Changes in Conduit Artery Blood Flow and Diameter Post Blood Flow Restriction

by

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A thesis

presented in the University of Waterloo

in fulfillment of the

thesis requirement for the degree of

Master of Science

in

Kinesiology

Waterloo, Ontario, Canada, 2011

Authors Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Abstract

Flow mediated dilation (FMD) is a non-invasive test that assesses endothelial health and nitric oxide bioavailability; it is commonly used to examine changes in vascular health due to disease or de-conditioning. Currently, a wide variety of protocols are being used to assess upper and lower extremity conduit artery health. The current project was embarked upon to gain a better understanding of the FMD protocols currently being used to asses conduit artery FMD and how these results impact our understanding of a participant's vascular health. More specifically occlusion duration, cuff placement and artery location were examined in three commonly examined conduit arteries. The FMD responses in the brachial artery (BA), superficial femoral artery (SFA), and popliteal artery (PA) of ten healthy men, mean age of 27 ± 4, after five and/or two-minutes of distal occlusion were examined. When the two-minute protocol was performed on the SFA and PA, low-resistance static calf exercise was added to augment the shear stimulus. It was hypothesized that percent FMD and shear stress responses of the SFA and PA would not be significantly different after five-minutes of occlusion, thereby allowing leg conduit artery FMD to be performed on either artery. It was further hypothesized that there would be no significant differences between the shear stress and percent FMD responses of the leg conduit arteries after five or two-minutes of occlusion; inferring that shorter occlusion durations when combines with ischemic muscle contractions can be used to assess SFA or PA FMD. With regards to comparisons between arm and leg conduit arteries, it was hypothesized that there would be significant between limb differences in baseline diameter, FMD and shear stress post five-minutes of distal occlusion. These differences will be used to better understand the effects of artery location and size on conduit artery FMD

responses. Limitations with the traditional edge-detection method of determining arterial diameter prompted the creation of a new method of measuring artery diameter, the center-based method. It was hypothesized that there would be no significant differences in the percent FMD and time to FMD after five-minutes of BA occlusion (n=7). The results of the current study demonstrated that five-minutes of calf occlusion elicited a significant PA FMD but not a significant SFA FMD. FMD post two-minutes of PA occlusion with exercise was not significantly different than that produced by five-minutes of occlusion. Conversely, two-minutes of calf occlusion with exercise was unable to elicit a SFA FMD response. Significant differences in shear stress and FMD were reported between arm and leg conduit arteries, demonstrating different responses to five-minutes of distal occlusion due to artery size and location. Finally, no significant differences were noted between FMD and time to FMD when the center-based or edge-detection method was used. This study has demonstrated that the calf occlusion protocol was unable to elicit a FMD response in the SFA FMD; this occlusion location is only able to elicit a PA FMD response. Furthermore, two-minutes of occlusion with one-minute of exercise can be used in place of the five-minute protocol to examine PA FMD but not SFA FMD. Differences between arm and leg conduit arteries are noted and it has been suggested that this is likely due to leg conduit artery adaptations to gravity. Lastly, preliminary data suggest that the center-based method is an appropriate method of measuring conduit artery diameter.

Acknowledgment

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List of Abbreviations

Ach	Acetylcholine
BA	Brachial artery
BP	Blood pressure
CAD	Coronary artery disease
CVD	Cardiovascular disease
EC	Endothelial cells
EDRF	Endothelial derived relaxation factor
eNOS	Endothelial nitric oxide synthase
FMD	Flow mediated dilation
GCX	Glycocalyx
L-NMMA	L-NG-monomethyl arginine citrate
MAP	Mean arterial pressure
MBF	Mean blood flow
MBV	Mean blood flow velocity
NO	Nitric oxide
PA	Popliteal artery
Q	Cardiac output
RA	Radial artery
RH	Reactive hyperemia
RTF	Return to flow
SFA	Superficial femoral artery
SNA	Sympathetic nerve activity
SNS	Sympathetic nervous system
SS_{AUC}	Shear stress area under the curve

Chapter 1: Introduction

Changes in arterial structure and function occur with aging, cardiovascular disease, spinal cord injury (67) and long-term microgravity exposure (9). Vascular health can be examined non-invasively in large conduit arteries such as the brachial artery (BA), superficial femoral artery (SFA) and popliteal artery (PA). These conduit vessels are primarily responsible for supplying underlying tissue with blood, oxygen and nutrients. These vessels show significant decreases in vascular tone regulation when one of the above-mentioned conditions is present. Assessing the arterial health of an individual is a good indicator of their overall cardiovascular health, because individuals with coronary artery disease or its associated risk factors have dramatic impairment of peripheral artery functioning (13).

All of a body's arteries, from large conduit arteries to small capillaries, are lined with a specialized layer of cells called endothelial cells (ECs). Furchgott & Zawadzki discovered the importance of the endothelium in regulating vascular homeostasis(23). It is now known that the endothelium is a large paracrine organ that not only plays an important role in vascular tone regulation but also helps regulate cell growth, inflammation and thrombosis (26). When blood flows past arterial walls it exerts a frictional force upon the walls termed shear stress. An increase in flow, and therefore shear stress, leads to the release of one or more endothelial derived relaxation factors (EDRF), such as nitric oxide (NO), which will induce vasodilatation. However, without a functional endothelium, alterations in diameter due to changes in flow will not occur. NO bioavailability is commonly measured as it has been reported to decrease with age and various disease states, such as cardiovascular disease. Once secreted, NO diffuses out of EC's and into vascular smooth muscle cells where it induces vasodilatation (68).

In a healthy individual, increases in blood flow are coupled with an increase in NO production leading to vasodilatation. This response can be examined in the laboratory with a flow-mediated dilation (FMD) test. This test measures changes in vessel diameter following an increase in blood flow; this response is significantly reduced or eliminated without a healthy and intact endothelium(72). Short duration occlusions of the RA, BA, SFA and PA produce a FMD response that is NO mediated (32; 36; 44). The magnitude of a FMD response can indicate endothelial function and NO bioavailability, which are both important predictors of cardiovascular health. A BA FMD response of less than 4% has been observed in patients with CVD and its associated risk factors(13). Research has demonstrated that endothelial impairment in the peripheral circulation occurs alongside coronary endothelial changes, suggesting that endothelial dysfunction is a systemic event. Therefore, using FMD to determine peripheral artery function is an appropriate surrogate marker of cardiac health(4).

Since the FMD protocol was first describe by Celermajer and colleagues in 1992 this test has become a common method of studying endothelial function; however, inconsistencies in study design and protocols have made comparisons of available literature difficult (Appendix 5: Table 6). Different research groups use different arteries, occlusion durations, pressures and cuff positions, among other variables. In 2002, Corretti and colleagues created a set of guidelines for examining BA endothelial function and NO bioavailability (14). This document, however, only provides guidance for conducting a FMD test on the BA and not other conduit arteries. The current study examined FMD responses of three conduit arteries the brachial, the superficial femoral and the popliteal, in order to better understand the role of baseline diameter and artery location on conduit endothelial responsiveness to distal occlusion. Furthermore, the two leg conduit arteries were subject to a five and two-minute occlusion in order to identify the most

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appropriate leg conduit artery, occlusion protocol and duration for evaluating leg endothelial function.

The subsequent chapter reviews the current literature and will examine the basic biology behind the FMD protocol, highlight the stimulus characteristics behind different FMD protocols and will compare the FMD protocols performed on different limbs. Lastly, the following section will discuss certain methodological issues that need to be taken into account when designing a FMD experiment. This review will form the basis for the hypotheses developed in chapter 2.9

Chapter 2: Literature Review

2.1: Vascular Structure and Function

The control of vascular tone and blood flow involves a complex integration of neural, physical and chemical stimuli. Appropriate arterial tone is required to maintain and regulate vascular homeostasis, which is essential to maintain blood pressure and meet the body's oxygen demands. Regulation of vascular tone is achieved by balancing arterial vasoconstriction and vasodilatation. Vasoconstriction can be increased due to elevated sympathetic nerve activity, the presence of vasoconstrictor autacoids or alterations in myogenic tone. Conversely, vasodilatation can be initiated by the presence of vasodilatory autacoids or via the attenuation of alpha-adrenergic tone (42). The ability to regulate vascular tone decreases with age, atherosclerosis, hypertension, diabetes, obesity and smoking (67).

Arteries are capable of responding to changes in blood flow and subsequently inducing vasoconstriction or vasodilatation due endothelial cells (ECs). These spindle-shaped cells line all of the body's arteries and are oriented parallel to the direction of blood flow (40). Their location and orientation allow them to modulate vascular tone in response to alterations in flow (26). ECs also play an important role in arterial remodeling, inflammation and thrombosis (26). The importance of this monolayer of cells was first documented by Furchgott & Zawadzki who reported that acetylcholine (Ach) infusion with an intact endothelium produced vasodilatation; however, without an intact endothelium, Ach infusion produced vasoconstriction (23). Since this pioneering study, scientists uncovered that Ach led to vasodilatation via the NO pathway (Figure 1a) (26), and that an intact endothelium is required for vasodilatation to occur (4).

The peripheral arteries of individuals with atherosclerosis tend to have a dysfunctional endothelium and a decreased ability to release NO. Therefore, these individuals have difficulty regulating vascular homeostasis. For this reason, a large amount of research is being conducted to better understand how changes in NO-mediated vasodilation relate to arterial health.

2.2 Nitric Oxide and Endothelial Cells

NO is a very important EDRF that plays a role in vascular tone, immune function, cell growth, neurotransmission, metabolic regulation, excitation-contraction coupling and inhibition of platelet aggregation (35). NO is secreted and released by ECs; within an EC the true interface with flowing blood is the endothelial glycocalyx (GCX). Through mechanotransduction, GCX membrane-bound proteins sense fluid shear stress and convert that force into a biochemical signal that can induce changes in diameter, according to the "glypican-caveolae-eNOS hypothesis" (68). The shear stress stimulus is transmitted to the caveolae (68) whereby endothelial nitric oxide synthase (eNOS) is phosphorylated (40). Once eNOS is phosphorylated it translocates to the cytolsol and converts L-arginine to NO and L-citruline (Figure 1a) (4; 28). Many factors can alter eNOS expression including: tumor necrosis factor-α (47), erythropoietin (74), hypoxia (41) and high concentrations of oxidized low-density lipoproteins (38). A discrepancy between the amount of eNOS mRNA present in ECs and eNOS protein production and activity suggests that post-translational and post-transcriptional control mechanisms can alter eNOS mRNA stability and thus decrease eNOS expression (28). Altering the expression of eNOS will alter the production of NO and can therefore be a factor in the regulation of vessel diameter.

Once NO is synthesized, it diffuses out of the EC and into vascular smooth muscle cells. Within the vascular smooth muscle cell NO can bind to guanylate cyclase increasing cyclic guanosine monophosphate which decreases intracellular calcium leading to vasodilatation (Figure 1a & 1b).
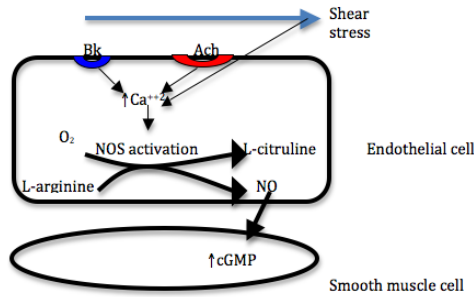
Figure 1a: Nitric oxide synthesis and diffusion into smooth muscle cell to induce vasodilatation (4)
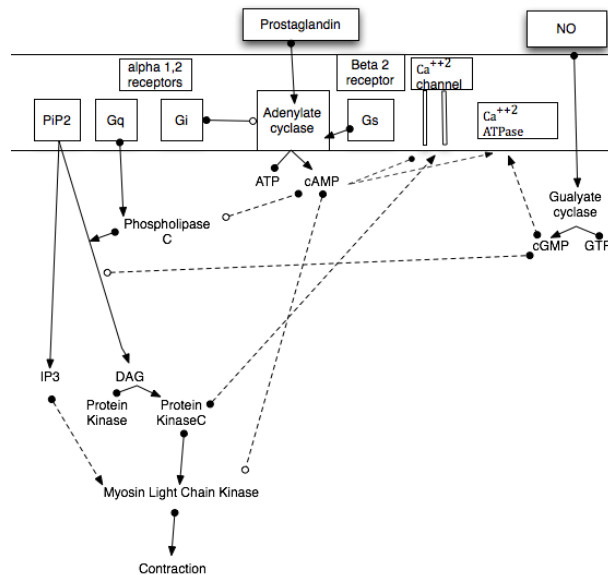
Figure 1b: Various pathways in which nitric oxide pathway can induce vasodilatation

Abbreviations: PiP2: Phosphatidylinositol 4,5-bisphosphate, Gq: class of G proteins, Gi, Gs: heterotrimeric G proteins, ATP: adenosine triphosphate, cAMP: cyclic adenosine monophosphate, cGMP: cyclic guanosine monophosphate, GTP:guanosine triphosphate, IP3: inositol triphosphate, DAG: diacylglycerol. (63)

The balance between vasodilator and vasoconstrictor autocoids regulates vascular smooth muscle tone. NO and other EDRF's, such as prostacyclin and endothelium derived

hyperpolarizing factor, can induce vasodilatation (40). Vasocontricting autocoids such as vasoconstrictor prostanoids, endothelin-1, angiotensin-II and reactive oxygen species like superoxide anion are also secreted by the endothelium (40) and can decrease arterial diameter. These factors will not be discussed further as their role in short duration shear stimuli in young healthy individuals is believed to be negligible (32; 44).

The production of NO and the ability for EC's to induce vasodilatation due to this EDRF is integral for proper vascular responses to increased shear stress. Research has demonstrated a decrease in NO production and peripheral artery function in patients with CVD and its associated risk factors (13). This has motivated researchers to create a non-invasive method of determining NO release and endothelial function as a predictor of CVD.

2.3: Non-invasive Assessment of Vascular Health

Prior to 1992 invasive tests, such as angiography, were the primary method used to detect the presence of cardiovascular disease and atherosclerosis. These tests are still being used, however now there is a non-invasive method available for measuring vascular and endothelial health. In 1992, Celermajer and colleagues validated the use of flow-induced changes in diameter as a useful non-invasive method of studying and predicting the health of the central and peripheral vascular system.

FMD is accomplished by inflating a pneumatic cuff to supra-systolic pressures to produce limb ischemia. This ischemic environment results in distal micro-vessel dilation caused by metabolite accumulation. Cuff deflation induces a short duration high blood flow state through the artery termed reactive hyperemia (RH); this elevated blood flow is due to a decrease in resistance of downstream-dilated resistance vessels (14). Furthermore, RH increases wall shear stress leading

to the release of one or more EDRF's from upstream arteries, which will produce significant changes in arterial diameter (13). Flow mediated dilation is calculated via the following equation:

$$\text{Equation 1: } FMD = \left(\left(D_{postdeflation} - D_{baseline} \right) \div D_{baseline} \right)$$

$D_{baseline}$ is measured prior to cuff inflation (71) and D_{post} is peak arterial diameter post cuff release.

The pioneering study by Celermajer et al. examined 100 participants, ages 8-67, and classified them as either: healthy, having at least one risk factor for cardiovascular disease, familial history of hypercholesterolemia or diagnosed coronary artery disease. A significant decrease in FMD in patients suffering from CVD and in young symptom free individuals with risk factors for cardiovascular disease was reported. The FMD in the SFA of control patients was $4 \pm 1\%$ while in patients with CVD, SFA FMD decreased to $1 \pm 0.3\%$. The BA FMD of controls was $11 \pm 2\%$, while in patients with CVD BA FMD significantly decreased to $0 \pm 1\%$. In children with a family history of hypercholesterolemia SFA FMD was $0 \pm 1\%$ while SFA FMD was $9 \pm 1\%$ in control children(13). These results are supported by Gocke et al. who examined 35 patients with known CVD, and 164 age matched controls with no CVD. They reported that BA FMD significantly decreased to approximately 4% in patients with a cardiac event, compared to 7% in those who did not have a cardiac event(27). Based on this evidence it has been suggested that a BA FMD response of less than 4% is a good predictor of decreased endothelial function and NO release.

Changes in peripheral artery vascular homeostasis are an important indicator of an individual's overall cardiovascular health. This is due to the fact that peripheral changes in FMD are seen in both patient with CAD and in individuals with CAD associated risk factors, inferring that

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peripheral vascular changes parallel those in the coronary circulation (4; 37). The creation and validation of FMD has been integral in the study of vascular and endothelial health in both healthy and diseased populations. There are two other non-invasive methods that can be used to infer endothelial function; venous occlusion plethysmography and pulse wave analysis. Briefly, venous occlusion plethysmography measures changes in limb volume and blood flow at rest or during limb occlusion, and pulse-wave analysis assesses global endothelial function based on measures of systemic arterial stiffness (2). Currently, FMD is the preferred method of examining endothelial function, over venous occlusion plethysmography or pulse-wave analysis, as it directly assesses conduit artery endothelial function and allows for repeated measurements (14).

2.4: Existing Flow Mediated Dilation Protocols

After conducting an extensive literature review (Appendix 5: Table 6) a limited number of articles using a distal occlusion protocol of the SFA and PA were found. Conversely, BA distal occlusions are commonly performed and a protocol was recommended by Corretti et al. (14). The International Artery Reactivity Task Force published *Guidelines for the Ultrasound Assessment of Endothelial-Dependent Flow-Mediated Vasodilatation of the Brachial Artery(14)*; this article describes the equipment, images, protocol and analysis that need to be performed when examining FMD within the BA. With regards to equipment, Corretti et al. explain that the ultrasound must be able to acquire two-dimensional images, color and spectral Doppler, in addition to being equipped with an internal ECG monitor and a high-frequency vascular transducer. These researchers suggest that M-mode sweeps of longitudinal BA images should be used and they recommended against cross-sectional images because of inadequate wall definition (14).

The guidelines recommend that after collecting a baseline diameter and velocity reading, the pneumatic cuff should be inflated to a pressure at least 50mmHg greater than the participant's systolic blood pressure for a "standard", undetermined, length of time. Diameter should be recorded continuously from 30 seconds prior to deflation to two-minutes post deflation while RH blood flow velocity should be recorded immediately post deflation (14). There are many missing pieces in the guidelines such as: cuff placement, the usefulness and effects of exercise during occlusion, limb differences, and how the FMD response should be normalized. Other groups have subsequently investigated these issues because they have been found to alter the magnitude of the shear response, which can alter the EDRF secreted and the magnitude of FMD response. Regardless of the limitations to the Corretti paper, it has been referenced by over 1200 articles since its publication and in the current study was used to generate the various protocols.

2.5: Nitric Oxide Mediated Flow Mediated Dilation

As mentioned above, endothelial NO production is decreased in patients with CVD and other disease states; therefore, when performing an FMD experiment researchers want to ensure the dilation is NO mediated and thus can infer NO bio-availability. To determine if NO and/or prostacyclin mediated FMD in peripheral arteries L-NMMA, a NO synthase inhibitor, or Aspirin, a prostacyclin inhibitor, was given to the participants prior to conducting a FMD test (32; 44). Joannides et al. demonstrated that L-NMMA abolished RA FMD while having no effect on the peak hyperemia response. When Aspirin was administered no effects on reactive hyperemia or FMD were noted (32). Mullen et al. confirmed these findings after manipulating RA blood flow; they reported that L-NMMA almost completely abolished (FMD= $0.7\pm0.7\%$) RA FMD, while Aspirin had no significant impact of the magnitude of the FMD response (44). This led Joannides et al. and Mullen et al. to conclude that dilation post short-duration occlusions

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are mediated by NO, not prostacyclin. These results hold true when the occlusion occurs distal, below, the site of measurement, but not when the occlusion is proximal, above the site of measurement (18).

There are a number of conditions under which NO does not mediate the FMD response, including proximal cuff placement and prolonged shear stress. The effect of cuff placement, relative to the site of measurement, on which EDRF mediates the FMD response was examined by Doshi et al. (18). They wanted to determine which EDRF is responsible for BA FMD post five-minutes of occlusion at 250 mmHg when the cuff is distal (placed around the wrist) or proximal (placed around the upper arm). This was accomplished by examining the FMD responses with and without the administration of L-NMMA. Doshi et al. concluded that L-NMMA infusion blocked the FMD response post distal occlusion of the BA but not post proximal occlusion; suggesting that FMD post distal occlusion is NO mediated and FMD post proximal occlusion is mediated by other EDRFs (18). Mullen et al. examined the effect of shear stress duration on the mediators of a FMD response. These researchers demonstrated that FMD responses post 15-minutes of occlusion are not solely mediated by NO as FMD was not attenuated with L-NMMA infusion (44).Therefore, FMD post five-minutes of distal occlusion is NO mediated (18; 32; 44) and can be used a measure of NO bioavailability and provide insight into a patient's vascular and endothelial health. More research is needed to determine which EDRFs influence FMD post proximal occlusions and occlusions of greater than five-minutes as it is clear that these occlusions result in FMD responses that are not solely mediated by NO.

Prior to 2008, NO-mediated FMD was only proven in the BA and RA. Due the heterogeneity of arm and leg vasculature, researchers needed to confirm these findings in leg conduit arteries such as the SFA or PA. Kooijman et al. performed distal occlusions of the SFA for five-minutes at

220 mmHg on eight healthy males (36). Continuous direct L-NMMA infusion into the SFA during occlusion and reactive hyperemia, at a similar dose to that used by Mullen et al., was used to inhibit NO mediated dilation. Percent FMD and FMD: SS_{AUC} were significantly blunted with the infusion of L-NMMA thereby confirming that leg conduit arteries FMD due to short (<five-minutes) duration occlusion is NO mediated.

Carlsson & Wennmalm found contradictory results using venous occlusion plethysmography (12). These researchers reported that forearm RH was significantly diminished when ibuprofen and/or naproxen, two cyclooxygenase inhibitors, were administered (12). This is an older study, in which a different technique was used; furthermore, no diameter changes were measured and no recent studies have confirmed these findings. Thus it can be concluded that for short duration (five minutes or less) distal occlusions, the FMD responses are primarily NO mediated in arm and leg conduit arteries.

2.6: Shear Stress

Shear stress is the primary mechanism mediating the increase in diameter post cuff deflation. To better understand shear stress a brief discussion on conduit artery blood flow is required. Arterial blood flows in a laminar fashion; therefore, velocity is greatest at the center of the artery and decreases as it approaches the arterial walls. In the laboratory, velocity can be measured using Doppler ultrasound and subsequent analysis allows vessel blood flow to be calculated using the following equation.

$$\text{Equation 2 (31): } Flow = blood\ flow\ velocity \times \pi r^2$$

Appropriate blood flow is essential for the supply of oxygen and nutrients to underlying tissue. Without altering mean blood flow velocity, average flow in one heart beat, blood flow can be modified via changes in arterial diameter (Equation 2).

Under normal resting conditions, flowing blood exerts a frictional force on the blood vessels, which is termed shear stress (32). The magnitude of arterial MBV is an important factor in determining shear stress (Equation 3).

$$\text{Equation 3 (15): } Shear\ stress = (8 \times MBV) \div Diameter$$

Shear stress is proportional to blood flow velocity while being inversely proportional to vessel diameter. The nature and magnitude of the shear stimulus has a direct impact on the FMD response (56). Pyke and Tschokovsky suggested that the rate of onset, magnitude, and duration of the shear stimulus affect the magnitude of a FMD response. The shear stress stimulus can be altered by occlusion duration, baseline diameter, the presence or absence of exercise, extremity, and concentrations of female sex hormones (56). Lastly, how the FMD data are normalized must be considered as it determines if a small FMD response is indicative of endothelial dysfunction or simply a small shear stimulus (56). Many of these issues were insufficiently examined by Corretti et al. (14) and must be further examined so that the results of future studies can more easily be compared.

2.7: Factors Affecting the Elicited Shear Stimulus and FMD Response

2.7.1: Shear Stress Duration

As previously discussed, short and long duration shear stimuli are mediated by different mechanisms. This phenomenon can potentially be explained by the "sequential recruitment of mechanisms" hypothesis. This hypothesis postulates that at the onset of a shear stimulus NO is released and is the primary mediator of FMD. If, however, an elevated shear stimulus is maintained, then non NO-mediated FMD occurs; currently the mediating mechanisms and the timing of the shift from NO to non NO-mediated FMD are still not well understood. Mullen et al. suggested that vasoactive prostanoids play a minimal role in vasodilation (44) and it is possible that non-endothelial dependent pathways could be involved in FMD due to long duration shear stress.

2.7.2: Baseline Diameter

The baseline diameter of the artery being examined will have an impact on the magnitude of the shear stimulus. Pyke & Tschokovsky reported that smaller conduit arteries show a larger FMD response to RH compared to larger arteries. They attributed this finding to the tendency for smaller conduit vessels to experience greater shear rates (Equation 3) (56). For example, Celermajer et al. reported that in healthy adults average baseline BA diameter is 0.37cm while SFA baseline diameter is 0.68 cm. Post four and one-half minutes of distal limb occlusion, BA FMD was 11% while the SFA FMD was 4% (13). Clearly there is a link between baseline diameter and the magnitude of the FMD; however, more research is still needed to better understand these interactions and how they affect the interpretation of a FMD test.

2.7.3: Cuff Placement

As previously mentioned (Section 2.5) the location of the pneumatic cuff, proximal or distal to the measurement site, will affect the EDRF responsible for the FMD response. Furthermore, cuff placement will affect the magnitude and duration of a FMD response. To reiterate, a proximal occlusion involves placing and inflating the blood pressure cuff above the site of measurement; whereas, a distal occlusion involves placing the blood pressure cuff below the site of measurement.

Using a proximal occlusion site may result in the artery under consideration becoming ischemic which would result in different/additional mechanisms governing the FMD response (18). Doshi et al. examined BA FMD post wrist (distal) and upper arm (proximal) occlusion and found that proximal occlusion produced a significantly larger FMD response (11.60% vs. 7.25%) although there was no significant difference in the peak blood flow stimulus post cuff release (18). The increased FMD, as seen with a proximal occlusion, could be a result of the synthesis and release of prostacyclin due to the hypoxic environment (1; 5). However, it is unclear if hypoxia occurs, as it is possible that there is enough oxygen in the trapped arterial blood to supply the underlying vascular smooth muscle for a five-minute occlusion. Another possibility is that a proximal occlusion causes a reduction in intra-arterial pressure, which would induce myogenic dilation (22) thereby altering the FMD response. Lastly, a proximal occlusion may result in a different shear stress stimulus than a distal occlusion thus altering NO release (6). Doshi et al. reported that FMD post proximal occlusions was only partially attenuated with the use of L-NMMA (11.62% vs. 7.51%), suggesting that proximal occlusions lead to the release of other EDRF in addition to NO (18). The possibility of a hypoxic environment or involvement of myogenic

dilation is not present with the use of distal occlusion; therefore it is recommended that a distal occlusion be used to measure endothelial function and NO bioavailability (6; 14).

2.7.4: Effect of Exercise on FMD

Exercise may be used during limb occlusion to augment the shear stimulus and decrease the occlusion duration. The concern when using an exercise protocol is the possibility of sympathetic nervous system (SNS) activation. The SNS is an important regulator of cardiovascular function (61) and patients with CVD have impaired FMD responses and increased sympathetic nerve activity (SNA) (13; 21). An increase in SNA in young healthy individuals might lead to a decrease in the magnitude of the FMD response. Dyson et al. demonstrated that only certain methods of elevating SNA result in an impaired FMD response (19). When SNA is elevated, mean arterial pressure (MAP) and reactive hyperemia responses are altered. For example, continuous plantar flexion exercise with both calves occluded, increased muscle chemoreflex activity, which in turn elevated SNA (19). If an exercise protocol is used in conjunction with limb occlusion it is possible that an increase in SNA would blunt the FMD response or lead to non NO-mediated dilation (19). To avoid this issue, MAP should be monitored to insure that it does not increase more than 20 mmHg, as that is a marker for SNA.

Another concern of using exercise during an FMD protocol is that conducted vasodilatation may play a role in conduit artery RH and FMD. Conducted vasodilatation is when arteriolar vasodilatation at the site of metabolic demand is transmitted to other arteries via endothelial cell-to-cell communication through their gap-junctions (62). Pyke et al. examined the effect of forearm exercise on BA FMD and determined that neither SNA nor conducted vasodilatation played a role in BA FMD (55). Therefore, the addition of exercise to a short duration occlusion

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(ex: two-minutes) may be an effective method of increasing the RH and shear stress responses to more closely match those of slightly longer (five-minute) occlusion durations.

2.7.5: Limb Differences

It has been well established that endothelial dysfunction is a systemic event, with changes in vascular and endothelial health in peripheral arteries mirroring changes in coronary vascular and endothelial health. Recent research has demonstrated a higher incidence of atherosclerotic lesions in the SFA compared to the BA (43). Therefore, if researchers and clinicians want to obtain a complete picture of a patient's overall vascular health the results and interpretations of leg conduit FMD responses are needed in conjunction of BA FMD data. There is significant heterogeneity between the upper and lower limb vasculature and research is ongoing to understand these differences and their usefulness as a predictor of overall cardiovascular health.

Arm and leg conduit arteries have noticeably different baseline diameters; BA diameter is approximately 0.4 cm while the PA and SFA are greater than 0.6 cm in diameter. Furthermore, while standing human legs experience large hydrostatic and transmural forces, which are not seen in the arms. Rowell estimated that the lower limb experiences 65mmHg more pressure than the arms (59). The increased blood pressure (+65mmHg) experienced in the legs induces changes in endothelial function (72) and phenotypic changes to vascular smooth muscle cells (30). Hill et al. suggested that differences in receptor expression, receptor activation, structural proteins and signal transduction proteins might be partially responsible for limb differences in FMD (30).

Nishiyama et al. documented greater vascular function in the PA (legs) than the BA (arms), where vascular function can be defined as the ability of an artery dilate in response to a controlled shear stimulus. They attributed this difference to either adaptive vascular stiffening in

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the legs or differences in limb conduit artery muscle bed perfusion (48). Using MRI data, Wu et al. reported that BA shear rates are approximately 50% greater than those of SFA (80). Using Doppler ultrasound data, Newcomer et al. reported an approximate 40% increase in BA shear stress (46) as compared to the femoral artery. Furthermore, Newcomer et al. reported a smaller increase in blood flow and vascular conductance in the leg as compared to the arm (45). When examining the differences in conduit artery FMD, there is a trend that legs have a lower FMD response than the BA (69). It has been postulated that the lower shear rate, blood flow and vascular conductance in the leg may contribute to the significantly higher incidence of atherosclerotic lesions found in lower limb vasculature (43). This evidence further highlights the importance of studying leg conduit artery FMD.

At rest and during exercise the legs require a higher absolute blood flow and percent cardiac output ($\dot{Q}$) due to their greater muscle mass and subsequently larger blood and oxygen demands. However, when blood flow is normalized to limb volume, the arms appear to have greater blood flow than the legs (11). More research is needed to clarify this issue and understand how it impacts our understanding of limb differences and FMD responses.

Regardless of the reason, arm and leg conduit arteries have different baseline diameter and blood flow and FMD responses. It is likely that the differences in shear stress are an important predictor in limb FMD differences. Leg conduit arteries are more susceptible to the development of atherosclerotic lesions than arm conduit arteries, which stress the importance of conducting and understanding leg FMD responses. More work is still needed to understand limb differences in FMD and how both arm and leg FMD responses can be used to gain a better and more comprehensive picture of a patient's overall vascular health.

2.7.6: Superficial Femoral vs. Popliteal Artery for Assessment of Lower Limb FMD

When assessing lower limb vascular function, researchers can examine either the PA or SFA. A review of the anatomy of the SFA and PA revealed that these arteries stem from the common femoral artery (CFA). The CFA originates in the femoral triangle and it receives blood from the external iliac artery. As the CFA descends the leg, it curves medially it branches into the deep artery of the thigh and the SFA. The SFA continues down the leg, dives deep and emerges behind the knee joint as the PA (79). MRI images of this artery, reveal no branching between the location where it is known as the SFA and where it is known as PA (79). In the current literature review three experiments examined the PA, and six studied the SFA. These papers were reviewed as all occlusions were distal to site of measurement.

Of the experiments where the PA was occluded, differences exist in how the participants were placed, the occlusion pressure and occlusion duration. Nishiyama et al. examined the PA, and required their participants to lie supine, on a gurney that was adapted to allow for access to the PA and performed a distal occlusion (pressure >250 mmHg for five-minute) (48). Conversely, Padilla et al. placed their subjects prone, and inflated the cuff to 220 mmHg for two-minutes (51). Thijssen et al. placed their subjects in the prone position, inflated the cuff to a pressure greater than 200 mmHg for five-minutes (69).

When Celermajer et al. first described the SFA FMD protocol they used distal thigh occlusions at a pressure of 300 mmHg for four and one-half minutes (13). This cuff placement has been continued in more recent publications; however, differences in occlusion pressure have been used. For example, Walther et al. inflated a thigh cuff for five-minutes to unspecified suprasystolic pressure (73), Silber et al. examined the femoral artery at a pressure 20 mmHg

greater than SBP for five-minutes (64) and Kooijman et al. chose a pressure of 220 mmHg for five-minutes (36) (see all SFA occlusion protocols in Appendix 5:Table 6). The above-mentioned studies have used different occlusion pressures and have reported a wide range of FMD values (between 2 and 6.9%) in healthy participants. The variety of protocols and the wide range of FMD values have made finding the optimal SFA FMD protocol difficult.

The PA FMD protocols placed the blood pressure cuff around the subjects' calf, whereas the SFA FMD has been studied using thigh cuffs. Wrapping the pneumatic cuff around the participant's thigh causes greater participant discomfort than wrapping the cuff around the calf. Furthermore, by placing the cuff around the thigh, a larger amount of muscle is being occluded and therefore it is possible that more metabolites are being built up leading to greater downstream resistance vessel dilation and therefore a larger shear stress response. From a logistical standpoint, using the SFA may be simpler than using the PA due to its location on the front of the leg and measurement at a shallower depth. Using the SFA would allow participants to lie comfortably on their back and the shallower depth would allow for clearer ultrasound images. The improved image quality would improve wall detection and therefore diameter determination. Currently no consistency or standard protocol exists for lower leg conduit artery FMD.

2.7.7: Time of Peak Diameter Measurement

According the BA FMD guidelines, arterial diameter should be continuously recorded from 30 seconds post deflation to two minutes post-deflation; however, this not always followed. Anderson et al. recommended that FMD be determined using diameter measures taken at 60 seconds post deflation (3). The issue with using one single time point is that it may not capture the actual peak diameter, which has been reported to occur anywhere from 60 seconds to three minutes post deflation. Black et al. reported that FMD reported using diameter calculated at 60 seconds post-deflation (FMD= 4.8%) was significantly lower than FMD reported using actual peak diameter (FMD= 7.3%) (7). This data further strengthens the recommendations of Corretti et al. that diameter needs to be continuously reported until two minutes post-deflation.

2.7.8.: Additional Factors that can Affect FMD

Other factors that need to be taken into consideration when conducting an FMD test are ambient temperature, recent exercise and alcohol and/or caffeine consumption. These factors have a reported vasoactive effect which can influence the magnitude of the FMD response (14).

2.7.9: Normalizing an FMD Response

The magnitude of a shear stress stimulus created with the RH response directly affects the magnitude of the FMD response and has implications for interpretations of vascular health. As discussed above, the magnitude of the shear stimulus is affected by many factors including, but not limited to, sex, cuff placement and occlusion duration. Furthermore, RH blood flow peaks and decays before peak diameter is seen; therefore, a quantification that can appropriately describe the stimulus is integral for understanding an FMD response. This will allow researchers to determine if the small percent FMD is due to a small shear stimulus or to an endothelial dysfunction.

Researchers are unable to agree on how best to normalize an FMD response. Currently, a FMD response may be normalized to the mean shear stimulus, peak shear stimulus and/or stimulus duration. Pyke and Tschakovsky and Padilla et al. suggested that shear stress area under the curve (SS_{AUC}) provides the most reliable interpretation of endothelial function from an FMD study (50; 57). Pyke & Tschakovsky found that when only exposed to peak shear, a small increase in diameter was seen. Conversely, when a conduit artery was exposed to more than 30 seconds of RH, a larger FMD response was observed. Therefore, Pyke & Tschakovsky recommended against normalizing the FMD response to peak shear and recommend normalizing to the total SS_{AUC} (57).

In a review article Pyke & Tschakovsky, argued that measuring shear stress and subsequently calculating SS_{AUC} until peak diameter is achieved is the ideal method of quantifying the total stimulus that contributes to the FMD response(53). To achieve this, an ultrasound machine must be capable of recording flow and diameter simultaneously. These machines are more expensive than those that measure one or the other. This fact might contribute to why few studies normalize

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their data to total SS_{AUC}. Therefore, Pyke et al. recommended that both the peak shear stress and SS_{AUC} until peak diameter should be recorded and reported when reporting a FMD repsonse (54).

2.8: Measuring Arterial Diameter

The majority of research using FMD has chosen to image the conduit artery in the longitudinal plane. This orientation was chosen because it allows for clear definition of the artery-lumen boundary on the near and far wall (14). Kao et al. demonstrated that imaging in the longitudinal plane is associated with decreased sensitivity and accuracy in determining arterial diameter. They illustrate that a 10-20% increase in diameter for an artery with a baseline diameter of 0.5 cm corresponds to a change of 0.5-1.0 mm. This significant change in diameter corresponds to a difference of a few pixels on an ultrasound image; therefore a sensitivity of greater than two to three pixels is needed in order to see a significant change in diameter (33). These changes can easily be masked due to slight movement of the transducer or the artery during deflation. Another limitation of using longitudinal images is that deviations in an artery from its traditional circular geometry may affect diameter measurements (33).

The above-mentioned limitations of using longitudinal plane images to determine arterial diameter are not present when using transverse/cross-sectional views of a conduit artery. Recent advances in ultrasound technology, including improved spatial resolution and lateral wall definition, have made cross-sectional imaging a possibility. Kao et al. captured cross-sectional images of the BA and were able to outline the arterial boundaries (33). They reported that using cross-sectional images, compared to longitudinal images, allows for increased pixel resolution and thus improving the accuracy of detecting the peak diameter increase post cuff deflation (33). Furthermore, imaging in the transverse plane allows the entire cross-sectional area of the artery to be visualized; allowing the researchers to insure that the ultrasound probe is in the

correct place at all times during testing. The usefulness of determining arterial diameter in the transverse plane was validated by Stroz & Fenster (66). They reported that artery diameter determined via cross sectional imaging is not significantly different from arterial diameter from longitudinal images. Furthermore, they reported that cross sectional diameter measurements are reproducible and that wall definition was not an issue. The above study used three-dimensional images, which is a feature not available on all ultrasound machines. More research using both two-dimensional and three-dimensional transverse images in FMD studies is required to validate the use of two-dimensional transverse imaging.

Wikstrand determined that vessel diameter should be calculated by measuring the distance between the leading edge of the intima-lumen echo on the near wall to that of the far wall. However, ultrasound visualization of the near wall intima-lumen surface is unreliable and thus the lumen-media echo is often used in its place (77) (Figure 2).
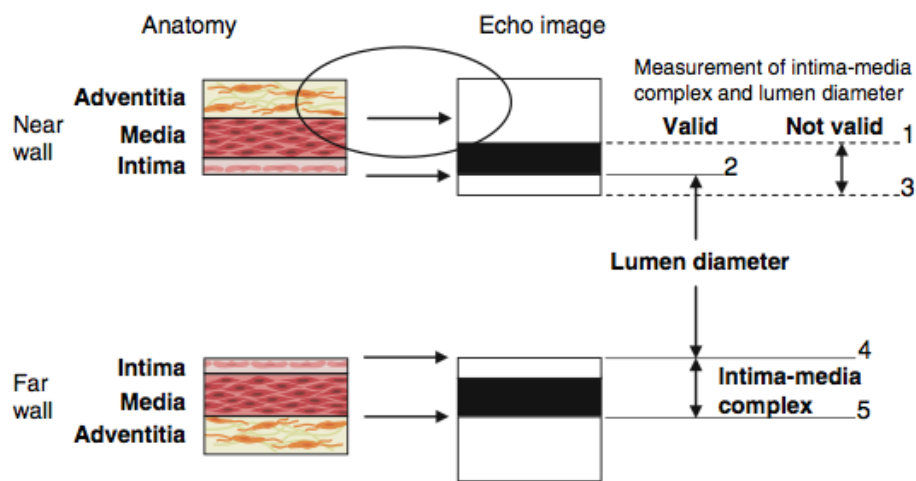
Figure 2: Schematic of the correlation between the anatomical structures and echoes generated images produced by the ultrasound to the carotid artery (77)

Currently when measuring arterial diameter to analyze FMD data the above-mentioned method is used (14). However, measuring arterial diameter from different structures on the near and far

wall is illogical. Furthermore, since defining the near wall structures is difficult, the issue of reliability and reproducibility using this method must be considered. In the current experiment, arterial diameter measurements were performed using both the current standard edge-detection method and using a new measurement method called the center-based method. The center-based method measures arterial diameter as the distance between the center of the wall (media layer) on the near to that on the far wall.

2.9: Hypothesis Generation

Endothelial cells play an integral role in the regulation of vascular tone. In response to short duration (< five-minute) occlusions, NO is released by EC's and directs vascular smooth muscle cells to dilate. Currently limited standardization of the FMD protocol, especially when testing the lower limb, limits our understanding of arm and leg conduit artery FMD and how it correlates to overall cardiovascular health.

To gain a better understanding of the possibilities and limitations of using lower limb FMD two large lower limb conduit arteries, SFA and PA, were examined. The SFA and PA were subjected to five-minutes of occlusion with no exercise. The first goal of the current study is to determine if the SFA can be used instead of the PA when measuring leg conduit artery function. The location and depth of the SFA make it the preferential leg conduit artery to examine. A five-minute occlusion at 220 mmHg was chosen as it follows the current guidelines used for BA FMD. It is hypothesized that there will be no significant difference in the shear stress, reactive hyperemia and FMD responses of the SFA and PA due to five-minutes of calf occlusion.

A two–minute occlusion protocol was examined because reducing the occlusion duration will not only decrease testing time but also eliminate excess discomfort to the participant. The SFA

and PA were subjected to a two-minute occlusion with one-minute of exercise imbedded within it. A shorter duration occlusion will result in a smaller shear stimulus; therefore, exercise was added in order to increase the elicited shear stimulus. The "traditional" five-minute occlusion of the SFA and PA were compared to the two-minute with one minute of exercise protocol. The resulting shear stress and FMD responses were compared to determine the usefulness of the shorter occlusion protocol. It is hypothesized that a two-minute occlusion containing one-minute of exercise will produce the same shear stress and FMD response as the five-minute protocol in the SFA and in the PA. Furthermore, the FMD and shear stress responses of the PA and SFA due to two-minutes of occlusion will not be significantly different.

The heterogeneity between arm and leg vasculature may be an important factor in the location and development of atherosclerotic lesions. Performing leg conduit artery FMD protocols allows for exercise-mediated changes to the trained area to be seen. To gain a better understanding of arm and leg conduit artery differences a five-minute occlusion was performed on the BA, SFA and PA. It is hypothesized that the BA will have a significantly larger shear stress, reactive hyperemia and percent FMD response than the SFA and PA.

Based on pilot data analysis, significant limitations to the current method used to measure arterial diameter, edge-detection method, were found including the fact that diameter is not measured between the same location on the top and bottom arterial wall. A new method of measuring diameter was created based on the premise of measuring diameter between the same location on the near and far arterial wall. The center-based method measures arterial diameter as the distance between the center of the media layer on the near wall to the center of the media layer on the far wall. The use of this method in determining BA FMD after five-minutes of

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occlusion was explored. It is hypothesized that the center-based method will demonstrate the same percent FMD and time to FMD as the edge-detection method.

Chapter 3: Methods

3.1: Participants

Men, ages 18 to 35, with no current or familial history of clotting disorders, cardiovascular or respiratory diseases were recruited for this study. Participants were non-smokers not currently taking any medications. A health status survey was filled out by all participants prior to testing to screen for eligibility.

Participants were required to abstain from caffeine, alcohol and exercise for 12 hours prior to testing. To confirm adherence, participants were asked by the examiners prior to beginning testing when and what their last meal was, the last time they drank caffeine and/or alcohol and last time they performed exercise. If the participant did not adhere to the above-mentioned restrictions, the FMD testing was rescheduled. All trials were performed in a quiet, dark, temperature controlled room (21-24°C). Prior to beginning the test, written informed consent was obtained from all participants. This study protocol was reviewed and approved by the University of Waterloo Office of Research Ethics (ORE #16079).

3.2: Experimental design

  Healthy young men who met the criteria specified in the "Participant" section were recruited for this study (Chapter 3.1). Potential participants were invited to visit the lab to fill out a medical screening form to filter out ineligible participants and to participate in a familiarization session. In addition, potential participants were given an information letter, approved by the ORE (#16079) to provide a better understanding of the current study and its potential risks.

  Participants were randomly assigned to either Treatment 1 (Table 1) or Treatment 2 (Table 2). Table 3 lists the limb (right vs. left) associated with the five and two-minute occlusions for treatment group 1 and 2. Care was taken to ensure that testing began at approximately the same time on each day of testing. Testing days were separated by at least 24 hours.

  When a participant arrived in the lab, his height, weight and previous meal were recorded. Participants were then connected to the electrocardiogram, Finometer^{TM}, and had two manual BP measurements taken. If a participant was in the "Treatment 1" group, they were asked to lie supine and three FMD protocols were performed on their first visit to the lab. On their second visit, two PA FMD protocols were performed during which they were asked to lie prone (Table 1). Conversely, if a participant was in "Treatment 2", they were instructed to lie prone and underwent two PA FMD protocols on their first visit to the lab. On their second visit they were asked to lie supine and a BA and two SFA FMD protocols were performed (Table 2). If a participant agreed to take part in the reproducibility study, they were required to return to the lab for a third day of testing (see chapter 4.0:"Reproducibility Study").

  In between each FMD protocol, a ten to fifteen minute break was given; this allowed the participant time to rest and provided time for the researchers to set up for the next test. Due to the

fact that the FMD protocols were being performed on different limbs (right arm and the right and left legs) there was no concern of a carry-over effect from the previous FMD protocol. Furthermore, on all testing days, the exercise FMD protocol containing one-minute of exercise was performed last to avoid any carry-over effects of exercise on arterial dilatory responses.

Table 1: Testing Protocol 1

Testing Day 2		First and second day of testing must be separated by at least one day	Testing Day 2	
Five minute BA FMD			Five-minute PA FMD	
Ten to fifteen minute break	Set up SFA FMD		Ten to fifteen minute break	Set up PA FMD on other leg
Five-minute SFA FMD			Two-minute PA FMD with one minute of exercise	
Ten to fifteen minute break	Set up SFA FMD on other leg			
Two-minute SFA FMD with one-minute of exercise				

Table 2: Testing protocol 2

Testing Day 1		First and second day of testing must be separated by at least one day	Testing Day 2	
Five minute PA FMD			Five-minute R-BA FMD	
Ten to fifteen minute break	Set up PA FMD on other leg		Ten to fifteen minute break	Set up SFA FMD
Two-minute with one-minute of exercise PA FMD			Five-minute SFA FMD	
			Ten to fifteen minute break	Set up SFA FMD on other leg
			Two-minute with one-minute of exercise PA FMD	

Table 3: Randomization of occlusion protocols

	Treatment 1 group					Treatment 2 group				
	S1	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**	**S8**	**S9**	**S10**
Tests										
R-BA	x	x	x	x	x	x	x	x	x	x
R-POP5						x	x	x	x	x
L-POP2						x	x	x	x	x
L-SFA5						x	x	x	x	x
R-SFA2						x	x	x	x	x
L-POP5	x	x	x	x	x					
R-POP2	x	x	x	x	x					
R-SFA5	x	x	x	x	x					
L-SFA2	x	x	x	x	x					
Reproducibility										
RBA						x	x	x	x	x
LSFA5						x	x	x	x	x
RPOP5						x	x	x	x	x

Abbreviations: L=left, R=right, SFA= superficial femoral artery, PA= popliteal artery, BA= brachial artery

For more information on the reproducibility study see Section 4.0.

3.3: Flow-Mediated Dilation Protocol

Endothelial vasomotor function of the BA, SFA and PA was assessed using FMD. The current protocol design was based on data acquisition and analysis by Celermajer et al. (13)and Corretti et al. (14). All occlusions performed in the current study were distal to the site of MBV and diameter measurements. The BP cuff was wrapped either around the forearm, just below the elbow, or around the lower leg, just below the knee joint. Once the cuff was in place but before inflation, one minute of baseline flow velocity and baseline arterial diameter were collected. After one minute of baseline, the cuff was inflated to 220 mmHg for either two or five minutes. The five-minute protocol did not contain an exercise component and the participants were instructed to remain still and avoid any leg or arm movements. The two-minute protocol contained one-minute of exercise; the subjects were instructed to press on a footplate until the sphygmomanometer reads plus 100 mmHg of their baseline pressure value.

After the allocated occlusion duration, the cuff was rapidly deflated. During the first 30 seconds post deflation, continuous MBV was recorded. Every 15 seconds from 45 seconds to 2.5 minutes post deflation, arterial diameter was determined (Figure 3 & 4).
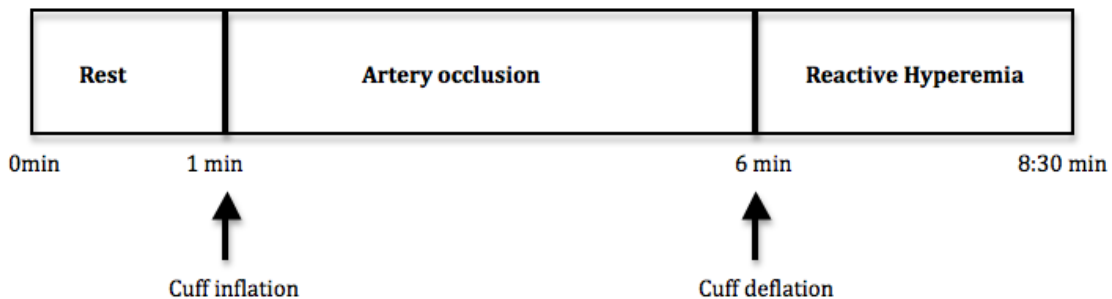
Figure 3: Five-minute FMD protocol for the SFA, BA, and PA.
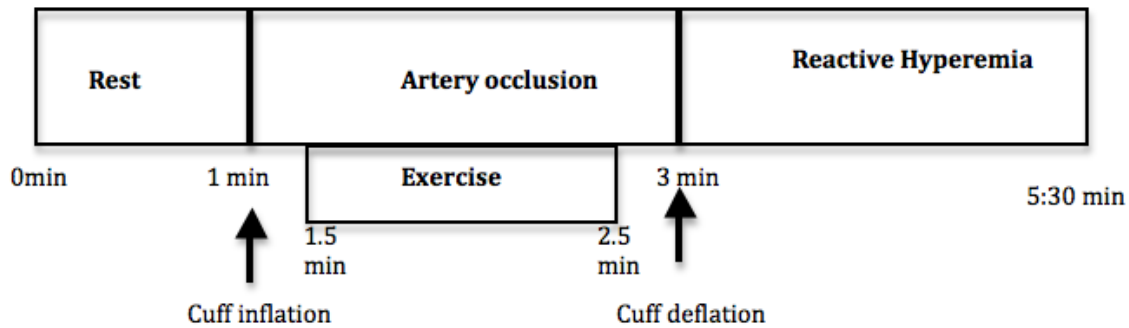
Figure 4: Two-minute with exercise FMD protocol for the SFA and PA

3.4: Physiological Measures

3.4.1: Cardiac Measures

Heart rate (HR) was measured via an electrocardiogram (Colin Pilot, San Antonio, Texas) by placing three electrodes on the participant. When the participant was in a supine position three electrodes were placed as follows; one below the right clavicle (negative lead), a second below the left clavicle (grounding lead) and a third on the left 12th rib (positive lead). When the participant was in the prone position, three electrodes were placed on their back as follows: one just above the spine of the right scapula (negative lead), a second just above the spine of the left scapula (grounding lead) and a third on the left 12th rib. The "R" peak from each heartbeat was used for beat-to-beat analysis of all the physiological variables.

Blood pressure (BP) and cardiac output ($\dot{Q}$) were estimated using an infrared photoplethysmographic cuff (Finometer, Finapres Medical Systems, Arnhem, Netherlands) placed on the participant's middle finger. The finger cuff detects small changes in arterial diameter and controls cuff pressure to oppose changes in arterial pressure. The Finometer™ uses the volume-clamp method of Penaz and the Physiocal criteria of Wesseling to continuously measure finger arterial pressure. Modelflow technology was employed to continuously estimate

$\dot{Q}$ from finger BP using a non-linear, self-adaptive model of aortic impedance (76). The non-linear equation used by the Finometer[TM] software employs a three-element (aortic impedance, Windkessel compliance and peripheral resistance) non-linear equation, which is dependent on the aortic pressure-area relationship. Finger arterial pressure was corrected to brachial BP using a return-to-flow (RTF) calibration. The RTF calibration was achieved by inflating the Finometer[TM] arm BP cuff to suprasystolic pressures and determining when the first pulsations pass under the cuff and are detected by the finger cuff. A height corrector was used by the Finometer[TM] to compensate for the finger not being at heart level. The Finometer[TM] accurately measures beat-to-beat variations in BP within the Association for the Advancement of Medical Instrumentation standards. To further ensure the accuracy of the Finometer[TM], two manual BP measurements were taken prior to the beginning of testing on each day of testing. If the Finometer[TM] reported a BP value that differed from the manual measurements by more than 15 mmHg, the Finometer[TM] BP data were corrected to match manual reading. This was accomplished by adjusting the Finometer[TM] BP data once it had been exported to excel to align it with the manual BP data.

3.4.2: *Measuring Arterial Diameter and Blood Flow Velocity*

Mean blood flow velocity (MBV) of the SFA, PA and BA was continuously measured by placing a 4 MHz Doppler probe (Multigon 500M, Mt. Vernon, New York) over the artery under consideration. The 4 MHz Doppler ultrasound probe directs its signal at a 45° angle relative to the skin. Arterial blood flow velocity was recorded using a computer via data acquisition software (Chart 5 and Powerlab, ADInstruments, Colorado). Artery specific mean blood flow (MBF) was calculated using the following equation:

$$\text{Equation 4: } MBF = MBV \times \pi \left(\frac{diameter}{2}\right)^2 \times \frac{60\ sec}{min}$$

This equation demonstrates how vessel diameter is an important determinant of flow. To allow for MBF comparisons between participants, MBF was adjusted to the participant's limb volume, per 100 ml of tissue using the following equation.

$$\text{Equation 5: } \frac{MBF}{100ml\ of\ tissue} = MBF\left(\frac{ml}{min}\right) \div limb\ volume(ml) \times 100ml$$

Arterial diameter was measured using a 15 to 6 MHz Doppler ultrasound linear array probe (Sonosite Micromaxx^{TM}, Bothell, Washington); cross-sectional arterial M-mode sweeps were used to capture arterial images. This method was chosen, contrary to the recommendations of Corretti et al., who recommended longitudinal B-mode images be used to measure diameter because pilot data demonstrated improved image resolution when images were captured in the transverse plane.

The use of cross-sectional and M-mode imaging has recently been validated. The use of M-mode over B-mode sweeps was substantiated by Kelly et al. (34). These researchers demonstrated, via a Bland-Altman analysis, a strong agreement between M-mode and B-mode diameter and FMD measures (34). Furthermore, Kelly et al. reported higher image resolution with M-mode as compared to B-mode images (34). The use of cross-sectional US images to determine artery diameter has recently gained support by Stroz & Fenster (66) and Kao et al. (33).

Ultrasound video was recorded to either a S-VHS videotape or captured directly to a computer using a video capture card. After testing, the video was uploaded to the computer and still images were taken at the desired time points. From these images end-diastolic diameter was calculated and analyzed using the program MATLAB.

3.4.3: Limb Volume

Forearm and lower leg volume was measured using water displacement. Forearm volume was measured from the base of the olecranon process to the head of the ulna. The participant was asked to submerge his arm, up to the olecranon, providing a volume for the arm and hand. Subsequently, his hand was submerged, up to the head of the ulna, and this volume was subtracted from the arm and hand volume to obtain forearm volume.

Lower leg volume was measured from the base of the medial malleolus to the top of the medial epicondyle. The participant was asked to submerge his leg up to the medial epicondyle and a volume for the leg and foot was obtained. Subsequently foot volume, up to the medial malleolus, was obtained and subtracted from the leg and foot volume to obtain leg volume.

3.5: Data analysis

The internal diameters of the three arteries were measured at rest and every 15 seconds from 45 seconds to 2.5 minutes post cuff deflation. All arterial diameters were measured at end-diastole, as it has been suggested that during systole the increase in pressure and volume produces an approximately 5% increase in reported artery diameter (17; 24).

After testing, a subject's ultrasound video was uploaded to a computer allowing still images of baseline and post deflation diameter to be captured and analyzed. The standard method of determining arterial diameter is via edge-detection. This involves determining the distance between the intima-lumen edge of the near wall and the intima-lumen edge of the far wall (Ch 2.8, Figure 2). As previously discussed, limitations with ultrasound technology make detection of the near wall intima layer indistinguishable from the adjacent media layer; therefore arterial

diameter is measured as the distance between the media-lumen edge on the near wall and the initma-lumen edge on the far wall (77).

Currently our lab measures arterial diameter using a MATLAB edge detection script called GDM. Pilot data analysis revealed many limitations with this software including trouble with reproducibility and inaccurate tracings of the edge of the top and bottom arterial walls. When edge-detection was performed using GDM manual corrections were needed to insure that the correct structures on the near and far arteries walls were being identified and used to calculate arterial diameter. Manual detection of arterial walls and thus arterial diameter is subject to significant experimental/observer error (78). Conversely, "computer-assisted" analysis would eliminate the above-mentioned source of error and has been shown to increase the precision of diameter and FMD measurements (78).

Based on the work of Woodman et al. and the pilot data analysis performed in the current study, two new "computer-assisted" MATLAB scripts written by Aaron Katz called the *centre-based method* and the *edge-detection method* were used to measure arterial diameter in this study. Prior to image analysis, the image under consideration will be "cleaned- up" using a standard pre-processing filter to smooth the image with a Gaussian kernel to reduce noise in the data and pre-condition the data.

The centre-based method calculates the numerical integral across each column of pixels, for a user-selected section of the image. The software then determines the location where the integrated intensity is half the total intensity, the point where one half of the intensity lies on either side of the program-generated line. This method was chosen as the resolution of the ultrasound system (US) (ignoring other parts of the system such as the image acquisition or digitization systems) is on the same order as the thickness of the wall (US: ~0.5-1.5mm, wall ~0.4-1mm) (10). Due to this correlation, wall substructures (intima, media and adventia) are difficult to distinguish without specific knowledge of the entire image generating system. Detection of the media wall can be difficult; therefore, based on the assumption that the response of the ultrasound is primarily due to the media rather than the intima or adventia (due to the low acoustic backscattering in these regions), the centre of the wall can be used instead. This method defines the centre of the wall not as the peak intensity but as the point at which half the density of tissue (taken to be equivalent to intensity) is located on either side (Figure 5).
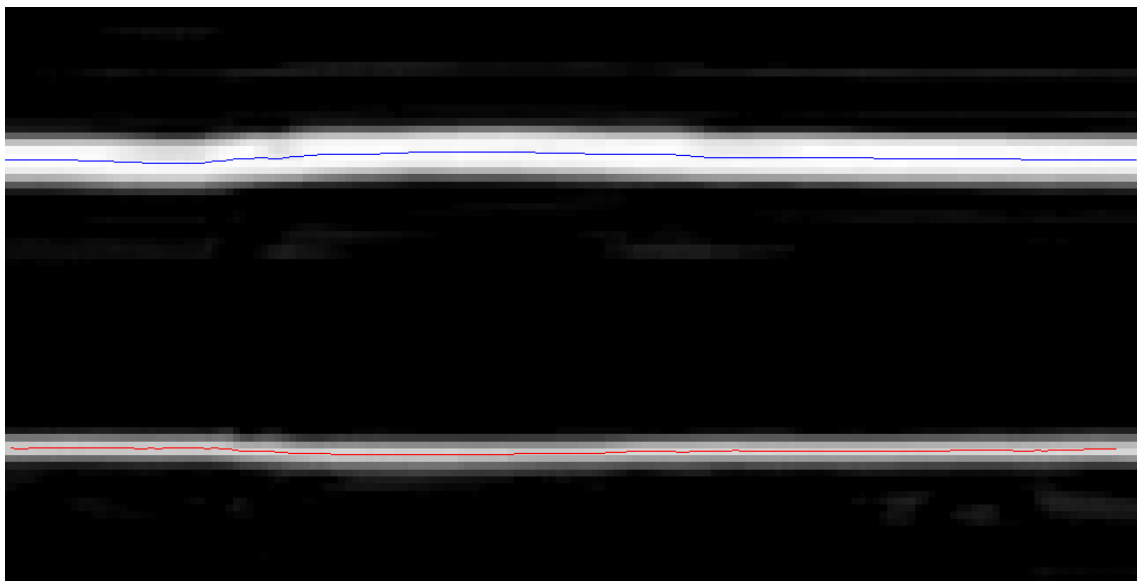
Figure 5: Crop of an M-mode sweep of a male superficial femoral artery whose diameter has been determined using the center-based method

3.5.2: Edge-detection Method

   This method is based on the definition of an edge as the maximum gradient of the intensity for the selected cropped image. To overcome the problem described above with ultrasound resolution, the gradient is weighted by the original intensity value (Katz, 2010). This effectively shifts the edge towards the high intensity section of the band, which matches the physiological structure that forms the image. The image undergoes a modification of a second order method; the media edge of the near wall is defined as second zero derivative and the intima edge of the far wall is found using the peak of the second derivative. Mathematically, the zero crossing for a second derivative is the same as the peak of a first derivative; however, due to the noise and granularity of the data it can be difficult to accurately identify the peak of the derivative. By assuming certain details of the shape of the data combined with smoothing using a Gaussian kernel, an estimate of the second derivative can provide an accurate sub-pixel position for an actual edge. This method differs from the "standard method" because the derivative is weighted by the intensity before the second derivative is taken (Figure 6 & 7). The major benefit of this edge-detection script over GDM is that manual corrections were not needed to accurately trace the desired wall structures on the near and far artery walls.
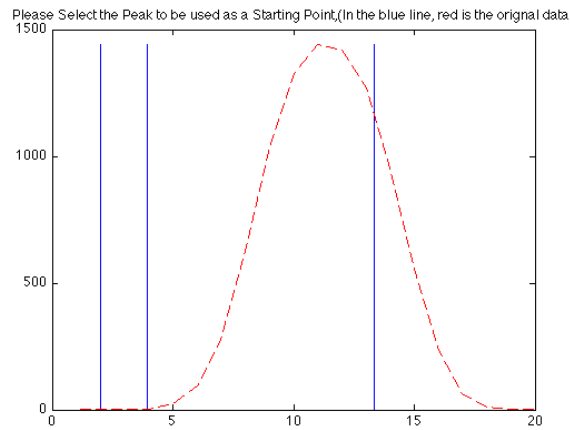
Figure 6: Initial point selection that guides the program to accurately track the edge under consideration

Figure 7: Crop of an M-mode sweep of a male superficial femoral artery whose diameter has been determined using the edge-detection method

Based on the diameters determined from the two methods described above, percent FMD will be determined by Equation 1(chapter 2.3). All reported diameter values are an average of two to three cardiac cycle end-diastolic diameters. Maximal arterial diameter post deflation, maximum FMD, will be determined using Equation 1, where D_{post} will be the largest recorded diameter

post deflation. Maximal FMD will be normalized to the elicited shear stimulus by calculating the ratio of percent FMD to SS_{AUC}.

Research has suggested that a BA FMD response of less than 4% is an indicator of endothelial dysfunction and reduced NO bioavailability (58). As young healthy males were examined in the current study a FMD of less than 4% was interpreted as the eliciting shear stimulus was unable to generate a FMD response. Therefore, the occlusion protocol was unable to elicit a strong enough shear stimulus to cause a significant increase in arterial diameter post cuff deflation.

3.5.3: Analysis of Hemodynamic Variables

Beat-to-beat analysis of systolic blood pressure, diastolic blood pressure, mean arterial pressure (MAP), cardiac output (Q) and heart rate (HR) were performed at rest and until two and one-half minutes post deflation. During the first 30 seconds post deflation MBF, vascular resistance (VR), conductance (VC) and shear stress (γ) were calculated using Equations 4-8.

$$\text{Equation 4: } MBF = MBV({cm}/{s}) \times \pi \times \left(\frac{diameter\ (cm)}{2}\right)^2 \times 60({sec}/{min})$$

$$\text{Equation 5: } \frac{MBF}{100ml\ of\ tissue} = MBF({ml}/{min}) \div limb\ volume(ml) \times 100ml$$

$$\text{Equation 6: } VR = MAP(mmHg) \div \frac{MBF}{100ml\ of\ tissue}$$

$$\text{Equation 7: } VC = \frac{MBF}{100ml\ of\ tissue} \times MAP\ (mmHg)$$

$$\text{Equation 8: } \gamma({1}/{sec}) = (8 \times MBV) \div Diameter$$

The shear stress area under the curve (SS_{AUC}) was calculated for the first 20 seconds post deflation. To better compare the participants' SS_{AUC}, the beat-by-beat data were converted into per second data.

3.6: Statistical analysis

Statistical Analysis Software (SAS Institute, Cary NC, USA) was used for all statistical procedures. Comparison of baseline diameter, shear stress, vascular resistance and vascular conductance was performed via a one-way ANOVA and subsequent Tukey Post Hock test. The effect of the five- and two-minute occlusion protocols on the SFA and PA were examined using a paired t-test (SFA5, SFA2, PA5 and PA2 respectively). The differences between baseline diameters, peak diameters, percent FMD, SS_{AUC} and $FMD:SS_{AUC}$ were examined. The SFA5 condition was compared with the PA5 and SFA2 conditions and the PA5 was compared to the PA2 condition. A Bonferoni correction was used to correct for the use of the same parameter in multiple comparisons; therefore, $\alpha=0.05/4$, resulted in a significance level of $p<0.0125$ being used to determine significance.

The effect of artery size and location was examined by comparing the FMD and SS_{AUC} data from five-minute distal occlusions on BA, SFA and PA using a one way ANOVA. Percent FMD and SS_{AUC} values for the BA, SFA, PA were compared at a significance level of $p<0.05$. Significant differences were noted, therefore a Tukey Post Hoc test was used to uncover which arteries, FMD and shear stress values were significantly different from one another. The effect of baseline arterial diameter on FMD and SS_{AUC} of the BA, SFA and PA were examined via a correlation analysis.

To compare the center and edge detection methods of determining artery diameter a one-way ANOVA of FMD and time to FMD was used. A significance level of $p<0.05$ was used. The BA was used for this analysis since the greatest body of literature exists concerning the expected range of FMD values and the underlying mechanisms responsible for these changes in diameter.

Chapter 4: Reproducibility Study

4.1: Reproducibility Study Methods

Five participants (S6-S10) were asked to participate in a reproducibility study. The participants came to the lab for one additional day of testing. On this day, three FMD protocols were performed: a five-minute BA FMD, a five-minute L-SFA FMD and a five-minute R-PA FMD (Table 4). The lower limb FMD protocols were performed on the same limb using the same procedure as on day one and day two.

Table 4: Reproducibility study protocol

Testing day 3	
Five-minute BA FMD -Using right arm	
Ten-fifteen minute break	Set up L-SFA FMD
Five-minute L-SFA FMD	
Ten-fifteen minute break	Set up R-SFA FMD
Two-minute with exercise R-SFA FMD	

Correlation analysis was run on baseline diameter, peak diameter, time-to-peak-diameter, peak shear stress, SS_{AUC}, and FMD. A correlation value, r, of greater than 0.90 was used to determine if the values were statistically similar between the different testing days. It should be noted that correlation analysis on calculated variables, such as FMD, peak shear stress or SS_{AUC}, is difficult as the differences in each of the variables is multiplied in the calculation of the desired value. For this reason, greater importance will be placed on direct measurements such as baseline diameter and peak diameter. Reproducibility was determined using both the Bland-Altman analysis and

correlation analysis. These tests examined baseline diameter, peak diameter, FMD, and shear stress area under the curve (SS_{AUC}) for the five-minute BA, SFA and PA FMD protocols.

4.2: Reproducibility Study Results

A correlation analysis revealed that PA baseline diameter, time to peak diameter and peak diameters were reproducible (r=0.981, 1.00 and 0.979 respectively) between testing day 1 and 3. A correlation analysis of the BA revealed that baseline diameter, peak diameter and time to peak shear were reproducible between testing day 2 and 3 (r= 0.971, 0.929 and 0.902 respectively). The SFA correlation analysis revealed that only baseline diameter and peak diameter (r=0.997 and 0.992 respectively). SS_{AUC}, and FMD were not reproducible in the SFA, BA or POP. Since peak shear and SS_{AUC} were not strongly correlated between testing days, it appears that it is difficult to elicit a similar shear stimulus on different testing days. (For more information refer to Appendix 6: Table 7)

The reproducibility of baseline and peak diameter of the BA, SFA and PA can be seen in the Bland-Altman plots in Appendix 7 Figures 8 through 13.

4.3: Reproducibility Study Discussion

The results of the reproducibility study demonstrate that the diameter values reported in the current experiment are an accurate representation of changes occurring in the BA, SFA and/or PA after different occlusion conditions. The reproducibility data demonstrate that baseline and peak diameters are reproducible in all three conduit arteries on different testing days.

The strong correlation of BA, SFA and PA peak diameters that was seen between both days of testing was not expected since SS_{AUC} and FMD were not correlated between the two testing days. The data emphasizes that a five-minute occlusion performed on different days can illicit different shear stimuli but produce similar peak diameters. The similarity of peak diameter values due to significantly different shear stimuli can be explained by the fact that the participants in this study were young healthy males and therefore even with a significantly lower shear stimulus near maximal vasodilatation was able to occur.

The lack of correlation in shear stress between testing days could be due to the uncontrolled nature of eliciting a shear stimulus (53). The magnitude of a shear stimulus, post temporary limb ischemia, is due in part of the magnitude of alterations down-stream vascular resistance and baseline conduit artery diameter. Pyke et al. suggested that the uncontrolled nature of the shear stimulus limits one's ability to reproduce a shear stimulus (53). Woodman et al. demonstrated that percent FMD was reproducible between visits, however shear stress data were not shown thus making any conclusions about shear stress reproducibility impossible (78). The above-mentioned research in conjunction with data obtained from the current study suggested that in young healthy individual's peak diameter was reproducible between visits; however, the eliciting shear stimulus was not consistently reproducible between visits.

The current study demonstrated that peak diameter was reproducible however FMD was not. It is likely that the lack of FMD correlation between testing days seen in the current study is due to mathematical limitations. There were small differences in both the baseline and peak diameter measurements. As a result when FMD was calculated and a correlation analysis was performed, those differences were magnified, creating a weaker correlation. FMD was examined both as a percent and as a ratio to SS_{AUC}. For the above-mentioned reasons, the ratio of FMD to SS_{AUC} was not put through a correlation analysis. Examination of the data revealed noticeable differences in FMD:SS_{AUC} (Appendix 6: Table 7)between testing days in the BA, SFA and PA for all participants. These results emphasize that the FMD response produced is a result of the elicited shear stimulus, produced after five-minutes of distal occlusion.

Taken together the results from the reproducibility study demonstrate that experimenter performed measures, such as diameter measurements, are reproducible. Limitations in reproducibility appear to lie in statistical limitations with calculated variables and not in procedural practices or experimental setup. Therefore the FMD and SS_{AUC} values obtained in the current study are an accurate measure of changes in conduit artery diameter due to increased shear stress.

Chapter 5: Results

5.1: Participant Characteristics

Participants were young, normo-tensive (resting BP<140/90 mmHg), active men with a mean age of 27 ± 4 years (range: 22 to 35 years, n=10). All participants were physically fit reporting an exercise frequency of three or more times per week. Average height was 182 ±7 cm and average weight was 83 ±12 kg. The average forearm volume was 1000±81 ml and average lower leg volume was 2379 ±763 ml, (Appendix 1: Table 1). Resting BP and HR were taken prior to testing day 1 and 2 after the subject was lying supine for approximately 20 minutes. Average resting SBP was 120 ±5 mmHg, resting DBP was 68 ±5 mmHg and average resting heart rate was 61 ± 11 bpm (Appendix 1: Table 1).

5.2: Cardiovascular Data

HR, BP and $\dot{Q}$ were recorded throughout the entire duration of testing. There were no biologically significant changes in BP, HR or $\dot{Q}$ between baseline, occlusion, and deflation for any participant in any of the arteries examined (Figures 8a & 8b). Furthermore, one-minute of exercise during occlusion, did not result in an increase in blood pressure of more than 20 mmHg or more for any subject (Appendix 2: Tables 2a-e).
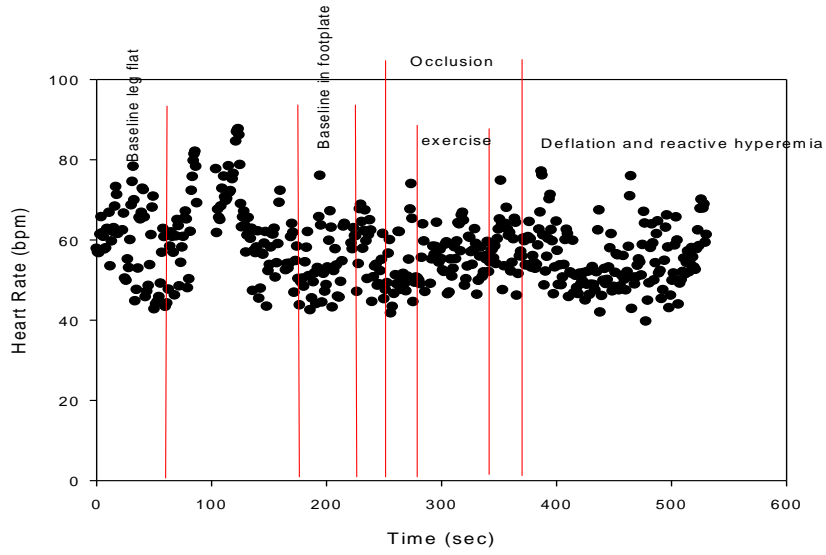
Figure 8a: A single participant's heart rate in beats per minute throughout the superficial femoral artery two-minute occlusion with one-minute of exercise protocol
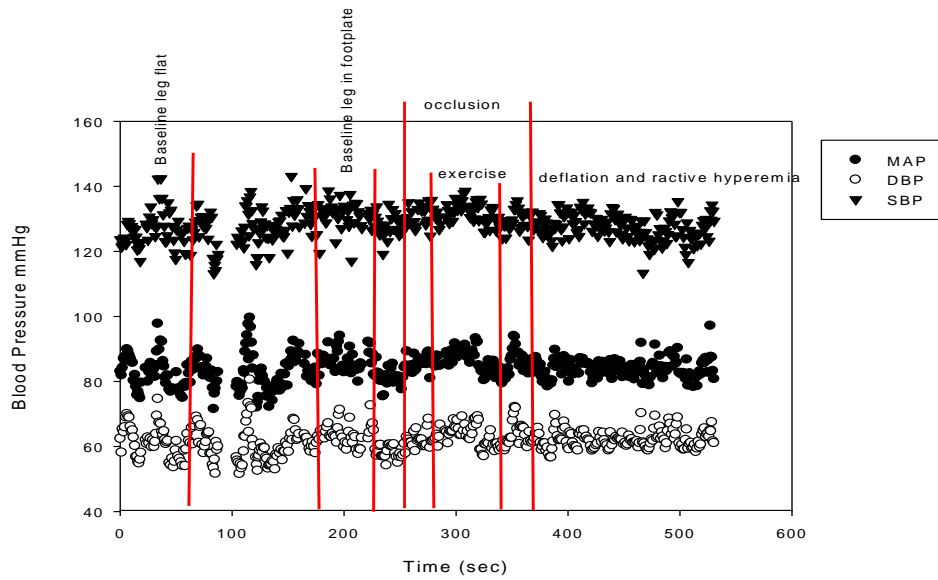
Figure 8b: A single participant's mean arterial pressure, systolic blood pressure and diastolic blood pressure during a two-minute occlusion with exercise protocol of the superficial femoral artery

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5.3: Arterial Baseline Parameters

A one way ANOVA and subsequent Tukey's test revealed that baseline BA diameter, conductance and shear stress were significantly different than baseline parameters of the PA and SFA ($p<0.05$). In fact, baseline BA shear stress was between three and one-half and four and one-half times larger than baseline SFA2, SFA5, PA2 or PA5 shear stress (Figure 9). BA vascular conductance was significantly higher than that of the SFA2, SFA5 and PA5; however, no significant difference was found between the BA and PA2. This could be due to the smaller sample size (n=8) in the PA2 condition as compared with the BA, where all 10 participants data could be analyzed (Figure 10). Vascular resistance was not significantly different between the three arteries examined. There was, however, a trend for slightly lower BA vascular resistance compared to the SFA2, SFA5, PA2 and PA5 conditions (Figure 11). Baseline diameter of the BA was significantly smaller than the SFA and PA. The BA was approximately 0.25 cm smaller than the two leg conduit arteries examined (Figure 12). Baseline BA diameter was 0.42$\pm$0.05cm, while average SFA5 artery diameter was 0.67$\pm$0.08cm, SFA2 was 0.69$\pm$0.09cm, PA5 diameter was 0.661$\pm$0.09cm and PA2 diameter was 0.66$\pm$0.09cm. There were no significant differences between the PA and SFA for either test for the above-mentioned parameters.
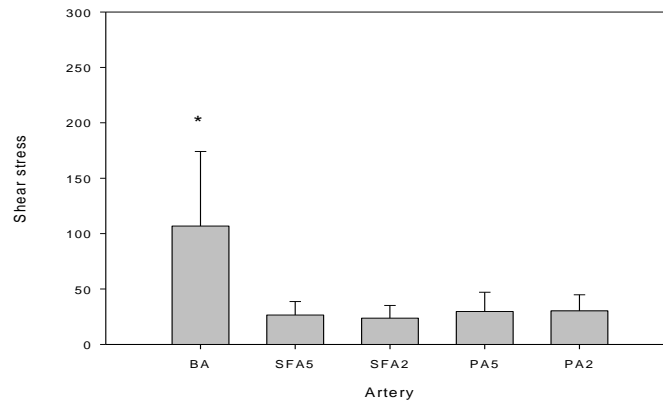
Figure 9: Average baseline shear stress in the brachial, superficial femoral and popliteal artery. Values are means ± standard deviation. * indicates significantly different than SFA2, SFA5, PA2 and PA5. Abbreviations: SFA5: superficial femoral artery five-minute occlusion, SFA2: superficial femoral artery two-minute occlusion, PA5: popliteal artery five-minute occlusion, PA2: popliteal artery two-minute occlusion

Figure 10: Average baseline vascular conductance of the brachial, superficial femoral and popliteal arteries before each occlusion protocol. Values are means ± standard deviation. * indicates significantly different than SFA2, SFA5, PA2 and PA5. Abbreviations: SFA5: superficial femoral artery five-minute occlusion, SFA2: superficial femoral artery two-minute occlusion, PA5: popliteal artery five-minute occlusion, PA2: popliteal artery two-minute occlusion.
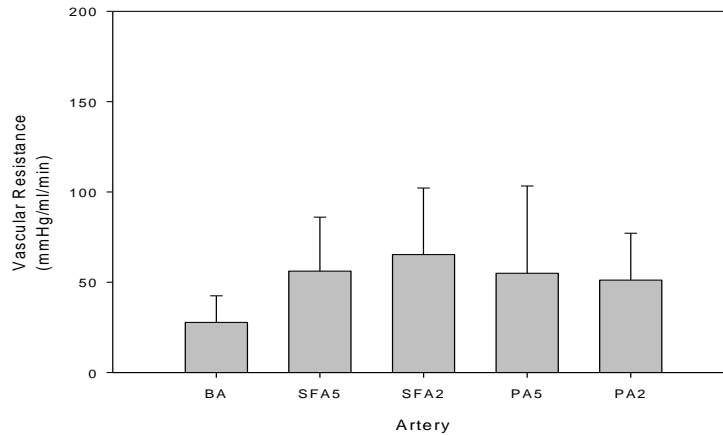
Figure 11: Average baseline vascular resistance of the brachial, superficial femoral and popliteal arteries before each occlusion protocol. Values are means $\pm$ standard deviation. Abbreviations: SFA5: superficial femoral artery five-minute occlusion, SFA2: superficial femoral artery two-minute occlusion, PA5: popliteal artery five-minute occlusion, PA2: popliteal artery two-minute occlusion.

Figure 12: Average baseline diameter of the brachial, superficial femoral and popliteal arteries before each occlusion protocol. Values are means $\pm$ standard deviation.* indicates significantly different than SFA2, SFA5, PA2 and PA5. Abbreviations: SFA5: superficial femoral artery five-minute occlusion, SFA2: superficial femoral artery two-minute occlusion, PA5: popliteal artery five-minute occlusion, PA2: popliteal artery two-minute occlusion

5.4: Two-and Five-Minute Occlusion of SFA and PA

As mentioned above there were no significant differences in baseline diameter, vascular resistance, vascular conductance or shear stress between the two leg conduit arteries examined, PA and SFA. Differences lay in the reactive hyperemia and FMD responses (Figure 13-16). As explained in chapter 3.6 a significance level of $p \leq 0.0125$ was used as the criterion for statistical significance. Any negative FMD value was considered as though no FMD i.e. FMD equal to zero; all positive percent FMD value were reported as is.

5.4.1Reactive Hyperemia and FMD Responses

5.4.1.1: SFA5 & PA5

Of the 10 men studied only two had a significant SFA FMD response ($FMD \geq 4\%$) following five-minutes of calf occlusion. Conversely, when examining the PA response to five-minutes of occlusion, only one participant did not have a FMD response of greater than 4% (Appendix 3:Table 3). There were statistically significant differences in peak shear, SS_{AUC} and FMD between the SFA5 and PA5 conditions (p=0.0037, 0.0108 and 0.0120 respectively, Figures 13-15). A borderline significant difference between SFA5 and PA5 FMD/SS_{AUC} was seen (p= 0.0349, Figure 16). No other significant differences were noticed when FMD was normalized, divided by, the elicited shear stimulus.

5.4.1.2: SFA5 and SFA2

Of the 10 participants examined in the current study, only nine sets of SFA2 - data could be analyzed. No participant demonstrated a SFA FMD response to two-minutes of calf occlusion (Appendix 3: Table 3). Furthermore, no significant differences in peak shear, SS_{AUC}, FMD, or FMD/SS_{AUC} were found between the SFA5 and SFA2 conditions (Figure 13-16). Therefore, neither SFA protocol was able to elicit a significant increase in shear stress or percent FMD.

5.4.1.3: PA5 and PA2

When two- minutes of calf occlusion with one-minute of exercise was performed on the PA only 8 participants' data could be analyzed. Of those, no significant increase in diameter was seen for three participants while the other five had an FMD response of greater than 4% (Appendix 3: Table 3). As mentioned above, 9 out of 10 participants had a significant FMD response to the PA5 protocol. With regards to peak shear, a borderline non-significant (p=0.0147) difference in peak shear was noticed. Based on the peak shear stress and SS_{AUC} values recorded (Appendix 3: Table 4), it is likely that there is a significant difference in peak shear between the five and two minute occlusion protocols on the PA (Figure 13). There was a significant difference (p=0.0079) in the post occlusion shear response, SS_{AUC}; however, there was no significant difference (p=0.396) in the percent PA FMD following either the five or two-minute occlusion protocol (Figures 14-16).

5.4.1.4: PA2 and SFA2

Comparing the two two-minute occlusion protocols revealed a few interesting findings. No participants had an FMD response in SFA2 protocol. Conversely, in the PA2 condition, 5 out of eight men had a significant FMD response. The baseline diameters between these two arteries were not significantly different (Figure 12, p= 0.3086). Furthermore, no significant differences in baseline vascular resistance, conductance or shear were observed (Figures 9-11). There was no significant difference in peak shear (Figure 13, p=0.0489), while there was a borderline significant difference in SS_{AUC} (Figure 15, p=0.0173). Therefore, when exposed to two-min of calf occlusion with one minute of exercise, the PA is more sensitive to the elicited shear stimulus and can produce a FMD response while the SFA cannot.
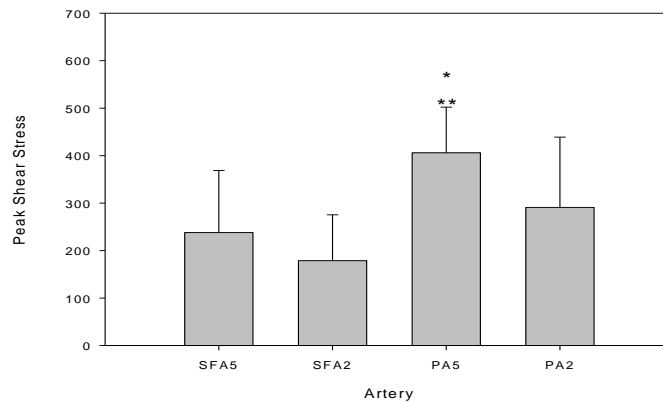
Figure 13: Peak shear stress in the superficial femoral and popliteal arteries after either two-or five minutes of occlusion. Values are means $\pm$ standard deviation. * indicates significant difference compared with SFA5. ** indicates significant differences compared with PA2. Abbreviations: SFA5: superficial femoral artery five-minute occlusion, SFA2: superficial femoral artery two-minute occlusion, PA5: popliteal artery five-minute occlusion, PA2: popliteal artery two-minute occlusion
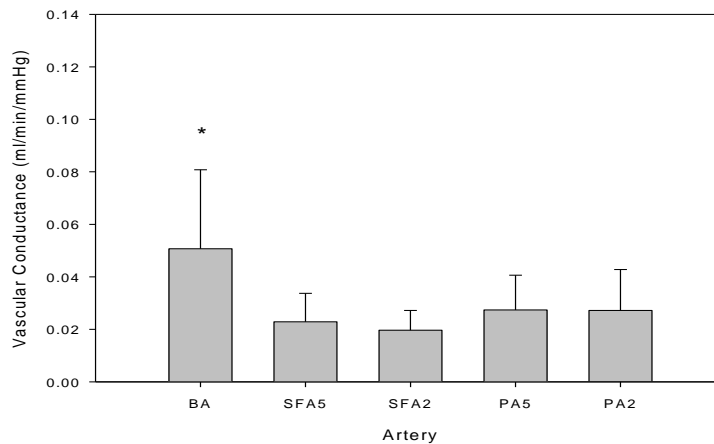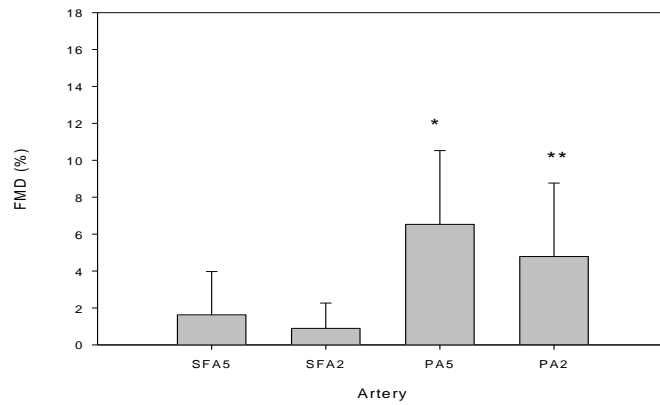
Figure 14: Percent flow mediated dilation in the brachial, superficial femoral and popliteal arteries after either two-or five-minutes of occlusion. Values are means ± standard deviation.* indicates significant difference compared to SFA5 ** indicates significant difference compared with SFA2. Abbreviations: SFA5: superficial femoral artery five-minute occlusion, SFA2: superficial femoral artery two-minute occlusion, PA5: popliteal artery five-minute occlusion, PA2: popliteal artery two-minute occlusion
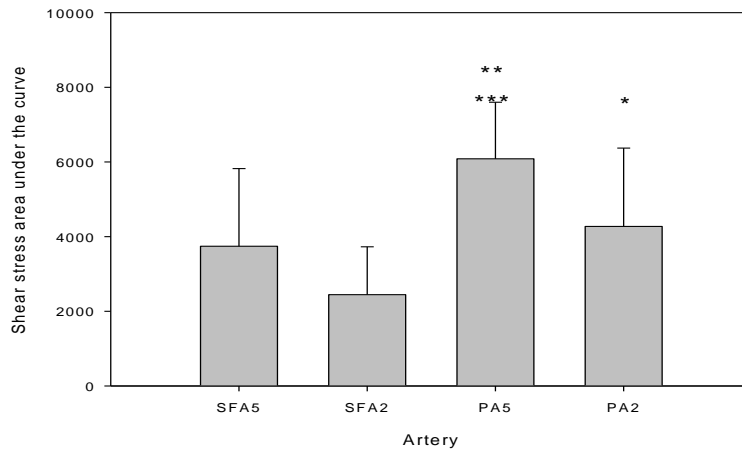

Figure 15: Shear stress area under the curve in the superficial femoral and popliteal arteries after either five-or-two-minute of occlusion. Values are means ± standard deviation * indicates a significant difference compared to SFA2. ** indicates a significant difference compared with SFA5. *** indicates a significant difference compared with PA2. Abbreviations: SFA5: superficial femoral artery five-minute occlusion, SFA2: superficial femoral artery two-minute occlusion, PA5: popliteal artery five-minute occlusion, PA2: popliteal artery two-minute occlusion
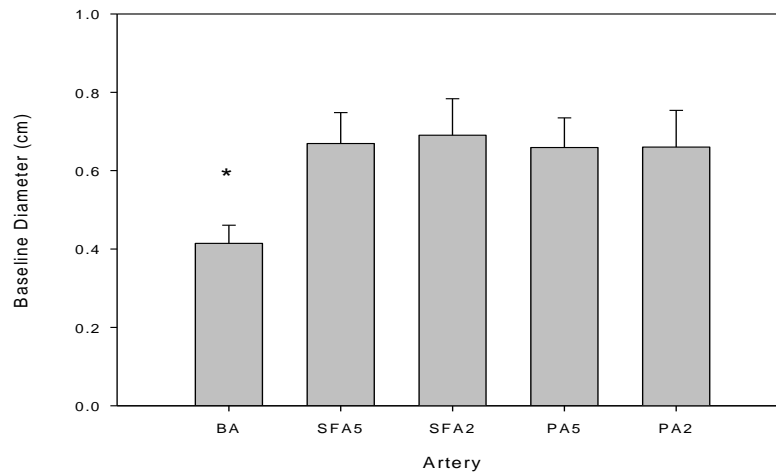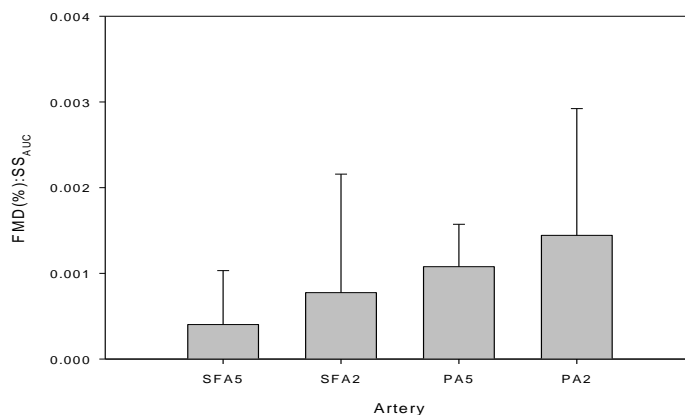
Figure 16: Ratio of percent flow-mediated dilation to shear stress area under the curve. Values are means $\pm$ standard deviation. Abbreviations: SFA5: superficial femoral artery five-minute occlusion, SFA2: superficial femoral artery two-minute occlusion, PA5: popliteal artery five-minute occlusion, PA2: popliteal artery two-minute occlusion

5.5: Five-Minute Occlusion of BA, SFA, PA

To examine the effect of artery location, i.e. upper and lower extremity, on the FMD response a five-minute occlusion was performed on the BA, SFA and PA. Baseline BA diameter was significantly smaller than that of the SFA5 and PA5; however, no difference between SFA5 and PA5 baseline diameter were found (Figure 12). Baseline shear stress and vascular conductance were significantly elevated in the BA compared with the SFA5 and PA5 (Figures 9 & 10), while there were no significant differences between the SFA5 and PA5. No significant differences in baseline vascular resistance were noted between the three arteries examined (Figure 11).

Using a one-way ANOVA, with significance determined for values of $p<0.05$, revealed significant differences between the FMD responses (F=7.51, p=0.0043), and SS_{AUC} (F=46.95, $p<0.001$) of the BA, SFA and PA. A post-hoc analysis, Tukey test, with a significance level of 0.05, revealed that that the FMD response of the BA and PA were not statistically different. However, the FMD responses of the BA and PA were significantly different than that of the SFA (Figure 17). Furthermore, all three arteries had statistically different SS_{AUC} (Figure 18). When

the FMD response was normalized to the elicited shear stimulus, FMD/SS$_{AUC}$, no significant differences were seen. There was a trend towards a lower FMD:SS$_{AUC}$ in the SFA5 compared with the BA and PA5 (Figure 19).
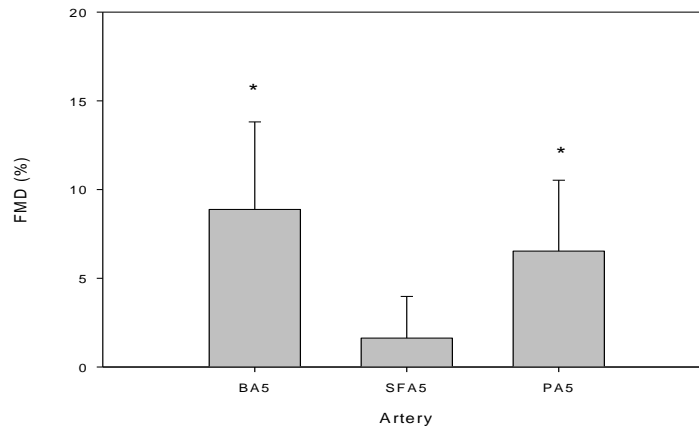
Figure 17: Percent flow-mediated dilation of the brachial, superficial femoral and popliteal arteries after five-minutes of distal occlusion. Values are means $\pm$ standard deviation.* indicates significant difference as compared to the SFA. Abbreviations: BA5: brachial artery five-minute occlusion, SFA5: superficial femoral five-minute occlusion, PA5: popliteal artery five-minute occlusion.
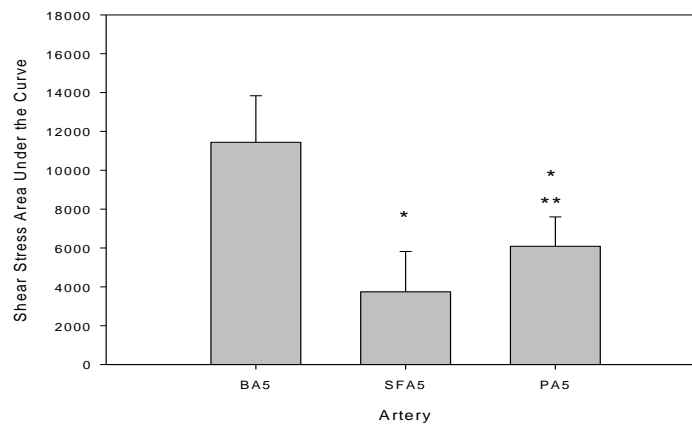
Figure 18: Shear stress area under the curve of the brachial, superficial femoral and popliteal arteries after five-minutes of distal occlusion. Values are means $\pm$ standard deviation. * indicates significant difference as compared with the BA. ** indicates significant difference as compared with the SFA. Abbreviations: BA5: brachial artery five-minute occlusion, SFA5: superficial femoral five-minute occlusion, PA5: popliteal artery five-minute occlusion.
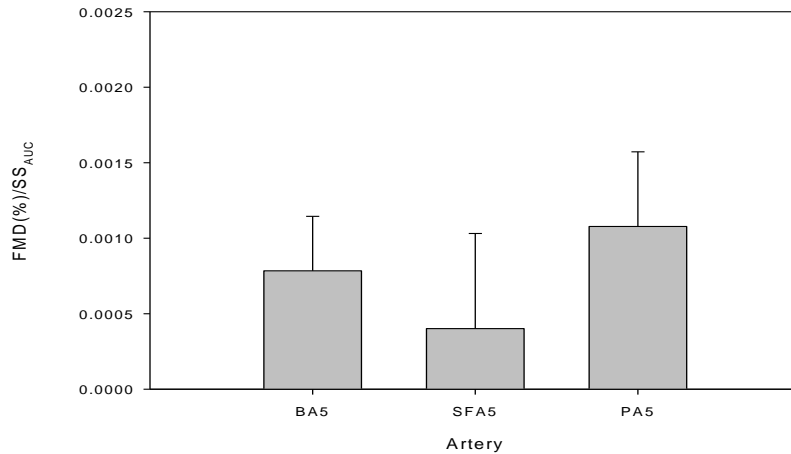
Figure 19: Ratio of percent flow-mediated dilation to shear stress area under the curve of the brachial, superficial femoral and popliteal artery after five-minutes of distal. Values are means $\pm$ standard deviation. Abbreviations: BA5: brachial artery five-minute occlusion, SFA5: superficial femoral five-minute occlusion, PA5: popliteal artery five-minute occlusion.

Within–artery comparisons were performed on the SFA, PA and BA. When examining the differences between arm and leg conduit arteries the PA was compared to the BA. SFA data were not compared to those of the BA, because the current protocol using five-minutes of calf occlusion was unable to elicit a FMD response larger than 4%.

When SS_{AUC} was plotted against diameter, a strong negative correlation was seen for both the BA and PA (BA r=-0.6321, PA r=-0.7782, p<0.05). These data suggest that the larger the arterial diameter the smaller the SS_{AUC} response (Figure 20). When percent FMD and FMD:SS_{AUC} are plotted against baseline diameter, no significant trends are reported (Figures 21 and 22).
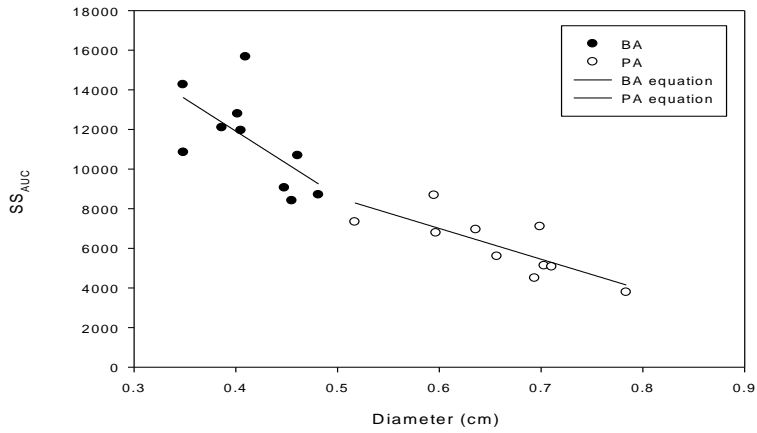
Figure 20: Shear stress area under the curve plotted against baseline diameter for the brachial and popliteal arteries. Data points represent individual subject values.
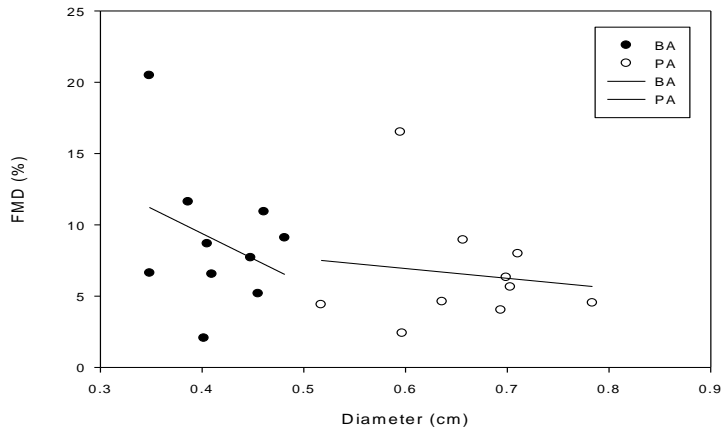
Figure 21: Percent flow-mediated dilation plotted against baseline diameter for the brachial and popliteal arteries. Data points represent individual subject values.
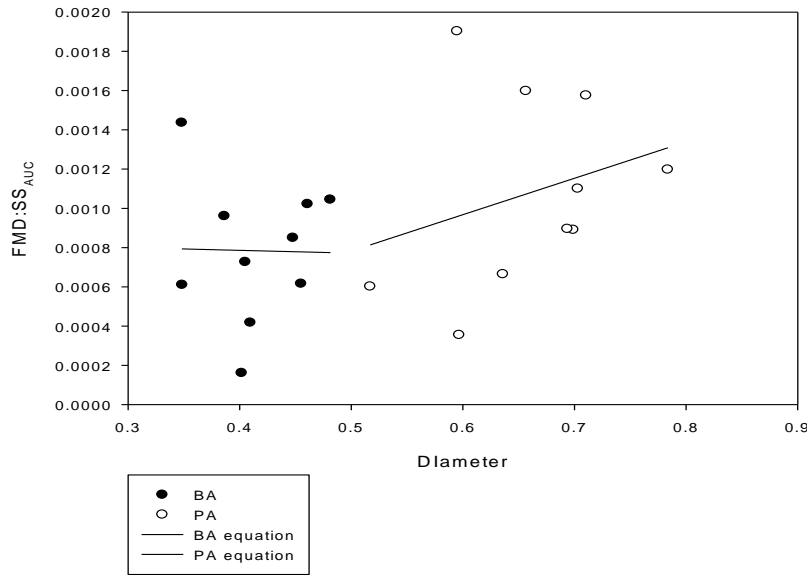
Figure 22: Flow-mediated dilation normalized to shear stress area under the curve plotted against baseline diameter of the brachial and popliteal arteries. Data points represent individual subject values.

Comparison of the two leg conduit artery responses to five-minutes of distal calf occlusion was examined via correlation plots. Correlation graphs of percent FMD, SSAUC and FMD:SS_{AUC} as a function of baseline diameter for the PA and SFA post five-minutes of calf occlusion are seen below (Figures 23-25). When SS_{AUC} was plotted against baseline diameter a strong negative correlation (r= -0.7782) was seen in the PA, while a weak negative correlation (r=-0.1768) was seen in the SFA. When percent FMD was plotted as a function of baseline diameter a weak negative correlation was seen for both the SFA and PA (PA r= -0.1296, SFA r=0.0250). Similarly, the plot of FMD:SS_{AUC} as a function of baseline diameter revealed weak correlation for the PA (r=0.284) and no noticeable trend for the SFA (r= -0.0596).
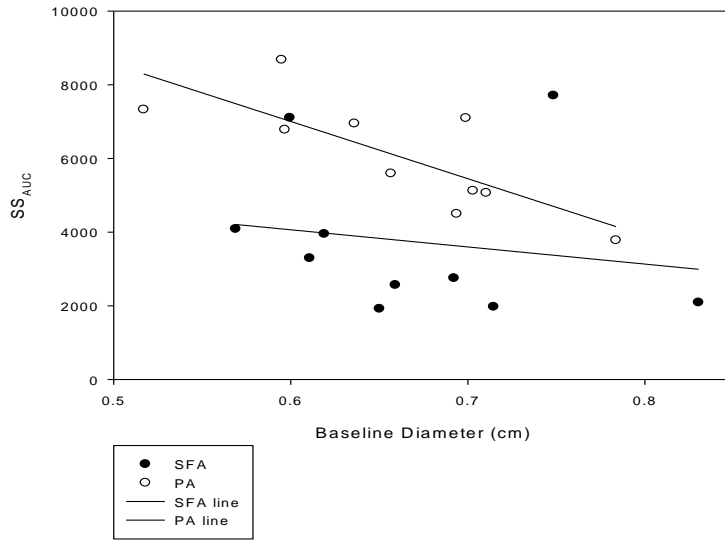
Figure 23: Shear stress area under the curve plotted against baseline diameter for the popliteal and superficial femoral arteries. Data points represent individual subject values.
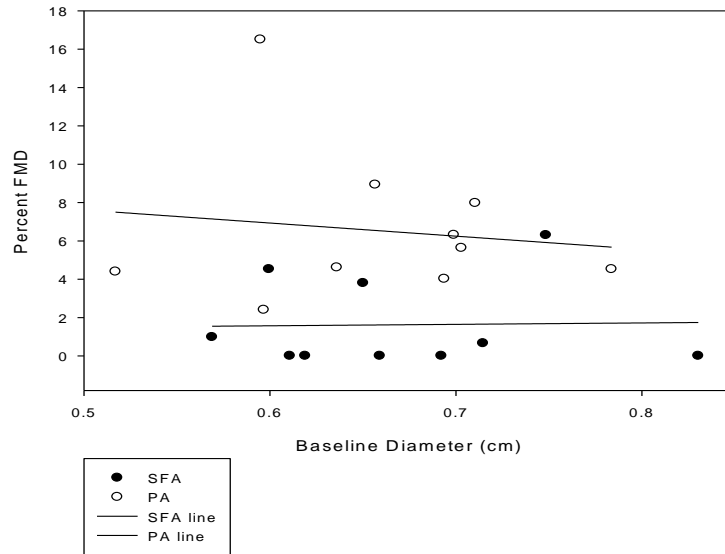
Figure 24: Percent flow-mediated dilation plotted against baseline diameter for the popliteal and superficial femoral arteries. Data points represent individual subject values
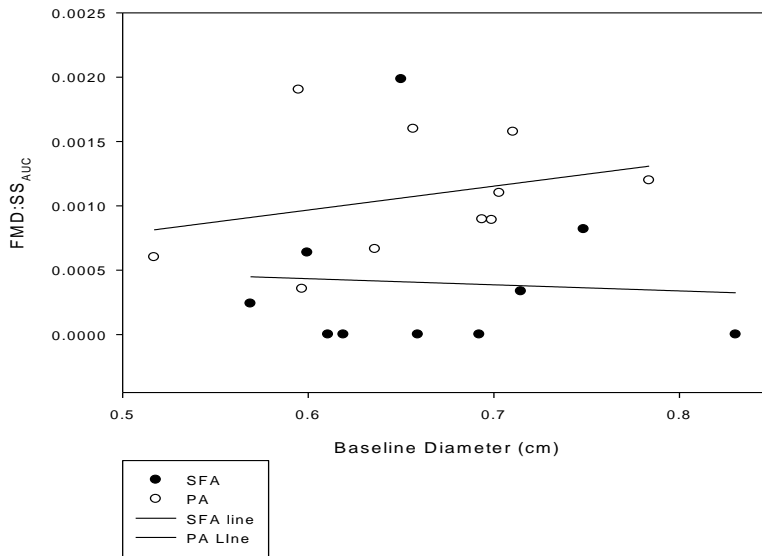
Figure 25: Flow-mediated dilation normalized to shear stress area under the curve plotted against baseline diameter for the popliteal and superficial femoral arteries. Data points represent individual subject values

5.6: Edge and Center Method of Determining Artery Diameter

In the current study seven participants BA were analyzed using both the traditional edge-detection method and the center-based method. The usefulness of the new method was compared using a paired T-test; a significance level of $p<0.05$ was used. Using the center-based method did not produce a significant difference in percent FMD as compared with the edge-detection method (F=0.05, p=0.8259, Figure 26). Furthermore, the time to FMD was not significantly different between the two methods (F=2.08, p=0.2996, Figure 27). An example of one subject's FMD profile can be seen in Figure 28. Although the center-based method did not result in a significantly different FMD response, using the center-based method resulted in a larger measure of diameter at all time points; the direction of this trend does not hold true for all subjects (See Appendix 4: Table 4).
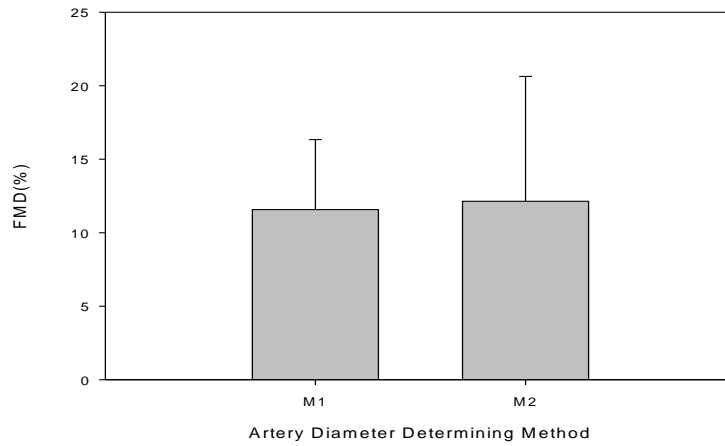
Figure 26: Percent flow-mediated dilation determined using the traditional edge detection method (M1) and the new center-based method (M2). No significant differences were seen.
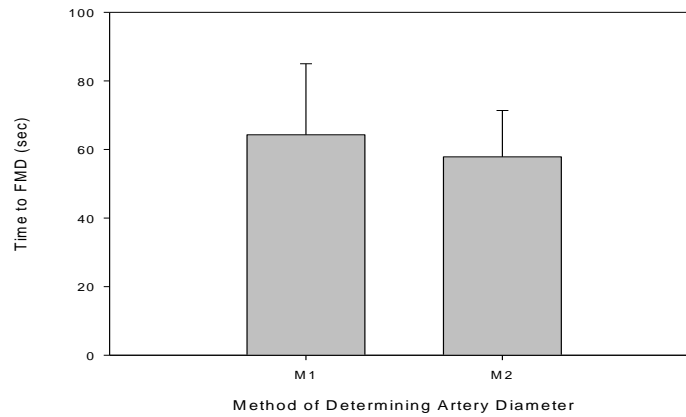
Figure 27: Time to flow-mediated dilation determined using the traditional edge-detection method (M1) and the new center-based method (M2). No significant differences were seen.
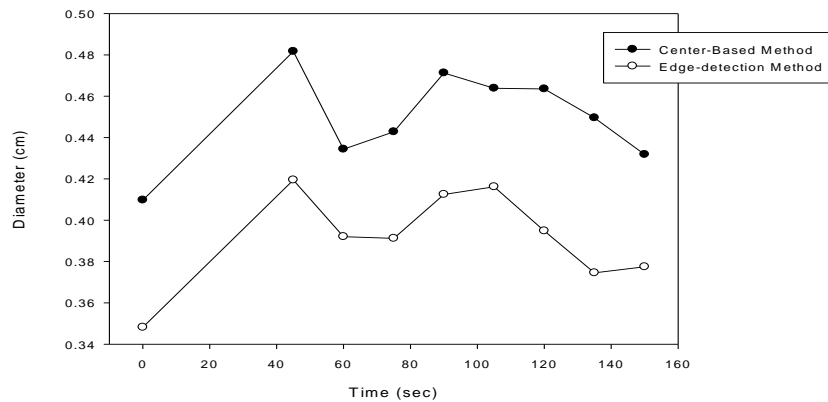
Figure 28: A single participant's diameters measured from baseline (time 0) through till 2:30min post deflation. The open circles represent diameters determined with the traditional edge detection method, while the closed circles represent diameters determined via the center-based method.

A Bland-Altman plot was used to compare the percent FMD results obtained using the center-based and edge-detection method (Figure 29); a full table associated with this plot can be seen in Appendix 4, Table 5. The Bland-Altman plot demonstrates that the majority of the data, except one data point, falls within the associated 95% confidence interval or two standard deviations of mean difference between the two methods. This plot further strengthens the usefulness of the center-based method of determining BA diameter.
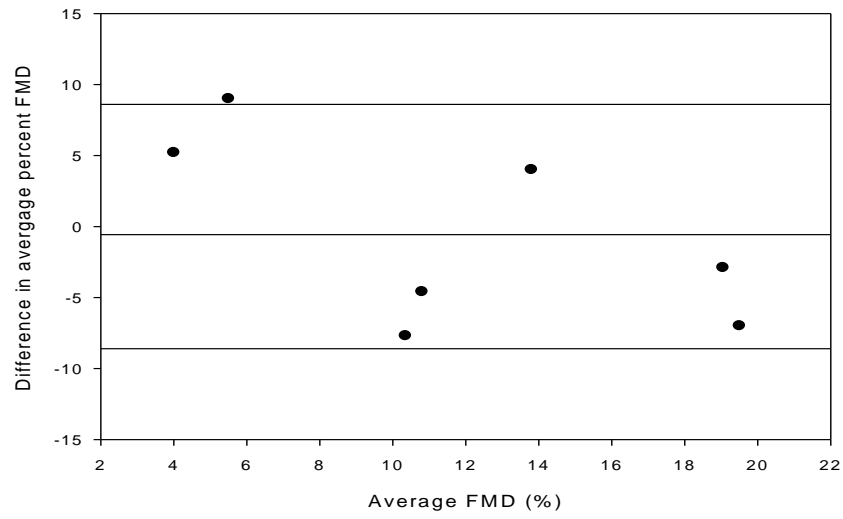
Figure 29: Bland Altman plot of the difference in percent FMD between the center-based and edge-detection methods of determining arterial diameter

Chapter 6: Discussion

Before the current study was undertaken a reproducibility study was performed which determined that the FMD results observed are reproducible. This was accomplished by testing the hypothesis that there would be no significant differences in baseline diameter, peak diameter, percent FMD, peak shear stress, SS_{AUC}, and time to FMD. Once the reproducibility study was completed the main study set out to examine the following hypotheses: First, the shear stress and FMD responses of the SFA and PA will not be significantly different due to a five-minute occlusion protocol, thereby permitting leg conduit artery FMD tests to be performed on the more accessible SFA. Secondly, no significant difference in FMD and shear stress responses between the five and two-minute occlusion protocols will be seen for either the SFA or the PA, allowing a shorter duration occlusion protocol to be used. Thirdly, no significant difference in the FMD and shear stress responses will be seen between the two-minute occlusion protocol performed on the SFA and PA. Fourthly, significant difference between BA, SFA and PA percent FMD and shear stress responses to five-minutes of occlusion will be used to examine the effects that baseline diameter and extremity have on endothelial function. Finally, the use of the center-based method of measuring artery diameter will be validated; no significant differences were expected in percent FMD and time to FMD using the edge-detection as compared with the center-based method.

6.1: Main Findings

The current project set out to answer the above-mentioned five hypotheses; the results of the current study support all of the hypotheses. The main findings of the current experiment are 1) Results of the reproducibility demonstrated that baseline and peak diameter measurements are reproducible (see Chapter 4); 2)The baseline characteristics (shear stress, diameter, vascular resistance and vascular conductance) of the BA are significantly different than those of the SFA and PA; however, there are no significant differences between SFA and PA baseline characteristics; 3) The reactive hyperemia shear stress and FMD responses of BA due to five-minutes of distal occlusion are significantly different than those of the PA and SFA; 4) Five-minutes of lower limb occlusion results in significantly different peak shear stress, SS_{AUC} and FMD responses between the SFA and PA; 5) When testing the PA, a two-minute occlusion containing one minute of exercise can be used instead of a five-minute occlusion; however, the same does not hold true for the SFA; 6) Preliminary testing demonstrated the usefulness of using the center-based method of measuring arterial diameter instead of the edge-detection method.

6.2: Baseline Characteristics

The BA, SFA and PA baseline diameter values reported in the current study are in line with previous research; BA baseline diameter was 0.42 cm, SFA was 0.67 cm and the PA was 0.69 cm. Previous research has reported PA baseline diameter ranges between of 0.60 and 0.7cm (48; 50; 69). With regards to the SFA a wider range of baseline diameters have been reported; Thijssen et al. reported SFA baseline diameter to be 0.77cm (69), while Walther et al. reported a SFA baseline diameter of 0.63 cm in young healthy normotensive males. BA baseline diameter has been reported to range from 0.40 cm to 0.47 cm (36; 69; 73).

In the current study no significant differences were seen between baseline diameters of the SFA and PA. Furthermore, a wide range of baseline SFA diameters have been reported in the literature. These findings can, at least partially, be explained by the location of diameter measurements. Previous SFA FMD protocols have used distal thigh occlusions, and therefore arterial diameter must have been measured farther up on a participant's thigh, closer to the hip. Conversely, in the current study a distal calf occlusion was used which allowed SFA diameter measurements to be taken lower on a participant's thigh, closer to the knee. The location of diameter measurement can have a significant impact on baseline and peak diameter. A MRI study performed by Wood et al. suggested that as the SFA descends the thigh diameter decreases by approximately 5% (79). In the current study, SFA diameter was measured approximately 11 cm or four inches from the medial epdicondyl. Thus, the lower SFA diameter reported in the current study compared to the work of Thijssen et al.(69) and Kooijman et al. (36) can largely be explained by the fact that in the current study SFA diameter was measured at a more distal location.

Based on available literature, baseline characteristics of the BA were expected, and in fact were, significantly different than the PA and SFA (Figure 9-12). Differences in baseline diameter between the upper and lower extremity has been attributed to adaptations to the force of gravity (72) and due to differences in the volume of tissue perfused by the arteries (48). The greater muscle mass perfused by the SFA and PA results in a higher absolute blood flow and percent Q in the legs compared to the arms. Exposure to a greater volume of blood and thus shear stress may also lead to vascular smooth muscle cell and EC adaptations.

An important factor regulating the differences in limb conduit artery baseline tone is vascular adaptations due to the effects of gravity(16). On average, North Americans spend greater than two-thirds of their day in an upright posture; which exposes the leg to 65 mmHg greater pressure than the arms (39). Eiken & Kolegard speculated that the lower limb has adapted to this increased gravitational pressure via "vascular stiffening" (20). This is reflected in the different factors mediating baseline vascular tone in the arms compared to the legs; leg conduit arteries experience greater SNS vasoconstrictor mediation (52), whereas the BA is more sensitive to circulating vasodilators (45). Leg "vascular stiffening" has been suggested to affect the decreased responsiveness due to increased shear stress (48) and the reason behind the increased susceptibility of the SFA to atherosclerotic lesion formation (51). More work is still needed to understand the importance of these differences and how they can affect FMD.

In the current study, baseline BA shear stress was approximately 75% larger than that of the PA or SFA. This is supported by baseline wall shear rate data from Wu et al., who reported that BA mean wall shear rate is approximately 50% larger than that of the SFA (80). Variation in the magnitude of the limb differences in baseline shear stress between current study and the data presented by Wu et al. are likely due to methodological differences; Wu et al. used MRI images

where as in the current study Doppler Ultrasound was used(80). Newcomer et al. confirmed the finding of Wu et al. using Doppler ultrasound. Newcomer and colleagues reported that when a subject is resting in the supine position, BA shear stress is significantly larger, 40%, than that of the SFA (46); furthermore, Nishiyama et al. reported that BA shear stress is 37.5% larger that of the PA (48). Animal research has suggested that lower shear stress may result in down-regulation of eNOS resulting in a decrease in NO available to stimulate underlying vascular smooth muscle cells. If this holds true for humans, then the lower resting shear stress of the PA and SFA would result in less NO being available to respond to the RH stimuli post limb occlusion and thus a smaller FMD response (25). It is apparent that differences in BA, SFA and PA baseline shear stress contribute to limb differences in FMD. More work is still needed to understand the pathways affected by the lower shear stress experienced by leg conduit arteries and how these changes relate to lower limb FMD responses.

6.3: Normalizing a Flow-Mediated Dilation Response

In the current study FMD responses are reported as a percent increase of baseline diameter and normalized to SS_{AUC} until 20 seconds post deflation. Pyke & Tschokovsky recommended that the change in diameter needs to be reported in reference to the magnitude of the shear stimulus, which is believed to be the primary mechanism guiding the increase in diameter (56). They recommended that the shear stimulus be recorded until the achievement of peak diameter. In the present study the ultrasound machine used was unable to capture simultaneous arterial diameter and velocity measurements. This technological limitation prohibited SS_{AUC} from being measured until peak diameter was achieved; instead SS_{AUC} was calculated until 20 seconds post deflation. This time point was chosen because it encompassed peak shear stress for all subjects. To elaborate, the post deflation increase in shear stimulus peaks and decays long before peak

diameter is achieved; however, shear stress does not return to baseline levels until a few minutes post deflation (56). Furthermore, research has demonstrated that both peak shear stress and the duration of the shear stimulus are determinants of peak FMD (31). Conversely, Dyson reported that peak shear is not an important determinant of a FMD response(19). As the importance of peak shear or total shear stress has yet to be uncovered, the current study calculated SS_{AUC} until just after peak shear was accomplished. Throughout the discussion, FMD will be reported as both a percent and normalized to SS_{AUC}.

6.4: Differences in Popliteal and Superficial Femoral Artery FMD Responses

6.4.1: PA & SFA FMD Responses to Five-Minutes of Occlusion

The current study examined the shear stress and FMD responses of the SFA and PA to the same occlusion protocol; a five-minute distal calf occlusion at 220 mmHg. Of the 10 participants tested in the current study, only two had a SFA FMD response greater than 4%. These findings are not in agreement with previous investigations that have used thigh cuffs. Previous investigations (13; 64; 65; 70; 73; 73) have reported SFA FMD between 4% and 7.5%, while one study (73) reported SFA FMD to be 2%, which is more in line with value obtained in the current study (Appendix 5: Table 6) post five-minutes of distal thigh occlusion. Conversely, the current study reported that PA FMD was 6.5%, and nine out of ten participants had a "clinically" significant (>4%) FMD response. The average PA FMD response found in the current study is in agreement with previous literature, that has reported PA FMD values of 6.1%(69), 4.5%(48) and 4% (51). In the current study, when FMD was normalized to SS_{AUC}, no significant differences between PA5 and SFA5 were noted; however, there is a noticeably lower

FMD/SS$_{AUC}$ of the SFA5 compared to the PA5. The lack of significance is most likely due to the large standard deviation associated with these values.

The inability of the distal calf occlusion protocol to elicit a SFA FMD greater than 4% may be due to differences in reactive hyperemia shear stress response produced post cuff release. Five-minutes of SFA occlusion elicited an average peak shear of approximately 200 s^{-1}, which is significantly lower than PA peak shear, approximately 400 s^{-1}. When the SS$_{AUC}$ was calculated, until 20 seconds post deflation, average SFA SS$_{AUC}$ was 3740 and average PA SS$_{AUC}$ was 6085. These data demonstrate that calf occlusions can elicit a strong enough shear stimulus in the PA but not in the SFA. Previous research has shown that thigh cuffs are able to elicit a strong shear stress reaction in the SFA; Thijssen et al. (2008) reported that SFA SS$_{AUC}$ until peak diameter (172 seconds post deflation), was 14541 (69). The work of Thijssen et al. suggested that peak SFA diameter, post cuff release, occurred almost two and a half minutes post deflation. In the current study, SFA diameter was not measured past two and half minutes post deflation(69), which could mean that peak diameter and thus the true FMD response was not measured. However, caution must be taken when analyzing the results of Thjissen et al. as these researchers reported a longer time to peak diameter for all arteries examined as compared to previous researchers. Briefly, Thijssen et al. reported that peak BA was reached at 85 seconds post deflation (69) whereas Padilla et al. reported that peak diameter was reached around 60 seconds post deflation (51). Most studies report peak diameter to occur well under two and a half minutes post deflation; furthermore, in the current study after peak diameter was seen arterial diameter began to return to baseline values (Figure 28). Based on these findings, it is unlikely that peak diameter was missed and therefore the lack of a SFA FMD response in the majority of participants might be attributed to the small shear stress response. The weak shear stress and low

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FMD response of the SFA to distal calf occlusions suggests that this occlusion protocol cannot be used to examine SFA endothelial health. If SFA function is to be examined then previous literature suggests that a distal occlusion with the cuff wrapped around the participant's thigh is the most appropriate protocol.

Based on the similarity in magnitude of SFA and PA FMD values reported by other researchers (36; 48; 51; 65; 69) it is tempting to conclude that either the SFA or PA could be used to examine lower limb vascular health. However, results from Walther et al. and Silber et al. contradict this idea by reporting small SFA FMDs of 2% (73) and 3.5% (64; 73) respectively. These values are much lower than the above reported PA FMD values and call in to question the ability of the SFA to be examined in place of the PA. Furthermore, results from the current study support the notion that the SFA cannot be used in place of the PA to examine lower limb conduit artery function.

The lack of a FMD response in the SFA when exposed to the same occlusion protocol as that used to examine PA FMD was an unexpected finding. Previous work examining SFA FMD placed the pneumatic cuff around the participant's thigh, just above the knee. Conversely, when examining the PA, the cuff is placed around the participant's calf, just below the knee joint (Appendix 5: Table 6). The location of diameter and velocity measures could explain the wide range of baseline SFA diameters reported in the literature and can partially explain the lack of SFA FMD reported in the current study. It is possible that there could be minor anatomical and physiological differences between the proximal and distal ends of the SFA that could alter the artery's ability to respond to shear stress and produce a FMD response to the reactive hyperemia following temporary limb ischemia. More work is needed to investigate this issue and determine

the optimal measurement site and stimulus protocol for the SFA when performing a FMD protocol.

As outlined in the BA FMD guidelines, inflating a pneumatic cuff to pressures at least 50 mmHg above systolic blood pressure results in tissue ischemia distal to the occlusion site. This ischemia results in dilation of downstream resistance vessels which drives the high flow state post deflation necessary to increase blood supply to the dilated resistance vessels (14). Placing the pneumatic cuff around the participant's thigh may result in a larger amount of tissue being ischemic compared to calf occlusions. The increased volume of occluded tissue may cause more metabolites to be produced and accumulate in downstream resistance vessels. The increased volume of occluded tissue could cause a greater reactive hyperemia and in turn larger shear stress stimulus upon cuff release. However, there is no branching in the SFA as it descends the thigh, suggesting that in the tissues supplied by the SFA there may not be an increase in ischemic tissue volume and a subsequently larger reactive hyperemia due to a thigh versus calf cuff placement. More work is needed to uncover if there are differences in distal micro-vessel resistance due to differences in cuff placements or whether there are differences in the properties of the SFA as it descends the leg and becomes the PA. It is possible that on top of a decrease in arterial diameter as the SFA descends the thigh (79) there could be differences in the receptor number and or function at different artery locations. It is important to study theses differences in order to uncover the possible contributor(s) to the lack of SFA FMD reported in the current study when a distal calf cuff was used. Therefore, it is possible that differences in cuff placement between the current study and other investigations (36; 65; 69), although all distal to the site of measurement, might explain why the SFA had a FMD response in previous studies but not in the current one.

In conclusion, the results of the current study demonstrate that temporary limb ischemia due to distal calf occlusion can elicit a PA FMD response but not a SFA FMD response. More work is needed to determine the optimal leg conduit artery FMD protocol so that researchers and clinicians can fully understand leg conduit FMD and its inferences for a participant's overall vascular health.

6.4.2: Comparison of PA FMD in Response to either a Five or Two-Minute Occlusion

Previous experiments on BA FMD have manipulated occlusion duration to determine the shortest occlusion protocol that can be used to achieve a significant FMD response. Nishiyama et al. manipulated occlusion duration using distal cuff placement. With a five-minute occlusion, FMD was 6.5%, conversely with a three-minute occlusion FMD was dramatically reduced to 0.7%; furthermore, this trend holds when FMD data were normalized the SS_{AUC} (48). These data suggest that shorter duration distal occlusions, without the addition of exercise, are insufficient to increase shear stress and elicit a significant BA FMD response. The above findings are supported by a recent study by Padilla et al.; they reported that occlusion durations less than four-minutes are unable to elicit a large enough shear stress to produce a FMD response similar to that produced after five-minutes of occlusion (50). However, when the FMD response was normalized to the SS_{AUC}, no differences due to occlusion duration were seen (50). Therefore, based on the findings of Padilla et al. (50) five-minutes of forearm occlusion is the shortest occlusion that can be used to assess endothelial function and NO bio-availability.

The current study aimed to examine if a shorter duration occlusion protocol could achieve significant FMD response. In the lower limb, the traditional five-minute occlusion was compared to a two-minute occlusion containing one-minute of exercise. One minute of exercise was added

in order to help augment the shear stimulus; participants were asked to apply a pressure of 100 mmHg to the footplate. As young healthy males were examined this amount of force was considered light exercise. PA FMD, both percent and normalized to SS_{AUC}, was not significantly different when the shorter duration protocol was used. The shorter occlusion protocol elicited a significant FMD response in five of the eight participants. It would be useful to examine two-minute occlusions containing a slightly harder exercise protocol in order to produce a slightly larger shear stress response and therefore a significant FMD response (>4%) in all participants.

Although peak shear stress and SS_{AUC} were significantly different between the five and two-minute protocols, the elicited stimulus in both cases was sufficient to cause a significant increase in diameter. When the ratio of FMD: SS_{AUC} was examined, no significant differences between the two protocols were seen. It appears that two-minutes of occlusion with one-minute of exercise can be used to instead of the traditional five minute protocol to examine PA FMD. The current study appears to be the first to demonstrate the usefulness of shorter duration occlusions of the PA to elicit a significant FMD response.

6.4.3: Comparison of SFA Responses to Five and Two-Minutes of Calf Occlusion

This study determined that five-minutes of calf occlusion only elicited a significant SFA FMD (>4%) in 20% of the subjects. When the two-minute protocol was performed on the SFA, exercise was added to augment the shear stimulus; however, the occlusion duration, even with an exercise component, was insufficient to elicit a significant increase in percent FMD. No significant difference between SFA2 and SFA5 peak shear and SS_{AUC} were noted; however, there was a trend for SFA2 to have a lower peak shear and SS_{AUC} than the SFA5 condition. It appears that this decrease was sufficient to abolish an FMD response from being produced. When the FMD: SS_{AUC} ratio was examined, no significant difference was seen; however, both data sets contained large standard deviations; therefore care must be taken when interpreting those data. Based on the above findings it can be concluded that both the two and five-minute calf occlusion protocols are unable to elicit a significant SFA FMD response.

In summary, five-or two-minute calf occlusions can be performed on the PA to elicit a significant FMD response. However, when examining the SFA, short duration calf occlusions are unable to elicit a sufficient stimulus to cause an FMD response. Therefore, based on previous research, examination of the SFA can only be performed when distal thigh occlusions are used.

6.5: Limb differences in Shear Stress and FMD after Five-Minutes of Occlusion

Previous research has validated the usefulness of studying BA FMD as the magnitude of the FMD response provides a good indicator of the participant's endothelial health and NO bio-availability. In patients with CVD, or its associated risk factors, BA FMD is found to be less than 4% (13). However, it is important to examine lower limb FMD responses because recent studies have reported that the SFA demonstrates a higher propensity than the BA of developing atherosclerotic lesions (43).

In the current experiment, the SFA, PA and BA were all subjected to five-minutes of distal occlusion at 220 mmHg. The BA (8.9%) and PA (6.5%) FMD values obtained in the current study are in line with those obtained by previous researchers (Appendix 5: Table 6). It is difficult to compare the SFA FMD results obtained in the current study to results from previous experiments because different cuff positions were used. In the present experiment SFA FMD was, 1.6% FMD which disagrees with previous literature reporting SFA FMD to range between 4 and 7.5%. As highlighted above (Chapter 6.4), the resulting stimulus from a calf occlusion was insufficient to significantly alter SFA diameter; therefore, when comparing limb differences, only the BA and PA values will be examined.

In the current study, although there was a trend for BA FMD (8.9%) to be higher than PA FMD (6.5%) this difference was not significant. Furthermore, when FMF:SS$_{AUC}$ data were compared between the BA and PA, no significant differences were reported. Previous investigations have confirmed the impact of baseline arterial diameter on the resulting percent FMD response (13; 29; 36; 60; 71). As an example, Thijssen et al. examined the femoral artery, SFA, PA, BA and RA and demonstrated that smaller conduit arteries have larger percent FMD responses than

larger arteries. In order of size, femoral artery FMD was 3.9%, SFA FMD was 6.9%, PA FMD was 6.1%, BA FMD was 7.0, and RA FMD was 9.4% (69). However, when FMD was corrected for the elicited stimulus, no significant differences between SFA, PA, BA and RA responses were found. These data demonstrate the relationship between baseline diameter and FMD responses; smaller conduit vessels produce larger FMD as compared to larger conduit arteries in response to five-minutes of distal occlusion (69).

Previous investigations have shown a correlation between baseline diameter and the magnitude of the increase in diameter. Pyke and Tschakovsky reported a correlation of $r^2 = 0.64(57)$, Thijssen et al. $r^2 = 0.33$ (69) and Pyke et al. $r^2 = 0.14$ (53). In the current experiment a correlation of $r^2 = 0.11$ was found, which is similar to the findings reported by Pyke and colleagues(53). When percent FMD was plotted as a function of baseline diameter, Thijssen et al. reported an attenuation of the slope with increases in baseline diameter (69). They concluded that baseline diameter may not be an important contributor to the magnitude of an FMD response in large conduit such as the SFA or PA. This trend was also seen in the current experiment; correlation decreased to $r^2 = 0.02$ for the PA artery which had a significantly larger baseline diameter than the BA. There are many limitations to using correlation analysis and many assumptions need to be made such as the assumption of normality and linearity(75). Care must be taken when employing a correlation analysis, as this analysis method might minimize the relationship between baseline diameter and the magnitude of the FMD response that has previously been reported (69). More work is needed to understand the link between baseline and peak diameter and how this relationship affects a researcher's interpretation of a FMD test.

Differences in the percent FMD reported between the BA and PA must be discussed in conjunction with the differences in shear stress post five-minutes of occlusion. Five-minutes of

distal occlusion resulted in significantly greater BA SS_{AUC} compared to the PA, which is supported by previous research. Nishiyama et al. reported a BA SS_{AUC} of 25,419 and PA SS_{AUC} of 8,089, therefore BA SS_{AUC} is three times greater than that of the PA (48). Although SS_{AUC} was calculated until a different time point in the current study, the trends still holds true; BA SS_{AUC} was found to be two times greater than PA SS_{AUC}. Research has suggested that the significantly larger shear stress and FMD responses of the BA, compared to the PA, are due to PA "vascular stiffening"(48). Eiken and Kolegard explained that leg conduit arteries have adapted to elevated levels of intramural pressure by increasing wall stiffness (20). Therefore, the structural differences in arm and leg vasculature are expected to result in different responses to five-minutes of distal occlusion.

Correlation analysis has been performed to examine the relationship between arterial baseline diameter and the shear stress responses post occlusion. To reiterate, shear stress is directly proportional to blood flow velocity and viscosity and inversely related to vessel diameter (15). The inverse relationship between diameter and shear stress was demonstrated in the current investigation, by a strong negative correlation between baseline diameter and SS_{AUC}, BA r=-0.6321, PA r=-0.7782, p<0.05 (Figure 20); Figure 20 demonstrates how shear stress post occlusion is strongly determined by baseline arterial diameter. The current experiment demonstrated a significantly lower PA SS_{AUC} than BA SS_{AUC} (Figure 18); this plot infers that larger conduit arteries exhibit lower post occlusion shear stress than smaller conduit arteries. Pyke and Tschakovsky also demonstrated this relationship by examining how blood flow velocity and shear stress are altered when flow is held constant between different size arteries. When flow is held constant, larger arteries will experience smaller blood flow velocities and shear rates compared to smaller conduit arteries. The increased shear stress exerted on the

smaller artery is an important factor mediating the larger FMD response produced to five-minutes of occlusion (56). Based on the results from the current study and the work of Pyke and Tschakovsky (56) it is clear that there is a strong link between baseline diameter and shear stress. It is likely that these differences in post occlusion shear stress are an important component of the limb differences in FMD responses.

The differences in shear stress and FMD responses between leg and arm conduit arteries can be attributed to a variety of factors. One likely culprit is the increase of 65 mmHg in pressure in the legs compared to the arms. The idea that gravity plays a role in the decreased lower limb vascular reactivity is supported by data collected from the Berlin bed rest study. In that project, 16 men were subjected to 52 days of head down bed-rest, a common model for ground based examinations of the effects of microgravity exposure. Post bed rest SFA FMD was increased inferring an improvement in endothelial function with the removal of the increased pressure otherwise placed on the lower limbs (8). The impact of increased intravascular pressure on endothelial-dependent vasodilatation can also be examined using an in-vivo model of aortic coarctation (49). Aortic coarctation is a condition in which the aorta narrows near the insertion of the ductus arteriosus. This model allows researchers to examine the effects that increased intravascular pressure have on proximal vascular beds. This increase in pressure has been reported to lead to endothelial and vascular smooth muscle remodeling; as well as functional changes to the endothelium and vascular smooth muscle cells (49). Padilla et al. examined the effects of increased pressure on vascular reactivity via arm hanging. This position leads to a 15 mmHg increase in intravascular BA pressure (51). Although this pressure is significantly lower than that exhibited in the legs it does allow researchers to study the effects of added pressure on endothelial function. Padilla et al. reported that a 15 mmHg increase in BA intravascular pressure

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resulted in a significant reduction in BA shear stress, percent FMD and FMD:SS$_{AUC}$(51). The above-mentioned studies suggest that the 65 mmHg additional pressure exerted on leg conduit arteries results in endothelial and vascular smooth muscle cell remodeling and functional changes. These changes will alter how the leg conduit arteries respond to an increase in shear stress when compared with arm conduit arteries (45). Therefore, EC and vascular smooth muscle cell adaptations result in different shear stress and FMD responses in the BA and PA due to five-minutes of distal limb occlusion.

6.6: Arterial Diameter

In the current study arterial diameter was measured using cross-sectional M-mode sweeps of arm and leg conduit arteries. Diameter was determined using a computer-assisted script called "edge-detection"; this script was run through MATLAB (see chapter 3.5.2 for more information). The use of computer-assisted diameter analysis over manual assessment was examined by Woodman et al (78). These researchers used both a phantom artery and an actual brachial artery and determined that computer-assisted analysis improves a researcher's ability to detect significant changes in arterial diameter by significantly reducing observer error (78). These findings support those found in the pilot data stage of the current study that noted significant limitations with an older tool for measuring arterial diameter that required manual corrections. Therefore, a computer-assisted program was used to measure arterial diameter which provides further confidence in the diameter measures obtained in the current study.

Previous research examining conduit artery FMD responses have chosen to use longitudinal images; however, Kao et al. demonstrated significant limitations with longitudinal images, which are not present when using cross-sectional images (33). The validity of capturing arterial diameter in the transverse plane was very recently validated by Stroz & Fenster(66). They reported no significant difference between measurements made in the longitudinal or transverse plane (66). Furthermore, the choice of M-mode sweeps, over traditional B-mode images was validated by Kelly et al.; these researchers demonstrated a close agreement in diameter and FMD measures captured using M-mode sweeps in comparison to B-mode images(34). Therefore, the decision to use two-dimensional cross-sectional M-mode sweeps of conduit arteries as a method of measuring arterial diameter is supported by previous literature.

In the current study, baseline diameters for the BA, SFA, and PA were 0.41, 0.67 and 0.66 cm respectively; similar values have also been reported by other investigators (6; 7; 51; 69; 71; 73). Previous literature has reported that baseline BA diameter ranging from 0.35 to 0.47 cm, SFA diameters between 0.63 to 0.78 cm and finally PA diameters between 0.60 and 0.70 cm (Appendix 5: Table 6). The baseline arterial diameter values measured in the current study are in agreement with previous studies which further increases confidence in the validity of cross-sectional measurements of arterial diameter.

Arterial diameter is measured between the media-lumen echo of the near wall to the intima-lumen echo of the far wall (13; 14; 77). Due to limitations in ultrasound image resolution, artery diameter is measured between different structures on the near and far wall of the artery. An alternate method of measuring diameter was devised that would measure the distance between the same point on the near and far arterial walls. This method is called the center-based method; it determines arterial diameter based on the distance between the center of the media layer on the near wall to the center of the media layer on the far wall. Analysis using this method was tested using BA baseline and post deflation images from seven subjects. Although this is a small sample it provides insight into the usefulness and limitations of this method. Images from the BA were chosen, over those of the SFA and PA, because there is more data available regarding the ranges of BA FMD in young healthy adults. A one-way ANOVA revealed no significant differences in percent FMD and time to FMD using the center-based method as compared to the traditional edge-detection method. No obvious trend regarding the magnitude and direction of the differences using the center based method were observed. These two methods were put through further analysis using a Bland-Altman plot of the percent FMD obtained using both methods. The plot reveals that arterial diameters obtained using the center-based method are in

agreement with those values obtained using the edge-detection method. Based on the above-described preliminary findings, further analysis of the usefulness of the center-based method is warranted.

6.7: Conclusions

The results of the current study have helped to further outline the possibilities and limitations that need to be considered when performing a FMD protocol. The current study demonstrated that five-or two-minutes of distal calf occlusion are unable to elicit a SFA FMD response; therefore when examining leg conduit artery health distal calf occlusions can only be used on the PA. With regards to the PA, the current study demonstrated that a two-minute distal occlusion containing one-minute of static calf exercise can be used instead of the five-minute occlusion. These results suggest that a shorter occlusion protocol can be used to examine leg conduit artery health with no loss of quality. The current study performed comparisons between arm and leg vasculature by comparing the shear stress and FMD responses of the BA and PA. These results highlight the differences in these arteries both at baseline and due to five-minutes of distal occlusions. Research has convincingly demonstrated that these differences are due to leg conduit arterial adaptations to the force of gravity. Lastly, preliminary findings from the current study suggest that the center-based method of measuring arterial diameter can be used in place of the traditional edge-detection methods.

6.8: Limitations

There are a few limitations associated with the current experiment. The current experiment was only performed on men, no women were examined. Therefore, the results of the current experiment can not be extrapolated to include all young healthy adults. Therefore more testing is needed to examine the different protocols in young adult females and in older men and women. This would allow researchers to gain a more complete picture of the ideal FMD protocol for testing leg conduit artery function and what this value infers of a patient's overall vascular health.

A second limitation of the current study is that the ultrasound machine used was unable to simultaneously measure arterial diameter and blood flow. As a consequence, SS_{AUC} could not be calculated until peak diameter was achieved as recommended by Pyke and Tschakovsky (56). Therefore in the current study SS_{AUC} was determined until 20 seconds post cuff deflation, this time point encompassed the participant's peak shear in every test and provided adequate time for the probes to be switched and a diameter measure to be captured at 45 seconds post deflation.

The current study required that all participants abstain from caffeine, alcohol and physical exercise for 12 hours prior to testing and to consume a light low fat meal. The only way that the experimenters could insure that these guidelines were followed was by verbal confirmation with the subject prior to testing. It is possible that they participants did not accurately report their diet and liquid consumption which therefore could have altered a participant's FMD response.

As noted in the reproducibility section (Chapter 4) baseline and peak diameter were reproducible between testing days, however FMD was not. FMD is a calculated variable, which when run through statistical analysis is drastically affected by the differences associated with

each of its measured parameters. As described in equation 1, FMD is derived from subtracting peak diameter post deflation from baseline diameter and dividing that value by baseline diameter. Therefore, when running a correlation analysis on FMD, the error associated with baseline and peak diameter get multiplied which negatively affected the FMD response and resulted in the finding that FMD was not reproducible between testing days.

6.9: Future Directions

The current experiment demonstrated the potential usefulness of the center-based method of determining arterial diameter. Future studies should expand upon this and analyze a greater number of FMD tests, not only those performed on the BA. By improving the analysis tools used to measure arterial diameter more accurately a study will be able to detect small yet significant changes in diameter and thus FMD.

In order for a FMD test to provide insight in a patient's peripheral and in turn coronary artery health, more work is needed to refine the available protocols and create a set of guidelines. An important protocol limitation that needs to be addressed is the ideal cuff placement. In the current experiment, calf cuffs were used to measure PA and SFA FMD responses. The lack of a SFA FMD due to this cuff position might be due to the fact that this occlusion protocol was unable to produce a significant reduction in downstream vascular resistance; therefore there was a smaller increase in blood flow/ shear stress post cuff release. The data demonstrate that this shear stimulus was unable to elicit a "biologically" significant FMD response. More work is needed to determine what factors contributed to the smaller shear response in the SFA post distal calf occlusion. This question would best be answered by future studies examining the difference in FMD responses due to distal occlusions using calf and thigh cuffs.

In further attempting to refine the available leg conduit artery FMD protocol determining the ideal occlusion duration and the usefulness of exercise is needed. The current study appears to be the first to demonstrate that a short two-minute occlusion with light intensity exercise can be used in place of the traditional five-minute protocol to examine PA vascular health. However, only five of the eight participants tested had a significant FMD response. Future work should examine a two-minute occlusion with a slightly harder intensity calf exercise to determine if that intensity can elicit a significant (>4%) FMD response in all participants. The more questions we answer regarding the FMD protocol, the more refined the guidelines can become, which would increase the clinical use and relevance of this non-invasive method of examining central and peripheral arterial health. More work is needed because a FMD test is a safe, reliable and reproducible measure of a participant's vascular and endothelial health.

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Appendix 1

Table 1: Subject characteristics and baseline heart rate and blood pressure for all 10 participants

Subject	Height (cm)	Weight (kg)	Age (yrs)	Resting SBP (mmHg)	Resting DBP (mmHg)	Heart Rate (BPM)	Forearm volume (ml)	Lower leg volume (ml)
AR	172	73	31	116	67	63	1030	2402.5
DZ	179	75	22	119	67	53	950	1410
CV	178	82	28	112	50	39	1000	1530
CS	189	87	23	122	63	71	1010	3305
AM	187	81	25	117	64	58	1090	1610
RV	182	80	35	124	77	58	870	1775
RS	183	72	26	120	70	63	1106	2670
JW	179	97	28	121	72	80	950	2945
BP	196	111	25	131	67	54	1090	3520
DG2	176	75	26	120	72	66	905	2622.5
Avg	182	83	27	120	68	61	1000	2379
SD	7	12	4	5.06	5.38	11	81	763
Min Value	172	72	22	112	59	39	870	1410
Max Value	196	111	35	131	77	80	1106	3520

Means ± standard deviation

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Appendix 2

Table 2a: Average baseline, occlusion and deflation blood pressure and heart rate during the brachial artery five-minute occlusion protocol (n=10)

Subject		MAP	DBP	SBP	Q	Heart rate
1	rest	96	77	126	5.17	54
	occlusion	95	76	126	5.33	54
	deflation	86	70	123	4.91	55
2	rest	77	61	123	7.57	72
	occlusion	80	63	125	7.36	72
	deflation	79	63	128	7.65	71
3	rest	83	64	122	4.61	38
	occlusion	87	68	127	4.83	41
	deflation	88	69	130	4.85	41
4	rest	84	66	115	6.25	59
	occlusion	85	67	121	6.37	56
	deflation	84	66	125	7.69	63
5	rest	89	70	126	6.03	62
	occlusion	96	70	124	5.63	63
	deflation	89	71	129	6.05	63
6	rest	99	77	122	5.35	52
	occlusion	99	75	123	5.55	52
	deflation	96	73	121	5.75	53
7	rest	87	70	117	7.02	57
	occlusion	89	72	123	7.07	54
	deflation	86	69	122	6.65	51
8	rest	79	55	126	7.90	58
	occlusion	87	63	132	7.75	58
	deflation	80	57	129	7.60	55
9	rest	83	62	119	6.83	64
	occlusion	82	62	118	6.39	60
	deflation	79	58	115	7.06	63
10	rest	90	68	124	10.06	77
	occlusion	95	72	132	10.51	78
	deflation	94	71	133	10.84	79

Table 2b: Average baseline, occlusion and deflation blood pressure and heart rate during the superficial femoral artery five-minute occlusion protocol (n=10)

Subject		MAP	DBP	SBP	Q	Heart rate
1	rest	89	71	130	6.61	55
	occlusion	87	70	123	6.47	57
	deflation	87	70	129	7.14	59
2	rest	84	67	123	5.31	50
	occlusion	92	72	131	5.54	52
	deflation	85	66	129	5.87	51
3	rest	84	66	120	4.37	41
	occlusion	91	73	128	4.45	41
	deflation	85	68	123	4.46	41
4	rest	87	69	123	5.87	52
	occlusion	90	71	128	6.17	53
	deflation	88	69	129	6.54	54
5	rest	92	73	130	5.41	59
	occlusion	90	71	128	5.22	56
	deflation	90	71	130	5.65	59
6	rest	96	74	134	6.37	58
	occlusion	97	74	137	6.60	59
	deflation	95	72	136	6.79	59
7	rest	90	73	124	6.18	48
	occlusion	92	75	128	6.54	51
	deflation	88	71	126	7.06	52
8	rest	79	57	124	7.41	59
	occlusion	85	63	129	7.08	57
	deflation	82	60	129	7.61	59
9	rest	82	61	117	6.32	60
	occlusion	84	63	119	6.66	63
	deflation	82	62	119	6.79	64
10	rest	98	76	137	9.78	76
	occlusion	100	77	140	10.4	79
	deflation	98	75	139	10.34	78

Table 2c: Average baseline, occlusion and deflation blood pressure and heart rate during the superficial femoral artery two-minute occlusion protocol (n=9)

Subject		MAP	DBP	SBP	Q	Heart rate
1	rest	86	72	117	3.81	49
	occlusion	88	74	119	4.11	54
	deflation	93	78	124	4.05	51
2	rest	101	80	138	5.47	54
	occlusion	104	82	146	6.49	60
	deflation	101	79	144	5.53	52
3	rest	89	72	125	3.87	37
	occlusion	92	73	130	4.19	38
	deflation	83	67	117	3.84	38
5	rest	85	66	121	4.45	54
	occlusion	87	68	125	4.68	57
	deflation	86	68	125	4.73	56
6	rest	99	74	141	6.31	59
	occlusion	101	75	143	6.22	59
	deflation	97	73	138	6.15	58
7	rest	92	75	125	5.70	45
	occlusion	96	80	131	5.58	47
	deflation	93	77	129	5.96	50
8	rest	84	63	127	7.20	61
	occlusion	86	63	131	6.92	56
	deflation	84	62	128	7.12	59
9	rest	81	63	113	6.11	64
	occlusion	82	63	115	6.24	62
	deflation	83	63	118	6.96	66
10	rest	98	75	136	10.39	78
	occlusion	100	78	136	10.30	83
	deflation	99	78	138	10.32	82

Table 2d: Average baseline, occlusion and deflation blood pressure and heart rate during the POP 5min occlusion protocol (n=10)

Subject		MAP	DBP	SBP	Q	Heart rate
1	rest	85	65	121	5.35	52
	occlusion	84	65	120	5.11	51
	deflation	82	64	119	5.51	54
2	rest	70	56	98	6.27	69
	occlusion	75	59	106	6.90	70
	deflation	73	57	105	7.13	70
3	rest	86	67	117	4.21	40
	occlusion	85	66	117	4.13	39
	deflation	82	64	118	4.31	40
4	rest	87	69	122	5.55	56
	occlusion	89	71	124	5.66	58
	deflation	87	69	124	6.18	58
5	rest	87	67	130	5.57	58
	occlusion	87	67	130	5.50	57
	deflation	84	64	130	5.77	58
6	rest	95	81	123	5.87	63
	occlusion	95	81	123	6.18	62
	deflation	95	80	126	6.72	65
7	rest	88	72	117	6.00	50
	occlusion	90	74	120	5.90	52
	deflation	88	73	124	6.22	51
8	rest	87	65	135	8.62	67
	occlusion	83	61	124	8.28	68
	deflation	80	59	119	8.16	69
9	rest	84	66	111	6.29	67
	occlusion	91	71	121	6.77	70
	deflation	90	70	121	6.8	71
10	rest	94	80	121	9.57	83
	occlusion	96	81	127	10.29	81
	deflation	93	79	122	10.01	81

Table 2e: Average baseline, occlusion and deflation blood pressure and heart rate during the POP 2min occlusion protocol (n=8)

Subject		MAP	DBP	SBP	Q	Heart rate
1	rest	94	74	127	4.85	50
	occlusion	90	72	127	4.96	52
	deflation	92	73	130	5.14	52
2	rest	79	59	119	5.58	69
	occlusion	80	61	117	6.72	71
	deflation	77	57	117	6.09	68
4	rest	92	73	131	5.82	56
	occlusion	88	70	126	5.73	60
	deflation	88	69	128	6.14	62
5	rest	89	69	127	4.99	56
	occlusion	87	68	126	5.00	56
	deflation	87	68	128	5.12	56
6	rest	95	73	134	6.01	60
	occlusion	99	78	141	5.94	65
	deflation	96	75	137	6.00	64
8	rest	76	57	116	7.15	59
	occlusion	82	61	127	8.37	66
	deflation	82	61	128	8.61	66
9	rest	84	66	110	6.66	71
	occlusion	90	70	119	5.99	63
	deflation	87	68	116	6.63	69
10	rest	93	80	121	9.63	78
	occlusion	92	81	124	9.98	90
	deflation	92	81	116	9.77	80

Appendix 3

Table 3: Percent flow-mediated dilation, shear stress and flow-mediated dilation normalized to shear stress area under the curve after five- and two-minutes of occlusions of the superficial femoral and popliteal arteries

Subject	FMD				Shear stress Area Under the Curve				FMD/SS$_{AUC}$				Peak Shear Stress			
	SFA5	SFA2	POP5	POP2	SFA5	SFA2	POP5	POP2	SFA5	SFA2	POP5	POP2	SFA5	SFA2	POP5	POP2
1	0	3.38	4.4	11	3290.86	1210.28	7324.18	3572.46	0	0.002793	0.0006	0.003079	202.95	72.31	467.46	213.90
2	0.66	0	6.31	0	1970.27	925.43	7092.02	4409.68	0.00033	0	0.00089	0	123.26	69.36	391.66	272.00
3	3.8	0	4.02		1915.74	1717.23	4492.60		0.00198	0	0.00089		137.64	127.16	282.53	
4	0		5.63	9.2	2561.50		5118.97	2604.82	0		0.0011	0.003532	153.11		337.06	204.48
5	0	2.40	4.52	5.61	2088.12	679.15	3776.09	1907.19	0	0.003534	0.0012	0.002942	138.67	51.12	274.11	132.67
6	0	2.25	16.50	4.74	3948.49	3512.25	8674.59	8139.68	0	0.000641	0.0019	0.000582	221.67	256.63	583.01	536.69
7	6.3	0	7.97		7703.47	3807.29	5061.40		0.00082	0	0.00157		491.36	242.02	373.89	
8	4.52	0.00	4.61	0.00	7100.57	3428.62	6946.59	2975.55	0.00064	0	0.00066	0	448.76	288.22	459.87	177.81
9	0.98	0.00	2.40	2.34	4082.72	3280.85	6777.92	4039.54	0.00024	0	0.00035	0.000579	271.94	257.04	486.82	296.30
10	0	0.00	8.93	5.40	2747.21	3446.85	5590.51	6541.88	0	0	0.0016	0.000825	190.09	243.84	403.11	493.82

Appendix 4

Table 4: Flow mediated dilation and time to peak flow mediated dilation determined using the edge-detection and center-based methods of determining arterial diameter

Subject	Measurement	FMD	TFMD
1	M1	10.0	75
1	M2	1.0	75
2	M1	17.6	45
2	M2	20.5	45
3	M1	16.0	90
3	M2	23.0	60
4	M1	6.5	90
4	M2	14.2	75
5	M1	15.8	45
5	M2	11.8	45
6	M1	6.6	60
6	M2	1.4	60
7	M1	8.5	45
7	M2	13.1	45

Table 5: Data associated with Bland-Altman plot (figure 29). Mean flow-mediated dilation using method 1 and method 2 as well as the difference between the two methods

Subject	M1	M2	Mean	Difference
1	10	1	5.5	9
2	17.6	20.5	19.05	-2.9
3	16	23	19.5	-7
4	6.5	14.2	10.35	-7.7
5	15.8	11.8	13.8	4
6	1.4	6.6	4	-5.2
7	8.5	13.1	10.8	-4.6

Appendix 5

Table 6: Literature review of flow-mediated dilation protocols

Authors	Subject	Subject Age	Artery	Cuff Position	Occ Press. (mmHg)	Occ Dur (min)	Inhibitor	% FMD	% FMD with blockade
Doshi et al. 2001	10 ♂	34 ± 5	BA	Distal	250	5	L-NNMA	7.25	0
				Proximal	250	5		11.62	7.51
Mullen et al. 2001	14♂ 4♀	35 ± 1.7	RA	Distal	300	5	L-NMMA	5.3	0.7
							Aspirin	4.7	4.9
Joannides et al. 1995	10♂ 6♀	24±1	RA	Distal	180	3	L-NMMA	3.6	-2.8
							Aspirin	3.9	3.9
Thijjsen et al. 2008a	20 ♂	31± 7	RA	Distal	> 200	5	N/A	9.4	N/A
			BA	Distal	> 200	5	N/A	7	N/A
			CFA	Distal	> 200	5	N/A	3.9	N/A
			SFA	Distal	> 200	5	N/A	6.9	N/A
			PA	Distal	> 200	5	N/A	6.1	N/A
Betik et al. 2003	8 ♂	20.5±0.9	BA	Wrist	200	5	N/A	1.1	N/A
				Forearm	200	5	N/A	3.4	N/A
				Upper Arm	200	5	N/A	6.6	N/A

Black et al. 2008	6 ♂ 6 ♀	26.2±3.3	BA	Distal	>200	5	N/A	7.8	N/A
	6 ♂ 6♀ trained	58±5.1	BA	Distal	>200	5	N/A	6.4	N/A
	6♂ 6♀ sed.	57.3±3.9	BA	Distal	>200	5	N/A	5.2	N/A
Thijjsen et al. 2008b	45♂	10±1	BA	Distal	>200	5	N/A	11.2	N/A
	31♂	28±6	BA	Distal	>200	5	N/A	7.3	N/A
	22♂	58±5	BA	Distal	>200	5	N/A	6.0	N/A
Sorsen et al. 1993	30	7 to 17	SFA	Distal	250	4.5	N/A	7.5	N/A
	30-FH	7 to 17	SFA	Distal	250	4.5	N/A	1.2	N/A
Walther et al. 2008	10 ♂	25±1	BA	Distal	supra-systolic	5	N/A	8	N/A
			SFA	Distal	supra-systolic	5	N/A	2	N/A
	10♂ swim	22±1	BA	Distal	supra-systolic	5	N/A	10	N/A
			SFA	Distal	supra-systolic	5	N/A	8	N/A
	10 ♂-cyclists	25±1	BA	Distal	supra-systolic	5		14	
			SFA	Distal	supra-systolic	5		5	

Kooijman et al. 2008	8 ♂	34±4	SFA	Distal	220	5	L-NMMA	4.2	1
	6 ♂	34±4				10	L-NMMA	~5	~5
	6 ♂ SCI	37±4	SFA	Distal	220	5	L-NMMA	8.2	2.4
Nishiyama et al. 2007	12 ♂	27 ±2	BA	Distal	>250	5		6.5	
			PA	Distal-calf	>250	5		4.5	
			BA	Distal	>250	1+Exer		7.6	
Padilla et al. 2009	10 ♂ 1 ♀	23.6±1.6	BA	Distal	Arm hanging, 220	5		~3.5	
			BA	Distal	220	5		~6	
			PA	Distal-calf	220	5		~4	
	8 ♂	23.6±1.6	PA-prone	Distal-calf	Seated, 220	5		~3.5	
Gaenzer et al. 2001	10♂	39.6±5.8	BA	Distal	250	4		7.7	
	8 ♂ smoker	38.9±4.1	BA	Distal	250	4		4.1	
Rakobowchuk et al. 2004	28♂	23±3.9	BA	Distal	200	4.5		7.5	

Padilla et al. 2008	10♂ 10♀	25.3±0.6	BA	distal	250	1		1.5	
						2		3	
						3		4.5	
						4		5.5	
						5		7	
Gokce et al. 2003	34♀ 130♂ NO CVD event	65±11	BA	Proximal	>200	5		7	
	11♀ 24♂ CVD event	70±10	BA	Proximal	>200	5		4.4	
Celermajer et al. 1992	10♂ 4♀	27±2	BA	Distal	300	4.5		11	
			SFA	Distal	300	4.5		4	
	7♂ 1♀ smokers	44±5	BA	Distal	300	4.5		4	
			SFA	Distal	300	4.5		0	
	6♂ 1♀ CAD	61±2	BA	Distal	300	4.5		0	
			SFA	Distal	300	4.5		1	
	9♂ 7♀	11.5±0.4	BA	Distal	300	4.5		9	
	4♂ 6♀ FH	11.3±0.8	BA	Distal	300	4.5		0	

| Silber et al. 2004 | 9♂ 15♀ | 27±6 | SFA | Distal | SBP+20 mmHg | 5 | | 3.5 | |
| | | | BA | Distal | SBP+20 | 5 | | 5.2 | |

Appendix 6

Table 7a: Correlation coefficients associated with data obtained from the reproducibility study

Artery	Baseline Diameter			Time to peak Diameter			Peak Diameter			FMD		
	Day 1	Day 2	r	Day 1	Day 2	r	Day 1	Day 2	r	Day 1	Day 2	r
BA	0.4056	0.4059	0.97	69	60	0.66	0.433	0.430	0.93	4.3	8.4	0.55
SFA	0.648	0.642	0.99	72	66	0.63	0.659	0.662	0.99	1.6	3.1	0.77
PA	0.648	0.653	0.98	56	56	1	0.692	0.690	0.98	6.7	5.5	0.66

Table 7b: Correlation coefficients associated with data obtained from the reproducibility study

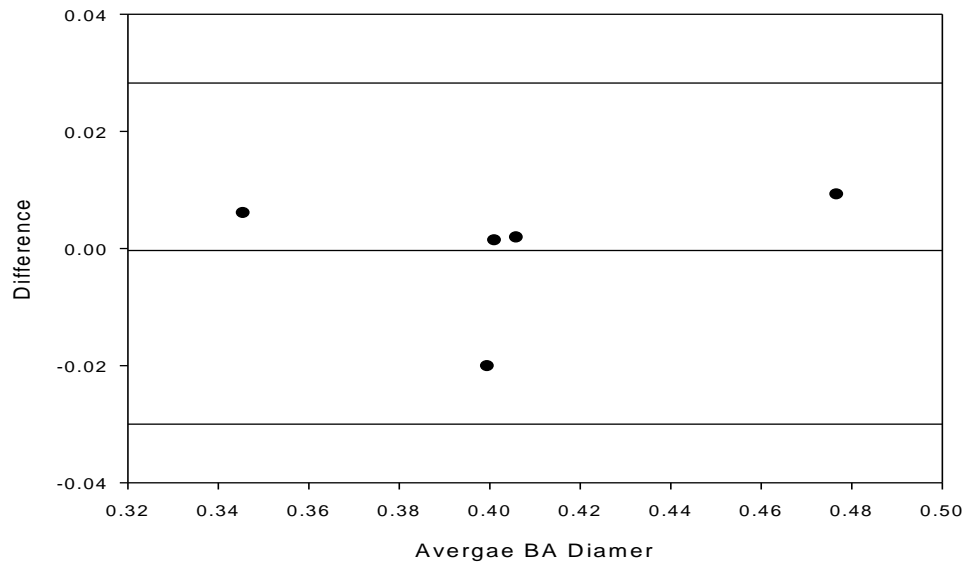| Artery	Peak Shear			Time to Peak Shear			AUC		
	Day 1	Day 2	r	Day1	Day2	r	Day1	Day2	r
BA	708	798	0.56	13	11.4	0.90	11555	12913	0.60
SFA	10.6	13.2	0.30	326	393	0.31	5116	5991	0.20
PA	433	358	0.73	9.3	10.8	0.57	6526	5682	0.58

Figure 8: Bland Altman plot of the difference in brachial artery baseline diameter as a function of the mean diameter between testing day one and two
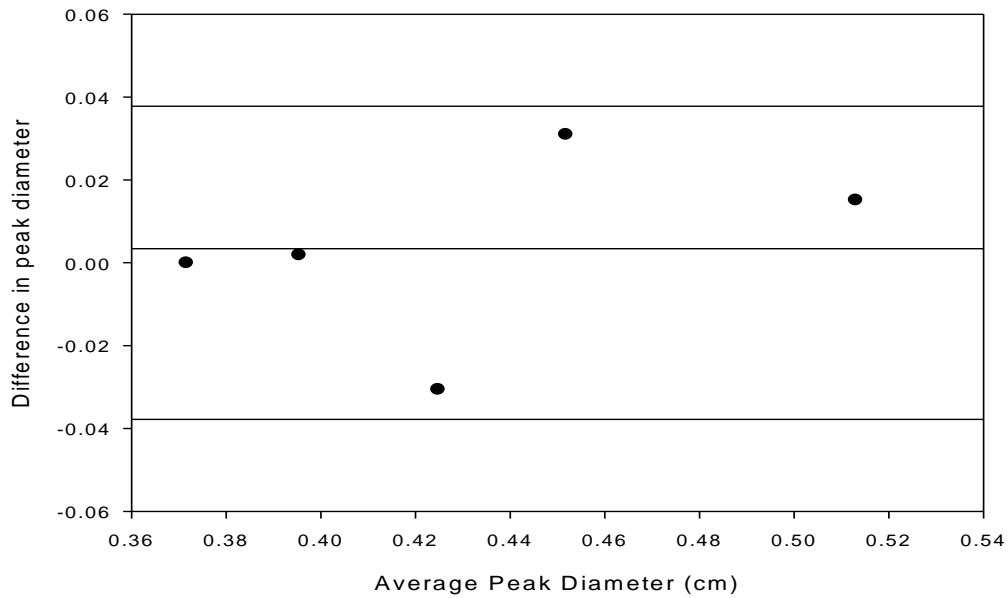

Figure 9: Bland-Altman plot of the difference in brachial artery peak diameter after five-minutes of distal occlusion as a function of the mean peak diameter between testing day one and two
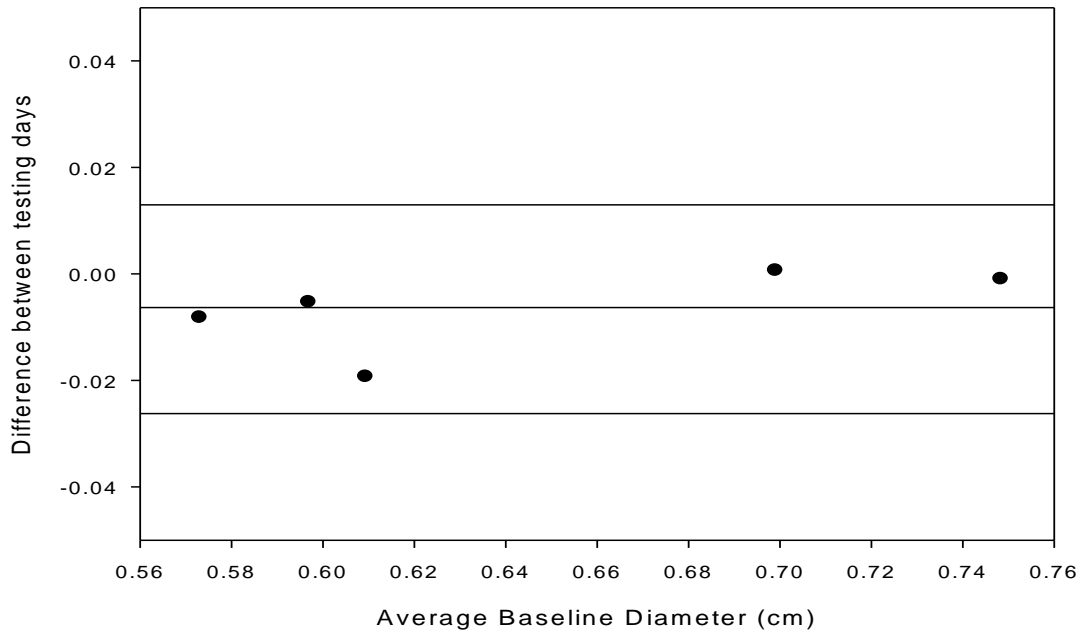
Figure 10: Bland-Altman plot of the difference in superficial femoral artery baseline diameter between testing days as a function of mean baseline diameter.
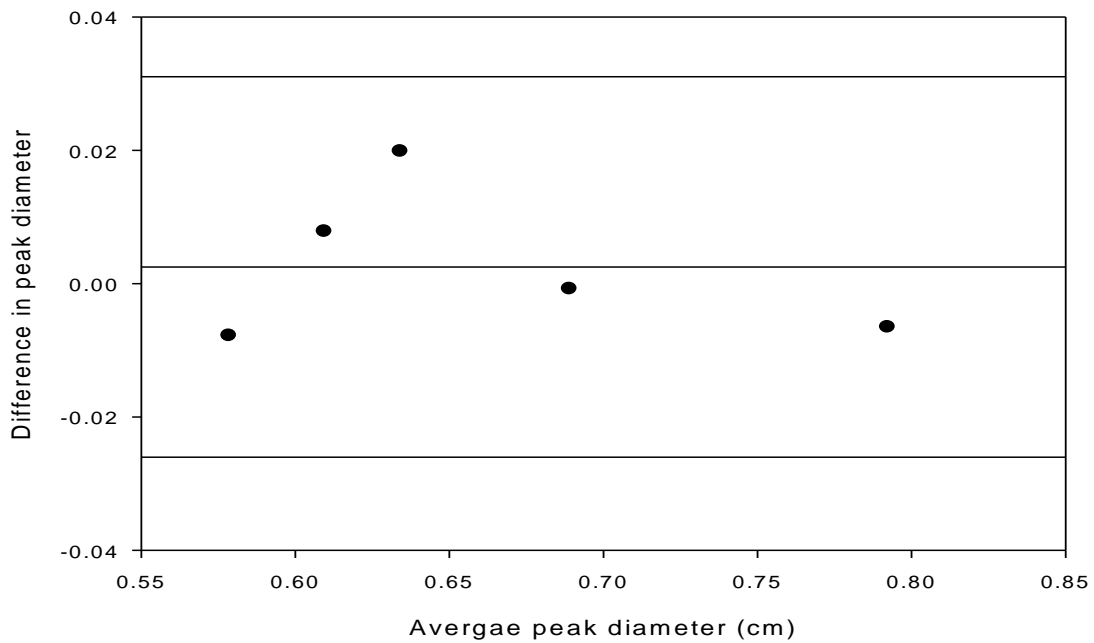

Figure 11: Bland-Altman plot of the difference in superficial femoral artery peak diameter as a function of mean peak diameter between testing days
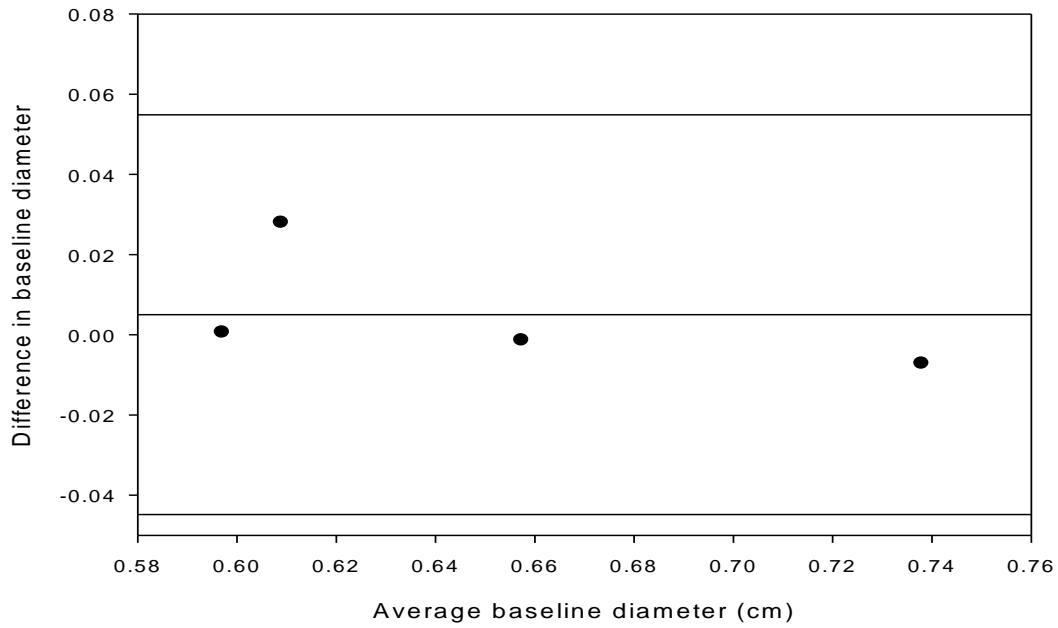
Figure 12: Bland-Altman plot of the difference in popliteal artery baseline diameter as a function of mean baseline diameter measured between testing days
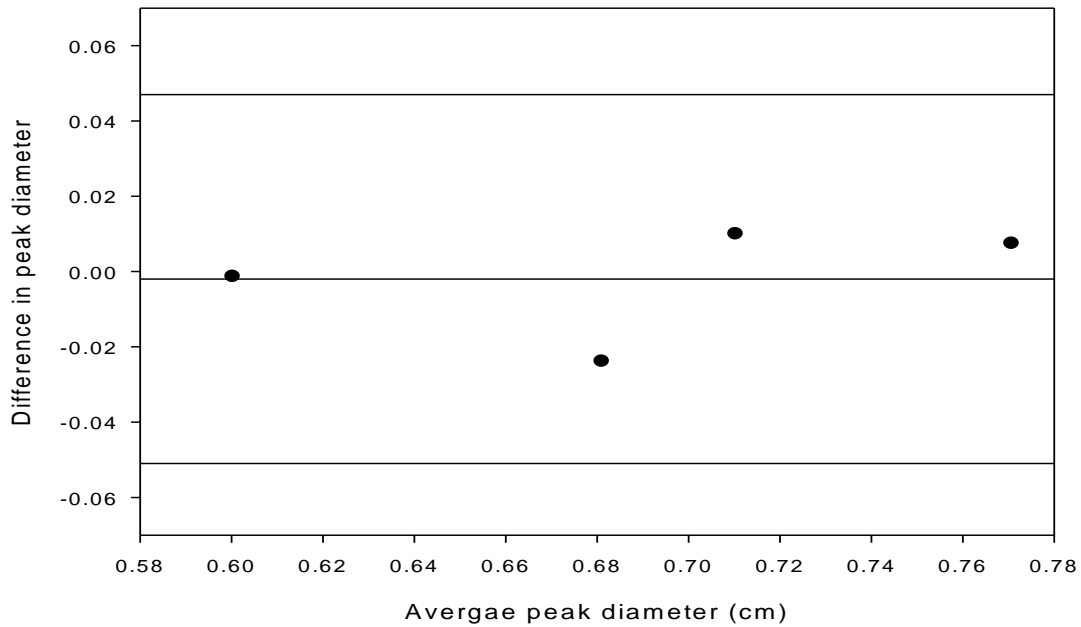

Figure 13: Bland-Altman plot of the difference in popliteal artery peak diameter as a function of mean peak diameter measured between testing days

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Appendix 7: Table 9a- Reproducibility study data for the Brachial, Superficial Femoral and Popliteal Artery

Artery		B1 (cm)	B2 (cm)	TP1 (s)	TP2 (s)	P1 (cm)	P2 (cm)	FMD1 (%)	FMD2 (%)
BA	S1	0.4068	0.4050	60	45	0.4095	0.4401	0.64	8.67
	S2	0.3895	0.4096	90	105	0.4672	0.4363	6.53	19.97
	S3	0.4813	0.4721	75	45	0.5205	0.5055	7.06	8.15
	S4	0.4018	0.4004	60	60	0.3963	0.3945	-1.49	-1.35
	S5	0.3485	0.3425	60	45	0.3716	0.3716	8.49	6.61
SFA	S1	0.6190	0.5997	75	60	0.6055	0.6133	-2.17	2.27
	S2	0.7488	0.7479	60	45	0.7955	0.7890	6.23	5.49
	S3	0.5995	0.5942	60	60	0.6242	0.6440	4.11	8.38
	S4	0.5771	0.5689	75	90	0.5824	0.5745	0.91	0.98
	S5	0.6987	0.6993	90	75	0.6894	0.6886	-1.33	-1.54
PA	S1	0.5949	0.6229	60	60	0.693	0.6691	16.49	7.42
	S2	0.7415	0.7344	45	45	0.7670	0.7745	3.44	5.46
	S3								
	S4	0.5967	0.5973	45	45	0.6010	0.5996	0.72	0.38
	S5	0.6580	0.6566	75	75	0.7055	0.7153	7.20	8.93

Appendix 7: Table 9b- Reproducibility study data for the Brachial, Superficial Femoral and Popliteal Artery

Artery		PS1 (s^{-1})	TPS1 (s)	PS2 (s^{-1})	TPS2 (s)	AUC1	FMD1/AUC	AUC2	FMD2/AUC
BA	S1	611.11	13	724.74	12	9786.01	6.59E-05	11942.56	7.26E-04
	S2	905.67	17	852.54	13	15664.32	4.17E-04	13798.44	1.45E-03
	S3	558.93	9	803.46	10	8701.41	8.12E-04	12858.47	6.34E-04
	S4	781.27	14	917.46	11	12783.96	-1.16E-04	14612.00	-9.26E-05
	S5	684.39	12	693.60	11	10841.36	7.84E-04	11354.09	5.82E-04
SFA	S1	221.67	10	296.11	12	3948.49	-5.51E-04	5065.62	4.49E-04
	S2	491.36	15	263.31	12	7703.47	8.09E-04	3979.65	1.38E-03
	S3	448.76	9	630.38	16	7100.57	5.79E-04	8958.67	9.35E-04
	S4	281.7	10	467	15	4082.72	2.22E-04	7278.29	1.35E-04
	S5	190.09	9	311.6	11	2747.21	-4.85E-04	4674.26	-3.29E-04
PA	S1	583.01	7	408.99	14	8674.59	1.90E-03	6880.11	1.08E-03
	S2	373.89	10	298.81	14	5061.40	6.80E-04	4984.45	1.10E-03
	S3								
	S4	373.89	10	328.33	8	6777.92	1.06E-04	4687.07	8.18E-05
	S5	403.11	10	396.97	7	5590.51	1.29E-03	6175.72	1.45E-03