Summer 8-19-2016

The Development of Transforming Growth Factor-Beta 1 Associated Fibrosis in the Gastrocnemius of Peripheral Artery Disease Patients and Response to Revascularization and Supervised Exercise Therapies

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THE DEVELOPMENT OF TRANSFORMING GROWTH FACTOR-BETA 1 ASSOCIATED FIBROSIS IN THE GASTROCNEMIUS OF PERIPHERAL ARTERY DISEASE PATIENTS AND RESPONSE TO REVASCULARIZATION AND SUPERVISED EXERCISE THERAPIES

By

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A DISSERTATION

Presented to the Faculty of

The Graduate College in the University of Nebraska

In Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

Department of Cellular and Integrative Physiology Graduate Program

Under the Supervision of Professors Iraklis P. Pipinos and George Casale

University of Nebraska Medical Center
Omaha, Nebraska

June, 2016

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ACKNOWLEDGEMENTS

When I embarked on my graduate studies, I never imagined myself working in a translational laboratory and studying human skeletal muscle tissue. I have been so fortunate to be under the supervision and mentorship of Dr. Iraklis Pipinos and Dr. George Casale, whose combined expertise have widened my research horizons and bridged my previous experiences in basic and clinical research. I am deeply grateful to Dr. Pipinos and Dr. Casale for their eagerness in introducing me to new fields and their patience in helping me become proficient at laboratory techniques that were novel to me. In these past couple of years, I have developed and nurtured a scientific mind and applied that towards scientifically sound experimental design and execution, manuscript publications, successful grant applications. Additionally, I have been able to artfully convey the story of my work at numerous local and national conferences that is also the stories of millions of Peripheral Artery Disease patients who suffer with this disease each and every day of their lives. Throughout this process, I have gained an appreciation for the importance translational research and a passion to advocate for such work in my own career path, but also that of those around me. I am certain that the doctoral training I have received will serve me well in my future as a clinician-scientist.

I sincerely believe that developing a scientist is like a village raising a child and my successes would not have been possible without the positive environments in which I conducted my work and most importantly, the people. I am so honored to have been a part of the Cellular and Integrative Physiology family at UNMC, where at each turn someone is there to lend a helping hand. Such a conducive

environment to graduate studies is due to leadership at the top and I want to acknowledge department chair Dr. Irving Zucker, and graduate program director, Dr. Pamela Carmines. The department is also blessed to have such committed administrative staff such as Cindy Norton, Pearl Sorensen, and Kim Kavan. I will remember the stimulating physiology courses, discussions at departmental seminar, and conversations at journal club with my fellow graduate students. I want to especially acknowledge Dr. Lie Gao and Dr. Erika Boesen for their dedication towards making journal club better and better each semester, and for taking time to individually mentor me on poster and oral presentation skills for conferences. Furthermore, I want to acknowledge Dr. Dele Davies and the other members of the Graduate Studies Council, whose leadership abilities and views on graduate education have inspired me to become a better teacher and mentor to others, as well as raise my standards as to what an excellent graduate student should accomplish. Last but not least, I would like to thank the directors of the M.D./Ph.D. Scholars program, Dr. Smith and Dr. Romberger, and Sonja Cox for their support throughout my journey.

My continual progress would not have been possible without the other members of my Graduate Supervisory Committee: Dr. Matthew Zimmerman, Dr. Kaushik Patel, and Dr. Geoffrey Thiele. I will remember the energy and attention to detail Dr. Zimmerman provided at each committee meeting, the expectations of Dr. Patel for me to reach for stellar rather than incremental knowledge, and kindness and collegiality of Dr. Thiele, whose advice and network of researchers have been so valuable to my work. I have always been appropriately encouraged,

supported, and also challenged by my committee in ways that have helped me constructively move forward.

I have survived tough days and weeks because of the people in my lab, who have become my second family away from home. Being an MD/PhD student with additional clinical responsibilities required of the program, I am so thankful to have such bright, caring, and reliable colleagues who consistently look after me. I have enjoyed learning about what inspires each of you to pursue you work and the experiences that have shaped your unique personalities and outlooks. I will take not only the science I have learned but also the life lessons.

I also want to thank our collaborators on the UNMC campus, and at the Veterans Affair Hospital, Creighton Exercise Lab, and the Biomechanics Core at the University of Nebraska in Omaha. Without the expertise of Dr. Nick Stergiou and Sara Meyers, it would have been extremely difficult for me to under the rationale of the biomechanics studies and the hands-on recording of functional measurements. I want to acknowledge research nurse Holly DeSpiegelaere for her efforts in recruiting patients and recording demographical and medical information. If not for Karen Dulaney, much of my time in lab would be sectioning tissues that probably would not have been as well done. I am grateful to Dr. Gleb Haynatzki for his statistical prowess that has allowed me to understand my data from multiple layers to unravel the complexities of Peripheral Artery Disease.

Finally, I would like to thank my family for raising me with unconditional love, teaching me to be successful but never at the expense of others, and always

believing in me. To my one and only sibling, Chi, whose thirst for life and adventure has always raised my spirits. To my 2-year old nephew, Tim, whose regular FaceTime calls at random hours brighten both my days and nights. And finally, to my best friends Alejandro Ramirez, Caronda Moore, Mina Hanna, Krupa Savalia, Alicia Diener, and Shamma Rahman, whose level shoulders and candidness have helped bring order, stability, and laughter into my life when I needed them most. To my parents for never pressuring me to pursue the profession they thought was best, but allowing me to carve out my own path, one that has been long and arduous for them as well as they continually provide me with moral support. I admire your courage and sacrifice for Chi and I to have a future filled with opportunities. I know how difficult it was to start over in a new country in your mid-thirties, but through living with humility, dignity, and grace, you have taught us how to live and how to live for others. You have instilled in Chi and I strong core values that have driven us to dream big, work hard, and persevere through any obstacles to realize our ambitions. I am forever indebted to you for your unwavering patience and unconditional support of my professional endeavors.

THE DEVELOPMENT OF TRANSFORMING GROWTH FACTOR-BETA 1 ASSOCIATED FIBROSIS IN THE GASTROCNEMIUS OF PERIPHERAL ARTERY DISEASE PATIENTS AND RESPONSE TO REVASCULARIZATION AND SUPERVISED EXERCISE THERAPIES

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Advisors: Dr. Iraklis Pipinos, Ph.D. and Dr. George Casale, Ph.D.

Peripheral Artery Disease (PAD) affects over 200 million people worldwide with a 5-year mortality rate as high as 30%. Atherosclerotic blockages impair blood flow to the lower limbs, causing ischemic leg pain and dysfunction upon exercise that later progresses to ulcerations and gangrene and often requires amputation. The standard therapies, revascularization and exercise, allow patients to walk further and longer but are at best maintained short-term. One explanation may be the development of myopathic changes in the limb that progress despite improved hemodynamics—a paradigm shift in the classic view of PAD pathophysiology as solely a mismatch between blood supply and demand. Thus, it is imperative that we characterize aspects of this myopathy, identify their functional consequences, and determine how their development is affected by standard therapies. Such knowledge is critical to developing adjunct therapeutic strategies that directly target the myopathy to improve the limb function of PAD patients. Excessive extracellular matrix deposition, or fibrosis, causes end-organ dysfunction in cardiovascular diseases. In the gastrocnemius of PAD patients, we have localized increased

expression of a key pro-fibrotic cytokine Transforming Growth Factor-Beta 1 (TGF-β1) to microvessels and demonstrated a direct relationship with collagen deposition through advancing disease stages of PAD. We determined that vascular smooth muscle cells and not macrophages, T cells, fibroblasts, or endothelia, are the primary producers. We also demonstrated an inverse relationship between vascular TGF-β1 expression and hemodynamics, which suggests that local ischemia-hypoxia may induce TGF-β1 expression. Our study of PAD patients with unilateral disease confirmed that TGF-β1 dependent myofibrosis is a localized response in the ischemic muscle without significant systemic contributions. Collectively, the data suggest that the development of myofibrosis in PAD is of a vascular etiology. To determine how myofibrosis is affected by standard therapies, patients either underwent revascularization, supervised exercise therapy, or received no interventions for 6 months. Biopsies and limb function measurements were obtained at baseline and 6 months. Without intervention, myofibrosis progressed in the gastrocnemius of PAD patients but did not alter limb function. Patients in the exercise group had increased myofibrosis despite improved limb function. However, myofibrosis did not progress in patients who underwent revascularization. These data suggest that long term benefits to the lower limb of PAD patients are better with revascularization than exercise by preventing myofibrosis progression. Moreover, benefits of exercise on limb function are not due to alterations in myofibrosis. Overall, the findings validate the need for adjunct therapies that directly decrease TGF-β1 expression, given that neither revascularization nor exercise reverses myofibrosis in PAD patients.

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4-HNE	4-hydroxynonenal
ABI	Ankle brachial index
CTGF	Connective tissue growth factor
HITAEC	Human internal thoracic artery endothelial cells
HITASMC	Human internal thoracic artery smooth muscle cells
ICC	Intraclass correlation coefficient
IFN-γ	Interferon-gamma
MHC	Myosin heavy chain
MSM	Multi-spectral microscopy
NADPH	Nicotinamide adenine dinucleotide phosphate
PAD	Peripheral artery disease
QFM	Quantitative fluorescence microscopy
ROS	Reactive oxygen species
SMC	Smooth muscle cells
SMWD	Six-minute walking distance
TGF-β1	Transforming growth factor-beta 1
TNF-α	Tumor necrosis factor-alpha
PAH	Pulmonary arterial hypertension
PDGF-BB	Platelet derived growth factor-BB
PWT	Peak walking time

CHAPTER I: INTRODUCTION & BACKGROUND

Atherosclerosis and Peripheral Artery Disease

Atherosclerosis is one of the leading causes of morbidity and mortality worldwide with important implications for public health and primary care[1]. This systemic disease affects the large and medium sized arteries and causes luminal narrowing due to the formation of plaques. These plaques are the accumulation of lipid and fibrous material between the intimal and medial layers of the vessels[1]. Peripheral artery disease (PAD) is the non-cardiac manifestation of atherosclerosis, where plaques are most commonly found in the aortoiliac and femoropopliteal regions of vasculature supplying the lower extremities[2]. Development of these plaques increasingly impair blood flow to the lower limbs, creating a supply and demand mismatch when patients exercise.

The development of PAD symptoms has been classified into four distinct disease stages[3]. PAD patients who are asymptomatic are at Fontaine Stage I. When patients experience the first symptoms of claudication, or ischemic leg pain and gait dysfunction upon exercise that is relieved by rest, they have progressed to Fontaine Stage II. At Fontaine Stage III, claudication occurs even at rest. When ischemia becomes critical at Fontaine Stage IV ulcerations and necrosis develop in the limbs and may require amputation. Transition between these stages often takes decades and as a result, PAD patients live with chronic debilitating pain, are unable to walk normally, and have poor quality of life[4].

The epidemiology of PAD in recent years has become alarming, both globally and in the United States, and is attributable to increased exposure to risk factors. In the past 20 years, there has been a worldwide increase in PAD-related death and disability[5,6]. PAD now affects over 200 million people worldwide and approximately 12 million in the United States alone. In addition to genetics and ethnicity, advancing age alone is a major underlying risk factor, where the prevalence of PAD increases from 1% to approximately 15% from age 40 to 70[7]. Avoidable risk factors are those related to lifestyle choices, which include tobacco, unhealthy diets, obesity, and lack of physical activity, and the management of co-morbid conditions. Given the causal linkage between tobacco and atherosclerosis, smokers have a greater relative risk for developing PAD (2.71), which is even higher than for coronary artery disease (1.67)[8]. Those who have hypertension or diabetes are at significantly greater risks of developing PAD due to the damaging effects of these conditions on vascular integrity and function. Hyperlipidemia is a constellation of abnormalities that include high cholesterol, triglycerides, and lipoproteins, all of which promote atherosclerosis and increases the risk of developing PAD. Finally, elevated levels of pro-thrombotic factors, inflammatory markers, and homocysteine in circulation are also known risk factors, which reflects the systemic nature of atherosclerosis, where the pathological factors from one plaque site can stimulate formation at other sites and affect the development and natural history of PAD in patients. The relative odds ratios for these risk factors for PAD is presented in **Figure 1.1**.

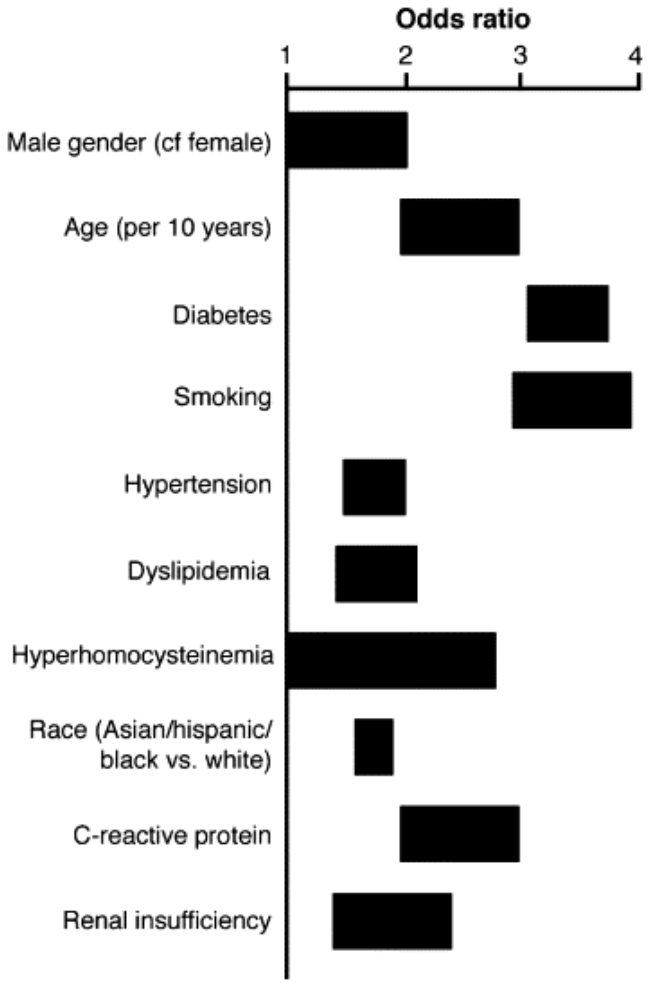


Figure 1.1: The Relative Contributions of Risk Factors to Development of Peripheral Artery Disease. Modified from: Intersociety Consensus for the Management of Peripheral Arterial Disease (TASC II)[9].

PAD patients are at increased risk of morbidity and mortality. The inability of PAD patients to walk normally due to claudication is, in itself, a risk factor for other co-morbid conditions. Since the majority of PAD patients are elderly and walking is the primary physical activity of the elderly, claudicating patients adopt a sedentary lifestyle that further escalates their risk for adverse health events. PAD patients have an increased risk for myocardial infarction, and renal and cerebrovascular disease and diabetes[9-11]. The consequence of this is highlighted by mortality studies. Those diagnosed with PAD have an average life expectancy of just 16 years that is reduced by almost 90% to 1.5 years either with a subsequent stroke or myocardial infarction, and this drastic drop is comparable to coronary artery disease and worse than cerebrovascular disease **(Table 1.1)**. Moreover, 5-year mortality rates for those diagnosed with PAD are as high as 30%[12].

Subsequent Adverse Event	**Diagnosed Condition or Adverse Event**		
	PAD	**AMI**	**Stroke**
None	16.0	13.9	8.80
AMI	1.60	0.40	4.20
Stroke	1.50	0.40	3.30

Table 1.1: The Life Expectancy, in Years, of PAD Patients with Subsequent Co-Morbid Adverse Events Relative to Other Cardiovascular Diseases.
Modified from the Framingham Study[13].

Given the epidemiology of PAD, this disease presents a significant financial burden on health care systems. PAD patients have recurring hospitalizations and outpatient care that incur substantial costs due to the chronicity of its natural history[14,15]. It is estimated that approximately $4 billion are paid by Medicare each year for PAD-related care that rivals congestive heart failure and cerebrovascular disease (4 billion each), and greater than cardiac dysrhythmias (2.7 billion). PAD patients are also costlier than the typical elderly Medicare beneficiary, with mean expenditures of about $14,000 compared to the average of $5800. The majority of these costs are incurred by inpatient care to treat severe manifestations of limb ischemia[14,15]. Thus, outpatient preventative care and alternative treatment strategies can drive down costs. Overall, PAD is a major health problem with high morbidity and mortality, and is very costly to the health care system.

The Pathophysiology of Limb Dysfunction in Peripheral Artery Disease

The pathophysiology of limb dysfunction in PAD is complex, being more than simply a mismatch between blood supply and demand[16,17]. A widely held view is that limb dysfunction is exclusively produced by claudication, during which metabolic demands rapidly increase but the blood flow required cannot be delivered due to the atherosclerotic blockages, causing the limb to become progressively more ischemic, painful and impaired. However, we now know that hemodynamic changes are not the sole contributor. Advanced biomechanical gait analysis of claudicating patients has shown that gait dysfunction is present even before onset of claudication. This suggests that a myopathy may be present that causes limb dysfunction in PAD patients.

A series of biomechanical studies have analyzed the kinetics and kinematics of the legs of claudicating patients. Lower extremity kinetics are an evaluation of the forces exerted by the weight bearing limbs of PAD patients on the ground[18]. These ground reaction forces are a direct application of Newton's third law of motion, in which the ambulating PAD patient pushes on the ground with a force and the ground exerts an equal and opposite force. This force can be measured with standard force plates as resolved into three components: vertical, anterior-posterior, and medial-lateral. Lower extremity kinematics, on the other hand, focuses on the angular displacement of the joints in the leg, measured by joint angles, moments, torques and powers[18]. Kinetic analyses of PAD patients demonstrate a significant flattening of the vertical force causing a lower and less fluctuant center of mass when ambulating[19,20]. PAD patients demonstrate

decreased propulsion forces in the anterior-posterior direction and increased forces in the medial-lateral direction that are consistent with a gait characterized by wider steps and an inability to swing their legs straight through[19,20]. Kinematic analyses of PAD patients have measured angles, torques and powers at the hip, knee, and ankle joints. PAD patients exhibit greater joint angles which manifests in gait as a “foot drop” upon heel touchdown[21-23]. Torque and power analysis demonstrate that PAD patients have decreased abilities of the knee and hip extensors and ankle plantar flexors, leading to difficulties in the control weight acceptance, support of the trunk, and forward propulsion while walking[21-23].

The defects in gait exhibited by PAD patients have been measured both before and after the onset of claudication and show that claudication is not the only factor contributing to limb dysfunction. Both kinetic and kinematic abnormalities were present before the onset of claudication, usually within the first 3-4 steps taken by patients[23]. The gait dysfunction is also present after onset of claudication and worsens. Presence of these alterations both before and after onset suggests that limitation of blood flow is not the sole cause and suggests that defects in the skeletal muscle of the limbs may also contribute. Additional hemodynamic and functional studies of the lower limb of PAD patients support the presence of myopathy. During the first few steps taken by PAD patients, tissue oxygenation[24] and blood flow[25,26] to the leg are very similar to that of age matched control patients. In light of the biomechanical studies, decreased blood flow and tissue oxygen levels are not the only mechanisms responsible for the gait dysfunction observed in PAD patients before the onset of claudication. This is further

corroborated by a study that measured muscle strength in PAD patients using a 5-second maximal isometric strength assessment that is independent of blood flow[27], given the short time frame and that isometric contractions produce no discernable joint motion. Muscle strength of PAD patients are significantly lower than that of age-matched control patients[27], again indicating that blood flow is not the only determinant of the dysfunction of claudicating muscle.

Lower limb myopathic changes in PAD patients may be initially caused by atherosclerotic blockages that produce chronic cycles of ischemia-reperfusion, which occur when patients walk and then have to rest due to claudication. Ischemia-reperfusion, and the resulting hypoxia-normoxia, is known to be highly damaging to end organs throughout the body[28]. Pathological mechanisms such as oxidative stress and mitochondrial dysfunction are initiated with the flux of stagnant blood filled with toxic metabolites that are harmful to the end-organ. In PAD, the ischemia-reperfusion injury to skeletal muscle may produce progressive decline in the integrity and function of myofibers, ultimately causing their degeneration and the inability of patients to walk normally. At some point in this progression, further decline in the hemodynamic state of the leg only further aggravates this myopathy. This is supported by the inability of interventions that restore blood flow to the legs of PAD patients to reverse functional impairment to the degree of improved blood flow[29]. Additionally, functional decline in PAD patients occurs to a greater degree than the progression of atherosclerosis in arteries supplying the lower extremities[30]. Thus, it is apparent that preventing future ischemia-reperfusion insults does not ameliorate the myopathic changes that have developed and likely

continue to progress largely independently of blood flow. This line of reasoning is supported by research on interventions against atherosclerosis in other vascular beds. Percutaneous revascularization for patients with coronary artery disease improves blood flow to the heart, chest pain, and quality of life, but does not increase survival rates or reduce the incidence of myocardial infarctions[31,32]. Similarly, percutaneous revascularization of atherosclerosis in renal arteries only confers clinical improvements in less than half of the patients, where it was postulated that concomitant presence of renal parenchymal disease was responsible[33]. Taken together, this body of research demonstrates that the pathophysiology of PAD limb dysfunction has evolved from a purely hemodynamic cause to one that is inclusive of myopathy in the lower limb of patients **(Figure 1.2)**. Such an evolution will provide greater insight into new therapeutic strategies to improve limb function of PAD patients.

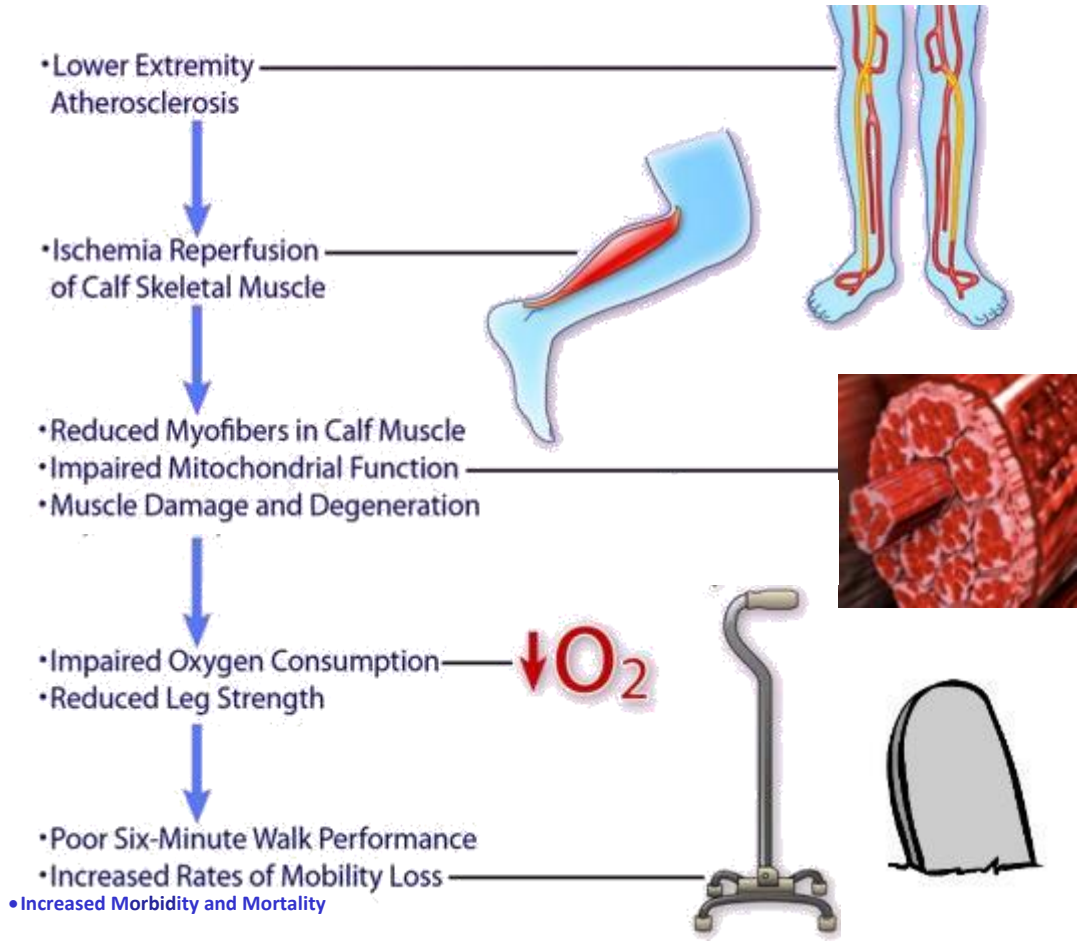


Figure 1.2: The Pathophysiology and Functional Implications of Leg Ischemia in Peripheral Artery Disease. Adapted from a Review Paper by Mary McDermott[34].

The Myopathy of Peripheral Artery Disease Decreases Limb Function

Skeletal muscle is a fascinating biological tissue that is able to transform chemical energy to mechanical work, allowing us to function as human beings. The functional unit that produces motion at a joint is comprised of the muscle belly and tendons that bind muscle to the bone. Within the muscle belly consists of an arrangement of muscle cells that produce contraction. The muscle as a whole is comprised of multiple fascicles that in turn are made up of myofibers, which are long cylindrical multinucleated cells filled with smaller units of filaments organized in parallel to the orientation of the myofiber. The largest filamentous structure is the myofibril that is composed of sarcomere subunits arranged end-to-end along the length of the myofibril. Within the sarcomere are myofilaments that contain actin and myosin proteins responsible for the sliding mechanism of muscle contraction, utilizing large amounts of oxygen in the process. When PAD patients exercise the demand for oxygen to fuel these muscular contractions increases and is not met by the restricted blood flow. As a result, specific muscle groups in the lower limbs of PAD patients do not perform optimally, producing biomechanical defects and limb dysfunction. The myopathy of PAD patients has been best characterized in the gastrocnemius muscle[16].

The gastrocnemius is the largest and most powerful muscle in the calf responsible for running, jumping, and walking, and the integrity and function of this muscle is compromised in PAD patients. Histomorphological changes observed by light microscopic evaluation include necrosis, phagocytosis, and presence of central nuclei in myofibers, all of which correlate with severity of occlusion[35,36].

Electron microscopy revealed ultrastructural changes in sarcomere that were highly disorganized and often disintegrated[37,38]. A more recent study found desmin, an intermediate filament of the myofibril, to abnormally accumulate in the gastrocnemius of PAD patients in a honeycombed and/or amorphous aggregate rather than its normal registration with Z-disks of sarcomeres[39]. The main function of desmin is to transmit forces produced by myofibrils by linking them into bundles and connecting them to specialized site of adhesions in the sarcolemma[40]. Thus, alterations in desmin will not allow myofibrillar forces to be transferred to the extracellular matrix and ultimately to the tendons for normal movement of the limb. Desmin also provides organizational support for the mitochondrial system and may contribute to mechano-chemical signaling between various compartments of the myofiber[41]. It is well established that cytoskeletal abnormalities are associated with muscle pathologies and decreased function of the sarcomere[42]. In PAD patients, desmin abnormalities strongly correlated with altered myofiber morphology, reduced mitochondrial respiration, and impaired walking ability in PAD patients[39].

The gradual degradation of gastrocnemius histology in PAD has been quantified non-invasively via imaging, but more recently, morphometric quantitative methods have allowed measurements of this progression within muscle biopsies in relation to limb function. Histologically, myofiber degeneration is not only characterized by decreased size, but also variation in shape, both of which become more abnormal with increasing PAD disease severity. Classically, myofiber cross-sectional area has been measured by computed tomography[43]. However, with imaging techniques, subtler changes in the shape of the myofibers

without significant change in size, is difficult to measure and may be early changes predictive of PAD development. Recently, morphometric analytical methods employing immunofluorescence staining of the sarcolemma of gastrocnemius biopsies from PAD patients, have allowed extraction of the myofibers into a binary mask, upon which changes in the size and shape of the myofiber can be measured with software algorithms. The morphological changes that were found to be progressively abnormal in the skeletal muscle include myofiber cross-sectional area, density, roundness, and distance of the major and minor axes. Using the combination of these parameters, discrimination of PAD patients at different Fontaine stages and from age-matched controls was achieved with an 88% accuracy[44]. Furthermore, these abnormalities in myofiber morphometrics are closely linked to the muscle strength and limb function of PAD patients[45]. The ability to measure myofiber morphology on a muscle biopsy also permits direct correlations with other aspects of myopathy, such as mitochondrial dysfunction and oxidative damage, in the same muscle biopsy.

The gastrocnemius of PAD patients, as well as other calf muscles, experience cycles of ischemia-reperfusion injury that produces oxidative stress and mitochondrial dysfunction[28,46]. During ischemia, the interruption of blood supply reduces oxygen and nutrient delivery to the myofibers. Generation of ATP by oxidative phosphorylation is disrupted to impair metabolism and energy dependent cellular functions[47]. In the lower limb of PAD patients, mitochondria demonstrate inefficient utilization of Kreb cycles components, impaired oxygen use, and have decreased amounts of electron transport chain components that

correlate with the extent of functional impairment[48,49]. Phosphorus-31 nuclear magnetic resonance spectroscopy studies of PAD claudicants suggested respiratory defects in mitochondrial bioenergetics[50], which was confirmed by studies that found Complexes I, III, and IV of the electron transport chain to be dysfunctional[51,52]. All of these changes in the mitochondria contribute to electrons being backed up where they may leak out and interact with the high influx of molecular oxygen that occurs with reperfusion to form reactive oxygen species (ROS)[53]. Reperfusion also introduces accumulations of circulating cells such as leukocytes, which have become activated to produce ROS via NADPH-oxidase. During ischemia, activation of cell survival programs and immune responses that promote angiogenesis, remodeling, and repair upregulate expression of adhesion molecules that aide transmigration and activation of leukocytes. The subsequent release of ROS from these leukocytes have cytotoxic effects on myocytes.

Additional ROS contributions in PAD skeletal muscle comes from increased activity of xanthine oxidase. During ischemia, inefficient mitochondrial respiration produces buildup of ADP that is metabolized into hypoxanthine and xanthine, both of which are substrates for xanthine oxidase[28,46]. When reperfusion occurs, xanthine oxidase is provided with ample molecular oxygen, the other substrate needed along with hypoxanthine or xanthine to produce superoxide anion[28,46]. Moreover, manganese superoxide dismutase, a major antioxidant enzyme is reported to be deficient in PAD gastrocnemius[51], which further permits the accumulation of ROS. Excessive ROS is known to induce damage of DNA and proteins in virtually all biomolecular in cells and tissues. In PAD, mitochondrial DNA

and protein may be targets of oxidative stress, which further exacerbates the myopathy and accelerates limb dysfunction in patients.

Further support for mitochondrial injury and oxidative damage can be found in histomorphological studies of PAD calf muscle. Electron microscopy has revealed mitochondria to be both qualitatively and quantitatively abnormal, demonstrating hypertrophy, hyperplasia, hypercristae, transverse orientation, paracrystalline inclusions, and lipid vacuolizations[37,38] all of which support the mitochondria being leaky to electrons that help form free radicals. The damage caused by ROS generated from the mitochondria is reflected by presence of oxidatively damage proteins, marked by carbonyl adducts, and lipids, reflected by the 4-hydroxynonenal content. Compared to age-matched controls, PAD claudicants had significantly higher carbonyl and 4-HNE in the myofibers, which was associated with fewer myofibers and more advanced Fontaine stages of disease[54]. Thus, ischemia-reperfusion via mitochondrial dysfunction and oxidative stress induce the degeneration of myofibers and limb dysfunction.

Myopathy caused by ischemia-reperfusion likely extends to encompass the reparative phase of ischemia-reperfusion injury. During ischemia, low concentrations of ROS can signal survival programs by increasing growth factors that promote angiogenesis, induce proliferation and differentiation of vascular smooth muscle cells for vascular remodeling, and activate inflammatory and pro-fibrotic cytokines that contribute to tissue remodeling and formation of scar tissue[28,46]. However, when ischemia-reperfusion is not relieved and is allowed to

develop over decades as in PAD patients, many of these beneficial adaptations are chronically activated, which actually produces pathological responses. Such findings have been reported in coronary and cerebrovascular manifestations of atherosclerosis and is likely a part of the myopathy of PAD[55]. Given the systemic nature of atherosclerosis, inflammatory factors in circulation have been thought to further induce disease[56]. In the serum of PAD patients, inflammatory factors such as C-reactive protein can help more accurately screen PAD patients[57], and predict disease severity[58], functional decline[59], and mortality[60]. Thus, they may influence the development and progression of PAD myopathy, such as by activating leukocytes that infiltrate into the muscle and release cytotoxic ROS. It has been shown in the heart and brain that as more cells dies, damage associated molecular patterns accumulate and chronically stimulate toll like receptors to activate innate immune responses that are associated with increased ROS, cell death, and fibrosis[61]. In PAD, it was recently shown that toll like receptors are upregulated in the muscle biopsies of PAD compared to age-matched control patients[62].

In addition to systemic inflammatory responses, cytokine profiling of the gastrocnemius of PAD claudicants is suggestive of local aberrant immune and fibrotic responses. Comparisons of the cytokines in serum and gastrocnemius show they are very different. First, the majority of pro-inflammatory (TNF-α, IFN-γ, IL-6, IL-8, and IL-12) and anti-inflammatory (IL-2, IL-10, and IL-13) cytokines were present in the gastrocnemius of PAD patients more often than in the serum, and also at much higher levels **(Table 1.2)**. The exceptions were the pro-inflammatory chemokine CCL5, which is known to recruit monocytes, and anti-inflammatory

cytokine Transforming Growth Factor-Beta 1 (TGF-β1) which was present in robust quantities both the muscle and serum. Yet, the levels in the serum are lower than muscle, indicating that the local cytokine milieu that drives myopathy cannot be accounted for by levels in the circulation. This is supported by a study of 17 PAD patients with unilateral disease that found the total neutrophil number and percent activated were higher in venous blood draining from the diseased limb than in arterial blood, differences that were not exhibited in the contralateral limb[63]. Another study of PAD patients with unilateral disease also demonstrate a venous-arterial difference of neutrophil myeloperoxidase, which is an index of neutrophil activation[64,65]. Myeloperoxidase uses nitric oxide which reduces its bioavailability to endothelia for proper vasodilatory functions and modulation of adhesion molecules, rendering endothelia dysfunctional[66]. Overall, such aberrant immune responses may adversely affect the microcirculation and skeletal muscle repair, which further compromises limb function.

	PAD MUSCLE (N=24)			PAD SERUM (N=24)		
	Number Positive	Mean (pg/mL)	Standard Error	Number Positive	Mean (pg/mL)	Standard Error
TNF-α	14	**11887**	1933	0	**ND**	N/A
IFN-γ	16	**3470**	844	0	**ND**	N/A
IL-17	14	**2844**	708	5	**680**	163
IL-12	7	**733**	162	2	**240**	N/A
IL-6	15	**2479**	453	0	**ND**	N/A
CCL5	24	**28589**	4977	24	**9737**	617
IL-8	9	**1004**	228	1	**154**	N/A
TGF-β1	24	**38531**	6718	24	**18547**	990
IL-2	3	**4824**	944	0	**ND**	N/A
IL-10	7	**1327**	281	1	**437**	N/A
IL-13	6	**2752**	292	0	**ND**	N/A

Table 1.2. **Cytokine Profile in Gastrocnemius and Serum of PAD Claudicants.** Unpublished laboratory data that is currently in preparation for manuscript submission. ND = Not Detected. N/A = Not Applicable.

This theory is highlighted by the findings of robust and consistent increases in CCL5 and TGF-β1 in PAD patients, both of which may contribute to myofibrosis. Compared to age-matched controls, CCL5 and TGF-β1 were at least 3-fold greater in the gastrocnemius of PAD compared to age-matched control patients **(Table 1.3)**. The role of CCL5 is to recruit monocytes, which are essential for the reparative phase of ischemia-reperfusion injury[67]. The role of TGF-β1 is multi-faceted, being released by immune cells to resolve inflammation but also induce wound healing[68]. The abnormally high levels of these two cytokines suggests a pathological fibrotic response and is consistent with maladaptations associated

with chronic activation of the reparative phase of ischemia-reperfusion injury in coronary artery and cerebrovascular disease[28,46]. Both indirect and direct evidence exists for pathological fibrosis in the gastrocnemius of PAD patients. Computed tomography of the lower limb of PAD patients and age-matched controls show increasing hypodense areas in between myofascicles that could be accumulation of extracellular matrix, and this is much more evident on hematoxylin and eosin histology **(Figure 1.3)**. However, the relationship between TGF-β1 and fibrosis in the lower limb skeletal muscle of PAD patients has never been examined. This unexplored aspect of PAD myopathy may have substantial functional consequences.

	PAD MUSCLE (N=24)			CONTROL MUSCLE (N=18)		
	Number Positive	Mean (pg/mL)	Standard Error	Number Positive	Mean (pg/mL)	Standard Error
TNF-α	14	**11887**	1933	1	**4092**	N/A
IFN-γ	16	**3470**	844	0	**ND**	N/A
IL-17	14	**2844**	708	0	**ND**	N/A
IL-12	7	**733**	162	0	**ND**	N/A
IL-6	15	**2479**	453	1	**2184**	N/A
CCL5	24	**28589**	4977	18	**3830**	759
IL-8	9	**1004**	228	0	**ND**	N/A
TGF-β1	24	**38531**	6718	18	**13518**	2681
IL-2	3	**4824**	944	0	**ND**	N/A
IL-10	7	**1327**	281	0	**ND**	N/A
IL-13	6	**2752**	292	0	**ND**	N/A

Table 1.3. **Cytokine Profile of PAD and Control Patient Gastrocnemius.** Unpublished laboratory data that is currently in preparation for manuscript submission. ND = Not Detected. N/A = Not Applicable.

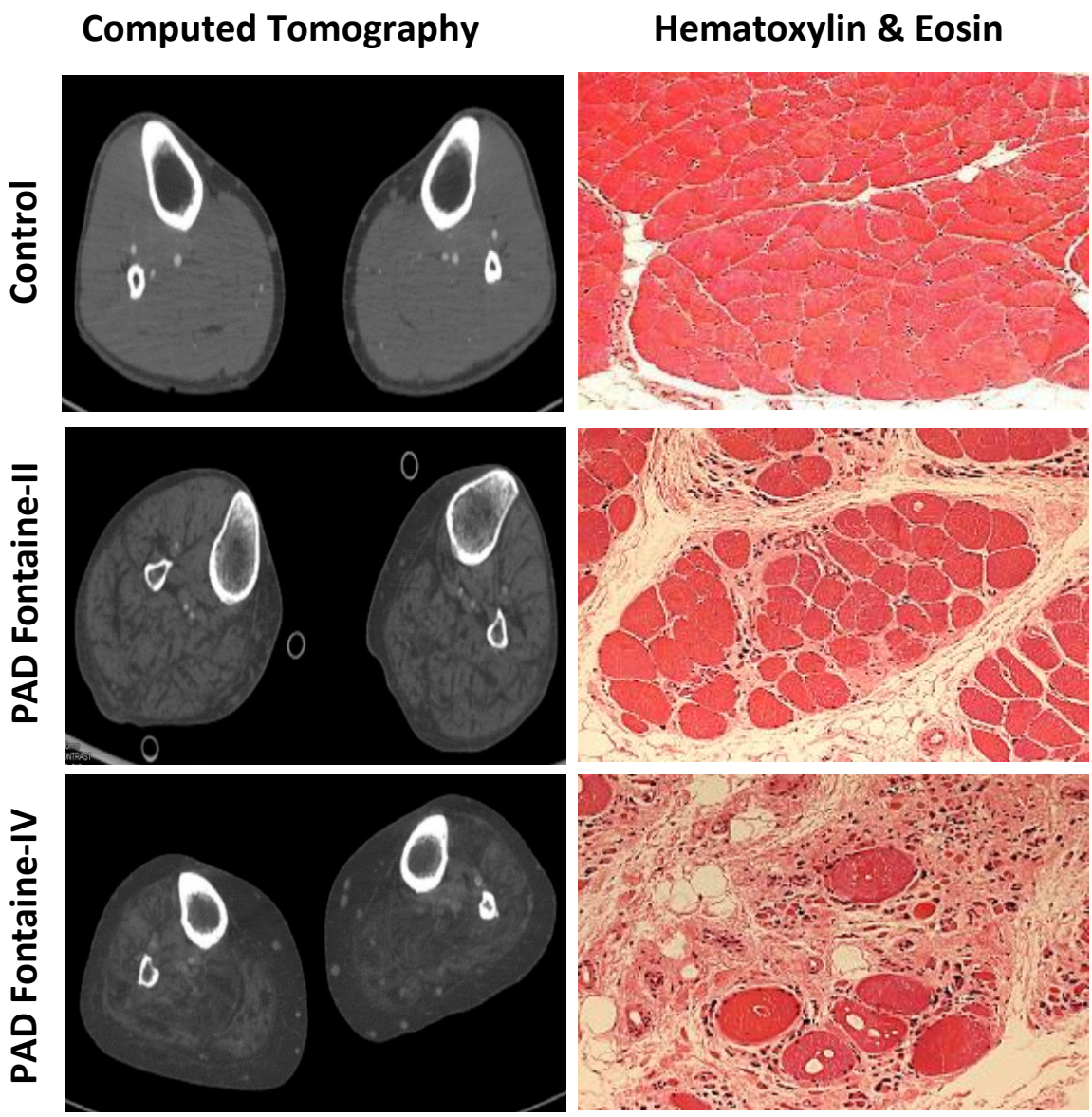


Figure 1.3. **Computed Tomography and Hematoxylin and Eosin Stain of PAD and Control Patient Gastrocnemius Demonstrating Increased Extracellular Matrix.** Unpublished laboratory data that is currently in preparation.

Roles of TGF-β1 in Skeletal Muscle Repair and Pathological Fibrosis

Fibrosis is a common pathobiological outcome of many chronic diseases, which includes the myopathy of PAD. In pulmonary fibrosis, liver cirrhosis, and cardiac remodeling, excessive deposition of extracellular matrix over time leads to loss of both tissue architecture and organ function. Despite their end organ diversity, all fibrotic processes are thought to arise from abnormalities of wound healing and regenerative responses[69]. In skeletal muscle, this occurs via an orchestrated response to sterile injury. Vascular and infiltrating immune cells release cytokines and growth factors that mediate survival and proliferation of living myocytes, while clearing remnants of dead ones[70,71]. Next, activation of satellite cells with stem cell capacity initiates formation of new myocytes to replace lost or damaged myofibers. Efficient repair also requires migration and proliferation of activated myofibroblasts that produce temporary extracellular matrix components, such as collagen, to stabilize healing tissue. Throughout the repair process, specific proteases and their inhibitors are activated to remove this temporary extracellular matrix, such that fibrotic components do not interfere with myofiber function once regeneration is complete. Fibrosis is thought to be the consequence of abnormal persistence of these coordinated processes, leading to excessive and irreversible extracellular matrix deposition[69]. Currently, pro-fibrotic cytokine interactions are active areas of research aimed at preventing and/or slowing progression of end-organ dysfunction.

TGF-β1 is potent pro-fibrotic cytokine elevated in numerous fibrotic diseases, where it induces myofibroblast activation[71]. In response to TGF-β1,

fibroblasts transform into myofibroblasts, which synthesize and release extracellular matrix and protease inhibitors that prevent enzymatic degradation of extracellular matrix[72-75]. The major mechanism by which TGF-β1 promotes fibrosis is signaling through the Smad family of transcription factors, which transactivate genes for collagen and inhibitors of collagenases[68]. Myofibroblasts are further characterized by increases in alpha-smooth muscle actin, cell proliferation, and migration during normal wound healing[76,77]. Following normal repair, myofibroblasts usually undergo apoptosis, but in pathological states, they persist within a milieu of elevated TGF-β1 to promote fibrosis[78-80]. Coordinated increases in TGF-β1, fibroblasts, and collagen were observed with a porcine model of cardiac remodeling caused by ischemia-reperfusion injury[81]. In patients undergoing coronary artery bypass after myocardial infarction, cardiac biopsies in proximity to the infarcts also revealed elevated TGF-β1 protein expression and mRNA in fibroblasts and macrophages[82]. Perhaps chronic ischemia-reperfusion injury in PAD also produces an increase in TGF-β1 that promotes myofibrosis.

Although not well studied in skeletal muscle, animal models of Duchenne Muscular Dystrophy and Marfan Syndrome have shown associations among TGF-β1 expression, myofibroblast activation, and myofibrosis[83,84]. In addition to fibroblasts, TGF-β1 can also induce transition of other cells into myofibroblasts[85,86]. In kidney and lung, TGF-β1 induces epithelial-to-mesenchymal transdifferentiation[87-89]. In liver, TGF-β1 induces pericytic stellate cells to transform into myofibroblasts, which accelerates liver cirrhosis[90]. More recently, others have shown that TGF-β1 induces endothelial-to-mesenchymal transdifferentiation that

increases the number of myofibroblasts in the heart and various other organs[91-94]. Although the mechanism by which TGF-β1 promotes fibrosis is being actively researched, cellular sources of TGF-β1 remain largely understudied.

Immune cells such as macrophages express TGF-β1 during fibrogenesis. Macrophages are innate immune cells that participate in inflammatory responses against pathogens, but are also intimately involved in responses to sterile injury, such as during chronic ischemia-reperfusion[95]. Depending on the cytokine environment mediated by specific T helper cells (pro-inflammatory Th1 and anti-inflammatory Th2), macrophage activation states range from a pro-inflammatory classical M1 phenotype to an anti-inflammatory alternative M2 state that promotes wound healing and follows resolution of inflammation[96]. Each phenotype is activated by specific Th1 and Th2 cytokines and in turn, produces specific cytokines. For example, IL-4 from Th2 can activate an M2 phenotype that produces IL-10 and TGF-β1 and is associated with fibrosis. Indeed, co-culture experiments with M2 cells that express elevated TGF-β1 and human fibroblasts demonstrated fibroblast proliferation and collagen synthesis[96]. In mouse models of cirrhosis[97] and renal fibrosis[98], a Th2 environment increased TGF-β1-expressing M2 cells that enhanced liver and renal fibrosis. Similarly, in patients with pulmonary fibrosis, alveolar macrophages exhibited an M2 phenotype that mediated collagen production by normal lung fibroblasts[99]. Although not well studied in skeletal muscle, fibrosis in a DMD mouse model was accompanied by increased numbers of M2 cells, TGF-β1 expression, and myofibroblasts activation[100,101]. Taken

together, these findings indicate macrophages may be the predominant cell type activated by chronic ischemia-reperfusion injury to induce pathological fibrosis.

Compared to immune sources of TGF-β1, vascular sources have not been well investigated. Rather, focus has been on how TGF-β1 signals vascular cells to mediate fibrosis[102], in hopes of developing anti-fibrotic therapies that block TGF-β1 receptors or its downstream signals in specific cell types. Such an approach, however, may overlook important vascular sources that continue to secrete TGF-β1, whose pleiotropic effects on many cell types may be difficult to modulate or prevent. This may explain the lack of successful TGF-β1 based anti-fibrotic therapies. Although TGF-β1 has been reported in vascular smooth muscle cells, myofibroblasts, and endothelial cells with vascular remodeling[72-74], vascular smooth muscle cells are the most plausible contributor to TGF-β1-mediated end-organ fibrosis in PAD.

Vascular smooth muscle cells can transition between a spindle-shaped contractile form to a cobblestone synthetic one capable of secreting extracellular matrix during vascular injury and atherosclerosis[76,77,103]. In such a context, it is possible that vascular smooth muscle cells upregulate TGF-β1 in response to chronic ischemia-reperfusion injury. Studies of human varicose veins, where remodeling also occurs, show increased TGF-β1 in vascular smooth muscle cells with age[104], which may not only contribute to venous insufficiency, but also end-organ fibrosis. If vascular smooth muscle cells indeed contribute to myofibrosis in PAD skeletal muscle, their phenotypes may be modulated towards the contractile

form to reduce or prevent fibrosis[76]. This may, in turn, have beneficial effects on the myofibers themselves, given that fibrosis physiologically and biologically prevents the regeneration of myofibers. Additionally, TGF-β1 can induce abnormal remodeling within vessels[105], which may compromise blood-flow mechanics, oxygen and nutrient distribution, and increase oxidative stress and mitochondrial dysfunction, all of which produce myofiber necrosis[16,53].

TGF-β1 may further promote fibrogenesis indirectly by inhibiting skeletal muscle regeneration in PAD[106]. The earliest study found, in culture, that TGF-β1 directs myoblasts to differentiate into fibrotic cells rather than myogenic cells and that when these cells were transplanted into whole muscle in mice, differentiates into myofibroblasts[107]. Furthermore, injection of TGF-β1 into healthy skeletal muscle of mice induces myoblasts to upregulate TGF-β1 and produce fibrosis within the injected area, and mouse skeletal muscle injured by laceration also contained myoblasts with increased TGF-β1 expression[107]. Later studies showed that TGF-β1 regulates the regenerative capacity of skeletal muscle. In mice that were subjected to freeze-injury of their skeletal muscle, regeneration became increasingly abnormal the longer TGF-β1 expression was upregulated in the muscle[108]. In rats, addition of neutralizing antibodies against TGF-β1 to platelet-rich plasma significantly reduced fibrosis and increased muscle regeneration, via prolonged satellite cell activation, when injected into sites injured by cardiotoxin[109]. Conditional overexpression of TGF-β1 in mouse skeletal muscle corroborates, demonstrating both increased endomysial fibrosis and myofiber atrophy[110]. Taken together, reducing the elevated levels of TGF-β1 in PAD skeletal muscle may not

only prevent or reverse fibrosis, but also induce regeneration, marked by increasing size of myofibers.

The increased expression of TGF-β1 in the gastrocnemius of PAD patients promotes myofibrosis, but through which cells types and by which cell types remains unknown. The myopathy that develops in PAD is initially caused by changes first experienced by the vasculature and thus, the vascular-myofiber interface may be where TGF-β1 is upregulated. In the context of atherosclerosis and its associated fibrosis, the most likely candidate would be vascular smooth muscle cells. However, in many myopathies, immune cells such as macrophages secrete elevated levels of TGF-β1 to promote fibrosis. With ischemia-reperfusion injury to PAD muscle, monocytes may be recruited and secrete TGF-β1 to induce fibrosis. Furthermore, the cell types that are activated by TGF-β1 to secrete the excessive amounts of extracellular matrix have not been well characterized in the skeletal muscle of PAD patients, although the classical fibroblast is likely to be the major cell type. Finally, the anti-regenerative effects of TGF-β1 may present additional barriers to normal repair following chronic ischemia-reperfusion injury that contributes to myofiber degeneration that paves the way for replacement by scar tissue in PAD skeletal muscle.

Diagnosis and Management of Peripheral Artery Disease

A detailed history and physical examination are necessary for the early detection of PAD that is critical for proper disease management. In addition to family history, risk factors, and symptoms of claudication, blood pressure measurements are performed on the arms and ankles to confirm the diagnosis of PAD. If the ratio between blood pressures in the ankle divided by those in the arm, known as Ankle-Brachial Index (ABI) is equal to or less than 0.90, there is a high sensitivity (90%) and specificity (98%) that the patient has PAD[111]. However, often times the ABI remains in the normal range, despite the presence of atherosclerosis in arteries supplying their lower limbs. Physical examination and ABI measurements of the patient while walking on a treadmill is then used to diagnosis PAD. If the ABI decreases by at least 20%, then they have PAD and are referred to vascular specialists who then perform imaging tests[111]. Based on the severity of the symptoms, duplex ultrasound, computed tomographic angiography, magnetic resonance angiography, and contrast angiography are performed to localize and determine the extent of the vascular blockages[112]. Failure to consider the history of the patients and risks factors for atherosclerosis often leads to the underdiagnosis of Fontaine stage I PAD patients who do not present with claudication. Early diagnosis is important to proper management, which may prevent exacerbation of ischemia-reperfusion to the legs, and the initiation of myopathy in the limbs of PAD patients, and preserve limb function.

Multiple modalities exist for the management of PAD patients. Patients are monitored by continual ABI measurements, which are known to correlate with

increasing Fontaine stages and declining limb function[113,114]. Imaging studies can determine the rate at which the atherosclerotic plaque is expanding. Both ABI and imaging can also be used to monitor the response of patients to therapies that include cardiovascular risk reductions related to lifestyle choices, medications, exercise therapy, and revascularization procedures[115,116]. Most PAD patients are treated medically by addressing risk factors, medication, and exercise training to manage the symptoms of claudication that allow patients to walk better. Diet and exercise in addition to cholesterol lower drugs like statins, better control of diabetes and hypertension, and quitting tobacco are examples of the recommendations to reduce risk factors. Additionally, two federally approved drugs, cilostazol and pentoxifylline, are commonly prescribed to alleviate claudication symptoms as they either dilate the arteries, decrease blood viscosity, or decrease platelet aggregation, all of which improves flow. However, these drugs do not directly target atherosclerosis or the myopathy. Despite these management efforts, many PAD patients still develop incapacitating claudication and they become candidates for revascularization procedures. As evident, the treatment algorithm for PAD patients focus on preventing or decreasing the symptoms, with none of them curing the disease[115,116]. However, supervised exercise and revascularization procedures are interventions that demonstrate robust improvements in limb function. Yet, in the long run limb function of patients often still declines and it is unknown how either intervention affects the myopathy that develops in the limbs of PAD claudicants.

Revascularization Improves Limb Function in PAD Patients

Interventional procedures aimed at restoring blood flow to the lower limbs have been well established to improve the limb function of PAD patients who present with critical limb ischemia or have claudication that significantly interferes with their quality of life. Revascularization can be achieved with open bypass surgery, where vein grafts are connected proximally and distally to the site of blockage[117]. Alternatively, endovascular procedures that include percutaneous balloon angioplasty and/or stenting can improve the patency of arteries[118]. Two of the most commonly assessed walking performance parameters are the six-minute walking distance (SMWD) and peak walking time (PWT). Each measures different aspects of limb function under conditions and have been used to study the effects of interventions[113,114]. Although revascularization increases both SMWD and PWT in PAD patients, the improved performance is often short-lived, and over a few years begins to decline. This may be because revascularization prevents further ischemia-reperfusion from injuring PAD muscle, but cannot reverse the myopathic changes that have already occurred. However, no study has examined how revascularization affects myopathic changes in PAD muscle.

Improvements in limb function following revascularization have been measured on treadmill. The classic protocol developed by Gardner-Skinner is standardly used and occurs in a very controlled environment[119]. PAD patients start walking on treadmill at 2 mph (3.2 km/hr) at a zero-degree incline, or grade. This work rate is typical of low-intensity activities of daily life that elderly patients would experience and is well tolerated by the vast majority of PAD patients. Then, every

2 minutes, the grade is increased by 2% by increasing the incline angle and this occurs until the patient becomes limited by maximally tolerated claudication symptoms. The longest time PAD patients can walk on this graded treadmill test is defined as the PWT. Thus, the walking velocity, and rate of increase in walking speed and intensity are highly regulated and as a consequence, the reproducibility is very high and variability is low[120]. Moreover, placebo effects, where performance in PAD patients is improved in the absence of interventions, is also not appreciable, ranging from 13% to 23% in 6 months[121].

PWT, therefore, is the typical primary outcome in clinical trials evaluating therapies, such as revascularization, that improve walking impairment[122]. One such study was CLEVER, which was a multi-centered randomized clinical trial of claudicating PAD patients. In that study, those who underwent endovascular revascularization procedures (41 patients) significantly improved their PWT by 2.5 minutes at 6 months compared to optimal medical treatment alone (18 patients), an improvement that endured at 18 months follow-up[123,124]. Similarly, revascularization by bypass surgery demonstrated a 5.6-minute increase in PWT in 14 PAD claudicants, but there was no sham operated control group for comparison due to obvious ethical reasons[29]. Other studies corroborate these findings, but measured maximal walking distance instead[125-127]. However, this is a surrogate measure of PWT, since greater distances covered on treadmill correlate with longer times.

Improvements in limb function following revascularization have been also measured by the SMWD test. This inexpensive test is performed under submaximal exercise conditions, where PAD patients are instructed to walk back and forth along a corridor that is at least 100 feet long and the distance covered is measured during six minutes[128]. Rest is allowed if discomfort and claudicating symptoms arise, but time continues to elapse. Like PWT, SMWD is a well validated measure of walking endurance and is sensitive to functional declines in PAD patients and therapeutic interventions, such as revascularization[129]. However, unlike PWT, SMWD is measured in an environment that more closely resembles walking in daily life[128,129] and predicts mobility loss and mortality[130,131]. Additionally, a minimally clinically important difference in SMWD of 30 meters has been established, albeit from studies of chronic lung disease, while it is more difficult to translate differences in PWT to clinical benefits[129]. However, SMWD has been criticized for being susceptible to environmental and intervention-independent factors because it is an endurance test under submaximal exercise conditions with no fixed physiological correlate[132]. Other disadvantages are that SMWD has been studied less in PAD patients compared to PWT and so factors such as applicability to a wide range of patients, and baseline values have not been well established[132]. As a result, SMWD data for revascularization intervention has only recently surfaced.

To date, only two studies have examined SMWD before and after revascularization and found significant improvements. In the first study, 153 PAD patients received percutaneous transluminal angioplasty followed by stenting[133].

The SMWD measured at baseline was 84.1 meters and significantly increased to 348 meters 6 months after revascularization. The ABI also significantly increased from about 0.5 to 1.0 in both limbs[133], a finding consistent with revascularization studies that measured PWT on treadmill as the endpoint[123,124]. The second study performed only angioplasty on 47 PAD patients and also found significantly improved function 6 months out, but by 12 months had declined below baseline[134]. As evident from these studies, SMWD can be used to measure improved limb function following revascularization and to determine if they last over time. In conjunction with PWT, SMWD may provide additional insights into the functional limitations of PAD patients, given SMWD only demonstrates a weak correlation with PWT (r^2 = 0.28)[135]. Similar studies have not been done for open bypass surgery because in the past decade, first line therapy has shifted from bypass surgery to endovascular interventions, which is less invasive with lower risks, and applicable to the majority of PAD patients who are indicated for revascularization.

The mechanism by which revascularization improves limb function is by restoring blood flow, but its effects on myopathy in the PAD limb remains largely unknown. Open bypass surgery and endovascular procedures acutely increases ABI, reduces ischemic leg symptoms, and immediately improves walking distance[136]. Thus, revascularization alleviates chronic ischemia-reperfusion injury to the limb and would be expected to improve or at least inhibit progression of myopathic changes, such as oxidative damage, mitochondrial dysfunction, myofiber degeneration, and fibrosis. It is important to determine this because defects not addressed by revascularization need to be targeted by novel

therapeutic strategies aimed at maintaining and further improving the walking abilities of PAD patients, especially those who are contraindicated for revascularization due to co-morbidities or overall health status.

Supervised Exercise Improves Limb Function in PAD Patients

Before PAD patients become candidates for revascularization, they are first prescribed exercise regimens that have been well established to relieve claudicating symptoms and improve functional status in PAD patients[137]. Other considerations for this recommendation include the risks associated with bypass surgery and endovascular procedures and the frequency with which restenosis of the arteries occur, as early as 6 months following revascularization[138]. Two types of exercise programs exist, unsupervised and supervised, and it has been established that supervised regimens, consisting of walking 3 times a week for 30-60 minutes over 12 weeks on treadmill, improves the walking ability of PAD patients better than unsupervised programs[139]. Reasons include lack of adherence and patients not performing the exercises properly to attain the maximal benefits. Supervised exercise therapy, however, is expensive and not covered by Medicare and insurance plans and is often cumbersome since patients have to travel to sites multiple times a week. Although supervised exercise improves limb function, the improvements are at best maintained short-term and like revascularization, is a management strategy and not a cure for PAD patients.

Given that exercise therapy is recommended before revascularization, more studies have been conducted to determine the impact of exercise therapy on limb function and how it directly compares with revascularization. A recent systematic review of 30 clinical trials found that compared to standard care, supervised exercise improved PWT by 4.51 minutes and maximum walking distances on graded treadmill by 109 meters in PAD patients[140]. Furthermore,

these improvements were greatest at 6 months and lasted up to two years[140]. Only two studies have been conducted to measure SMWD in PAD patients before and after supervised exercise. The first study was conducted in the United States and found that in 156 PAD patients, SMWD significantly increased by 35.9 meters at 6 months follow-up after supervised treadmill exercise[141]. A subsequent smaller European study of 51 PAD patients reported SMWD to increase by 100 meters after 3 months of supervised exercise[142]. Although follow-up at 6 months still revealed a significant increase like the American study, there was a trend towards declining function that resulted in performance similar to baseline at 12 months[142]. Thus, improvements in SMWD by exercise therapy may not last beyond six months. Two comprehensive systematic reviews have concluded that the ability of PAD patients to walk longer and further after supervised exercise is comparable to revascularization[143,144]. A single-site randomized clinical trial published after these two systematic reviews corroborates their conclusion. PAD patients were followed seven years out and no differences were found in walking distances or quality of life between the two interventions[145]. However, a more recent multi-site trial CLEVER reported that supervised exercise was superior to revascularization stenting for PWT for at least 18 months[123,124], prompting comparisons of exercise with revascularization against exercise therapy alone.

The few clinical trials that have compared supervised exercise and revascularization against exercise alone demonstrated that combination therapy was superior for limb function. The ERASE trial studied 212 PAD patients and found at 1 year follow-up that combined endovascular procedures with supervised

exercise increased the treadmill walking distance on the graded treadmill test significantly more than supervised exercise alone, by 282 meters[127]. An earlier study MIMIC randomized 127 patients to similar groups, with angioplasty as an adjuvant therapy for PAD patients who were already partaking in supervised exercise programs[146]. Compared to supervised exercise alone, combination therapy produced significantly greater maximal treadmill walking distance at 6 months when the supervised exercise ended, and the improvements were maintained at 2 years[146]. However, other studies put into question how long the improved limb function lasts. A trial that studied 178 PAD patients found that combined exercise and angioplasty increased PWT significantly more than exercise therapy alone at 3 months, but there were no differences at 12 months[126]. Overall, the benefits of combined therapy *versus* exercise alone probably represent the best improvements for PAD patients at approximately 6 months. Benefits are largely maintained up to 2 years, but does not progressively improve throughout that period of time, pointing to the need for adjunct therapies. Adjunct therapies are also especially needed, since PAD patients with co-morbidities or overall poor health are precluded from either supervised exercise or revascularization, thus limiting the maximal benefit that they can achieve in walking performance. A better understanding of the mechanisms by which exercise improves limb function and how it affects the developing myopathy in the limbs of PAD patients may provide novel paths towards such adjunct therapies.

The mechanisms by which exercise therapy improves the walking abilities of PAD patients are distinct from revascularization and are not as well

characterized. Unlike the immediate functional improvements seen with revascularization, supervised treadmill exercise improves performance gradually, with the earliest benefits at approximately 1 to 2 months[136]. Despite these improvements, the blood flow does not improve. A systematic review of 33 clinical trials found that exercise therapy did not increase the ABI[140], which demonstrates that the mechanisms of supervised exercise on limb function are not directly blood flow dependent. The following theories have been implicated by exercise studies of PAD patients.

In healthy people, exercise training induces an array of adaptations that include increased muscle mitochondrial content, improvements in the ability to generate energy with the amount of oxygen that is delivered, increased perfusion of blood in the microcirculation, and improved walking economy[132]. This increased metabolic efficiency may improve the mitochondrial dysfunction and oxidative stress found in the muscle of PAD patients. Studies in PAD patients following 12 weeks of supervised exercise have shown that the oxidative capacity of calf skeletal muscle was increased, whereby greater oxygen extraction per unit of blood occurred[147]. In patients who completed 12 weeks of treadmill training that intentionally produced claudication pain, the buildup of Kreb cycle components decreased, indicating improved muscle metabolism[48]. Similarly, the increased blood perfusion that results from exercise training in healthy individuals may allow the blood that does arrive to the lower limb move more efficiently into the muscle. In PAD patients, exercise-induced angiogenesis and enhanced nitric oxide-dependent vasodilation in endothelia of the microcirculation, and decreased blood

viscosity have all been demonstrated[148,149]. The increased efficiency of blood to the muscle, coupled with improved oxygen extraction and muscle metabolism, may underlie the improved walking economy of PAD patients following supervised exercise therapy. After four months of exercise training, PAD patients use less oxygen at a given workload[150] and this biomechanical efficiency likely translates into improved walking performance. It may be that exercise reverses the oxygen cost created by the gait abnormalities that patients adopt in response to leg pain, mainly those that favor increased stability at the expense of velocity[151-154].

Much less well studied are the effects of exercise therapy on systemic and local inflammation and fibrosis in the muscle of PAD patients, which are the reparative defects produced by chronic ischemia-reperfusion injury[65]. One study found that exercise training improved claudication symptoms and concomitantly reduced inflammatory markers in the circulation, such as C-reactive protein[155]. Inflammatory markers in the leg muscle may also be altered to promote resolution of chronic inflammation and pathological fibrosis. More research is needed to know how all the physiological benefits of exercise specifically extend to PAD myopathy. For example, peripheral nerve function is improved following exercise therapy, but it is unclear how this may affect the myopathy. The potential mechanisms by which exercise therapy improve limb function are summarized in **Figure 1.4**. Overall, how the biological mechanisms improved by supervised exercise therapy directly relate to the myopathy present in the legs of PAD patients is unclear. What is clear though, is that understanding how supervised exercise affects PAD myopathy will point to novel therapeutic targets that improve limb function of PAD patients.

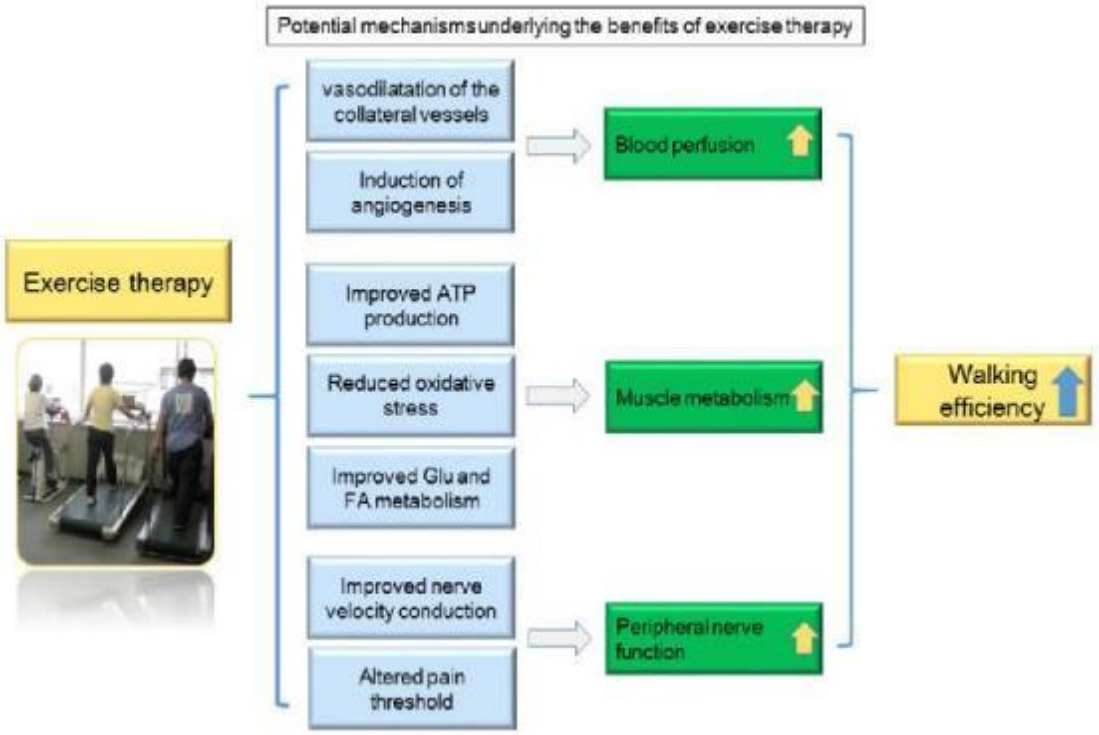


Figure 1.4: Schema of Potential Mechanisms Underlying the Benefits of Exercise Therapy on Walking Performance in Peripheral Artery Disease. Obtained from the European Society of Cardiology Online Article "Exercise Therapy for Intermittent Claudication in Peripheral Artery Disease. http://www.escardio.org/Guidelines-&-Education/Journals-and-publications/ESC-journals-family/E-journal-of-Cardiology-Practice/Volume-13/exercise-therapy-for-intermittent-claudication-in-peripheral-artery-disease

Hypothesis and Specific Aims

Based on data from the literature regarding the pathophysiology of PAD, it is clear that a myopathy develops in the lower limb skeletal muscle of these patients and causes limb dysfunction. Various components of this myopathy, such as oxidative stress, mitochondrial dysfunction, and myofiber degeneration have been characterized, while others, such as fibrosis, have not. Although increased extracellular matrix in the lower limb skeletal muscle of PAD patients has been described by our laboratory and others, the etiology of its development is unknown, such as which biological factors play an important role and its pathobiological link to atherosclerosis. The robust increase in TGF-β1, a potent inducer of fibrosis, in the gastrocnemius of PAD patients was greater than other cytokines and also greater than TGF-β1 in serum, both of which suggest its critical role in promoting myofibrosis locally in the muscle. In various organs, fibrosis is highly detrimental to function. Altering the development of myofibrosis may be a mechanism by which the standard therapies, revascularization and supervised exercise, are improving limb function. The following hypotheses and specific aims were developed to determine if local hemodynamic changes produce a TGF-β1 associated myofibrosis and whether or not this myofibrosis is targeted by revascularization and exercise therapies to improve limb function in PAD patients.

The overall hypothesis is that decreased hemodynamics is tightly linked to a local TGF-β1 associated increase in collagen deposition within the gastrocnemius of PAD patients that increases with disease severity, and

that this myofibrosis is reversed by revascularization but not exercise therapy in association with improved limb function. We propose that the etiology of myofibrosis in PAD is from hemodynamic changes that are directly a part of the pathophysiology of PAD, rather than solely as a consequence of myofiber degeneration, characteristic of other myopathic diseases. Furthermore, the myofibrosis that develops will be dependent on the extent of atherosclerosis and hemodynamic state of the limb and not pathological systemic changes that are associated with PAD. Finally, revascularization procedures that directly improve hemodynamics will reduce expression of TGF-β1 and collagen deposition in the gastrocnemius of PAD patients. Exercise therapy, on the other hand, is not expected to decrease TGF-β1 associated myofibrosis, since it does not directly alleviate compromised hemodynamics. Addressing this hypothesis will provide insight into the natural history of PAD myopathy and response to standard therapies, as well as strategies that directly target development of myofibrosis to maintain or improve limb function in PAD patients.

This hypothesis will be tested by implementation of three specific aims that determine 1) the presence and etiology of a TGF-β1 associated myofibrosis, 2) whether myofibrosis represents a local response to ischemia or a response to systemic events and 3) how revascularization and exercise therapy affect the progression of this TGF-β1 associated myofibrosis in relation to limb function.

Specific Aim 1: Investigate the relationships among limb hemodynamics, and both TGF-β1 expression and collagen deposition in the gastrocnemius of PAD patients at advancing disease stages to establish the presence and potential etiology of myofibrosis. Using multi-spectral microscopy (MSM) of gastrocnemius specimens stained with Masson Trichrome, we measured collagen density and area to determine the extent of fibrosis in gastrocnemius biopsies of PAD patients at Fontaine Stage II and Fontaine Stage IV stages of disease compared to age-matched control patients. Expression of TGF-β1 in the same patient groups was measured by quantitative fluorescence microscopy (QFM) after immunofluorescence labeling for TGF-β1. Co-localization studies using specific markers for vascular and immune cells was used to determine the cellular source of TGF-β1 expression that is indicative of the etiology of myofibrosis. Immunohistochemistry for expression of TE-7 positive fibroblasts further explored the mechanism of myofibrosis, whereby increased TGF-β1 expression activates fibroblasts to deposit extracellular matrix to promote pathological fibrosis. The hemodynamic state of the limbs from which biopsies were obtained was measured as the Ankle-Brachial Index (ABI). Correlational analyses were performed among ABI, TGF-β1 expression, and collagen density to determine if a TGF-β1 associated myofibrosis exists and whether or not it is associated with reduced hemodynamics in the lower limb of PAD patients.

Specific Aim 2: Determine whether TGF-β1 associated myofibrosis in the gastrocnemius of PAD patients is a localized response in the ischemic muscle or response to systemic events. The previous aim established that myofibrosis in the gastrocnemius of PAD patients is associated with increased TGF-β1 expression of vascular origin, leaving the possibility that the observed myofibrosis is induced by circulating factors related to atherosclerosis in addition to local hemodynamic changes produced by atherosclerosis. Gastrocnemius biopsies were obtained from both limbs of Fontaie Stage II PAD patients with unilateral disease, who had significantly greater atherosclerosis in the diseased compared to contralateral limbs as reflected by the signifiantly lower ABI. Biopsies were assessed for TGF-β1 expression at the protein level by QFM and transcript level by qPCR, and for collagen density by MSM. Correlational analyses were performed between the limbs for the expression of TGF-β1 and collagen density. Furthermore, correlational analyses between TGF-β1 expression and collagen density were performed separately in the diseased and contralateral limbs. Greater myofibrosis in the diseased compared to contralateral limb, with no relationships between the limbs would indicate a local response to atherosclerosis as the etiology, while no difference in myofibrosis between the limbs and a direct relationship between the limbs would indicate that systemic contributions exist.

Specific Aim 3: Determine changes in myofibrosis in relation to limb function, hemodynamics, and myofibers size, in PAD patients who have undergone revascularization or exercise therapy. Revascularization and supervised exercise are standard therapies that improve limb function in PAD patients but whether or not it is by improving the TGF-β1 associated myofibrosis is unknown. Fontaine Stage-II PAD patients underwent revascularization by open bypass surgery or endovascular procedures that involved balloons and/or stents, or participated a supervised exercise therapy program for 6 months. Biopsies were obtained at baseline and 6 months. Limb functional assessments were also performed at those time points. Six Minute Walking Distance (SMWD) and Peak Walking Time (PWT) were measured. Additionally, ABI and morphometric analysis for myofiber size was determined. Correlational analyses were performed among TGF-β1 expression, collagen deposition, SMWD, PWT, ABI, and myofiber size. A subset of patients received no intervention and served as a control for direct comparison against revascularization and exercise therapies.

CHAPTER II: TGF-β1 ASSOCIATED MYOFIBROSIS DEVELOPS WITH INCREASING PAD DISEASE SEVERITY AND IS ASSOCIATED WITH REDUCED HEMODYNAMICS

Introduction

Lower leg ischemia, myopathy and limb dysfunction are distinguishing features of Peripheral Artery Disease (PAD) caused by atherosclerotic blockages of the arteries supplying the legs. The myopathy in the affected legs of PAD patients is characterized by myofiber degeneration, elevated pro-fibrotic cytokines, and increased fibrosis[35,36,44,54,156,157]. In Chapter I, the development of myofibrosis with increasing PAD disease stage was suggested by observations on computed tomography and hematoxylin and eosin histology of the gastrocnemius of PAD patients at Fontaine Stage II and IV compared to age-matched controls **(Figure 1.3)**. Cytokine profiling of gastrocnemius homogenates from Fontaine Stage-II PAD patients and age-matched controls revealed that transforming growth factor-beta 1 (TGF-β1), which is considered the most potent inducer of fibrosis, is the most robustly and consistently elevated cytokine in PAD patients **(Table 1.1)**. The expression of TGF-β1, however, was not evaluated in Fontaine Stage IV patients and its relationship to fibrosis with advancing disease in PAD has not been examined. Moreover, the etiology of the increased TGF-β1 is unknown. Chapter II explores the relationships between TGF-β1, collagen deposition in PAD patients at different disease stages to establish that TGF-β1 associated myofibrosis is part of PAD myopathy. Furthermore, we investigate the cellular sources of TGF-β1 in relation to ABI limb hemodynamics to gain insight into the etiology of PAD myofibrosis.

The etiology of myofibrosis in PAD may be immune or vascular. Classically, in response to acute injury and inflammation, TGF-β1 is secreted by immune cells, such as macrophages and T cells, during the resolution of phase of inflammation to reduce inflammation and initiate physiological wound healing[73,74]. TGF-β1 inhibits T cells and activates myofibroblasts to deposit extracellular matrix, of which a major component is collagen. In many chronic diseases of the muscle, such as the dystrophies, TGF-β1 is highly elevated and persistently activates myofibroblasts to deposit excessive extracellular matrix around the myofibers and associated microvessels which interferes with oxygen and nutrient delivery, producing myofiber degeneration[70,71,158,159]. Additionally, TGF-β1 can inhibit regeneration in skeletal muscle to promote PAD myopathy by inducing myoblasts to differentiate into myofibroblasts rather than new myofibers[70,71,158,159]. In this setting, it has been demonstrated that TGF-β1 is produced by immune cells to induce pathological fibrosis[69-71].

However, other cells besides immune cells have also been reported to upregulate TGF-β1 expression and contribute to fibrosis in various organ systems, and these include vascular cells[67,72,160-162]. For example, in pulmonary arterial hypertension (PAH), vascular smooth muscle cells (SMC) transition from a contractile to a synthetic phenotype that produces TGF-β1[76,77,163,164]. In PAH, chronic hypoxia induces substantial thickening of vascular intima, media and adventitia caused by extensive proliferation and hypertrophy of vascular SMC that exhibit increased expression of TGF-β1, and deposition of extracellular matrix; features that extend to normally non-muscularized microvessels[164,165]. In the

context of PAD, vascular cells may contribute to the expression of TGF-β1, since PAD is a chronic disease originating from the arteries. A similar transitioning of vascular SMC may be occurring in response to exercise-induced ischemia and the chronic intermittent hypoxia that are caused by atherosclerosis of the large vessels supplying the legs. If that is true, we expect this transitioning to also be related to the degree of hemodynamic compromise of the affected extremity and the clinical stage of PAD.

This study addresses **Specific Aim 1** by evaluating the cellular expression of TGF-β1 in the gastrocnemius of control and PAD patients with advancing Fontaine Stage, and its relationship to deposited collagen, fibroblast accumulation, and Ankle-Brachial Index (ABI). We hypothesize that there is a TGF-β1 associated myofibrosis that is increased with PAD severity and is of vascular origin, whereby TGF-β1 is expressed by vascular cells and inversely correlates with ABI.

Methods

Characteristics of Human Subject Groups

The experimental protocol was approved by the Institutional Review Boards of the Veterans Affairs Nebraska-Western Iowa and University of Nebraska Medical Centers. All subjects gave informed consent.

PAD Groups: We recruited 25 Fontaine Stage-II PAD patients (PAD-II) and 20 Fontaine Stage-IV patients (PAD-IV). PAD-II patients presented with intermittent claudication, but no rest pain or tissue loss. PAD-IV patients presented with non-healing ulcers and/or gangrene and had lower extremity operations. Diagnosis for each patient was established on the basis of medical history, physical examination, decreased ankle brachial index (ABI < 0.9), and computerized or standard arteriography that revealed stenotic and/or occluded arteries supplying the lower extremities.

Control Group: We recruited 20 control patients (CTRL) who were undergoing lower extremity operations for indications other than PAD. Control patients led sedentary lifestyles with no history of PAD symptoms and had normal blood flow to their lower limbs, as indicated by normal lower extremity pulses at examination, and normal ABI at rest and after exercise.

Patient Demographics: Data for CTRL, PAD-II, and PAD-IV patients are presented in **Table 2.1**. On average, PAD-IV patients were 6 years older than PAD-II ($p = 0.047$) and 8 years older than CTRL ($p = 0.006$) patients. More PAD-

IV patients had diabetes than PAD-II and CTRL patients ($\chi 2 = 5.55$, $p = 0.006$; both $p < 0.05$). Age and diabetes were treated as covariates in all subsequent statistical analyses.

Biopsy and Histological Preparation

Gastrocnemius samples weighing approximately 250 mg were obtained from the anteromedial aspect of the muscle belly, 10 cm distal to the tibial tuberosity. All biopsies were obtained with a 6 mm Bergstrom needle. Some of the samples were frozen in liquid nitrogen for biochemical analysis while others were placed immediately into cold methacarn. After 48 hours, the specimens were transferred to cold 50% ethanol, and subsequently embedded in paraffin.

Quantitative Assessment of Collagen Deposition

Masson Trichrome Stain: Paraffin-embedded biopsies sectioned at 4 microns were stained with a Masson Trichrome Kit according to manufacturer's protocol (Fisher Scientific Richard Allan Scientific #22-110-648, Pittsburg, PA, USA). Briefly, slide specimens were deparaffinized in xylene, hydrated and then incubated at room temperature in Bouin's fixative for 16 hours. Nuclei were stained with Weigert's Hematoxylin, myofiber cytoplasm with Scarlet Red, and collagen with Aniline Blue dye, with washes in-between steps. Stained slide specimens were dehydrated and mounted with Permount (Fisher Scientific #SP15-100, Pittsburg, PA, USA).

	CTRL	PAD-II	PAD-IV	p-value
Number of Patients	20	25	20	N/A
Mean Age (years)	62.1 ± 5.40	64.1 ± 7.80	69.9 ± 9.40*	0.011
Gender (male/female)	19/1	24/1	20/0	0.983
Height (m)	1.75 ± 0.07	1.76 ± 0.06	1.77 ± 0.06	0.576
Weight (kg)	91.0 ± 17.4	87.5 ± 18.8	85.0 ± 21.1	0.598
Body Mass Index	29.9 ± 6.40	28.2 ± 5.16	28.4 ± 6.61	0.593
Obesity† (%)	45	36	25	0.416
Smoking (%)	50.0	56.0	30.0	0.202
Diabetes Mellitus (%)	25.0	20.0	65.0*	0.004
Dyslipidemia (%)	55.0	80.0	60.0	0.166
Coronary Artery Disease (%)	20.0	36.0	35.0	0.701
Myocardial Infraction (%)	0.00	8.00	0.00	0.192
Hypertension (%)	65.0	84.0	90.0	0.116
Statins Medication (%)	70.0	84.0	65.0	0.317
Renal Insufficiency‡ (%)	5.00	12.0	20.0	0.352
Ankle Brachial Index§ **(Minimum – Maximum)**	1.04 ± 0.11* (0.79–1.20)	0.55 ± 0.22* (0.10–0.95)	0.22 ± 0.13* (0.00–0.44)	<0.001

Table 2.1: Demographics of Study Groups[157].

† Obesity: Body mass index > 30
‡ Renal Insufficiency: Creatinine clearance < 60 ml/min/1.73m^2
§ABI: Data presented as Mean ± Standard Deviation

CTRL = Controls, PAD-II = Fontaine Stage II, PAD-IV = Fontaine Stage IV
* $p < 0.05$ *versus* other two groups by post-hoc Bonferroni adjusted t-tests

Image Acquisition and Multi-Spectral Microscopy: Microscopic fields of the five most fibrotic regions were collected from each Masson Trichrome stained slide (20x objective). Muscle tissue completely filled each chosen microscopic field. Imaging was implemented by multispectral wide-field microscopy, using a Leica microscope (North Central Instruments DMRXA2 Model, Plymouth, MN, USA) coupled with the Nuance EX Multispectral Imaging System (PerkinElmer N-MSI-EX Model, Waltham, MA, USA) that incorporates a CCD camera and liquid crystal tunable filter. This system generates an absorbance spectrum at each pixel of a two-dimensional spatial image of the specimen. The Nuance software quantitatively extracts the grey scale image of deposited collagen, which is then transferred to the Image-Pro® Plus image analysis software (Media Cybernetics, Warrendale, PA, USA) for quantification of collagen area and density. Collagen density was determined as area-weighted mean pixel intensity (12-bit grey scale). Quantitative analysis was performed blinded from the group status. **Figure 2.1** depicts the processing of one representative region of interest.

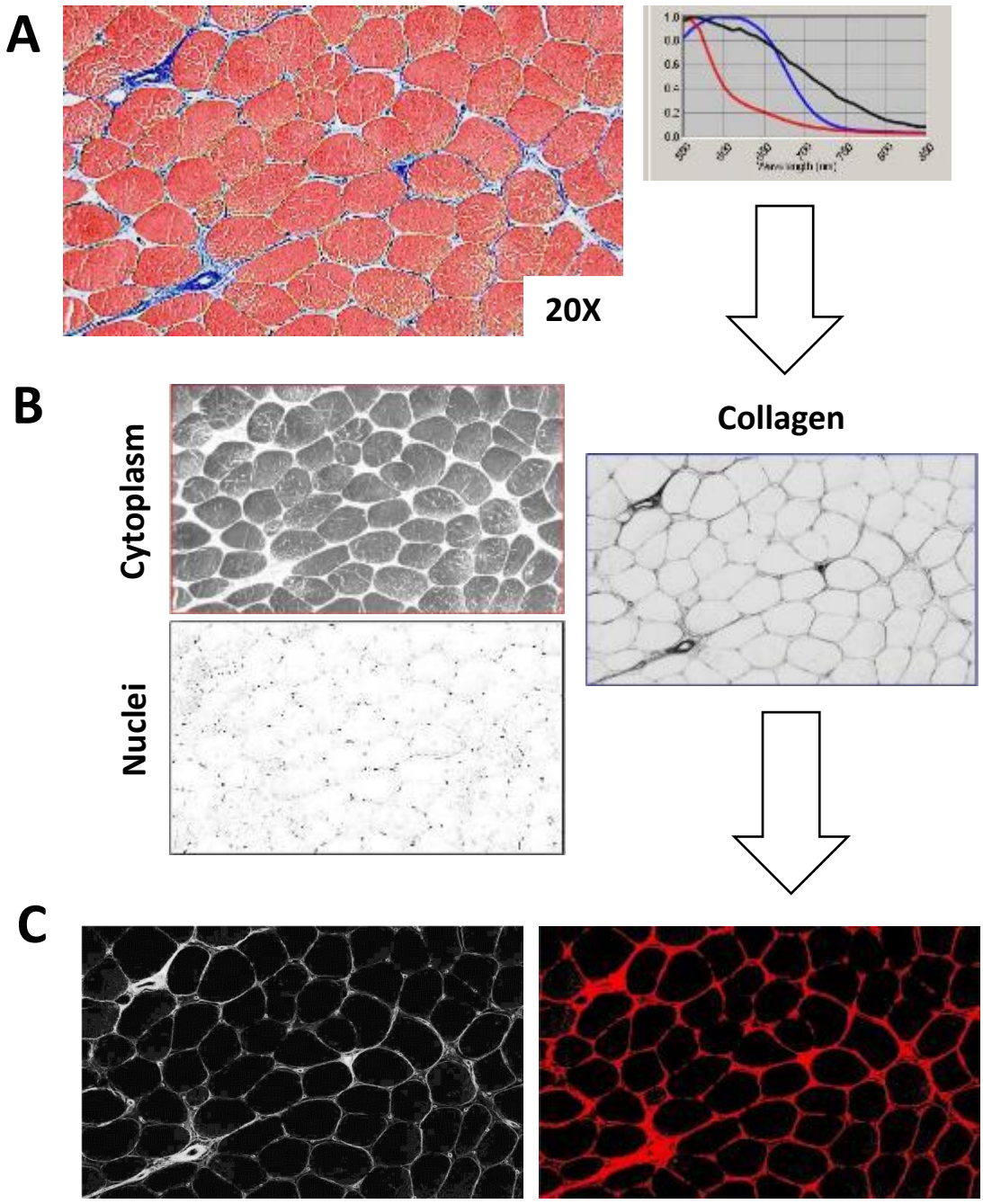


Figure 2.1: Multi-Spectral Microscopy of a Representative Fibrotic Region of PAD Patient Gastrocnemius[157]. (A) Slide specimens were stained for Masson Trichrome, which labels collagen blue, myofiber cytoplasm red, and nuclei black. Fibrotic regions of muscle were image and Nuance Software generated a spectral library containing each component. **(B)** Each component was extracted as grey scale images. **(C)** The gray scale image for collagen was inverted in ImagePro Plus Software and intensity of labeling was measured as collagen density, while collagen area is the number of pixels analyzed (red).

Validation of Spectral Analysis: Spectral analysis was validated by the hydroxyproline assay for collagen content of human gastrocnemius specimens[166]. Four PAD-IV, three PAD-II, and three CTRL subjects were selected for analysis based on their wide range of collagen density determined by wide-field multi-spectral microscopy. Briefly, approximately 10 mg of liquid nitrogen preserved gastrocnemius muscle from each patient was hydrolyzed in hydrochloric acid (Fisher Scientific #SA56-1, Pittsburg, PA, USA) overnight for 12 hours at 130° C. Then, contents were adjusted to neutral pH by potassium hydroxide (Sigma #C9887, St Louis, MO, USA) using a solution of phenolphthalein (Sigma #34607, St Louis, MO, USA) as indicator, and buffered with Sodium Borate that is composed of Sodium Hydroxide (Fisher Scientific #S318-500, Pittsburg, PA, USA) and Boric Acid (Fisher Scientific #BP168-500, Pittsburg, PA, USA). Hydroxyproline was oxidized by the Chloramine T solution, which is comprised of chloramine T (Sigma #C9887, St Louis, MO, USA), citric acid anhydrous (Sigma #C0759, St Louis, MO, USA), glacial acetic acid (Fisher Scientific #A38-212, Pittsburg, PA, USA), sodium acetate trihydrate (Sigma #S7670, St Louis, MO, USA), sodium hydroxide (Fisher Scientific #S318-500, Pittsburg, PA, USA), and 2-mthoxyethanol (Sigma #284467, St Louis, MO, USA). Then, the reaction was stopped by Sodium Thiosulfate (Sigma #217263, St Louis, MO, USA). Impurities were removed by addition of toluene (Sigma #244511, St Louis, MO, USA), inversion and centrifugation, and aspiration of the resulting toluene layer. Next, the oxidized product was extracted by inversion, centrifugation, and collection of the toluene layer. Finally, Ehrlich's Reagent, a mixture of sulfuric acid (Sigma #339471, St

Louis, MO, USA), ethanol (Decon Labs #2701, King of Prussia, PA, USA), and 4-dimethylaminobenzaldehyde (Sigma #156477, St Louis, MO, USA) was added to the extraction to initiate a colorimetric reaction and incubated at room temperature for 30 minutes. These samples and hydroxyproline standards (0.0 - 8.0 µg; Sigma #H54409, St Louis, MO, USA) were analyzed at 560-nm wavelength on a spectrophotometer. The hydroxyproline content of muscle was expressed as µg per mg muscle (wet weight). A robust positive correlation ($r = 0.907$; $p < 0.001$) was detected between hydroxyproline (µg per mg muscle) and optical density from spectral images, for 10 patients who encompassed a wide range of collagen spectral densities **(Figure 2.2A)**.

Reproducibility of Spectral Analysis: To determine intersession reliability, slide specimens of biopsy samples from 10 patients were stained by Masson's Trichrome in two separate analytical sessions and assessed by spectral analysis. The Intraclass Correlation Coefficient (ICC) was estimated by using a two-way mixed ANOVA with absolute agreement. An ICC value of less than 0.40 indicates poor reproducibility, between 0.40 and 0.75 indicates fair to good reproducibility, and greater than 0.75 indicates excellent reproducibility. The ICC (0.865) indicated an excellent reproducibility **(Figure 2.2B)** with an average difference from the mean of the two analytical sessions of 4%.

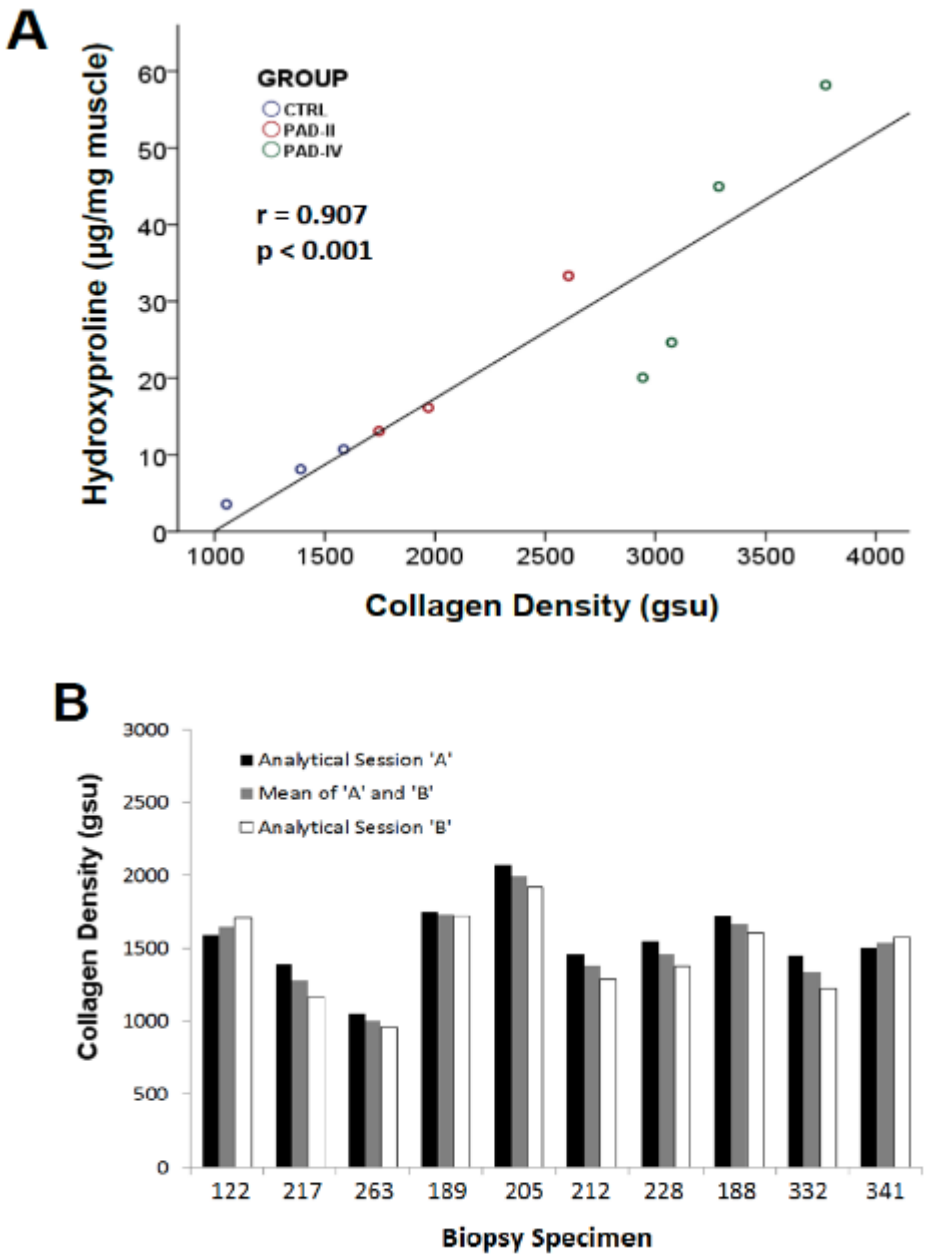


Figure 2.2: Validation of Multi-Spectral Microscopic Analysis of Collagen Deposition[157]. (A) Pearson correlation demonstrating the relationship between collagen density measurements by multi-spectral microscopic analysis and hydroxyproline content in muscle homogenate, as measured by the hydroxyproline assay. **(B)** Reliability of multi-spectral measurements of collagen density in independent, analytical sessions A and B was determined as an intraclass correlation (ICC) value of 0.865, representing excellent reliability (ICC > 0.75 is excellent, 0.75 ≥ ICC ≥ 0.40 is good, and ICC < 0.40 is poor).

Quantitative Assessment of TGF-β1 Expression

Quantitative Fluorescence Microscopy: We describe a novel application of quantitative fluorescence microscopy (QFM) to measure TGF-β1 expression in the gastrocnemius of PAD patients. QFM is a technique developed by our laboratory to measure biomarkers of PAD and prostate cancer [54,167-170].

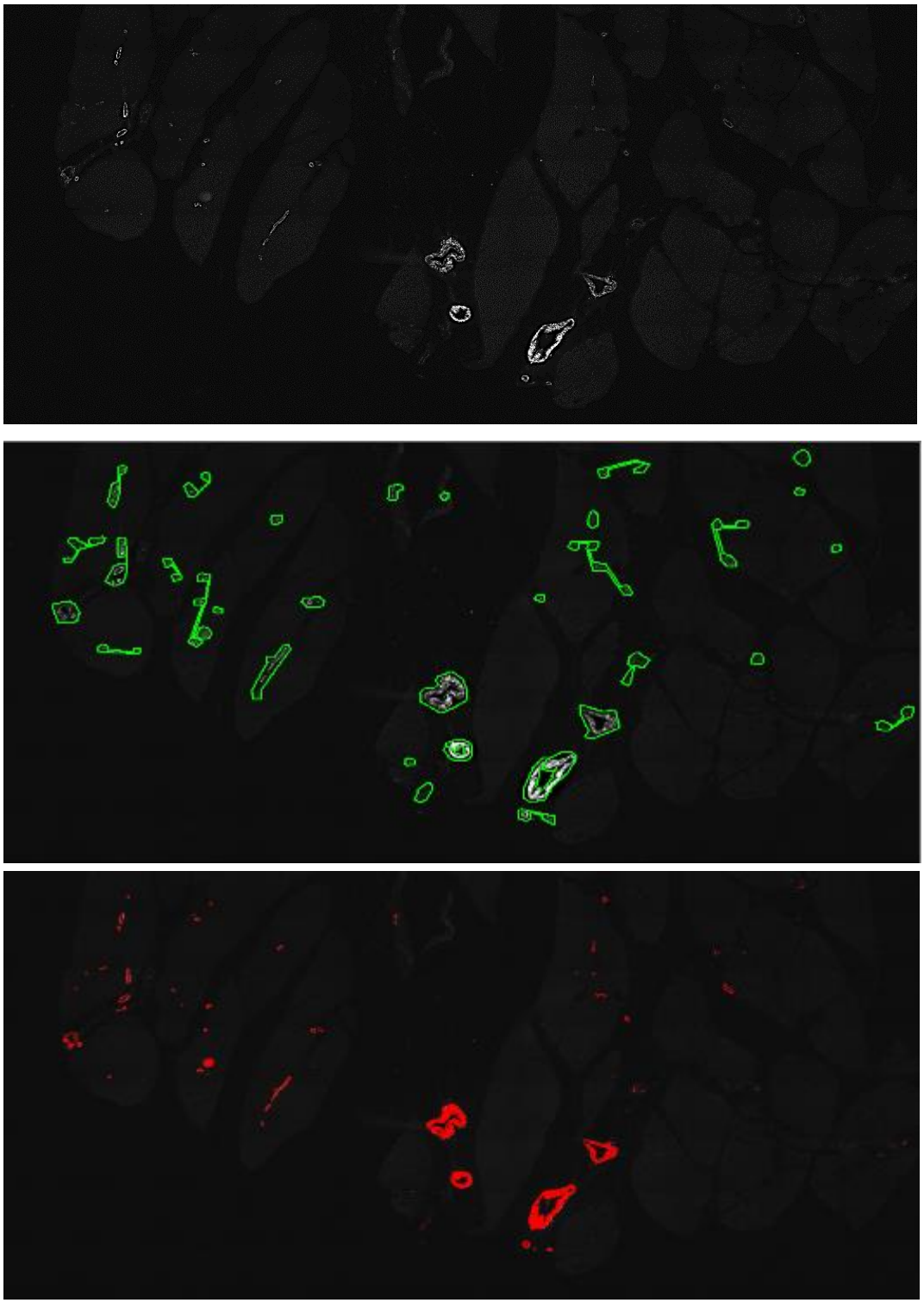
Duplicate slide specimens of gastrocnemius biopsies were exposed to a rabbit anti-TGF-β1 antibody (2.5 µg/mL; Abcam Ab53169, Cambridge, MA, USA) and a mouse anti-CD31 antibody (1:50 dilution; Abcam Ab9498, Cambridge, MA, USA; to validate identification of microvessels). Slides were then treated with both goat anti-rabbit IgG secondary antibody coupled with Alexa Fluor® 555 and goat anti-mouse IgG secondary antibody conjugated with Alexa Fluor® 555 (Life Technologies #A21429, Carlsbad, CA, USA). Isotype control slides were treated with rabbit IgG (Vector Laboratories #I-1000, Burlingame, CA, USA) and mouse IgG1 (eBioscience #14-4714-85, San Diego, CA, USA) at the same concentration as the anti-TGF-β1 and anti-CD31 antibodies, respectively. For epitope recovery, Tris buffer (pH 9.0) was used. All labeling procedures were performed with a fully programmable, robotic autostainer (BioGenex i6000 Model, Fremont, CA, USA). The labeled specimens were mounted in ProLong Gold® anti-fade medium containing 4',6-Diamidino-2-phenylindole (DAPI; a nuclear stain) (Life Technologies #P36931, Carlsbad, CA, USA). Fluorescence images were captured with a Leica epifluorescence wide-field microscope (10x objective) (North Central Instruments, DMRXA2 Model, Plymouth, MN, USA) and CCD camera

(Hamamatsu Photonics, Orca C4742 Model, Bridgewater, NJ, USA), with Hamamatsu software (HCImage 4.0). All fields of each specimen are captured and a montage of the gray scale images was generated for analysis with Image-Pro® Plus (Media Cybernetics, Bethesda, MD, USA). TGF-β1 positive events in the microvasculature are partitioned and both event area and mean pixel intensity are determined for each (12-bit grey scale). The sum of the products of area and mean pixel intensity of all positive events per microscopic field was computed and normalized to the total area of muscle tissue specimen analyzed. Quantitative analysis was performed blinded from the group status. **Figure 2.3** depicts a representative montaged region of interest and its analysis.

Validation of Quantitative Fluorescence Microscopy: QFM measurements of TGF-β1 were validated by comparing results with ELISA and qPCR measurements of muscle homogenates from PAD-II and CTRL patients (N=13 in each group). Additionally, the TGF-β1 expression patterns were confirmed with a second anti-TGF-β1 antibody (Abcam Ab170736, Cambridge, MA, USA).

ELISA: Conventional sandwich-based ELISA measurements of TGF-β1 expression in PAD gastrocnemius was carried out according to manufacturer instructions as part of a customized Human Inflammatory Cytokine Multi-Analyte ELISArray Kit (Qiagen, Valencia, CA, USA). TGF-β1 expression by QFM significantly correlated with TGF-β1 by ELISA ($r = 0.602$; $p = 0.001$; **Figure 2.4A**).

A



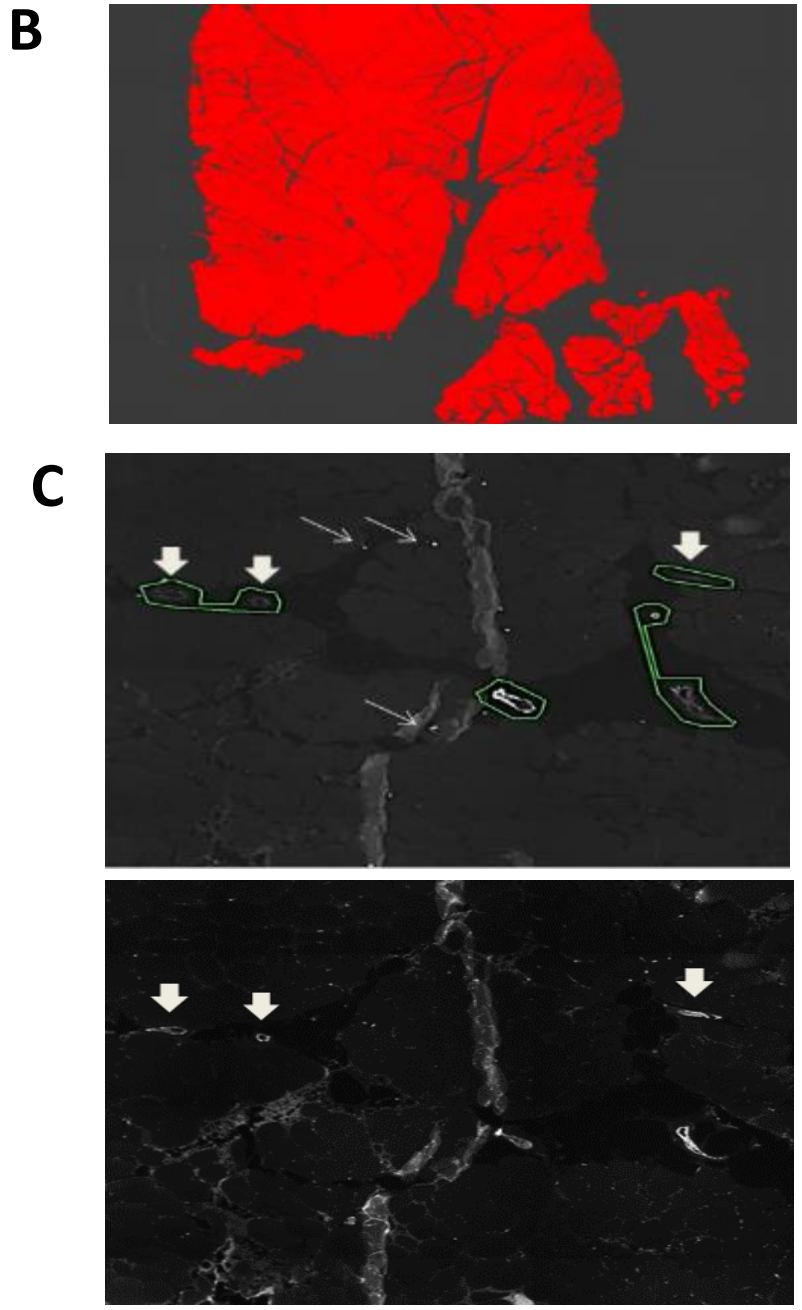
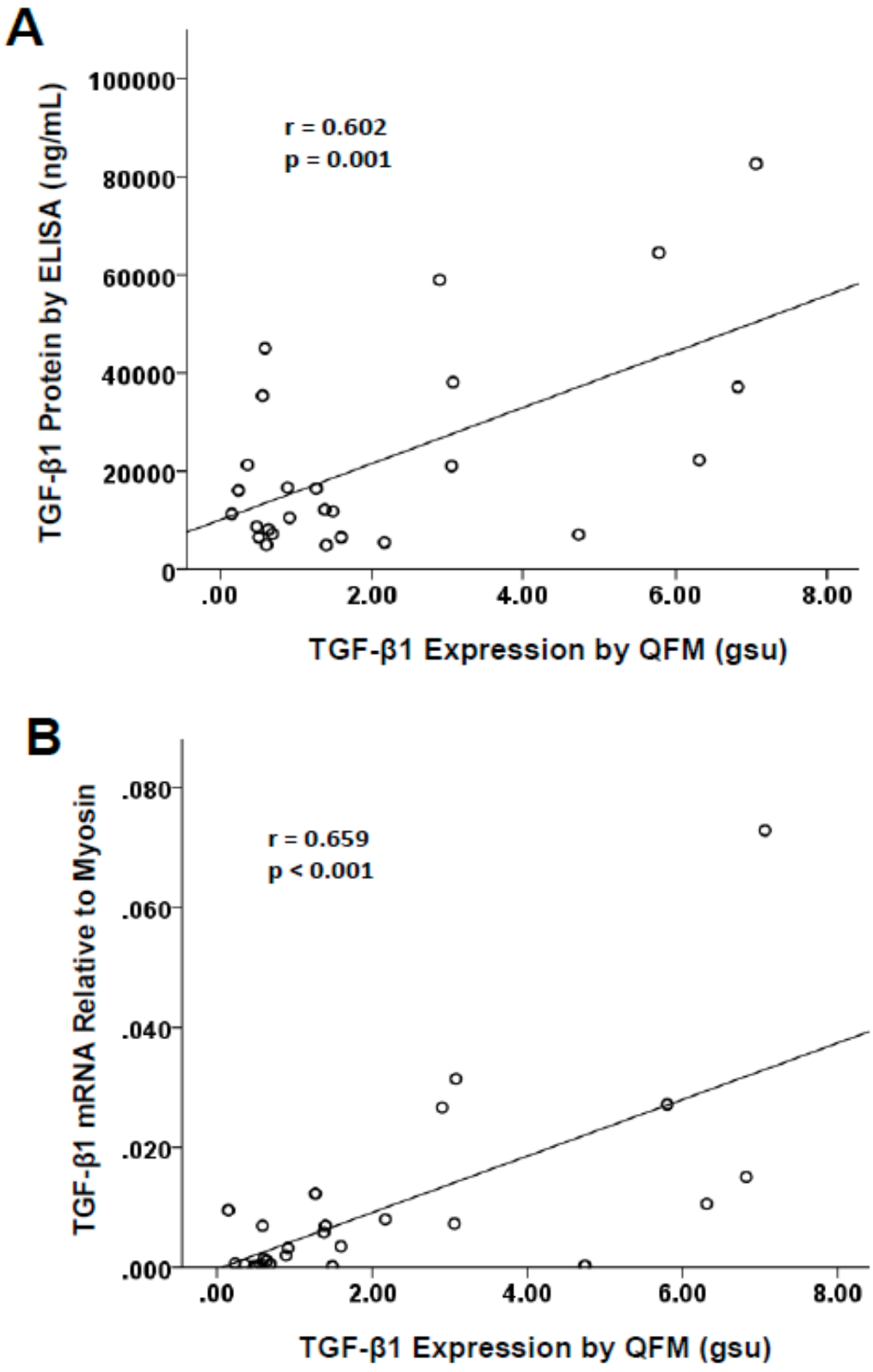


Figure 2.3: Quantitative Fluorescence Microscopy of a Representative Montaged Image of PAD Gastrocnemius[157]. (A) Slide specimens were labeled with TGF-β1 antibody by immunofluorescence and imaged at 10X as individual gray scale images that were then montaged into larger regions and transferred into ImagePro Plus Software. All vessels were manually selected and the intensity of labeling within each vessel was measured as the expression of TGF-β1. **(B)** TGF-β1 expression was normalized to the total myofiber area. **(C)** The same slide specimens were labeled for CD31, a marker of endothelia, to aide in the identification of microvessels (thick arrows) from potential artifacts (thin arrows).

Quantitative PCR: TGF-β1 RNA transcripts in skeletal muscle biopsies stored in liquid nitrogen were extracted, reverse transcribed, and quantified by qPCR, as previously described[39,171]. Levels of TGF-β1 transcripts were then normalized to myosin gene transcripts. The following TGF-β1 primers (188 bp, NCBI, NM_000660) were used: forward, 5'-CCGCAAAGACTTTTCCCCAGACC-3'; reverse, 5'- ACCTAGATGGGCGCGATCTGGTA-3'. The following myosin primers (199 bp; NCBI, NM_005963) were used: forward, 5'-TCCGAAAGTCTGAAAGGGAGCGAA-3'; reverse, 5'-GAGGGTTCATGGGGAAGACTTGGT-3'. TGF-β1 expression by QFM demonstrated a significant positive correlation with TGF-β1 transcripts as determined by qPCR ($r = 0.659$; $p < 0.001$; **Figure 2.4B**).

Reliability of Quantitative Fluorescence Microscopy: The intersession reliability was determined for TGF-β1 from the averages of patient biopsy specimens, analyzed a second time in the next analytical session. The two-session mean of each biopsy specimen was determined and each session mean was expressed as the absolute difference from the two-session mean. The ICC was estimated by a two-way mixed ANOVA with absolute agreement. The ICC (0.993) indicated excellent reproducibility **(Figure 2.4C)**, with an average absolute difference from the mean of the two analytical sessions of 5.3%. Overall, QFM is a reliable method to measure expression of TGF-β1 in skeletal muscle biopsy specimens. The intrasession reliability was high (ICC = 0.957), with a 6% average absolute difference of each slide from the mean of its duplicate pair.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TGF-β1 Protein by ELISA (ng/mL)
100000
80000
60000
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r = 0.602
p = 0.001
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6.00
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TGF-β1 Expression by QFM (gsu)
B
TGF-β1 mRNA Relative to Myosin
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.060
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TGF-β1 Expression by QFM (gsu)

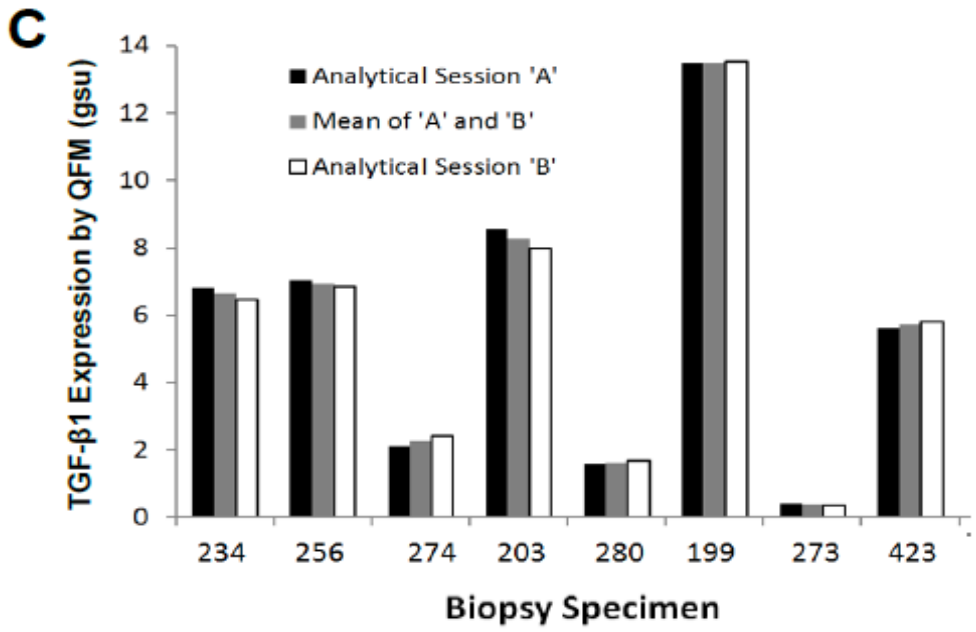


Figure 2.4: Validation of Quantitative Fluorescence Microscopic (QFM) Analysis of TGF-β1[157]. The relationships between TGF-β1 expression determined by QFM and TGF-β1 expression determined by **(A)** ELISA, and **(B)** qPCR in 13 controls and 13 PAD-II patients were analyzed by Pearson correlation. **(C)** Intersession reliability of normalized TGF-β1 values obtained with slides from the same patient specimens in two independent analytical sessions A and B was determined as an intraclass correlation (ICC) value of 0.918, representing excellent reliability (ICC > 0.75 is excellent, 0.75 ≥ ICC ≥ 0.40 is good, and ICC < 0.40 is poor).

Localization of TGF-β1 Expression by Immunofluorescence

Individual slides were treated with primary anti-TGF-β1 antibody (Abcam Ab53169, Cambridge, MA, USA) and primary antibody specific for each of the following cell types. For endothelia, we used an antibody against CD31 (1:50 dilution) (Abcam Ab9498, Cambridge, MA, USA); for vascular smooth muscle cells, an antibody against high molecular weight caldesmon (1 µg/mL) (Abcam Ab1826, Cambridge, MA, USA); for fibroblasts, an antibody for TE-7 (1:10 dilution) (Millipore

CBL271, Billerica, MA, USA); for macrophages, antibodies for CD163 (2.5 µg/mL) (Abcam Ab156769, Cambridge MA, USA) and CD68 (1 µg/mL) (Fisher Scientific Thermo MS-397, Pittsburg, PA, USA); for T cells, an antibody for CD3 (1ug/mL) (Abcam Ab699, Cambridge, MA, USA). To detect proliferative cells, we labeled with an antibody against Ki-67 (5 µg/mL) (Abcam Ab15580, Cambridge, MA, USA) and ProLong Gold® anti-fade medium with DAPI nuclear stain (Life Technologies #P36931, Carlsbad, CA, USA). For all labels, we used Tris buffer (pH 9.0) for epitope recovery, except for Ki-67 where we used citrate buffer (pH 6.0). Isotype control slides were stained with rabbit IgG (Vector Laboratories #I-1000, Burlingame, CA, USA) or mouse IgG1 (eBioscience #14-4714-85, San Diego, CA, USA), IgG2a (eBioscience #14-4724-85, San Diego, CA, USA), or IgG2b (eBioscience #14-4732-85, San Diego, CA, USA) at the same concentration as their respective primary antibodies. Primary antibodies to TGF-β1 and Ki-67 were labeled with a goat anti-rabbit IgG secondary antibody coupled with Alexa Fluor® 555 (Life Technologies #A21429, Carlsbad, CA, USA) and all other primary antibodies were labeled with a goat anti-mouse IgG secondary antibody coupled with Alexa Fluor® 647 (Life Technologies #A21236, Carlsbad, CA, USA). All labeling procedures were performed with a fully programmable, robotic autostainer (BioGenex i6000 Model, Fremont, CA, USA).

Detection of Fibroblast Accumulation by Immunohistochemistry

Duplicate slide specimens were deparaffinized and heated in Tris buffer (pH 9.0) for epitope recovery. Specimens were blocked with 10% goat serum for 5 minutes, treated with primary antibody against TE-7 (1:10 dilution) (Millipore

CBL271, Billerica, MA, USA), a highly specific marker of fibroblasts[172-174], and incubated for 14 hours. An isotype control treated with the same concentration of mouse IgG1 (EBioscience #14-4714-85, San Diego, CA, USA) was included with each duplicate antibody-treated pair of slide. Specimens were treated with peroxidase-conjugated secondary antibody according to instructions from DAB Polink 2 Kit (GBI Labs #D22-60D, Bothell, WA, USA). Hematoxylin (Fisher Scientific Richard Allan Scientific #72511, Pittsburg, PA, USA) was used as the counterstain for 4 minutes. Slides were dehydrated and mounted in Permount (Fisher Scientific #SP15-100, Pittsburg, PA, USA). Staining was implemented with a fully programmable, robotic autostainer (BioGenex i6000 Model, Fremont, CA, USA).

Statistical Analyses

The baseline characteristics between PAD and controls subjects were compared using general linear models for continuous variables and chi-square test for categorical variables to determine confounders. Confounding variables were covariates in subsequent analyses. For all biological parameters, group differences were determined by analysis of covariance (ANCOVA) and evaluated post-hoc by Bonferroni adjusted t-tests. Correlations were assessed by the Pearson test. All data are expressed as mean ± SD. All statistical analyses were performed with SPSS 20 (IBM, Armonk, NY, USA) using a confidence level of 95%.

Results

PAD Gastrocnemius Exhibits Increased Collagen Deposition with Advancing Disease

Spectral imaging revealed increased collagen density with higher Fontaine stage, which became diffuse in Stage IV muscle **(Figure 2.5A)**. Collagen density and area in PAD muscle was increased between the myofibers and around the lumen of microvessels. The most noticeable pathological change was dense collagenous investment of the microvessels of PAD muscle **(arrows)**. Spectral analysis established increased collagen density **($p < 0.001$)** and area **($p < 0.001$)** at the higher Fontaine stage. Collagen density in the PAD-IV patients **(2708.8 ± 612.3 gsu)** was 40% and 75% greater than in PAD-II and CTRL patients, respectively **(1969.9 ± 277.5 gsu and 1551.7 ± 232.8 gsu; both $p < 0.001$)**, while collagen density was 25% greater in PAD-II compared to CTRL **($p = 0.015$; Figure 2.5B)**. Collagen area was approximately twice as great in PAD-IV **(241179 ± 133159 mm^2)** compared to either PAD-II or CTRL gastrocnemius **(109179 ± 56481 mm^2 and 118624 ± 99559 mm^2; both $p < 0.01$)**, with no difference between PAD-II and CTRL **(Figure 2.5C)**. These data suggest that increased collagen deposition occurs first around microvessels and then expands throughout the extracellular matrix between myofibers and myofascicles as PAD advances.

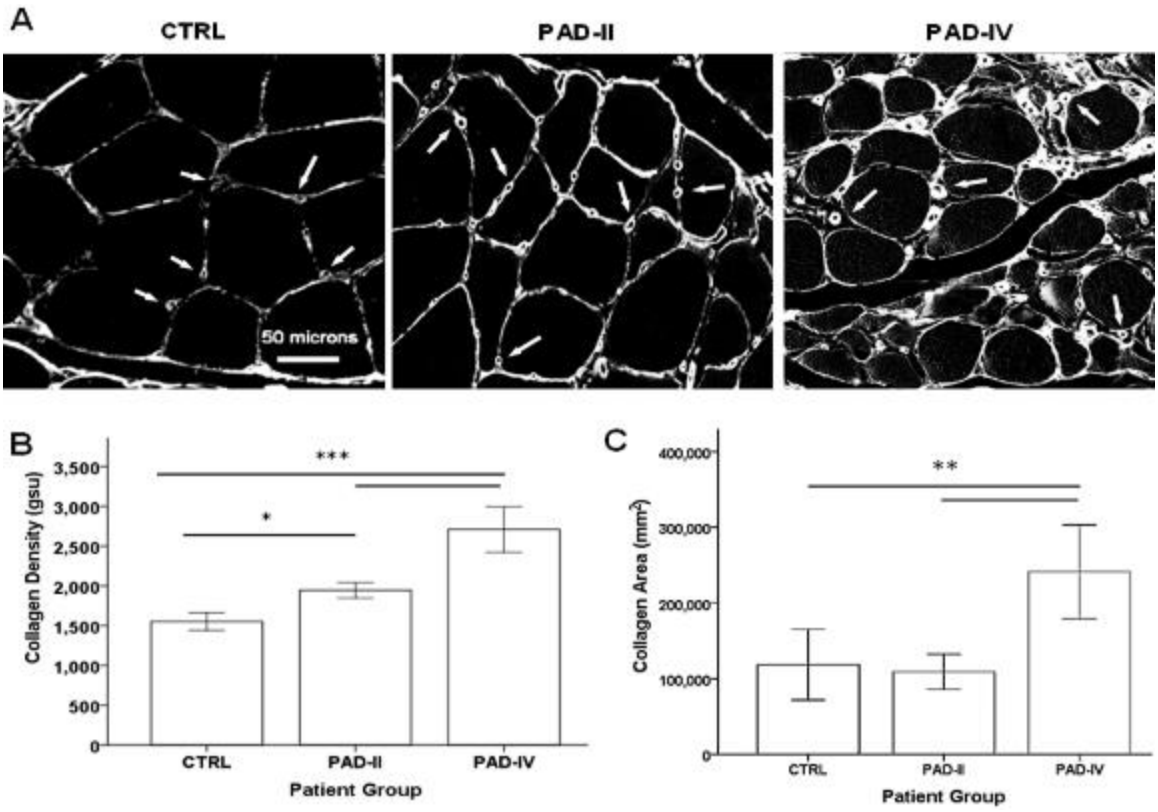


Figure 2.5: Collagen Deposition in the Gastrocnemius of CTRL and PAD Patients with Claudication and Tissue Loss[157]. (A) Representative gray scale images of gastrocnemius specimens stained with Masson Trichrome were captured by multi-spectral, bright-field microscopy at 20x objective. Specimens were collected from control (CTRL) and Fontaine Stage-II and Fontaine Stage-IV PAD patients (PAD-II and PAD-IV respectively). Myofibers delineated by collagen staining appear black. Collagen density and area are represented by the intensity and extent of the bright pixels, respectively. Arrows point to collagen deposition associated with microvessels. **(B)** Collagen density and **(C)** collagen area in specimens of CTRL (n=20), PAD-II (n=25), and PAD-IV (n=20) gastrocnemius were analyzed by quantitative multi-spectral microscopy. Collagen density was calculated as area-weighted mean intensity of all collagen events per specimen. Data are presented as mean ± SEM. Significance is denoted as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

TGF-β1 Expression is Tightly Linked to Myofibrosis of PAD Gastrocnemius

QFM imaging localized TGF-β1 expression to the vasculature of both CTRL and PAD muscle, with no detectable labeling outside of the vascular walls **(Figure 2.6A)**. TGF-β1 expression was uniformly low in CTRL muscle and exhibited a progressive increase in PAD-II and PAD-IV muscle. PAD-IV gastrocnemius had approximately 2.5 and 8 fold greater expression of TGF-β1 compared to PAD-II and CTRL patients **(6.56 ± 3.12 gsu vs. 2.89 ± 2.12 gsu and 0.842 ± 0.399 gsu, respectively; both $p < 0.001$)**, while PAD-II had 3.5 fold more TGF-β1 than CTRL **($p < 0.05$; Figure 2.6B)**. Vascular TGF-β1 expression positively correlated with collagen density across all subjects (N=65) in this study **($r = 0.798$, $p < 0.001$; Figure 2.6C)**. Separate analysis of the PAD patients alone (PAD-II and PAD-IV; N=45) and the CTRL subjects alone (N=20) revealed a significant correlation between TGF-β1 and collagen density in PAD group **($r = 0.854$, $p < 0.001$)**, but not in the CTRL group **($r = 0.119$, $p = 0.618$)**, indicating that the pathological fibrosis in PAD is driven by TGF-β1 expression. These findings identify increased microvascular TGF-β1 expression as a characteristic of PAD muscle and establish an association between increased TGF-β1 expression and muscle fibrosis and between increased TGF-β1 expression and advancing disease stage.

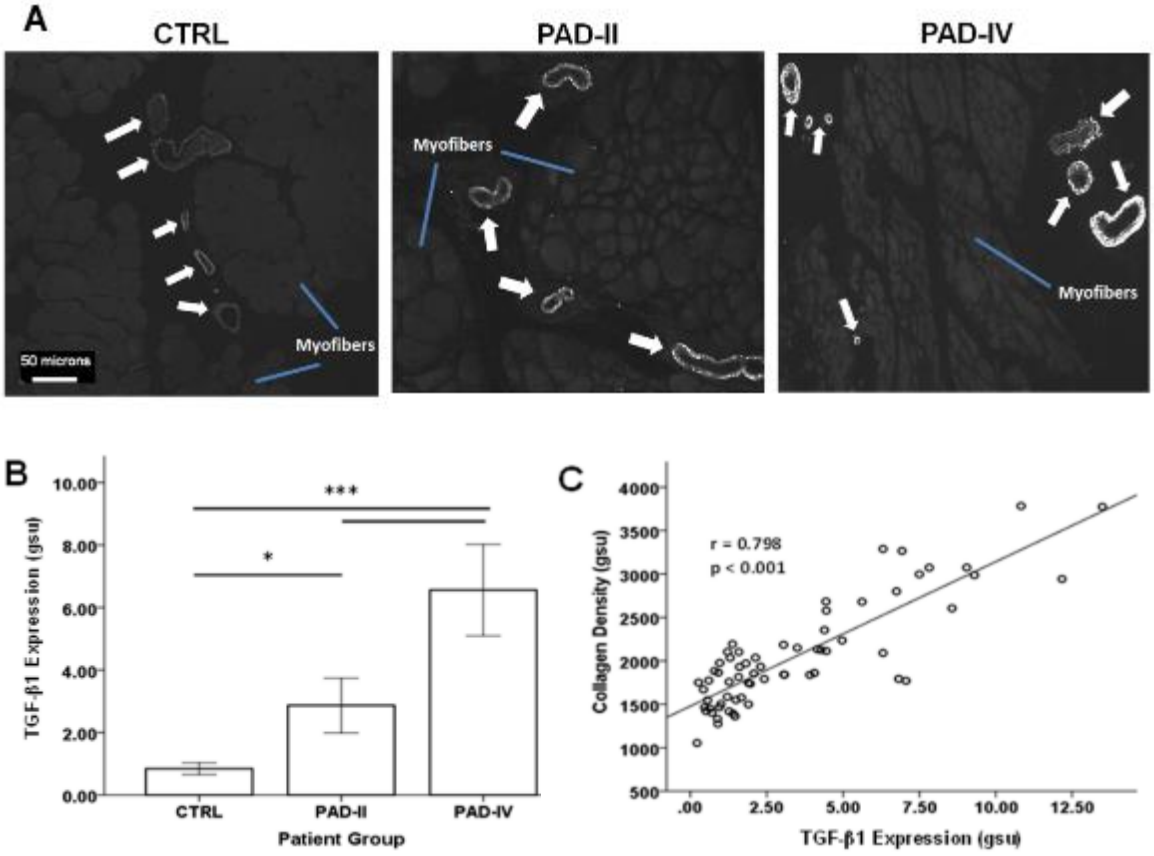


Figure 2.6: TGF-β1 Expression in CTRL and PAD Gastrocnemius and its Relationship with Collagen Density and ABI[157]. (A) Representative gray scale images of gastrocnemius microvessels positive for TGF-β1 labeling (arrows) were captured with a wide-field fluorescence microscope (10x objective). Unlabeled myofibers (blue line) appear grey against black background. Specimens collected from Control (CTRL) and Fontaine Stage-II and Fontaine Stage-IV PAD patients (PAD-II and PAD-IV respectively) were labeled with primary antibody specific for TGF-β1 and a fluorescent secondary antibody. Both the intensity and extent of TGF-β1 labeling were increased in the microvessels of PAD *vs.* CTRL and PAD-IV *vs.* PAD-II specimens. **(B)** TGF-β1 expression was determined by Quantitative Fluorescence Microscopy and defined as the sum of the products of area and mean pixel intensity of all positive events per microscopic field, normalized to the total area of specimen in the same field. **(C)** The relationships between TGF-β1 expression and collagen density. Data are presented as mean ± SEM. Significance is denoted as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Hemodynamics Predicts TGF-β1 Associated Myofibrosis in PAD Limbs

Across all subjects (N=65) in this study, vascular TGF-β1 expression increased with decreasing ABI **(r = -0.694, p < 0.001; Figure 2.7A)**, which is an indicator of blood flow (hemodynamic) compromise and increased ischemia of the lower limbs. The relationship between TGF-β1 and ABI remained significant when analyzing PAD patients alone **(r = -0.543, p < 0.001)**. Similarly, collagen density increased with decreasing ABI across all patients **(r = -0.734, p < 0.001; Figure 2.7B)** and the relationship remained significant when analyzing PAD patients alone **(r = -0.632, p < 0.001).** These findings show that as the blood flow (and presumably oxygenation) to the leg is decreased by atherosclerotic blockages in the arteries supplying the legs (reflected by decreasing ABI) there is a corresponding increase in the expression of TGF-β1 expression in the microvessels and collagen deposition in the muscles of the affected leg.

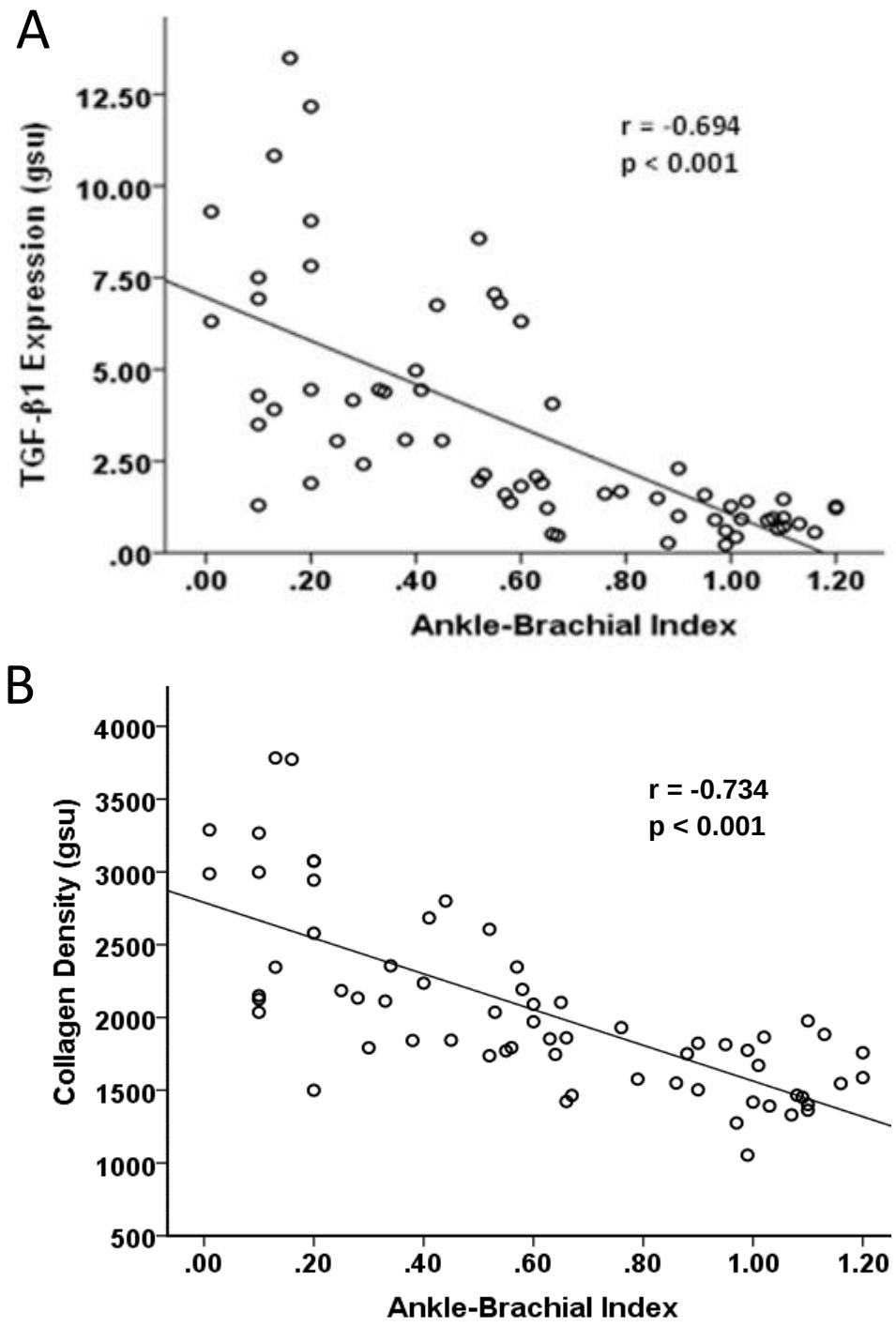


Figure 2.7. Hemodynamics Predicts TGF-β1 Associated Myofibrosis in PAD Limbs[157]. (A) Pearson correlation between Ankle-Brachial Index and TGF-β1 expression across all patients. **(B)** Pearson correlation between Ankle-Brachial Index and collagen density across all patients. The threshold for significance was $p < 0.05$.

Increased Expression of TGF-β1 is Associated with Accumulation of Fibroblasts and Collagen Deposition in PAD Gastrocnemius

To further evaluate the concept of a TGF-β1 associated myofibrosis in PAD, we selected three adjacent gastrocnemius sections and stained the first section for collagen with Masson Trichrome, the second for TE-7 positive fibroblasts by immunohistochemistry, and the third for TGF-β1 by immunofluorescence. Images from a PAD-IV patient with significant fibrosis are shown in **Figure 2.8**. In areas of dense collagen deposition **(ellipse, Figure 2.8A)**, we observed a high density of fibroblasts **(ellipse, Figure 2.8B)**. This is in contrast to areas containing relatively little collagen **(circle, Figure 2.8A)**, which had very few fibroblasts **(circle, Figure 2.8B)**. Vessels near the heavily fibrotic areas **(high magnification of the square in Figure 2.8)** contain a high density of fibroblasts **(Figure 2.8C)** in association with intense TGF-β1 labeling **(Figure 2.8D)** and dense adventitial collagen **(square, Figure 2.8A)**. These observations are consistent with the pro-fibrotic activity of TGF-β1, where TGF-β1 activates motile fibroblasts that deposit collagen[73,74], and establish a spatial association of increased TGF-β1 expression, fibroblast accumulation, and collagen deposition as a characteristic of PAD pathophysiology. They also further demonstrate that the microvessels of ischemic legs play a key role in the pathophysiology of PAD myofibrosis.

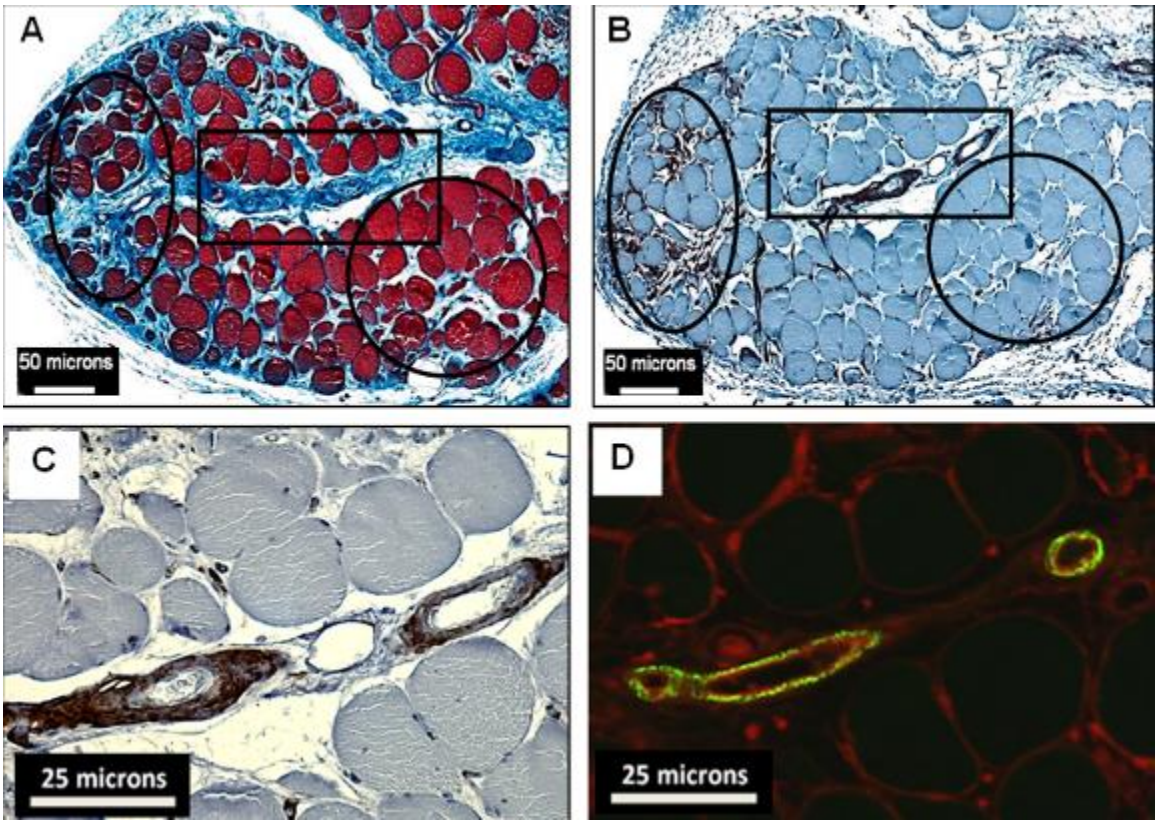
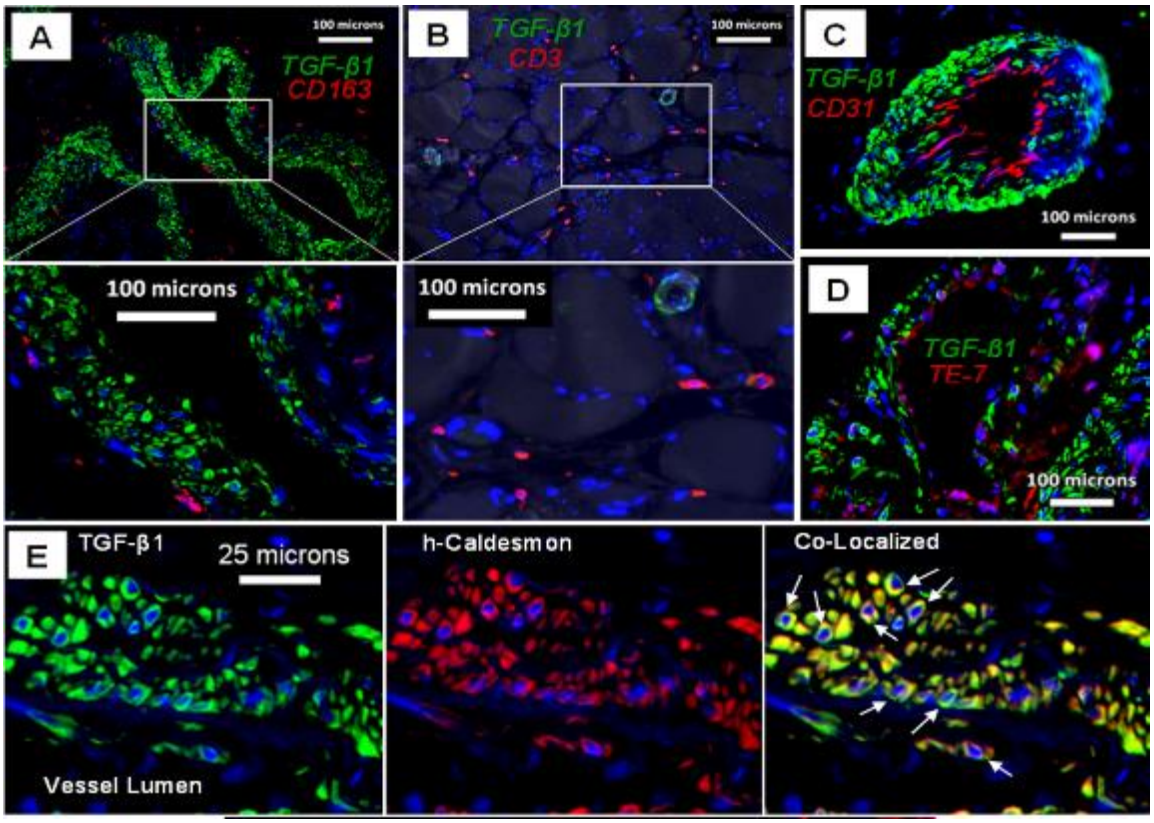


Figure 2.8: Association of TGF-β1 Expression with Fibroblast Accumulation and Collagen Deposition in Gastrocnemius of PAD Patients[157]. All images are of gastrocnemius of a representative Fontaine Stage-IV PAD patient. **(A)** Masson Trichrome staining reveals highly fibrotic regions (blue labeling) around myofibers (oval) and microvessels (rectangle), and a region with relatively little fibrosis (circle). **(B)** A neighboring 4-micron section was labeled by immunohistochemistry with anti-TE-7 antibody, a fibroblast marker which identified fibroblasts in the same three regions of interest. **(C)** High magnification of the rectangular region of interest reveals the extent of fibroblast accumulation with microvessels. **(D)** A neighboring section labeled by immunofluorescence for TGF-β1 (green fluorescence) shows the intensity and extent of TGF-β1 labeling of microvessels within the rectangular region of interest. Wheat Germ Agglutinin (WGA; red fluorescence) labeled membranes and was used to delineate myofibers.

Vascular Smooth Muscle Cells are the Producers of TGF-β1 in the Microvessels of PAD Gastrocnemius

To determine the specific cellular source of TGF-β1 expression in the microvessels of PAD gastrocnemius, we implemented co-localization studies with antibodies for highly specific markers of candidate vascular cells, including endothelial cells, SMC, fibroblasts, macrophages, and T cells **(Figure 2.9)**. TGF-β1 labeling did not co-localize with CD163 positive macrophages **(Figure 2.9A)**, CD3 positive T cells **(Figure 2.9B)**, or CD31 positive endothelia **(Figure 2.9C)**. TE-7 positive fibroblasts located in the adventitia were not positive for TGF-β1, nor were cells stained with TE-7 in the intima, which may represent endothelial-mesenchymal transition **(Figure 2.9D)**. TGF-β1 co-localized with cells expressing high molecular weight caldesmon, a marker of SMC that is not found in macrophages or fibroblasts[175,176] **(Figure 2.9E)**. Prominent co-localization was observed in rhomboidal-shaped SMC located in the subendothelial region (arrows). This has been reported for other vascular fibrotic pathologies, where SMC are activated toward the pro-fibrotic synthetic phenotype, including in atherosclerotic arterial plaques. Many of the rhomboidal SMC stained positive for Ki-67 in the nuclei **(arrowheads; Figure 2.9F)**, indicating they are proliferative and activated. The finding that TGF-β1 is derived from locally proliferative SMC rather than immune cells in PAD myofibrosis is novel.



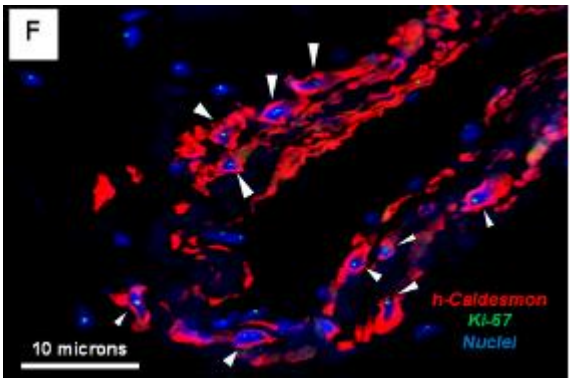


Figure 2.9: Evaluation of Candidate Vascular Cells for Expression of TGF-β1[157]. All immunofluorescence images are from PAD patients who present with tissue loss and are representative of all diseased patients in our study. **(A)** TGF-β1 labeling (green) does not co-localize with CD163 positive macrophages (red) present at relatively high density in the adventitia of microvessels. High magnification of the boxed region reveals cellular labeling of CD163 around DAPI stained nuclei (blue). **(B)** TGF-β1 labeling (green) does not co-localize with CD3 positive T cells (red) that are located typically around myofibers near microvessels. High magnification of the boxed region reveals cellular labeling of CD3 around DAPI stained nuclei (blue). **(C)** TGF-β1 labeling (green) does not co-localize with CD31 positive endothelial cells (red) that are characteristically located in the intima of microvessels. **(D)** TGF-β1 labeling (green) does not co-localize with TE-7 positive fibroblasts (red). **(E)** TGF-β1 labeling (green) co-localizes with high molecular weight caldesmon (h-Caldesmon) a specific marker of smooth muscle cells (red). Arrows point to rhomboidal morphology characteristic of secretory SMC. **(F)** The proliferation marker Ki-67 (green) is expressed in nuclei (blue) of h-Caldesmon positive SMC (red) that highly express TGF-β1 as determined with a neighboring tissue section.

Discussion

This study established that vascular TGF-β1 is strongly associated with myofibrosis during the progression of PAD. Using rigorous quantitative methods, we have shown that vascular expression of TGF-β1 and collagen deposition in PAD gastrocnemius increased in parallel with advancing disease severity. Qualitatively, TGF-β1 production was limited to cells in the walls of the microvessels in affected PAD muscle. In advanced disease, these cells expressing TGF-β1 were present at high density and were associated with accumulation of fibroblasts and increased deposition of collagen around the vessels and throughout an expanded interstitium. Our findings suggest that increased vascular TGF-β1 induces PAD myofibrosis by activating fibroblasts that proliferate and either stay locally around the microvessels or migrate between myofibers and around myofascicles into areas of myofiber degeneration, thus being responsible for the perivascular and endomysial/perimysial fibrosis we and others[177,178], have shown in the affected leg muscles of PAD patients. Accumulation of fibrous tissue in the PAD muscle can affect the muscle both by compromising the function of its myofibers and myofascicles but also by interfering with the function of its microvessels. The fibrosis of microvessels in PAD muscle has been shown by us and others[177,178] to involve both collagenous thickening of the capillaries and muscularization and fibrosis of normally non-muscularized microvessels and is known to impair diffusion of gases and small molecules between the vasculature and end organ parenchyma, probably enhancing the tissue hypoxia and inducing myofiber degeneration in the affected PAD legs. TGF-β1 may also be contributing

to PAD myopathy by suppressing skeletal muscle regeneration by inducing myoblasts to differentiate into myofibroblasts rather than new myofibers[158,159]. This model of PAD myofibrosis is consistent with the contribution of TGF-β1 to fibrosis and muscle degeneration seen in other skeletal myopathies[71] and myocardiopathies[179,180].

Another novel finding of this study is that TGF-β1 is expressed exclusively by vascular SMC in PAD muscle. Our co-localization studies show conclusively that TGF-β1 is produced by vascular SMC and not in the associated fibroblasts, suggesting that these fibroblasts are activated by TGF-β1 produced in the SMC. These observations again point to a widely reported mechanism of fibrosis in human fibrotic diseases, in which TGF-β1 activates fibroblasts to myofibroblasts. Our co-localization studies also revealed that macrophages and T cells, which are reported to be the source of TGF-β1 in other skeletal myofibroses, did not express detectable TGF-β1 in PAD muscle[70,71]. This is likely due to the different etiology of PAD myofibrosis compared to other skeletal myopathies, of which the muscular dystrophies are the best studied. The primary cause of PAD myopathy is atherosclerotic stenoses and occlusions of large arteries that supply the legs, which produce ischemia and hypoxia in the lower extremities[181]. Over time, chronic hypoxia may damage the muscle myofibers to produce their characteristic degeneration and induce SMC of the microvessels to increase TGF-β1 expression and cause myofibrosis. In contrast, muscular dystrophies originate from mutated genes that code for defective skeletal muscle proteins primarily involved in transmitting sarcomeric forces to the extracellular matrix[70,71]. In muscular

dystrophies, repeated cycles of myofiber degeneration and regeneration induce a chronic inflammatory response that includes TGF-β1 production by activated immune cells and produces the myofibrosis. Progressive fibrosis in PAD muscle appears not to be a response to chronic inflammation. We have profiled cytokines in the gastrocnemius of moderately diseased PAD patients and, indeed, did not find a generalized inflammatory signature[157]. The unique cytokine milieu produced by chronic ischemia in PAD gastrocnemius may explain why we did not observe detectable TGF-β1 in macrophages and T cells as is seen, e.g., in muscular dystrophies. Overall, the vascular etiology of PAD myofibrosis points to the possibility that chronic hypoxia of microvessels causes increased expression of TGF-β1 by SMC.

In PAD, hypoxic injury may cause transitioning of SMC from a contractile to a synthetic phenotype that is pro-fibrotic. Vascular SMC are normally contractile, with a spindle-shaped morphology and an abundance of contractile proteins that allow them to regulate vessel diameter. Under various pathological conditions marked by hypoxia, SMC can acquire a synthetic phenotype marked by rhomboidal morphology, decreased contractile proteins, and the ability to proliferate, migrate, and deposit collagen[76,77]. In this study, we found that patients with lower ABI had greater expression of TGF-β1 across Fontaine Stages of disease, which suggests that hypoxia induces SMC to increase TGF-β1 expression. This is supported by our observation that many SMC with increased TGF-β1 expression were proliferative and exhibited a rhomboidal morphology that is characteristic of synthetic SMC.

The responses of vascular SMC to hypoxia, in pulmonary arterial hypertension (PAH), are likely similar to those in the ischemic muscle of PAD patients and may provide insight into the mechanisms by which TGF-β1 expression is increased in PAD muscle. PAH is a condition characterized by substantial thickening of the vascular wall caused by extensive proliferation of SMC that exhibit increased TGF-β1 expression and deposition of collagen[164,165]. Hypoxia alone can stimulate proliferation of human pulmonary SMC in culture[164]. Alternatively, growth factors released by cultured endothelial cells exposed to hypoxia also can stimulate proliferation of SMC[182]. Either or both mechanisms may operate in vivo causing SMC to shift towards a more proliferative, synthetic phenotype in PAH. In PAD gastrocnemius, SMC with intense TGF-β1 labeling were observed frequently in the sub-endothelial region, suggesting that hypoxic insult to endothelial cells may stimulate secretion of growth factors that cause SMC to increase TGF-β1 expression. Given the histopathological similarities between PAH and PAD, future mechanistic studies of PAD myofibrosis should determine whether chronic hypoxia directly induces TGF-β1 expression by SMC, and/or activates endothelial cells to increase production of pro-fibrotic factors that cause SMC to increase expression of TGF-β1.

Understanding the mechanism by which chronic hypoxia stimulates SMC production of TGF-β1 can lead to development of animal models that recapitulate the myofibrosis observed in patients with PAD. Such models will allow for testing of novel anti-fibrotic therapies that modulate the cellular phenotypes and growth factors released from specific cell types to reduce SMC expression of TGF-β1 and

myofibrosis. Importantly, we can determine side effects in these animals that may hamper the success of therapeutic strategies. Additionally, limb function can be assessed over time and in relation to changes in myofibrosis.

A limitation of this study is the number of human subjects, however our power calculations indicated enough statistical power to analyze each of the biological parameters. The main barriers against recruitment of additional human subjects are the rigorous inclusion and exclusion criteria used to reduce confounding variables, and the demands of obtaining muscle biopsies. PAD patients included in our study must have had exercise-limiting claudication established by history and direct observation during a screening walk test administered by a vascular surgeon. We enrolled only those patients who did not exhibit concurrent symptoms of heart, lung, musculoskeletal (mainly arthritis), and neurologic (mainly back pain and sciatica) ailments that would affect their walking ability and their performance in the screening walk test. Furthermore, we excluded individuals with 1) asymptomatic PAD, i.e. patients with occlusive arterial disease who do not have claudication symptoms or tissue loss/gangrene, 2) acute lower extremity ischemic events secondary to thromboembolic disease or acute trauma, and 3) exercise capacity limited by conditions other than claudication including leg (joint or musculoskeletal and neurologic) and systemic (heart and lung disease) pathology. The invasiveness of a muscle biopsy and the time required often discourages participation of PAD patients and control subjects especially when the muscle biopsy cannot be performed during their routine care (at the time of a leg operation) which was frequently the case for most PAD-II and control patients. With

the 65 patients recruited, post-hoc power sample analysis using the data (mean and SD) for collagen density and area, and TGF-β1 expression, revealed adequate statistical power. Power sample analysis for an ANCOVA of TGF-β1 expression demonstrated that a total sample size of N=65 (20, 25 and 20 subjects per group) assured at least 99% power to detect differences between groups adjusting for two covariates with a conservative R2 value of 0.20. Similarly, for an ANCOVA of collagen density and area, our sample size assured at least 99% and 93% power, respectively, to detect differences between the groups adjusting for two covariates with a conservative R2 value of 0.20. The power and sample size determination package PASS (PASS, Number Cruncher Statistical Systems, Kaysville, UT) was used for the analysis.

An additional limitation is the correlational nature of the study, but the high quality human data that we have presented for PAD myofibrosis provide a basis for future mechanistic studies, development of disease models, and improved therapies and prognosis. In this study, we established an association between ischemia and increased TGF-β1 production by microvascular SMC. We have identified microvascular SMC in PAD muscle as the exclusive producer of TGF-β1, making it a specific target for anti-fibrotic therapies for PAD. Candidate therapeutic drugs will be those capable of shifting the pro-fibrotic synthetic phenotype of SMC back to the contractile form to decrease TGF-β1 expression and prevent or slow down the progression of PAD myofibrosis. Moreover, TGF-β1 production by SMC may be a potential biomarker for determining efficacy of therapeutics, including anti-fibrotic interventions. Finally, our finding of worsened

myofibrosis with advancing Fontaine Stage in PAD suggests that the patients who are optimal for anti-fibrotic intervention are those with moderate disease, since 1) a relatively small proportion of vascular SMC express TGF-β1, 2) fibroblasts are present at relatively low density, and 3) collagen deposition is largely limited to microvessels with little expansion into the interstitium.

Fibrosis is often viewed as an adaptive response to injury and tissue degeneration, but as we have shown in this study, can be part of the pathophysiology of chronic disease. We have established that increased expression of TGF-β1 by microvascular SMC in the gastrocnemius of PAD patients correlates with Fontaine Stage and increasing collagen deposition. The pattern of vascular TGF-β1 expression, fibroblast accumulation, and collagen deposition points to pathological changes in microvessels as the immediate cause of PAD myofibrosis. The contribution of hypoxia was suggested by a strong negative correlation between ABI and vascular TGF-β1 expression and presence of locally proliferative rhomboidal SMC in microvessels of PAD gastrocnemius that are indicative of the pro-fibrotic synthetic phenotype of SMC known to be induced by hypoxia. Collectively, these findings provide insight into the development of PAD myofibrosis and direction for future mechanistic studies, and consequently, basis for improved diagnosis and treatment for PAD patients.

CHAPTER III: TGF-β1 ASSOCIATED MYOFIBROSIS IS A LOCALIZED RESPONSE IN THE ISCHEMIC MUSCLE OF PAD PATIENTS

Introduction

The work presented in Chapter II established myofibrosis as part of the myopathy of PAD, but whether or not it arises predominantly as a local response to ischemia or also as a consequence of systemic changes could not be determined. This is because studies of PAD patients with bilateral disease mask the contributions of systemic factors to local myopathy. In the context of myofibrosis, it is conceivable that pathological factors in circulation, either directly related to atherosclerosis or not, induced the expression of TGF-β1 expression by vascular SMC and associated increase in collagen deposition[56]. It is important to determine if systemic contributions exist because this knowledge will allow for the development of appropriate strategies that decrease TGF-β1 expression in microvessels to reverse or prevent myofibrosis in PAD patients. For example, if systemic contributions do exist, focusing solely on relieving ischemia may not alter TGF-β1 expression and lead to novel therapeutic targets in circulation being overlooked. Determination of systemic contributions to other aspects of myopathy have been achieved by studies of PAD patients with unilateral disease, in which significant atherosclerosis and decrease in ABI is present in only one of the limbs. In unilateral PAD patients, presence of myopathy in only the hemodynamically comprised limb would indicate that the myopathy is a direct consequence of atherosclerosis in that limb, with minimal systemic contributions.

Previous studies of the abnormal bioenergetics in the gastrocnemius of unilateral PAD patients revealed systemic contributions and further demonstrate the need for these studies for myofibrosis. Metabolic changes in PAD skeletal muscle have been defined with features similar to those of mitochondrial myopathies[48,183,184]. In unilateral disease PAD patients, mitochondrial DNA deletions are increased in both the affected and contralateral limb compared to controls, but are not significantly different from each other[185,186]. This suggests that mitochondrial injury may not have a primary role in the pathogenesis of PAD myopathy. Furthermore, there was a significant correlation between the degree of mitochondrial deletion in the affected and contralateral limbs[185,186] which indicates that the contralateral limb reflects a subclinical phenotype of the ischemic limb. Similarly, in a separate study of unilateral disease PAD patients, skeletal muscle carnitine metabolism was shown to exhibit a similar pattern[49]. Taken together, these findings suggest that abnormal bioenergetics in the skeletal muscle of PAD may not be a direct consequence of atherosclerosis and the local ischemia produced in that limb, since abnormalities lateralized between the limbs. Given that atherosclerosis is a systemic disease associated with oxidative stress[187-189], biological factors such as free radicals in the circulation may be responsible for the impaired bioenergetics in both limbs, especially since the same types of mitochondrial deletions in skeletal muscle were also found in atherosclerotic lesions[190]. However, the opposite interpretation may have been made had only the limbs of bilateral PAD patients been studied. Thus, it is imperative that unilateral PAD patients are studied to determine if systemic contributions contribute to

myofibrosis or if it is a response to local ischemia produced by atherosclerosis in that specific limb.

In Chapter II, TGF-β1 expression was inversely correlated with ABI measurements, indicating that the decreased blood flow, and consequent ischemia-hypoxia may be stimulating SMC to increase production of TGF-β1. Preliminary data in our laboratory demonstrates a cytokine profile in the muscle that is different from serum in PAD patients, with TGF-β1 being significantly higher in muscle than serum (**Chapter I, Table 1.2)**. This suggests that the fibrotic effects of TGF-β1 may arise from local ischemia-reperfusion injury. Studies of neutrophils, one of the major producers of cytokines that can circulate into end organs, found that in unilateral disease PAD patients, number of neutrophils and percentage activated was higher in venous than arterial blood[65]. Moreover, compared to the diseased limb, this difference was not found in the contralateral limb, further suggesting a unique local cytokine response. These findings provide rationale for the following study that addresses **Specific Aim 2.**

To test the hypothesis that TGF-β1 expression and the subsequent collagen deposition is a localized response in the ischemic muscle of PAD patients, we compared myofibrosis between the limbs of PAD patients with unilateral disease and explored correlative relationships between the limbs for TGF-β1 expression and collagen density. If myofibrosis is a localized response, then the diseased limb will have significantly greater TGF-β1 expression and collagen density compared to the contralateral limb, with no correlation between the limbs in either

measurement. However, if systemic contributions exist, then TGF-β1 expression and collagen density will be similar between the limbs with a direct relationship between the limbs in both measurements.

Methods

Characteristics of Human Subjects

We studied a total of 10 Fontaine Stage-II PAD patients with unilateral disease, where only one of the two limbs has atherosclerosis significant enough to produce claudication. The diagnosis for each PAD patients was established on the basis of medical history, physical examination, decreased ankle-brachial index (ABI < 0.9), and computerized or standard arteriography that revealed stenotic and/or occluded arteries supplying the lower limbs. ABI was measured by first taking blood pressure measurements from both brachial arteries and from both the dorsalis pedis and posterior tibial arteries after patients have been at rest in the supine position for 10 minutes. The ratio is calculated by taking the averaged ankle blood pressures and dividing by the averaged blood pressures obtained from the arm. Using ABI measurements, we confirmed that the diseased leg had significantly lower ABI than the contralateral leg **(mean ± SD; 0.49 ± 0.21 *vs.* 0.99 ± .10, respectively; p = 0.005)**. All of the diseased legs had ABI < 0.9, while only one of the contralateral legs had an ABI < 0.9, demonstrating that these patients are suitable for this study. We did not examine demographics such as co-morbidities as potential confounders since each patient served as his or her own control. The experimental protocol was approved by Institutional Review Boards from the Veterans Affairs Nebraska-Western Iowa and University of Nebraska Medical Centers, and all patients gave informed consent.

Quantitative Assessment of Collagen Density

Multi-spectral imaging analysis measured the density of collagen in both the diseased and contralateral limbs as previously described in Chapter II Methods.

Quantitative Assessment of TGF-β1 Expression

Quantitative fluorescence microscopy measured the expression of TGF-β1 expression in both the diseased and contralateral limbs as previously described in Chapter II Methods. Quantitative PCR was performed on both the diseased and contralateral limbs and normalized to Myosin Heavy Chain transcript levels as described in Chapter II Methods.

Statistical Analysis

All data are expressed as mean ± SEM. Comparison of ABI between the groups was performed using the non-parametric Wilcoxon Rank-Signed Test. Similarly, TGF-β1 expression and collagen density were analyzed by the Wilcoxon Rank-Signed Test. All correlational analyses were performed using the non-parametric Spearman test. All statistical analyses were performed with SPSS 20 (IBM, Armonk, NY, USA) using a confidence level of 95%.

Results

TGF-β1 Expression is Independently Greater in the Diseased Compared to Contralateral Leg of PAD Patients with Unilateral Disease

Compared to the contralateral limb, labeling of TGF-β1 appeared brighter in microvessels of all sizes, both those located in the perimysial and endomysial regions of PAD gastrocnemius **(Figure 3.1A)**. QFM measured a greater than 4-fold expression of TGF-β1 in the diseased compared to contralateral limb **(mean ± SEM; 3.27 gsu ± 0.74 *vs.* 0.76 gsu ± 0.20 respectively; p = 0.013; Figure 3.1B)**. Correlational analysis revealed no relationship between the diseased and contralateral limbs for TGF-β1 expression **(r = 0.181; p = 0.543)**.

Quantitative PCR measured changes in TGF-β1 expression at the transcript level between the limbs and results were similar to QFM measurements of TGF-β1 expression at the protein level. Compared to the contralateral limb, there was an approximately 7.5-fold greater expression of TGF-β1 transcripts, normalized to myosin heavy chain, in the diseased limb **(0.00465 ± 0.00095 *vs.* 0.00061 ± 0.00016; p = 0.007; Figure 3.1C)**. Correlational analysis revealed no relationship between the diseased and contralateral limbs for mRNA levels of TGF-β1 **(r = 0.006; p = 0.987**). Correlational analysis was also performed between protein and transcript TGF-β1 levels across all limbs. A direct relationship demonstrates that QFM and qPCR cross-validated one another **(r = 0.720; p < 0.001; Figure 3.1D)**.

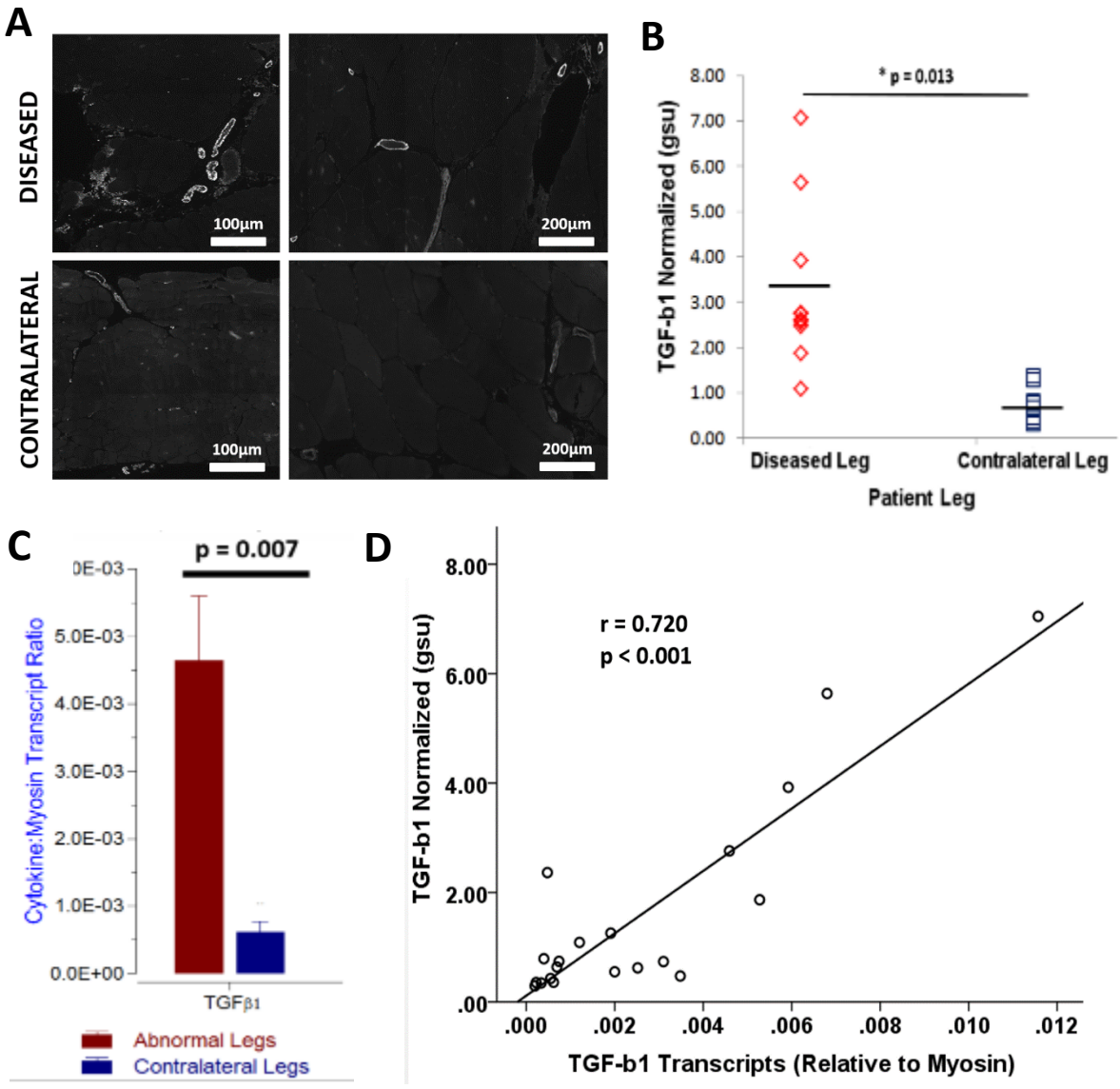


Figure 3.1: TGF-β1 Expression is Independently Greater in the Diseased Compared to Contralateral Leg of PAD Patients with Unilateral Disease. (A) Representative gray scale images of gastrocnemius microvessels positive for TGF-β1 labeling were captured with a wide-field fluorescence microscope (10x objective). Higher magnification images were taken (20x objective) to highlight differences present in microvessels located between myofibers. Specimens collected from both legs of PAD patients at Fontaine Stage II with unilateral disease. **(B)** Quantitative Fluorescence Microscopy measured the expression of TGF-β1 expression in both limbs. **(C)** Quantitative PCR measured transcript levels in the gastrocnemius of both limbs, normalized to myosin heavy chain transcripts. (D) Spearman correlation between QFM and qPCR measurements of TGF-β1. Data are presented as mean ± SEM and significance threshold is $p < 0.05$.

Collagen Density is Independently Greater in the Diseased Compared to Contralateral Leg of PAD Patients with Unilateral Disease

Compared to the contralateral limb, the labeling of collagen appeared brighter in the gastrocnemius of PAD patients **(Figure 3.2A)**. Quantitative muti-spectral microscopy measured a 25% increase in the density of collagen in the diseased compared to contralateral limb **(mean ± SEM; 2162 gsu ± 99 *vs.* 1739 gsu ± 73 respectively; p = 0.013; Figure 3.2B)**. Correlational analysis did not reveal a significant relationship between the diseased and contralateral limbs for TGF-β1 expression **(r = 0.254; p = 0.368**).

Relationship between TGF-β1 Expression and Collagen Density in the Limbs of PAD Patients with Unilateral Disease

Correlational analysis revealed a significant relationship between vascular TGF-β1 expression and collagen density in the diseased limbs **(r = 0.69; p = 0.03; Figure 3.3A)**, but such a relationship did not exist in the contralateral limbs of PAD patients **(r = 0.42; p = 0.23; Figure 3.3B)**.

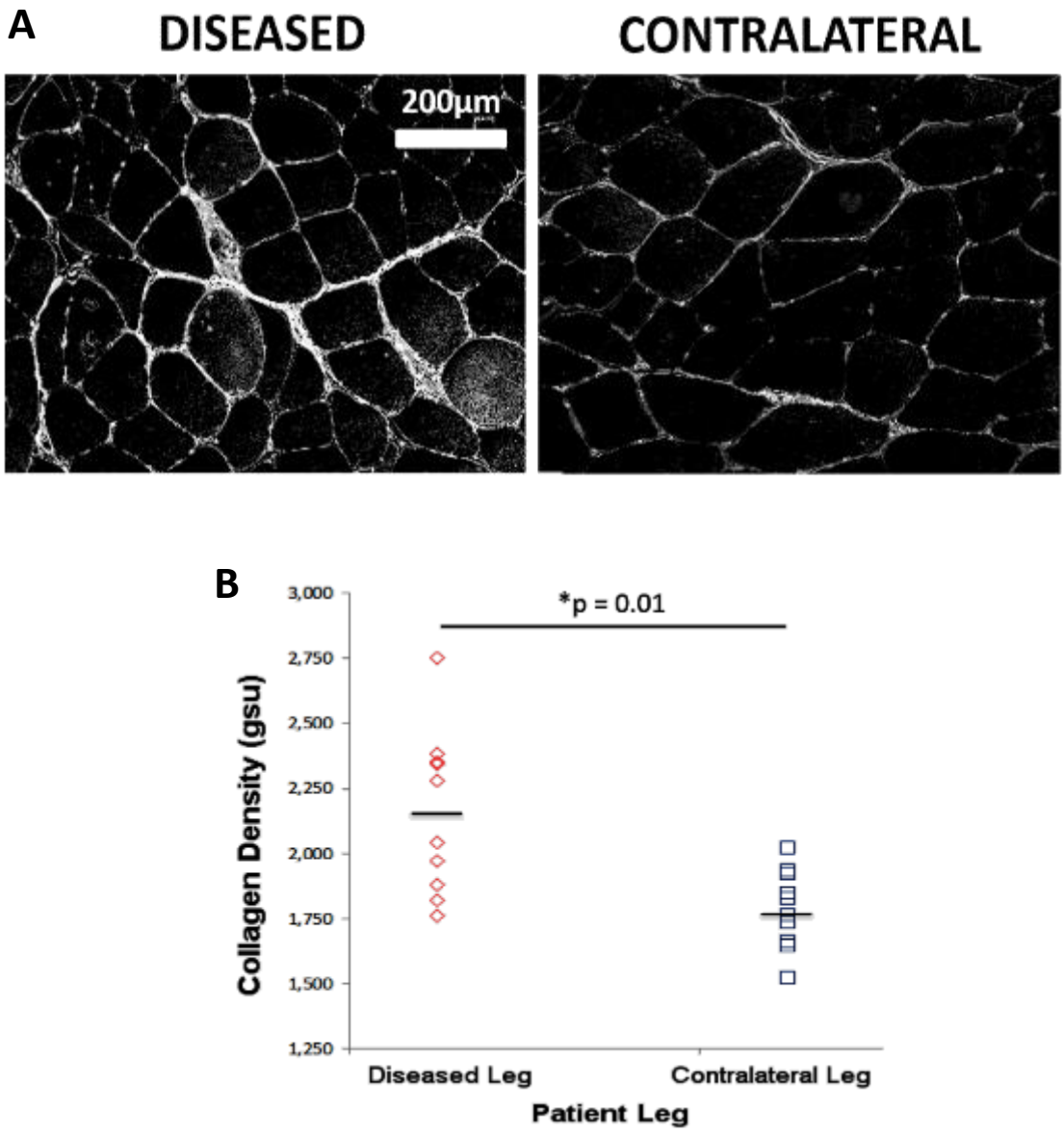


Figure 3.2: Collagen Density is Independently Greater in the Diseased Compared to Contralateral Leg of PAD Patients with Unilateral Disease. (A) Representative gray scale images of gastrocnemius specimens stained with Masson Trichrome were captured by multi-spectral, bright-field microscopy (20x objective). Specimens were collected from both legs of PAD patients at Fontaine Stage II with unilateral disease. Myofibers delineated by collagen staining, appear black. Collagen density and area are represented by the intensity and extent of the bright pixels, respectively. **(B)** Collagen density was measured by quantitative multi-spectral microscopy as area-weighted mean intensity of all collagen events per specimen. Data are presented as mean ± SEM and the significance threshold is $p < 0.05$.

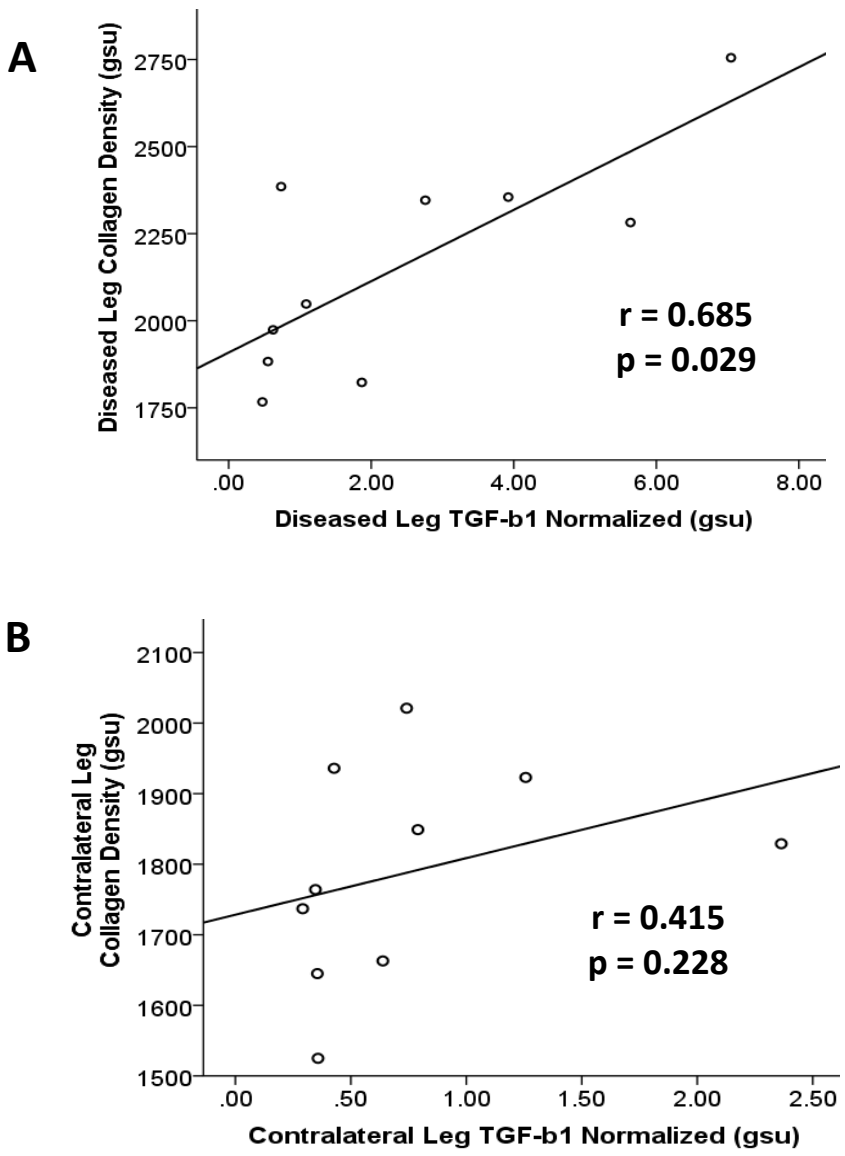


Figure 3.3: Relationship between TGF-β1 Expression and Collagen Density in the Limbs of PAD Patients with Unilateral Disease. (A) Spearman correlation in diseased limbs. **(B)** Spearman correlation in contralateral limbs. A threshold of $p < 0.05$ was considered significant.

Discussion

Examination of myofibrosis in the limbs of unilateral disease PAD patients revealed that it is a localized response in ischemic muscle without significant systemic contribution. The greater vascular TGF-β1 expression and collagen density in the diseased compared to contralateral limb indicates that the presence of significant atherosclerosis and ischemia is the predominant driver of myofibrosis. This is further corroborated by correlational analyses that showed no relationship of either TGF-β1 expression or collagen density between the limbs. Lack of a relationship between myofibrosis in the limbs makes it highly unlikely that a pathological factor from the diseased limb circulates and induces myofibrosis in the contralateral limb. Such a pattern is different from previous studies of bioenergetic abnormalities in PAD skeletal muscle, where lateralization between the limbs did occur, reflected in the correlation of mitochondrial deletions between the limbs[185,186]. A direct relationship between TGF-β1 expression and collagen density was also detected in the diseased but not contralateral limb, further demonstrating that the presence of significant atherosclerosis is needed for the development of TGF-β1 associated myofibrosis. With aging, vascular TGF-β1 expression is known to increase[105], which may account for vascular TGF-β1 in the contralateral limb and thus, it is logical that those levels are not accompanied by increased collagen density in the gastrocnemius. Overall, myofibrosis is a local myopathy that develops and is not a consequence of systemic pathological changes. Thus, therapeutic strategies that alleviate ischemia-hypoxia and reduce TFG-β1 expression will likely have direct improvements in the myopathy of PAD.

These findings also have important implications regarding improvement of limb function, since biomechanical studies in PAD patients with unilateral disease demonstrate that both limbs are dysfunctional. Comparison of joint torques and powers in both limbs of unilateral disease PAD patients while walking showed significant gait impairments in both limbs that are present before the onset of ischemic pain[191]. Moreover, the dysfunction of the contralateral limb is significantly greater than in the limbs of age matched controls[191]. In a separate study, energy output in the diseased and contralateral limbs at rest showed that both legs performed less work than healthy controls and were not significantly different from each other[192]. Perhaps this is a functional sequelae of the bioenergetics abnormalities in the PAD muscle that are associated with both local ischemia and systemic factors. If so, treatments that do not address both aspects, may not yield benefits to limb function. In contrast, targeting TFG-β1 expression locally will likely produce direct benefits to limb function, since there is not a significant systemic contribution. An alternative explanation of the lateralization of biomechanical dysfunction is that compensation from the contralateral limb may be occurring that renders it dysfunctional[191,192]. In this context, targeting TGF-β1 expression in the diseased limb may lead to improved myopathy and limb function that reduces need for compensation by the contralateral limb. Overall, findings in unilateral PAD patients show that what is happening pathologically in the one limb does not affect the other, and that pathological changes in the atherosclerotic plaques do not significantly contribute to myofibrosis. Rather it is likely the local ischemia-hypoxia that the atherosclerotic blockages produce that induces myofibrosis in PAD.

CHAPTER IV: REVASCULARIZATION BUT NOT EXERCISE THERAPY PREVENTS PROGRESSION OF TGF-β1 ASSOCIATED MYOFIBROSIS IN PAD WHILE IMPROVING LIMB FUNCTION, HEMODYNAMICS, AND MYOFIBER SIZE

Introduction

Fibrosis develops in the gastrocnemius of PAD patients with increasing disease severity, but it is not known if there are functional consequences. In Chapter II, we demonstrated increased collagen density with increasing disease stage that appears first around microvessels and then surrounds degenerating myofibers. These fibrous changes strongly correlate with expression of Transforming Growth Factor-Beta 1 (TGF-β1), a well-established pro-fibrotic cytokine. Interestingly, production of TGF-β1 was predominantly localized to vascular smooth muscle cells within microvessels of the gastrocnemius and inversely correlated with ankle-brachial index, a measurement of blood flow. This suggests that atherosclerosis in the large vessels produces ischemia in the smaller vessels that upregulates expression of TGF-β1 in smooth muscle cells to stimulate pathological fibrosis that is known to be detrimental to end organ function. However, due to the cross-sectional design and lack of functional measurements in patients of that study, it cannot be determined how the development of fibrosis in the skeletal muscle of claudicating PAD patients alters limb function.

How the development of fibrosis in PAD is affected by the two conventional therapies, revascularization and supervised exercise therapy has also not been studied. Both treatment modalities produce significant improvements in the

walking ability of PAD patients at 6 months[122,127,141,193]. However, whether these improvements are sustained at longer time points is not known. On the other hand, in patients with PAD who receive no intervention, a decrease in the walking ability has been documented[194,195]. It is possible that the myopathy of PAD progressively worsens in patients who receive no intervention and this worsening is responsible for the decrease in their walking ability. Similarly, it is possible that the two standard therapies (revascularization and exercise) produce an improvement in the myopathy of PAD, which is then reflected in the walking distances at 6 months.

A better understanding of how revascularization and supervised exercise affects fibrosis development may point to novel therapeutic strategies that can sustain, if not enhance, the improved limb function. For example, if the therapies do not alter or only modestly decrease expression of TGF-β1 and collagen deposition, then fibrosis is an aspect of the myopathy that likely contributes to the long-term decline in limb function. Given the strong negative correlation between ABI and both TGF-β1 expression and collagen deposition presented in Chapter II, revascularization likely will reverse or at least prevent further progression of PAD myofibrosis. Additionally, the findings of Chapter III demonstrate that the TGF-β1 associated myofibrosis is a local response directly related to the extent of atherosclerosis of that particular limb. Taken together, it would be expected that bypass surgery and endovascular procedures, all of which directly improve blood flow, decreases ABI in the revascularized leg of PAD patients[136], and thus ischemia-reperfusion injury to the muscle, has beneficial effects on PAD myopathy. Exercise therapy, on the other hand, does not improve ABI despite demonstrated

systemic cardiovascular benefits[140]. Moreover, the mechanism by which exercise improves limb function remains unclear and may be mediated predominantly by systemic changes, which the studies of Chapter III showed did not significantly contribute to PAD myofibrosis. Based on the differential effects that revascularization and exercise have on ABI, benefits may be greater with revascularization than exercise for the myofibrosis in PAD patients. If either therapy does not significantly reverse myofibrosis, strategies that directly decrease TGF-β1 in the muscle are warranted so that adjunct therapies can be developed.

In this study, we addressed **Specific Aim 3** by evaluating the development of fibrosis over a period of 6 months in claudicating PAD patients who were treated by revascularization or supervised exercise and compared the changes with those who were not clinically suitable for either intervention. The relationship between TGF-β1, collagen deposition, and assessments of limb function were examined to determine the functional consequences of fibrosis development in PAD. We hypothesized that revascularization but not supervised exercise therapy improves limb function in association with improved TGF-β1 associated fibrosis.

Methods

Characteristics of Human Subjects

We recruited a total of 72 claudicating PAD patients classified as early stage disease, presenting with only intermittent claudication, but no rest pain or tissue loss (Fontaine Stage II). The diagnosis for each PAD patients was established on the basis of medical history, physical examination, decreased ankle-brachial index (ABI < 0.9), and computerized or standard arteriography that revealed stenotic and/or occluded arteries supplying the lower limbs. ABI was measured as described in Chapter II Methods. The experimental protocol received Institutional Review Board approval from the Veterans Affairs Nebraska-Western Iowa and University of Nebraska Medical Centers and all patients gave informed consent.

The patient demographics for CTL, RVS, and EXE patients are presented in **Table 4.1**. There were no differences in age and baseline ankle-brachial index among the groups. Smoking history, coronary artery disease, hypertension, dyslipidemia, statins medication, myocardial infarction, and diabetes mellitus were also were not confounding factors.

Experimental Timeline and Design

The 72 patients were divided into the Revascularization (RVS; N=30), Exercise (EXE; N=25), and No Intervention (CTL; N=17) groups. All patients received a baseline and second biopsy 6 months later. Lower limb functional and biomechanical analyses were also performed at baseline and at 6 months.

Gastrocnemius samples weighing approximately 250 mg were obtained from the anteromedial aspect of the muscle belly, 10 cm distal to the tibial tuberosity, using a 6mm Bergstrom needle. The muscle specimen was immediately placed into cold methacarn, and after 48 hours, transferred to cold 50% ethanol, and subsequently embedded in paraffin. At the start of the 6-month period, patients in the RVS group underwent open bypass or endovascular interventions immediately after the baseline biopsy. Patients in the EXE group walked on treadmill 3 times per week for 45 minutes each, while CTL patients did not receive any interventions.

Quantitative Assessment of Collagen Density

Multi-spectral imaging analysis measured the density of collagen as previously described in Chapter II Methods.

Quantitative Assessment of TGF-β1 Expression

Quantitative fluorescence microscopy measured the expression of TGF-β1 expression as previously described in Chapter II Methods.

	CTL	RVS	EXE	p-value
Number of Patients	17	30	25	N/A
Gender (male/female)	16/1	29/1	25/0	0.983
Mean Age (years) §	63.2 ± 1.94	62.9 ± 1.38	64.4 ± 1.39	0.733
Height (m) §	1.75 ± 0.01	1.76 ± 0.01	1.75 ± 0.02	0.965
Weight (kg) §	89.7 ± 2.61	85.2 ± 3.33	85.6 ± 3.35	0.635
Body Mass Index§	29.3 ± 0.58	27.5 ± 0.98	27.9 ± 1.14	0.481
Ankle Brachial Index§	0.66 ± 0.07	0.56 ± 0.05	0.56 ± 0.04	0.313
Obesity† (%)	29	20	16	0.570
Smoking (%)	53	45	68	0.380
Diabetes Mellitus (%)	29	30	21	0.331
Dyslipidemia (%)	88	85	89	0.949
Coronary Artery Disease (%)	24	30	42	0.768
Myocardial Infarction (%)	6	5	5	0.914
Hypertension (%)	94	75	84	0.394
Statins Medication (%)	88	75	95	0.874
Renal Insufficiency‡ (%)	12	13	16	0.921

Table 4.1: Demographics of Claudicants

† Obesity: Body mass index > 30
‡ Renal Insufficiency: Creatinine clearance < 60 ml/min/1.73m2
§: Data presented as Mean ± Standard Error
CTL = No Intervention, RVS = Revascularization, EXE = Supervised Exercise

* $p < 0.05$ compared to each of the other two groups by post-hoc Bonferroni adjusted t-tests

Lower Limb Functional Assessments

Six Minute Walking Distance: Patients walked back and forth along a 100-meter corridor and the distance that each patient can quickly walk in a period of six minutes was measured in meters. The pace was determined by the patient and when a patient stopped due to claudication, the measured time continued. Overall, this is a measurement of submaximal endurance[196,197].

Peak Walking Time: Patients performed a progressive, graded treadmill protocol that consisted of a constant speed of 3.2 km-h-1 at 0° grade incline with subsequent 2% increases in speed and incline every 2 minutes. Patient walked until maximal claudication pain was induced whereby ambulation could no longer continue. Walking time, measured in seconds, was when the patient could no longer walk on treadmill[198,199].

Morphometric Analysis of Myofiber Size

Greyscale (12-bit) fluorescence images (at 1344 × 1044 resolution) were captured with a wide-field, epifluorescence microscope (Leica DMRXA2; North Central Instruments, Plymouth, MN) (×10 objective; 0.5128 μm/pixel), a black and white CCD camera (Orca ER C4742-95; Hamamatsu Photonics, Bridgewater, NJ), and HCImage Hamamatsu software (64-bit version 4.2.5; Hamamatsu Photonics). An acquisition matrix was programmed into the HCImage software to cover the whole muscle specimen area, acquiring 100–150 microscopic frames per specimen yielding images of 134,400 × 104,400 to 201,600 × 156,600 pixels. At each location, images were collected in fluorescence channels corresponding to

each fluorophore; 1) 4′,6-diamidino-2-phenylindole (nuclei) with an excitation maximum at 358 nm and emission maximum at 461 nm, 2) Alexa Fluor 488 (myosin heavy chains) with an excitation maximum at 495 nm and emission maximum at 519 nm, and 3) Alexa Fluor 647 (sarcolemma) with an excitation maximum 650 nm and emission maximum at 668 nm. The frames corresponding to each fluorophore were montaged in one large image to represent the whole muscle specimen. Image segmentation was done with a custom-made Matlab algorithm (R2012a; MathWorks Inc, Natick, MA), using thresholding, edge detection, regionprops, and a set of heuristics as previously described[44,45]. Labeled sarcolemma acquired at 668 nm provided a binary image that outlined each myofiber and was superimposed on the image of myosin heavy chains, allowing precise identification of the myofibers for analysis. For each specimen, we extracted the measurement of myofiber area. The methods describing how these parameters were measured have been described in detail and are illustrated in **Figure 4.1**[44,45]. Briefly, the myofiber area was calculated as the cross-sectional area (μ^2), from the number of pixels enclosed within a segmented myofiber. Biologically, myofiber area can objectively and quantitatively measure pathological changes in the muscle, including myofiber degeneration, necrosis, apoptosis, and regeneration[39,45].

Statistical Analysis

The baseline characteristics among the subject groups were compared using general linear models for continuous variables and chi-square tests for categorical variables to determine confounders, which if present, were treated as

covariates in subsequent analyses. For TGF-β1 expression and collagen deposition comparisons at baseline and 6 months, the Wilcoxon Rank-Signed Test was used. Correlations were assessed by the Spearman test. The change in each of the biological parameters were compared among all three groups using a Kruskal-Wallis 1-way ANOVA and difference between pairs of groups were analyzed by the Mann-Whitney U test with Bonferroni correction. All statistical analyses were performed with SPSS 20 (IBM, Armonk, NY, USA) using a confidence level of 95% for the Wilcoxon Rank-Signed, Spearman, and Kruskal-Wallis tests, and a 98.33% confidence level for the Mann-Whitney U test.

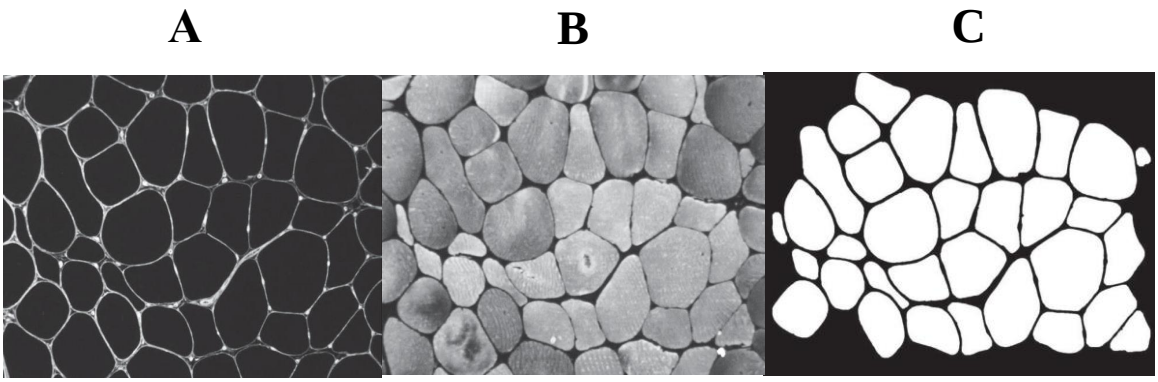


Figure 4.1: Morphometric Analysis of Myofiber Size. (A) Sarcolemma labeling provided an outline of each myofiber. **(B)** Myosin labeling served as a confirmation that a given sarcolemma outline encompassed a myofiber. **(C)** The final binary segmented image contained the geometry of each myofiber. Note that myofibers on the edge of the image were removed, as accurate morphometric parameters could not be extracted from only part of the myofibers.

Results

Development of Myofibrosis in Six Months among PAD Patient Groups:

QFM imaging revealed increased microvascular TGF-β1 expression in the gastrocnemius of CTL patients after six months without interventions, as evident by the increased intensity of labeling in the microvessels. A pattern of increased TGF-β1 was observed in the EXE patients. In both groups, there are increasing numbers of vascular cells that upregulate TGF-β1 after 6 months, forming a more uniformly labeled vascular wall. This is consistent with our previous cross-sectional study of advanced disease PAD patients (Fontaine Stage IV) compared to claudicating PAD patients (Fontaine Stage II). However, TGF-β1 labeling remained similar in the RVS group after 6 months **(Figure 4.2A)**. Quantitatively, CTL patients had an approximately 2.5-fold greater expression of TGF-β1 after a period of 6 months **(mean ± s.e.; 3.31 gsu ± 0.49 vs. 1.26 gsu ± 0.17; $p < 0.001$)**, while EXE patients increased in TGF-β1 about 2-fold **(4.46 gsu ± 0.59 vs. 2.35 gsu ± 0.52, $p < 0.001$)**. Patients in the RVS group had TGF-β1 expression levels relatively unchanged **(2.17 gsu ± 0.33 vs. 2.14 gsu ± 0.42)** after 6 months **(Figure 4.2B)**. Baseline expression of TGF-β1 among the three groups were similar **($p = 0.564$)**, showing that differences among the groups at baseline are not likely a confounding variable for the changes observed.

The difference in TGF-β1 expression before and after six months were compared among the groups and differences were detected **(χ^2 (2) = 24.0, $P < 0.001$)**. Post-hoc tests demonstrated that the change in TGF-β1 in RVS patients

was significantly than lower both EXE **(U(2) = 620, Z = 4.15, p < 0.001)** and CTL **(U(2) = 71.5, z = -4.06 p < 0.001)** patients, with no difference between EXE and CTL patients **(U(2) = 222, Z = 0.24, p = 0.808; Figure 4.2C)**.

Multi-spectral imaging revealed increased collagen density in the gastrocnemius of CTL patients after six months without interventions, as evident by the increased intensity of labeling in the extracellular matrix between myofibers and around microvessels. A similar increase in TGF-β1 was observed in EXE patients. In both groups at baseline, there exists a unique pattern of collagen deposition, where density of collagen is most intense around microvessels compared to collagen that surrounds the myofibers. After six months, collagen density continues to increase around microvessels as well as around myofibers. Again, this pattern is consistent with our previous cross-sectional study of advanced disease PAD patients (Fontaine Stage IV) compared to claudicating PAD patients (Fontaine Stage II)[201]. However, these changes in collagen intensity were not present in the RVS group after 6 months **(Figure 4.3A)**. Quantitative analysis determined CTL and EXE patients both had an approximately 25% increases in TGF-β1 after a period of 6 months **(2196 ± 84.2 vs. 1761 ± 56.7 and 2302 ± 122 vs. 1904 ± 123 respectively; both p < 0.001)**, while RVS patients had relatively unchanged collagen density **(1697 ± 77.2 vs. 1662 ± 60.5; p < 0.001)** after 6 months **(Figure 4.3B)**. Baseline collagen density among the three groups were similar **(p = 0.351; data not shown),** showing that baseline differences are not likely a confounding variable for the changes observed.

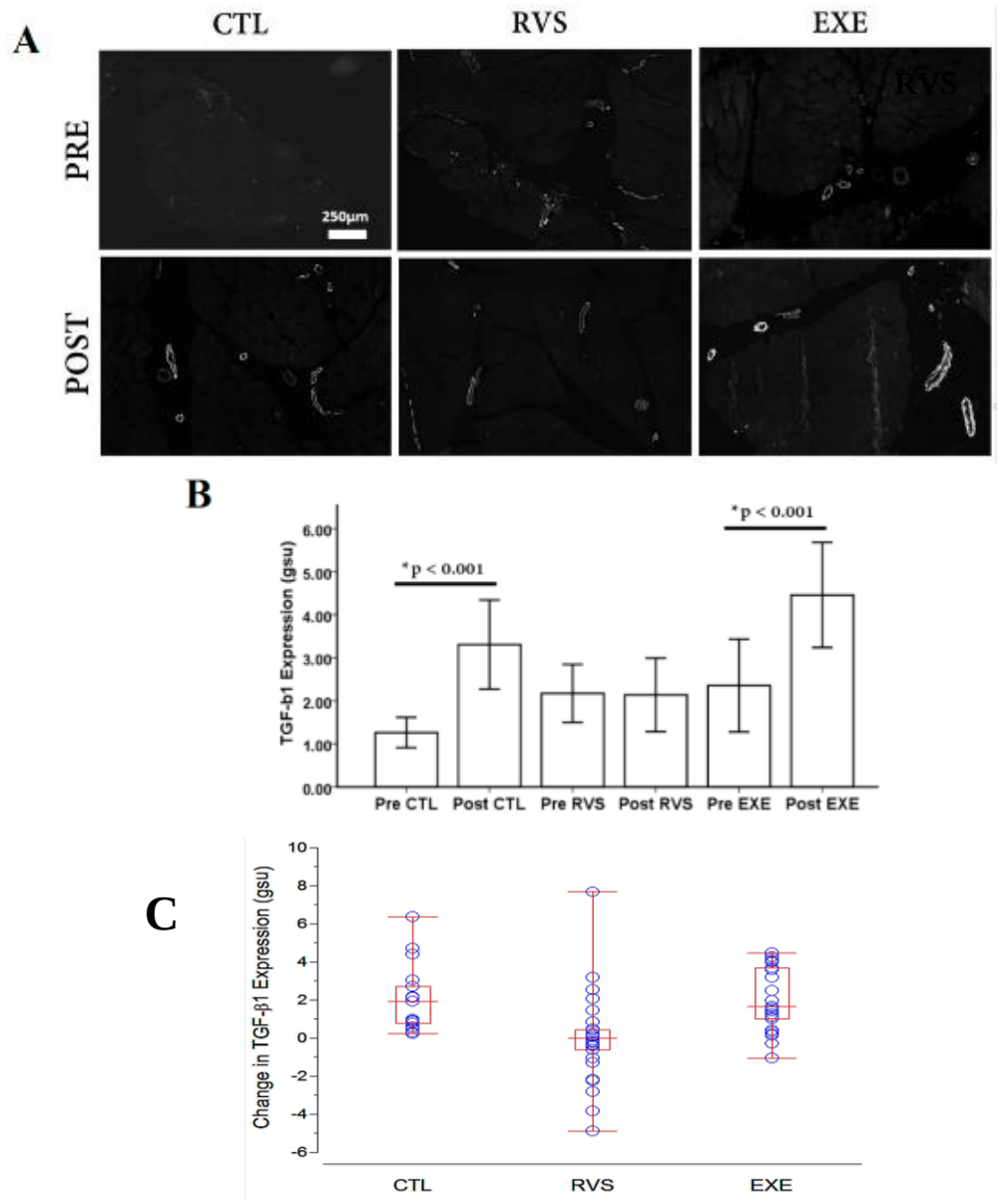
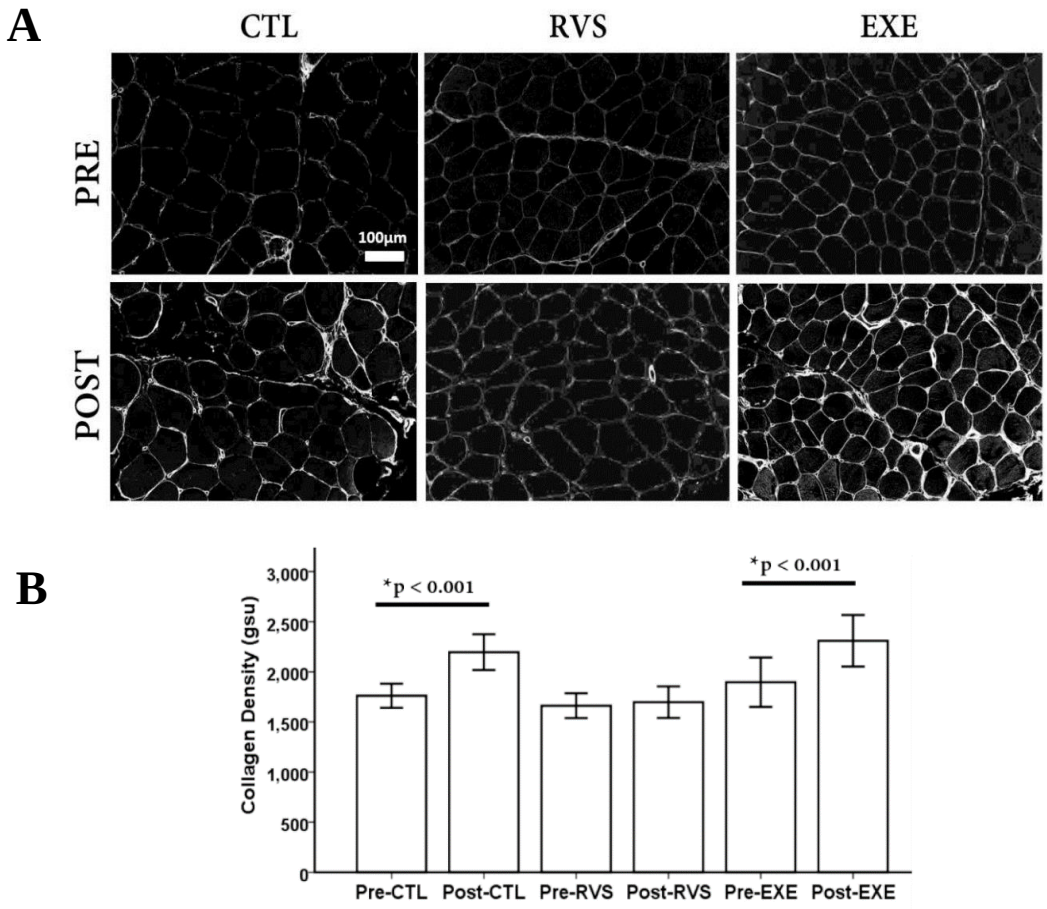


Figure 4.2: Changes in TGF-β1 Expression among Patient Groups. (A) Representative gray scale images of gastrocnemius microvessels positive for TGF-β1 labeling at baseline and after 6 months of no intervention, revascularization, or supervised exercise therapy, were captured with a wide-field fluorescence microscope (10x objective). Unlabeled myofibers appear grey against a black background. **(B)** TGF-β1 expression was determined by Quantitative Fluorescence Microscopy and defined as the sum of the products of area and mean pixel intensity of all positive events per microscopic field, normalized to the total area of the specimen in that field. **(C)** The change in TGF-β1 expression compared among the groups. There were significant differences between RVS and both CTL and EXE, but not between CTL and EXE patients. Significance threshold for all analyses was $p < 0.05$, except post-hoc tests where is $p < 0.013$. Significance is denoted as * $p < 0.001$ and ** $p < .013$.

The change in collagen density were significantly different among the groups **(χ^2 (2) = 20.8, p = 0.001)**. Post-hoc tests show that the change in collagen density in RVS patients was significantly lower than both CTL **(U(2) = 61.0, z = -4.30 p < 0.001)** and EXE **(U(2) = 572, Z = 3.32, p = 0.001)** patients, with no difference between EXE and CTL patients **(U(2) = 290, Z = -0.46, p = 0.648; Figure 4.3C)**. Across all times points and patient groups, there was a significant positive correlation between TGF-β1 expression and collagen density **(r = 0.670; p < 0.001)**, consistent with the previous finding of a robust relationship between TGF-β1 and collagen across disease stages **(Figure 4.3D)**.



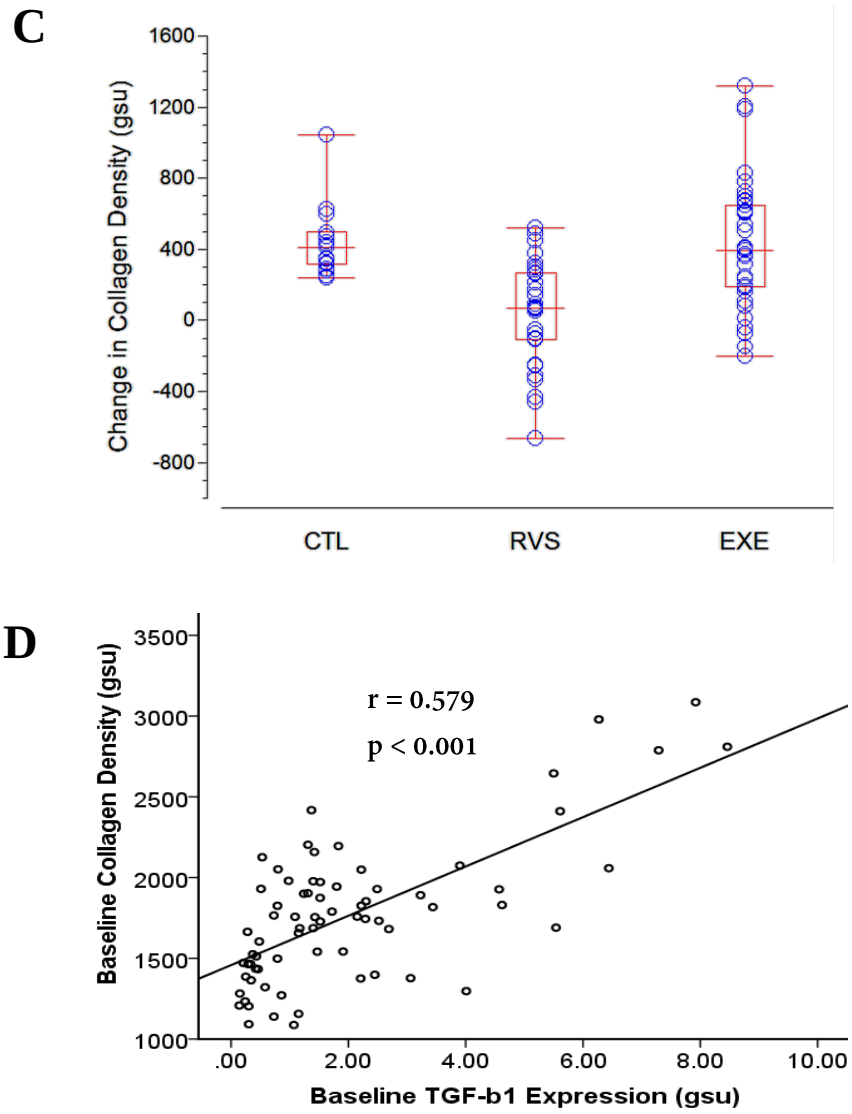


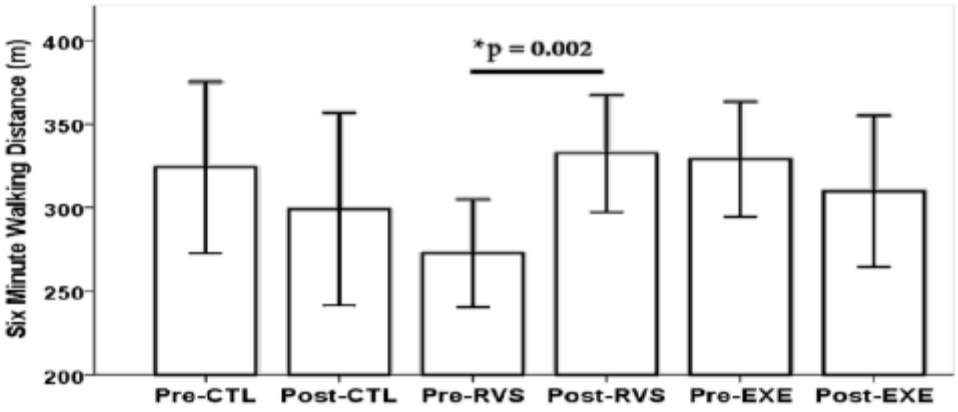
Figure 4.3: Development of TGF-β1 Associated Myofibrosis among Patient Groups. (A) Representative gray scale images of gastrocnemius specimens stained with Masson Trichrome were captured by multi-spectral, bright-field microscopy (20x objective) at baseline and after 6 months of no intervention, revascularization, or supervised exercise therapy. Myofibers delineated by collagen staining, appear black. Collagen density is represented by the intensity of the bright pixels. **(B)** Collagen density was analyzed by Multi-Spectral Microscopy and calculated as area-weighted mean intensity of all collagen events per specimen. **(C)** The change in collagen density compared among the groups by post-hoc tests. There were significant differences between RVS and both CTL and EXE, but not between CTL and EXE patients. **(D)** The relationship between TGF-β1 expression and collagen density at baseline determined by Spearman correlation. Significance threshold was $p < 0.05$, except for post-hoc tests where is $p < 0.013$. Significance is denoted as * $p < 0.001$ and ** $p < .013$.

Limb Function among Patient Groups and Relationship to Myofibrosis:

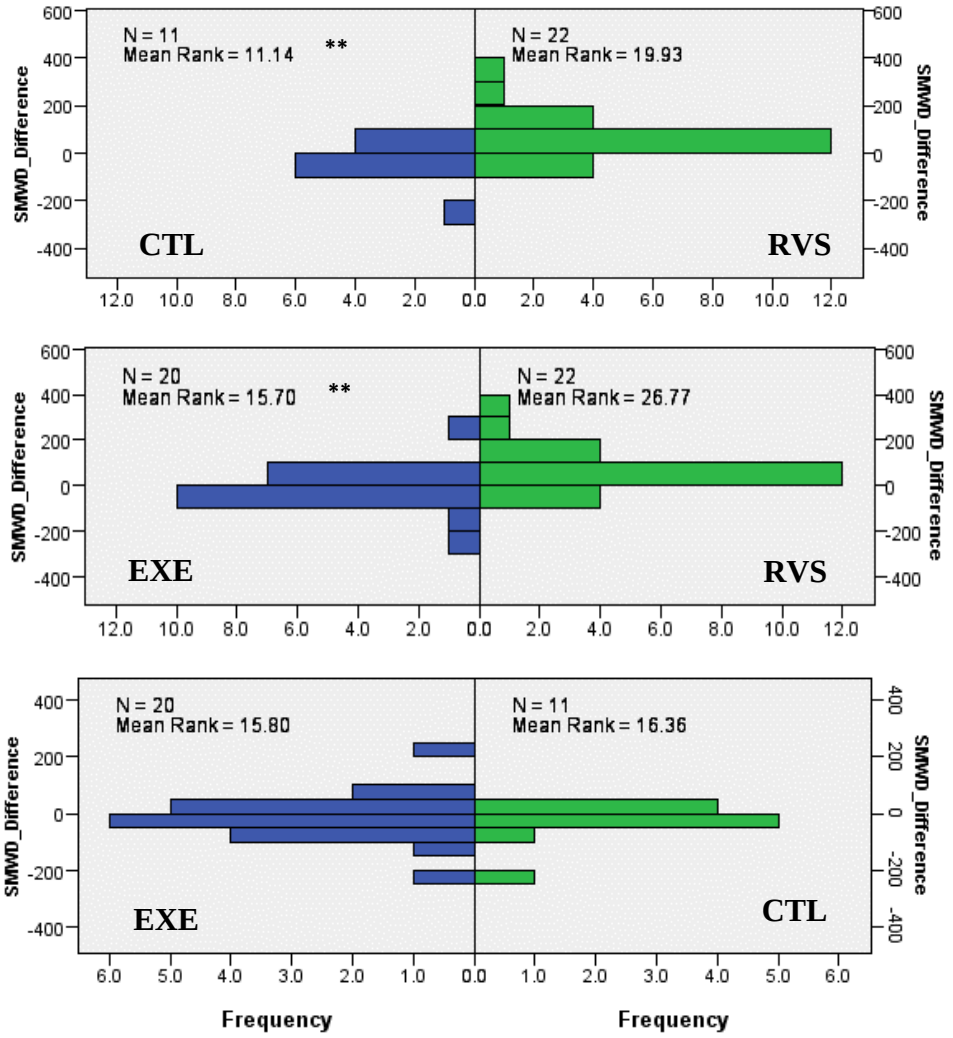
PAD patients in the CTL and EXE groups did not perform differently before and after 6 months on the SMWD, and only RVS patients walked further **(Figure 4.4A)**. The change in SMWD among the groups were significantly different **(χ^2 (2) = 10.6, p = 0.005)**. Post-hoc tests show that the change in SMWD in RVS patients was significantly higher than both CTL **(U(2) = 185, Z = 2.46 p = 0.012)** and EXE **(U(2) = 104, Z = -2.92, p = 0.003)** patients, with no difference between EXE and CTL patients **(U(2) = 106, Z = -0.17, p = 0.887; Figure 4.4B)**.

Correlation analyses were performed for TGF-β1 expression and collagen density against SMWD. At baseline, there was a significant inverse relationship between TGF-β1 expression and SMWD **(r = -0.245, p = 0.026; Figure 4.4C).** Unlike TGF-β1, collagen density did not correlate with SMWD at baseline **(r = -0.129; p = 0.298)**.

A


*p = 0.002
Six Minute Walking Distance (m)
400
350
300
250
200
Pre-CTL
Post-CTL
Pre-RVS
Post-RVS
Pre-EXE
Post-EXE

B


N = 11
Mean Rank = 11.14
**
N = 22
Mean Rank = 19.93
CTL
RVS
N = 20
Mean Rank = 15.70
**
N = 22
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EXE
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N = 20
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N = 11
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EXE
CTL
SMWD_Difference
Frequency

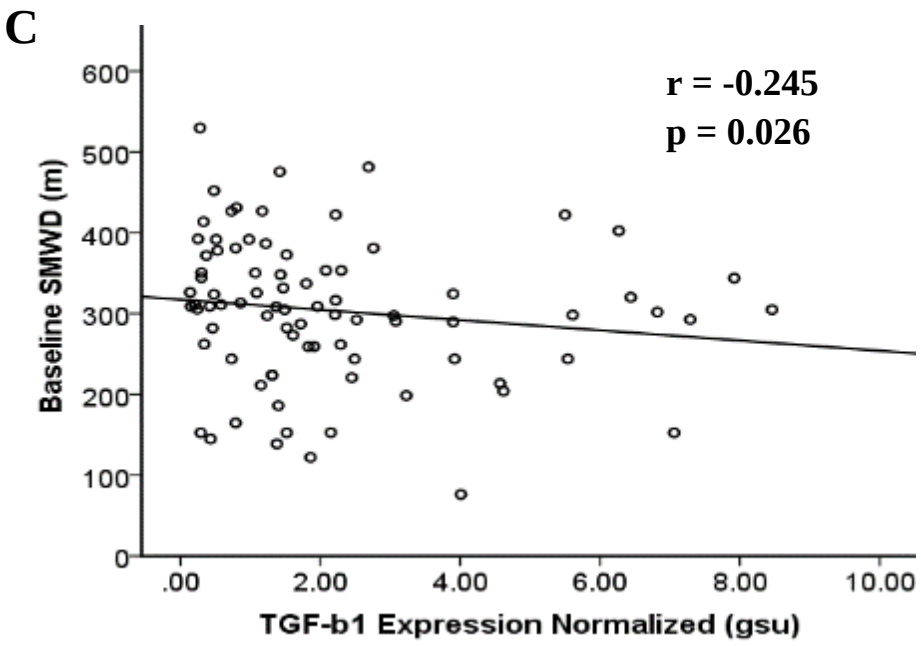

Figure 4.4: Six-Minute Walking Distance among Patient Groups and Relationship to Myofibrosis. (A) The six-minute walking distance, in meters, walked by PAD patients who underwent revascularization, exercise therapy, or neither intervention. **(B)** The change in SMWD compared among the groups by post-hoc tests. There were significant differences between RVS and both CTL and EXE, but not between CTL and EXE patients. **(C)** Correlation between TGF-β1 and SMWD at baseline. Significance threshold for all analyses was $p < 0.05$, except post-hoc tests where is $p < 0.013$. Significance is denoted as * $p < 0.001$ and ** $p < .013$.

PAD patients in the CTL group did not perform differently before and after 6 months in their PWT, while both RVS and EXE patients walked longer on the graded treadmill test **(Figure 4.5A)**. The change in PWT among the groups after six months demonstrated a trend towards significance **(χ^2 (2) = 5.22, p = 0.073)**. However, post-hoc tests did not demonstrate significant differences between any of the individual patient groups **(all p > 0.025),** although again there was a trend towards significantly improved PWT, between both RVS and EXE compared to CTL patients **(p = 0.028 and p = 0.055 respectively; Figure 4.5B)**.

Correlation analyses were performed for TGF-β1 expression and collagen density against PWT. At baseline, there was no correlation between TGF-β1 expression and PWT **(r = -0.136, p = 0.283)**. Again, collagen density did not correlate with PWT at baseline **(r = -0.132; p = 0.297)**.

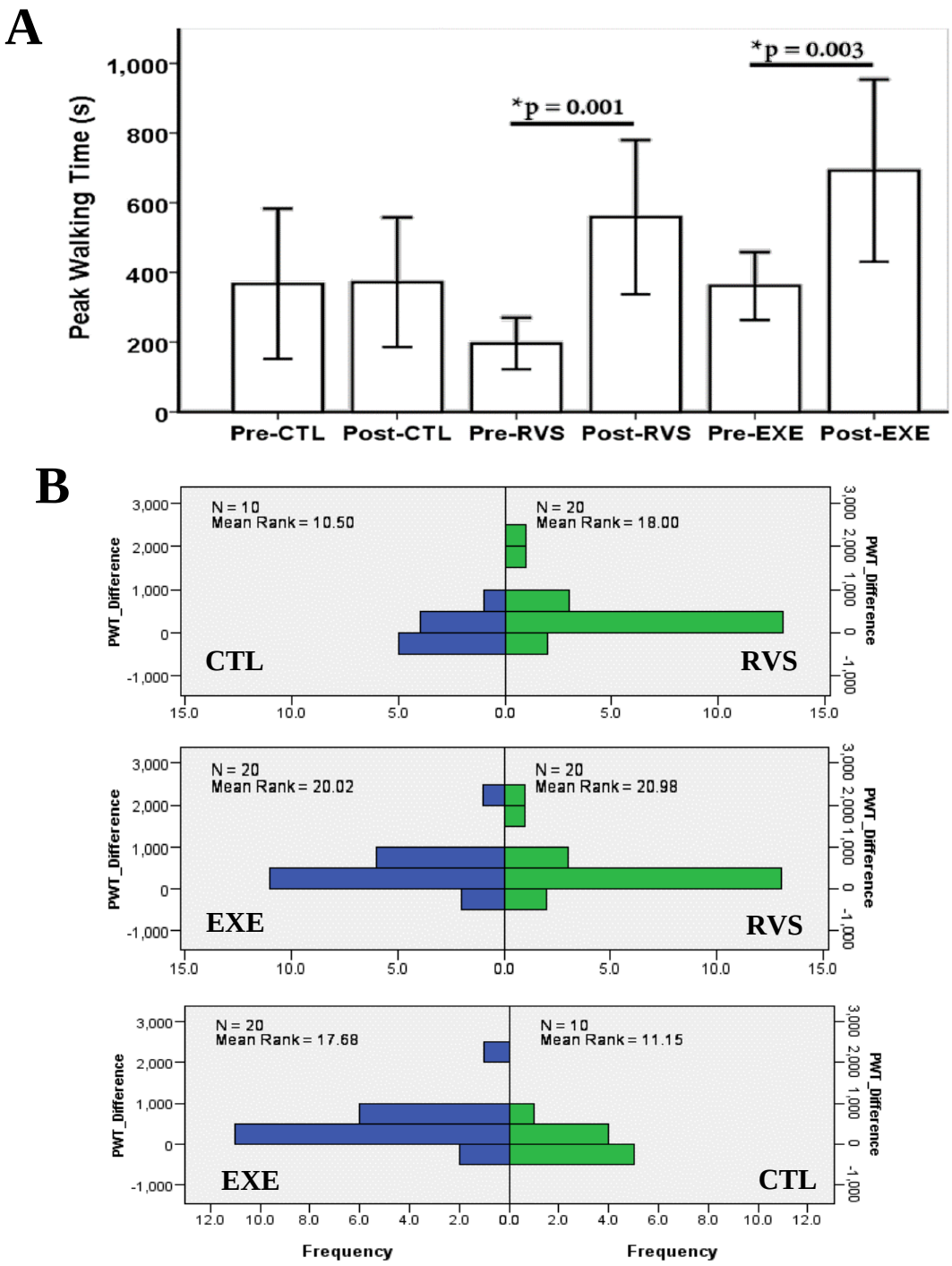

Figure 4.5: Peak Walking Time among Patient Groups and Relationship to Myofibrosis. (A) Peak walking time, in second, on graded treadmill test by PAD patients who underwent revascularization, exercise therapy, or neither. **(B)** The change in PWT compared among the groups by post-hoc tests. There were no significant differences among the groups. Significance threshold for all analyses was $p < 0.05$, except post-hoc tests where is $p < 0.013$. Significance is denoted as * $p < .013$.

Hemodynamics among Patient Groups and Relationship to Myofibrosis:

Ankle-Brachial Index (ABI) is the ratio of blood pressure recorded at the ankle compared to that of the arm, which reflects blood flow to the lower limbs and is considered surrogate measurement of ischemia-hypoxia in PAD. In 6 months, the ABI remained relatively stable in both the CTL and EXE patients, but significantly increased in RVS patients **(Figure 4.6A)**. Revascularization removes atherosclerotic blockages and directly improves blood flow, while exercise therapy is thought to improve circulation despite allowing blockages to persist. In these patients, ABI was inversely correlated with TGF-β1 expression **(r = -0.245, p = 0.003; Figure 4.6B),** consistent with our previous study of claudicating and critically ischemic PAD patients[23]. In patients with lower ABI, or greater ischemia-hypoxia, there is increasing TGF-β1 expression in microvessels. Collagen density, however, did not correlate with ABI **(r = -0.075, p = 0.377)**.

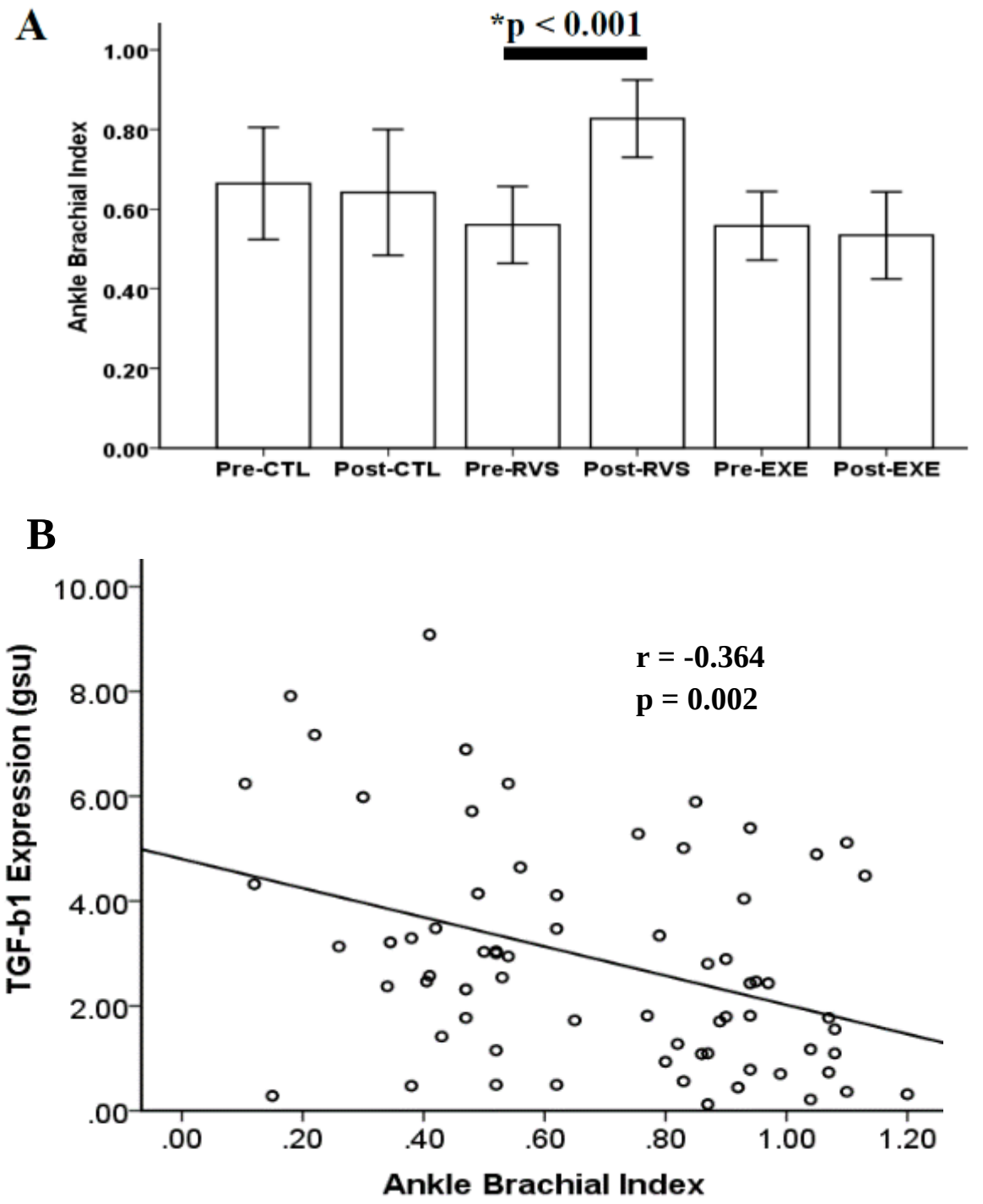

Figure 4.6: Hemodynamics among Patient Groups and Relationship to Myofibrosis. (A) Ankle Brachial Index in PAD patients before and after 6 months of revascularization, exercise therapy, or no intervention. **(B)** Correlation between Ankle Brachial Index and expression of TGF-β1 at baseline. The significance threshold for all analyses was $p < 0.05$.

Myofiber Area among Patient Groups and Relationship to Myofibrosis:

Myofiber cross-sectional area demonstrated a trend towards decreasing size in CTL patients, and significantly decreased in EXE patients **(Figure 4.7A)**. Patients in the RVS group, however, had increased myofiber area **(Figure 4.7A)**. At baseline, expression of TGF-β1 negatively correlated with myofiber area **(r = -0.247, p = 0.007; Figure 4.7B)**, while collagen density demonstrated a trend towards such a relationship **(r = -0.160, p = 0.081; Figure 4.7C)**. In skeletal muscle, TGF-β1 is anti-regenerative, so decreasing TGF-β1 may promote myofiber regeneration. Less collagen also prevents a physical barrier to myofiber regeneration.

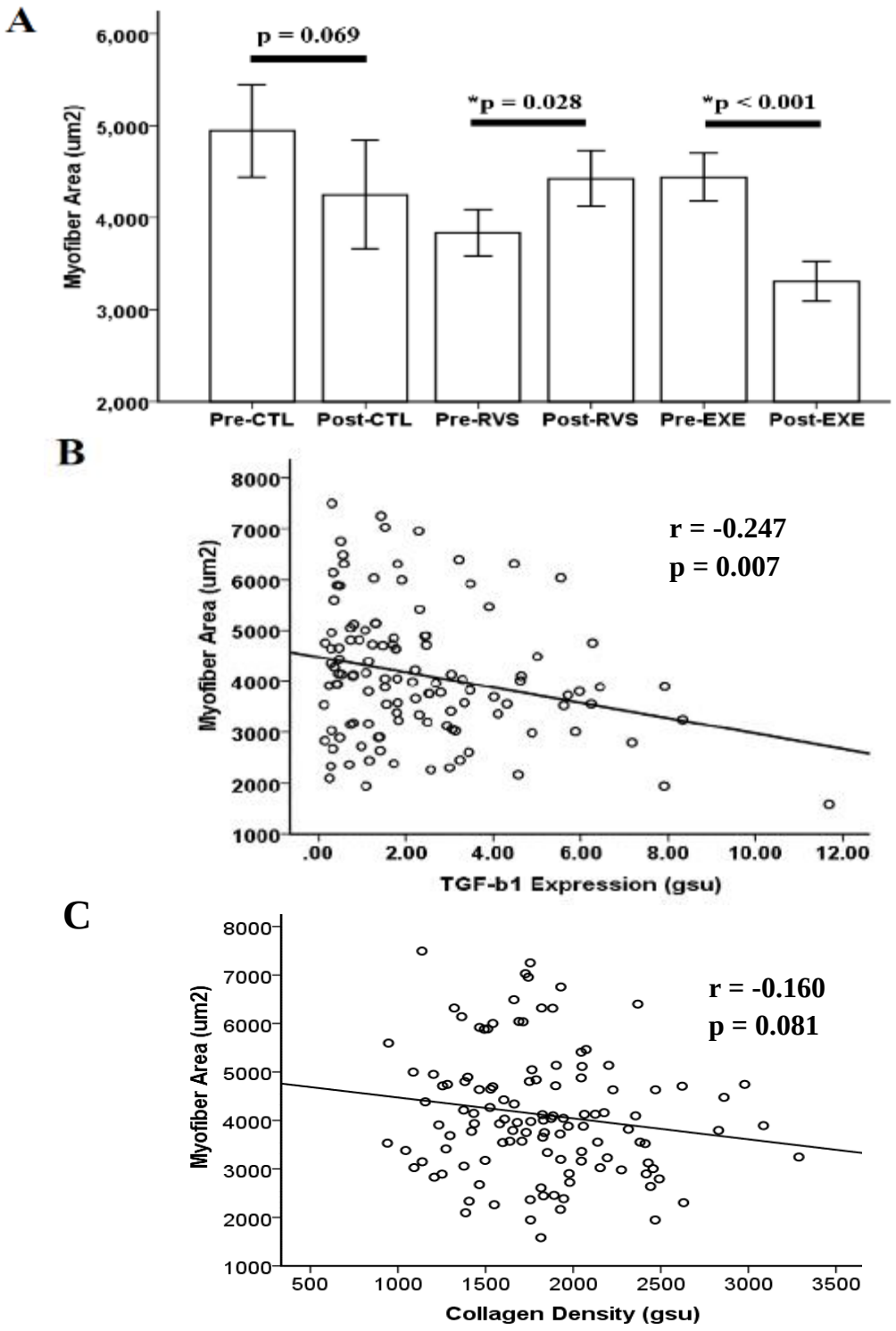

Figure 4.7: Myofiber Area among Patient Groups. (A) Myofiber cross-sectional area in PAD patients before and after 6 months of revascularization, exercise therapy, or no intervention. Correlation between **(B)** TGF-β1 expression and myofiber area and **(C)** collagen density and myofiber area at baseline. The significance threshold for all analyses was $p < 0.05$.

Discussion

This study examined, for the first time, the effects of two standard therapies for PAD patients on the development of myofibrosis over a period of 6 months. Overall, there were three important findings. First, in the span of 6 months, there was a significant increase in both vascular TGF-β1 expression and collagen density in the gastrocnemius of PAD patients who received no interventions. Second, revascularization prevented myofibrosis progression in the gastrocnemius of PAD patients while improving their walking times and distances. In these patients, both the ABI and myofiber cross-sectional area were increased, indicating potential mechanisms by which limb function improved. Finally, supervised exercise therapy improved only peak walking time, and was associated with progression of myofibrosis in the gastrocnemius, similar to PAD patients who received no interventions.

This study is also the first to evaluate the association of TGF-β1 expression and collagen density to limb function in PAD patients. At baseline, there was a significant inverse correlation between TGF-β1 and SMWD, but no correlation between collagen density and SMWD. There was not a significant relationship between TGF-β1 or collagen and PWT. Overall these data suggest first, that TGF-β1 expression is more closely related to limb function than collagen density. TGF-β1 expression may be a rapid response to hypoxia, which would have direct negative effects on muscle and limb function. For example, hypoxia is expected to compromise energy production in the myofibers. On the other hand, collagen

deposition, which is mediated by TGF-β1, may be viewed as a somewhat delayed response to hypoxia. Alternatively, TGF-β1 may have a direct negative effect on myofiber regeneration and contribute to the reduced limb function in these patients. Second, the data suggest that TGF-β1 expression better reflects SWMD than PWT performance. Since PAD patients are often tested on treadmill, inherent training effects may mask TGF-β1 associated detrimental effects on PWT. Although the relationship between TGF-β1 expression and SMWD was significant, it is not robust, suggesting one or both of two possibilities. First, the suppressive effects of TGF-β1 on muscle regeneration account for only a small fraction of limb dysfunction. Second, if both TGF-β1 and limb dysfunction, e.g., caused by a deficit in energy metabolism, are driven by hypoxia, the strength of their responses to hypoxia may be significantly different.

Without interventions, claudicating PAD patients experience a significant increase in TGF-β1 expression and collagen density in their gastrocnemius in just 6 months, although this was not associated with a significant decline in limb function. The lack of limb function decline in PAD patients without intervention may reflect the significant but not robust relationship between TGF-β1 expression and limb function. This is in line with PAD being a chronic disease, where it likely takes multiple myopathic changes over time to reach a threshold of damage that will produce limb dysfunction. In addition to promoting fibrosis, the increased TGF-β1 expression may have a direct suppressive effect on muscle regeneration or may be functioning as a biomarker associated with other pathogenic events that decrease limb function. This is supported by our finding that in 6 months, patients

who did not receive either revascularization or exercise therapy present with a trend towards decreasing myofiber cross-sectional area and myofiber degeneration. Furthermore, decreased myofiber area correlated with increased expression of TGF-β1. TGF-β1 is known to stimulate myoblasts to differentiate into myofibroblasts rather than new myofibers[70,71,158] and may limit regeneration of the chronically ischemic skeletal muscle. Accumulation of fibrotic tissue offers an unfavorable environment for myogenesis and decreases the efficiency of force transmission from the sarcomere to the extracellular matrix[70,71,158]. Indeed, there was a trend towards an inverse relationship between myofiber area and collagen density. Thus, these findings demonstrate the importance of an intervention that may be able to target TGF-β1 production by SMCs in PAD muscle and potentially improve limb function.

Claudicating PAD patients treated with revascularization demonstrated an arrest in the progression of myofibrosis, which was associated with improved limb function. Revascularization prevented an increase in vascular TGF-β1 expression and collagen density that was detected in the gastrocnemius of PAD patients who received no interventions. Preventing increased TGF-β1 expression may contribute to improvements in SMWD by a mechanism that spares myofiber regeneration. RVS patients were the only group of patients that had increased myofiber area, which is indicative of regeneration. The robust inverse relationship of TGF-β1 expression to myofiber area further supports regeneration as a mechanism. However, future studies should evaluate for markers of regeneration, such as MyoD, to verify[200]. Both the increased limb function and myofiber cross

sectional area may be due to improved hemodynamics and tissue oxygenation, since RVS patients were the only group of patients that had increased ABI. At baseline, ABI strongly correlated with TGF-β1 expression, suggesting that increased blood flow and thus, reduced ischemia-reperfusion injury contributes to preventing the progression of myofibrosis and improving limb function with revascularization.

A 6-month supervised exercise regimen improved the walking ability of PAD patients, but did not alter the development of myofibrosis that was observed in untreated PAD patients. Patients who participated in supervised exercise walked longer on treadmill, as reflected by greater PWT, but exhibited no change in their SMWD. The increased PWT has been widely reported in the literature[140], but only two studies have examined changes in SMWD and both demonstrated increases with exercise[141,142]. An explanation may be that our study is underpowered compared to those two studies which had at least twice as many PAD patients. The estimated effect size for SMWD (0.70) is relatively smaller than that of PWT (1.01) and would require more patients to demonstrate a statistical significance[132], which is consistent with the findings of this study. An alternative explanation is that in our study, patients were recruited when they were referred to vascular surgery as opposed to the other two studies that recruited patients from various social outlets, such as advertising. PAD patients in our study may have more advanced disease despite falling into the same Fontaine Stage II classification and improvements in submaximal endurance may be minimized. Such an effect may not similarly apply to PWT since the graded treadmill provides a very similar

environment to the supervised exercise therapy they receive. Thus, a part of the improvement may be due to a training effect, in which the leg muscles of these PAD patients have been trained to perform treadmill exercise and develop tolerance that allows them to improve their performance further. SMWD, however, is more reflective of how patients would exercise in their daily lives. Finally, exercise is thought to improve conditioning of the body for maximal exertion such as that on the graded treadmill test, and thus, is where the benefits may be best observed rather than endurance with SMWD.

Both TGF-β1 expression and collagen density increased in patients who participated in exercise therapy and their relative increases were similar to those of the control group. This suggests that exercise does not change the natural history of myofibrosis in the skeletal muscle of PAD patients. With exercise, there was no change in ABI and there was a decrease in myofiber area. These findings demonstrate that the benefits of exercise may be independent of blood flow, TGF-β1 associated myofibrosis, and myofiber regeneration. The mechanism may be through systemic cardiovascular benefits, increased perfusion of blood at the microvascular level, or a different aspect of PAD myopathy such as increasing energy metabolism in the muscle[201,202]. However, improvements have been attributed to training effects that improve the tolerance of PAD patients to claudication symptoms while performing the graded treadmill test, and psychologically patients feel they should improve performance having been trained on the treadmill[128,129]. Exercise may actually be physically damaging to the skeletal muscle by increasing the bouts of ischemia-reperfusion, which may

counterbalance potentially positive effects on myofibrosis. Overall, the functional improvements with exercise therapy appear unrelated to myofibrosis.

One reason why revascularization and not exercise therapy prevents myofibrosis progression could be that revascularization directly targets blood flow and thus ischemia-reperfusion injury, while exercise at best, indirectly targets ischemia-reperfusion. Among the study groups, only patients receiving revascularization improved their ABI. Exercise therapy, on the other, does not increase ABI in PAD patients of this study, which is not unexpected given the blockages are left intact[138]. ABI, in turn, predicts microvascular TGF-β1 expression, such that as ABI decreases, which reflects increasing ischemia and hypoxia, TGF-β1 levels increase. Revascularization restores blood flow to the microvessels, which should in theory prevent further production of TGF-β1 by vascular smooth muscle cells. Yet, TGF-β1 levels are not reduced as would be expected by the inverse relationship between ABI and TGF-β1 expression. This recapitulates the insidiousness of PAD myopathy and points to the major problem with revascularization as a conventional therapy. Previous biomechanical, blood flow, and oxygen kinetic studies show that even before the skeletal muscle of PAD patients becomes ischemic, that they already present with gait dysfunction[22-26]. It seems that once ischemia-reperfusion has induced increased vascular TGF-β1 expression, restoration of blood flow does not significantly reduce TGF-β1 expression and reverse myofibrosis. Although as a whole group, RVS patients had unchanged TGF-β1 expression, many patients did have decreased TGF-β1 expression and correspondingly less dense collagen. This demonstrates that at

this early stage of PAD progression, myofibrosis may be reversible in some patients. Further studies aimed at understanding the characteristics of these “responders” and the circumstances under which revascularization may reverse fibrosis should be rigorously pursued.

Another benefit of preventing the increased TGF-β1 levels detected in untreated patients is that the compensatory regeneration response is allowed to proceed more effectively. Patients who underwent revascularization were the only ones who had increased myofiber area, which in the context of PAD, points to myofiber regeneration occurring. Although these new myofibers have better contractile abilities and will improve overall limb function, they are vulnerable to the pro-fibrotic environment that persists and may become atrophic and degenerate in the future. PAD patients who claudicate, do not maintain their limb function and succumb to sedentary lifestyles, further aggravating other cardiovascular co-morbidities. With myofibrosis still a hindrance and the possibility of these additional insults to regenerating myofibers, the likelihood of aging PAD patients preserving their limb function and quality of life is low. Thus, given the anti-regenerative properties of TGF-β1, adjunct therapies that decrease its expression may preserve healthy myofibers that contribute to improved limb function in PAD patients.

In conclusion, this study reinforces TGF-β1 associated myofibrosis as an important marker of PAD myopathy and disease progression, and reveals how the two conventional therapies, revascularization and supervise therapy, affect development of this pathology. Fibrosis has been linked to end organ dysfunction

and when it occurs in the skeletal muscle, is highly disruptive[69]. Around myofibers it restricts normal contractile properties and in microvessels, interferes with oxygen and nutrient delivery, all of which predisposes the muscle to dysfunction and degeneration[70,71,158]. Although this study demonstrated only a weak, although significant, relationship between TGF-β1 and limb function, the myofibrosis that TGF-β1 induces can leave the skeletal muscle vulnerable to development of additional myopathies, which once progressing together, decreases limb function. We further demonstrated that neither of the two conventional therapies can reverse myofibrosis. However, revascularization but not supervised exercise therapy prevented progression of myofibrosis in PAD gastrocnemius. This suggests that, clinically, long-term benefits to the lower limb of PAD patients are better with revascularization than exercise and that preventing myofibrosis progression is a contributing mechanism. Thus, adjunct therapies that can reverse or further inhibit development of myofibrosis may help maintain or improve the limb function and quality of life of PAD patients.

CHAPTER V: CONCLUSION

Model of PAD Myofibrosis

The work presented in this dissertation provides insight into the development of myofibrosis in PAD patients, its functional consequence, and how current standard therapies affect this myofibrosis. The overarching hypothesis was that decreased hemodynamics drives a local TGF-β1 associated increase in collagen deposition within the gastrocnemius of PAD patients that increases with disease severity, and that this myofibrosis is reversed by revascularization but not exercise therapy in association with improved limb function. Three specific aims were developed to test this hypothesis. The first aim was to investigate the relationships among limb hemodynamics, and both TGF-β1 expression and collagen deposition in the gastrocnemius of PAD patients at advancing disease stages to establish the presence and potential etiology of myofibrosis. The second aim was to determine whether TGF-β1 associated myofibrosis in the gastrocnemius of PAD patients is a localized response in the ischemic muscle or response to systemic events. The third aim was to determine changes in myofibrosis in relation to limb function, hemodynamics, and myofibers size, in PAD patients who have undergone revascularization or exercise therapy.

Studies that addressed the first specific aim established a TGF-β1 associated myofibrosis by the robust correlation between TGF-β1 and collagen density that increases in the gastrocnemius with the severity of disease in PAD

patients (Fontaine Stage II to IV). The increased production of TGF-β1 was determined to be predominantly by vascular SMC, rather than immune cells or endothelia, suggesting a vascular etiology and downstream consequence of atherosclerotic blockages. The relationship between ABI and SMC-derived TGF-β1 is further suggestive that altered hemodynamics and the resulting hypoxia, caused by the atherosclerotic blockages in the named vessels, may induce expression of TGF-β1 by SMC at the microvascular level. Activation of SMC from a contractile to a synthetic phenotype capable of promoting fibrosis was evidenced by its proliferative state and cobblestone, rather than spindle shaped morphology. Consistent with the well-known mechanism of fibrosis, whereby TGF-β1 activates myofibroblasts to secrete excessive extracellular matrix, TGF-β1 was found closely associated with fibroblastic cells around the microvessels, along with increased collagen density and area in the adjacent myofiber tissue. Ischemia and hypoxia experienced by SMC of microvessels may be driving the increased production of TGF-β1 by SMC, which activates fibroblastic cells. These activated fibroblasts have the known ability to migrate to areas around the myofibers and deposit extracellular matrix. Indeed, we localized fibroblasts to collagenous areas around myofibers. Overall the vascular etiology of TGF-β1 associated myofibrosis is in line with PAD disease being a vascular disease.

Studies that addressed the second specific aim established that the TGF-β1 associated myofibrosis is a localized response that develops with early disease (Fontaine Stage II) in the presence of atherosclerosis in the diseased limb, rather than a response to systemic changes associated with atherosclerosis. Although

the increased production of TGF-β1 by microvascular SMC could be a response to local ischemia and hypoxia of the diseased leg, systemic contributions cannot be ruled out, such as pro-fibrotic factors in circulation that increase with disease severity that may also be inducing the production of TGF-β1 by SMC. Since the majority of PAD patients have bilateral disease, it is difficult to know if systemic contributions exist, but this can be determined by comparing the limbs of PAD patients with unilateral disease. The greater expression of TGF-β1 and collagen density in the diseased compared to contralateral leg and the lack of correlation between the limbs in both measures, indicated that systemic effects were minimal. Furthermore, a TGF-β1 associated myofibrosis did not exist in the contralateral compared to the diseased leg, further suggesting that atherosclerosis and its hemodynamic consequences on the microvessels is needed to produce TGF-β1 associated myofibrosis. These findings demonstrate that TGF-β1 associated myofibrosis is primarily a localized response in the ischemic muscle.

Finally, studies that addressed the third specific aim characterized the progressive development of this TGF-β1 associated myofibrosis over a 6 month period in early stage PAD patients, and determined its response to the standard therapies of revascularization and supervised exercise and associations with changes in limb function. Our previous studies comparing age-matched controls with early and advanced PAD disease was cross-sectional and did not allow us to determine the progressive development of TGF-β1 associated myofibrosis in PAD patients. This prospective study found that the gastrocnemius of PAD patients who had no interventions during the 6 month period demonstrated a significant increase

in TGF-β1 associated myofibrosis in their gastrocnemius, but this did not produce changes in the limb function measurements of PWT or SMWD. With supervised exercise, there was still a significant increase in TGF-β1 associated myofibrosis, despite improved PWT, although there was no change in SMWD performance. PAD patients who underwent revascularization did not exhibit an increase in TGF-β1 associated myofibrosis, and performed significantly better on both PWT and SMWD. This may be attributed to the improved hemodynamics, reflected by significant increase in ABI, measured in revascularization patients that was not found in patients who had no interventions or participated in exercise therapy. Alternatively, it may be attributable to increased myofiber regeneration, since only in the revascularization group was there an increase in the myofiber size compared to the no intervention and exercise groups.

Taken together, the findings of these three specific aims tested the overarching hypothesis. Hemodynamics is tightly linked to the increased TGF-β1 expression by SMC, which is strongly correlated with collagen deposition that increased with advancing Fontaine disease stage in PAD patients. These myofibrotic changes are indeed a local response that is associated with the decreased hemodynamics of the affected limb caused by atherosclerosis. Finally, as hypothesized, supervised exercise therapy did not reverse myofibrosis, but neither did revascularization, although it did halt the progression of myofibrosis in 6 months. Preventing myofibrosis progression was accompanied by improvements in both the ability of PAD patients to walk further and longer. Interestingly, however, TGF-β1 but not collagen density was associated with these improvements,

particularly walking distance. Perhaps TGF-β1 is an early response directly associated with ischemia-reperfusion and the consequently hypoxic environment that is detrimental to the muscle, while deposition of collagen is a later response induced by TGF-β1 that requires more time to impact limb function in Fontaine Stage II PAD patients. Alternatively, TGF-β1 may impact function by additional biological roles in the skeletal muscle of PAD patients besides fibrosis, such as inhibiting regeneration. Overall, these studies show that the best that standard therapies can do for TGF-β1 associated fibrosis is to halt progression and thus it is imperative to understand the mechanism driving the increased TGF-β1 expression by vascular SMC to develop therapeutic strategies that directly target the development of myofibrosis.

In summary, this dissertation adds knowledge to an aspect of myopathy that has not been well studied and links it to the known pathophysiology of PAD. The lower limbs of PAD patients experience cycles of ischemia-reperfusion that impairs limb function. However, how altered hemodynamics and oxygenation levels may affect the skeletal muscle of PAD patients to produce a myopathy that contribute to the decreased limb function is unknown. The finding that increased TGF-β1 expression by SMC of microvessels is inversely correlated with ABI suggests that the changes produced by atherosclerosis of the named vessels can adversely activate pro-fibrotic changes in the smaller vessels. Increased TGF-β1 is strongly associated with collagen deposition with significant progression in 6 months during early stages of disease, and this relationship holds over years to more advanced stages. Furthermore, the development of this myofibrosis is a local response rather

than caused by systemic effects. Progression of myofibrosis was halted by revascularization, but supervised exercise did not affect this change, as it did not improve or worsen the progression. This model of PAD myofibrosis is illustrated in

Figure 5.1.

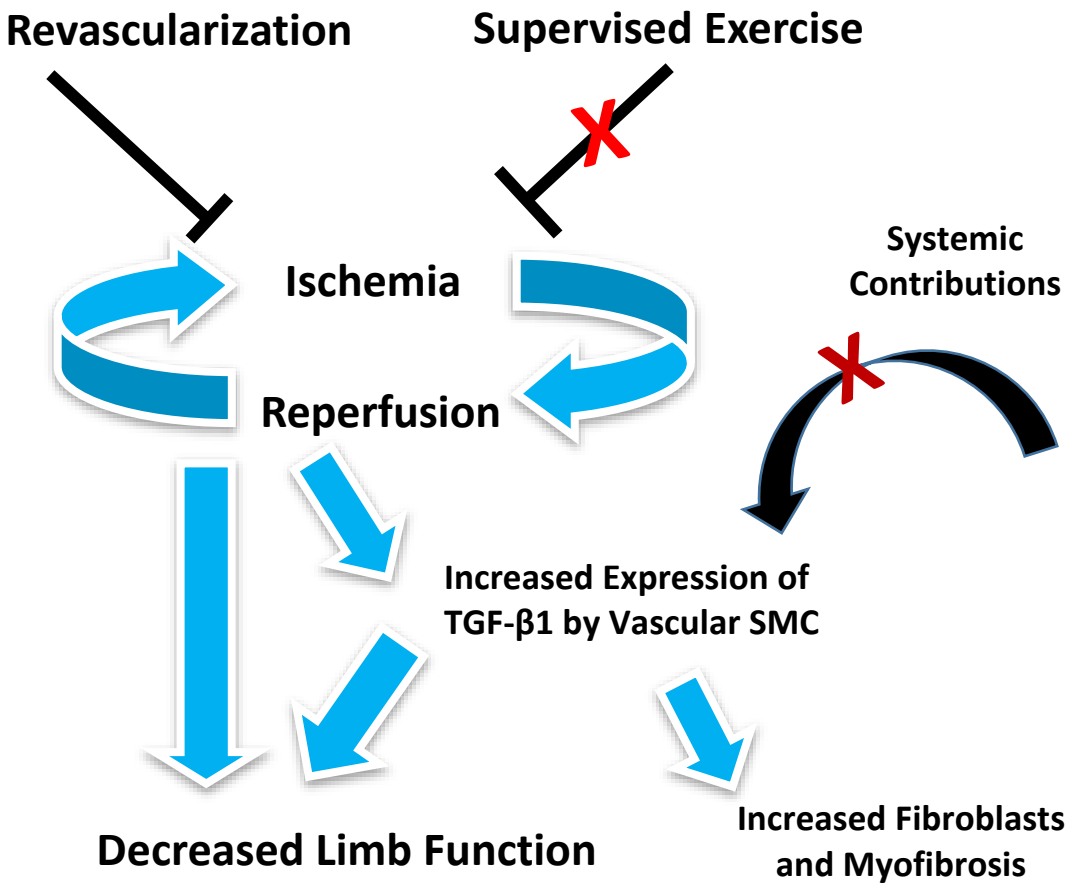

Figure 5.1: Model of Myofibrosis in PAD.

Future Directions

In addition to better understanding the development of another component of the myopathy afflicting PAD patients, the work presented in this dissertation also points to future potential mechanisms to be studied. Central to these studies should be understanding how TGF-β1 expression by SMC of microvessels is increased, given it is an early marker of the disease that progresses with stage, significantly correlates with limb function performance, and is not decreased by revascularization or supervised exercise. One potential inducer could be cycles of ischemia-reperfusion, which can be modeled by alternating hypoxia and normoxia conditions. Such hypoxia-normoxia may directly shift the phenotype of SMC from contractile to the synthetic form that is characterized by increased expression of TGF-β1. In future *in vitro* studies, we will examine if alternating cycles of hypoxia-normoxia can induce the expression of TGF-β1 in human internal thoracic artery smooth muscle cells (HITASMC) and shift the phenotype from a contractile to a synthetic one.

Alternatively, cycles of hypoxia-normoxia may induce endothelia to secrete pro-fibrotic factors that activate SMC to produce TGF-β1. This is supported by preliminary observations in our laboratory of the close physical association of SMC and endothelia in the subendothelial region, suggestive of pathological signaling interactions. Two potential candidate pro-fibrotic markers that endothelia may release are Platelet Derived Growth Factor (PDGF)-BB and Connective Tissue Growth Factor (CTGF)[70,71]. In future studies, we will first label for PDGF-BB and

CTGF in the gastrocnemius of Fontaine Stage II and IV PAD patients along with age matched controls to determine if there is increased expression of either factor in endothelia, and if these changes correlate with the expression of TGF-β1 expression in the same biopsy sample. If results are promising of their involvement, then *in vitro* studies will be conducted to determine if cycles of hypoxia-normoxia induce PDGF-BB and CTGF expression in human internal thoracic artery endothelial cells (HITAEC), if transfer of supernatant to HITASMC will induce increased TGF-β1 expression, and if addition of a neutralizing antibody to each factor will reverse the findings. Overall, these studies on the mechanisms by which TGF-β1 is increased will point to novel therapeutic targets. If any of these mechanisms prove to be important, then these cells from the microvessels of PAD patient gastrocnemius can be isolated and similar experiments performed.

Ultimately, the goal will be to understand how atherosclerotic blockages in the named vessels leads to the pathophysiological changes not just in the microvessels of the gastrocnemius but of vessels of various sizes in between. It is still unknown how the increase in TGF-β1 in SMC that occurs focally at the atherosclerotic sites spreads downstream. Additionally, the context with which TGF-β1 is increased and the consequences appear to be different as well, where SMC within a plaque proliferate and migrate into the intimal layer to promote formation of a fibrous cap, whereas in the microvessels proliferate within the media and promote fibrotic thickening of that layer but not many are seen promoting plaque formation or even significant thickening of the intimal wall. Beyond the media layer of microvessels, TGF-β1 activates fibroblastic cells to promote

myofibrosis, and it has been suggested that by an autocrine mechanism may stimulate SMC nearby along the vessel wall to increase TGF-β1 expression. Perhaps this is a similar mechanism that occurs around the vessel segment with atherosclerosis. However, ischemia-hypoxia may not play a critical role distal to the atherosclerosis compared to microvessels. Other potential mechanisms may include disturbed flow that can activate sensors in endothelia that is known to promote vascular remodeling, which may include induction of TGF-β1 expression by SMC. And for vessels further downstream, perhaps both disturbed flow and ischemia-hypoxia are important. Finally, circulatory factors originating from the atherosclerotic plaque may flow through the vasculature distal to the blockage and contribute to development of TGF-β1 associated myofibrosis of that particular leg. Understanding how pathological changes are propagated down the vasculature distal to a blockage that results in elevated TGF-β1 expression from SMC in the end organ skeletal muscle is necessary to interdict myofibrosis at its root cause.

This is not to say that understanding the additional effects of TGF-β1 in the skeletal muscle, such as myofiber regeneration, are not important. TGF-β1 has known anti-regenerative effects in skeletal muscle and its increased expression by the microvessels may reflect a compensatory mechanism early on to increase the force of contraction for adequate delivery of blood to the lower extremities. However, with progression of the disease, may serve as a means to wall off flow and nutrient delivery, as well as gas and waste exchange, to skeletal muscle that is beyond repair. Such a theory may also be applicable to the skeletal muscle itself, where activation of increased activation fibroblasts by TGF-β1 is to directly

stimulate the repair process, and thus bypass the immune mediated mechanism that requires resolution of inflammatory responses that may release cytotoxic ROS. If adequate flow is not restored in a timely manner and myofibers are beyond repair, the excessive amounts of TGF-β1 then triggers a fibrotic response that fills in the space left behind by the dying skeletal muscle for stabilization of the lower limb. It may be that at some point, even if adequate flow is restored this sequelae will still continue, despite the limb being able to be salvaged and that is where interventions that directly reduce TGF-β1 may help. If this theory is true, how the vasculature and skeletal muscle coordinates such a response will need to be explored, since this may affect our understanding of the development of fibrosis and end organ damage in other parts of the body. During the development of PAD, the increasing TGF-β1 may act as a molecular switch to turn off regenerative processes that would waste valuable resources and divert myoblasts to accelerate the fibrotic response that stabilizes the leg with scar tissue.

Beyond the mechanistic aspects, uncovering TGF-β1 as a novel marker of disease progression that develops early in PAD pathophysiology has important prognostic value. One of the most important findings from this dissertation is that the early increase in TGF-β1 is strongly linked to hemodynamic compromise. Our laboratory and others have been reported increased oxidative damage in the skeletal muscle of PAD patients, and we have observed increased carbonyl and 4-hydroxynonenal damage in cells of the microvessels in the gastrocnemius of Fontaine Stage II PAD patients. Along with markers of mitochondrial dysfunction and myofiber morphometrics, carbonyl and 4-hydroxynonenal oxidative damage

and TGF-β1 can form a biomarker panel that may be able to predict the decline of limb function of patients with no interventions and outcomes of revascularization and supervised exercise therapies. It would also be interesting to determine if increasing TGF-β1 levels predict the other biomarkers and whether any predicts the others, which would provide clues as to the earliest trigger of PAD myopathy. It would also test the theory above that TGF-β1 is first a compensatory mechanism to supply more blood to the limbs but at some point becomes a molecular switch that promotes pathological fibrosis. Finally, staging of PAD patients, such as with the Fontaine stages, remain a crude designation of disease development. Fontaine stages are solely based on overt symptoms, and often the decision to revascularize or start supervised exercise therapy does not take into account the myopathy present in the legs of PAD patients. Future development of guidelines for prognosis and for optimal use of current therapeutics taking into account myopathy will be beneficial to the management of PAD patients.

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