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Non-invasive determination of vascular reactivity: a new approach to an important measure

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A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Kinesiology

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ABSTRACT

Vascular impairments at both the macro- and the micro-circulatory level are known to be associated with cardiovascular disease (CVD) and may be one of the earlier signs of disease. As such, it is important to have a means of non-invasively assessing vascular reactivity, however accurate assessment remains challenging. Tests of flow-mediated dilation (FMD) have been widely used to non-invasively determine endothelium-dependent vasodilation in humans; however, the use of this technique poses some challenges. Thus, the purpose of this thesis was to explore new strategies to non-invasively determine the magnitude and dynamic adjustment of vascular responses in humans. Using near-infrared spectroscopy (NIRS)-derived measures of tissue oxygen saturation (StO_2) following vascular occlusion it was demonstrated that: 1) the reperfusion rate (Slope 2 StO_2) was significantly correlated to the widely used measure of vascular function, FMD (Chapter II); and 2) that the measure of Slope 2 StO_2 had a strong reliability both within and between testing days (Chapter III). Allometric scaling has been proposed to address one of the challenges associated with the FMD measurement, however this allometric scaling, and the development of a *new scaling* calculation, to account for variability in baseline diameter between subjects did not improve the measure (Chapter IV). Additionally, the application of allometric scaling may not be necessary for homogenous groups. In Chapter V a “dose-response” like curve was constructed for the Slope 2 StO_2 measurement across a range of different occlusion durations, to determine differences in vascular responsiveness, and demonstrated differences in trained versus untrained individuals. Collectively, these data suggest that the NIRS-derived measure of Slope 2

StO₂ obtained directly at the microvascular level is reflective of the vascular responsiveness as measured by FMD. The strong reliability, both intra- and interday, of the NIRS-derived Slope 2 StO₂ measure make this technique suitable for the assessment of vascular responsiveness, and it is particularly well-suited for determining differences between groups or before and after an intervention. Slope 2 StO₂ is sensitive to different occlusion durations and changes in this sensitivity of the measure to a variety of ischemic challenges can be used to detect differences between groups. Therefore, NIRS-derived measures of StO₂ show promise for assessing vascular responsiveness between groups, or for monitoring changes in vascular responses to various interventions.

Keywords: Vascular responsiveness, Near-infrared spectroscopy, Tissue oxygen saturation, Flow-mediated dilation

CO-AUTHORSHIP

This thesis includes versions of the following manuscripts that were submitted and accepted for publication or are in preparation to be submitted for publication:

1. **McLay, K.M.**, Fontana, F.Y., Nederveen, J.P., Guida, F.F., Paterson, D.H., Pogliaghi, S., & Murias, J.M. Vascular responsiveness determined by near-infrared spectroscopy measures of oxygen saturation. (Experimental Physiology. 101(1): 34-40; 2016).
2. **McLay, K.M.**, Nederveen, J.P., Pogliaghi, S., Paterson, D.H., & Murias, J.M. Repeatability of vascular responsiveness measures derived from near-infrared spectroscopy. (Physiological Reports. In press)
3. **McLay, K.M.**, Nederveen, J.P., Koval, J.J., Paterson, D.H., & Murias, J.M. Is allometric scaling necessary for the accurate assessment of flow-mediated dilation in young healthy men?
4. **McLay, K.M.**, Gilbertson, J.E., Pogliaghi, S., Paterson, D.H., & Murias, J.M. Vascular responsiveness measured by tissue oxygen saturation reperfusion slope is sensitive to different occlusion durations.

K.M. McLay, J.M. Murias and D.H. Paterson contributed to the design of all studies. The design of studies 1, 2, 3 and 4 were also largely contributed to by S. Pogliaghi (studies 1, 2 and 4) and J.J. Koval (study 3). All data were collected and analyzed by K.M. McLay with collection assistance provided by J.P. Nederveen, F.F. Fontana and J.E. Gilbertson. The original manuscripts comprising this thesis were written by K.M. McLay with feedback provided by the co-authors.

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LIST OF ABBREVIATIONS

ACh	acetylcholine
ANOVA	analysis of variance
ATP	adenosine triphosphate
ATT	adipose tissue thickness
AUC	area under the curve
BSLN StO ₂	baseline tissue oxygen saturation
Ca ⁺⁺	calcium
CAD	coronary artery disease
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
COX	cyclooxygenase
CVD	cardiovascular disease
CV	coefficient of variation
D _{base}	baseline artery diameter
D _{peak}	peak artery diameter
ECG	electrocardiogram
EDHF	endothelium-derived hyperpolarizing factor
eNOS	endothelial nitric oxide synthase
FMD	flow-mediated dilation
GTP	guanosine triphosphate
Hb	hemoglobin
HbO ₂	oxygenated hemoglobin
HHb	deoxygenated hemoglobin
ICC	intraclass correlation coefficient
L-NMMA	N ^G -monomethyl-L-arginine
LSD	least significant difference
Mb	myoglobin
Min StO ₂	minimum tissue oxygen saturation
NIRS	near-infrared spectroscopy

NO	nitric oxide
O ₂	oxygen
Peak StO ₂	peak tissue oxygen saturation
PG	prostaglandin
PO	power output
PO _{peak}	peak power output
RI	ramp incremental
SD	standard deviation
StO ₂	tissue oxygen saturation
Sw	within-subject standard deviation
T	trained
TA	tibialis anterior
UT	untrained
VOT	vascular occlusion test
$\dot{V}O_2$	oxygen uptake
$\dot{V}O_{2max}$	maximal oxygen uptake
W	watt
%FMD	percent flow-mediated dilation
μ_s	absorption coefficient
μ_a	scattering coefficient

Importance of Vascular Function

Cardiovascular disease (CVD) is presently the leading cause of morbidity and mortality (Lloyd-Jones *et al.*, 2010; Green *et al.*, 2011) and although most traditional risk factors (i.e., high blood lipids, hypertension, diabetes, etc.; Mora *et al.* 2007, Green *et al.* 2008) are not detected until later in life, changes in macro- and micro-vascular functions, which have been proposed as predecessors of CVD, have been shown to be present as early as adolescence (Celermajer *et al.*, 1992; Khan *et al.*, 2003). It has been well established that exercise decreases cardiovascular risk, and that the risk reduction is greater than would be expected based on the reductions observed in traditional risk factors (Mora *et al.*, 2007). Vascular impairments at both the macro- and the micro-circulatory level are known to be associated with CVD (Lloyd-Jones *et al.*, 2010; Seals, 2014), and it is proposed that direct effects on the vessel wall may account for some of the exercise-related risk reduction independently of the factors listed above (Green *et al.*, 2008).

More specifically, endothelial dysfunction, commonly expressed as deficiencies in nitric oxide (NO) production and release by the endothelial cells, and the consequent reduction in vascular responsiveness has been proposed to play a pivotal role in the advancement of CVD (Langham & Wehrli, 2011), independent of changes in smooth muscle function. Studies in CVD patients with more severe disease have shown that changes in smooth muscle function do occur and have suggested that these changes occur subsequent to changes in the endothelium (Maiorana *et al.*, 2003; Green *et al.*, 2004; Thijssen *et al.*, 2010; Seals, 2014). This has led to a proposed “hierarchy of dysfunction”, insinuating that impairments in vascular function begins with the endothelium and can

migrate to the rest of the vessel wall with disease progression (Green *et al.*, 2011). In this context, non-invasive assessment of endothelium-dependent vascular responses becomes an important instrument for early detection of pre-clinical dysfunction, diagnosis, monitoring of treatment and possibly even prevention of CVD.

Structure of the Vasculature

Blood flows through the peripheral vasculature via a closed network circuit of conduit arteries and arterioles, ultimately terminating at the capillaries before entering the venous circulation. The conduit arteries, are elastic vessels and the smooth muscles of the arterial walls of these arteries contract or relax to regulate the flow of blood through their lumen. Blood flow through the vessel is governed by Poiseuille's law;

$$Blood\ Flow = \frac{\Delta P \pi r^4}{8 \eta l} \quad (1.1)$$

Where ΔP is the pressure gradient across the vessel, r is the vessel radius, η is the viscosity of the blood and l is the vessel length. As blood flow is related to r raised to the fourth power, small changes in vascular diameter have substantial effects on flow. In this way, the arterioles can control how much blood flows to different parts of the body under various cardiovascular challenges (i.e., ischemia, exercise, etc.).

Arteries are composed of several cell types that are integral to this regulation of vascular tone. The outer adventitial layer (tunica externa) consists of perivascular nerves and the extracellular matrix. The middle layer (tunica media) contains the vascular smooth muscle cells, which are oriented perpendicular to the lumen of the vessel to provide circumferential force allowing for changes in lumen diameter. The innermost layer (tunica intima) consists of the internal elastic lamina and endothelium. The internal elastic lamina

separates the smooth muscle layer from the endothelium. The innermost layer of the blood vessel is composed of a single layer of endothelial cells oriented longitudinally to sense shear forces associated with blood flow through the lumen.

Function of the Endothelium

The endothelium is a single layer of cells lining all the blood vessels in the body. In the case of the smallest blood vessels, the capillaries, endothelial cells constitute the entire wall structure. It controls many important functions including maintenance of blood circulation and fluidity as well as regulation of vascular tone, coagulation and inflammatory responses. The endothelium responds to flow and shear stress which initiates a cascade of events that leads to the production of nitric oxide (NO) and resultant vasodilation. This flow-mediated, endothelium dependent mechanism has been extensively researched and is the principle that supports a method to non-invasively assess endothelial function/ dysfunction commonly termed flow-mediated dilation (FMD).

Vascular Anatomy of the Leg

Figure 1.2 illustrates the major arteries of the lower limb. Blood flows through the femoral artery into the popliteal artery, the major conduit responsible for the perfusion of the lower leg. The popliteal artery descends down the posterior thigh, giving off genicular branches that supply the knee joint. The popliteal artery moves through the popliteal fossa and terminates by dividing into the anterior and posterior tibial arteries.

The posterior tibial artery continues inferiorly (through the back of the leg), along the surface of deep muscles (i.e., flexor muscles) and ultimately enters the sole of the foot. It also gives rise to the peroneal artery which supplies the lateral peroneal muscles of the leg. The other division of the popliteal artery, the anterior tibial artery, passes between the

tibia and fibula and runs through the anterior compartment of the leg. It runs down through the entire length of the leg and into the foot, supplying the extensor muscles along the way (including the tibialis anterior muscle).

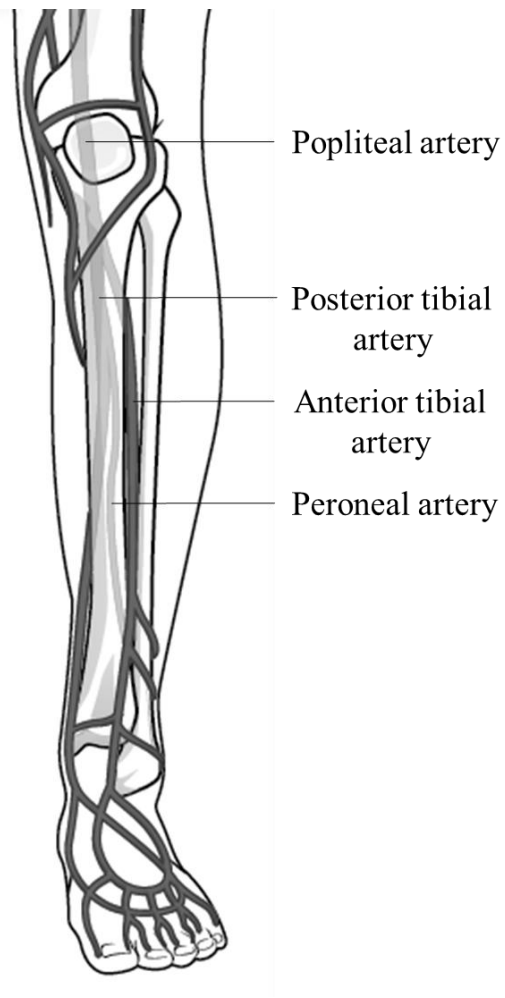


Figure 1.1. Major arteries of the lower leg; anterior view of the left leg.

Flow-mediated Dilation

The reactive hyperemia endothelial function test, commonly referred to as an FMD test was first proposed in 1992 (Celermajer *et al.*, 1992) and involves Doppler ultrasound imaging of conduit artery responses to shear stress-induced FMD, consequent to a brief period of ischemia. This technique generates a shear stress stimulus, resulting in a dilation of downstream resistance vessels following the occlusion of blood flow to the limb with a pressure cuff. Upon cessation of the occlusion, inflow of blood through the conduit artery is transiently increased (reactive hyperemia) and acts as the stimulus for FMD.

In humans FMD is typically assessed in the large peripheral conduit arteries and is considered representative of the response in the more clinically relevant coronary circulation (Takase *et al.* 1998). As a result of the close relationship to the coronary circulation, the FMD test has become widely used as a measure of endothelial dysfunction in both clinical and asymptomatic patients. Experimental and clinical studies suggest that endothelial dysfunction is an important feature of vascular disease and is strongly associated with several cardiovascular conditions, including atherosclerosis (Ross, 1999), hypertension (Taddei *et al.*, 1993; Perticone *et al.*, 2001; Modena *et al.*, 2002), and coronary and peripheral artery disease (Yataco *et al.*, 1999; Zhang *et al.*, 2000; Neunteufl *et al.*, 2000; Kuvin *et al.*, 2001; Brevetti *et al.*, 2003), and that endothelial dysfunction can predict cardiovascular events in these groups (Gokce *et al.*, 2002; Widlansky *et al.*, 2003).

Animal studies established that FMD in arteries was dependent on the presence of an intact endothelial lining (Smiesko *et al.*, 1985; Pohl *et al.*, 1986) and that in response to shear stress, the endothelium releases a substance now known as NO (Rubanyi *et al.*, 1986; Moncada *et al.*, 1988). Therefore, reductions in FMD are widely assumed to reflect

diminished NO production as several pivotal human studies have concluded that FMD is, at least in part, dependent on an NO pathway (Joannides *et al.*, 1995; Mullen *et al.*, 2001; Doshi *et al.*, 2001; Jiang *et al.*, 2011). Studies involving the administration of NO blockade, such as N^G-monomethyl-L-arginine (L-NMMA), have confirmed the major role that NO plays in the regulation of vascular tone. Joannides *et al.* (Joannides *et al.*, 1995) found that radial artery dilation following 3 minutes of ischemia was abolished in the presence of L-NMMA. Similarly, Mullen *et al.* (Mullen *et al.*, 2001) found that NO blockade decreased the radial artery FMD response to 5 minutes of ischemia from 5.3% to 0.7% dilation, with no difference in hyperemic stimulus, concluding that it was unlikely that stimulus magnitude was responsible for the abolished FMD response.

Figure 1.3 details the pathway of endothelial-dependent vasodilation in response to increases in blood flow, or fluid shear stress. Immediately following cuff release, the resultant increase in blood flow creates a shear stress stimulus causing deformation of mechanosensitive structures on the endothelial cell membrane, such as membrane proteins (glycocalyx), primary cilia and mechanosensitive ion channels (Pyke & Tschakovsky, 2005; Davies, 2009). The acute response to the shear stress stimulus is the opening of calcium (Ca⁺⁺)-activated potassium channels, causing hyperpolarization of the endothelial cell (Olesen *et al.*, 1988; Cooke *et al.*, 1991; Miura *et al.*, 2001). This results in an increased driving force for Ca⁺⁺ entry into the cell. The increased [Ca⁺⁺] triggers phosphorylation of endothelial nitric oxide synthase (eNOS) which in turn increases the conversion of L-arginine to L-citrulline and produces NO (Pohl *et al.*, 1986; Joannides *et al.*, 1995). Over prolonged periods of shear stress stimulus, the mechanosensitive structures signal increases G-protein expression and resultant phosphorylation of eNOS (Corson *et al.*, 1996;

Dimmeler *et al.*, 1999). This increase in eNOS activity increases the production of NO, even at low concentrations of calcium. Vasodilators diffuse from the endothelial cell into the tunica media (composed of smooth muscle), which in the case of NO activates the enzyme guanylate cyclase. This increases the conversion of guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP), which acts to decrease the concentration of intracellular $[Ca^{++}]$ and induces relaxation of the smooth muscle and subsequent vasodilation.

As mentioned previously, there is some redundancy in the mechanisms controlling vasodilation. In addition to multiple mechanosensitive structures on the endothelial cell surface, there are also multiple vasodilatory pathways within the endothelial cell which may contribute to FMD (Sun *et al.*, 1999; Mullen *et al.*, 2001; Doshi *et al.*, 2001; Pyke *et al.*, 2009; Parker *et al.*, 2011). Studies have shown that in the absence of NO there are other pathways that can be upregulated to ensure continued functioning, at least in part, of the endothelium. For example, a study using blood vessels from mice, genetically engineered to lack eNOS, found they still responded to shear stress by dilating (Sun *et al.*, 1999). Other animal models have determined prostaglandins (PGs) and endothelial-derived hyperpolarizing factor (EDHF) may also contribute to endothelial-dependent vasorelaxation (Huang *et al.*, 1998; Pak *et al.*, 2002; Scotland *et al.*, 2005). More recently, Pyke *et al.* (Pyke *et al.*, 2009) were unable to reduce radial artery FMD with a large dose of L-NMMA and concluded that there may be heterogeneous vasodilator phenotypes which affect the contribution of NO to FMD. Although other vasodilators can be released by the endothelium, reviews in the area have demonstrated that FMD of conduit arteries in humans is, at least in part, mediated by NO (for meta-analysis see Green *et al.* 2014).

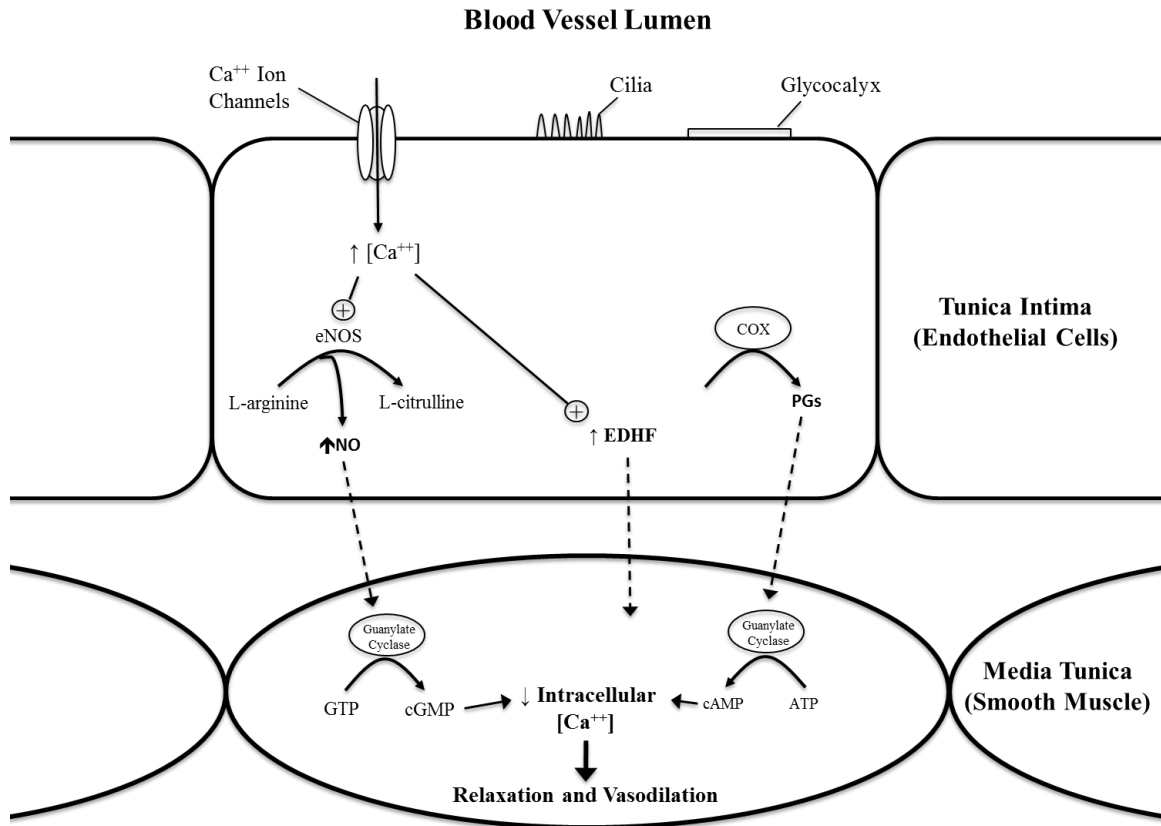


Figure 1.2. Schematic representation of the pathways involved in flow-mediated dilation (FMD) from the initial fluid shear stress stimulus to the resultant change in vessel diameter. Adapted from McLay, 2012.

Changes in FMD with Interventions

FMD is presently the most widely used non-invasive method to assess endothelial function and has been used to detect changes in responsiveness of the vasculature following various interventions. The majority of studies undertaken in subjects with CVD or risk factors report that exercise training interventions improve vasodilator function of resistance and conduit arteries (Thijssen *et al.*, 2010). For example, Maiorana *et al.* (Maiorana *et al.*, 2001a) reported an increased FMD response in the brachial artery following eight weeks of a combined aerobic and resistance training program in patients with type 2 diabetes. Additionally, they reported improved forearm blood flow following acetylcholine (ACh) infusion, indicative of enhanced endothelial-dependent vasodilation of the resistance vessels as well. Similarly, coronary artery disease (CAD) patients, who had lower brachial FMD responses compared to healthy controls, showed an improved conduit vasodilatory response with eight weeks of exercise training (Walsh *et al.*, 2003). Following training, the reported FMD responses in the CAD patients were similar to those reported for the healthy controls.

Studies examining the influence of exercise training in healthy populations have reported more inconsistent findings, however, these inconsistencies appear to be a result of the various training stimuli. Studies that reported improvements in FMD following training interventions suggest that a moderate to higher intensity endurance-type exercise may be required to improve endothelial function in these healthy individuals (Green *et al.*, 2004; Birk *et al.*, 2013). It has also been suggested, it has been reported that improvements in vascular function with exercise training can occur rapidly (Tinken *et al.*, 2008), and may then be succeeded by adaptations in arterial structure.

Limitations with FMD Assessment of Vascular Function

The FMD test offers a non-invasive measure of conduit artery function, which can provide valuable prognostic information; however this technique is not without limitation. First, the use of Doppler ultrasound for the determination of FMD requires costly equipment and skilled personnel for reliable data acquisition. Secondly, although FMD provides insight into peripheral conduit artery vasoreactivity in response to the shear stress stimuli, this measure largely negates other signaling pathways and vasodilator mechanisms that may also contribute to the response. In addition, there is no assessment of downstream hyperemia within the tissue itself. Finally, the results from the FMD test primarily focus on the changes in conduit artery dilation from a baseline to a post-occlusion value expressed as a percent dilation. Not only have concerns about the statistical bias of this calculation been recently broached (Atkinson *et al.*, 2013), but this commonly reported value also neglects the dynamic adjustments of the response.

As outlined previously, changes in vascular function with interventions such as exercise training may follow a relatively rapid time-course that is succeeded by structural adaptations. For example, Tinken *et al.* (Tinken *et al.*, 2008) reported functional changes (i.e., improved FMD) two weeks into an eight week exercise training intervention. These improvements in FMD were sustained at four weeks, however returned toward pre-training values with six to eight weeks of training. In this case, it is not that individuals exhibit endothelial dysfunction as the training program progressed, but that the structural adaptations that occur impede the ability of FMD to accurately assess vascular function. Therefore, this measure may not be ideally suited for detecting differences across exercise training interventions.

Near-infrared Spectroscopy

Near-infrared spectroscopy (NIRS) is a non-invasive optical method used for measuring tissue oxygenation. Measurements are based primarily on the absorption of light at wavelengths in the NIR range (700-900 nm), because oxygenated hemoglobin (HbO₂) and deoxygenated hemoglobin (HHb) display different light absorption characteristics. Specifically, NIR light is transmitted from an emitting diode to a light-detecting optode after passage through tissue, where the penetration depth is approximately half the distance of the inter-optode spacing (Kalliokoski *et al.*, 2006). Because HbO₂ and HHb display these different light absorption characteristics, by emitting light at several specific wavelengths in the NIR range of the spectrum and detecting changes in these received signals (i.e., after passing through tissue), precise separation and quantification of changes in these compounds is made possible. The changes in HbO₂ and HHb can be measured continuously, and tissue oxygen saturation (StO₂) can be extrapolated (defined as $[\text{HbO}_2] / [\text{HbO}_2 + \text{HHb}]$) (Ferrari *et al.*, 2004).

The microcirculation can be isolated because the light emitted into the larger vessels (arteries and veins) is almost completely absorbed by the larger relative molar concentration of hemoglobin (Hb), and thus any detectable changes in absorption can be attributed to the microcirculation. One limitation of the NIRS technique is the overlapping absorption spectra of muscle myoglobin (Mb) and Hb; this makes separation of these absorbers difficult. However, the contribution of Mb to light absorption changes is estimated to be ~10%, which leads to the interpretation that light absorption changes with NIRS are attributable mainly to the oxygenation status of Hb (Kalliokoski *et al.*, 2006).

By precisely separating and quantifying changes in [HbO₂] and [HHb], NIRS has been used to provide an index of O₂ extraction during exercise in humans and has become widely used to obtain measures of tissue oxygenation and hemodynamics at rest and during different exercise modalities and intensities (McCully *et al.*, 1994; De Roia *et al.*, 2012; Murias *et al.*, 2012; Bellotti *et al.*, 2013). More recently, it has been reported that static and dynamic measures of StO₂ during a vascular occlusion test (VOT) can help in evaluating peripheral perfusion in some clinical settings (Kragelj *et al.*, 2001; Doerschug *et al.*, 2007; Creteur *et al.*, 2007a; Mayeur *et al.*, 2011; Lipcsey *et al.*, 2012; De Backer *et al.*, 2013); specific studies in which NIRS was used in this way are discussed below.

StO₂ Measurements in Clinical Populations

NIRS is by no means a new technology, however advancements relating to the technology have led to the increasing use, especially within clinical populations. Early measures of StO₂, to evaluate peripheral perfusion, suggested that regional oxygenation measured by NIRS was able to non-invasively detect progressive hypovolemia. In trauma patients, suffering from this condition of low blood volume, absolute values of StO₂ were demonstrated to have a strong prognostic value such that low StO₂ values during initial exams were associated with larger transfusion requirements (Moore *et al.*, 2008; Smith *et al.*, 2008; Beekley *et al.*, 2010), increased risk of infection (Ikossi *et al.*, 2006), organ failure (Ikossi *et al.*, 2006; Cohn *et al.*, 2007) and higher mortality rates (Cohn *et al.*, 2007). The research surrounding the measurement of absolute StO₂ values in trauma patients indicated that NIRS provided valuable prognostic information, however, in septic conditions this association was less clear (Leone *et al.*, 2009; Shapiro *et al.*, 2011). Patients with septic shock exhibit diverse microcirculatory effects, such that the same vascular bed

can have ischemic and highly oxygenated regions ultimately leading to “normal” oxygenation measures of that area. Patients with this condition appear to have lower StO₂ values compared to healthy controls however there was a vast overlap between the two groups (Lipcsey *et al.*, 2012). These observations lead to a shift in focus away from measurements of absolute StO₂ values and lead to exploration of the dynamic StO₂ parameters derived from a vascular occlusion test (VOT).

Vascular Occlusion Test

The VOT consists of an arterial occlusion, similar to that of the FMD test, however occlusion occurs proximal to the NIRS probe site. The StO₂ response is measured throughout the duration of the test (before, during and after the ischemic challenge) and provides dynamic information which has been used to evaluate peripheral perfusion in some clinical settings (Kragelj *et al.*, 2001; Doerschug *et al.*, 2007; Creteur *et al.*, 2007a; Mayeur *et al.*, 2011; Lipcsey *et al.*, 2012; De Backer *et al.*, 2013). Figure 1.4 depicts the StO₂ response to a VOT.

A parameter of particular interest is the reperfusion slope, or the rate of re-saturation immediately following cuff release. This slope likely reflects the vascular reactivity of the microvasculature throughout the 5 minute vascular occlusion. While the FMD response to ischemia (macrovascular reactivity) is largely attributed to a single dilatory pathway (Green *et al.*, 2014), the mechanisms governing the control of microvascular blood flow distribution are not fully understood. There are likely multiple mechanisms influencing the microvascular response, one of which is shared with the FMD measure (i.e., endothelial-dependent nitric oxide-mediated vasodilation). The endothelium is likely to play a major role both detecting oxygen concentration in the capillary and by inducing

vasodilation by releasing NO (Greenberg & Kishiyama, 1993). However, additional pathways that may be influencing the StO₂ reperfusion slope may include metabolic pathways and even red blood cells themselves have been identified as regulators of oxygen delivery and distribution rather than just transporters (Bergfeld & Forrester, 1992). That being said, no study has compared the reperfusion slope with that of the widely used FMD assessment of endothelial function.

As mentioned previously, several studies have examined the dynamic adjustment of StO₂ response in combination with the VOT in clinical settings (For complete reviews see Mesquida *et al.* 2013, Boezeman *et al.* 2016). For example, Doerschug *et al.* (Doerschug *et al.*, 2007) reported the reperfusion rate during the hyperemic response of the thenar eminence muscle was significantly slower in septic subjects than in controls and this impairment was accentuated in those with more severe organ failure. Similarly, Creteur *et al.* (Creteur *et al.*, 2007a) found that the reperfusion slope was steeper in patients with severe sepsis who survived than in non-survivors. Additionally, they reported that the reperfusion slope tended to increase in survivors across the 48 h observation period but not in non-survivors. Taken together, these studies suggest that NIRS-derived measures of StO₂, specifically the reperfusion slope, can monitor differences in vascular responsiveness.

Very few studies to date have explored the use of the NIRS-derived StO₂ response to a VOT in healthy populations, and each of these studies have related to methodological approaches to the technique such as probe spacing (Bezemer *et al.*, 2009), comparing measurement sites (Bezemer *et al.*, 2009; Fellahi *et al.*, 2014) and modelling of the reperfusion response (Bopp *et al.*, 2011). The sensitivity of the measurement has been

clearly established in clinical populations to detect differences in vascular responsiveness compared with controls. However, it remains unclear whether the more subtle differences expected in vascular function between two healthy populations (i.e., trained and untrained) can be detected.

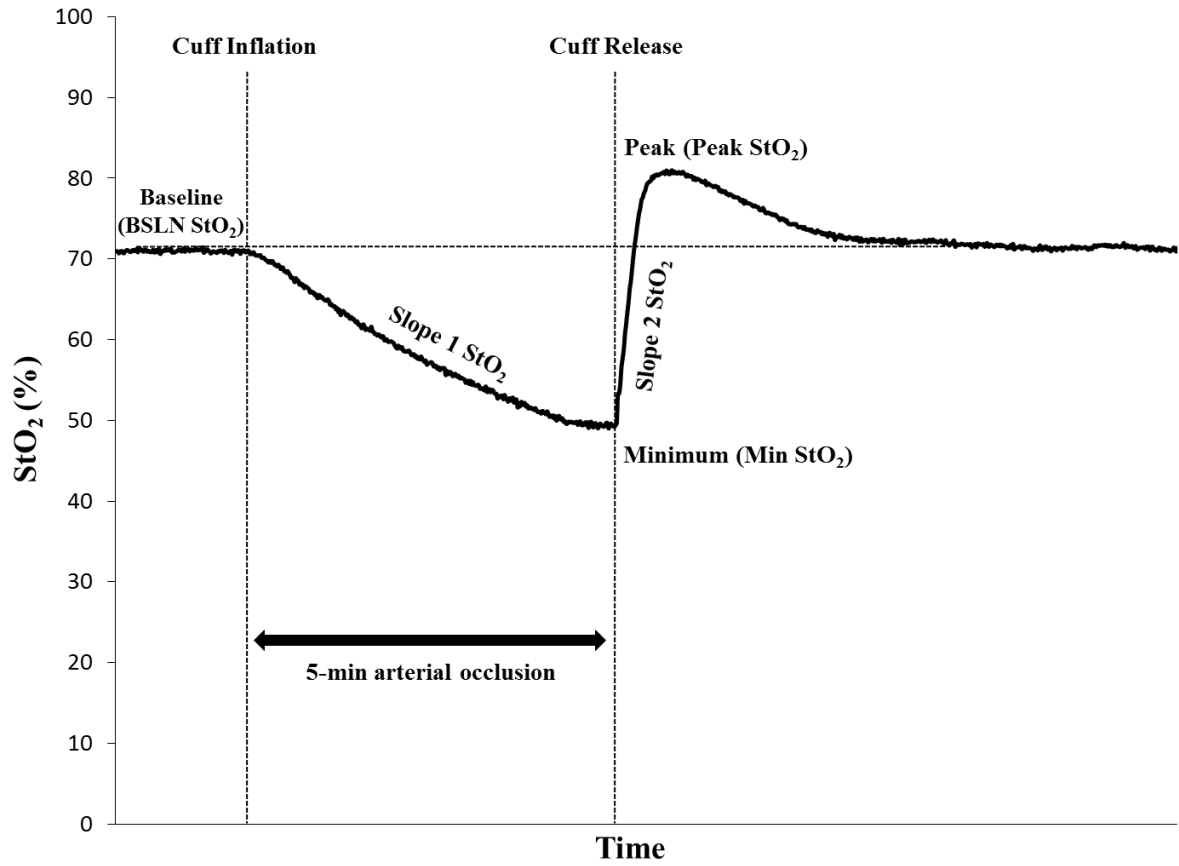


Figure 1.3. The NIRS-derived profile of % tissue oxygen saturation (StO_2) throughout a vascular occlusion test. BSLN StO_2 (%), baseline StO_2 prior to ischemia; Slope 1 StO_2 (%/s), StO_2 downslope following cuff inflation is considered to reflect muscle metabolism; Min StO_2 (%), absolute value for lowest StO_2 value reached during ischemia; Slope 2 StO_2 (%/s), StO_2 reperfusion slope following cuff release and is considered to be dependent on the accumulation of metabolites during the ischemic phase; Peak StO_2 (%), highest absolute StO_2 value reached following cuff release. Adapted from McLay *et al.*, 2016.

Summary, Overall Purpose of the Thesis

Vascular impairments at both the macro- and the micro-circulatory level are known to be associated with CVD and may be one of the earlier signs of disease. Using *In vivo* animal models, direct determination of endothelium-mediated vascular responsiveness is possible even at different levels of vascular organization (Muller-Delp, 2006; Murias *et al.*, 2013). However, accurate assessments of vascular reactivity are more challenging in humans. Tests of FMD have been widely used to non-invasively determine endothelium-dependent vasodilation in humans, and they are well documented to detect differences in vascular responsiveness in different groups such as trained versus untrained individuals (Allen *et al.*, 2003; Green *et al.*, 2003, 2010; Rakobowchuk *et al.*, 2008; Black *et al.*, 2009; Thijssen *et al.*, 2011*b*; Currie *et al.*, 2012; Birk *et al.*, 2013). However, the use of this technique poses some challenges. Thus, the overall purpose of this thesis is to explore new strategies to non-invasively determine the magnitude and dynamic adjustment of vascular responses in humans.

Overview of Studies

Chapter II will compare the more recently developed NIRS-derived measure of vascular function to that of the widely used ultrasound-derived flow-mediated dilation technique. The subsequent studies that comprise this thesis will build on the development and application of this technique as a reliable measure sensitive to differences in vascular responsiveness, and address some of the challenges with the FMD technique. The specific purposes of this thesis were:

1. To evaluate if NIRS technology can be used as a measure of vascular responsiveness by establishing a correlation between the NIRS-derived StO_2 reperfusion slope and the ultrasound-derived measure of flow-mediated dilation.
2. To examine the test-to-test reliability (variability between repeated tests within a single day) and day-to-day reliability of the NIRS-derived measure Slope 2 StO_2 , and compare it to the widely used measurement of flow-mediated dilation.
3. To examine if the employment of a new approach to allometrically scale values for flow-mediated dilation can improve the ability to obtain individual values that are corrected for variations in baseline diameter between subjects.
4. To determine if the NIRS-derived measure of StO_2 reperfusion slope (Slope 2 StO_2), which reflects vascular responsiveness, is sensitive to a range of ischemic conditions (i.e., various occlusion durations) and to determine if differences exist between two groups that would be expected to have different vascular responsiveness (i.e., trained and untrained individuals).

Hypotheses

This thesis specifically addressed the following hypotheses:

1. There would be a significant correlation between the ultrasound-derived measure of vascular responsiveness (FMD) and NIRS-derived reperfusion rate (Slope 2 StO_2).
2. That the reliability of the NIRS-derived measure for the reperfusion rate (Slope 2 StO_2) would be better than that of flow-mediated dilation for three tests repeated in a single day and five tests performed across five separate days.

3. Individual FMD values obtained from the new allometrically scaled calculation for flow mediated dilation would more closely resemble those typically presented for FMD calculated in the traditional manner, while still obtaining the benefits of controlling for variations in baseline diameter.
4. The reperfusion rate (Slope 2 StO₂) would progressively increase with the increase in occlusion duration and there will be differences in the reperfusion rate of trained and untrained individuals, such that trained individuals would have a steeper Slope 2.

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CHAPTER II: Vascular responsiveness determined by near-infrared spectroscopy measures of oxygen saturation

Introduction

Cardiovascular disease (CVD) is presently the leading cause of morbidity and mortality (Lloyd-Jones *et al.*, 2010; Green *et al.*, 2011). Vascular impairments at both the macro- and the micro-circulatory level are known to be associated with CVD (Lloyd-Jones *et al.*, 2010). More specifically, deficiencies in nitric oxide (NO) production and release by the endothelial cells, and the consequent reduction in vascular responsiveness have been proposed to play a pivotal role in the advancement of CVD (Langham & Wehrli, 2011). As such, proper assessment of endothelium-dependent vascular responses becomes an important instrument for early detection of pre-clinical dysfunction, diagnosis, monitoring of treatment efficacy and possibly prevention of CVD.

Using animal models, direct determination of endothelium-mediated vascular responsiveness is possible even at different levels of vascular organization (Muller-Delp, 2006; Murias *et al.*, 2013). However, accurate assessments of vascular reactivity are more challenging in humans. Tests of flow-mediated dilation (FMD) have been widely used to determine endothelium-dependent vasodilation in humans as they are considered the “gold standard” for non-invasively measuring this response; FMD is well documented to detect differences in vascular responsiveness in different groups such as trained versus untrained (Allen *et al.*, 2003; Green *et al.*, 2003, 2010; Rakobowchuk *et al.*, 2008; Black *et al.*, 2009; Thijssen *et al.*, 2011b; Currie *et al.*, 2012; Birk *et al.*, 2013) or older versus young (Parker *et al.*, 2006; Black *et al.*, 2009; Green *et al.*, 2010) individuals. However, the use of this technique poses some challenges. The use of Doppler ultrasound for the determination of

FMD requires high cost equipment and skilled personnel for data acquisition. In addition to this, although FMD provides insight into peripheral conduit artery vasoreactivity, there is no assessment of downstream hyperemia within the tissue itself. Moreover, the results from the FMD test primarily focus on the changes in conduit artery dilation from a baseline to a post-occlusion value expressed as a percent dilation, which neglects the dynamic adjustments of the response. Thus, exploring new strategies to non-invasively determine the magnitude and dynamic adjustment of vascular responses in humans is warranted.

A recent study has examined different dynamic parameter estimates of oxygenated hemoglobin (HbO₂) and blood flow velocity following cuff occlusion of the femoral artery using multi-echo gradient-recalled sequence (Langham & Wehrli, 2011). Although this novel approach considered the dynamic effect of the post-occlusion hyperemic responses, the methodology used in the study makes data collection and analysis difficult to incorporate into a clinical setting. Within the last decade, near-infrared spectroscopy (NIRS) has become widely used to obtain measures of tissue oxygenation and hemodynamics at rest and during different exercise modalities and intensities (McCully *et al.*, 1994; De Roia *et al.*, 2012; Murias *et al.*, 2012; Bellotti *et al.*, 2013). More specifically, it has been recently reported that static and dynamic measures of StO₂ during a vascular occlusion test (VOT) can help in evaluating peripheral perfusion in some clinical settings (Kragelj *et al.*, 2001; Doerschug *et al.*, 2007; Creteur *et al.*, 2007a; Mayeur *et al.*, 2011; Lipcsey *et al.*, 2012; De Backer *et al.*, 2013). The VOT is commonly performed on the thenar eminence (Doerschug *et al.*, 2007; Gómez *et al.*, 2008; Bezemer *et al.*, 2009; Mayeur *et al.*, 2011; Bopp *et al.*, 2011; Lipcsey *et al.*, 2012) with very few studies applying this technique on other sites (Kragelj *et al.*, 2001; Fellahi *et al.*, 2014). Although the thenar

eminence is useful for bed-side measures with intensive care patients, exploring different measurement sites of StO₂ may provide more insight into the relationship between macro- and micro-circulatory responses to occlusion for a region that may be more sensitive to evaluate the effect of interventions (i.e., acute and chronic exercise interventions) on vascular reactivity.

Therefore, the purpose of the present study was to evaluate if NIRS can be used as a measure of vascular responsiveness by establishing a correlation between the NIRS-derived StO₂ reperfusion slope and FMD. Based on the expected relationship between macrovascular responsiveness (FMD) and reperfusion rate (Slope 2 StO₂), we hypothesized that these two variables would be significantly correlated.

Methods

Participants

20 healthy young (age, 26 ± 3 yr; height, 177 ± 7 cm; weight, 82 ± 10 kg; $\text{VO}_{2\text{max}}$, 46 ± 5 ml·kg⁻¹·min⁻¹) adult men volunteered and gave written consent to participate in the study. This was a multi-site project with subjects being recruited and tested in London, Ontario, Canada and in Verona, Italy. All procedures conformed to the standards set by the latest revision of the Declaration of Helsinki and were approved by The University of Western Ontario Research Ethics Board for Health Sciences Research Involving Human Subjects and the Ethical Committee of the Department of Neurological and Movement Sciences, University of Verona (REB file number 102273). All participants were recreationally active and non-smokers. Additionally, all subjects were normotensive (Systolic: 125 ± 9 ; Diastolic: 72 ± 9) and no subjects were taking medications that would affect hemodynamic responses.

Protocol

Two FMD tests were performed on each participant, each separated by a 30 minute rest period to allow blood flow and arterial dilation to return to resting conditions (Harris *et al.*, 2006). Resting blood pressure was measured in triplicate prior to each FMD test, and was taken as the average of the three measurements.

Popliteal artery assessments

FMD of the popliteal artery was assessed in accordance with previously published guidelines for the current standardized methodology (Corretti *et al.*, 2002; Thijssen *et al.*, 2011a). All participants were instructed to refrain from caffeine, alcohol and exercise for 12 hours prior to their scheduled visit. Following at least 10 minutes of supine rest,

participants were instructed to lie prone as ultrasound imaging was performed on the back of the knee. A small pillow was placed under the participant's ankle for comfort and optimization of the knee angle so there was no leg movement throughout the cycles of the FMD tests. The left popliteal artery was imaged immediately proximal to the bifurcation (usually at or slightly above the popliteal fossa), and a pneumatic cuff (Flexiport; Welch Allyn Inc., Skaneateles Falls, NY, USA) was placed around the calf (approximately 5 cm distal to the popliteal fossa). Heart rate was continuously monitored with a three-lead ECG to allow for consistent and accurate selection of arterial diameter measurements at the end of the diastolic phase of the cardiac cycle.

The popliteal artery was imaged with a 10-MHz multifrequency linear-array transducer attached to a Doppler ultrasound machine (VingMed System FiVe, GE Medical Systems, Horten, Norway). All scans were performed by an experienced investigator. All scans were made with similar ultrasound settings and all images were recorded on an external video camera (HDD Everio; JVC, Canada) for later offline analysis. Baseline diameter was recorded prior to manual inflation of the pneumatic cuff. The cuff was then inflated for 5 minutes to an occlusion pressure of 250 mmHg, during which diameter was not recorded. Fifteen seconds prior to release of the cuff the video camera resumed recording and at exactly 5 minutes after inflation, the pneumatic cuff was released and arterial diameter was continuously monitored for 5 minutes post-release.

Diameter measurements, defined as the distance between the media and intima interface of the near wall and far wall, were obtained using a caliper that converted image pixels to millimeters. Triplicate measurements of diameter were taken for each of five baseline images and averaged to determine the baseline diameter (D_{base}) of the artery.

Similarly, triplicate measurements of diameter were averaged for images taken every 15 seconds following cuff release. Peak diameter (D_{peak}) was determined as the post-occlusion image with the largest diameter and percent flow-mediated dilation (%FMD) was then calculated as the percent change in diameter from D_{base} .

Near-infrared spectroscopy.

StO₂ of the tibialis anterior muscle was monitored continuously with a frequency-domain multi-distance near-infrared spectroscopy (NIRS) system (Oxiplex TS, ISS, Champaign, Ill., USA). Briefly, the system was composed of a single channel consisting of 8 laser diodes operating at 2 wavelengths ($\lambda = 690$ and 828 nm, 4 at each wavelength), which were pulsed in rapid succession, and a photomultiplier tube. The lightweight plastic NIRS probe (connected to laser diodes and a photomultiplier tube by optical fibers) consisted of 2 parallel rows of light emitter fibers and 1 detector fiber bundle; the source-detector separations for this probe were 2.0, 2.5, 3.0, and 3.5 cm for both wavelengths. The probe was placed on the belly of the muscle, was secured in place with an elastic strap tightened to prevent movement and was covered with an optically dense, black vinyl sheet, thus minimizing the intrusion of extraneous light. An elastic bandage was applied to further minimize intrusion of extraneous light and probe movement. By measuring changes in light absorption at different wavelengths, changes in oxyhemoglobin (HbO₂) and deoxyhemoglobin (HHb) can be measured continuously, and StO₂ can be extrapolated (defined as $[\text{HbO}_2] / [\text{HbO}_2 + \text{HHb}]$). NIRS measurements were collected continuously for the entire duration of each FMD test (2 min baseline, 5 min occlusion and 5 min post-release) plus an additional 3 minutes following cuff release (for a total of 8 minutes post-release).

The NIRS device was calibrated at the beginning of the first test session following an instrument warm-up period of at least 20 min. The calibration was done with the probe placed on a calibration block (phantom) with absorption (μ_a) and reduced scattering coefficients (μ_s') previously measured; thus, correction factors were determined and were automatically implemented by the manufacturer's software for the calculation of the μ_a and μ_s' for each wavelength during the data collection. Calculation of [HbO₂] and [HHb] reflected continuous measurements of μ_s' made throughout each testing session (i.e., constant scattering value not assumed). The probe remained secured to the leg throughout the duration of the visit to ensure measurement consistency between both FMD tests. Data were stored online at an output frequency of 2 Hz, but were reduced to 1 s bins for all subsequent analyses within the present study.

The NIRS parameters calculated are depicted in Figure 2.1. Baseline StO₂ (BSLN StO₂, %) was calculated as the average StO₂ prior to ischemia. The desaturation rate was quantified as the downslope of StO₂ (Slope 1 StO₂, %/S) measured from 30 to 150 seconds following cuff inflation. Minimum StO₂ (%) was calculated as the lowest StO₂ value attained during ischemia. The StO₂ reperfusion rate was quantified as the upslope of a 10 second window following cuff release of the StO₂ signal (Slope 2 StO₂, %/s). Peak StO₂ (%) was calculated as the highest StO₂ value reached following cuff release.

Statistical Analysis

All data were imported into SigmaPlot 11 (Systat Software, Inc., SanJose, CA, USA). Correlation coefficients (Pearson product moment) were used to determine relationships of FMD and NIRS-derived Slope 2 StO₂, as well as the relationships between NIRS-derived parameters. Statistical significance was declared when $p < 0.05$. All data

are presented as means \pm standard deviation. The coefficients of variation (CV) were calculated for %FMD and the NIRS-derived Slope 2 StO₂.

Results

FMD: Group means and standard deviation for baseline diameter, peak diameter and %FMD are reported in Table 2.1.

NIRS: Group means and standard deviation for StO₂ parameters are listed in Table 2.1.

%FMD was significantly correlated with Slope 2 StO₂ ($R = 0.63$, $df = 19$, $p = 0.003$; Figure 2.2). There was no significant correlation between Slope 2 StO₂ and the minimum StO₂ ($R = 0.34$, $df = 19$, $p = 0.15$) or between Slope 2 StO₂ and Slope 1 StO₂ ($R = 0.35$, $df = 19$, $p = 0.13$).

The CVs for %FMD and Slope 2 StO₂ were $29.3 \pm 13.3\%$ and $11.0 \pm 7.8\%$, respectively.

Table 2.1. Ultrasound- and NIRS-derived measurements throughout a vascular occlusion test.

	Young Men (n = 20)
Ultrasound-derived Measures	
Baseline diameter (mm)	6.7 ± 0.5
Peak diameter (mm)	7.1 ± 0.6
Flow-mediated dilation (%)	6.7 ± 3.4
NIRS-derived Measures	
Baseline StO ₂ (%)	71.3 ± 2.9
Minimum StO ₂ (%)	44.8 ± 8.6
Slope 1 StO ₂ (%·s ⁻¹)	-0.10 ± 0.03
Peak StO ₂ (%)	82.6 ± 2.3
Slope 2 StO ₂ (%·s ⁻¹)	1.63 ± 0.5

Notes: Values are means \pm standard deviations.

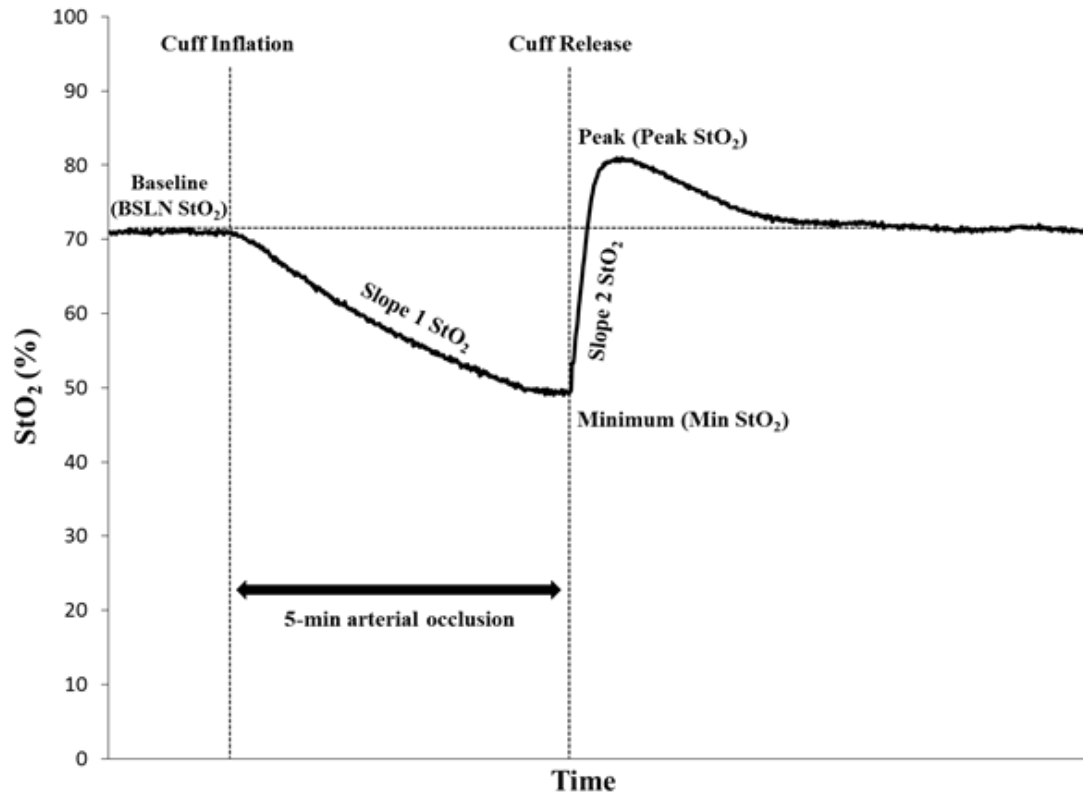


Figure 2.1. NIRS-derived profile of % tissue oxygen saturation (StO₂) for a representative subject measured during a vascular occlusion test.

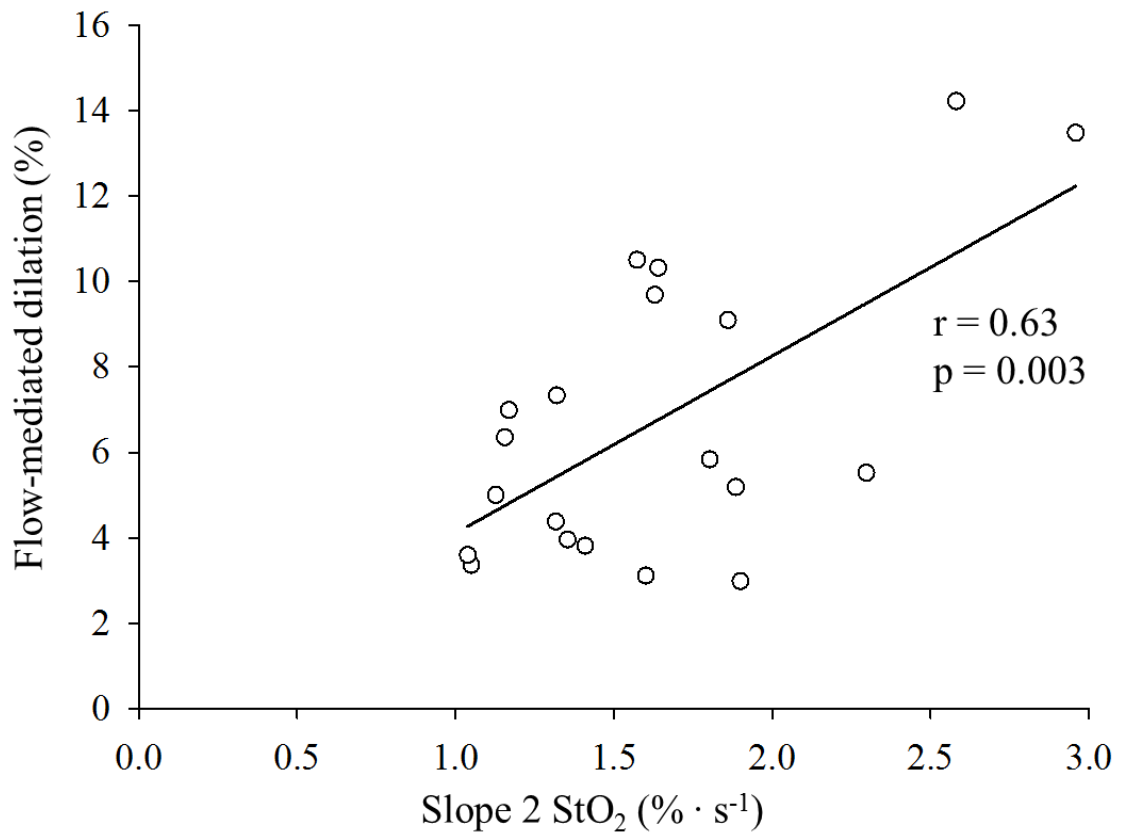


Figure 2.2. Individual values of flow-mediated dilation, expressed as percentage of the baseline diameter value (%FMD), are plotted as a function of the speed of re-oxygenation (Slope 2 StO₂) after cuff release during the vascular occlusion test in young adults.

Discussion

The main goal of this study was to determine if a relationship existed between NIRS-derived StO₂ reperfusion slope (microvascular response) and FMD (arterial vascular responsiveness) measured in healthy young adult men. The main finding was that the NIRS-derived measure of tissue O₂ saturation, Slope 2 StO₂, was significantly associated with the FMD response, such that the Slope 2 StO₂ provides a measure of vascular reactivity.

During a vascular occlusion test the reperfusion phase following cuff release, results in an immediate increase in blood flow to the ischemic limb, characterized by Slope 2 StO₂. The present study is the first known to the authors to establish a correlation between the “gold-standard” technique for non-invasive assessment of vascular function and the more recently proposed NIRS technique for assessing vascular responsiveness. Slope 2 StO₂ was significantly correlated with %FMD likely related to the fact that both FMD and Slope 2 StO₂ are, at least in part, dependent upon the increase in metabolism as a result of ischemia. Although FMD by definition is the dilatory response to an increase in flow, downstream accumulation of catabolites during the ischemic period results in a rapid dilation of smaller vessels (ie. 3A and terminal arterioles) (Berg *et al.*, 1997). This downstream dilation (i.e. the drop in local resistance) elicits the resultant hyperemic response that stimulates the FMD in the upstream conduit artery. Thus, this significant correlation between %FMD and Slope 2 StO₂ is important from a physiological perspective.

The present study is the first to our knowledge to utilize NIRS during vascular occlusion in healthy young adults and attempt to associate the reperfusion slope of the StO₂

with endothelial function. However, this technique has been previously used in clinical populations (i.e., patients with sepsis and septic shock) to assess peripheral perfusion (Kragelj *et al.*, 2001; Doerschug *et al.*, 2007; Creteur *et al.*, 2007a; Mayeur *et al.*, 2011; Lipcsey *et al.*, 2012; De Backer *et al.*, 2013). For example, Doerschug *et al.* (Doerschug *et al.*, 2007) reported the reperfusion rate during the hyperemic response of the thenar eminence muscle was significantly slower in septic subjects than in controls and this impairment was accentuated in those with more severe organ failure. Similarly, Creteur *et al.* (Creteur *et al.*, 2007a) found that Slope 2 StO₂ was higher in patients with severe sepsis who survived than in non-survivors. Additionally, they reported that Slope 2 StO₂ tended to increase in survivors over the observation period but not in non-survivors. These studies utilized patients with severe sepsis, an inflammatory response that can alter blood flow distribution, and suggested that NIRS-derived measures of StO₂, specifically the reperfusion slope, could monitor differences in hemodynamic responses, which aligns with results from the present study.

An important aspect of the present study is the strong and significant correlation that was reported between Slope 2 StO₂ and %FMD, in spite of the high variability for FMD. The %FMD measure has been shown to have low reliability, which is associated with a large error (Hijmering *et al.*, 2001; Brook *et al.*, 2005; Magda *et al.*, 2012). This would tend to reduce the chances of finding a strong correlation. This high variability of the %FMD measure, in part, inspired the use of NIRS measures of StO₂ in combination with a VOT for assessing microvascular reperfusion and reactivity. An advantage of using NIRS measures is that they have been shown to be highly reliable both within and between days. Gomez *et al.* (Gómez *et al.*, 2008) studied the repeatability of NIRS measurements

of StO₂ in combination with a VOT in healthy controls. They reported high reproducibility of StO₂ for three VOTs performed within the same day (CV $14.2 \pm 9.2\%$), as well as high reliability for tests 6 months apart (CV $21.2 \pm 16.4\%$). The present study found similar values for the CV of Slope 2 StO₂ (CV $11.0 \pm 7.8\%$), which were lower than those for %FMD (CV $29.3 \pm 13.2\%$). Another advantage of utilizing the NIRS technique is the ease with which changes in microvascular reactivity can be assessed at the level of the muscle instead of solely relying on conduit artery estimates of vascular reactivity.

At the onset of vascular occlusion (i.e. cuff inflation) the metabolic activity of the muscle leads to a linear decline in StO₂, Slope 1 StO₂, which is said to reflect the oxygen extraction (i.e., the metabolic rate) of the tissue. The minimum StO₂ attained during occlusion is considered to indicate the extent of ischemia attained during the set occlusion period. Mayeur *et al.* (Mayeur *et al.*, 2011) reported that Slope 2 StO₂ is influenced by the extent of StO₂ desaturation, showing a strong correlation between the minimum StO₂ and Slope 2 StO₂ during a 3 minute occlusion period in healthy young adults. Previously, De Blasi *et al.* (De Blasi *et al.*, 1994) reported that Slope 2 StO₂ is metabolism-dependent and strongly correlates to Slope 1 StO₂, such that a greater metabolic accumulation during ischemia results in a greater reperfusion response of Slope 2 StO₂. The present study did not find a significant correlation between Slope 2 StO₂ and either the minimum StO₂ or Slope 1 StO₂. This is likely due to the longer occlusion period used in the present study. The 3 minute occlusion period used in previous studies allowed for a range of desaturation levels to be attained, however all of the participants in the present study reached what might be considered a maximal desaturation level by the end of the 5 minute occlusion period. All of the participants reached a StO₂ close to 40%, which is the lowest value typically

reported in the literature (Doerschug *et al.*, 2007; Mayeur *et al.*, 2011). For a correlation to be shown a range of occlusion periods would need to be used to reach varying levels of desaturation. In the present study a 5 minute occlusion period was used as that is the standardized occlusion period recommended for optimal determination of the endothelial-dependent vasodilatory response of FMD (Thijssen *et al.*, 2011a).

The present study was performed using healthy young adult men with no history of vascular impairment. Although results from previous research using patient populations support the present results, it is possible that this technique may not be applicable to other clinical populations (i.e., patients with a flow-limiting stenosis). In addition to the patient conditions, there are other factors to consider that may alter the StO₂ response such as blood viscosity and alterations in hemoglobin that would change the indirect relationship of StO₂ and endothelial function as assessed by FMD. Although these results are promising, further research is required to confirm that the results from the present study would extend to other populations.

In conclusion, this study was able to establish a significant correlation between the NIRS-derived measure of Slope 2 StO₂ and ultrasound-derived %FMD. The data support the notion that NIRS-derived measures of StO₂ obtained directly at the microvascular level are reflective of the microvascular reperfusion and reactivity as measured via FMD in the conduit artery. The ease of use, good reproducibility, cost-efficiency and low invasiveness of this technique make it a promising application for assessing vascular responsiveness and for monitoring changes in vascular responses to various interventions (i.e., exercise training, diet or pharmacological) in the leg, as previously proposed for other regions of the body.

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CHAPTER III: Repeatability of vascular responsiveness measures derived from near-infrared spectroscopy

Introduction

Impaired endothelial-dependent vasodilation is an important feature of vascular disease and is strongly associated with several chronic cardiovascular conditions (Neunteufl *et al.*, 2000; Perticone *et al.*, 2001; Kuvin *et al.*, 2001; Gokce *et al.*, 2002; Modena *et al.*, 2002; Widlansky *et al.*, 2003). The reactive hyperemia endothelial function test, commonly referred to as a flow-mediated dilation (FMD) test, is a widely used, non-invasive technique which provides insight into peripheral conduit artery vasoreactivity and information about the integrity and function of the endothelium (Vita & Keaney, 2002).

The FMD technique, typically assessed in peripheral conduit arteries such as the brachial (Betik *et al.*, 2004), radial (Brook *et al.*, 2005), superficial femoral (Kooijman *et al.*, 2008), and popliteal (Green *et al.*, 2010), has increasingly been applied in both clinical and physiological studies. Although FMD is an important tool in assessing vascular and endothelial function, there is some concern regarding the reliability of the measurement. Some studies have reported that the test-retest repeatability of %FMD, as measured by the coefficient of variation (CV) statistic, can be markedly worse than that of the baseline and peak diameters (Herrington *et al.*, 2001). Additionally, other studies have shown that %FMD may be a satisfactory (Peretz *et al.*, 2007), or a very poor (Brook *et al.*, 2005), indicator of vascular function due to high variability between repeated measures.

A new approach to assess vascular responsiveness has emerged with the use of near-infrared spectroscopy (NIRS). Recently, tissue oxygen saturation (StO₂) was measured distal to the occlusion site during a FMD test and the NIRS-derived reperfusion

slope immediately following ischemia (Slope 2 StO₂) was indicated to be a good measure of vascular responsiveness (McLay *et al.*, 2016a), which could be used to test responses to various interventions (i.e., exercise training, diet or pharmacological). Additionally, this new approach to assess vascular function can be easily employed in the leg, something that is more difficult with FMD due to the small diameter changes relative to the large vessel diameter. This is an important feature as being able to evaluate changes in vascular responsiveness in the regions where those changes are often expected to occur (e.g., in the lower limbs before and after an exercise training intervention or chronic adaptations to exercise) might contribute to a better characterization of this response. Furthermore, measurements of StO₂ have been previously conducted in clinical settings and have reported results that suggest that NIRS-derived measures, specifically the reperfusion rate, were able to monitor differences in hemodynamic responses. Creteur *et al.* (Creteur *et al.*, 2007a) found that the reperfusion slope was higher in patients with severe sepsis who survived than in non-survivors. Additionally, they reported that the reperfusion slope tended to increase in survivors over the observation period but not in non-survivors.

With the increasing use of noninvasive techniques to assess vascular responsiveness both in clinical settings and in physiological research, it is important to have a better understanding of the reliability of measures being used. To our knowledge, no study to date has systematically evaluated the day-to-day (interday) and test-to-test (intraday) reliability of the recently proposed measurement of vascular reactivity (Slope 2 StO₂) in the leg, or compared the reliability to that of %FMD in the popliteal artery, which is a necessary step if this new approach is to be applied to compare responses before and after an intervention or simply at different time points. Therefore, the main purpose of the

present study was to examine the test-to-test reliability (variability between repeated tests within a single day) and day-to-day reliability of the NIRS-derived measure Slope 2 StO₂; by calculating the repeatability, which is a measure of absolute reliability and used to examine the influence of measurement errors (Bland & Altman, 1996). A secondary aim of this study was to compare the reliability of Slope 2 StO₂ to that of the widely used FMD measurement.

Methods

Participants

Nine healthy young men (mean \pm SD, age: 26 ± 3 yr; mass: 82 ± 8 kg; height: 178 ± 4 cm) volunteered and gave written consent to participate in the study. All procedures were approved by The University of Western Ontario Research Ethics Board for Health Sciences Research Involving Human Subjects. All participants were recreationally active (i.e., meeting the recommended guidelines for physical activity (Tremblay *et al.*, 2011)) and non-smokers. Additionally, all subjects were normotensive (mean blood pressure \pm SD: systolic 124 ± 7 mmHg; diastolic 66 ± 7 mmHg) and no subjects were taking medications that would affect hemodynamic responses.

Study Design

A series of FMD tests were performed on each participant over five consecutive days, with NIRS-derived measures of StO₂ obtained throughout the duration of each test. All tests were performed in an environment where temperature was controlled throughout the testing protocol (20-22°C) and at the same time each day to minimize diurnal effects. All participants were instructed to refrain from caffeine, alcohol and exercise for >12 hours prior to their scheduled visit. One FMD test was performed on each of four days, with a fifth day involving three FMD tests. The day when three FMD tests were performed was randomized between subjects and each of the three FMD tests were separated by a 30 minute rest period to allow blood flow and arterial dilation to return to resting conditions (Harris *et al.*, 2006). Each FMD test was performed with an occlusion pressure of 250 mmHg.

Near-infrared spectroscopy

StO₂ of the tibialis anterior muscle was monitored continuously throughout each FMD test with a frequency-domain multi-distance NIRS system (Oxiplex TS, ISS, Champaign, Ill., USA). Briefly, the system was composed of a single channel consisting of 8 laser diodes operating at 2 wavelengths ($\lambda = 690$ and 828 nm, 4 at each wavelength), which were pulsed in rapid succession, and a photomultiplier tube. The lightweight plastic NIRS probe (connected to laser diodes and a photomultiplier tube by optical fibers) consisted of 2 parallel rows of light emitter fibers and 1 detector fiber bundle; the source-detector separations for this probe were 2.0, 2.5, 3.0, and 3.5 cm for both wavelengths. The probe was placed on the belly of tibialis anterior the muscle (midway between the knee and the ankle), was secured in place with an elastic strap tightened to prevent movement and was covered with an optically dense, black vinyl sheet, thus minimizing the intrusion of extraneous light. An elastic bandage was applied to further minimize intrusion of extraneous light and probe movement. A pneumatic cuff (Adult 11 long, Flexiport; Welch Allyn Inc., Skaneateles Falls, NY, USA) was placed just below the knee, above but not over the secured NIRS probe. By measuring changes in light absorption at different wavelengths, changes in oxyhemoglobin (HbO₂) and deoxyhemoglobin (HHb) can be measured continuously, and StO₂ can be calculated (defined as $[\text{HbO}_2] / [\text{HbO}_2 + \text{HHb}]$). NIRS measurements were collected continuously for the entire duration of each FMD test (2 min baseline, 5 min occlusion and 5 min post-release) plus an additional 3 minutes following cuff release to ensure StO₂ fully returned to baseline levels (for a total of 8 minutes post-release).

The NIRS device was calibrated at the beginning of the first test session following an instrument warm-up period of at least 20 min. The calibration was done with the probe placed on a calibration block (phantom) with absorption (μ_a) and reduced scattering coefficients (μ_s') previously measured; thus, correction factors were determined and were automatically implemented by the manufacturer's software for the calculation of the μ_a and μ_s' for each wavelength during the data collection. Calculation of [HbO₂] and [HHb] reflected continuous measurements of μ_s' made throughout each testing session (i.e., constant scattering value not assumed). The probe remained secured to the leg throughout the duration of the visit to ensure measurement consistency between both FMD tests. Data were stored online at an output frequency of 2 Hz, but were reduced to 1 s bins for all subsequent analyses within the present study.

Baseline StO₂ (%) was calculated as the average of 1 min of StO₂ prior to ischemia. Minimum StO₂ (%) was calculated as the lowest StO₂ value attained during ischemia. The StO₂ reperfusion rate was quantified as the upslope of a 10 second window immediately following cuff release of the StO₂ signal (Slope 2 StO₂, %·s⁻¹); the reperfusion rate immediately following cuff release is a relatively linear response which allows for a simple slope calculation. Peak StO₂ (%) was calculated as the highest StO₂ value reached following cuff release (McLay *et al.*, 2016a).

Popliteal artery assessments

FMD of the popliteal artery was assessed in accordance with previously published guidelines for the current standardized methodology (Corretti *et al.*, 2002; Thijssen *et al.*, 2011a). Following at least 10 minutes of supine rest, participants were instructed to lie prone as ultrasound imaging was performed on the back of the knee. A small pillow was

placed under the participant's ankle for comfort and optimization of the knee angle so there was no leg movement throughout the cycles of the FMD tests. The left popliteal artery was imaged immediately proximal to the bifurcation (usually at or slightly above the popliteal fossa), and a pneumatic cuff (Flexiport; Welch Allyn Inc., Skaneateles Falls, NY, USA) was placed around the calf (approximately 5 cm distal to the popliteal fossa). Heart rate was continuously monitored with a three-lead ECG to allow for consistent and accurate selection of arterial diameter measurements at the end of the diastolic phase of the cardiac cycle.

The popliteal artery was imaged with a 10-MHz multifrequency linear-array transducer attached to a Doppler ultrasound machine (VingMed System FiVe, GE Medical Systems, Horten, Norway). All scans were performed by an experienced investigator. All scans were made with similar ultrasound settings and all images were recorded on an external video camera (HDD Everio; JVC, Canada) for later offline analysis. Baseline diameter was recorded prior to manual inflation of the pneumatic cuff. The cuff was then inflated for 5 minutes to an occlusion pressure of 250 mmHg, during which diameter was not recorded. Fifteen seconds prior to release of the cuff the video camera resumed recording and at exactly 5 minutes after inflation, the pneumatic cuff was released and arterial diameter was continuously monitored for 5 minutes post-release.

Diameter measurements, defined as the distance between the media and intima interface of the near wall and far wall, were obtained using a caliper that converted image pixels to millimeters. Triplicate measurements of diameter were taken for each of five baseline images and averaged to determine the baseline diameter of the artery. Similarly, triplicate measurements of diameter were averaged for images taken every 15 seconds

following cuff release. Peak diameter was determined as the post-occlusion image with the largest diameter and percent flow-mediated dilation (%FMD) was then calculated as the percent change in diameter from baseline.

Statistical Analysis

All statistical analyses were performed using SPSS software, version 19 (SPSS Inc., Chicago, Ill., USA) and Microsoft Excel 2010 (Microsoft, Seattle, Wash., USA).

Group mean, standard deviation (SD) and coefficient of variation ($CV = SD/\text{mean} \times 100$) were calculated for NIRS- and ultrasound-derived parameters for each test. A one-way repeated measures analysis of variance (ANOVA) was used to determine if there were significant differences within the variables of the five NIRS and FMD tests performed over consecutive days, and the three NIRS and FMD tests performed within the same day. The repeatability, also known as the coefficient of repeatability, of each variable for the comparisons was calculated by multiplying the within-subject standard deviation (S_w) by 2.77 [or $(1.96 \times \sqrt{2}) \times S_w$] (Bland & Altman, 1996). The repeatability represents the critical value at which a measurable change is observed in a given participant between tests. Reliability of three FMD tests repeated in a single day and the five tests performed over consecutive days were assessed using the intraclass correlation coefficient (ICC), which was based on the repeated measures ANOVA with testing session as the independent variable (Shrout & Fleiss, 1979). For statistical tests $p < 0.05$ was considered significant.

Results

NIRS: Group means and standard deviation for StO₂ parameters for within and between-day comparisons are listed in Tables 3.1 and 3.2, respectively. There was no significant difference in Slope 2 StO₂ between tests performed within a single day, or across five days. Repeatability values for the intraday and interday comparisons were 0.43 and 0.48, respectively; which represents that 33% and 36% of the mean value could be attributed to measurement error. ICC for the intraday and interday comparisons were 0.92 and 0.94, respectively. CV for the intraday and interday comparisons were $9 \pm 4\%$ (range 3 – 15%) and 14 ± 5 (range 9 – 24). Figure 3.1 shows the average profile for each of three tests performed in a single day (Panel A), as well as the variation in Slope 2 StO₂ values for each individual and group means (Panel B). Figure 3.2 shows the average profile for each of the five tests performed over five days (Panel A), as well as the variation in Slope 2 StO₂ values for each individual and group means (Panel B).

FMD: Group means and standard deviation for baseline diameter, peak diameter and %FMD for within and between-day comparisons are listed in Tables 3.1 and 3.2, respectively. There was no significant difference for %FMD between tests performed within a single day, or across five days. Repeatability values for the intraday and interday comparisons were 5.62 and 4.82, respectively; which means that greater than 100% of the mean value for FMD could be attributed to measurement error both within and between days. ICC for the intraday and interday comparisons were 0.36 and 0.25, respectively. CV for the intraday and interday comparisons were $44 \pm 24\%$ (range 7 – 79%) and $40 \pm 22\%$ (range 3 – 88%). Figure 3.3 shows the variation in %FMD values for each individual, as well as group means, for three tests within a single day (Panel A) and five tests across five

days (Panel B).

Table 3.1. Ultrasound- and NIRS-derived measurements throughout a vascular occlusion test for three tests performed in a single day.

	Test 1	Test 2	Test 3
Ultrasound-derived Measures			
Baseline diameter (mm)	6.5 ± 0.7	6.7 ± 0.5	6.6 ± 0.8
Peak diameter (mm)	6.8 ± 0.7	6.9 ± 0.6	6.9 ± 0.8
Flow-mediated dilation (%)	4.5 ± 2.5	3.1 ± 2.0	5.6 ± 3.5
NIRS-derived Measures			
Baseline StO ₂ (%)	69.6 ± 1.7	71.1 ± 2.4	70.7 ± 2.9
Absolute Minimum StO ₂ (%)	46.0 ± 6.6	46.2 ± 7.5	45.9 ± 7.5
Absolute Peak StO ₂ (%)	81.5 ± 1.8	82.1 ± 1.4	82.2 ± 2.0
Slope 2 StO ₂ (%·s ⁻¹)	1.24 ± 0.25	1.32 ± 0.38	1.32 ± 0.33

Notes: Values are means \pm standard deviations.

Table 3.2. Ultrasound- and NIRS-derived measurements throughout a vascular occlusion test for five tests performed across five days.

	Day 1	Day 2	Day 3	Day 4	Day 5
Ultrasound-derived Measures					
Baseline diameter (mm)	6.7 ± 0.7	6.7 ± 0.5	6.7 ± 0.6	6.5 ± 0.5	6.7 ± 0.5
Peak diameter (mm)	6.9 ± 0.6	6.9 ± 0.6	7.0 ± 0.6	6.8 ± 0.5	7.0 ± 0.4
Flow-mediated dilation (%)	3.1 ± 2.0	3.5 ± 1.6	4.8 ± 1.8	4.5 ± 2.3	4.5 ± 2.0
NIRS-derived Measures					
Baseline StO ₂ (%)	71.1 ± 2.4	71.7 ± 3.6	72.3 ± 2.6	70.4 ± 4.0	70.4 ± 3.5
Absolute Minimum StO ₂ (%)	46.2 ± 7.5	46.6 ± 9.9	49.2 ± 5.9	46.6 ± 8.0	46.1 ± 7.7
Absolute Peak StO ₂ (%)	82.1 ± 1.4	83.4 ± 2.7	82.7 ± 1.7	82.8 ± 2.5	81.5 ± 4.5
Slope 2 StO ₂ (%·s ⁻¹)	1.32 ± 0.38	1.38 ± 0.41	1.37 ± 0.35	1.39 ± 0.40	1.30 ± 0.40

Notes: Values are means \pm standard deviations.

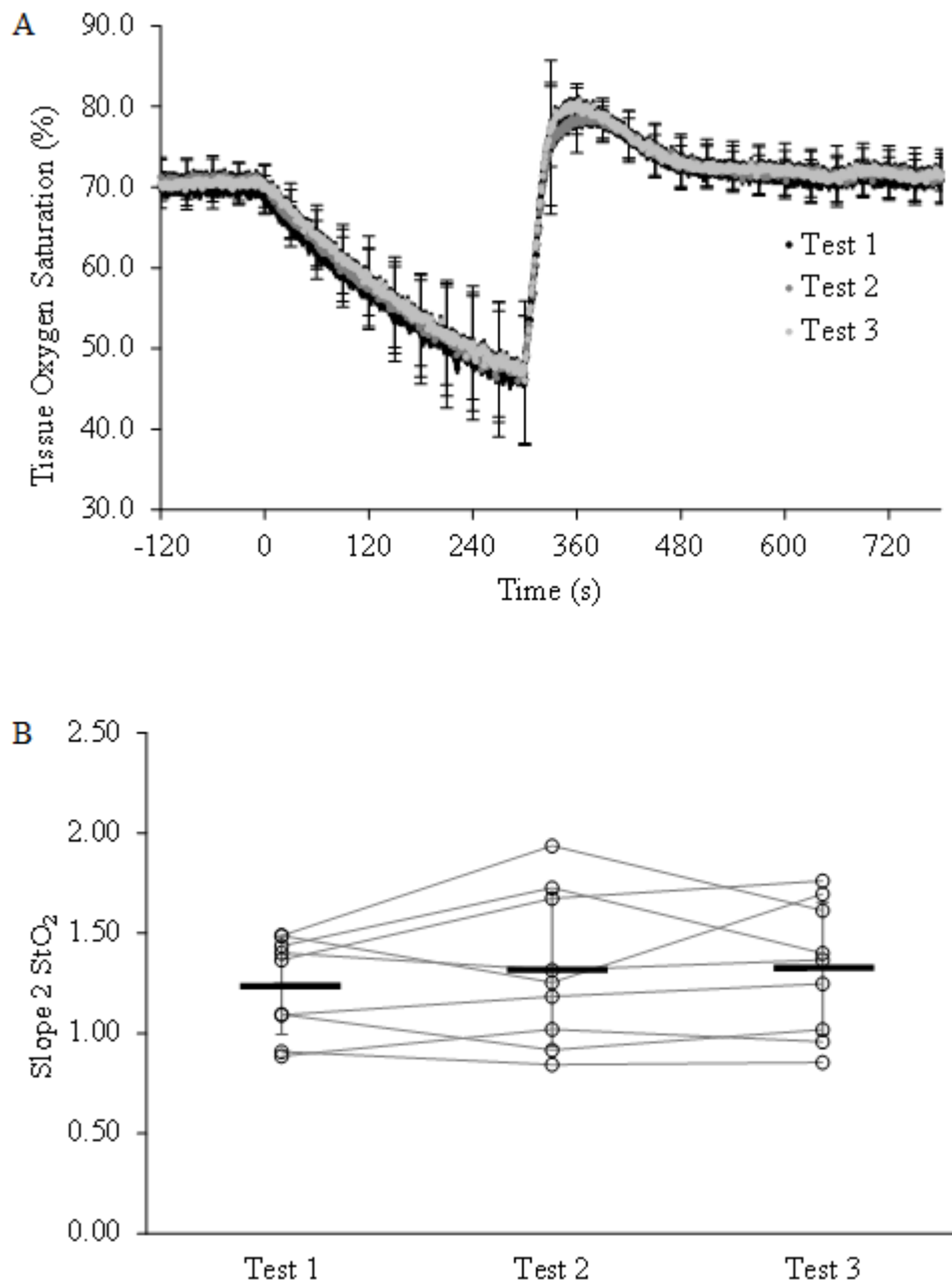


Figure 3.1. A) Group average tissue oxygen saturation profile for three vascular occlusion tests performed within the same day; B) Group mean values for Slope 2 StO₂ each day are represented by the thick black lines, with each open circle and connecting line representing an individual subject.

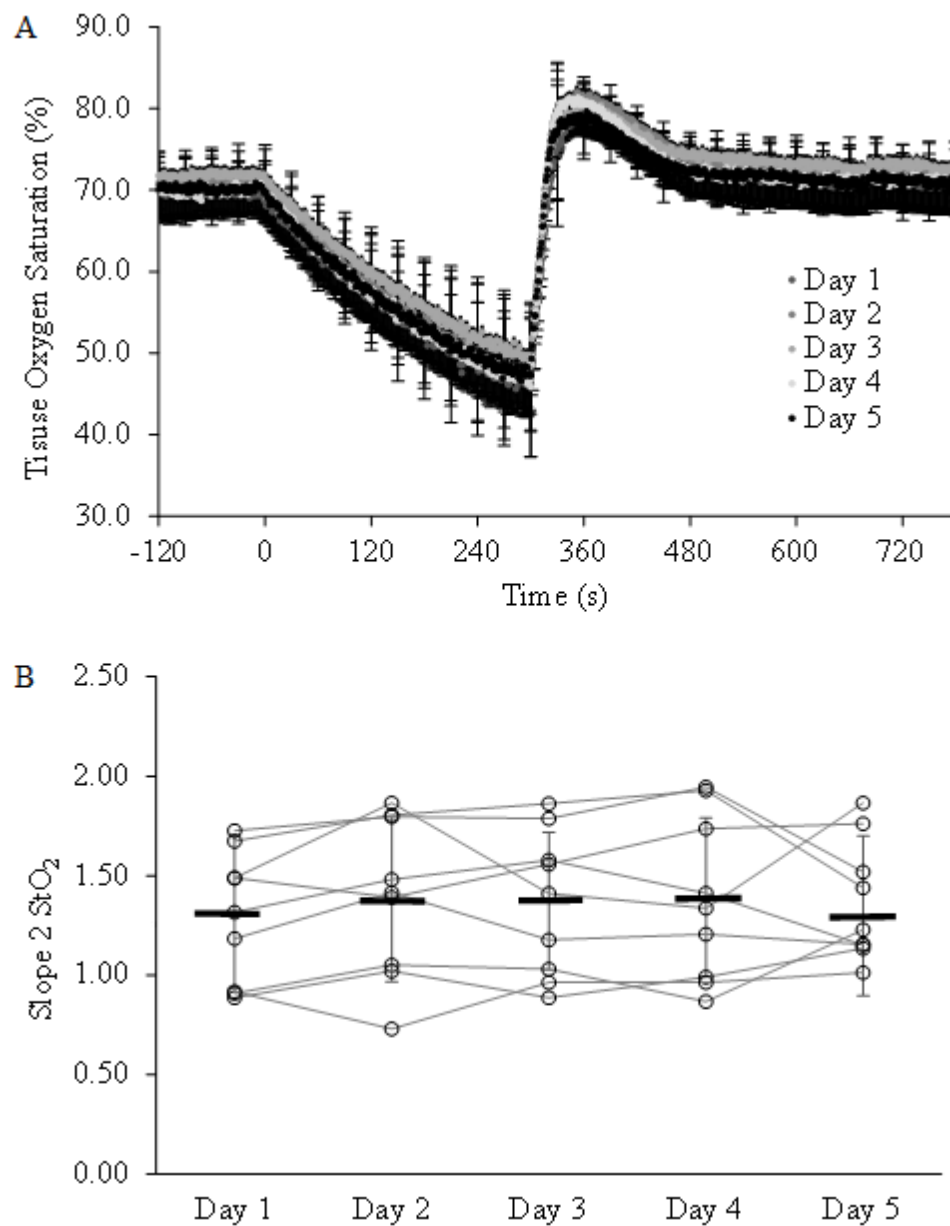


Figure 3.2. A) Group average tissue oxygen saturation profile for each of five vascular occlusion tests performed on separate days; B) Group mean values for Slope 2 StO_2 each day are represented by the thick black lines, with each open circle and connecting line representing an individual subject.

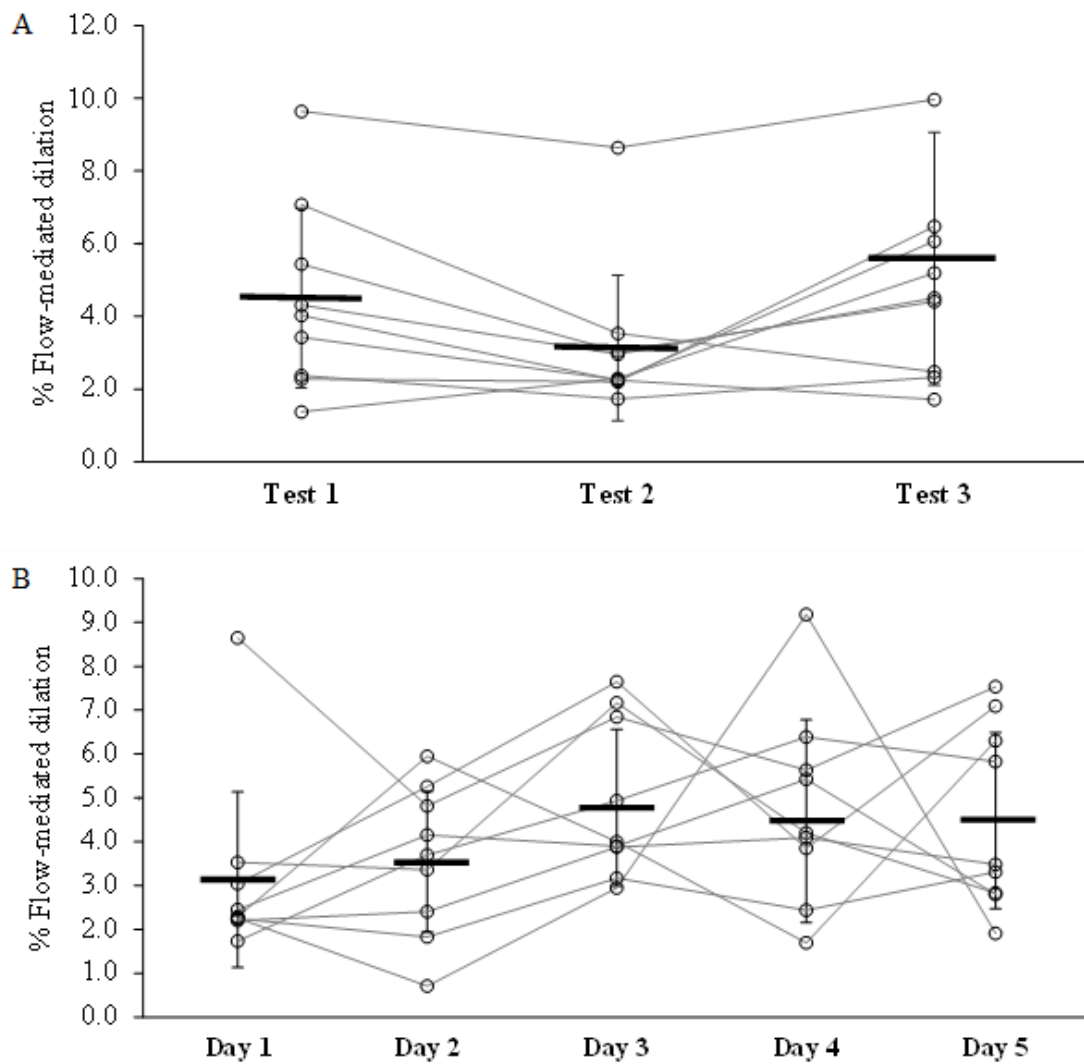


Figure 3.3. A) Group mean values for each of three FMD tests performed in a single day are represented by the thick black lines, with each open circle and grey line representing an individual subject; B) Group mean values and individual data for each of five FMD tests performed across five days.

Discussion

The main goal of this study was to investigate the test-to-test (intraday) and day-to-day (interday) reliability of the NIRS-derived Slope 2 StO₂ and compare it to that obtained from ultrasound-derived %FMD of the popliteal artery. The main findings were as follows: 1) the reliability of the Slope 2 StO₂ measure was strong both within and between testing days (low repeatability values, high ICCs, and low CV); 2) the reliability of %FMD, at least in the artery measured in this study, was poorer than that observed for the StO₂, both within and between testing days, as indicated by high repeatability values, low ICCs, and high CV.

Although NIRS-derived measures of StO₂ have been shown to reflect vascular responsiveness (McLay *et al.*, 2016a), and they have been used to measure StO₂ in healthy and clinical populations previously (Doerschug *et al.*, 2007; Creteur *et al.*, 2007a), the present study was the first to comprehensively examine the reliability of the NIRS-derived parameter Slope 2 StO₂ in the leg. This is important as repeatability is used to examine the influence of measurement errors on data analysis and is an indicator of absolute reliability (Bland & Altman, 1996), which is necessary to know when interpreting changes in response to an intervention or as a consequence of factors such as training status, disease, etc. In the present study, small values for repeatability indicated strong reliability of the measures. Repeatability values were indicative of strong reliability of the measure for both intraday (0.43) and interday (0.48) comparisons, with approximately 30% of the measurement potentially being influenced by measurement error. This study also reported the ICC, which provides a measure of relative reliability. As suggested by Portney and Watkins (Portney & Watkins, 2000), ICC values >0.75 are considered to be reliable. In

the present study, ICC values for intraday (0.92) and interday (0.94) reliability of Slope 2 StO₂ indicated very strong reliability of the technique. Similarly, CV was indicative of strong reliability of the measure both within ($9 \pm 4\%$) and between days ($14 \pm 5\%$). Comparable CV values for Slope 2 StO₂ in the leg were previously reported by McLay *et al.* (McLay *et al.*, 2016a); however, of note, in that study the CV was derived from only two tests performed in a single day. The present study examined the reliability not only within a single day (with three repeats) but also across multiple days, something of particular value for intervention-type studies. These results are consistent with a previous study that reported the CV of StO₂ in combination with vascular occlusion in the thenar eminence of healthy controls. Gómez *et al.* (Gómez *et al.*, 2008) reported high reliability of StO₂ for three occlusion tests performed within the same day (CV $14.2 \pm 9.2\%$). The present study examined the reliability of the Slope 2 StO₂ in a more systematic way than Gómez *et al.* and also reported strong day-to-day reliability for tests performed on five consecutive days.

In this study, Slope 2 StO₂, a relatively new approach to assessing vascular responsiveness which has been shown to significantly correlate to %FMD (McLay *et al.*, 2016a), showed better reliability than that of the widely used measure of FMD. Repeatability values were high for intraday and interday FMD such that a difference of 5.6% and 4.8% respectively would be needed to observe a change in FMD that would not be associated with measurement error of the technique. These values for repeatability are as large as the mean FMD measures themselves. Similarly, ICC values for both intraday (0.36) and interday (0.25) comparisons were indicative of poor reliability. It has to be acknowledged that the present study utilized the popliteal artery for FMD measures, which

could result in poorer reliability as compared to what might be observed in arteries that are more commonly assessed, such as the brachial or the radial artery. However, it has to be noted that poor reliability has been reported in other measurement sites as well. Hardie *et al.* (Hardie *et al.*, 1997) demonstrated that reproducibility of brachial artery FMD was poor and likely to provide inaccurate measurements for two FMD tests separated by an average of 90 days. Repeatability calculated from reported values for within subject SD indicated that changes in FMD in the brachial artery would need to be approximately 19% to be able to detect differences that could not be attributed to measurement error. Similarly, Brook *et al.* (Brook *et al.*, 2005) assessed intra- and interday reliability for two FMD tests performed in the same day and two tests performed approximately 7 days apart. The repeatability values were high for both intraday (11%) and interday (11%). Thus, even though the relevance of the %FMD as a measure of vascular responsiveness is undeniable, this lack of reliability that is often acknowledged in the literature and that it is also supported by this current experimental data set, might be one of the factors contributing to the lack of differences in %FMD between groups that were expected to show different responses (Birk *et al.*, 2013; Atkinson & Batterham, 2013). A measure such as the Slope 2 StO₂ can contribute to the study of non-invasive assessment of vascular responsiveness not only by providing another technique that can estimate vascular reactivity within the microvasculature, but also by offering the possibility of detecting between group differences more easily (i.e., needing a lower number of participants) thanks to the greater reliability of this measure.

The measurement of StO₂ throughout a vascular occlusion has been previously used in clinical settings to monitor recovery of patients. Studies have shown significant

differences between the reperfusion rate of the StO₂ signal (Slope 2 StO₂) of septic patients and healthy controls, with the septic patients having a much slower reperfusion following cuff release than that of controls (Doerschug *et al.*, 2007). Additionally, repeated measurements of Slope 2 StO₂ in intensive care patients has shown that slopes increase over time in surviving patients, but not in non-survivors (Doerschug *et al.*, 2007). These studies, in combination with recent demonstration of the association between Slope 2 StO₂ and %FMD (McLay *et al.*, 2016a), demonstrate the ability of this measure to detect differences in vascular and hemodynamic responses.

Vascular impairments at both the macro- and the micro-circulatory level are known to be associated with cardiovascular disease (CVD) (Lloyd-Jones *et al.*, 2010). As such, proper assessment of vascular responsiveness becomes an important instrument for early detection of pre-clinical dysfunction, diagnosis, monitoring of treatment efficacy and possibly prevention of CVD. The use of NIRS allows for changes in microvascular reactivity to be assessed at the level of the muscle instead of solely relying on conduit artery estimates of vascular reactivity. This is an important feature as some forms of CVD have been indicated to originate with functional limitations within the microcirculation (Seals, 2014). Thus, detecting problems where they originate may help early detection of future cardiovascular complications. In the present study, assessing vascular responsiveness in the leg was an important factor when selecting the area of FMD and NIRS interrogation as several training intervention are predominately lower limb exercises (such as cycling and running) and thus, even though it is acknowledged that changes in vascular reactivity can extend to other areas, it is important to be able to assess vascular responsiveness in the region that is more affected by the proposed intervention. Nonetheless, with any

measurement technique it is important to understand the degree to which differences in measures may be attributed to physiological adaptations to various interventions or clinically meaningful changes instead of reflecting measurement error. The strong reliability of this new technique to measure vascular responsiveness, taken together with the previously established capability of tracking changes in hemodynamic responses, makes it a promising application for assessing vascular responsiveness, complimenting the assessment of endothelial health, and in monitoring responses to various interventions.

A criticism of the present study could be that %FMD was calculated from diameter measurements made every 15 seconds, as the most recent recommendations for the analysis of FMD stipulate the use of edge-tracking software for the assessment of arterial diameters for each cardiac cycle in the post-occlusive period. A previous analysis comparing manual and edge-tracking software (data not presented) found that there was no difference between FMD values obtained through the two analysis methods. Additionally, although the automated edge-tracking software does include more diameters for the determination of %FMD, there is more noise in the data as a result of the variability in the greater number of diameters which results in poorer reliability of the measure derived from the edge-tracking software. In this context, the values reported here are derived from the less variable %FMD of the manual analysis to avoid exacerbating the low repeatability of the FMD measure. The present study did not focus on the reliability of the FMD measure but instead emphasizes the strong reliability of the new approach to assessing vascular reactivity (i.e., NIRS-derived measures of Slope 2 StO₂).

The present study also reports values for Baseline StO₂, Minimum StO₂ and Peak StO₂ as a reference for future studies. It should be noted that the static values for StO₂ are

not “corrected” for adipose tissue thickness (ATT), which has been suggested to influence the measurement of these values (Niwayama *et al.*, 2000). Nevertheless, while ATT may influence the absolute values of StO₂ obtained for certain parameters it has been reported that dynamic changes, such as Slope 2 StO₂, should not be affected as the changes are independent of absolute values (Bopp *et al.*, 2011). That being said, the TA muscle offers an ideal sight of measurement for NIRS as there is often less adipose tissue overlying the muscle compared to other muscles in the leg.

In conclusion, this study demonstrated that the NIRS-derived Slope 2 StO₂, a measure established to reflect vascular reactivity (Doerschug *et al.*, 2007; Creteur *et al.*, 2007a; McLay *et al.*, 2016a), has strong reliability. The reliability of this new approach for non-invasively assessing vascular responsiveness has important implications for the assessment of vascular responsiveness as it might contribute in determining differences between groups or before and after an intervention that would be otherwise difficult to establish.

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CHAPTER IV: Is allometric scaling necessary for the accurate assessment of flow-mediated dilation in young healthy men?

Introduction

Impaired endothelial-dependent vasodilation is an important feature of vascular disease and is strongly associated with several chronic cardiovascular (CV) conditions (Neunteufl *et al.*, 2000; Perticone *et al.*, 2001; Kuvin *et al.*, 2001; Gokce *et al.*, 2002; Modena *et al.*, 2002; Widlansky *et al.*, 2003). The reactive hyperemia endothelial function test, commonly referred to as a flow-mediated dilation (FMD) test, is a widely used, non-invasive technique which provides insight into peripheral conduit artery vasoreactivity and provides information about the integrity and function of the endothelium (Vita & Keaney, 2002). The FMD technique, typically assessed in peripheral conduit arteries such as the brachial (Betik *et al.*, 2004; Pyke *et al.*, 2008; Weissgerber *et al.*, 2011; Szigyarto *et al.*, 2013), radial (Pyke *et al.*, 2009), superficial femoral (Kooijman *et al.*, 2008; Totossy de Zepetnek *et al.*, 2014), and popliteal (Parker *et al.*, 2006; McLay *et al.*, 2016a), has increasingly been applied in both clinical and physiological studies. The FMD test has been documented to correlate with invasively assessed endothelial function in the coronary arteries (Takase *et al.*, 1998a), and appears to be predictive of CV events in asymptomatic subjects (Fathi *et al.*, 2004) and those with established cardiovascular disease (CVD) (Neunteufl *et al.*, 2000). The primary outcome measure of the FMD test is the change in arterial diameter from baseline (D_{base}) to a post-ischemic peak diameter measurement (D_{peak}). This change (D_{diff}) is then expressed as a percentage of D_{base} (Eq. 1) and that is the value (%FMD) commonly reported by researchers over the past two decades.

$$\%FMD = \frac{(D_{peak} - D_{base})}{D_{base}} \times 100 \quad (1)$$

Although FMD is a widely used measure, limitations have been acknowledged such as poor reliability (Hardie *et al.*, 1997; Brook *et al.*, 2005) and bias towards D_{base} , such that the D_{base} of the vessel is going to influence the %FMD response. In this regard, Atkinson *et al.* (2013) have suggested that the traditional calculation of FMD as a % change from baseline violates statistical assumptions and introduces an element of error, because the dilatory response is very small in relation to the D_{base} . D_{base} has been negatively correlated to %FMD (Thijssen *et al.*, 2008) such that individuals with larger values for D_{base} (i.e., diseased populations) have lower %FMD, and vice versa. However, although D_{base} is often higher in diseased versus healthy populations (Yeboah *et al.*, 2007, 2009), D_{base} can also become larger in response to many interventions, including regular exercise (Birk *et al.*, 2013). This may account for the difficulty in explaining certain results; for example, trained and untrained individuals having similar %FMD values. Atkinson *et al.* (2013) have proposed the use of allometric scaling of FMD to eliminate the dependency of %FMD on D_{base} . The *Atkinson scaling* technique employs a log-linked generalized linear model with the absolute change in diameter (D_{diff}) as the outcome and logarithmically transformed D_{base} as a covariate. This approach yields ‘corrected’ group mean values for %FMD by dividing the covariate-adjusted mean of D_{diff} by the antilog of D_{base} , for conventional interpretation in percentage terms. Although the ANCOVA model to generate ‘corrected’ group means has value when assessing FMD in different populations, with different D_{base} , this technique is also being proposed for application in populations where no difference in D_{base} exists.

Additionally, the ANCOVA model only generates ‘corrected’ group mean values for %FMD and a single standard deviation (SD) shared by all groups with no indication of FMD from the individual tests. To allow for the calculation of scaled FMD values for individual tests the authors also outline a calculation which requires the slope of the relationship between logarithmically transformed D_{base} and D_{peak} (Atkinson *et al.*, 2013). This slope value, m , is proposed as the correct allometric exponent for scaling purposes in a given population, such that individually scaled FMD for each participant can be calculated by dividing D_{peak} by D_{base}^m (eq. 2).

$$Atkinson\ scaling\ FMD = \frac{D_{peak}}{D_{base}^m} \quad (2)$$

Preliminary analysis from individual data indicated that these individual values were substantially different from the ‘corrected’ means for FMD derived from the ANCOVA model, such that the values for the individuals were larger than would make physiological sense based on previously reported data. This led us to further investigate the use of the *Atkinson scaling FMD* calculation for individual participants and, eventually, to consider a new approach for individually scaling FMD (eq. 3).

$$New\ scaling\ FMD = \frac{D_{peak} - D_{base}}{D_{base}^m} \times 100 \quad (3)$$

Thus, the purpose of the present study was to examine if the employment of the *new scaling FMD* calculation improved the ability to obtain individual FMD values that are corrected for variations in D_{base} . We hypothesized that individual FMD values obtained from the *new scaling FMD* calculation would yield FMD values that were close to those typically presented for FMD, while still obtaining the benefits of controlling for D_{base} .

Methods

Participants

Eighteen healthy young men (mean \pm SD, age: 26 ± 3 yr; mass: 82 ± 7 kg; height: 181 ± 5 cm) volunteered and gave written consent to participate in the study. All procedures conformed to the standards set by the latest revision of the Declaration of Helsinki and were approved by The University of Western Ontario Research Ethics Board for Health Sciences Research Involving Human Subjects. All participants were recreationally active (i.e., meeting the recommended guidelines for physical activity (Tremblay *et al.*, 2011)) and non-smokers. Additionally, all subjects were normotensive (mean blood pressure \pm SD: systolic 124 ± 7 mmHg; diastolic 68 ± 6 mmHg) and no subjects were taking medications that would affect hemodynamic responses.

Experimental Protocol

FMD of the popliteal artery was assessed in accordance with previously published guidelines for the current standardized methodology (Corretti *et al.*, 2002; Thijssen *et al.*, 2011a). All participants were instructed to refrain from caffeine, alcohol and exercise for >12 hours prior to their scheduled appointments and all tests were performed in an environment where temperature was controlled throughout the testing protocol (21 °C). Following 10 minutes of supine rest, participants were instructed to lie prone as ultrasound imaging was performed on the back of the knee; a small pillow was placed under their ankle for comfort and optimization of the knee angle so there was no leg movement throughout the cycles of the FMD tests. The left popliteal artery was imaged immediately proximal to the bifurcation (usually at or slightly above the popliteal fossa), and a pneumatic cuff (Flexiport; Welch Allyn Inc., Skaneateles Falls, NY, USA) was placed

around the calf (approximately 5 cm distal to the popliteal fossa). Heart rate was continuously monitored with a three-lead ECG to allow for consistent and accurate selection of arterial diameter measurements at the end of the diastolic phase of the cardiac cycle.

The popliteal artery was imaged with a 10-MHz multifrequency linear-array transducer attached to a Doppler ultrasound machine (VingMed System FiVe, GE Medical Systems, Horten, Norway). All scans were performed by the same investigator who had completed specific training for the performance of FMD tests. Ultrasound settings were kept the same between all FMD tests and all images were recorded on an external video camera (HDD Everio; JVC, Canada) for later offline analysis. Baseline diameter was recorded prior to manual inflation of the pneumatic cuff. The cuff was then inflated to 250 mmHg for 5 minutes, during which diameter was not recorded. At 15 seconds prior to release of the cuff the video camera resumed recording. At exactly 5 minutes after inflation, the pneumatic cuff was released and arterial diameter was continuously monitored for 5 minutes post-release.

Popliteal Artery Diameter Analysis

Commercially available software (Sante DICOM Editor, Version 3.2.1; Santesoft, Athens, Greece) was used to extract the end-diastolic frames, determined by the R-spike of the ECG trace, and stack them in a new DICOM file for determination of popliteal artery diameters. Diameters at end-diastole were determined with a semi-automated edge detection software program (Artery Measurement System Image and Data Analysis, Gothenburg, Sweden). Baseline diameter (D_{base}) was determined from the average of ten end-diastole frames. Post-release diameters were averaged into 5-second time bins (Origin,

OriginLab Corp., Northampton, Massachusetts, USA). Peak diameter (D_{peak}) was determined as the largest post-occlusion diameter.

FMD was calculated in each of three ways, where D_{peak} is the largest post-ischemic diameter, D_{base} is the average diameter prior to cuff-inflation, and m is the slope of the relationship between logarithmically transformed D_{base} and D_{peak} ;

$$1) \text{ Traditional calculation: } \%FMD = \frac{(D_{peak} - D_{base})}{D_{base}} \times 100$$

$$2) \text{ Atkinson scaling: } \%FMD = \frac{D_{peak}}{D_{base}^m}$$

$$3) \text{ New scaling: } \%FMD = \frac{D_{peak} - D_{base}}{D_{base}^m} \times 100$$

Statistical Analysis

Group means and SD were calculated for the FMD statistics derived from each of the three different calculations. Correlations (Pearson product moment) between D_{base} and each of the three FMD calculations were calculated using SigmaPlot 11.0 (Systat Software, Inc., SanJose, CA, USA).

Results

The means and SD for D_{base} , D_{peak} and each of the three FMD calculations are listed in Table 4.1. The slope of the relationship between logarithmically transformed D_{base} and D_{peak} was 0.94 (Figure 4.1).

Figure 4.2 illustrates the relationship between D_{base} and each of the three FMD calculations. The *traditional FMD* had a very weak correlation ($r = 0.0161$). Employment of the *Atkinson scaling FMD* resulted in more positive correlation ($r = 0.2104$). The *New scaling FMD* resulted in a weaker correlation than that of the *traditional FMD* calculation ($r = 0.0029$).

Table 4.1. Variables measured during the flow-mediated dilation protocol based on individual values.

	n = 18
Baseline diameter, D_{base} (mm)	6.96 ± 0.62
Peak diameter, D_{peak} (mm)	7.36 ± 0.68
<i>Traditional FMD</i> Calculation (%)	5.82 ± 2.58
<i>Atkinson scaling FMD</i> Calculation (%)	19.25 ± 2.98
<i>New scaling FMD</i> Calculation (%)	6.56 ± 2.91

Values are presented in means \pm standard deviation

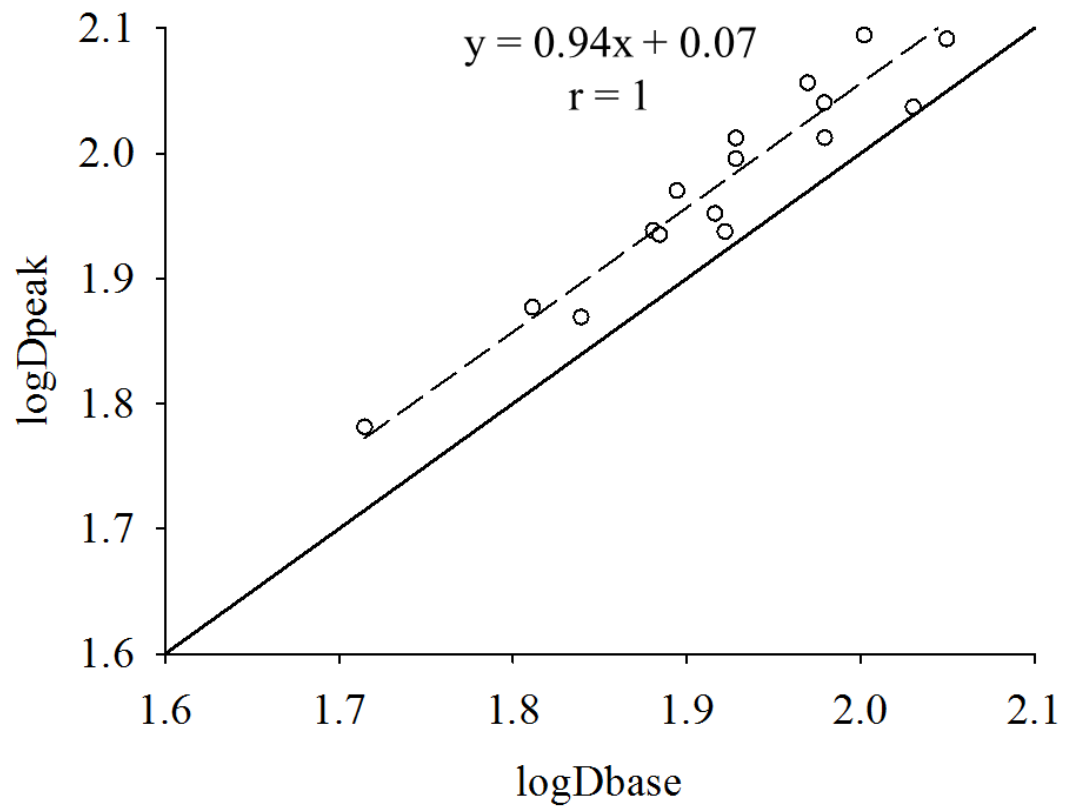


Figure 4.1. The relationship between logarithmically transformed values for baseline and peak diameter. The equation for the regression is plotted on the graph; such that the regression slope is 0.94.

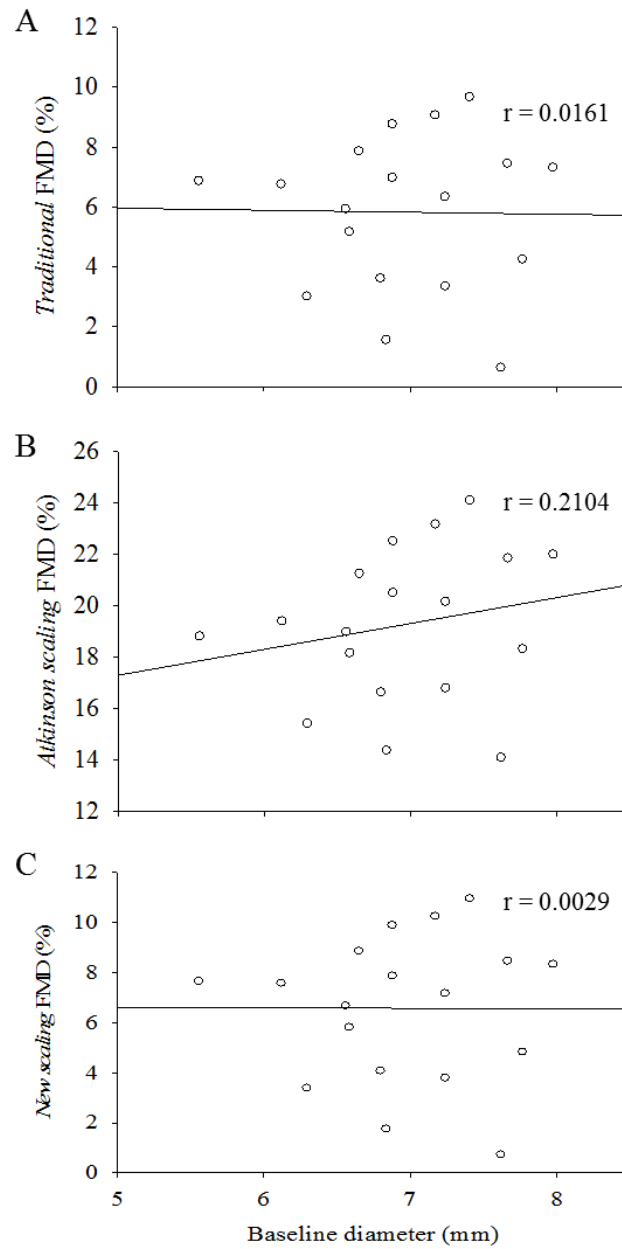


Figure 4.2. The correlation between values of D_{base} and calculated %FMD for the *traditional FMD* (A), *Atkinson Scaling FMD* (B) and *New scaling FMD* (C). There is no negative correlation between D_{base} and the *traditional FMD*, which results in a more positive correlation with the employment of the *Atkinson scaling*. The *new scaling FMD* results in a correlation less positive than the *traditional FMD* calculation.

Discussion

The main goal of this study was to examine the relationship between the *Atkinson scaling FMD* and *traditional FMD* values and determine if the employment of the *new scaling FMD* calculation improved the ability to obtain individual FMD values that are corrected for variations in D_{base} . The main finding was that the average %FMD from the individual values derived from the *Atkinson scaling FMD* calculation were 3-4 fold larger than meaningful %FMD values that should be expected for this measure. Additionally, the *new scaling* calculation for FMD resulted in values that were within the expected %FMD response for this measure, but did not correct for variation in D_{base} as well as the *Atkinson scaling FMD* calculation.

FMD is a very common assessment of endothelial function however concerns with the specificity of the measure in providing useful information has been recently identified, as the measure is heavily dependent upon D_{base} . This concept was identified by leading laboratories in the area of FMD (Atkinson *et al.*, 2013; Stoner *et al.*, 2013) and ultimately lead to the proposed use of allometric scaling. Although the scaling method may have value for evaluating group changes and estimating mean %FMD, the suggestion of applying it on an individual basis for prognostic and diagnostic purposes has deficiencies. The individual values derived from the *Atkinson scaling* calculation are 3-4 times larger than those of the *traditional FMD*. The high values of FMD are a concern when FMD is used as a prognostic or diagnostic tool, because it becomes difficult to establish what a clinically meaningful differences in %FMD would be. Additionally, the averages of the individually scaled values differ from those calculated in preliminary analysis from the proposed ANCOVA model for group means. This discrepancy in the mean values is likely

due to the fact that mean values are more easily corrected than individual values with this method of scaling. The ANCOVA model calculates mean values for D_{diff} using D_{base} as the covariate, and these values are then back-transformed into percentages for conventional interpretation. However, the individual FMD values are allometrically scaled for the participant's D_{base} value which is raised to the power of a slope derived from group data. The difference between the two mean values, suggested a need to modify the *Atkinson scaling* calculation in order to obtain values of FMD that were closer to what would be expected from this physiological response, while still being scaled for variations in D_{base} .

The use of the *new scaling* calculation for %FMD did result in mean values for each FMD test that approximated those of the original calculation and those produced by the ANCOVA model, however the calculation does not account for variation in D_{base} as well as the *Atkinson scaling*. %FMD has been shown to negatively correlate with D_{base} , as %FMD is based on the premise that D_{diff} will vary in proportion with D_{base} (Atkinson & Batterham, 2013). Employment of the *Atkinson scaling* reduces this bias towards D_{base} and resultant negative correlation, ultimately producing a more positive correlation. Although the present study did not find a strong negative correlation between %FMD and D_{base} , employment of the *Atkinson scaling* resulted in a slightly more positive correlation indicating a reduction of a dependency on D_{base} (Figure 4.2). The relationship between D_{base} and the *new scaling FMD* produced a similar correlation to that of the *traditional FMD* calculation, thereby indicating that the *new scaling FMD* does not adequately scale for the variation in D_{base} .

Allometric scaling may have value for evaluating differences between groups with expected differences in D_{base} (i.e., comparing children to adults, or diseased and healthy

populations), however the question remains as to whether this analysis technique should be adopted as a standard practice in all FMD studies. As outlined earlier in the discussion, the present study found no negative correlation between the *traditional %FMD* and D_{base} , which would suggest that variations in D_{base} are not strongly influencing the %FMD values in this sample group. The present study utilized a rather homogeneous population (i.e., healthy young men) in which very little difference in D_{base} would be expected; however, studies involving similar population demographics are being asked to employ allometric scaling practices to account for variations in D_{base} . The results of the present study would indicate that although allometric scaling has usefulness comparing groups where differences in D_{base} exist, there appears to be no additional value to the application in studies of a similar population or studies quantifying within-subject effects where no arterial structural adaptations are expected.

Technical considerations: Although we acknowledge that the FMD technique is more commonly used to assess endothelial function of the brachial artery, the present study examined %FMD responses in the popliteal artery. Two reasons prompted this decision: 1) as shown by Parker *et al.* (2006), the average dilatory response of the popliteal is markedly less than that of the brachial ($8.1 \pm 1.5\%$ compared to $14.6 \pm 1.9\%$) which should result in variation in D_{base} having a larger influence on the calculation of popliteal artery %FMD; 2) several exercise intervention studies prescribe primarily lower body exercises (i.e., cycling and running). Thus, more information surrounding the assessment of FMD at a more localized level may be of additional value in interventions that mainly involve lower limb muscle activity.

In conclusion, although allometric scaling offers some benefits in that it reduces the bias of D_{base} on the FMD measurement, individual values cannot be calculated, which reduces the prognostic value of the approach and limits the ability to examine the statistical relevance of the measure. Additionally, the present study found no advantage to employing the allometric scaling approach in this population of healthy young individuals. Thus, recommendations related to the advantages of allometrically scaling the %FMD measure in homogeneous sample groups should be made with caution until research clearly establishes the benefits of this approach.

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CHAPTER V: Vascular responsiveness measured by tissue oxygen saturation reperfusion slope is sensitive to different occlusion durations in healthy trained and untrained young men

Introduction

Cardiovascular disease (CVD) is presently the leading cause of morbidity and mortality in so-called developed countries (Lloyd-Jones *et al.*, 2010; Green *et al.*, 2011). Vascular impairments at both the macro- and the micro-circulatory level are known to be associated with CVD (Lloyd-Jones *et al.*, 2010). More specifically, endothelial dysfunction, commonly expressed as deficiencies in nitric oxide (NO) production and release by the endothelial cells, and the consequent reduction in vascular responsiveness has been proposed to play a pivotal role in the advancement of CVD (Langham & Wehrli, 2011). Therefore, it is important to have a means to accurately and non-invasively assess vascular responsiveness, for early detection of impairment.

Flow-mediated dilation (FMD) via Doppler ultrasound is currently the most widely used technique to non-invasively assess vascular responsiveness in humans, and has been well documented to detect differences in vascular responsiveness following interventions in healthy and diseased individuals (Maiorana *et al.*, 2001a; Modena *et al.*, 2002; Walsh *et al.*, 2003; Green *et al.*, 2006; Currie *et al.*, 2012). Nonetheless, FMD is dependent upon the use of high cost equipment, skilled personnel required for data acquisition, and limited by a high coefficient of variation upon repeated measures (Hijmering *et al.*, 2001; McLay *et al.*, 2016a).

Recently, near-infrared spectroscopy (NIRS) in combination with vascular occlusion testing (VOT) has emerged as an easily applicable, non-invasive measure of tissue oxygenation (Gerovasili *et al.*, 2010). Immediately following an ischemic challenge,

the NIRS-derived tissue oxygen saturation signal (StO₂) rapidly increases. This dynamic adjustment of the StO₂, or the reperfusion slope, has been shown to significantly correlate to the ultrasound-derived measure of FMD (McLay *et al.*, 2016a). The relationship between FMD (macrovascular responsiveness) and NIRS-derived reperfusion slope (microvascular responsiveness) likely arises from a shared vasodilatory pathway. However, while the FMD response to ischemia may be largely attributed to a single dilatory pathway (i.e., endothelium-dependent vasodilation), the mechanisms governing the control of microvascular blood flow distribution, as assessed by the reperfusion slope of StO₂, are not fully understood and there are multiple different mechanisms influencing the microvascular response. Through the employment of NIRS and VOT, previous studies have thus differentiated patient outcome, mortality, and response to pharmacological intervention in vascular disease-states based on patient StO₂ reperfusion rate (Creteur *et al.*, 2007; Doerschug *et al.*, 2007; Nanas *et al.*, 2008; Skarda *et al.*, 2007).

Given the complexities of data analysis associated with FMD testing, a standardized occlusion duration of five minutes is recommended for the measurement of FMD, in order to observe the greatest endothelial-dependent dilatory response (Thijssen *et al.*, 2011a). Although this is a logical approach, recording the highest endothelium-dependent vasodilatory response to a single occlusion time might limit the ability of establishing differences between groups that are expected to show dissimilar vascular responsiveness. For example, Maiorana *et al.* (Maiorana *et al.*, 2001b) were unable to detect differences between a trained and untrained group of middle-aged subjects. Metabolite build-up (including nitric oxide, ATP, and prostaglandins) is known to influence the vascular responsiveness (Joannides *et al.*, 1995; Toth *et al.*, 2007; Kooijman

et al., 2008; Crecelius *et al.*, 2011) and it is possible that the 5 minute occlusion duration may elicit a maximal dilatory response in all individuals. Therefore, a single long duration occlusion period may not best reflect vascular differences between different subject populations.

In opposition to this single measure approach, animal models typically assess vascular reactivity by measuring a dose-response curve with exposure to a vasodilatory substance such as acetylcholine (ACh). For example, Haram *et al.* (2006) reported an increased sensitivity to lower doses of ACh following a single bout of exercise, as well as after six weeks of endurance training, compared to age-matched controls. However, they observed that at higher doses of ACh both exercised and control rats had similar responsiveness to ACh. Thus, although individuals may have similar dilatory responses to high levels of a stimulus, it is possible that improved sensitivity to lower doses may highlight differences in vascular responsiveness. This concept introduced the idea that differences in sensitivity to vasodilatory responses could be non-invasively evaluated by monitoring changes in the slope of the StO₂ reperfusion signal to different occlusion times during vascular occlusion challenges. In this model, a “dose-response” like curve could be built using the occlusion time as the vasodilatory stimulus.

Therefore, the purpose of this study was to determine if the NIRS-derived measure of StO₂ reperfusion slope (Slope 2 StO₂) was sensitive to a range of ischemic conditions (i.e., various occlusion durations), and to determine if differences exist between two groups that would be expected to have different vascular responsiveness (i.e., trained and untrained individuals). We hypothesized the reperfusion slope would progressively increase with the increase in occlusion duration, and that there would be differences in the reperfusion rate

of trained and untrained individuals, such that trained individuals were more sensitive to shorter occlusion durations.

Methods

Participants

Nine healthy young trained (T, mean \pm SD, age: 25 ± 3 yr) and nine healthy young untrained (UT, mean \pm SD, age: 21 ± 1 yr) men volunteered and gave written informed consent to participate in the study. All procedures conformed to the standards set by the latest revision of the Declaration of Helsinki and were approved by the Conjoint Health Research Ethics Board of the University of Calgary (REB15-3117). All subjects were normotensive (T: systolic 115 ± 13 mmHg and diastolic 71 ± 8 mmHg; UT: systolic 112 ± 6 mmHg and diastolic 72 ± 5 mmHg) and no one was taking medications that would affect cardiorespiratory or hemodynamic responses to exercise. Subjects had no history of cardiovascular, respiratory or musculo-skeletal diseases.

Participants were classified into two groups, trained and untrained, based on self-reported cardiovascular (CV) training and results of a ramp incremental (RI) exercise test. All T individuals reported regular CV training over a number of years and their CV fitness attested to their reports. It should be noted that all T individuals had a maximal oxygen uptake ($\dot{V}O_{2\max}$) greater than $55 \text{ ml}\cdot\text{min}^{-1}\cdot\text{kg}^{-1}$ (DePauw *et al.*, 2013). Conversely, UT individuals reported participation in some physical activities but did not participate in any regular CV training programs or athletic training and their $\dot{V}O_{2\max}$ was less than $55 \text{ ml}\cdot\text{min}^{-1}\cdot\text{kg}^{-1}$.

Study Design

Participants reported to the laboratory on two separate occasions. On the first visit, subjects performed a RI exercise test (20 W baseline for 4 min followed by a 25 W min^{-1} ramp) on a magnetically braked cycle ergometer (Velotron RacerMate Inc., Seattle, WA,

USA) for determination of $\dot{V}O_{2\max}$. The $\dot{V}O_{2\max}$ was defined as the greatest 20 s pulmonary $\dot{V}O_2$ computed from a rolling average, and peak power output (PO_{peak}) was defined as the PO achieved at termination of the RI test.

Participants returned to the laboratory on a separate day and a series of VOTs were performed with different occlusion durations; 30 s, 1 min, 2 min, 3 min and 5 min. All VOTs were performed on the same day and participants were instructed to refrain from caffeine, alcohol and exercise for >12 hours prior to their scheduled visit. Prior to the VOTs, adipose tissue thickness (ATT) was measured over the belly of the tibialis anterior (TA) muscle in each participant. Following ATT measurements, subjects were instructed lie down for at least 10 minutes of supine rest, the NIRS probe was secured on the tibialis anterior muscle and a pneumatic cuff (Flexiport; Welch Allyn Inc., Skaneateles Falls, NY, USA) was placed around the calf (proximal to the NIRS probe). For each VOT the cuff was inflated to an occlusion pressure of 250 mmHg for the specified occlusion duration. The order of the tests was randomized between subjects and each test was separated by a 30 minute rest period to allow blood flow to return to resting conditions.

Near-infrared spectroscopy

StO₂ of the tibialis anterior muscle was monitored continuously throughout each VOT test with a frequency-domain multidistance NIRS system (Oxiplex TS, ISS, Champaign, Ill., USA). Briefly, the system was composed of a single channel consisting of 8 laser diodes operating at 2 wavelengths ($\lambda = 690$ and 828 nm, 4 at each wavelength), which were pulsed in rapid succession, and a photomultiplier tube. The lightweight plastic NIRS probe (connected to laser diodes and a photomultiplier tube by optical fibers) consisted of 2 parallel rows of light emitter fibers and 1 detector fiber bundle; the source-

detector separations for this probe were 2.0, 2.5, 3.0, and 3.5 cm for both wavelengths. The probe was placed on the belly of the TA muscle, was secured in place with an elastic strap tightened to prevent movement and was covered with an optically dense, black vinyl sheet, thus minimizing the intrusion of extraneous light. An elastic bandage was applied to further minimize intrusion of extraneous light and probe movement. The elastic strap and bandage were tightened just enough to secure the NIRS probe in place, such that there would be no pressure to disrupt perfusion in the area of interrogation. By measuring changes in light absorption at different wavelengths, changes in oxyhemoglobin (HbO_2) and deoxyhemoglobin (HHb) can be measured continuously, and StO_2 can be calculated (defined as $[\text{HbO}_2] / [\text{HbO}_2 + \text{HHb}]$). NIRS measurements were collected continuously for the entire duration of each VOT test (2 min baseline, pre-determined occlusion duration and 8 min post-release).

The NIRS device was calibrated at the beginning of the first test session following an instrument warm-up period of at least 20 min. The calibration was done with the probe placed on a calibration block (phantom) with absorption (μ_a) and reduced scattering coefficients (μ_s') previously measured; thus, correction factors were determined and were automatically implemented by the manufacturer's software for the calculation of the μ_a and μ_s' for each wavelength during the data collection. Calculation of $[\text{HbO}_2]$ and $[\text{HHb}]$ reflected continuous measurements of μ_s' made throughout each testing session (i.e., constant scattering value not assumed). The probe remained secured to the leg throughout the duration of the visit to ensure measurement consistency between all tests. Data were stored online at an output frequency of 2 Hz, sampling every 0.5 s, but were reduced to 1 s time bins for all subsequent analyses within the present study to facilitate noise reduction.

Baseline StO₂ (% a.u.) was calculated as the average of 1 min of StO₂ prior to ischemia. Minimum StO₂ (% a.u.) was calculated as the lowest StO₂ value attained during ischemia. The StO₂ reperfusion rate was quantified as the upslope of the StO₂ signal (Slope 2 StO₂, % a.u.·s⁻¹) during a 10 second window immediately following cuff release. It should be noted that immediately after cuff release there is a linear increase in the StO₂, followed by a brief plateau around the Peak StO₂. This linear response is sustained for greater than 10 seconds which allows for an accurate slope to be easily calculated when including the initial reperfusion phase immediately following cuff release. Peak StO₂ (% a.u.) was calculated as the highest StO₂ value reached following cuff release (McLay *et al.*, 2016a). The ‘metabolic accumulation’ for each occlusion duration was determined as the integrated area under the curve (AUC) (Origin, OriginLab Corp., Northampton, Massachusetts, USA) during the occlusion period, taken from cuff inflation to cuff release. For a schematic representation of each parameter see Figure 1.

Statistical Analysis

All statistical analyses were performed using SPSS software, version 23.0 (SPSS Inc., Chicago, Ill., USA) and Microsoft Excel 2010 (Microsoft, Seattle, Wash., USA). All results are presented as means ± standard deviation (SD). The estimates for the NIRS-derived parameters were analyzed with a two-way analysis of variance (ANOVA) for repeated measures. Significant interactions and main effects were analyzed using a LSD post hoc test. Pearson product–moment correlation coefficient was used to determine relationship of occlusion AUC and Slope 2 StO₂. Statistical significance was accepted at $P < 0.05$.

Results

Subject characteristics for both groups are listed in Table 5.1. Those in the T group all had a $\dot{V}O_{2\max}$ exceeding $55 \text{ ml}\cdot\text{min}^{-1}\cdot\text{kg}^{-1}$ with a mean of $63.4 \text{ ml}\cdot\text{min}^{-1}\cdot\text{kg}^{-1}$ (range: 57.8 to $76.5 \text{ ml}\cdot\text{min}^{-1}\cdot\text{kg}^{-1}$), whereas the untrained group averaged $46.6 \text{ ml}\cdot\text{min}^{-1}\cdot\text{kg}^{-1}$ (range: 42.8 to $50.1 \text{ ml}\cdot\text{min}^{-1}\cdot\text{kg}^{-1}$) with all individuals below $55 \text{ ml}\cdot\text{min}^{-1}\cdot\text{kg}^{-1}$.

The StO_2 profiles for trained and untrained group mean data are depicted in Figure 1; which also illustrates the NIRS parameters calculated for each VOT. Group mean and SD for StO_2 parameters measured for each VOT duration are listed in Table 2 for both groups. Slope 2 StO_2 was significantly steeper in T compared to UT ($P = 0.01$) at all occlusion durations. Slope 2 StO_2 was progressively steeper as the occlusion time increased (Figure 2, $P < 0.001$), indicating greater vascular reactivity during longer occlusion times, however there was a significant interaction effect ($P = 0.01$). For the trained group the slope 2 StO_2 at 30 s was significantly less steep than at 1 min, 2 min, 3 min and 5 min ($P < 0.001$, $P < 0.001$, $P < 0.001$ and $P < 0.001$, respectively). Similarly, slope 2 StO_2 at 1 min was less steep than all other longer durations (2 min, $P < 0.001$; 3 min, $P < 0.01$; 5 min, $P < 0.001$). Slope 2 StO_2 following 2 min occlusion was similar to 3 min ($P = 0.92$); however, they both were less steep than following 5 min of occlusion (2 min, $P < 0.01$; 3 min, $P = 0.01$). For the untrained group, slope 2 StO_2 at 30 s was smaller than at 1 min, 2 min, 3 min and 5 min ($P = 0.02$, $P = 0.01$, $P = 0.01$ and $P = 0.02$, respectively). One minute of occlusion resulted in a slope 2 StO_2 that was significantly less steep than following 2 min and 3 min ($P = 0.04$ and $P = 0.03$, respectively), albeit not different from 5 min ($P = 0.07$).

Baseline StO₂ was not different between groups ($P = 0.14$) or occlusion durations ($P = 0.49$) (Table 2). AUC during the occlusion period was progressively larger as the occlusion duration increased ($P < 0.001$) and although there was no difference between groups ($P = 0.05$), there was a trend for occlusion AUC to be larger in T compared to UT (Figure 3). Min StO₂ was significantly lower in T compared to UT ($P = 0.01$) at all occlusion times, and Min StO₂ significantly decreased as occlusion duration increased ($P < 0.001$) (Table 2). Peak StO₂ was similar between groups ($P = 0.78$) with no difference for changes in Peak StO₂ (main effect for occlusion duration ($P < 0.001$) with no interaction effect (0.19)). Mean Peak StO₂ was higher as the occlusion time increased, such that 30 s was smaller than all longer occlusion times (Table 2, $P < 0.001$). Similarly, Peak StO₂ following 1 min and 2 min of occlusion was significantly smaller than following 3 min ($P < 0.001$ and $P = 0.04$, respectively) and 5 min of occlusion (Table 2; $P < 0.001$ and $P = 0.01$, respectively).

Table 5.1. Subject characteristics and peak exercise responses.

	Untrained (n = 9)	Trained (n = 9)
Age (yr) [#]	21 ± 1	25 ± 3
Weight (kg)	74 ± 9	76 ± 5
Height (cm)	183 ± 9	180 ± 4
Relative VO _{2max} (ml·kg ⁻¹ ·min ⁻¹) [#]	46.6 ± 2.5	63.4 ± 5.6
Peak Power Output (W) [#]	340 ± 45	414 ± 30
Systolic Blood Pressure (mmHg)	112 ± 6	114 ± 12
Diastolic Blood Pressure (mmHg)	72 ± 5	71 ± 8
Adipose Tissue Thickness (mm)	8.6 ± 2.0	7.4 ± 0.8

Values are means ± standard deviations.

[#]Significant (P < 0.05) difference between groups

Table 5.2. Average parameter estimates for StO₂ following different durations of vascular occlusions.

		30 s	1 min	2 min	3 min	5 min
Trained	BSLN StO ₂ (% a.u.)	68.0 ± 3.3	69.5 ± 2.3	68.8 ± 3.1	68.5 ± 2.8	68.4 ± 3.5
	Min StO ₂ (% a.u.)	60.9 ± 2.7	57.0 ± 3.5*	48.1 ± 5.2*†	42.4 ± 4.5*†‡	34.3 ± 5.0*†‡§
	Peak StO ₂ (% a.u.)	74.6 ± 2.0	78.1 ± 1.8*	80.4 ± 2.7*	81.2 ± 3.0*†‡	81.9 ± 1.4*†‡
	Slope 2 StO ₂ (% a.u.·s ⁻¹)	1.11 ± 0.29	1.63 ± 0.30*	2.34 ± 0.33*†	2.33 ± 0.37*†	2.83 ± 0.48*†‡§
	Occlusion AUC (% a.u.·s)	54.8 ± 35.9	267.8 ± 118.8*	889.2 ± 116.6*†	1817.4 ± 196.5*†‡	4305.4 ± 530.2*†‡§
Untrained	BSLN StO ₂ (% a.u.)	71.9 ± 4.4	71.7 ± 4.0	71.1 ± 4.2	71.9 ± 4.6	70.7 ± 3.2
	Min StO ₂ (% a.u.) [#]	67.7 ± 3.6	64.9 ± 3.5*	59.5 ± 3.9*†	56.4 ± 4.4*†‡	50.0 ± 5.2*†‡§
	Peak StO ₂ (% a.u.)	75.9 ± 3.3	77.3 ± 4.1*	77.9 ± 4.3*	80.2 ± 3.2*†‡	79.9 ± 2.8*†‡
	Slope 2 StO ₂ (% a.u.·s ⁻¹) [#]	0.61 ± 0.19	0.84 ± 0.19*	1.15 ± 0.31*†	1.21 ± 0.30*†	1.26 ± 0.28*
	Occlusion AUC (% a.u.·s)	62.5 ± 49.4	198.0 ± 61.5*	702.6 ± 200.1*†	1463.7 ± 374.1*†‡	3474.0 ± 715.3*†‡§

Values are means ± SD.

[#] Significant (P < 0.05) difference between groups at all occlusion durations.

* Significant (P < 0.05) difference from 30 s.

† Significant (P < 0.05) difference from 1 min.

‡ Significant (P < 0.05) difference from 2 min.

§ Significant (P < 0.05) difference from 3 min.

62.5 ± 49.4 198.0 ± 61.5* 702.6 ± 200.1*† 1463.7 ± 374.1*†‡ 3474.0 ± 715.3*†‡§

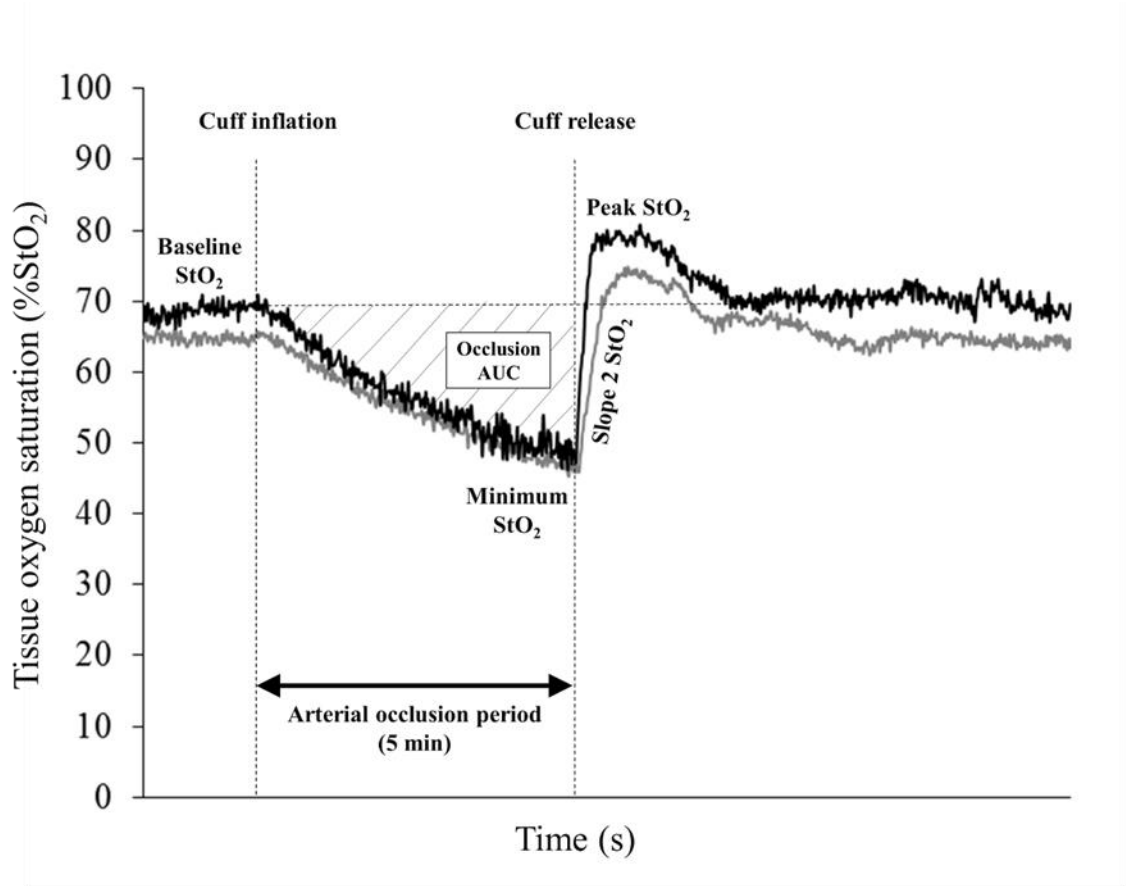


Figure 5.1. NIRS-derived profiles of % tissue oxygen saturation (StO_2) measured during a 5 minute vascular occlusion test for one trained (black line) and one untrained (grey line) representative subject. NIRS parameters are marked up on the trained subject's profile to illustrate what was calculated for each participant. Slope 2 StO_2 for the trained subject shown here was $2.06 \text{ \% a.u.} \cdot \text{s}^{-1}$ and for the untrained subject was $1.14 \text{ \% a.u.} \cdot \text{s}^{-1}$ following the 5 min occlusion duration.

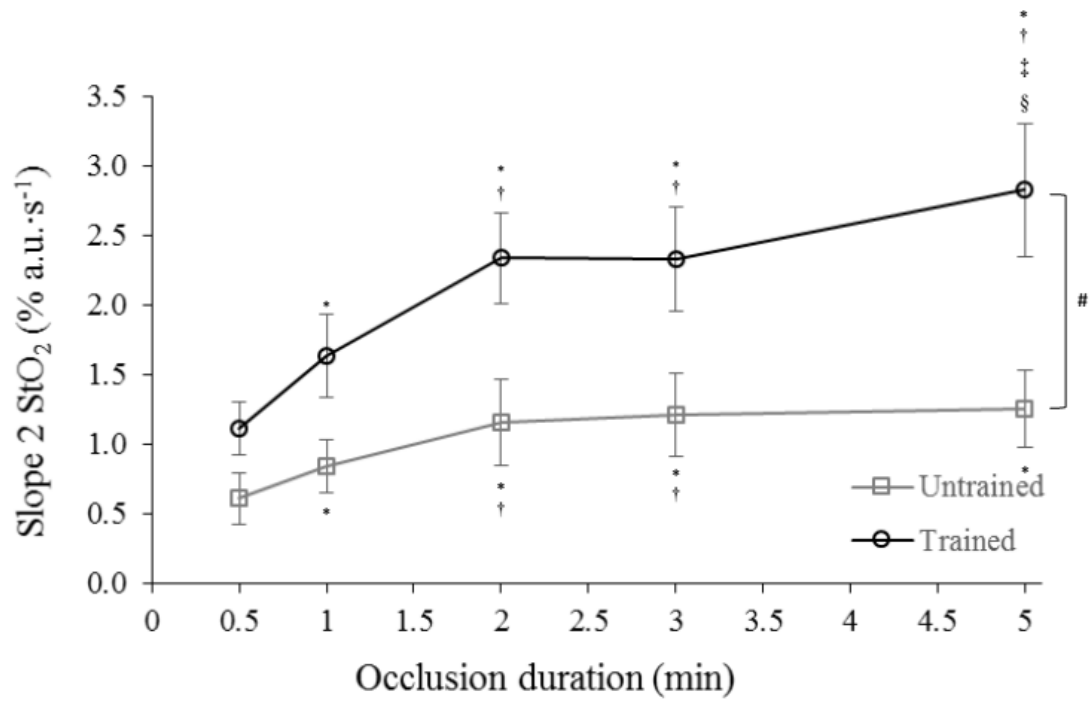


Figure 5.2. Trend of Slope 2 StO₂ following different durations of vascular occlusion.

Significant ($P < 0.05$) difference between groups at all occlusion durations; * Significant ($P < 0.05$) difference from 30 s; † Significant ($P < 0.05$) difference from 1 min; ‡ Significant ($P < 0.05$) difference from 2 min; § Significant ($P < 0.05$) difference from 3 min.

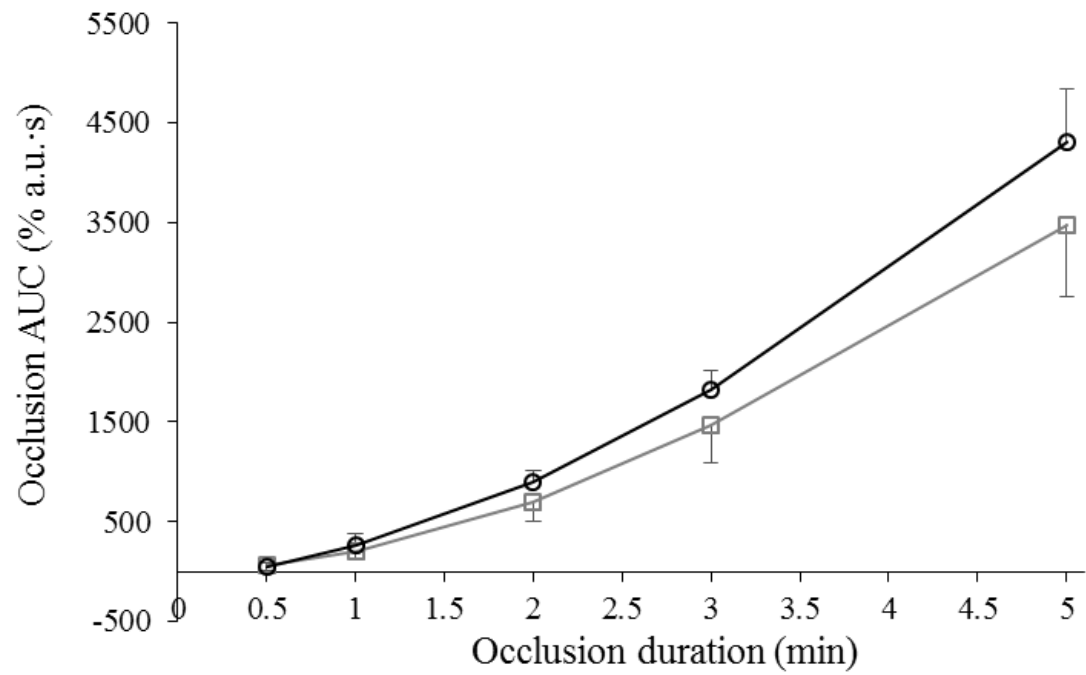


Figure 5.3. Group mean occlusion area under the curve (AUC) for trained (black open circles) and untrained (grey open squares) groups following each occlusion duration.

Discussion

This study used a novel approach to non-invasively evaluate vascular responsiveness by examining the sensitivity of the StO₂ reperfusion slope across a range of occlusion durations, and examined if differences in vasodilatory responses between trained and untrained individuals could be determined using this approach. The main findings were: (i) Slope 2 StO₂ was sensitive to different occlusion durations, such that the Slope 2 StO₂ was progressively steeper with the increase in occlusion duration; (ii) trained individuals had a steeper Slope 2 StO₂ compared to untrained following all occlusion durations, supporting the notion that NIRS-derived measures of StO₂ can be used to detect differences in vascular responsiveness between groups.

A major finding of the present study was that the reperfusion slope, which has been shown to positively correlate to FMD (McLay *et al.*, 2016a) and to reflect vascular reactivity, was sensitive to various ischemic conditions, such that the longer occlusion durations resulted in the steepest reperfusion slopes. This behavior was present in both groups, however the present data indicate that trained individuals were more responsive to progressively larger stimuli such that significant differences were more noticeable at a wider range of occlusion durations. This sensitivity of the reperfusion slope of the StO₂ measure may have important implications for assessing vascular responsiveness not only for group comparisons, but also within an individual. For example, the longer occlusion time typically used in FMD measurements or previously proposed for the slope 2 StO₂ analysis (McLay *et al.*, 2016a), may result in a stimulus that will produce the largest level of vascular responsiveness. This high level of stimulus may challenge the ability to detect changes following an intervention-type study in some particular populations. Additionally,

even if differences at the highest level of stimulus already exist, there are situations in which analysis at lower levels of stimulus provide valuable information from a vascular responsiveness perspective. For example, in older and/or diseased populations it could be that examining a “dose-response” like profile would provide a more detailed analysis that would inform about differences that occur at a variety of levels of stimulus (i.e., level of ischemia), that could be analogous to different intensities of exercise. In this context, an increased vascular responsiveness at a lower stimulus could have important functional implications, as people often perform activities of daily living at lower relative intensities of exercise. This practice of measuring a “dose-response” to a vasodilatory stimulus has been well-established in animal models to assess vascular reactivity (Ulker *et al.*, 2003; Muller-Delp, 2006; Rodrigues *et al.*, 2008). For example, Haram *et al.* (Haram *et al.*, 2006) found that exercise training in healthy rats resulted in an increased sensitivity to lower doses of ACh; however, at higher [ACh] there appeared to be no differences in endothelium-dependent vasodilation between trained and untrained rats. The data from the present study mimics those from these animal models such that the reperfusion slope is sensitive to lower “doses” of ischemia and provides a novel non-invasive and more comprehensive way of estimating vascular responsiveness in humans.

Another important finding of the present study was that the reperfusion slope is not only sensitive to different occlusion durations, but that differences between groups can be detected with this technique. This study showed that the Slope 2 StO₂ was significantly steeper in the trained group compared to the untrained group across all occlusion durations, such that for a given ischemic challenge (occlusion duration) trained individuals were more responsive compared to untrained. Vascular reactivity has been commonly measured by

FMD and it has been well established through previous studies that exercise training improves endothelial function (Rakobowchuk *et al.*, 2008; Tinken *et al.*, 2008; Black *et al.*, 2009; Thijssen *et al.*, 2011b; Currie *et al.*, 2012). Additionally, FMD has been shown to differentiate between clinical populations and healthy controls (Maiorana *et al.*, 2001a; Modena *et al.*, 2002; Walsh *et al.*, 2003; Green *et al.*, 2006; Currie *et al.*, 2012); however, some studies using FMD have failed to find differences in groups that were expected to show them. Using the reperfusion slope, or Slope 2 StO₂, which has been shown to correlate with FMD responses (McLay *et al.*, 2016a), group changes in vascular responsiveness might be easier to detect in the sense that differences can be established across a wide range of stimuli. The present study is the first to show that non-invasive NIRS-derived measures of StO₂, specifically the reperfusion rate, can be used to detect differences in vascular responsiveness between two groups that are expected to differ in this response.

The present data also suggest that this “dose-response” like approach may be sensitive to detect more subtle differences in vascular health that might occur in other populations. Constructing a dose-response curve is commonly employed in animal studies examining vascular responsiveness, for example, in response to vasodilatory substances such as ACh (Ulker *et al.*, 2003; Muller-Delp, 2006; Haram *et al.*, 2006; Rodrigues *et al.*, 2008). This provides insight into the extent of vasodilation to a given dose, but also the sensitivity to different levels of stimuli. Although there was a significant difference between groups at each occlusion duration in the present study, indicating that a single vascular occlusion may be sufficient to identify differences between these two groups, this may not be the case between different populations. It is possible that in other populations

changes might be small and only detectable at 1 or 2 of the occlusion times, eliciting a left- (or right-) shift in the curve. For example, the effects of a short-term exercise training intervention might not be apparent, or might not trigger the changes with longer occlusion durations, however differences might appear with the shorter occlusion durations. In other words, even though it was not evident in the present study, building a “dose-response” like curve for measurement of vascular responsiveness at different levels of stimuli should facilitate detection of changes in vascular reactivity that might be missed otherwise. This is important as the FMD technique, which is widely used for estimating vascular responsiveness in humans, uses a single occlusion duration as the technique is associated with challenges such as high cost of equipment, technical skill and time for analysis. In opposition to this, NIRS measures have been shown to be repeatable (Gómez *et al.*, 2008; McLay *et al.*, 2016b), the equipment is easy to operate and the subsequent data analysis is simple such that assessments of vascular responsiveness can be rapidly obtained.

The present study reported that AUC during the occlusion period increased with the lengthening of occlusion duration and that, although there was no significant difference between groups, there was a trend toward significance such that the trained group may have a greater occlusion AUC. The desaturation rate, or in the case of the present study the occlusion period AUC, has been indicated to represent metabolic accumulation during ischemia (De Blasi *et al.*, 1994), such that greater accumulation of metabolites is thought to result in a more rapid rate of reperfusion, or Slope 2 StO₂. In the present study it appears that multiple mechanisms play a role in the reperfusion response of StO₂. The microvascular responsiveness, as assessed by StO₂ reperfusion rate, is at least in part reliant on metabolic mechanisms of vascular control; however, there are likely other mechanisms

related to endothelial-dependent dilation that are also contributing to this response and the combination of mechanisms is ultimately reflected by upstream vasodilation, typically measured by FMD. In contrast, the FMD response (macrovascular responsiveness) being measured proximal to the site of occlusion is thought to be largely reliant on a single vasodilatory pathway, endothelium-dependent nitric oxide-mediated vasodilation.

In the present study NIRS-derived parameters were not normalized for ATT, as the saturation signal is calculated as a ratio that is independent of absolute amplitudes that are influenced by ATT (Koga *et al.*, 2011). However, while ATT has been shown to influence the absolute measurement values obtained for the oxy- and deoxyhemoglobin concentrations it has been suggested that dynamic changes should be unaffected as the changes are independent of absolute values (Bopp *et al.*, 2011). That being said, the TA muscle offers an ideal sight of measurement for NIRS as there is often less adipose tissue overlying the muscle compared to other muscles in the leg. Choosing a muscle in the leg was an important factor when selecting the area of NIRS interrogation as several training studies are predominately lower limb exercises (such as cycling and running) and thus, it is important to be able to assess vascular responsiveness in the region that is more affected by the proposed intervention. In addition to measuring vascular reactivity in the leg, NIRS allows for assessment of responsiveness of smaller vessels at a level of the microcirculation within the muscle. FMD is assessing vascular function of conduit arteries and this provides a challenge, particularly with the larger vessels in the leg compared to the more commonly assessed vessels of the arm. More specifically, the relatively small changes in conduit artery diameter following a 5 min occlusion in comparison to the large vessel diameters can affect the calculation of FMD, such that differences in vascular function can be

undetectable with this technique (Maiorana *et al.*, 2001*b*). Although measuring StO₂ in other muscles of the leg such as the quadriceps would also be valuable, the occlusion pressure required to fully occlude blood flow through the upper thigh would often need to be higher and may be associated with more discomfort for individuals. Occlusion of the lower leg is more easily achieved, while the TA muscle can also be easily isolated from other muscles in the area which allows for a greater level of confidence in which vascular bed is being measured. It is important to note that in some instances changes in skin blood flow may also influence the NIRS-derived StO₂, however that would not be the case with the NIRS system used in the present study (Koga *et al.*, 2014), but should be considered for future applications. Additionally, the exchange of oxygen across muscle capillary beds is facilitated by increases in both blood velocity and increasing microvascular blood volume through greater capillary perfusion. The NIRS-derived StO₂ is measuring a combination of these two factors which both contribute to the reperfusion rate of the StO₂ response. In the future, the use of dynamic quantitative contrast-enhanced ultrasound in combination with NIRS, may provide insight into differentiating between blood velocity and blood volume through high resolution imaging of blood perfusion of the muscle.

In conclusion, the present study demonstrated that the reperfusion rate of StO₂ is sensitive to different occlusion durations, and that the changes in the sensitivity of the StO₂ measure to a variety of ischemic challenges can be used to detect differences between two groups (i.e., trained and untrained). These data support the notion that NIRS-derived measures of StO₂, specifically the reperfusion slope following a vascular occlusion, can be used as a measure of vascular responsiveness.

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CHAPTER VI: General Discussion and Conclusions

Cardiovascular disease (CVD) is presently the leading cause of morbidity and mortality in industrialized societies and changes in vascular function at both the macro- and micro-vascular level have been proposed as precursors to CVD (Lloyd-Jones *et al.*, 2010). Therefore, it is important to have a means to accurately and non-invasively assess vascular responsiveness to facilitate early detection of impairments, or track changes with interventions (i.e., lifestyle, pharmacological, etc.). Presently, the most widely used technique to assess endothelial dysfunction is the Doppler ultrasound measure of flow-mediated dilation (FMD). This method allows for non-invasive imaging of a conduit artery and is used to monitor changes in diameter following a hyperemic response to ischemia. Although this measure is widely used, it is technically challenging to perform, analyze and in some cases interpret. Additionally, while the FMD technique may provide insight into the endothelial function of a conduit artery, the vascular responsiveness of downstream vessels responsible for perfusion of tissues remains unclear. This thesis explored new strategies to non-invasively determine the magnitude and dynamic adjustment of vascular responses in humans.

Summary of Studies

The studies comprising this thesis relied on two non-invasive techniques. The first, the FMD technique, involves continuous Doppler ultrasound imaging of a conduit artery to measure changes in vessel diameter following 5 min of distal arterial occlusion. The reactive hyperemia subsequent to ischemia results in a flow-mediated dilatory response which can be quantified as the greatest percent change in diameter (%FMD). The second

technique involves the continuous measurement of tissue oxygen saturation (StO_2), via near-infrared spectroscopy (NIRS), before, during and after proximal arterial occlusion. While the FMD technique has been widely used in diverse populations, the newly emerging NIRS-derived measure of StO_2 has only been applied to clinical populations for measurement of small tissue areas (i.e., thenar eminence). It remained unclear whether this technique could be applied to the larger muscles of the leg, or in healthy populations to assess vascular responsiveness, and to detect more subtle differences in vascular function.

In Chapter II the aim was to compare the more recently developed NIRS-derived measure of vascular reactivity to that of the widely used ultrasound-derived FMD technique. StO_2 over the tibialis anterior (TA) muscle was continuously measured throughout a vascular occlusion test (VOT). While several parameters can be measured, we focused on the reperfusion rate of saturation (Slope 2 StO_2) following 5 min of vascular occlusion of the popliteal artery to evaluate if a correlation could be established with FMD. We were able to establish a significant correlation between the NIRS-derived measure of Slope 2 StO_2 and the ultrasound-derived measure of %FMD. These data supported the notion that NIRS-derived measures of StO_2 , specifically Slope 2 StO_2 , obtained directly at the microvascular level were reflective of the vascular responsiveness as measured by FMD.

After establishing the correlation between the NIRS-derived reperfusion rate and the currently used measure of vascular function, %FMD, it was important to establish the reliability of the new technique. Therefore, Chapter III examined the test-to-test (intraday) and day-to-day (interday) reliability of the Slope 2 StO_2 . As the FMD technique is presently the most widely used measure of vascular function we compared the reliability

of Slope 2 StO₂ to that of %FMD. We found that the Slope 2 StO₂ measure had strong reliability both within and between testing days, which was in fact markedly better than that of %FMD. These data suggested that the strong reliability, both intra- and interday, of the NIRS-derived Slope 2 StO₂ measure would make this technique a more suitable one for the assessment of vascular responsiveness, as it might contribute in determining differences between groups or before and after an intervention that would be otherwise difficult to establish.

Although the measure of %FMD is widely used, limitations have been acknowledged such as poor reliability, as shown in Chapter III, and bias towards baseline diameter (D_{base}). In regard to the latter, the D_{base} of the vessel is proposed to influence the calculation of %FMD (as *traditional* FMD is calculated as the change in diameter relative to D_{base}); such that individuals with a larger D_{base} will have a smaller %FMD for a given change in diameter compared to someone with a smaller D_{base} . In this regard, Atkinson et al. (2013) proposed the use of allometric scaling of FMD to eliminate the dependency of %FMD on D_{base} . These authors proposed the use of an ANCOVA model to generate ‘corrected’ group mean values for %FMD and also outlined a new calculation for scaling individual %FMD values (*Atkinson scaling* FMD). Using our data, preliminary analysis of individual values for *Atkinson scaling* FMD resulted in values that were substantially different from the ‘corrected’ group mean values, as well as the original values, and were not physiologically meaningful based on previously reported data. Chapter IV examined if the employment of a new approach (*New scaling* FMD) to allometrically scale values for FMD could improve the ability to obtain individual values that were corrected for variations in baseline diameter between subjects. Results demonstrated that the average

from individual values derived from the *Atkinson scaling* FMD calculation were 3-4 fold larger than meaningful %FMD values that were to be expected. Unfortunately, while the *new scaling* calculation for FMD resulted in values that were within the expected %FMD response for this measure, it did not correct for variation in D_{base} as well as the *Atkinson scaling* FMD calculation. These results demonstrated that while allometric scaling offers some benefits in that it reduces the bias of D_{base} on the FMD measurement, individual values cannot be calculated. This reduces the prognostic value of the measure and limits the ability to examine its repeatability. Finally, these data showed no advantage of employing the allometric scaling approach in a relatively homogenous population of healthy young individuals. Thus, recommendations related to the advantages of scaling %FMD in similar sample groups should be interpreted with caution until the benefits can clearly be established.

With limitations of the FMD technique being addressed in Chapters III and IV, Chapter II and III established and evaluated the reliability of a new approach to non-invasively assess vascular reactivity with the use of NIRS. The VOT used in Chapters II and III was for a fixed duration, as a standardized occlusion duration of 5 minutes is recommended for the measurement of FMD. Chapter V explored the idea that differences in sensitivity to vasodilatory responses could be non-invasively evaluated by monitoring changes in the reperfusion rate following different occlusion durations during VOT. This “dose-response” like curve could be built using occlusion duration as the vasodilatory stimulus. An additional aim of the study in Chapter V was to determine if differences existed between two groups that would be expected to have different vascular responsiveness (i.e., trained and untrained individuals). Results demonstrated that a “dose-

response” like curve can be constructed, as Slope 2 StO₂ was sensitive to changes with the increase in occlusion duration. Additionally, trained individuals had a steeper Slope 2 StO₂ compared to untrained individuals following each of the occlusion durations. These data demonstrated that the reperfusion rate, Slope 2 StO₂, is sensitive to different occlusion durations, and that these changes in the sensitivity of the measure to a variety of ischemic challenges can be used to detect differences between two groups (i.e., trained and untrained).

In summary, this series of studies explored new strategies to non-invasively determine vascular responsiveness in humans. While the ultrasound-derived FMD technique is currently the most widely used measure of vascular function, NIRS-derived measures of StO₂ can be easily obtained and correlate to %FMD. The reperfusion rate, Slope 2 StO₂, of the StO₂ signal throughout a VOT can be used as a measure of vascular responsiveness and is a reliable measure both within and between days. This measure of Slope 2 StO₂ is sensitive to different occlusion durations and changes in this sensitivity can be used to detect group differences in vascular reactivity. Therefore, NIRS-derived measures of StO₂ show promise for assessing vascular responsiveness between groups, or for monitoring changes in vascular responses to various interventions (i.e., training, diet or pharmacological).

Assumptions and Limitations

The studies comprising this thesis used non-invasive means to assess vascular responsiveness in humans and, as with most non-invasive measures, the interpretation of these data were based on several assumptions:

First, the ultrasound-derived measure of %FMD is based on the assumption that increases in blood flow following a brief period of ischemia elicit a shear stress force on the endothelial cells lining the conduit artery being insonated. This shear stress force causes a resultant dilatory response that is thought to be primarily nitric oxide (NO) mediated. Studies that infused NO blockades upstream from an insonation sight reported that dilatory responses to ischemia and shear stress were almost or completely abolished (Joannides *et al.*, 1995; Mullen *et al.*, 2001; Doshi *et al.*, 2001). However, it is important to note that not all studies have been able to inhibit the FMD response using NO blockades (Pyke *et al.*, 2009). A recent meta-analysis of literature examining the contribution of NO in FMD of conduit arteries revealed that with the current standardized technique of FMD ~72% of the dilatory response is mediated by NO, with the remainder of the FMD response being influenced by the role of other vasoactive substances (Green *et al.*, 2014). Additionally, while the FMD technique assesses conduit endothelial function pathways stimulated by shear stress, some interventions, such as exercise training, may induce changes in many signaling pathways and vasodilator mechanisms other than those induced by FMD which might make isolating any one pathway is difficult.

Secondly, the NIRS-derived StO₂ reperfusion rate as a measure of vascular responsiveness was evaluated against another non-invasive measure, the FMD technique. The FMD technique is widely accepted as an index of vascular function and has been shown to closely relate with coronary vasomotor response to infusion of dilatory substances (Anderson *et al.*, 1995; Takase *et al.*, 1998b). However, the ideal validation of the NIRS technique would be to assess vasodilatory responses to intra-arterial infusion of a vasoactive substance such as acetylcholine (ACh). The dilatory response to infused ACh

can be assessed in peripheral vessels by strain-gauge plethysmography. Although this invasive measure is associated with fewer risks than the assessment of endothelial function in coronary arteries by coronary angiography, the invasive nature of the measure prevented its applicability in the studies comprising this thesis. Comparison of the StO₂ reperfusion rate with different ACh infusion across a range of doses would provide further validation to this newly developed non-invasive measure.

Thirdly, there are various limitations associated with NIRS for the determination of StO₂. Near-infrared light has the capacity to penetrate skin and bone and reach muscle tissue. In the tissue, light is absorbed by chromophore molecules, the most important of which is hemoglobin. As discussed previously, absorption characteristics of deoxyhemoglobin (HHb) and oxyhemoglobin (HbO₂) differ. When light of different wavelengths is emitted through muscle tissue, it is absorbed to different degrees depending on the relative concentrations of HHb and HbO₂. With the use of scattering and absorption coefficients in combination with the modified Beer-Lambert law, saturation of the microvasculature of the respective tissue can be calculated. However, before the NIR light reaches the muscle tissue it must first pass through different layers, such as the epidermis and dermis. Within these layers chromophores are present which can cause light absorption independent of Hb saturation such as melanin, bilirubin and cytochrome c oxidase (Madsen & Secher, 1999). Within the muscle tissue itself another chromophore, myoglobin (Mb), is present (Ferrari *et al.*, 2004). Therefore the absorption calculated by the NIRS system may not be solely reflecting that of HHb and HbO₂. Additionally, the models used to quantify Hb concentrations can only approximate the amount of scattered NIR light. The models used do not predict nonlinear paths of light, which are likely to be present in human

tissue (Hiraoka *et al.*, 1993). It is also important to note that the NIRS-derived StO₂ is an average of the saturation of hemoglobin in all vascular compartments (including arterioles, capillaries and venules) within the tissue. Additionally, NIRS-derived measures of tissue oxygen saturation are made over a small region of the TA muscle. This places a heavy reliance on that small area of NIRS “interrogation” to be reflective of different portions of the muscle, however it has been shown in other muscle groups that this may not be the case (Koga *et al.*, 2007).

Future Directions

Chapter II established that a significant correlation exists between NIRS-derived Slope 2 StO₂ and %FMD indicating that Slope 2 StO₂ can be used as a measure of vascular responsiveness. As mentioned previously, the FMD technique is a non-invasive measure that is assumed to reflect endothelial function of central and peripheral vasculature. However, future studies should look toward evaluating the non-invasive NIRS-derived measure of StO₂ against the gold standard for assessing endothelial function, intra-arterial infusion of ACh. This line of research could be particularly valuable with regard to the “dose-response” like approach to assessing vascular reactivity developed in Chapter V of this thesis. Additionally, the administration of pharmacological vasodilatory agents and blockades (for example N^G-monomethyl-L-arginine (L-NMMA); a common NO blockade) in combination with the measurement of StO₂ throughout a vascular occlusion test may be beneficial in elucidating the governing mechanisms of microvascular responsiveness.

While Chapter V established that the differences in the NIRS-derived Slope 2 StO₂, a measure of vascular responsiveness, can be used to detect differences in vascular

reactivity between two groups, these findings should be further substantiated. In Chapter V, it was demonstrated that healthy young trained individuals have a significantly greater rate of reperfusion immediately following ischemia across a range of occlusion durations. However, it remains unclear whether differences in vascular reactivity between other groups can be detected with this technique. Future studies need to examine the reperfusion rate, Slope 2 StO₂, in a diverse group of individuals across the lifespan. It has been shown that endothelial function declines with age (Muller-Delp *et al.*, 2002; Parker *et al.*, 2006; Black *et al.*, 2009) and therefore it would be of interest to compare healthy young, middle-aged and older adults. Additionally, it was discussed earlier in this thesis that bedside measures of StO₂ and the reperfusion rate have been previously examined in clinical populations suffering from severe sepsis and septic shock. It would be interesting to apply this technique to assess vascular reactivity in other clinical populations with more hemodynamically stable conditions but where impaired vascular reactivity may still be present (i.e., diabetes, peripheral artery disease, etc.). Finally, the influence of sex on this measure remains to be ascertained. The studies comprising this thesis all examined this measure of vascular reactivity in young men. As sex hormones, and fluctuations of hormones in women, are known to impact endothelial function (Huang *et al.*, 1998; Pak *et al.*, 2002; Black *et al.*, 2009), then differences in vascular responsiveness would be expected. Future studies need to examine both men and women and consider that the vascular reactivity for the two sexes may be different. With further research, it may be possible to establish if sex-related differences exist, and if so, to characterize the wide spectrum of vascular reactivity for each group to provide references for clinical relevance.

In addition to the cross-sectional approach to comparing the vascular reactivity of different groups detailed above, it would also be of value to track differences in any or all of the groups throughout an intervention, like exercise training. Although Chapter V showed significant differences in the reperfusion rate (i.e., Slope 2 StO₂) between two groups with markedly different cardiovascular fitness levels, it has yet to be established if this approach to assessing vascular reactivity can be used to track changes in an individual.

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APPENDIX I: Ethics approvals



Western
Research

Research Ethics

Use of Human Participants - Ethics Approval Notice

Principal Investigator: Dr. Donald Paterson
File Number: 102273
Review Level: Delegated
Approved Local Adult Participants: 22
Approved Local Minor Participants: 0
Protocol Title: How reliable are repeated measures of flow-mediated vasodilation and what are the effects of different occlusion pressures on the flow-mediated dilatatory response?
Department & Institution: Health Sciences/Kinesiology, Western University
Sponsor: Natural Sciences and Engineering Research Council

Ethics Approval Date: August 08, 2013 Expiry Date: January 31, 2014
Documents Reviewed & Approved & Documents Received for Information:

Document Name	Comments	Version Date
Revised Study End Date		

This is to notify you that The University of Western Ontario Research Ethics Board for Health Sciences Research Involving Human Subjects (HSREB) which is organized and operates according to the Tri-Council Policy Statement: Ethical Conduct of Research Involving Humans and the Health Canada/ICH Good Clinical Practice Practices: Consolidated Guidelines; and the applicable laws and regulations of Ontario has reviewed and granted approval to the above referenced revision(s) or amendment(s) on the approval date noted above. The membership of this REB also complies with the membership requirements for REB's as defined in Division 5 of the Food and Drug Regulations.

The ethics approval for this study shall remain valid until the expiry date noted above assuming timely and acceptable responses to the HSREB's periodic requests for surveillance and monitoring information. If you require an updated approval notice prior to that time you must request it using the University of Western Ontario Updated Approval Request Form.

Members of the HSREB who are named as investigators in research studies, or declare a conflict of interest, do not participate in discussion related to, nor vote on, such studies when they are presented to the HSREB.

The Chair of the HSREB is [REDACTED]. The HSREB is registered with the U.S. Department of Health & Human Services under the IRB registration number IRB 00000940.

Signature

Ethics Officer to Contact for Further Information

This is an official document. Please retain the original in your files.

Western University, Research, Support Services



Western
Research

Research Ethics

**Western University Health Science Research Ethics Board
HSREB Annual Continuing Ethics Approval Notice**

Date: January 30, 2015

Principal Investigator: Dr. Donald Paterson

Department & Institution: Health Sciences/Kinesiology, Western University

HSREB File Number: 105246

Study Title: Effect of acute exercise training on VO₂ kinetics and the relationship to endothelial function in young and older adult men.

Sponsor: Natural Sciences and Engineering Research Council

HSREB Renewal Due Date & HSREB Expiry Date:

Renewal Due -2015/07/31

Expiry Date -2015/08/07

The Western University Health Science Research Ethics Board (HSREB) has reviewed the Continuing Ethics Review (CER) Form and is re-issuing approval for the above noted study.

The Western University HSREB operates in compliance with the Tri-Council Policy Statement Ethical Conduct for Research Involving Humans (TCPS2), the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use Guideline for Good Clinical Practice (ICH E6 R1), the Ontario Freedom of Information and Protection of Privacy Act (FIPPA, 1990), the Ontario Personal Health Information Protection Act (PHIPA, 2004), Part 4 of the Natural Health Product Regulations, Health Canada Medical Device Regulations and Part C, Division 5, of the Food and Drug Regulations of Health Canada.

Members of the HSREB who are named as Investigators in research studies do not participate in discussions related to, nor vote on such studies when they are presented to the REB.


The HSREB is registered with the U.S. Department of Health & Human Services under the IRB registration number IRB 00000940.

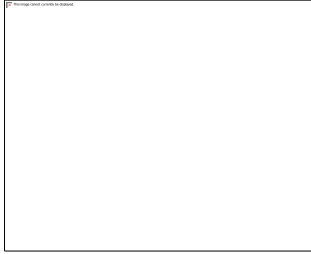

Ethics Officer, on behalf of Dr. Joseph Gilbert, HSREB Chair

Ethics Officer to Contact for Further Information



This is an official document. Please retain the original in your files.

Western University, Research, Support Services Bldg. 



Conjoint Health Research Ethics Board
Research Services Office

Calgary
Telephone: [REDACTED]

CERTIFICATION OF INSTITUTIONAL ETHICS REVIEW

This is to certify that the Conjoint Health Research Ethics Board at the University of Calgary has examined the following research proposal and found the proposed research involving human participants to be in accordance with University of Calgary Guidelines and the *Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans* 2010 (TCPS 2). This form and accompanying letter constitute the Certification of Institutional Ethics Review.

Ethics ID: REB15-3117_MOD1
Principal Investigator: Juan Murias
Co-Investigator(s): There are no items to display
Student Co-Investigator(s): James Gilbertson
Study Title: Vascular Occlusion Duration and the NIRS-Derived Reperfusion Rate of Tissue Oxygen Saturation in Healthy Subjects
Sponsor (if applicable):

Effective: 1/4/2016

Expires: 1/4/2017

Restrictions:

This Certification is subject to the following conditions:

1. Approval is granted only for the project and purposes described in the application.
2. Any modification to the authorized study must be submitted to the Chair, Conjoint Health Research Ethics Board for approval.
3. An annual report must be submitted within 30 days prior to expiry date of this Certification, and should provide the expected completion date for the study.
4. A final report must be sent to the Board when the project is complete or terminated.

Approved By:



Chair , CHREB

Date:

January 18, 2016



LETTER OF INFORMATION

How reliable are repeated measures of flow-mediated vasodilation and what are the effects of different occlusion pressures on the flow-mediated dilatory response?

Principal Investigator: Donald H Paterson, PhD

MSc Student: Kaitlin McLay, BSc

Purpose of Study: You are being invited to participate in a research study that examines the effects of different occlusion pressures on the flow-mediated dilation (FMD) response in the popliteal artery. Flow-mediated dilation (or the dilation of blood vessels in response to an increase in blood flow) will be measured using echo Doppler ultrasound on the surface of the skin. The increase in blood flow is achieved by temporarily restricting blood flow to the artery. Upon release of the occlusion cuff, blood rushes back into the artery which produces a friction against the walls of the artery. The friction, also termed shear stress, produces dilation of the artery which can be detected using ultrasound technology.

Guidelines for the methodological and physiological assessment of FMD have been developed for most aspects of the procedure, however the occlusion pressure used for the assessment of FMD varies between laboratories. The effects of these different pressures remain unknown, making it difficult to compare studies in the literature. Theoretically any pressure above resting systolic pressure should be sufficient to restrict blood flow however no research has been done to look at the effects of different pressures used on the FMD response.

Additionally, the present study will examine the day-to-day variability and test-to-test repeatability within the ultrasound measure of FMD. As well we will use near-infrared spectroscopy (as described later) to assess microvascular deoxygenation to also reflect muscle blood flow following cuff occlusion

Participation in this study involves visits to the research laboratory at the Canadian Centre for Activity and Aging (Arthur and Sonia Labatt Health Science Centre, [REDACTED]) on a maximum of 5 different occasions (total time commitment = approximately 11 hours). Each testing session is expected to take no longer than 2 hours to complete, however the day when 3 tests are performed will take approximately 3 hours.

Up to 22 young adult men will be invited to participate in this study. In order to participate you must be between 18-40 (young) years of age and healthy. You will not be able to participate in the study if you have been previously diagnosed with any respiratory, cardiovascular, metabolic or musculoskeletal disease; or you are currently on medication affecting cardiovascular responses to exercise; or you are a smoker. If you are participating in another study at this time, please inform the investigator right away to determine if it is appropriate for you to participate in this study.

Research Testing Protocol:

If you agree to participate in this study you will perform a series of flow-mediated dilation (FMD) tests in the popliteal artery for five consecutive days. Two FMD tests will be performed each of four days, with the fifth day involving three tests. Each test will be separated with a 30 minute rest period to allow blood flow and arterial dilation to return to resting levels. The first FMD test of each day will be done with a different occlusion pressure found in literature; 175, 200, 225, 250 or 300 mmHg. The second FMD test of each day will be performed with an occlusion pressure of 250 mmHg, resulting in five tests performed in five consecutive days at the same pressure – allowing us to make conclusions regarding day-to-day variation. On the day when two FMD tests are performed with an occlusion pressure of 250mmHg, a third test at that pressure will be performed allowing us to determine the test-to-test repeatability within the same day. The order that the first tests will be performed in will be randomized.

Measurements: All measurement techniques have been approved previously and are used as routine measures in our laboratory. You will lie on the ultrasound bed for 10 minutes prior to testing to eliminate any effects of walking around or climbing stairs on resting blood flow status. Blood pressure will be taken prior to the start of each test. FMD will be measured non-invasively using B-mode echo Doppler ultrasound. We will make continuous measures of your popliteal artery diameters for 5 minutes following the release of a 5 minute period of lower leg (calf) circulatory occlusion.

During each of the FMD tests, the oxygenation of your lower leg muscle will be continuously measured non-invasively using near-infrared spectroscopy (NIRS). NIRS projects light into a specific location of your leg muscles and measures the amount of light coming out at another location. The amount of light that is reflected back and detected by the NIRS probe is used to measure muscle oxygenation. A small piece of equipment (the NIRS probe) will be placed on your lower leg approximately midway between your ankle and your knee. It will be secured with tape, covered to prevent light from entering or leaving the area, and bound with elastic bandage to minimize movement.

Heart rate and rhythm will be continuously monitored by electrocardiogram. One electrode will be placed on each of the following areas: left chest, right chest, and left side under your ribs and connected to an electrocardiograph. The electrodes use adhesive tape to secure to the skin. There are no known risks or discomforts associated with this procedure.

Possible Risks and Discomforts:

When the occlusion cuff is inflated to restrict blood flow, there may be some associated discomfort in the lower leg.

Participation in this study requires a time commitment that may be inconvenient for you at some point during the study.

Benefits of Participation:

This is a basic physiology/biochemistry study and, as such, there will be no direct benefits received as a consequence of participating in the study. If you are interested, the rationale for conducting the research and the theory and significance of each of the tests will be explained, as will your individual results from each of the tests.

Other Pertinent Information:

You are encouraged to ask questions regarding the purpose of the study, specific measures or outcomes of your exercise test, or overall findings and conclusions from this research study.

Confidentiality:

Records from this study are confidential and will be stored securely at the Canadian Centre for Activity and Aging, Sonia Arthur Labatt Health Sciences Building. Your records will be identified by a number rather than your name. The data will be available for analysis within the research group. Published reports resulting from this study will not identify you by name. We would like to keep and use your data in future, as of yet unknown analyses. There is a check box on the consent form to indicate your choice. You will be able to withdraw your data at any time by contacting the Principal Investigator, Dr. Donald H. Paterson at [REDACTED]. Representatives of the University of Western Ontario Health Sciences Research Ethics Board may contact you or require access to your study-related records to monitor the conduct of the research.

Voluntary Participation:

Participation in this study is voluntary. You may refuse to participate, refuse to answer any questions or withdraw from the study at any time with no effect on your academic or employment status.

You will be given a copy of this letter of information and signed consent forms. You do not waive any legal rights by signing the consent form. If you have any questions regarding this study please contact Dr. Donald Paterson [REDACTED] at the Canadian Centre for Activity and Aging, Sonia and Arthur Labatt Health Sciences Building, The University of Western Ontario, London. If you have any question about the conduct of this study or your rights as a research subject you may contact the Director of the Office of Research Ethics, The University of Western Ontario, [REDACTED].



LETTER OF INFORMED CONSENT

How reliable are repeated measures of flow-mediated vasodilation and what are the effects of different occlusion pressures on the flow-mediated dilatory response?

Principal Investigator: Donald H Paterson, PhD

MSc Student: Kaitlin McLay, BSc

I have read the Letter of Information, have had the nature of this study explained to me and I agree to participate. All questions have been answered to my satisfaction.

- ☐ I consent to having my data kept for future as of yet unknown analyses.
- ☐ I do not consent to having my data kept for future as of yet unknown analyses.

Participant:

Name (please print)

Signature

Date

Investigator (i.e. Person Responsible for Obtaining Informed Consent):

Name (please print)

Signature

Date



LETTER OF INFORMATION

Effect of acute exercise training on VO₂ kinetics and the relationship to endothelial function in young and older adult men.

Principal Investigator: Donald H Paterson, PhD

PhD Student: Kaitlin McLay, MSc

Purpose of the Study:

You are being invited to participate in a research study that examines the effects of short term exercise training on oxygen (O₂) uptake and blood flow distribution. During the transition from rest or light-intensity exercise to higher intensities, the rate of adjustment of O₂ use (called “VO₂ kinetics”) may depend on how rapidly certain enzymes in the muscle are activated or on how quickly blood flow increases to supply O₂ to the active muscle. In general, VO₂ kinetics is slower in older adults compared to young adults. Endurance training has been shown to speed VO₂ kinetics in as little as two training sessions in young adults; however the effect of short-term training in older adults has not been studied. This study will compare VO₂ kinetics in older and young adult males following short-term exercise training. Additionally, the dilation of blood vessels in response to an increase in blood flow will be examined during and following the exercise training to assess changes in the ability of those vessels to dilate.

Participation in this study involves visits to the laboratory at the Canadian Centre for Activity and Aging (Arthur and Sonia Labatt Health Sciences Centre, [REDACTED]) on a maximum of 10 different occasions, with each visit taking a maximum of 2.5 hours. Although the first two visits are not time sensitive and can be scheduled flexibly, the other 8 need to follow a specific time frame (see Figure 2).

A total of 24 male adults will be invited to participate in this study (12 young; 18-40 yrs and 12 older adults; 60-85 yrs). In order to participate you must be a healthy male. You will not be able to participate in the study if you have been diagnosed previously with any respiratory, cardiovascular, metabolic, neurological or musculoskeletal disease; or you are currently on medication that will affect your cardiovascular response to exercise*; or you are a smoker; or you respond to the exercise protocol in an irregular manner (i.e. chest pains, dizziness, shortness of breath, nausea etc.) or cannot tolerate the exercise protocol. If you are participating in another study at this time, please inform the investigator right away to determine if it is appropriate for you to participate in this study.

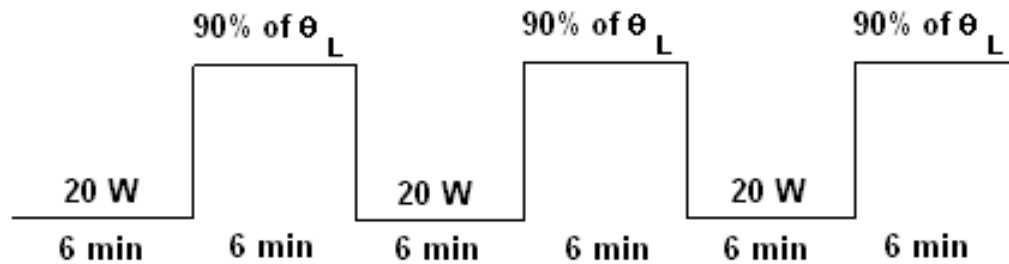
* These include, but are not limited to: Angiotensin-converting enzyme inhibitors (eg. Benazepril, Enalapril, Fosinopril); Diuretics (Hydrochlorothiazide, Metolazone, Chlorthalidone); Beta blockers (Metoprolol, Propranolol, Bisoprolol), Calcium channel blockers (Amlodipine, Diltiazem, Nifedipine, Verapamil hydrochloride); and Angiotensin II receptor blockers (Candesartan, Irbesartan, Losartan). The medications listed are the pharmacological names of the drugs not the brand names. Please note this is not a complete list and if you are unsure of the cardiovascular response to any medications, the researchers will complete a search for the associated effects of each medication on the cardiovascular system.

Research Testing Protocol:

During the first visit to the laboratory, you will complete an incremental exercise test (VO_2 max test) to your limit of tolerance until you will be physically unable to continue exercising because the intensity is either too high or too uncomfortable. The exercise will consist of leg cycling on a cycle ergometer (a stationary bicycle) while in the upright, seated position. The test will begin with the exercise intensity being very light (very little resistance). After several minutes the exercise intensity will increase until you are unable to continue because of fatigue, or until you wish to stop. This visit should last approximately one hour, and will be used as a guideline for all subsequent bike tests.

On a separate day but prior to the commencement of training, dilation of blood vessels in the arm and leg will be measured using echo Doppler ultrasound on the surface of the skin. The order of the two ultrasound tests will vary for each testing session. A blood pressure cuff will be placed below the site of the ultrasound probe (either on the calf or the forearm), inflated to restrict blood flow and then released to monitor dilation for 5 minutes following release. Additionally, you will perform a series of 3 moderate-steps at ~90% of your lactate threshold (as determined by your first visit). This series of exercise steps will be performed on the cycle ergometer and will involve transitions from very light work (ie. 20 watts; an intensity similar to slow walking) to moderate intensity (exercise in the moderate domain could theoretically be performed indefinitely and should not produce signs of fatigue). Repeated moderate-steps are required in order to reduce normal biological variability associated with such testing and increase the underlying signal-to-noise ratio which means the amount of accurate data points to the amount of errant data points from non-uniform breathing. The lactate threshold (θ_L) is the exercise intensity at which lactic acid starts to accumulate in the blood stream. The moderate-steps are performed just below the lactate threshold because it is an intensity that can be maintained without significant blood lactate accumulation and is therefore in the moderate-intensity exercise domain. You will exercise while seated in the upright position on a cycle ergometer at a workload of 20 watts (W) (baseline) and after an accommodation period, the resistance will be increased to 90% of lactate threshold (see Figure 1). Moderate step tests and ultrasound measurements will also be completed following the second training session and 24, 48, 72 and 120 hours after the final training session to assess acute effects of training (see Figure 2). During testing sessions, height, weight and blood pressure measurements will be taken.

Figure 1.



Measurements: All measurement techniques have been approved previously and are used as routine measures in our laboratory. You will lie on the ultrasound bed for 10 minutes prior to testing to eliminate any effects of walking around or climbing stairs on resting blood flow status. Blood pressure will be taken prior to the start of each test. Dilation of the two blood vessels will be measured non-invasively using B-mode echo Doppler ultrasound. We will make continuous measures of your artery diameters for 5 minutes following the release of a 5 minute period of blocked forearm or lower leg (calf) circulation. The order of the two ultrasound tests will vary between visits, and an accommodation period of 30 minutes will separate the two tests to ensure no effect of the previous test on the later.

During each of the ultrasound tests, the oxygenation of your forearm or lower leg muscle will be continuously measured non-invasively using near-infrared spectroscopy (NIRS). NIRS projects light into a specific location of your leg muscles and measures the amount of light coming out at another location. The amount of light that is reflected back and detected by the NIRS probe is used to measure muscle oxygenation. A small piece of equipment (the NIRS probe) will be placed on your forearm or lower leg. It will be secured with tape, covered to prevent light from entering or leaving the area, and bound with elastic bandage to minimize movement. Heart rate and rhythm will be continuously monitored by electrocardiogram. One electrode will be placed on each of the following areas: left chest, right chest, and left side under your ribs and connected to an electrocardiograph. The electrodes use adhesive tape to secure to the skin. There are no known risks or discomforts associated with this procedure.

During each of the exercise tests you will be required to wear a nose-clip (to prevent you from breathing through your nose) and a rubber mouthpiece (similar to breathing through a snorkel or diving mask); nose-clips and mouthpieces are disinfected before each test. This will enable us to measure the volume of air that you breathe in and out, and measure the gas concentration in that air.

During each of the exercise tests, the oxygenation of your leg muscle will again be measured using NIRS. The probe will be placed on your leg approximately midway between your hip and your knee. It will be secured with tape, covered to prevent light from entering or leaving the area, and bound with elastic bandage to minimize movement. You might experience a bit of discomfort

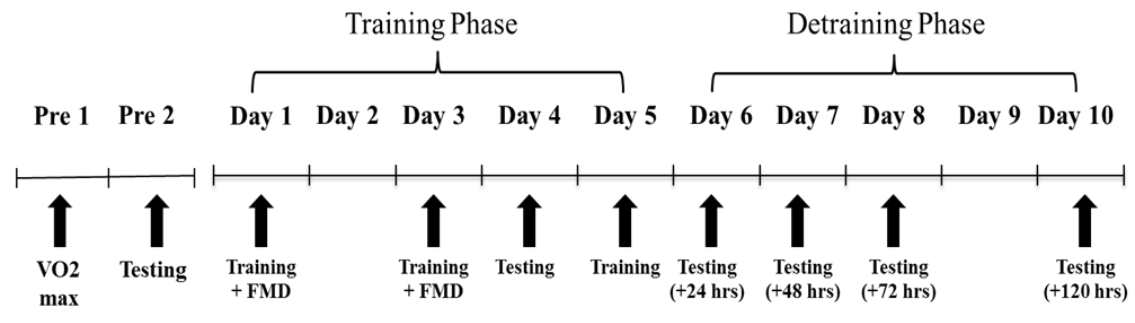
by having this equipment secured to your leg during the exercise period. However, this is a non-invasive procedure.

Arterial blood saturation will be monitored non-invasively and continuously using an oximeter with a cuff on your ear. Heart rate and rhythm will be continuously monitored by electrocardiogram. One electrode will be placed on each of the following areas: left chest, right chest, and left side under your ribs and connected to an electrocardiograph. The electrodes use adhesive tape to secure to the skin. There are no known risks or discomforts associated with this procedure.

Training Protocol:

You will complete three exercise training sessions, separated by 1 day of rest. Each training session will involve 30-45 minutes of continuous exercise training at 70% of your maximal oxygen uptake (as determined by your first visit). Immediately following the first and second training days, an ultrasound test will be performed on either the brachial or popliteal artery. The artery that is not used on the first day will be examined immediately following the second training session, such that only one ultrasound test is performed on each of the two training days. You must refrain from starting any exercise training program during this study and must not be concurrently participating in any other research studies that may interfere with the results of this study.

Figure 2.



Possible Risks and Discomforts:

When the blood pressure cuff is inflated to restrict blood flow, there may be some associated discomfort in the forearm or lower leg, however this sensation will disappear immediately upon cuff release. You may experience some minor discomfort from wearing the nose-clip and rubber mouthpiece, and by having the NIRS probes secured to your leg during the exercise period. These sensations often become less noticeable with time during the exercise.

Any exercise carries a slight risk of a heart attack or may be uncomfortable if you are unfit or not used to exercise. There may be some minor discomfort during the exercise training and testing. You may experience increased awareness of breathing, muscle fatigue and soreness, increased

sweating, or a general feeling of fatigue or nausea, none of which are unexpected consequences of exercise.

All testing procedures will only be conducted when a lab technician or research assistant that is certified in CPR is present. In the case of an emergency, 911 will be called using the telephone located in the testing laboratory. An automatic external defibrillator is also available within the testing building. If a heavy pressure sensation or pain develops in your chest or down your left arm it is important that you discontinue the exercise immediately and report these sensations to the exercise supervisor, or seek medical attention if you have left the exercise area.

Participation in this study requires a time commitment that may be inconvenient for you at some point during the study.

Benefits of Participation:

This is a basic physiology/biochemistry study and, as such, there will be no direct benefits received as a consequence of participating in the study. If you are interested, the rationale for conducting the research and theory and significance of each of the tests will be explained, as will your individual results from each of the tests.

Other Pertinent Information:

You are encouraged to ask questions regarding the purpose of the study, specific measures or outcomes of your exercise test, or overall findings and conclusions from this research study.

Confidentiality:

Records from this study are confidential and will be stored securely at the Canadian Centre for Activity and Aging, Sonia Arthur Labatt Health Sciences Building. Your records will be identified by a number rather than your name. The data will be available for analysis within the research group. Published reports resulting from this study will not identify you by name. We would like to keep and use your data in future, as of yet unknown analyses. There is a check box on the consent form to indicate your choice. If you consent, following the removal of all personal identifiers (only linked to a research number), data are stored indefinitely as information may be used later in other research comparisons of aging or included as part of a database on aging held in the Canadian Centre for Activity and Aging. All written and password-protected computer files are filed under a research number and are secured at the Canadian Centre for Activity and Aging Laboratory in a locked filing cabinet accessible only to investigators and research assistants. You will be able to withdraw your data at any time by contacting the Principal Investigator, Dr. Donald H. Paterson at [REDACTED]. Representatives of the University of Western Ontario Health Sciences Research Ethics Board may contact you or require access to your study-related records to monitor the conduct of the research.

Voluntary Participation:

Participation in this study is voluntary and there is no compensation for participating in the study. You may refuse to participate, refuse to answer any questions or withdraw from the study at any time with no effect on your academic or employment status.

You will be given a copy of this letter of information and signed consent forms. You do not waive any legal rights by signing the consent form. If you have any questions regarding this study please contact Dr. Donald Paterson [REDACTED] at the Canadian Centre for Activity and Aging, Sonia and Arthur Labatt Health Sciences Building, The University of Western Ontario, London. If you have any question about the conduct of this study or your rights as a research subject you may contact the Director of the Office of Research Ethics, The University of Western Ontario, [REDACTED]



LETTER OF INFORMED CONSENT

Effect of acute exercise training on VO₂ kinetics and the relationship to endothelial function in young and older adult men.

Principal Investigator: Donald H Paterson, PhD

PhD Student: Kaitlin McLay, MSc

I have read the Letter of Information, have had the nature of this study explained to me and I agree to participate. All questions have been answered to my satisfaction.

- ☐ I consent to having my data kept for future as of yet unknown analyses.
- ☐ I do not consent to having my data kept for future as of yet unknown analyses.

Participant:

Name (please print)

Signature

Date

Investigator (i.e. Person Responsible for Obtaining Informed Consent):

Name (please print)

Signature

Date

LETTER OF INFORMED CONSENT



UNIVERSITY OF
CALGARY
KINESIOLOGY

**Vascular Occlusion Duration and the NIRS-Derived
Reperfusion Rate of Tissue Oxygen Saturation in
Healthy Subjects**

Principal investigator: Juan M. Murias, PhD

This consent form is only part of the process of informed consent. It should give you the basic idea of what the research is about and what your participation will involve. If you would like more detail about something mentioned here, or information not included here, please ask. Take the time to read this carefully and to understand any accompanying information. You will receive a copy of this form.

BACKGROUND

You are being invited to participate in a study that examines the blood flow response to different occlusion periods. Previously near-infrared spectroscopy (NIRS) has been widely used to determine changes in oxygen saturation during exercise. More recently, NIRS has been used to measure tissue oxygen saturation in response to a brief period of blood flow restriction, termed a vascular occlusion test (VOT). The VOT has been used in healthy and clinical populations to determine the response of blood flow and may be useful identifying the responsiveness of small vessels.

WHAT IS THE PURPOSE OF THE STUDY?

The goal of this study is to compare 6 different blood flow restriction durations (30 seconds, 1, 2, 3, 5 and 10 minutes) in healthy young individuals.

WHAT WOULD I HAVE TO DO?

To be part of the study, the following admission criteria will be considered: you will have to be healthy, non-smoker, non-obese, normotensive, with no peripheral vascular occlusive disease, not taking medications that are known to affect cardiovascular or hemodynamic responses to exercise (e.g., β -blockers, blood pressure medication, anti-inflammatories, anti-coagulants, etc.).

Once accepted in to the study, you will be required to come into the laboratory for two testing sessions:

1) During this 3.5-hour testing session, you will complete a series of blood flow restriction tests for 6 different durations (30 seconds, 1, 2, 3, 5 and 10 minutes). Blood flow restriction will be achieved by inflating a blood pressure cuff to 250 mmHg, a pressure above your resting systolic blood pressure to prevent flow below the cuff. The order of the tests will be randomized and each test will be separated by a 30 minute rest period to allow blood flow following cuff release to return to baseline levels.

2) During this visit you will complete an incremental exercise test ($\text{VO}_{2\text{max}}$ test) to your limit of tolerance until you will be physically unable to continue exercising because the intensity is either too high or too uncomfortable. The exercise will consist of cycling on a cycle ergometer (a stationary bicycle) while in the upright, seated position. The test will begin with the exercise intensity being very light (very little resistance). After several minutes the exercise intensity will gradually increase until you are unable to continue because of fatigue, or until you wish to stop. This visit should last approximately 45 minutes.

Measurements: All measurement techniques are used as routine measures in our laboratory. During each of the 6 tests described in the first point above, the oxygenation of the tibialis anterior and gastrocnemius muscles will be continuously measured non-invasively using near-infrared spectroscopy (NIRS). NIRS projects light into a specific location of your leg muscles and measures the amount of light coming out at another location. The amount of light that is reflected back and detected by the NIRS probe is used to measure muscle oxygenation. A small piece of equipment (the NIRS probe) will be placed on your lower leg approximately midway between your ankle and your knee. It will be secured with tape, covered to prevent light from entering or leaving the area, and bound with elastic bandage to minimize movement. During the test described in the second point above, you will be required to wear a mask connected to a volume turbine so that gas concentration in the air and ventilatory rates can be measured. This will enable us to measure the volume of air that you breathe in and out, and measure the gas concentration in that air. Masks and turbines are disinfected before each test. Additionally, heart rate will be continuously monitored by a heart rate monitor. There are no known risks associated with any of these technical procedures.

WHAT ARE THE RISKS?

There might be some minor pain and discomfort experienced during the blood flow restriction periods of testing. These sensations will immediately disappear once the cuff is released.

Blocking blood flow for any period of time may increase your risk for developing a blood clot, due to increased blood pooling in the extremities. In extremely rare cases, this may lead to the development of a severe blood clot, known as a deep-vein thrombosis (DVT) requiring immediate medical attention, and potential further complications. If you have any pre-existing medical conditions, it is essential that you inform the researchers prior to testing, as your participation may carry additional risks.

Note that no studies known to date have shown NIRS to have harmful effects on health. However, research on rats has suggested that prolonged visual exposure to near-infrared light can cause eye damage.

Any exercise carries a slight risk or may be uncomfortable if you are unfit or not used to doing exercise. The risk of a cardiac event (heart attack, dysrhythmias, etc.) in a mixed

subject population (healthy low risk and unhealthy high risk patients together) is approximately 6:10,000; however, the risk decreases in a previously healthy (i.e. young moderately active) population (adapted from ACSM's Guidelines for Exercise Testing and Prescription). There might be some minor discomfort during the exercise testing. You may experience increased awareness of breathing, muscle pain and/or fatigue, increased sweating, or a general feeling of fatigue or nausea, all of which are not unexpected consequences of exercise. You may experience some minor discomfort from wearing the breathing mask necessary for VO₂ measurements

Additionally, know that participation in this study requires a significant time commitment that may be inconvenient for you at some point in the study.

All testing procedures will only be conducted when a lab technician or research assistant that is certified in CPR is present. In the case of an emergency, 911 will be called using a telephone available in the testing laboratory. An automatic external defibrillator is also available within the testing building.

WILL I BENEFIT IF I TAKE PART?

This is a basic physiology/biochemistry study and, as such, there will be no direct benefits received as a consequence of participating in the study. If you are interested, the rationale for conducting the research and theory and significance of each of the tests will be explained, as will your individual results from each of the tests. You will also have the opportunity to learn about and better understand your physiological responses to an exercise situation. You are encouraged to ask questions regarding the purpose of the study, specific measures or outcomes of your exercise test, or overall findings and conclusions from this research study.

DO I HAVE TO PARTICIPATE?

Your participation in this research project is entirely voluntary. You can withdraw anytime by sending an email to [REDACTED] or by expressing this desire verbally to the investigators.

You might be withdrawn from the study for the following reasons:

- Changes in your status so that you do not fit within the admission criteria for this study.
- You cannot complete all testing sessions within the proposed period of the study.
- You are not able to comply with the instructions prior to each testing session.

If new information becomes available that might affect your willingness to participate in the study, you will be informed as soon as possible.

WHAT ELSE DOES MY PARTICIPATION INVOLVE?

Your participation in this study does not involve anything else beyond what is specified on this informed consent.

WILL I BE PAID FOR PARTICIPATING, OR DO I HAVE TO PAY FOR ANYTHING?

You will not be paid for your participation in this research project.

WILL MY RECORDS BE KEPT PRIVATE?

Information obtained during this research project is confidential. Nobody except the researchers will have access to your personal information. Your records are listed according to an identification number rather than by your name. Published reports resulting from this study will not identify you by name. Thus, your right to privacy will be retained. If you require it, you will be given a summary of your results and the average results for all participants in this study.

IF I SUFFER A RESEARCH-RELATED INJURY, WILL I BE COMPENSATED?

In the event that you suffer injury as a result of participating in this research, no compensation will be provided to you by the University of Calgary, or the Researchers. You still have your legal rights. Nothing said in this consent form alters your right to seek damages.

SIGNATURES

Your signature on this form indicates that you have understood to your satisfaction the information regarding your participation in the research project and agree to participate as a participant. In no way does this waive your legal rights nor release the investigators or involved institutions from their legal and professional responsibilities. You are free to withdraw from the study at any time without consequences. If you have further questions concerning matters related to this research, please contact:

Dr. Juan M. Murias



If you have any questions concerning your rights as a possible participant in this research, please contact the Chair, Conjoint Health Research Ethics Board, University of Calgary at



Participants Name

Signature and Date

Investigator's name

Signature and Date

Witness' name

Signature and Date

The University of Calgary Conjoint Health Research Ethics Board has approved this research study.

A signed copy of this consent form has been given to you to keep for your records and reference.

PERMISSION TO REPRODUCE PREVIOUSLY PUBLISHED MATERIALS

Chapter II:

Prior permission from *Experimental Physiology* is not required if the purpose of the reproduction is for inclusion in a thesis dissertation.

Chapter III:

Prior permission from *Physiological Reports* is not required if the purpose of the reproduction is for inclusion in a thesis dissertation.

Chapter V:

Prior permission from *Experimental Physiology* is not required if the purpose of the reproduction is for inclusion in a thesis dissertation.

CURRICULUM VITAE

Kaitlin M. McLay
School of Kinesiology, Faculty of Health Sciences
Canadian Centre for Activity and Aging
The University of Western Ontario
London, Ontario, Canada

Education

- 2012 - 2016 **Doctor of Philosophy (Ph.D.)**, Integrative Physiology of Exercise, *The University of Western Ontario, London, Ontario*
- 2010 - 2012 **Master of Science (M.Sc.)**, *The University of Western Ontario, London, Ontario*
- 2006 - 2010 **Honors Bachelor of Science (B.Sc.H.)**, Biological Sciences, *University of Guelph, Guelph, Ontario*

Research

Peer-Reviewed Original Research Publications (4)

1. **McLay, K.M.**, Fontana, F.Y., Nederveen, J.P., Guida, F.F, Paterson, D.H., Pogliaghi, S. & Murias, J.M. (2016) Vascular responsiveness determined by near-infrared spectroscopy measures of oxygen saturation. *Experimental Physiology*. Jan 1;101(1):34-40.
2. **McLay, K.M.**, Nederveen, J.P., Pogliaghi, S., Paterson, D.H. & Murias, J.M. (2016) Repeatability of vascular responsiveness measures derived from near-infrared spectroscopy. *Physiological Reports*. Mar 4 (9):e12772
3. **McLay, K.M.**, Gilbertson, J.E., Pogliaghi, S., Paterson, D.H. & Murias, J.M. (2016) Vascular responsiveness measured by tissue oxygen saturation reperfusion slope is sensitive to different occlusion durations in healthy trained and untrained young men. *Experimental Physiology*, In press.
4. Maturana Mattioni, F., Keir, D.A., **McLay, K.M.** & Murias, J.M. (2016) Can measures of critical power precisely estimate the maximal metabolic steady state? *Applied Physiology, Nutrition, and Metabolism*, In press.

Refereed Abstracts (12)

1. **McLay, K.M.**, Mattioni Maturana, F. & Murias J.M. (2016). Experienced cyclists predict the highest sustainable intensity of exercise more effectively than critical power testing. *Medicine & Science in Sports & Exercise*
2. Mattioni Maturana, F., **McLay, K.M.** & Murias J.M. (2016). Can measures of critical power precisely estimate the maximal metabolic steady state? *Medicine & Science in Sports & Exercise*
3. **McLay, K.M.**, Gilbertson, J.E., Paterson, D.H. & Murias, J.M. (2016). Vascular responsiveness measured by tissue oxygen saturation reperfusion slope is sensitive to different occlusion durations in healthy trained and untrained young men. *Okanagan Cardiovascular and Respiratory Symposium*.
4. **McLay, K.M.**, Nederveen, J.P., Pogliaghi, S., Paterson, D.H. & Murias, J.M. (2016) Repeatability of vascular responsiveness measures derived from near-infrared spectroscopy. *Okanagan Cardiovascular and Respiratory Symposium*.
5. **McLay, K.M.**, Paterson, D.H. & Murias, J.M. (2015). Effects of short-term exercise training and detraining on $\dot{V}O_2$ kinetics in young and older adults. *Applied Physiology, Nutrition, & Metabolism Suppl.*
6. **McLay, K.M.**, Fontana F.Y., Nederveen, J.P., Guida, F.F, Paterson, D.H., Pogliaghi, S. & Murias, J.M. (2015). Vascular responsiveness determined by near-infrared spectroscopy measures of oxygen saturation. *Applied Physiology, Nutrition, & Metabolism Suppl.*
7. **McLay, K.M.**, Fontana F.Y., Pogliaghi, S., Paterson, D.H. & Murias, J.M. (2014). Can NIRS derived measures of oxygen saturation differentiate vascular responsiveness in young and older adults? *Applied Physiology, Nutrition, & Metabolism Suppl.*
8. **McLay, K.M.**, Murias, J.M., Nederveen, J.P. & Paterson, D.H. (2013). Analysis of flow-mediated dilation in the popliteal artery using manual detection compared with edge-tracking software. *Applied Physiology, Nutrition, & Metabolism Suppl.*
9. **McLay, K.M.**, Murias, J.M., Nederveen, J.P. & Paterson, D.H. (2013). Day-to-day reliability of flow-mediated dilation measures in the popliteal artery. *Medicine & Science in Sports & Exercise*:
10. **McLay, K.M.**, Murias, J.M., Nederveen, J.P. & Paterson, D.H. (2012). Test-to-test repeatability of FMD measures in healthy young adult males. *Applied Physiology, Nutrition, & Metabolism* 37:S24.

11. **McLay, K.M.**, Dogra, S.K. & Paterson, D.H. (2012) Responsiveness of the Popliteal Artery in Older Trained and Untrained Women. *Medicine & Science in Sports & Exercise: Suppl.*
12. Spencer, M.D., Murias, J.M.; Gravelle, B.M.R., **McLay, K.M.**, Kowalchuk, J.M. & Paterson, D.H. (2011) Does Improved Local O₂ Distribution Explain Faster VO₂ Kinetics During Smaller Compared To Larger Moderate-intensity Transitions? *Medicine & Science in Sports & Exercise: Suppl.* Vol 43 (5):387.

Grants and Awards

Grants

- | | |
|-------------|--|
| 2015 - 2016 | Ontario Graduate Scholarship (OGS) , Ministry of Training, Colleges and Universities – funded for 3 terms |
| 2012 - 2016 | Western Graduate Research Scholarship , University of Western Ontario Institutional Scholarship – 2012-16 academic year(s) |
| 2011 – 2012 | Queen Elizabeth II Graduate Scholarship in Science and Technology (QEIIST) , Ministry of Training, Colleges and Universities – funded for 3 terms |
| 2010 - 2012 | Western Graduate Research Scholarship , University of Western Ontario Institutional Scholarship – 2010-12 academic year(s) |

Travel Grants and Other Awards

- | | |
|------|--|
| 2016 | Faculty of Health Sciences Graduate Student Conference Travel Award , The University of Western Ontario |
| 2015 | Faculty of Health Sciences Graduate Student Conference Travel Award , The University of Western Ontario |
| 2015 | School of Kinesiology Travel Award , The University of Western Ontario |
| 2014 | Faculty of Health Sciences Graduate Student Conference Travel Award , The University of Western Ontario |
| 2014 | School of Kinesiology Travel Award , The University of Western Ontario |

- 2013 **Faculty of Health Sciences Graduate Student Conference Travel Award**, The University of Western Ontario
- 2013 **School of Kinesiology Travel Award**, The University of Western Ontario
- 2013 **Graduate Thesis Research Award**, The University of Western Ontario
- 2012 **School of Kinesiology Travel Award**, The University of Western Ontario
- 2012 **Faculty of Health Sciences Graduate Student Conference Travel Award**, The University of Western Ontario