# FUNCTIONAL VASODILATION IS IMPAIRED IN ARTERIALIZED CAPILLARIES IN THE SPINOTRAPEZIUS

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ABSTRACT

FUNCTIONAL VASODILATION IS IMPAIRED IN ARTERIALIZED CAPILLARIES IN THE SPINOTRAPEZIUS

Joshua Cutts

Ischemic diseases are the result of atherosclerotic plaques, which occlude conduit arteries. Ischemic disease in different tissues leads to different conditions, such as coronary artery disease (CHD), cerebrovascular disease (CVD), and peripheral arterial occlusive disease (PAOD). Patient vasculature architecture is variable; some patients having many collateral vessels, which are connect one arterial branch to another, and readily serve as natural bypass routes to atherosclerotic occlusions, to enlarge and provide blood flow to tissue distal to the occlusion. Patients with many natural collateral vessels are ischemia protected. Unfortunately, not all patients have collateral arterioles to remodel into conduit vessels and provide blood flow to distal tissue. It would therefore be advantageous to stimulate the arterialization of collateral capillaries, capillaries that connect adjacent arterial branches, to remodel and form conduit collaterals. Unfortunately, just having a robust collateral network is not sufficient to provide effective revascularization of tissue; it requires that collaterals have the ability to regulate blood flow, which is hypothesized to be impaired during collateral growth. Therefore the reactivity of arterialized capillaries was examined to determine if arterialized capillaries can regulate blood flow into the ischemic zone of tissue. Vascular reactivity of arterialized capillaries was examined using intravital microscopy with functional vasodilation seven days after surgical ischemia was induced. The reactivity of arterialized capillaries was significantly impaired (2% ± 2% vs. 29% ± 12% $p<0.05$) when compared to sham operated terminal arterioles of a similar diameter. Thus, arterialized capillaries were unable to efficiently regulate blood flow in response to endogenous muscle stimulation. Ineffective regulation of blood flow could be caused by a synthetic smooth muscle cell phenotype, which is proliferative and unable to regulate tension in contrast to a contractile phenotype which can regulate its contractile state. More research will be conducted to determine the duration and cause of the limited ability of arterialized capillaries to vasodilate in order to cultivate arterialized capillary treatment as a therapy for patients with occlusive ischemic diseases.

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"Our greatest glory is not in never failing, but in rising up every time we fail"

-Ralph Waldo Emerson

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## CHAPTER 1: Introduction

### 1.1 Clinical Relevance: Ischemic Disease

Ischemic diseases are the leading cause of mobidity and motality in the developed world.[1] Ischemic diseases are caused by occlusion of conduit arteries by atherosclerotic plaques, restricting blood flow to distal tissue and resulting in ischemic, potentially hypoxic tissue and necrosis. Occluded arteries cause ischemic disease; the location of the atherosclerotic obstruction determines the classification of ischemic diseases. Coronary heart disease (CHD) is the leading cause of death in the western world and it is caused by occluded coronary arteries.[2] Cerebrovascular disease (CVD) is a leading cause of lasting disability and the third leading cause of death in industrialized countries, responsible for 5.5 million deaths per year,[3,4] and is caused by occluded cerebral arteries. Peripheral arterial occlusive disease (PAOD) causes a severe impairment in the quality of life of affected patients, affects 8 million individuals in the US and has an increased prevalence with age,[5,6] and is caused by plaque occlusion in the arteries to the limbs, most commonly the legs (**Figure 1**). [7]

The most common symptom of PAOD is intermittent claudication, which refers to transient pain, numbness, aching, or heaviness in the leg muscles during locomotion or physical activity that is relieved by rest.[5,8,9] PAOD can develop into critical limb ischemia (CLI), a more severe form of PAD characterized by more severe atherosclerotic blockage and pain at rest, which can result in amputation. Although most patients with PAOD are asymptomatic, and only 10-35% of patients are exhibit intermittent claudication, it is important to diagnose and treat patients with PAOD since it can lead to amputation and increased risk for other ischemic diseases.[10,11]

Variation in the severity of injury following ischemic events in patients, in addition to the low percentage of patients that exhibit symptoms of PAOD suggest variation in microvascular phenotype. [12] Although intermittent claudication may be mainly due to a poorly developed collateral circulation, the fact that patients are asymptomatic at rest and experience claudication

during physical activity suggests that the vasculature fails to increase blood flow in response to an increased metabolic demand. This implies impaired vasodilation as the main cause of the symptoms of ischemia, rather than merely a lack of a collateral circulation.[11,13] This observation is supported by work in an experimental model of hindlimb ischemia that showed no difference in resting blood flow between ischemic and non-ischemic models at rest, but a severe impairment in exercise induced hyperemia between groups.[13,14]

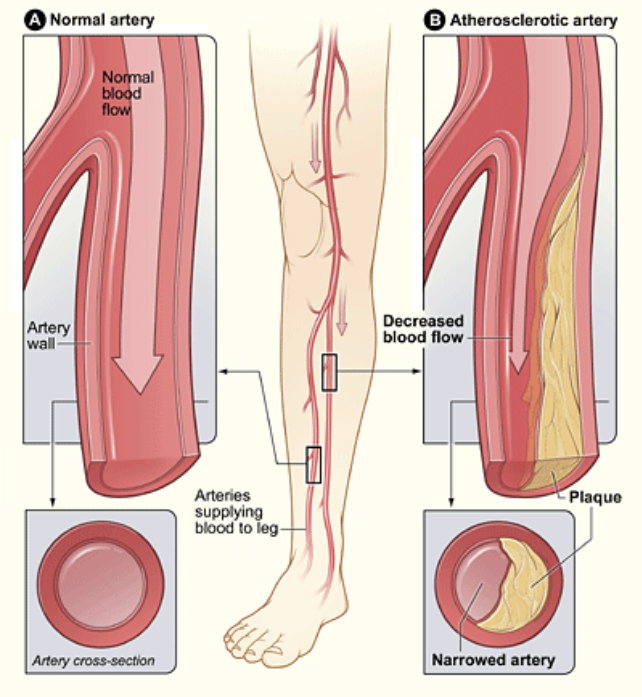


**Figure 1: Peripheral Artery Disease.** Shows an atherosclerotic plaque occluding arteries in the leg resulting in PAOD. [7]

The main risk factors for PAOD are age (over 40 years), smoking, diabetes mellitus (DM), hyperlipidemia, and hypertension. [9] Smoking and DM are the strongest risk factors and increase the risk of more severe PAOD.[15] Diabetic patients respond especially poorly to current treatments for PAOD and have higher rates of complications following endovascular or surgical intervention, resulting in lower amputation free survival.[16] This is especially problematic considering the total prevalence of DM in the US is expected to double from 2005 to 2050. This necessitates the development of new therapeutics for ischemic diseases, especially PAOD.[1]

### 1.2 Current Treatments for Peripheral Artery Disease

Treatments for PAOD attempt to slow the progression/severity of the disease by reducing the development of atherosclerotic plaques, managing symptoms, and restoring blood flow in occluded arteries. Current therapies include lifestyle modifications, pharmaceutical interventions, and endovascular/surgical procedures.

Lifestyle modifications include cessation of smoking, diet adjustment, and exercise. Exercise is one of the most effective treatments for PAOD, demonstrating efficacy superior to anti-platelet therapy and equal to percutaneous angioplasty.[17] Although exercise therapy is an effective treatment for PAOD, it is most effective in a supervised environment compared with at-home exercise programs. In addition, supervised exercise treatments are unlikely to be covered by insurance and patient experienced claudication may limit compliance with this therapy, which may limit the efficacy of exercise treatments for PAOD.[8,18]

Should lifestyle modifications be insufficient to slow the progression of PAOD, pharmaceutical interventions may be prescribed. These include anti-platelet drugs to prevent thrombosis and occlusion in atherosclerotic region and vasodilators to treat the main symptom of PAOD, claudication. The most commonly prescribed anti-platelet therapy is aspirin, but clopidogrel and ticlopidine have some efficacy as alternatives.[9,15] Another pharmacologic approach is to treat claudication with vasodilators such as Cilostazol and Pentoxifylline, both phosphodiesterase inhibitors.[15]

In patients where both lifestyle modification and pharmacological therapy are insufficient to reduce symptoms or improve quality of life, endovascular and/or surgical interventions may be required.[15] Endovascular interventions are minimally invasive and are recommended for less

severe plaque occlusions. Endovascular techniques include percutaneous transluminal angioplasty (PTA), typically the first recommended endovascular intervention, and peripheral stenting.[17] PTA may be performed with or without a stent, but stent placement reduces the chance of vessel collapse after the plaque is compressed. Initial attempts to transfer the success of coronary stenting in CHD have not yet been successful.[15] Although the placement of a stent in coronary arteries has proved effective in preventing elastic recoil associated with PTA, peripheral artery stenting is complicated due to the unique mechanical strain that these stents experience due to locomotion, which makes these stents more prone to fracture.[15] While endovascular techniques are advantageous due to the minimal invasive nature of the procedure, the requirement for local vs. general anesthesia, and faster recovery times, more severe plaques such as those seen in CLI may necessitate surgical revascularization with a bypass grafting procedure.[19] Bypass grafting is commonly performed using an autologous saphenous vein, although if the patient lacks a viable autologous conduit, artificial conduits may be used.

All treatments for PAOD are palliative, focusing on slowing the progression of the disease or providing temporary revascularization. In addition, not all patients are eligible for endovascular or surgical intervention, especially those who have many co-morbidities or especially severe occlusions. Attempts to restore distal tissue perfusion using endovascular and surgical techniques are also prone to restenosis. Due to the limited efficacy of available treatments novel therapeutic strategies need to be developed, potential strategies include stimulating the growth and reactivity of collateral arterioles to enhance blood flow.

### 1.3 Arteriogensis

Arteriogenesis is defined as the outward remodeling of pre-existing collaterals (**Figure 2)**, it is one potentially efficacious treatment for patients with PAOD. Arteriogenesis is caused by an occlusion,

commonly an atherosclerotic plaque, which decreases downstream pressure, increasing blood flow through the collateral circulation and initiating the remodeling process. Increased blood flow increases shear stress across the endothelium and activates it. Activated endothelial cells present adhesion molecules and cytokines, which recruit and activate monocytes, causing their extravasation. Extravasated macrophages secrete: matrix metalloproteinases (MMPs) to degrade the basement membrane of the pre-existing collateral, vascular endothelial growth factor (VEGF) to promote endothelial proliferation and migration, and PDGF/TNF- α to elicit vascular smooth muscle cell (VSMC) dedifferentiation to a synthetic proliferative phenotype.[4,20] This series of growth factors secreted by macrophages enable the outward remodeling of pre-existing collaterals to larger caliber vessels, a process that can minimize the severity of symptoms of ischemic disease by providing sufficient blood flow to distal tissue. Unfortunately there is significant variability in the existence of patient pre-existing collaterals.[21]

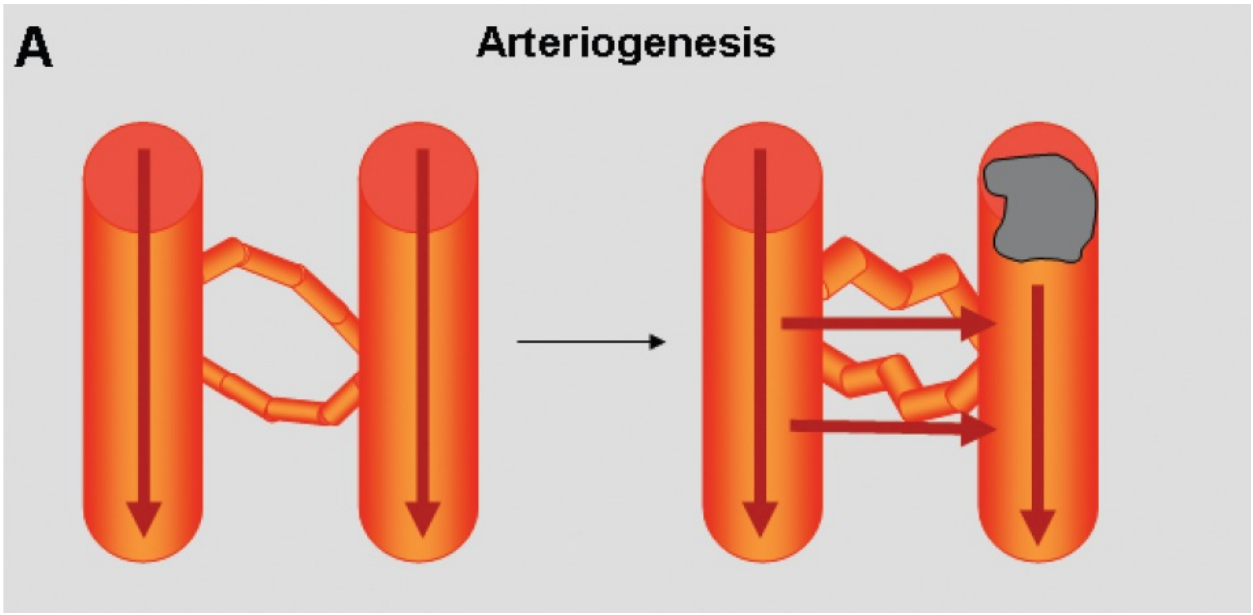


**Figure 2: Arteriogenesis.** Arteriogenesis is induced after occlusion of an artery (shown here in grey). Increased fluid shear stress, caused by redirected blood flow through pre-existing collateral anastomoses serves as the initial trigger. Growth of collaterals proceeds by remodeling of pre-existing arteriole anastomoses. Adapted from [20]

## 1.4 Collaterals Confer Ischemic Protection

Patients with collateral anastomoses that undergo arteriogenesis and bypass the occlusion sites can resupply distal tissue with blood flow.[20] Collaterals in these patients confer protection from symptoms of ischemia, and are therefore called ischemia-protected.[12] Work done in animal models shows inter-individual differences in number of pre-existing collaterals, based on genetic background, which suggests that such differences are reflected in the patient population as well.[12,20] The extremes of the two types of vascular phenotypes, ischemia-protected and ischemia vulnerable, can be easily observed in figures 3 and 4. A dog heart has a high number of pre-existing collateral anastomoses which would provide protection from ischemia in the case of occlusion, while the pig heart has a dendritic vascular network architecture that makes it more susceptible to symptoms of ischemia in the event of an occlusion (**Figure 3**).[20] A similar difference is observed in two strains of inbred mice (**Figure 4**).[12] C57 mice are similar to dog hearts in that they possess a high number of pre-existing collateral anastomoses, while Balb/c mice exhibit a dendritic phenotype that lacks pre-existing collaterals similar to the pig heart.

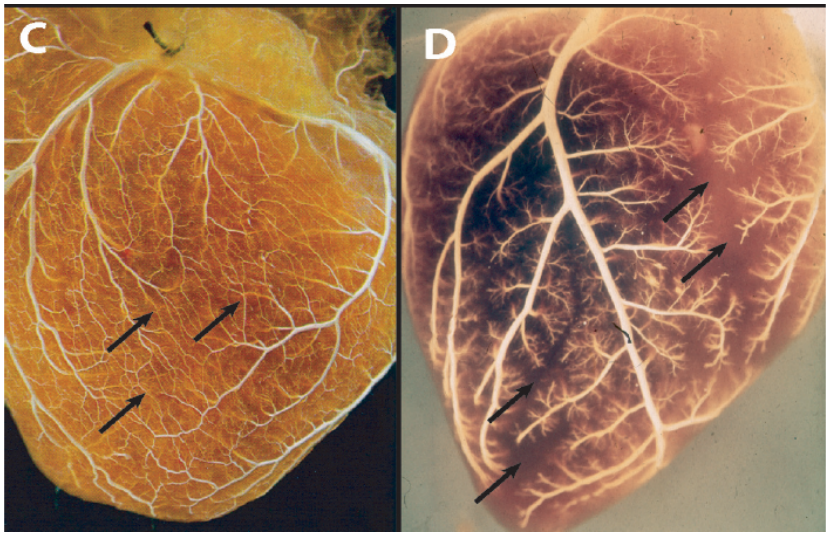


**Figure 3: Variability in pre-existing vascular architecture.** On the left, an image of a dog heart demonstrating the extent of pre-existing collateral anastomoses (black arrows)**.** On the right, an

image of a pig heart that lacks collateral anastomoses. Black arrows indicate where collaterals would be located.[20]

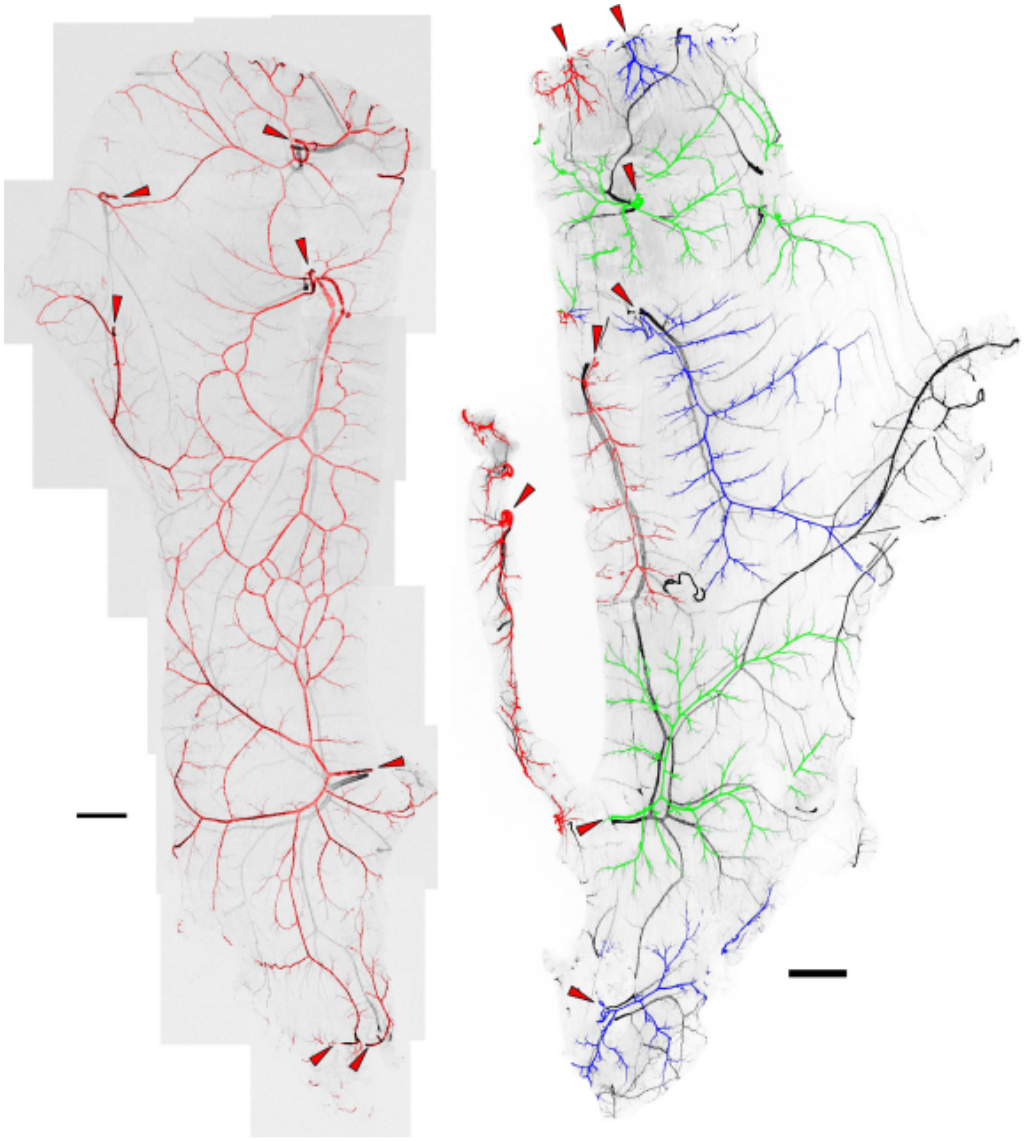

**Figure 4: Murine Spinotrapezius Vascular Phenotype.** On the left, an image of the C57 murine spinotrapezius demonstrating the ischemia-protected phenotype with many pre-existing collateral anastomoses**.** On the right, an image of the Balb/c spinotrapezius which lack pre-existing collateral anastomoses.[12]

Differences in vascular phenotype reflect differences in the severity of symptoms experienced in experimental models of ischemia.[12] Combined with the observation of varying severity of symptoms following ischemic events in human patients, suggests inter-individual differences in the degree of

pre-existing collateral anastomoses.[12] This makes stimulating the growth of new collateral anastomoses a potentially valuable and novel therapeutic avenue, which could improve the prognosis for patients with ischemic vascular diseases.

### 1.5 Objective

An arteriolar surgical ligation model was previously developed in the murine spinotrapezius, which is very thin, flat, easily accessible, and highly vascularized muscle.[12,22] Previous studies demonstrated the formation of arterialized capillaries in response to arteriolar ligation, but the vascular reactivity of these vessels is unknown.[12,22] The aims of this study are as follows:

1. Develop an intravital microscopy protocol with functional vasodilation to assess the reactivity of small caliber vessels in the murine spinotrapezius
2. Assess the reactivity of arterialized capillaries in the murine spinotrapzius, seven days following arteriolar ligation, to determine if these vessels are capable of regulating blood. It was hypothesized that day seven arterialized capillary functional vasodilation will be impaired compared similarly-sized control arterioles.

## CHAPTER 2: Methods

### 2.1 Animal Husbandry

Experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of California Polytechnic State University. Male Balb/c (Harlan) mice were maintained in a temperature controlled room with a 12 hour light/dark cycle in microisolater cages containing enrichment with ad libitum access to feed and water.

### 2.2 Spinotrapezius Ligation Surgery

Chronic ischemia was induced in the murine spinotrapzius as previously described.[12,22,23] Briefly, instruments and consumable materials were sterilized and the surgical area was disinfected using chlorohexidine diacetate. Mice were anesthisized with 1-3% isoflurane gas at a flow rate of 0.8-1. 2 $l \cdot min^{-1}$. Hair on the anterior dorsal aspect of the animal was removed using depilatory cream and the incision site was sanitized using chlorohexidine diacetate. Once the animal was placed on the surgical stage with a heating pad, a rectal thermistor probe was inserted to maintain rectal temperature at 35°C. A straight incision (~5 mm) was made about 5 mm caudal to the bony prominence of the shoulder blade, lateral to and parallel to the spine where the fat pad meets the spinotrapezius. Under a dissecting microscope the spinotrapezius was identified and separated from the overlying fat pad. It was then reflected back to revel the lateral feed artery-vein pair (**Figure 5**). Forceps (#7 FST fine curved) were then used to separate the vein from the artery. Two ligatures of silk suture were placed around the separated artery, and the artery was transected between them. Skin incisions were closed with 6-0 polypropylene suture. Sham surgeries were performed on the contralateral muscle, and involved an identical incision and blunt dissection to identify the ligation site. Immediately before and after surgery, animals were given buprenorphine analgesic (0.075 $mg \cdot kg^{-1}$) subcutaneously and observed until ambulatory.

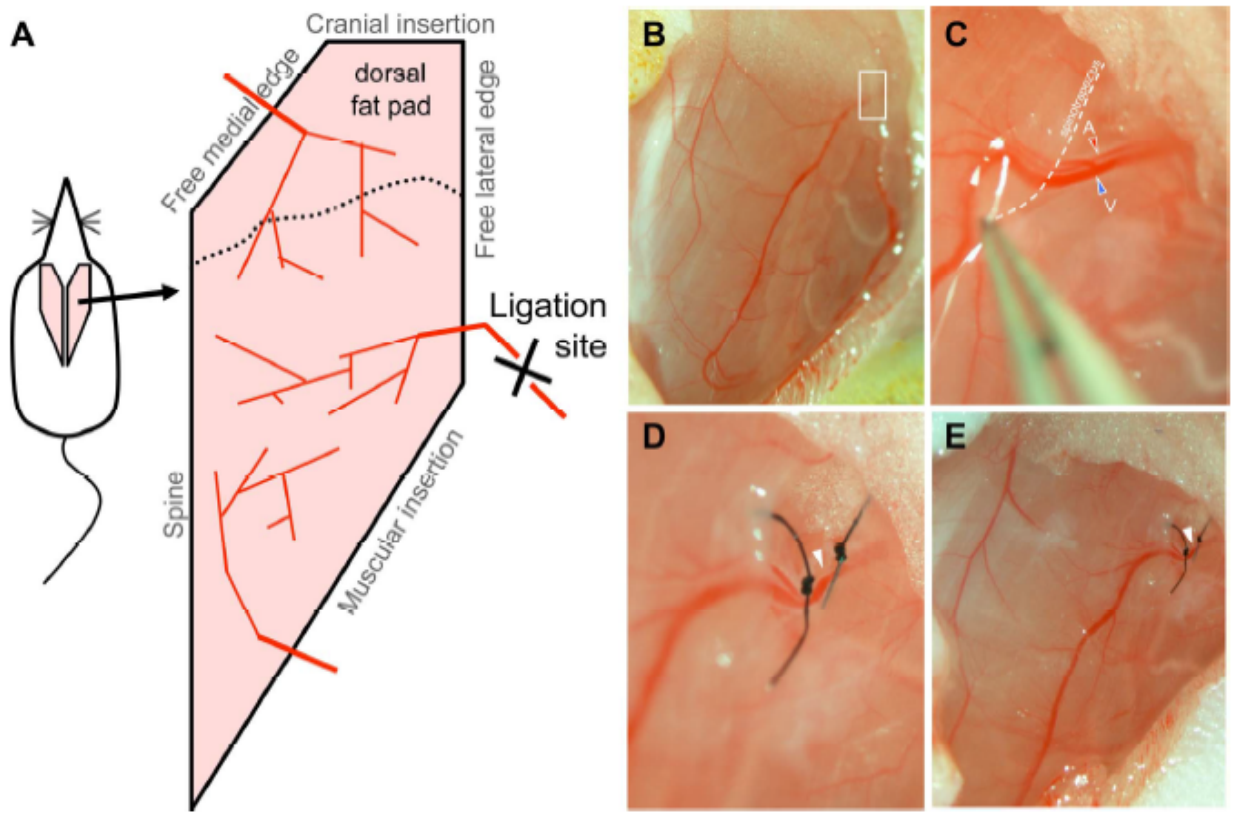


**Figure 5: Arteriolar Ligation Procedure. A)** Schematic of the spinotrapezius**.** White dotted line indicates edge of the dorsal fat pad, which is retracted to expose the target ligation site. **B)** Intravital image of murine spinotrapezius, identical orientation to A. **C)** Close-up of target ligation site, artery and vein pair. **D)** Having separated the artery from the vein, arteriole was then ligated twice and transected between the ligatures. **D)** Ligature placement in relation to entire muscle. Adapted from [12]

### 2.3 Spinotrapezius Intravital Microscopy with Functional Vasodilation

A protocol was developed to assess endogenous vasodilation, using an intravital microscopy protocol with functional vasodilation. Seven days after arteriolar ligation this protocol was used to assess the vascular reactivity of arterialized capillaries.

The protocol used side-stream dark field microscopy (SDF) (Microscan) to visualize in vivo vessel architecture. Two tungsten microelectrodes were used to create field stimulation to elicit contraction of the spinotrapezius muscle, and metabolic vasodilation. To perform field

stimulation, the entire spinotrapezius was exposed, overlying facia was gently removed and tungsten microelectrodes were placed lateral to the spine at the caudal end of muscle (Figure 6).

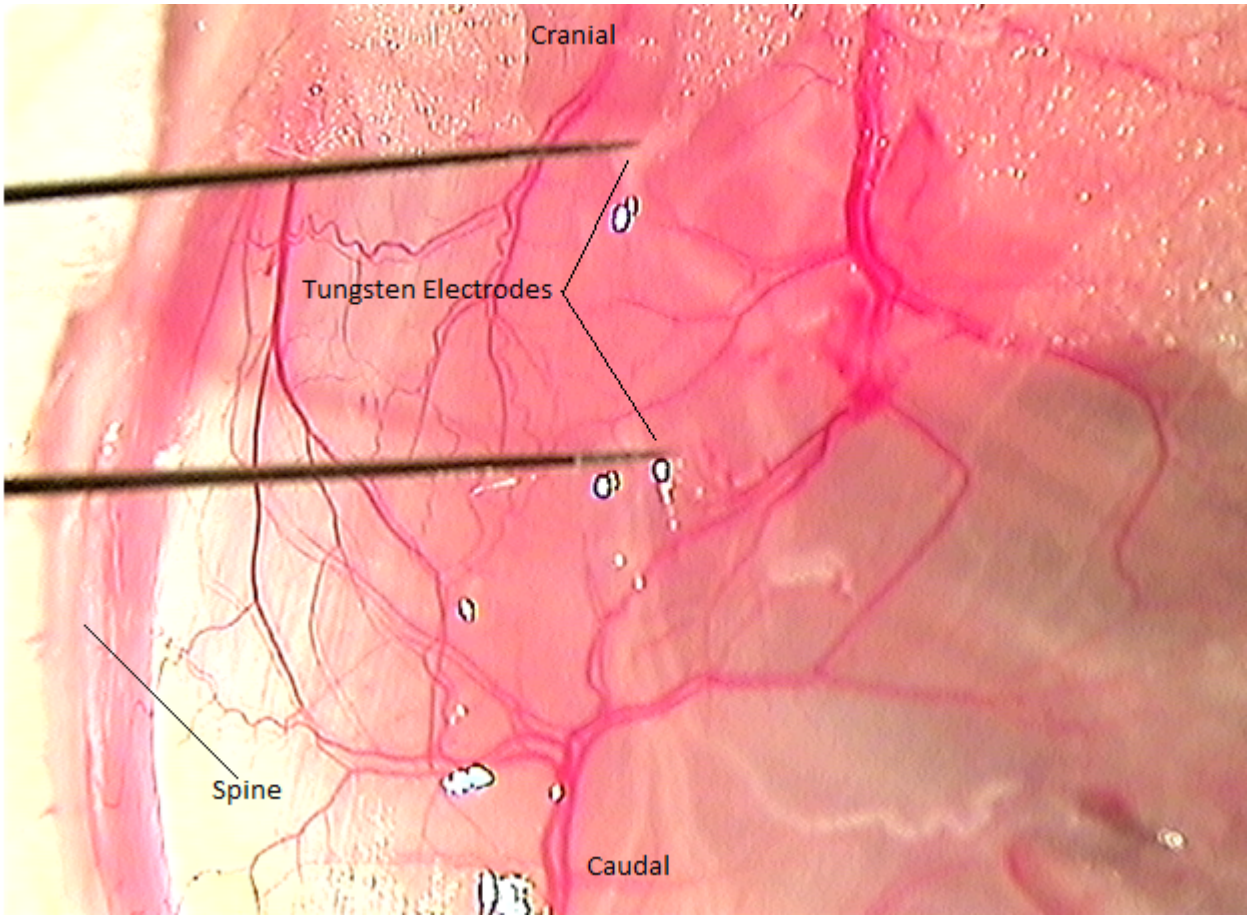


**Figure 6: Spinotrapezius Field Stimulation.** Demonstrates placement of tungsten microelectrodes as outlined in text above. Application of stimulating current results in robust muscle contraction which causes metabolic vasodilation.

A short test stimulation (1Hz, 200 µs, 2mA) was applied using LabChart Software and a PowerLab Data Acquisition System (ADInstrument) to verify maximal muscle contraction after initial placement of the electrodes. Following verification, plastic wrap was placed over the muscle to minimize oxygen diffusion and prevent dessication. The intravital miscroscope was placed over the spinotrapezius and phosphate buffered saline (PBS) was applied directly between the plastic wrap the intravital 'water'-immersion microscope lens. Arterialized capillaries were

identified in the “watershed” area fed by the ligated artery by visually following the ligated arteriole caudally and identifying the area between adjacent watershed regions (Figure 7). Arterialized capillaries were selected by identifying an arteriole in this area that runs parallel to muscle fibers and connects two adjacent watersheds (Figure 8). After an arterialized capillary was selected the intravital microscope was positioned over the vessel and the preparation was given 30 minutes to equilibrate, at which point a video was captured to measure the baseline diameter of arterialized capillaries. LabChart was then used to contract the spinotrapezius for 90 seconds (8Hz, 200 μs, 2mA); a video was captured immediately after stimulation and every minute after until the vessel returned to its resting diameter. A similar procedure was followed in the sham-operated contralateral muscle, but instead of an arterialized capillary, a similarly sized terminal arteriole in the contralateral muscle was imaged to serve as the control. Terminal arterioles in the contralateral muscle were identified by following the similar feed artery branch as was ligated in the operated muscle until an arteriole was identified that branched into capillaries.

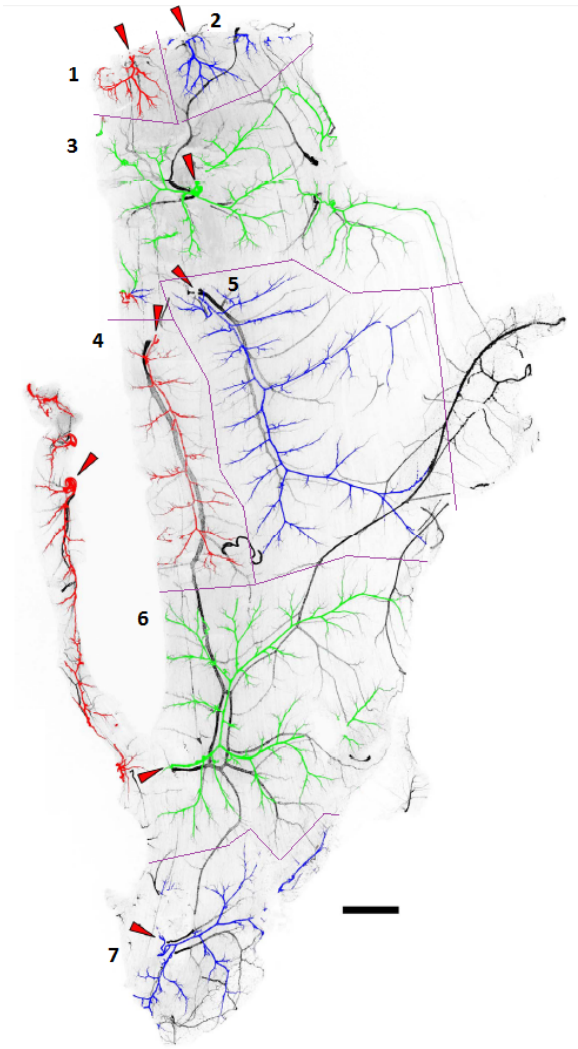


**Figure 7: Watersheds in the Balb/c Spinotrapezius** Arterioles have been pseudocolored in red, green, and blue; venules in grey. Arterioles entering the tissue are indicated by red arrowheads. Scale bar, 1mm. Watershed areas are indicated by numbers and outlined with purple lines. Adopted from [12].

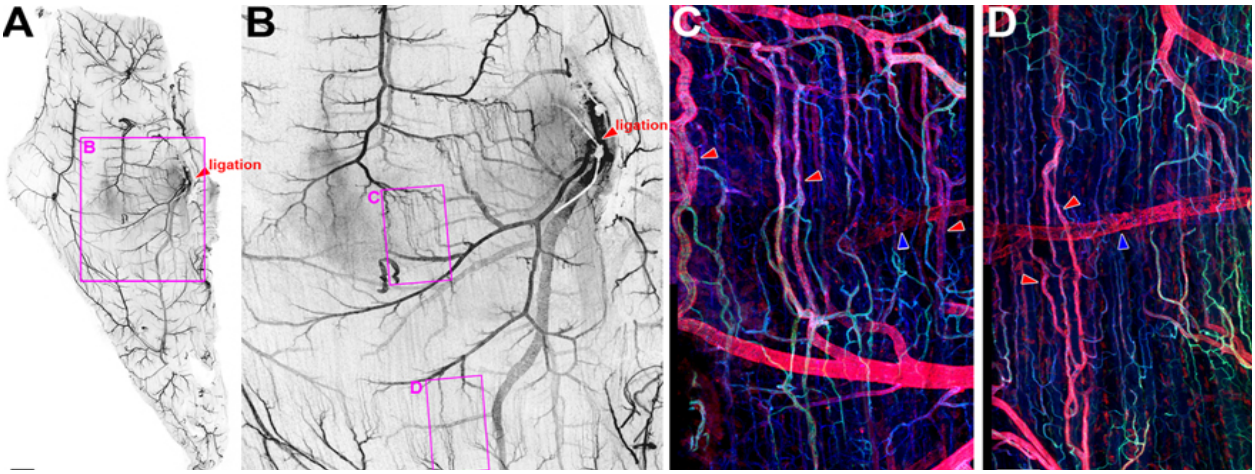


**Figure 8: Identification of Arterialized Capillaries** . Balb/c spinotrapezius muscles show the presence of arterialized capillaries that are connecting the targeted (ligated) arteriolar tree to a neighboring, perfused network, seven days after ligation. Montage of smooth muscle α-actin images. Ligation site indicated by red arrowhead. **C and D,** Close-up of the arteriolar structures in B. Red, smooth muscle α-actin; green, perfused lectin; blue, superfused lectin. Red arrowheads, arterialized capillaries; blue arrowheads, veins. Scale bars, 100µm [12]

### 2.4 Statistics

Diameter measurements were made using automated vascular analysis (AVA) software. Diameters before and immediately after muscle stimulation were measured and the percent change was calculated, which indicated the vasodilation response.

An independent t-test was used to determine statistical significance between pre and post stimulation measurements for both the ischemic and sham procedures, and between arterialized capillaries and sham operated muscle terminal arterioles. A p-value less than 0.05 indicated statistical significance. Data are presented as the mean ± SE.

## CHAPTER 3: Results

### 3.1 Protocol Development – Intravital Microscopy with Functional Vasodilation

The first objective was to develop an intravital microscopy protocol with functional vasodilation to assess the reactivity of small caliber vessels in the murine spinotrapezius. Field stimulation at the caudal end of the muscle was verified to cause muscle contraction. The resting diameter of terminal arterioles was found to be 8.0 ± 0.9 μm and 15.2 ± 4.0 μm post 90s muscle contraction (8Hz, 200 μs, 2mA); resulting in a percent change of 88 ± 31%. There was a significant increase in post stimulation diameter (Figure 9).

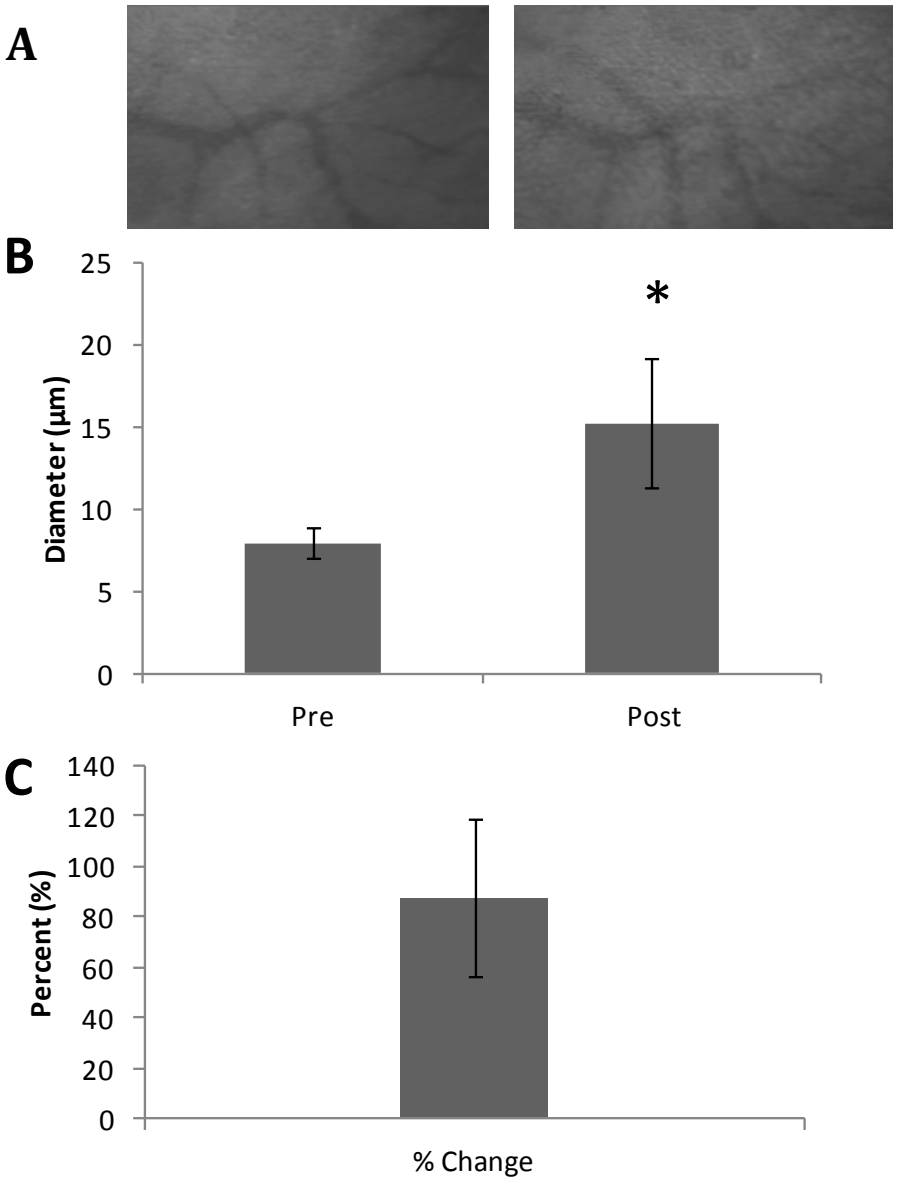


**Figure 9: Pilot study of Intravital Microscopy Functional Vasodilation in the Murine Spinotrapezius.** A) Representative images of small arterioles imaged and Analyzed using an intravital microscope and AVA analysis software. B) Diameter in microns of arterioles pre and post 90s muscle stimulation, measured using AVA analysis software. C) Percent change of arterioles. n=6. * indicates $p<0.05$.

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### 3.2 Vascular Reactivity of Arterialized Capillaries

The second objective was to assess the reactivity of arterialized capillaries in the Balb/c spinotrapzius, seven days following arteriolar ligation to determine if these vessels are capable of regulating blood flow. It was hypothesized that day seven arterialized capillary functional vasodilation would be impaired compared similarly sized arterioles. The average resting diameter of arterialized capillaries measured was 8.0 ± 0.7 μm and after muscle contraction was 8.2 ± 1.0 μm; the percent change was 2 ± 1.5 %. The average diameter of terminal arterioles in the sham operated muscle was 8.9 ± 0.6 μm and after muscle contraction it was 11.0 ± 0.9 μm giving a percent change of 29 ± 11%. (Figure 10)

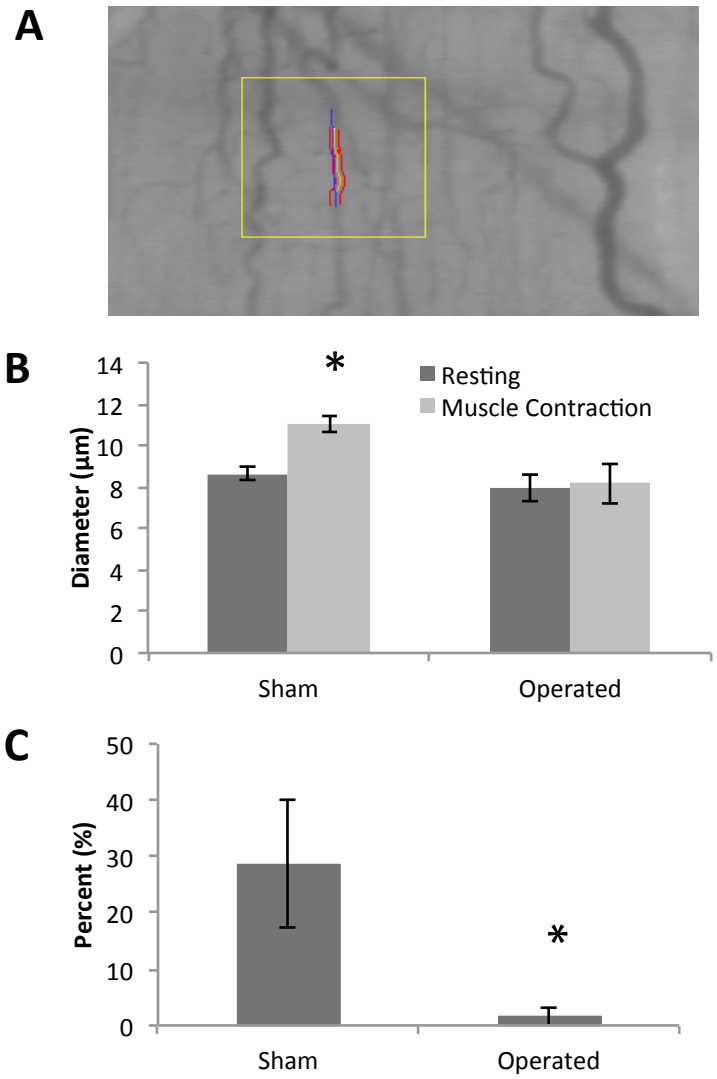


**Figure 10 Vascular Reactivity of Day 7 Arterialized Capillaries**. A) Representative image of arterialized capillary images and measured using microscan microscope and AVA anlysis. B) Diameter in microns of terminal arterioles in sham operated animals and arterialized capillaries in operated animals pre and post 90s muscle contraction. C) Percent change of vessels. n=5 ; * indicates p<.05 t-test

## CHAPTER 4: Discussion

### 4.1 Protocol Development – Intravital Microscopy with Functional Vasodilation

Arterioles in the spinotrapezius exhibited a significant increase in diameter in response to muscle contraction. Terminal arterioles (measuring 5-11 μm) dilated 88 ± 31% in response to muscle contraction caused by field stimulation with tungsten microelectrodes at 8 Hz, 200 μs, 2mA. Xiang et al found a similar functional vasodilation response (72 ± 1%) in exteriorized rat spinotrapezius, third order arterioles, (9-15 μm) stimulated (4-5 V, 1Hz, 2min) with 2 hooked silver-silver chloride electrodes placed at either end of the rat (Sprague-Dawley) spinotrapzezius muscle.24 Although Xiang et al surgically exteriorized the spinotrapezius for intravital measurements, Bailey et al demonstrated that there is no difference in spinotrapezius muscle microcirculatory function due to surgical exteriorization and this work confirms this finding.25 Lash et al used a similar surgical exteriorization and functional vasodilation stimulation protocol in the rat spinotrapezius and observed similar reactivates in 3A arterioles (14 ± 0.7 μm) .26 They measured the functional vasodilation in 3A arterioles at 8V and 0.2 ms duration, at a range of frequencies from 1 Hz to 8 Hz. They observed approximately 20%, 60%, 110%, 170% dilations at 1 Hz, 2 Hz, 4 Hz, and 8 Hz respectively. The stimulation frequency used in our work was 8 Hz, which was selected to elicit a maximal contraction. The percent vasodilation observed in terminal arterioles in our study was slightly lower than that of 3A arterioles in the study performed by Lash et al. The results observed in this study correlate more closely with those seen in the study performed by Xiang et al showing a 72 ± 1% dilation in response to muscle contraction in the rat spinotrapezius, although their stimulation parameters were 4-5V at 1 Hz.

Overall our results correlate well with similar protocols that examine the functional vasodilation of arterioles in skeletal muscle. Slight variations in the stimulation parameters may account for

the slight variability in the percent vasodilation observed in terminal arterioles compared to other studies. Our protocol demonstrates that field stimulation of the spinotrapezius results in robust contraction of the muscle and ensuing vasodilation.

**4.2 Vascular Reactivity of Arterialized Capillaries**

The protocol developed to assess the reactivity of terminal arterioles in the murine spinotrapezius described above, was then applied towards assessing the reactivity of arterialized capillaries seven days following arteriolar ligation. Arterialized capillaries 7.9 ± 0.3 μm in diameter dilated 2.3 ± 1.5% while terminal arterioles in the contralateral muscle 8.6 ± 0.6 μm in diameter dilated 28 ± 11%. Terminal arterioles dilated significantly in response to muscle contraction caused by field stimulation with electrodes, similar to results from experiments conducted during protocol development. However, this dilation was much lower than that observed during the protocol development, 28 ± 11% vs. 88 ± 31%. This may have been due damage to the feed artery during the sham operation on the contralateral muscle. A very thorough sham surgery was performed in the contralateral spinotrapezius that involved every aspect of the actual surgical ligation except for the actual ligation of the feed artery, including threading the silk suture under the feed artery to be ligated. The surgeon performing the feed artery ligations was relatively inexperienced and may have damaged the feed artery, resulting in altered blood flow leading to remodeling and thus impaired vasodilation.

Most interestingly however was the significant impairment in vasodilation in arterialized capillaries compared to that of terminal arterioles in the contralateral spinotrapezius. Capillary arterialization occurs when capillaries recruit smooth muscle cells, and is thought to occur for a diverse set of reasons including normal growth and maturation, skeletal muscle stretch, hemodynamic changes, electrical stimulation, and exercise training.27 In the case of these

experiments, pre-existing capillary collaterals experience increased hemodynamic flow in response to arteriolar ligation, and resupply blood flow to the ligated watershed through the recruitment of smooth muscle cells and the formation of arterialized capillaries. Multiple hypotheses exist to explain the recruitment of smooth muscle cells to form arterialized capillaries including: pericytes, which cover the abluminal side of capillaries and may be vascular smooth muscle precursors, smooth muscle cells on upstream arterioles that may proliferate and migrate toward soon to be arterialized capillaries, or smooth muscle cells that may differentiate from a yet unidentified precursor in the parenchyma.28 The observation that in response to exogenous thrombin delivery, which enhances and accelerates capillary arterialization, smooth muscle cell coverage occurs in patches, suggests that the smooth muscle cell recruitment is not due to proliferation and migration in a linear fashion.29 An additional hypothesis to the above-mentioned sources of smooth muscle cell recruitment in arterialized capillaries, is an endothelial to mesenchymal transition (EMT), which may occur in endothelial cells and has been demonstrated in vitro.30 Additionally, circulating myeloid progenitor cells may differentiate into smooth muscle cells.31 It is not known how smooth muscle cells are recruited to form arterialized capillaries, but it may be that the smooth muscle cells that are recruited in a synthetic, proliferative phenotype that lack contractile proteins in favor of more subcellular organelles involved in protein synthesis.32 The observed impairment in vasodilation could be explained by synthetic smooth muscle cells lining the surface of the newly-formed arterialized capillaries.

### 4.3 Future Work

Future work will focus on determining the cause of impaired vasodilation in arterialized capillaries at day-7 following arterial ligation. One possible explanation for the decreased

dilation capacity could be the immaturity of newly recruited smooth muscle cells. Smooth muscle cells regulate arteriole diameter through their contractile state and it is thought that during outward remodeling smooth muscle cells dedifferentiate from a contractile phenotype into a proliferative synthetic state. Synthetic smooth muscle cells lack the contractile machinery to regulate their contractile state and therefore are unable to regulate vessel diameter. It is our hypothesis that the smooth muscle cells that form arterialized capillaries are in a synthetic state and are therefore unable to regulate their diameter in response to endogenous pathways for vasodilation.

It would therefore be useful to examine cellular-specific vasodilation pathways by assessing the reactivity of arterialized capillaries through the application of pharmacological agents to the experimental preparation.33 Specifically, the smooth muscle- and endothelial-dependent vasodilation pathways could be examined using sodium nitroprusside (SNP) and acetylcholine, respectively. Additionally, time course experiments could be performed to determine if synthetic smooth muscle cells return to a contractile phenotype over time; the functional vasodilation protocol used in this study could be used at later time points. Pharmacological superfusion of vasoactive drugs could also be performed in a time course fashion to determine cell-dependent vasodilation responses.

Stimulating the growth of arterialized capillaries could be a novel and efficacious treatment for patients with limited therapeutic options to treat ischemic diseases. FUNCTIONCurrent evidence suggests that the worst symptoms of ischemic diseases are due to impaired vasodilation, not just a lack of natural collateral anastomoses. Therefore it is important to not only understand how to stimulate the growth of arterialized capillaries, but determine why their ability to regulate blood flow is impaired. If we can understand how to stimulate the growth of reactive arterialized

capillaries, this could be developed into a potentially efficacious new treatment for ischemic disease.

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## APPENDIX

Spinotrapezius Ligation Protocol

Date _____ Spinotrapezius Ligation Initials _____

**Mouse Information**
DOB: _____
Sex: _____
Tag: _____
Genotype/strain: _____
Cage: _____
Weight: _____

**Materials**
Instruments
____1. Standard Pattern Forceps (1)
____2. Iris Scissors (1)
____3. S & T (2)
____4. 5/45 (1)
____5. Dumont #7's (1)
____6. Microdissection scissors (1)
____7. Needle Holders (1)

Pre-sterilize in autoclave
____8. Cotton gauze (4)
____9. Cotton swabs (4)
____10. 6.0 silk suture (2x1 in)
____11. Surgical Drape

Obtained in surgery suite
____12. Petri dish w/ sterile saline
____13. Gloves
____14. Sterile 7-0 prolene suture
____15. FST heat pad w/ rectal probe
____16. heat pad
____17. Recovery bin and weigh boat
____18. Depilatory cream
____19. non-sterile cotton swabs
____20. non-sterile cotton gauze
____21. Isolation mask & cap
____22. Analgesic (buprenorphine)

**Surgery Preparation**
____23. Spray surgery area with Nolvasan
____24. Weigh animal in weight boat
____25. Place animal in anesthesia box
____26. Open the oxygen cylinder and set anesthesia-machine flow meter to ~3 $l \cdot min^{-1}$
____27. Anesthetize animal w/ 5% isoflurane
____28. Reduce flow rate to 0.5-1.0 $l \cdot min^{-1}$ and the isoflurane to 1-3%
____29. Lay animal prone with nose in nose-cone
____30. Remove hair on anterior dorsal aspect of the animal with clippers and depilatory cream.
____31. Apply vetinary ointment to eyes to avoud drying during the procedure
____32. Give the animal subcutaneous injection of buprenorphine
____33. Lay animal prone on circulating heat pad (w/ 4x4 on top) w/ nose in nose-cone
____34. Insert rectal probe and set thermo-controller to 37°C
____35. Insert rectal probe and set thermo-controller to 35 ° C
____36. Adjust focus, lighting, and center mouse
____37. Don isolation mask, bonnet, and scrubs
____38. Open sterile surgical instrument pack
____39. Open sterile pack and dump in surgical instrument field
____40. Place sterile petri dish in surgical field and fill with sterile saline
____41. Don sterile gloves
____42. Transfer the content of sterile pack to surgical instrument sterile field (leave 1 4x4 on bench for non-sterile hands)

**Surgery**
____43. Make 5mm incision parallel to spine at intersection of fat pad and muscle
____44. Dissect skin overlying the spinotrapezius muscle
____45. Search for lateral feed artery vein pair jumping from the underlying fat pad to the spinotrapezius
____46. Blunt dissect fat pad to reveal arteriole-venule pair, using lidocaine as a vasodilator to aid in visualization
____47. Blunt dissect arteriole away from venule
____48. Pass a separated strand from 6-0 silk suture below the arteriole and tie off
____49. Repeat again about 1mm above previous ligature
____50. Cut arteriole between the two ligatures with micro dissection scissors
____51. Suture incision with 7-0 prolene suture
____52. Repeat for contralateral side to perform sham, but do not tie off ligature

**Post-Surgical**
____53. Give the animal a subcutaneous injection of buprenorphine
____54. Place the animal in the recovery bin, on a blue bench cover, above a heat pad and allow to recover
____55. Turn the flow meter down to 0, turn off the isoflurane, and close the oxygen cylinder
____56. Indicate surgery on cage card.

**Notes**
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Spinotrapezius Intravital Microscopy with Functional Vasodilation Protocol

Date_____ Spinotrapezius Intravital Microscopy Initials_____

**Mouse Information**
DOB: _____
Sex: _____
Tag: _____
Genotype/strain:_____
Cage: _____
Weight: _____

**Materials**
Instruments
____1. forceps (2)
____2. fine forceps (2)
____3. ultrafine forceps (1)
____4. fine scissors (1)

Obtained in surgery suite
____5. Petri dish w/ sterile saline
____6. gloves
____7. FST heat pad w/ rectal probe
____8. heat pad
____9. non-sterile cotton swabs
____10. non-sterile cotton gauze

**Surgery Preparation**
____11. Weigh animal in weight boat
____12. Place animal in anesthesia box
____13. Open the oxygen cylinder and set anesthesia-machine flow meter to ~3 $l \cdot min^{-1}$
____14. Anesthetize animal w/ 5% isoflurane
____15. Reduce flow rate to 0.5-1.0 $l \cdot min^{-1}$ and the isoflurane to 1-3%
____16. Lay animal prone with nose in nose-cone
____17. Remove hair on anterior dorsal aspect of the animal with clippers and depilatory cream.
____18. Lay animal prone on circulating heat pad w/ nose in nose-cone
____19. Insert rectal probe and set thermo-controller to 37°C
____20. Apply veterinary ointment to eyes to avoid drying during procedure

**Protocol**
____21. Make an incision (1cm) at the caudal end of the spinotrapezius
____22. Extend the incision cranially to the fat pad, creating a horse shoe incision
____23. Blunt dissect the subcutaneous connective tissue to maximize surgical exposure
____24. Place electrodes as close together as possible in clay and place electrodes lateral to the spine, at the caudal end of the muscle
____25. Stimulate the muscle at frequency 1 Hz, duration 200 us, and 2 mA to ensure electrode placement stimulates the muscle.
____26. Place saran wrap over the exposed muscle and skin flap

**Microscan**
____27. Place microscan in stand and ensure proper connectivity to computer
____28. Open AVA instruments and create a new folder containing the date of the procedure and make this the directory save location
____29. Select capture on AVA main menu for microscan imaging
____30. Label patient I.D. with the number of patient first followed by mus for experimental muscle or sham for control
____31. Locate arterialized capillaries by following the ligated branch downstream and adjust the microscan for best resolution
____32. Allow a 30 minute time period to pass between test stimulation and taking the first measurement
____33. Capture video file of arterialized capillary to measure resting diameter
____34. Set frequency to 8 Hz and keep other setting s the same in Labchart
____35. Stimulate the muscle for 90s
____36. Immediately capture video and continue to capture every minute until the vessel has returned to resting diameter
____37. Perform the same procedure on the control limb

**Analysis**
____38. Open analysis section in AVA and open file of interest
____39. Pick a relatively stable period of collection and stabilize file for measurement
____40. Analyze vessel diameter by manually drawing diameter and chaining sections together
____41. Record results in provided table

**Post-Surgical**
____42. Cervical dislocation to euthanize animal

**Notes**
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