † indicates P < .05 compared to RCCA value at the same cross-sectional location (tested via paired t-test; n=7 in all groups).

5.3.3 Temporal progression of arterial cross-sectional areas
Arterial cross-sectional areas at both diastole and systole were assessed pre-op, and at post-op day 27. Since no differences among spatial positions of either the LCCA or RCCA were
noted, strain values for all regions were analyzed together. Diastolic cross-sections in all spatial regions (2, 3 and 4 mm proximal to the carotid bifurcation) on the focal stenosis side were decreased at post-op day 27 compared to pre-op area (p=0.003). On the contralateral (right) side, diastolic areas increased from pre-op to post-op day 27 (0.10 ± 0.01 mm² vs. 0.14 ± 0.01 mm²; p<0.001).

Systolic cross-sectional areas were the same for the LCCA and RCCA at pre-op (0.15 ± 0.01 mm² vs. 0.15 ± 0.01 mm²; p=0.50; Power: 0.05). Systolic cross-sectional areas in the LCCA were decreased compared to pre-op at post-op day 27 (0.08 ± 0.01 mm²; p<0.001). In the RCCA, systolic cross-section increased compared to pre-op at day 27 (0.20 ± 0.01 mm²; p=0.01) (Figure 5-4).

Figure 5-4: Changes in (A) diastolic and (B) systolic lumen area. *Indicates P<.05 compared to pre-op values (tested via paired t-test; n=8 in all groups)
5.3.4 Relationship between Day 27 strain and Histological outcome

As the results above demonstrate, we have found that there are no regional differences in circumferential wall strain amongst the 2mm, 3mm and 4mm locations proximal to the focal stenosis on either the stenosis vessel or contralateral control vessel. Additionally, we found no regional differences in either wall thickness or intimal thickness histological outcome amongst any of the regions. Consequently, we analyzed the relationship between day 27 strain and histological outcome as an aggregate of all of the regions, ignoring distance from the focal stenosis.

Qualitative analysis of a plot of circumferential wall strain vs. intimal thickness shows that all regions have reduced strain values at day 27. Further, regions with high amounts of intimal hyperplasia appear to have lower strains compared to those with no intimal hyperplasia which exhibit a wide range of strains. Finally, we once again observed no trends toward differences amongst the three regions.

Given the qualitative results in Figure 5-5, the differences in intimal thickness outcome were analyzed between regions with low strain and those with high strain. By examining a histogram of day 27 strains (Figure 5-6A), it was observed that the majority of strain values are in the range of 0.01- 0.08 and appear to be normally distributed. In Figure 5-6B, the neointima formation in the 4 regions that have high strains (>0.08) was compared. Although no statistical differences occur between the low and high strain groups in either the wall thickness (p=0.357) measure or intimal thickness (p=0.145) measure, there appears to be a trend toward increased intimal and wall thickness in the low strain group. Since only 4 regions fell into the high strain
group, lack of statistical significance here could be due to a type 2 statistical error given that statistical power for both tests was only 0.05.

**Figure 5-5: Relationship between day 27 strain and day 28 intimal thickness.** Although there is a wide range of regions with low intimal thickness, regions with significant neointima formation are confined to the low strain range.

**Figure 5-6: Quantitative relationship between day 27 strain and neointima formation.** (A) Histogram analysis of wall strains in all regions at day 27. (B) Analysis of wall thickness and intimal thickness between regions of low strain (<0.1) and those of high strain (>0.1).
5.4 Discussion

In Objective 1 (Chapters 3 and 4), we demonstrated that our novel methods are capable of measuring circumferential wall strains in the murine vasculature. In the present chapter we demonstrate that there is a relationship between regional circumferential wall strain and the formation of intimal hyperplasia. In a previous study in our collaborator’s lab, we found that using the murine focal stenosis model results in a ~85% reduction in wall shear stress and a subsequent formation of IH in the vessel 1 month later.\textsuperscript{37} This finding is consistent with the large body of literature demonstrating a correlation between low wall shear stress and IH.\textsuperscript{82, 104, 134, 162, 216-224} Additionally, in this previous study, we showed that immediately after creation of the focal stenosis, there may be a trend toward strain reduction (as measured by an optical method) in the region proximal to the stenosis.\textsuperscript{37} In this objective, we go on to demonstrate that a reduction in strain exists in nearly all regions at Day 27 post focal stenosis creation.

Oscillatory wall strain is a measure of how much the vessel wall dilates from diastolic to systolic pressure in the average cardiac cycle. Given that the stress in the vessel wall is related to strain through the mechanical properties of the wall, reductions in mechanical strain observed herein are likely due to either changes in wall stiffness or changes in wall stress. Increases in vascular wall stiffness would result in the vessel wall stretching less between the diastolic and systolic blood pressures—thereby reducing oscillatory wall strain. Alternatively, the blood pressure itself or the wall thickness could change and affect the wall stress, resulting in changes in wall strain. Increases in wall thickness or decreases in pulse pressure could both result in reductions in wall strain. Interestingly, changes in mechanical strain are not observed in the contralateral control artery. This finding is confounded by the data in Figure 5-4 that shows an
increase in both contralateral diastolic and systolic areas. This suggests that although the vessel area is increasing, both diastolic and systolic area are increasing proportionally to maintain a constant cyclic wall strain. On the stenosis (left) side, a decrease in both systolic and diastolic area was observed in addition to the reduction in strain, although the decrease appears to be greater in systolic area compared to diastolic area.

A major limitation of the studies in this chapter is that the blood pressure and arterial wall thickness were not assessed. Ideally, blood pressures and wall thicknesses would be measured along with strain on both the experimental and contralateral arteries. Due to the small size of the murine blood vessels, the outer wall of the carotid artery cannot be differentiated from the surrounding tissues via contemporary ultrasound. Additionally, measurement of blood pressures within the vessel is limited by the arterial size. Given that the mouse carotid artery is typically about 300-400μm in lumen diameter\textsuperscript{37, 225} Insertion of even the smallest commercially available pressure catheters (300 microns in diameter, Fiso LS 0.9 French, Harvard Apparatus, Holliston, MA) into the carotid artery would significantly impede blood flow and likely alter the entire \textit{in vivo} model.

Despite the limitations of this study, the results show a promising trend toward a relationship between mechanical strain and intimal hyperplasia in our murine model. The data in Figure 5-6 indicate a division of regions showing low or high strain and a trend toward low strain regions having more intimal hyperplasia. Given that nearly 2/3 of the regions analyzed demonstrated IH at day 28, it is possible that a more clear relationship could be found at earlier time points when less IH formation had occurred. In Specific Aim 3 (Chapters 6 and 7), we will further assess the temporal relationship between IH and cyclic wall strain.
6 Objective 3A: Determine the temporal progression of wall strain in the focal stenosis model

Portions of this section are taken from The Journal of Vascular Surgery, In Press, John T Favreau, Chengwei Liu, Peng Yu, Ming Tao, Christine Mauro, Glenn R. Gaudette, C. Keith Ozaki, “Acute reductions in mechanical wall strain precede the formation of intimal hyperplasia in a murine model of arterial occlusive disease”, Copyright 2013, with permission from Elsevier (See Appendix A).

6.1 Introduction

In chapter 5 of this dissertation, we demonstrate that there is a relationship between reductions in mechanical strain and the formation of IH in a murine model. Although significant differences as a function of distance proximal to the focal stenosis were not observed in either strain or IH measurements, our data suggest that there may be a relationship between mechanical strain and IH in this model. To further understand this relationship, in this chapter we will aim to further delineate the progression of wall strain in this model. To accomplish this, wall strain was assessed at time points earlier than 28 days to determine when strain reductions occur in relation to IH at 28 days post-op. By examining more time points, we show how early detectable changes in strain occur in this model and gain a better understanding of when strains are reduced.

6.2 Materials and methods

6.2.1 Experimental Design

We will re-use histological data collected for the animals in Objective 2 and assess ultrasound data collected in the same animals at the day 4 timepoint. Arterial wall strains were assessed in the same locations described in Chapter 5 using our ultrasound-based methodology at day 4 after the surgical procedure. Day 4 strain outcomes were compared to day 28
histological outcomes presented in Objective 2. The experimental design for this aim is shown in Figure 6-1.

![Figure 6-1: Schematic of Aim 3A study. A focal stenosis will be created using the methodology described in Chapter 5. Early strains, at day 4, will be compared to histological outcome at day 28.](image)

6.2.2 Relationship between early strain and eventual intimal hyperplasia
Animals from Objective 2 are re-used for this chapter to determine the temporal progression of wall strains in this model. Ultrasound cine loops from day 4 post-op were collected and strain analyses were performed as described in Section 5.2.4. Cyclic wall strain, diastolic lumen area and systolic lumen area were assessed for the day 4 time point.

6.2.1 Statistics
Data are presented as mean and standard error of the mean. All statistical analyses were completed in Sigmmaplot version 11 (Systat Software Inc., San Jose, CA, USA). Statistical comparisons amongst spatial regions for strain, histological measurements and diameters were performed using a one way ANOVA followed by a Tukey post-hoc test. Differences between pre-op, post-op day 4 and post-op day 28 time points were performed using a repeated measures ANOVA. Differences between contralateral control and experimental artery were
determined using a paired t-test (paired by animal). Statistical powers are reported for all negative statistical results. P<0.05 was considered significant for all tests.

6.3 Results
All animals survived surgery to tissue harvest. Three of the 10 mice had occluded LCCAs on harvest and their morphology data were not included in any of the analyses. Histology was available on all of the remaining animals. Due to technical difficulties related to visualizing the relatively low flow artery and edema, some day 4 strain data was unusable and thus is missing. The baseline data for these animals was included in the strain analyses. Table 6-A summarizes the data available and used for the following analyses.

Table 6-A: Sample sizes used for strain and histological analyses on the LCCA. *The lumen of some vessels were not visible via ultrasound and were therefore excluded. † 3 arteries were fully occluded at day 28 and therefore were not used for histological analysis

<table>
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<th>3mm Strain (# of Regions)</th>
<th>4mm Strain (# of Regions)</th>
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<td>Day 28</td>
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</table>

6.3.1 Wall strain analysis
Wall strains at baseline (pre-op) were consistent in all regions of both the left and right common carotid artery with no significant differences (Figure 5-2; p=0.56; Power: 0.05). At day 4, all regions on the focal stenosis (left) side were significantly reduced compared to pre-op strains (p<0.001) while no changes in strain occurred on the contralateral (right) side (p=0.90);
Power: 0.05). At day 27, strains remained reduced compared to pre-op measurements but did not significantly reduce further compared to strains at day 4 (p=0.2). Strains on the contralateral artery remained unchanged compared to pre-op strains at day 27. No regional differences in strain were seen amongst either the stenosis or contralateral locations at any time point.

6.3.2 Temporal progression of arterial cross-sectional areas
Arterial cross-sectional areas at both diastole and systole were assessed pre-op, and at post-op day 4 and day 27. Since no differences among spatial positions of either the LCCA or RCCA were noted, strain values for all regions were analyzed together. Diastolic cross-sections in all spatial regions (2, 3 and 4 mm proximal to the carotid bifurcation) on the focal stenosis side remained unchanged from pre-op to post-op day 4 (0.10 ± 0.01 mm² vs. 0.12 ± 0.01 mm²; p=0.19; Power: 0.96). However, diastolic cross-sectional areas were decreased at post-op day 27 compared to both pre-op area and post-op day 4 area (0.07 ± 0.01 mm²; p<0.001). On the contralateral (right) side, diastolic areas increased from pre-op to post-op day 4 (0.10 ± 0.01 mm² vs. 0.13 ± 0.01 mm²; p=0.009) and remained increased at post-op day 27 compared to pre-op (0.14 ± 0.01 mm²; p<0.001). Diastolic cross-sectional areas were statistically the same for the focal stenosis and contralateral arteries at pre-op and day 4, but contralateral cross-section was larger at post-op day 27 (p<0.001).
Regional differences in strain. Wall strains were assessed on the left (partially stenosed) and right (unaltered) common carotid artery in the regions shown in Figure 5-1. Average for each side indicates the average of all regions. No differences amongst spatial locations were found on either side at any time point. * indicates P<.05 compared to pre-op values (n=8 in all groups).

Systolic cross-sectional areas were the same for the LCCA and RCCA at pre-op (0.15 ± 0.01 mm\(^2\) vs. 0.15 ± 0.01 mm\(^2\); p=0.50; Power: 0.05). Systolic cross-sectional areas in the LCCA were unchanged compared to pre-op at post-op day 4 (0.14 ± 0.01 mm\(^2\); p=0.64; Power: 0.9) but were decreased at post-op day 27 (0.08 ± 0.01 mm\(^2\); p<0.001). In the RCCA, systolic cross-section increased compared to pre-op at day 4 (0.17 ± 0.01 mm\(^2\); p=0.004) and continued to increase at post-op day 27 (0.20 ± 0.01 mm\(^2\); p<0.01) (Figure 5-4).
6.3.3 Relationship between acute strain and subsequent intimal thickness

Analysis of the wall strain values in all regions at post-op day 4 using a histogram demonstrated a range of results (Figure 6-4A). To further analyze the acute changes in wall strain produced by this model, the LCCA data were divided into regions with strain >0.10 (n=13) and those with strain ≤0.10 (n=13). The median was utilized as the threshold for the two
groups (the same cut off used in Aim 2). Of those regions with strains ≤0.10 at post-op day 4, all regions went on to form IH or to completely occlude by day 28. Regions with strain >0.1 at post-op day 4, only 31% (4 regions) went on to form any detectable IH by day 28.

Diastolic cross-sections remained unchanged in the strain >0.1 group from pre-op to day 4 (0.10 ± 0.01 mm² vs. 0.09 ± 0.01; P=.93; Power: 0.51) but were significantly reduced compared to pre-op at post-op day 27 (0.06 ± 0.01 mm²; P=.04). Diastolic areas in the strain ≤0.1 group were increased from pre-op to post-op day 4 (0.10 ± 0.01 mm² vs. 0.15 ± 0.02 mm²) but returned to their pre-op levels at day 27 (0.09 ± 0.01 mm²; P=.40 compared to pre-op; Power: 0.86; Figure 6-4B). Systolic areas, however, remained unchanged from pre-op to day 4 in both >0.1 (0.15 ± 0.01 mm² vs. 0.12 ± 0.02 mm²; P=.13; Power: 0.99) and ≤0.1 (0.16 ± 0.01 mm² vs. 0.17 ± 0.02 mm²; P=.89; Power: 0.94) groups and both decreased below pre-op baseline by day 27 (P=.005 and P=.01, respectively; Figure 6-4C). The strain >0.1 group had significantly smaller intimal thickness than the strain ≤0.1 group (8 ± 4 μm vs. 32 ± 7 μm; P=.006). Similarly, the strain >0.1 group had both a smaller wall thickness (82 ± 7 μm vs. 111 ± 6 μm; P=.009) and intima:media area ratio (0.19 ± 0.10 vs. 0.81 ± 0.19; P=0.006; Figure 6-4D).
Figure 6-4: Differences in LCCA regions with low (<0.1) and high (>0.1) day 4 strain. (A) Histogram analysis showing the distribution of strain values amongst regions with cut off value. (B) Histological outcome of LCCA regions with low and high strain. ‡ indicates P<.05 vs. low strain values. (C) Changes in LCCA area at diastole over time in low and high strain groups. (D) Changes in LCCA area at systole over time in low and high strain groups. (E) Linear fit between wall strain at day 4 and intimal thickness at day 28 * indicates P<.05 compared to pre-op values.

6.4 Discussion
Given the results of Chapter 5, we believe that the temporal progression of strains may be important in regards to IH formation. Since we suspect that IH does not form before day 4, it is
important to determine exactly how early strain reductions occur and how early IH forms in this model. By experimentally demonstrating that strains reduce before IH forms, these experiments could effectively support our overall hypothesis: that reductions in oscillatory wall strain precede the formation of IH in a murine model of intimal hyperplasia.

Herein, we find that vessels with early (four days after focal stenosis) low wall strain consistently develop IH 28 days after creation of an outflow focal stenosis. Furthermore, only 31% of arteries that demonstrated high strain early went on to develop a measurable amount of IH. Such an analytical approach stands as a reasonable strategy to understand this small dataset. However, via multiple linear regression (intimal thickness as a dependent variable, wall strain and location proximal to the focal stenosis as independent variables), IH can be modeled as a linear combination of wall strain and distance proximal to the stenosis. Wall strain appears to account for the ability to predict IH (P=0.007) while location does not (P=0.343). Again, application of multiple linear regression to these data is limited by the small sample size and goodness of fit was suboptimal (R^2= 0.36).

Wall strains measured herein were oscillatory, meaning that stretch from diastole to systole was measured. In order to better understand how wall strains are reduced in the short-term, diastolic and systolic areas were assessed. In the low strain group, a short-term reduction in diastolic lumen area was shown while no changes in systolic area were found. In the high strain group, no changes in diastolic or systolic area were observed. Our results demonstrate a decrease in LCCA vessel diameter in all mice by day 27 post-op, a finding that was previously reported at 2 weeks post-operatively in a similar flow model in rabbits. However, we reveal at
4 days that diastolic diameter increases significantly in animals that will eventually form IH, a phenomenon that has not been previously reported.

We speculate that the lower level of strain early is likely associated with altered vessel wall properties (biologic and mechanical). Though they have high heart rates, mice are known to have blood pressures similar to humans. Assuming a systolic pressure of 120 mmHg and a diastolic pressure of 80 mmHg, the 28 day wall modulus of elasticity (using a thin wall model based on the Law of Laplace and assuming linear vessel properties) yielded 152 ± 27 kPa for the low strain group and 100 ± 17 kPa in the high strain cohort. These computed elasticities fall in the same order of magnitude as those computed using more accurate (pressure based) measurements in previous studies.227 Although there was a trend toward higher modulus of elasticity in the low strain group, there is no statistical difference between the two groups (P=.10). These changes in strain could be directly related to changes in ECM or elastin content in the vessel wall. In particular, similar mechanical responses have been observed in vitro due to application of elastase to the vessel wall.228 Additionally, others have reported that expression of GAGs such as hyaluronic acid can be changed during an intimal hyperplastic response.229

The early reductions in mechanical strain demonstrated in this chapter have important implications to the relation between mechanical strain and IH in this model. Given that we do not expect significant IH to occur by the day 4 timepoint, it is possible that the early reductions in strain could be a result of important biological changes in the wall that occur as a result of the focal stenosis. In order to better understand the underlying cause of this reduction in strain, Chapter 7 will examine the temporal changes in IH and the acute (immediately post-op) changes in mechanical strain.

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Chapter 6: Objective 3A
Chapter 7: Objective 3B: Determine the temporal progression of intimal hyperplasia in the focal stenosis model

7.1 Introduction

The results presented in Chapter 5 (Objective 2) and Chapter 6 (Objective 3A) of this dissertation demonstrate compelling evidence for a temporal relationship between changes in wall strain and intimal hyperplasia in our murine model. In Chapter 6, we demonstrated that 100% of regions with low mechanical strain (<0.1) at day 4 went on to form IH by post-op day 28. In contrast, only 31% of regions with high mechanical strain (>0.1) went on to form IH. These findings suggest a promising link between changes in mechanical strain and IH.

From a mechanical standpoint, the reductions in mechanical strains seen at post-op day 27 (Chapter 5) were most likely due to the increased wall thickness, changes in diastolic diameter and/or changes in wall mechanical properties. Reduced strains observed at post-op day 4, however suggest a more mechanistic role of strain reduction in the formation of IH. Although it was not confirmed in the previous studies, we believed that it is highly unlikely that significant changes in wall thickness due to IH have occurred by post-op day 4, thus eliminating increased wall thickness as a key regulator of strain. An idea that is supported by previous literature in other murine models of IH. 230-232 In this chapter, we will assess IH formation via histology at day 4 to determine if the wall thickness has significantly changed by day 4 post-op.

A second possible cause of reduced strains at day 4 would be that placement of the suture immediately and directly causes reductions in mechanical strain. To assess this possibility, we measure strains immediately after focal stenosis creation and determine if reductions occur immediately upon focal stenosis creation. In this chapter, we aim to further understand the
relationship between oscillatory strain reduction and IH in order to present a clearer picture of the temporal progression of both mechanical strain and IH in this model.

### 7.2 Materials and methods

#### 7.2.1 Experimental design

The validated focal stenosis model used in the previous aims was once again used in this objective. Thirty wild-type mice (C57BL/6J, The Jackson Laboratory) were randomly split into sham control (10 mice) and experimental (20 mice) groups. The experimental timeline for all Aim 3B studies is shown in Figure 7-1.

![Figure 7-1: Experimental design for Aim 3B studies. Aim 3B animals were assessed via ultrasound at pre-op, immediately post-op and at post-op day 4. Animals were euthanized for histological analysis at day 4 to determine if significant IH is present at post-op day 4.](image)

#### 7.2.2 Ultrasound data acquisition

All sham and experimental animals were anesthetized and scanned using ultrasound 2-3 days prior to operation, immediately post-op and at post-op day 4 as described in section 5.2.3. Ultrasound cine loops were recorded at 2mm, 3mm and 4mm proximal to the carotid bifurcation on both the RCCA and LCCA. Data recorded in this Aim were collected using an MS700 ultrasound transducer and Visualsonics Vevo2100 system.

Each carotid was first visualized in longitudinal section to reveal the location of the bifurcation. At all time points, color Doppler cine loops were recorded in longitudinal section to
verify blood flow distal to the focal stenosis. After centering the probe on the carotid bifurcation, the probe was rotated 90 degrees to reveal a cross-sectional view of the artery. A stage micrometer was then used to move the probe 2mm, 3mm and 4mm with respect to the animal. At each location, 1000 frame cine loops were recorded at 534 frames per second for strain analysis. Ultrasound cross-sectional cine loops were analyzed using VevoVasc software using the same methodology described in Section 5.2.4.

7.2.3 Focal stenosis model
For experimental animals the same focal stenosis model employed in Chapter 5 was used (see section 5.2.2). For sham animals, the LCCA was surgically exposed following the same procedure used for experimental animals. After LCCA exposure, sham animal surgical sites were closed without manipulating the LCCA. All sham and experimental animals were scanned using ultrasound immediately post-op.

At post-op day 4, experimental, and sham animals were anesthetized and scanned via ultrasound for a final time. Animals were then transported to the O.R. and perfusion fixed as described in section 5.2.5.

7.2.4 Quantification of Intimal Thickness and Wall Thickness
In order to quantify wall morphology, a custom, semi-automated MatLab (Mathworks) script was developed to measure the cross-sectional area and average wall thicknesses of individual vascular layers. Sections of each vessel at 2, 3 and 4 mm proximal to the carotid bifurcation were stained using Masson’s Trichrome (see Appendix B for protocol). Trichrome sections were imaged on a Leica DMLB2 upright microscope fitted with a DFC 480 c-mount microscope camera (Leica Microsystems, Buffalo Grove, IL). The resulting images were loaded into MatLab. To determine lumen area, the images were converted to grayscale and
automatically thresholded to yield a black and white image. The lumen was selected and the pixel area was automatically quantified. Internal elastic lamina, external elastic lamina and outer vessel perimeters were then manually traced in MatLab. Cross-sectional areas of each region (in pixels) were then converted to physical areas (based on a scaled image taken on the same scope camera and objective). Wall thickness and intimal thickness were computed as described in section 5.2.5. Thickness values were averaged from 3 sections in each artery. Appendix D provides further details on the methods used to determine intimal and wall thicknesses.

7.2.5 **Assessment of cell type markers**

In order to determine which cell types are present in the arterial wall at day 4, a triple immunohistochemical stain was developed to assess 3 different cell types related to the vascular wall and IH. Myosin heavy chain, a marker of contractile smooth muscle cells, was stained along with CD31 (PECAM-1), a marker of vascular endothelial cells. Finally, the presence of leukocytes was assessed using CD45, an antigen common to all leukocytes. Briefly, tissue sections were rehydrated through several changes of xylenes and ethanols. Heat induced epitope retrieval was completed using a pH 6.0 Citrate buffer and pressure cooker. Sections were permeabilized with triton-x, blocked with normal goat serum and simultaneously treated with mouse anti-myosin heavy chain, rabbit anti cd-31 and rat anti-mouse cd45 overnight at 4°C. Sections were then simultaneously treated with Alexa-fluor 488 goat anti-rat, Alexa-fluor 568 goat anti-rabbit and Alexa-fluor 633 goat anti-mouse. Sections were then counter stained with Hoechst 33342 dye and coverslipped (for details of the staining process used here, see
Healthy mouse heart sections and intestine sections were run simultaneously as positive and primary delete controls.

Stained sections were imaged on a laser confocal microscope (Leica TCS SP5, Leica Microsystems, Buffalo Grove, IL) with 4 photomultiplier tubes set to sequential mode. Maximum projection images of sections were imaged at 40x and 63x oil where appropriate.

7.2.6 Assessment of cell proliferation
To assess cellular proliferation, sections were immunostained simultaneously for alpha smooth muscle actin and ki-67, markers of smooth muscle cells and proliferating cells respectively. Briefly, tissue sections were rehydrated through several changes of xylenes and ethanol. Heat induced epitope retrieval was completed using a pH 9.0 tris-EDTA buffer and pressure cooker. Sections were permeabilized with triton-x, blocked with normal goat serum and simultaneously treated with mouse anti alpha smooth muscle actin and rabbit anti ki-67 overnight at 4°C. Sections were then simultaneously treated with Alexa-fluor 488 goat anti-mouse and Alexa-fluor 568 goat anti-rabbit. Sections were then counter stained with Hoechst 33342 dye and coverslipped (for details of the staining process used here, see Appendix B). Mouse intestine controls were run simultaneously as positive and primary delete controls. Sections were imaged with confocal microscopy as described in section 7.2.5.

7.2.7 Assessment of glycosaminoglycan synthesis
To assess GAG synthesis, the protocol described by Shukla and colleagues was used. Briefly, tissue sections were rehydrated through several changes of xylenes and ethanol and permeabilized with triton-x. Negative controls were digested with hyaluronidase in a sodium acetate buffer at 37°C for 1 hr. Tissue sections were then blocked with goat serum and bovine serum albumin and incubated simultaneously with hyaluronic acid binding protein and rabbit
anti-mouse versican overnight at 4°C. Sections were then treated with Alexafluor 488 goat anti-rabbit and Alexafluor 568 Streptavidin for 1 hr at room temperature. Sections were counterstained with Hoechst dye and coverslipped. Sections were imaged with confocal microscopy as described in section 7.2.5.

7.2.8 Statistical Analyses
Data are presented as mean and standard error of the mean. All statistical analyses were completed in Sigmaplot version 11 (Systat Software Inc., San Jose, CA, USA). Statistical comparisons amongst spatial regions for strain, histological measurements and diameters were performed using a one way ANOVA followed by a Tukey post-hoc test. Differences between pre-op, post-op day 4 and post-op day 28 time points were performed using a repeated measures ANOVA. Differences between contralateral control and experimental artery were determined using a paired t-test (paired by animal). Statistical powers are reported for all negative statistical results. P<0.05 was considered significant for all tests.

7.3 Results
All animals survived to tissue harvest and ultrasound data acquisition for all regions and all time points was successful. Additionally, blood flow was evident distal to all focal stenoses at day 4 as assessed using color Doppler ultrasound. Strain data for all animals and regions was available and is reported below. Histology for half of the animals discussed in the methods section was sent to another lab for additional analysis. Thus, histology for 10 experimental animals and 5 sham animals is reported below.

7.3.1 Ultrasound strain analysis
Mean oscillatory wall strains in sham controls (sham LCCA), contralateral controls (experimental RCCA) and experimental LCCAs were consistent at pre-op (0.27 ± 0.01 vs 0.29 ±
0.01 vs 0.28 ± 0.01, p=0.50; Power: 0.05). Shortly after focal stenosis placement (post-op day 0), LCCA strains did not change compared to pre-op in either the sham or experimental group (sham: 0.24 ± 0.02, p=0.24 (Power: 0.12) vs. pre-op; experimental: 0.26 ± 0.01, p=0.43 (Power: 1.0)). At post-op day 4, experimental LCCA strains were significantly reduced (0.18 ± 0.01) compared to pre-op values (p<0.0001), sham controls (0.26 ± 0.01; p<0.001) and contralateral controls (0.24 ± 0.01; p<0.001). Strain data averaged by animal are shown in Figure 7-2.

Data were also analyzed by spatial region (2mm, 3mm and 4mm proximal to the bifurcation). No differences were found amongst the three spatial regions at any timepoint in either the sham control LCCA or the experimental LCCA. Experimental LCCA strains were reduced in all 3 regions at day 4 compared to pre-op, post-op day 0 and sham post-op day 4 data. Figure 7-3 shows the regional changes in mechanical strain.

![Graph showing average oscillatory wall strain](image)

**Figure 7-2: Average oscillatory wall strain.** Average wall strain at Pre-op, Post-op Day 0 and Post-op day 4. Averages are for Sham control (n=10), Contralateral RCCA control (n=20) and Experimental LCCA (n=20). * indicates p<0.001; n=10 in sham group and n=20 in contralateral control and experimental group.
Figure 7-3: Average regional oscillatory wall strains. Regional wall strains were consistent amongst the three regions assessed at all time points. * indicates p<0.05 vs. sham control. † indicates p<0.05 vs. Pre-op. ‡ indicates p<0.05 vs. post-op day 0. ; n=10 in sham group and n=20 in experimental group.

As in the data presented in Aim 3A, strains were significantly reduced in many animals at day 4. Figure 7-4 shows the changes in strain levels over time in the experimental animals vs. both controls. Individual data points (for each animal) are plotted to demonstrate progression of strain levels.

Figure 7-4: Progression of strain in control and experimental arteries. Strains in both control groups remain approximately constant throughout the study. Strains in experimental arteries, however, are reduced at day 4 post-op.
Figure 7-5: Histogram analysis of strain data at each time point. Strain levels were fairly consistent in all time points and groups except for the strain levels in the experimental animals at day 4 post-op. Dotted line through post-op day 4 data indicates the minimum control strain cut off used to analyze strain in the document.

7.3.2 Changes in wall cross-sectional area

Wall cross-sectional areas were measured at diastole and systole using ultrasound. Diastolic areas were the same in sham, contralateral and experimental arteries at pre-op (0.13 ± .02 mm² vs. 0.14 ± 0.02 mm² vs. 0.15 ±0.02 mm²; p=0.36; Power: 0.06). Similarly, systolic areas were the same in sham, contralateral and experimental arteries at pre-op (0.20 ± .03 mm² vs. 0.22 ± 0.03 mm² vs. 0.22 ±0.03 mm²; p=0.17; Power: 0.18). Sham control arteries remained unchanged from pre-op at both post-op day 0 and day 4. At post-op day 0, contralateral control diastolic and systolic areas were both significantly increased compared to pre-op (0.18 ± 0.04 mm², p=0.002 and 0.26 ± 0.04 mm², p<0.001 respectively). Conversely, experimental diastolic and systolic areas were significantly decreased at post-op day 4 compared to pre-op (0.12 ± 0.02 mm², p=0.17 and 0.17 ± 0.03 mm² p<0.001 respectively). At post-op day 4, contralateral control diastolic and
systolic areas returned to their pre-op levels (0.15 ± 0.04 mm², p=0.80 vs. pre-op; Power: 0.91 and 0.22 ± 0.06 mm², p=0.99 vs pre-op, Power: 0.80; respectively). Experimental artery diastolic areas increased back to pre-op levels at day 4 but still had significantly reduced systolic areas (0.13 ± 0.04 mm², p=0.32 vs. pre-op, Power: 0.58; and 0.17 ± 0.05 mm², p<0.001 vs pre-op; respectively).

Figure 7-6 shows the changes in diastolic and systolic area over time throughout the Aim 3B experiments.

Figure 7-6: Changes in arterial cross-sectional area throughout the experiment A) Changes in diastolic cross-sectional area over time. B) Changes in systolic area over time. * indicates p<0.05; n=10 in sham group and n=20 in contralateral control and experimental group.
**7.3.3 Histological analysis of intimal thickness**

Masson’s trichrome stain on day 4 tissues was used to assess the mean intimal thickness, and wall thickness for all vessels. Figure 7-7 shows representative images of vessels from each group with the manual tracing overlaid.

Intimal thickness at day 4 was consistent amongst the three groups with no statistical differences amongst the groups. Intimal thickness for sham, contralateral and experimental arteries were 2.5 ± 0.3 μm, 2.1 ± 0.3 μm and 2.6 ± 0.1 μm respectively (p=0.12; Power: 0.25). Intimal thicknesses are plotted in Figure 7-8A. Additionally, no significant differences in total wall thickness were observed between control and experimental arteries. Sham, contralateral and experimental arteries had total wall thicknesses of 53.9 ± 3.5 μm, 64.6 ± 4.8 μm and 58.3 ± 3.4 μm respectively (p=0.69; Power: 0.05; Figure 7-8B).

![Figure 7-7: Use of Masson’s Trichrome to find thickness of vessel layers. Representative sections are shown with overlaid green tracings of the two lamina and the outer extent of the vessel. Wall thickness values were computed using these tracings. A) Sham control, B) Contralateral control, C) Experimental LCCA. Scale bar: 100μm](image)
7.3.4 Relationship between strain and diameter
Strain magnitudes were divided into high and low strains in two different ways. In Figure 7-9 A-C (left), strain was cut off based on the lowest observed control strain value (0.16, see dotted lines in Figure 7-5). A trend toward increased diastolic and systolic areas (Figure 7-9B, C) was observed between pre-op and post-op day 4. This trend was not present in the high strain group. Strain was also cut off at 0.1 (the cutoff used in Aim 3A of this work). The lower cutoff clearly exacerbated the increase in areas. Given that only 2 animals fell below the 0.1 cutoff, statistical measures were not employed on areas.
Figure 7-9: Differences in cross-sectional area between low and high strain groups. A) Histogram of day 4 strain values with strain cut off at 0.16 (minimum control value). B) Diastolic areas for low strains (<0.16) and high strains (>0.16). C) Systolic areas for low strains (<0.16) and high strains (>0.16). D) Histogram of day 4 strain values with strain cut off at 0.10 (value used in aim 3A). E) Diastolic areas for low strains (<0.1) and high strains (>0.1). F) Systolic areas for low strains (<0.1) and high strains (>0.1).

7.3.5 Immunohistochemical analysis

The medial layer of the vessel appeared normal at day 4, with consistent expression of myosin heavy chain throughout. Similarly, consistent expression of CD-31 was visible along the luminal surface suggesting that the endothelium was intact at day 4. Interestingly, cells expressing CD-45 were also observed at the luminal surface in 40% of experimental arteries.
examined but not in control arteries. At some points, these cells appear to be disrupting the layer of CD-31 positive cells, while other regions demonstrate co-expression of CD-31 and CD-45. Based on IHC analysis of only 1-2 sections per artery, however, there does not appear to be a correlation between CD45 expression and low strain magnitude. Figure 7-10 shows a representative image of an experimental artery stained for expression of all three markers.

**Figure 7-10: Myosin heavy chain, CD-31 and CD-45 expression.** A) Hoechst dye indicating nuclei (blue), B) Myosin Heavy Chain indicating contractile smooth muscle cells (green), C) CD-31 a marker expressed by endothelial cells, D) CD-45, an antigen common to leukocytes, E) Merge of all images. All scale bars: 25 μm.

In addition to cell types, expression of Ki-67, a marker of proliferating cells was assessed. Ki-67 expression was found in the vessel wall or on the luminal surface in 90% of animals assessed (n=10). Qualitative assessment of Ki-67 positive cells suggests that cells are recruited to both the lumen and adventitial layer of the vessel wall. Ki-67 positive cells were not found at the wall of any control sections.
Figure 7-11: Ki-67 and αSMA expression in control and experimental arteries. A) Sham control artery, B) Contralateral control artery, C) Experimental artery. Immunofluorescence stain show nuclei in blue (Hoechst 33342), alpha smooth muscle actin in green, and ki-67 in red. White arrows indicate cells at the vessel wall expressing ki-67.
Finally, synthesis of the GAGs hyaluronic acid and versican was assessed. No versican synthesis was observed in arteries in any of the experimental groups. HA expression was seen in experimental animals but no obvious qualitative differences in expression levels were observed between control and experimental arteries (Figure 7-12).

Figure 7-12: Hyaluronic acid and versican expression in control and experimental arteries. A) Baseline (no surgery) control artery. B) Sham control artery. C) Contralateral control artery. D) Experimental artery. Stain shows nuclei (Hoechst) in blue, versican in green and hyaluronic acid in red.
7.4 Discussion

In the earlier chapters of this dissertation, we demonstrated a promising relationship between early reductions in mechanical strain and the eventual formation of intimal hyperplasia. The major assumption made in those chapters, however, was that significant intimal hyperplasia had not formed by the day 4 timepoint. In this chapter, we both verify our assumption from previous chapters and enhance our understanding of early changes in the vessel wall at the cellular level.

In support of our findings in chapter 6, we observed a significant reduction in mechanical strain at day 4 compared to pre-op in our experimental group but no changes in the control groups. Interestingly, the reductions in strain observed in this study were not as severe as those seen in the chapter 6 study (with day 4 strains in this chapter of 0.18 ± 0.01 and the chapter 6 strains of 0.11 ± 0.02). We suspect that this difference between the two studies is likely due to biological variability and variability due to different surgeons performing the surgery. Another interesting difference between this study and the previous study is that changes in strain here appear to be due to decreases in systolic area rather than increases in diastolic area. Despite the minor differences between the day 4 outcomes between this study and the chapter 5 study, it remains clear that reductions in strain occur at day 4 in this model.

In addition to pre-op and day 4 data, we also assessed strains immediately after creating the stenosis. Reductions in oscillatory strain were not observed immediately after placement of the stenosis. This suggests that reductions in strain are not an immediate mechanical effect of focal stenosis creation, but rather a change in mechanical properties caused a biological reaction to focal stenosis creation.
Diastolic and systolic areas were also assessed using ultrasound. Data in Figure 7-6 demonstrates that, shortly after stenosis formation, the experimental cross-sectional area at both diastole and systole are decreased while the contralateral control areas are increased. By day 4, however, the contralateral control arteries return to their baseline levels while the experimental systolic area remains reduced. To better compare the data from this aim to the data in Aim 3A, area data was stratified using a strain cutoff. In Figure 7-9, strains were cut off using the lowest control artery strains at day 4, as well as the 0.1 cutoff used in Aim 3A. These data show a similar trend toward increase in diastolic area at day 4 to that found in Aim 3A. Additionally, systolic areas are seen to decrease in low strain groups at day 4. These data demonstrate the importance of assessing oscillatory wall strain at day 4. Although vessel diameters may vary from animal to animal and need to be assessed before IH formation. Strain, on the other hand, can provide evidence of changes in wall properties based on data from only one time point.

Intimal and wall thicknesses were assessed at day 4 to improve our understanding of the temporal progression of IH in this model. The data in this chapter support our assumption in previous chapters that IH has not formed at the day 4 timepoint in this model. No significant differences in wall thickness or intimal thickness were observed at day 4 amongst the three groups. We do, however, observe differences between control arteries and experimental arteries at the cellular level. Recruitment of leukocytes to the vascular wall appears to occur at or before the day 4 timepoint as well as some interruption in the expression of CD-31 at the arterial lumen; both hallmark signs of the beginnings of IH. Additionally, staining for Ki-67 demonstrates some proliferating cells in the vicinity of the vessel wall.
The lack of significant IH at day 4 observed in this chapter, combined with the day 4 reductions in strain observed in chapter 5 and this chapter suggest an important mechanistic role for strain reduction in the formation of IH. Since we demonstrate that wall thickness increases have not occurred at day 4 and other research suggests that pressure reductions will not occur in the region proximal to the stenosis, the culmination of our data suggests that early changes in wall mechanical properties drive the reductions in strain we observed. By observing these changes the methods described herein have potential to help predict the formation of IH in this model.
8 Conclusions and Future Work
8.1 Conclusions

Coronary and peripheral artery diseases remain a major cause of morbidity and mortality in the United States.\(^1\) Although the underlying causes of these diseases remain largely unknown, evidence suggests that a major initiating factor of these diseases is Intimal Hyperplasia (IH).\(^3\)\(^,\)\(^6\)\(^-\)\(^9\) A number of researchers have correlated local differences in the mechanical environment with neointimal formation and arteriosclerosis.\(^14\)\(^,\)\(^19\)\(^,\)\(^20\) Despite the extensive evidence suggesting the role of shear stress in IH formation, others have demonstrated that shear based explanations alone fail to predict IH.\(^27\)\(^,\)\(^28\) In this dissertation, we demonstrate that arteries form IH in response to changes in a different mechanical force, oscillatory wall strain. Herein, we hypothesized that reductions in oscillatory wall strain precede the formation of IH in our murine model of IH.

In order to test this hypothesis, we developed a novel method to noninvasively measure mechanical strains on the murine arterial wall. Although the murine model is attractive as a research tool, mouse studies are complicated by both the small vessel size and high heart rates seen in the murine model. To overcome these issues, we first adapted the combination of high frequency ultrasound with speckle tracking from the cardiac literature \(^40\)\(^-\)\(^42\) for use in the measurement of oscillatory wall strains in the mouse artery.\(^44\) In the first objective of Aim 1, we used an \textit{in vitro} vascular phantom to show a correlation between ultrasound-based strains and a previously validated optics-based strain measurement system.\(^181\)\(^,\)\(^183\) To test the \textit{in vivo} measurement capabilities of our strain measurement approach, in objective 1B, we applied our approach to a mouse model of aortic aneurysms. In this \textit{in vivo} study, we demonstrated the ability of our measurement approach to measure significant changes in aortic strains due to a vascular disease in the murine model.

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Chapter 8: Conclusions and Future Work
The methods and data presented in this dissertation provide compelling evidence suggesting a correlation between early reductions in mechanical strain and the eventual formation of IH. The animal model discussed herein and developed by Tao et al.\textsuperscript{37} is unique in that it does not directly injure or occlude any blood vessel unlike most other models.\textsuperscript{230-232} Interestingly, we still reveal a similar intimal hyperplastic response to those studied previously.\textsuperscript{161, 230, 231} Given that both the original model study,\textsuperscript{37} and subsequent study\textsuperscript{238} using this model both report that only a subpopulation of mice given a focal stenosis go on to form IH, we suspect that biological variation amongst the animals plays a role in clinical outcome at day 28. Herein we suggest that changes in the mechanical environment, particularly circumferential wall strain, play a key role in determining neointimal formation.

We found that reductions in strain at 27 days after focal stenosis occur in the regions of the carotid artery with the most hyperplasia, while those with higher strains had less neointima formation. Even though this phenomenon supports our hypothesis, the significant increase in wall thickness seen at day 28 likely played a role in causing strain reductions. From a mechanical standpoint, we expect that one of three changes will result in mechanical strain reduction. Increases in wall thickness directly increase the load bearing cross-sectional area over which wall forces are applied; our data in Aim 2 demonstrate that increased cross-sectional area has occurred at day 28. Decreases in luminal pulse pressure could also play a role in decreasing vascular strain. Since strains measured in this studied are measured proximal to the focal stenosis, we do not expect that there is any change in pressures in these animals. Finally, an increase in wall stiffness could cause a decrease in wall strain. At day 27, we expect this to be true as well due to the reported increase in collagen content in our model.\textsuperscript{37} To assess whether
the strain reductions were caused by increased wall thickness or by other factors, we assessed
strains and IH at earlier time points.

Herein, we probed the temporal changes in both IH and mechanical strain in the focal
stenosis model. In Objective 3A, we re-examined histological data from Aim 2 and compared
histological outcome at day 28 to strains at just 4 days after focal stenosis creation. The results of
this objective demonstrated that the reductions in strain we saw at day 28 had already occurred
at day 4 post-op. This finding suggests that the changes in strain may precede the formation of
IH. The results of Objective 3A also revealed that, on a region by region basis, some regions had
low strain at day 4 while others had higher strains (close to baseline). Further, we discovered
that there was statistically lower IH formation at day 28 post-op in regions with high day 4
strains vs. regions with low day 4 strain.

The final piece of evidence needed to fully support the overall hypothesis of this
dissertation was to show that IH does not form by day 4 post-op. In objective 3B we examined
both neointimal formation at post-op day 4 and the changes in strain found immediately post-
op. Our quantification of intimal and wall thickness at day 4 post-op demonstrated no
significant changes in gross wall morphology at day 4. These data suggest that the reductions in
strain both precede IH in this model and are not due solely to increases in wall thickness.
Additionally, post-op day 0 data (Objective 3B) demonstrated that no immediate decrease in
strain occurs after focal stenosis creation. This evidence suggests that changes in wall
morphology between post-op day 0 and day 4 results in the strain reductions we find in this
model.
Previous models of IH in the mouse model have identified that cellular proliferation and inflammatory cell recruitment to the region are the events that initiate eventual intimal hyperplasia.\textsuperscript{14, 237} In this study, we find that, similar to other studies, leukocytes (CD45+ cells) are recruited to the endothelial wall by day 4. This suggests that the beginning of an inflammatory response is occurring. One group has demonstrated that a circulating cell type, the fibrocyte, which is known to express cd45 is recruited to the vascular wall at the onset of intimal hyperplasia.\textsuperscript{239} Additionally, Varcoe \textit{et al.} identified that these fibrocytes also expressed cd31 when recruited to the vascular wall, a phenomenon we observed in Aim 3B.\textsuperscript{240} Similarly, we observed cells in the vascular wall that co-expressed CD31 and CD45.

To support our cellular findings, we also examined ki-67 expression to probe proliferation at the vessel wall. Our findings demonstrate that the majority of experimental arteries at day 4 contain cells that express Ki-67, a marker of proliferating cells. This finding is significant because cellular proliferation is considered a hallmark of intimal hyperplastic responses.\textsuperscript{14, 86} In addition to cellular recruitment, we assessed expression of GAGs in the vascular wall at day 4. Looking at both versican and hyaluronic acid (HA) expression, we observed no obvious differences in the expression of either GAG at day 4. Chajara \textit{et al.} previously demonstrated that rat aortas express HA in the neointima 14 days after balloon injury as well as expression in the adventitia at baseline.\textsuperscript{229} Given that our day 4 histology was completed prior to neointima formation, we did not see HA synthesis at the wall. Versican expression was also assessed. At day 4, we did not observe versican expression in any experimental or control arteries. This suggests that versican expression does not occur in this model or that it occurs downstream of strain reduction.
The clinical relevance of this model differs from that of previous models in that it does not focus on deliberate endothelial injury as the underlying cause of IH. Many previous studies focus on the effects of either balloon angioplasty or wire injury (or denudation) of the endothelium on subsequent IH. Interestingly, we and others have found that IH forms without causing any apparent injury to the endothelium. As a result, the relevance of both this model and the subsequent work in this dissertation is mainly to the underlying mechanisms of IH, without endothelial injury. Based on the data at hand, we suspect that reductions in luminal flow lead to structural changes in the vessel wall between the focal stenosis creation and day 4 post-op. These changes result in oscillatory strain reduction and eventually lead to leukocyte infiltration, cellular proliferation and intimal hyperplasia.

![Figure 8-1: Summary of the results of this dissertation.](image)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Pre-op</th>
<th>0</th>
<th>4</th>
<th>8</th>
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<td>↓</td>
<td>↓</td>
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<tr>
<td>CD45 expression</td>
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<td>*</td>
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<td></td>
<td></td>
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<tr>
<td>Ki-67 expression</td>
<td>↔</td>
<td>*</td>
<td>↑</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Key:
- Baseline levels
- Increased
- Decreased
- Not assessed

Figure 8-1: Summary of the results of this dissertation.
8.2 Limitations

The experiments in this dissertation are limited by a number of factors. First and foremost, the present study did not directly decouple the effects of other mechanical forces on the wall from mechanical strain. Namely, we did not compute wall tensile stress or wall shear stress in these studies. In order to assess wall stress, it would have been necessary to measure luminal pressures in the carotids during the study. Unfortunately, given the relatively large size of the smallest commercially available pressure catheters (0.9 French size/300 μm) compared to the inner diameter of the mouse carotid artery (300-400 μm),225 insertion of a pressure catheter would likely impede or change blood flow in the artery. Despite this limitation, others have assessed systemic pressure changes in similar flow reduction models and have not observed significant pressure changes in the proximal region.235 Blood flow through the stenosed blood vessel was also not assessed. In the published study describing the focal stenosis model, an average of 85% reduction in blood flow was reported immediately after stenosis.37 We have not, however, determined if a correlation exists between different levels of blood flow and resultant IH in this model. Given that all of the comparisons made in this study are between animals of the same age and strain, with focal stenoses made by the same lab team, it is not likely that measurable differences in blood flow through the arteries would be observed.

The results in Objective 1A demonstrated a large difference between strains measured via ultrasound at the tubing lumen and strains measured via HDM at the tubing outer wall. Theoretical analysis of these relations assuming constant tubing wall volume demonstrated this difference in strains was largely due to the thickness of the tubing. Although others have used
thin wall assumptions to simplify modelling of carotid arteries\textsuperscript{241} our results suggest that some arteries must be considered as thick walled vessels depending on the ratio of vessel wall thickness to radius. In our silicone tubing vessel analog, the ratio of wall thickness to outer radius was 0.25 while sham arteries at day 4 had a ratio of only 0.13±0.02 (by histology). This much smaller ratio would likely allow for accurate determination of wall strains using thin wall assumptions. In our day 28 arteries, however, the ratio of wall thickness to wall radius averaged 0.46±0.13. In the case of day 28 arteries, thick wall assumptions should apply. Future studies should carefully consider the wall thickness of arteries with IH to determine if thin wall assumptions apply. Despite this limitation in approach, we expect the luminal surface of the vessel, the region where we measured strain with ultrasound, to have the highest wall stress and strain. \textsuperscript{242}Limitations to our strain measurement methodologies are also recognized. Only 1 dimensional strain was assessed in this study. Given the lack of contrast between the outer wall of the carotid artery and the surrounding tissue, we were only able to measure luminal strains in the circumferential direction and longitudinal strains were not assessed. Given that the circumferential strain is known to be the most prominent strain on the vessel wall, it is likely that the greatest changes in strains will be observed in the circumferential direction. Finally, our ultrasound approach is limited by our ability to orient the probe perpendicular to the artery in 3 dimensions. Variations in probe angle can lead to smaller or larger diameter measurements using ultrasound, which may increase animal to animal variability in diameter measurements. Since we measure oscillatory strains based on systolic and diastole without changing probe angles, we do not suspect this variation significantly affected strain our measurements.

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Chapter 8: Conclusions and Future Work
8.3 Future work

In this dissertation, we developed a novel method of measuring murine arterial strains noninvasively and demonstrate a promising correlation between oscillatory strain reduction and IH formation in this model. We identified that changes in wall tensile stress and wall shear stress may also play a role in IH formation in this model. Consequently, it will be important in future studies to directly assess these mechanical factors and determine their relation to IH outcome in this model. As a result of these limitations, a crucial future step would be to measure pressure simultaneously with strain measurements. Since we believe that insertion of a pressure catheter into the carotid would result in extreme changes in local hemodynamics, simultaneous systemic pressure recordings should be used (in either the aorta or femoral artery) to estimate carotid pressure. Additionally, future studies should use either pulsed wave Doppler ultrasonography or a flow probe to measure flow through the carotid artery.

In the results of this dissertation we identified that changes in mechanical strain, particularly those driven by increased diastolic area, lead to IH formation. This finding suggests that compositional changes in the arterial wall are causing changes in its mechanical properties. The major component of the arterial wall that contributes to its ability to distend under pressure is elastin. The mechanical results presented herein suggest that, assuming luminal pressure does not change significantly, the stiffness of the artery may be increasing in the first few days after creation of the focal stenosis. Interestingly, in vitro research into abdominal aortic aneurysms has demonstrated that elastin degradation assays result in increased vascular stiffness and increased vessel diameters. This similarity between in vitro elastin degradation and our in vivo results suggests a mechanism that may drive strain changes in our model.
Future studies should look in greater detail into the structural changes in elastin in our model of IH. Others have accomplished this using multi-photon microscopy and have demonstrated that fibers can be extracted and angles can be quantified. Application of such a tool to our model could allow for direct comparison of elastin structural changes to reductions in mechanical strain. In collaboration with Yanhang Zhang, Ph.D. at Boston University, we have begun to analyze the elastin and collagen structure of blood vessels from our murine model using two-photon microscopy (Figure 8-2). The results of these preliminary analyses demonstrate that elastin structure at the vessel’s medial surface appears to be less organized at day 4 in experimental animals compared to baseline arteries. Others have demonstrated that inhibition of matrix mettaloproteinases (MMPs) prevents elastin breakdown and minimizes IH in arteriovenous grafts. This evidence suggests that release of MMPs from leukocytes recruited to the vessel wall may result in the elastin breakdown observed by the Zhang lab; which could produce the strain reductions presented in this dissertation. Future studies should focus on quantifying the elastin organization and orientation using two-photon microscopy images.
In addition to assessment of elastin structure, the mechanisms behind IH in this model could be better understood by determining the point at which IH cannot be reversed. Given that our results suggest that creating a focal stenosis leads to structural changes and mechanical strain reductions and leads to IH, it may be important to understand if removal of the suture at day 4 would return the artery to its initial strain levels and if removal of the suture would prevent formation of IH at day 28.

Another major finding in this dissertation is the presence of leukocytes and proliferating cells at the vessel wall as early as day 4. Since the immunohistochemical analyses presented herein are decidedly preliminary, it will be necessary for future studies to analyze the cellular activity in greater detail. In particular, regional differences in leukocyte recruitment (differences along the length of the vessel) should be assessed. Additionally, leukocytes and proliferating cells should be counted in each region of the vessel and correlated with oscillatory wall strain levels. This can be accomplished by counting nuclei that either co-express Ki67 or which are

Figure 8-2: Two-photon microscopy analysis of medial elastin and collagen View of the internal surface of a murine artery showing collagen and elastin structure. A) Baseline artery demonstrating wavy, structured elastin structure and B) Day 4 experimental artery showing less structured elastin interrupted by collagen. Tissue processing and image acquisition courtesy of Ming-Jay Chow, Raphael Turcotte and Yanhang Zhang Ph.D. from Boston University

Chapter 8: Conclusions and Future Work
surrounded by CD45 staining. Future work should also aim to assess the specific cell types seen proliferating at the wall and the specific type of leukocytes seen at the wall (i.e. markers that identify macrophages such as CD68).

Further, this dissertation could provide the groundwork for future therapies for treating IH. The data herein suggest that mechanical strain reductions play a role in IH formation. Given the elastin degradation observed by the Zhang lab using two-photon imaging, an important first therapeutic step would likely be to increase the cyclic stretch on the vessel wall by either increasing elastin content in the vascular wall or providing mechanical support to the vessel via some other means, such as an external cuff. A number of previous studies have demonstrated that external reinforcement of vein grafts can reduce intimal hyperplasia and medial thickening.\textsuperscript{246-249} The data presented herein suggest that by adding external support to arteries may reduce IH as well. Additionally, our data suggest that the compliance of the external supporting the vessel may be important. By changing the mechanical stretch applied to the vessel wall, our data suggest that IH formation could be reduced.

Finally, the results of this study could provide the foundation for future clinical applications. Ultrasound imaging is commonly used in the clinical setting to assess patients with vascular diseases. The approach described herein could be used on top of existing clinical US scans to provide noninvasive measurements of arterial wall strains. The ability to determine the structural properties of arteries based on noninvasive strain measurements could be applied as a first level determination of susceptibility to IH and the vascular diseases that result from it. Vessels with inherently low strain could have altered mechanical properties that make them prone to failure. In the work described herein, we demonstrate that strains below around 10 -
14% in the murine model may cause susceptibility to IH. If a similar strain threshold level could be determined in human arteries, this parameter could provide additional data to physicians and aid in determination of the appropriate vascular intervention. If this correlation exists in human arteries, it may allow for more efficient prediction of arterial susceptibility to occlusion. For example, patients with low oscillatory wall strain will likely need an intervention to overcome the rapid progression of IH. Patients with high oscillatory wall strain may not need an immediate intervention. The relationships demonstrated in this dissertation could be used to improve the diagnosis of vascular disease.
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Appendix B: Staining protocols and controls
Masson’s Trichrome

This protocol was provided courtesy of the WPI Gateway Park histology core.

Biebrich Scarlet Acid Fuchsin stock solution (Red)

Biebrich scarlet aqueous 1% 90.0ml
Acid Fuchsin aqueous 1% 10.0ml
Glacial Acetic Acid 100% 1.0ml

Biebrich scarlet aqueous 1%

Biebrich Scarlet 1.0gm
DH2O 99.0ml

Acid Fuchsin aqueous 1%

Acid fuchsin 1.0gm
DH2O 99.0ml

Phosphomolybdic-Phosphotungstic Acid Solution (Yellow)

Phosphomolybdic acid 5.0gm
Phosphotungstic acid 5.0gm
DH2O 200.0ml

Aniline Blue solution (Blue)

Aniline blue 2.5gm
Glacial Acetic acid 100% 2.0ml
DH2O 100.0ml

1% Glacial Acetic Acid solution

Glacial acetic acid 100% 1.0ml
DH2O 100.0ml

Hematoxylin Solution A

Hematoxylin crystals 1.0gm
95% Alcohol 100.0ml

Hematoxylin Solution B

Ferric Chloride, 29% aqueous 4.0ml
DH2O 95.0ml
Hydrochloric acid, concentrated 1.0ml

Ferric chloride 29%
- Ferric chloride 29.0gm
- DH2O 100.0ml

Weigerts Iron Hematoxylin solution
- Equal parts of solution A and B

Run a control slide.
- Control tissue: Heart, uterus, skin.

Safety equipment: Work under a hood with lab coat, gloves, and glasses.
1. De-paraffin slides in xylene (1) 2 minutes (re-use)
2. De-paraffin slides in xylene (2) 2 minutes (re-use)
3. Clear slides in 100% alcohol 2 minutes
4. Clear slides in 100% alcohol 2 minutes
5. Hydrate slides in 95% alcohol 2 minutes
6. Hydrate slides in running water 5 minutes
7. Bouin’s at 60 degrees Celsius 1 hour (re-use)
8. Wash in running water 10 minutes
9. Weigert’s Hematoxylin (make fresh) 10 minutes (dump)
10. Wash in running water 10 minutes
11. Biebrich Scarlet Acid Fuchsin 10 minutes (re-use)
12. Rinse in running water 2 minutes
13. Phosphomolybdic / Phosphotungstic acid 10 minutes (re-use)
14. No rinse, goes directly into blue
15. Aniline Blue 10 minutes (re-use)
16. Rinse in running water 1-2 minutes
17. 1% Glacial Acetic Acid 5 minutes (re-use)
18. Dehydrate in 95% alcohol 1 minute
19. Dehydrate in 95% alcohol 1 minute
20. Dehydrate in 100% alcohol 1 minute
21. Dehydrate in 100% alcohol 1 minute
22. Dehydrate in 100% alcohol 5 minutes
23. Clear in xylene (4) 2 minutes (re-use)
24. Clear in xylene (5) 5 minutes (re-use)

Results:
- Nuclei- **Black**
- Cytoplasm, keratin, muscle fiber and intercellular fibers- **Red**
Collagen- **Blue**
Weigerts Iron Hematoxylin:
   Equal parts of solutions A and B – 20ml and 20ml for tall glass staining coplin jar.
**Ki-67 and alpha Smooth Muscle Actin**

**Control tissue**
- Mouse intestine (+)
- Use primary delete negative control

Sample positive control below (left) and negative control (right) with alpha smooth muscle actin in green, Ki-67 in red and nuclei in blue

---

**Deparaffinize**

**Materials**
- Xylene
- Ethanol
- DI water
- Tap water

**Procedure**
1. Xylene 1 for 3 minutes
2. Xylene 2 for 3 minutes
3. 100% ethanol for 3 minutes
4. 100% ethanol for 3 minutes
5. 95% ethanol for 3 minutes
6. 70% Ethanol for 3 minutes
7. 50% ethanol for 3 minutes
8. Rinse in running tap water for 5 minutes

**Heat induced epitope retrieval**

**Materials**
- Tris-EDTA buffer (pH 9.0)
1.21 grams Tris HCl (MP Biomedical, 816124)
0.37 grams Ethylenediaminetetraacetic acid (EDTA. Sigma, ED4SS)
1000 mL Distilled water
0.5 mL Tween 20

Mix Tris, EDTA and DI water. pH should be near 9.0 but adjust as necessary. Add Tween 20

**Procedure**

While deparaffinizing slides, bring 1L Tris-EDTA buffer to a boil in the unsealed pressure cooker. Once buffer is at a rolling boil, transfer slides to a metal slide rack and place in the boiling buffer solution. Seal the pressure cooker and wait until it reaches full pressure (gas escapes pressure release valve). Time 3 minutes from onset of full pressure. After 3 minutes, transfer pressure cooker to sink, release pressure and run cold water into pressure cooker for 10 minutes.

**Staining protocol**

**Materials**

- Phosphate buffered saline (PBS)
- 0.25% Triton-X 100 in DI H2O
- 5% Goat serum in PBS
- 3% Goat serum in PBS
- Primary:
  - Mouse anti Alpha, smooth muscle actin monoclonal 1:50 (Abcam,AB7817)
  - Rabbit anti Ki-67 monoclonal 1:100 (Sigma,AB16667)
  - In 3% goat serum
- Hoechst dye 1:6000 in PBS
- Secondary:
  - Alexa-fluor 488 goat anti-mouse 1:400 (Invitrogen A11029)
  - Alexa-fluor 568 goat anti-rabbit 1:400 (Invitrogen A11036)
  - In 3% goat serum
- Cytoseal-60

**Procedure**

1. Wash 3x in PBS for 5 mins
2. Permeabilize with 0.25% Triton X for 20 mins
3. Block with 5% goat serum in PBS for 30 mins
4. Incubate in primary antibody overnight at 4 degC
5. Wash 3x in PBS for 5 mins
6. Incubate in secondary antibody for 1 hr at room temperature
7. Wash 3x in PBS for 5 mins
8. Counterstain with Hoechst for 5 minutes
9. Wash 3x in PBS for 5 mins
10. Coverslip with cytoseal-60
**Hyaluronic acid and Versican**

**Control tissue**
- Mouse heart valve (+)—Use longitudinal sections of the mouse heart
- Use primary delete negative control

Sample positive control below (left) and negative control (right) with versican in green, hyaluronic acid in red and nuclei in blue

---

**Deparaffinize**

**Materials**
- Xylene
- Ethanol
- DI water
- Tap water

**Procedure**
1. Xylene 1 for 3 minutes
2. Xylene 2 for 3 minutes
3. 100% ethanol for 3 minutes
4. 100% ethanol for 3 minutes
5. 95% ethanol for 3 minutes
6. 70% Ethanol for 3 minutes
7. Rinse in running tap water for 5 minutes

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**Staining protocol**

**Materials**
- Control tissues: Artery (+) and (-), Heart with valves visible (+) and (-)
- PBS
- 5% Goat serum in PBS
- Sodium acetate buffer
  - NaOAc 50 mM (0.41%)
• NaCl 0.15 M (0.87%)
• pH 6.0 in D.I. water

- Primary hyaluronidase (-) control
  - 20 units/mL Hyaluronidase from Bovine testes (Sigma, H3506)
  - In Sodium Acetate Buffer

- Primary:
  - Hyaluronic acid binding protein-biotinylated 3 ug/mL, 1:30 (Sigma, H9910)
  - Rabbit anti-mouse Versican 1:150 (Millipore, AB1033)
  - 0.1% BSA, 3% Goat serum in PBS

- Secondary:
  - Alexa-fluor 488 goat anti-rabbit 1:400 (Invitrogen, A-11008)
  - Alexa-fluor 568 Streptavidin 1:400 (Invitrogen, S-11226)
  - 0.1% BSA, 3% goat serum in PBS

- Hoechst 33342 dye 1:6000 in PBS
- Cytoseal-60

**Procedure**
1. Wash 3x in PBS for 5 mins
2. Permeabilize with 0.25% Triton X for 20 mins
3. Wash 3x in PBS for 5 mins
4. Perform Enzyme digestion of HA in negative controls (Leave positive controls in Sodium acetate buffer) for 1 hr at 37 degC
5. Wash 3x in PBS for 5 mins
6. Block with 5% goat serum/1% BSA in PBS for 1 Hour
7. Incubate in primary antibody overnight at 4 degC
8. Wash 3x in PBS for 5 mins
9. Incubate in secondary antibody for 1 hour at room temp
10. Wash 3x in PBS for 5 mins
11. Counterstain with Hoechst for 5 minutes
12. Wash 3x in PBS for 5 mins
13. Coverslip with cytoseal

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Appendices
Myosin Heavy Chain, CD-31 and CD-45

Controls

Intestine control (CD-45 in yellow shows up clearly) Positive control (right) and primary delete control (left)

Heart control (Myosin heavy chain in green and CD-31 in red). These two stains are clearly visible in the blood vessels in the heart. Note that surrounding myocardium is not stained.

Deparaffinize

Materials

- Xylene
- Ethanol
- DI water
- Tap water

Procedure

1. Xylene 1 for 3 minutes

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Appendices
2. Xylene 2 for 3 minutes
3. 100% ethanol for 3 minutes
4. 100% ethanol for 3 minutes
5. 95% ethanol for 3 minutes
6. 70% Ethanol for 3 minutes
7. 50% ethanol for 3 minutes
8. Rinse in running tap water for 5 minutes

Antigen retrieval

Materials
- Citrate Buffer (1mM Citric Acid, 0.05% Tween 20, pH 6.0)
  - Citric acid (anhydrous) 1.92 g
  - Distilled water 1000 ml

Mix to dissolve. Adjust pH to 6.0 with 1N NaOH and then add 0.5 ml of Tween 20 and mix well. Store this solution at room temperature for 3 months or at 4°C for longer storage.

- Pressure cooker

Heat induced epitope retrieval

Materials
- Citrate Buffer (1mM Citric Acid, 0.05% Tween 20, pH 6.0)
  - 1.21 grams Tris HCl (MP Biomedical, 816124)
  - 0.37 grams Ethylenediaminetetraacetic acid (EDTA. Sigma, ED4SS)
  - 1000 mL Distilled water
  - 0.5 mL Tween 20

Mix Tris, EDTA and DI water. pH should be near 9.0 but adjust as necessary. Add Tween 20

- Pressure cooker

Procedure
- While deparaffinizing slides, bring 1L Tris-EDTA buffer to a boil in the unsealed pressure cooker. Once buffer is at a rolling boil, transfer slides to a metal slide rack and place in the boiling buffer solution. Seal the pressure cooker and wait until it reaches full pressure (gas escapes pressure release valve). Time 3 minutes from onset of full pressure. After 3 minutes, transfer pressure cooker to sink, release pressure and run cold water into pressure cooker for 10 minutes.

Procedure
1. Place citrate buffer in coplin jar
2. Add coplin jar with citrate buffer to water bath on hot plate
3. Heat to 90-100°C, incubate for 20-40 minutes
4. Remove and allow sections to cool in citrate buffer for 20 minutes at room temperature
Staining protocol

Materials

- Control tissues: Aorta, heart
- PBS
- 0.25% Triton-X 100 in DI H2O
- 5% Goat serum in PBS
- Primary:
  - Mouse monoclonal anti smooth muscle myosin heavy chain 1:400 (Abcam, AB683)
  - Rabbit anti CD-31 monoclonal 1:50 (Abcam 28364)
  - Rat anti Mouse CD-45 1:50 (BD 550539)
  - 3% goat serum
- Hoechst dye 1:6000 in PBS
- Secondary:
  - Alexa-fluor 488 goat anti-rat 1:400 (Invitrogen A11029)
  - Alexa-fluor 568 goat anti-rabbit 1:400 (Invitrogen A11036)
  - Alexa-fluor 633 goat anti-mouse
  - 3% goat serum
- Cytoseal-60

Procedure

1. Wash 3x in PBS for 5 mins
2. Permeabilize with 0.25% Triton X for 20 mins
3. Block with 5% goat serum in PBS for 30 mins
4. Incubate in primary antibody overnight at 4 degC
5. Wash 3x in PBS for 5 mins
6. Incubate in secondary antibody for 1 hr at room temperature
7. Wash 3x in PBS for 5 mins
8. Counterstain with Hoechst for 5 minures
9. Wash 3x in PBS for 5 mins
10. Coverslip with cytoseal
Appendix C: Derivation of theoretical strain relationship

Definitions

\( E_i \)  Green's Strain at inner wall

\( E_o \)  Green's Strain at outer wall

\( \lambda_i \)  Stretch ratio at inner wall

\( \lambda_o \)  Stretch ratio at outer wall

\( r_o \)  Outer radius at zero stress state

\( r_i \)  Inner radius at zero stress state

\( R_o \)  Outer radius at stressed state

\( R_i \)  Inner radius at stressed state

\( L \)  Length of tubing

Known relationships

Definition of Stretch ratio

\[ \lambda_o = \frac{R_o}{r_o} \quad Eq \ 1 \]

\[ \lambda_i = \frac{R_i}{r_i} \quad Eq \ 2 \]

Definition of Green's Strain in 1D

\[ E_o = \frac{1}{2}(\lambda_o^2 - 1) \quad Eq \ 3 \]

\[ E_i = \frac{1}{2}(\lambda_i - 1) \quad Eq \ 4 \]

Conservation of tube wall Volume

\[ L \left( \pi r_o^2 - \pi r_i^2 \right) = L \left( \pi R_o^2 - \pi R_i^2 \right) \quad Eq \ 5 \]
**Solution**

\[
E_o = \frac{1}{2} \left( \frac{R_o^2}{r_o^2} - 1 \right) \quad E_i = \frac{1}{2} \left( \frac{R_i^2}{r_i^2} - 1 \right)
\]

Combine Eq1, Eq3
Combine Eq2, Eq4

\[
R_o^2 = r_o^2 \left( 2 \cdot E_o + 1 \right) \quad R_i^2 = r_i^2 \left( 2 \cdot E_i + 1 \right)
\]

Solve for \( R_o^2 \) and \( R_i^2 \)

\[
r_o^2 - r_i^2 = r_o^2 \left( 2 \cdot E_o + 1 \right) - r_i^2 \left( 2 \cdot E_i + 1 \right)
\]

Simplify Eq5 and input values for \( R_o^2 \) and \( R_i^2 \)

\[
E_o = \frac{r_i^2}{r_o^2} \cdot E_i
\]

Simplify and solve for \( E_o \)
Appendix D: Determination of vessel layer thickness

1. Original image:

2. Thresholded image:
3. Extraction of lumen edge:

4. User selection of IEL, EEL and vessel extent:

5. Convert pixel areas to area in microns²:
R = 0.540 \frac{\mu m}{\text{pixel length}} \quad \text{(Known value for our scope, camera and objective)}

A_{\text{micron}} = A_{\text{pix}} R^2

6. For each area, calculate ideal radius:

r_{\text{ideal}} = \sqrt{\frac{A_{\text{micron}}}{\pi}}

7. Compute vessel parameters from ideal radii:
   - Intimal thickness:
     \[ t_I = r_{\text{ideal}} - r_{\text{lumen}} \]
   - Wall thickness:
     \[ t_{\text{wall}} = r_{\text{extent}} - r_{\text{lumen}} \]