

DEVELOPING AN ALTERNATIVE METHODOLOGY FOR  
MEASURING REGIONAL SKELETAL MUSCLE BLOOD  
FLOW

by

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A THESIS

Presented to the Department of Human Physiology  
and the Robert D. Clark Honors College  
in partial fulfillment of the requirements for the degree of  
Bachelor of Science

June 2017

## **An Abstract of the Thesis of**

Marisa Polonsky for the degree of Bachelor of Science  
in the Department of Human Physiology to be taken June 2017

Title: Developing an Alternative Methodology for Measuring Regional Skeletal  
Muscle Blood Flow

Approved: \_\_\_\_\_

John Halliwill, PhD

Previous studies have indicated that sustained post-exercise vasodilation is mediated by the activation of histamine receptors, but the specific signal responsible for driving the release of histamine in response to exercise remains unknown. Tissue manipulation and vibration have been hypothesized to be key mediators of this histamine release. In order to test this and related hypotheses, skeletal muscle blood flow in response to passive limb movement must be measured. This study compared two methodologies for doing so: Doppler ultrasound and near infrared spectroscopy-indocyanine green (NIRS-ICG). A low correlation ( $r_{ratio} = .238$ ,  $r_{percent\ difference} = .278$ ) between methods was found. However, clear changes in blood flow were observed through visual analysis of graphical representations of individual subject data. The knowledge developed in this study will enable future investigation of the relationship between passive limb movement and histamine through the measurement of regional skeletal muscle blood flow.

## **Acknowledgements**

I would like to thank the entire Exercise and Environmental Physiology lab team for their support in the completion of this thesis. The time I have gotten to spend interacting with members of the lab has greatly enhanced my undergraduate experience and aided me in determining my future career and academic goals. I would like to thank Dr. John Halliwill for leading such a great group of people, encouraging scientific inquiry and always being available as a resource. I would also like to thank Dylan Sieck for his wisdom, humor, and many hours invested in supporting me through this project. I would also like to thank Keeley Debar, a fellow undergraduate in the lab who is also writing a thesis this year, for her partnership in navigating this process together.

Beyond members of the lab, I would like to thank Dr. Joseph Fracchia for serving on my thesis committee and teaching some of the best courses of my undergraduate career. I feel very privileged to have learned and grown under his guidance. I would also like to thank the faculty and staff of the University of Oregon, Human Physiology Department, and Clark Honors College for sharing their passions in the classroom and beyond. I would further like to thank my parents, Jon and Jessica Polonsky, for offering encouragement, love, and support, always.

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## **Introduction**

### **The Exercise and Environmental Physiology Lab**

Over the past fifty years, no shortage of research has been done on exercise and its benefits. However, the same amount of attention has not been paid to the post-exercise period. The Exercise and Environmental Physiology (EEP) Lab at the University of Oregon, led by Dr. John Halliwill, is very interested in this period. Even more specifically, the period after exercise when the body is returning to resting state. The EEP Lab investigates this state by focusing on examining cardiovascular changes related to exercise. Two changes in the cardiovascular system during the post-exercise period are of particular interest. First, post-exercise hypotension, which refers to a decrease in blood pressure (Halliwill, Buck, Lacewell, & Romero, 2013). This response is mediated by the baroreflex, which works to maintain blood pressure within optimal ranges under resting conditions (Hall, 2010). After exercise, the baroreflex adjusts these operational ranges towards lower pressures, leading to a drop in blood pressure known as post-exercise hypotension. The second important change is sustained post-exercise vasodilation, a dilation of the blood vessels (Halliwill et al., 2013). After exercise, blood vessels, specifically arteries, that lead to previously active skeletal muscle become vasodilated. Arteries are lined with smooth muscle which can contract or relax to allow for vasoconstriction or vasodilation. When vasodilation occurs, more blood can be delivered to this previously active skeletal muscle. This vasodilation is considered a sustained response because it lasts for longer than 100 minutes (Lockwood, Wilkins, & Halliwill, 2005).

The underlying mechanisms behind these two post-exercise phenomena have been a focus of the EEP Lab and other research groups in recent years. Found to be related to these responses is an organic compound, histamine, which is involved in local immune response and found in humans and most animals. Previous studies have indicated that sustained post-exercise vasodilation is mediated by the activation of histamine receptors, but the specific signal responsible for driving the release of histamine in response to exercise remains unknown (Halliwill et al., 2013). A multitude of potential causes for driving histamine release have been explored, which are based on an understanding of two potential sources of intramuscular histamine in response to exercise. Both mast cell degranulation and de novo formation of histamine from decarboxylation of histidine are triggered by exercise (Halliwill et al., 2013). Previous studies have explored exercise related factors that may induce mast cell degeneration. These include increased temperature, cytokine release, and oxidative stress. Other factors currently being explored include heat, soluble factors and physical stimuli like shear stress, vibration and muscle compression. As of now, these hypotheses remain under investigation as part of the search to fully understand the story of histamine and the role it plays in mediating post-exercise cardiovascular changes.

It is important to determine the upstream signal that causes the release of histamine so we can eventually progress to manipulation of that response. The long term goal, once the cause of localized histamine release in skeletal muscle is known, would be to develop a dose-response curve for prescribing exercise and develop interventions to facilitate this ability. This plays into a larger goal of developing

interventions to promote vascular health in humans, by making better use of exercise or mimicking the benefits of exercise for those who cannot participate.

### **Passive Limb Movement**

Of the many hypothesized factors that may act as upstream signals for histamine release post-exercise, one current focus is the response to physical stimuli like vibration and mechanical stress. Passive limb movement has been shown to increase blood flow and cause passive stretching without increasing metabolic demand or significantly increasing oxygen uptake (Hellsten et al., 2008). For these reasons, it serves as an effective experimental model to test triggers of histamine release focused on physical stimuli independent of other exercise related changes in oxygen uptake, metabolism and temperature. Passive limb movement has been shown to lead to up to a 3-fold increase in blood flow to the leg (Mortensen, Askew, Walker, Nyberg, & Hellsten, 2012). This response is reduced in elderly subjects and those with peripheral artery disease, and is dependent on nitric oxide (Mortensen et al., 2012). Based on this response, our lab has hypothesized that physical manipulation of skeletal muscle will cause a release of histamine.

In order to test this hypothesis, reliable methodologies that are sensitive enough to measure changes in blood flow after passive limb movement must be developed and validated. Thus, the focus of this thesis is to assess a methodology that will meet this need. It will serve as a precursor to later work that explores the relationship between histamine and passive limb movement. If the passive movement protocol employed by this study leads to measurable changes in blood flow, the next step would be to use a repeated measures design to investigate the role of histamine. The subject would likely



be put through the same protocol explained here twice; comparing responses to the control day and a study day where a histamine blockade would be used. This would ideally allow conclusions to be drawn about the overlap between physical manipulation of tissue, histamine release and sustained post-exercise vasodilation.

### **Measuring Blood Flow**

Scientific interest in human blood flow dates back to the mid-19<sup>th</sup> century. Jean Poiseuille was a French physiologist and physician at the time, who developed an equation to explain blood flow and explore its relationship to different factors. His equation includes three changing variables that are all parameters that impact blood flow: viscosity, vessel length and radius.

$$Q = \frac{\Delta P \pi r^4}{8 L \eta}$$

As indicated by the radius variable being raised to the fourth power, changing radius of the vessel is the most likely of the three factors to dramatically impact blood flow, as it can be modulated by vasoconstriction or vasodilation (Hall, 2010).

For the past 50 years, Doppler ultrasound has utilized the Doppler frequency shift principle to determine blood velocity (Buck, Sieck, & Halliwill, 2014). Ultrasound works by transmitting sound waves through a probe and gel and into the body, then collecting the sound waves that bounce back. Using a computer program that analyzes these waves, an image is produced. Ultrasound is used across many areas of the body to assess cardiovascular function and blood flow. Based on the images and recordings produced, blood flow can be calculated by multiplying mean velocity and cross-sectional area (Buck et al., 2014).

As shown by this history, blood flow has long been of scientific interest and ultrasound has been relied on to measure it. Examples of the use of ultrasound to assess blood flow include observing changes after maximal exercise (Harms et al., 1997), investigating the impact of aging on limb blood flow (Donato et al., 2005), and looking at changes in blood flow due to the introduction of acetylcholine and nitric oxide (Brock et al., 1998). Outside of the research setting, blood flow measurement is also used clinically. For example, ultrasound can assess endothelial dysfunction as a predictor of various cardiovascular pathologies, such as atherosclerosis (Celermajer et al., 1992). These examples highlight the common practice of measuring blood flow through ultrasound; it serves as a measure of cardiovascular system function and is impacted by a wide range of factors that are relevant to clinical and research practices.

### **Understanding NIRS-ICG**

In addition to using ultrasound to measure blood flow, specifically femoral blood flow, the focus of this study will be employing an alternative methodology for assessing blood flow. This methodology utilizes two tools that, when used together, allow for measurement of blood flow in the vastus lateralis. The vastus lateralis is a muscle that runs near the femoral artery in the lower extremity, as shown in Figure 1. The alternative methodology that will be used to measure blood flow in this muscle is known as near infrared spectroscopy-indocyanine green (NIRS-ICG).

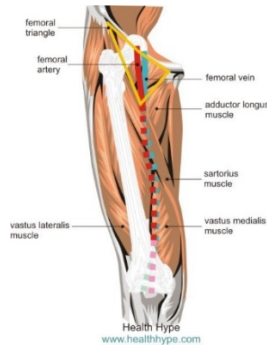


Figure 1: Lower extremity muscles and vasculature.

Indocyanine green (ICG) is a dye whose passage through vasculature of the body can be monitored by NIRS after intravenous injection. ICG has an absorption peak of 805 nm, which is within the near-infrared range. When ICG enters the blood stream via injection it follows the path of blood throughout the body; it enters through venous blood, flows to the heart, through the lungs, and then back through the heart until it is circulating systematically as arterial blood. As it is traveling, ICG binds to albumin, a protein in the blood plasma. The presence of ICG in the body is transient as it is cleared from blood through uptake by the liver and biliary excretion (Kuebler et al., 1998).

Near infrared spectroscopy (NIRS) works in conjunction with ICG. The spectrometer uses two laser diodes that generate light at two distinct wavelengths, 728 nm and 828 nm. ICG absorbs some of the emitted near-infrared light at its absorption peak, and the spectrometer collects the scattered light that is reflected back. The NIRS spectrometer is attached to the leg using bandages that are impermeable to light. The attenuation of light is measured as absorbance, which reflects the appearance of ICG and thus acts as a measure of local skeletal muscle perfusion. Absorbance is a representation of the concentration of ICG represented as an arbitrary unit. Thus, there is a clear relationship between absorbance and ICG: the more ICG present, the greater

absorbance, and the more blood has traveled to that area. Figure 2 shows a representation of how NIRS is used to take measures of the appearance of ICG after infusion, based on a study that assessed blood flow in the calf after plantar flexion (Boushel et al., 2000).

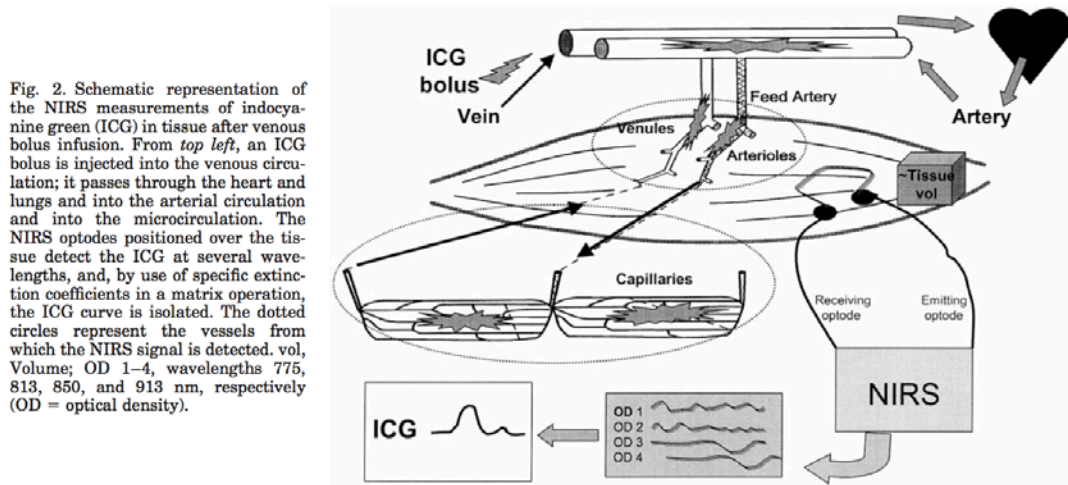


Figure 2. Schematic of NIRS-ICG measurements.

### Previous Use of NIRS-ICG

The use of NIRS-ICG as a technique to measure blood flow is not novel in this study. However, there are differences between the protocol and data analysis techniques presented here compared to previous work. The technique of using NIRS to noninvasively measure blood flow was first described in 1977 (Jöbsis, 1977). It is typically used to monitor levels of chromophores, such as hemoglobin. Using NIRS-ICG, the oxygen-dependent absorption of deoxygenated and oxygenated hemoglobin is measured. Then, chromophore absorption can be calculated using the Lambert-Beer law:

$$OD_{\lambda} = \text{Log} \left( \frac{I_0}{I} \right) = \epsilon_{\lambda} \cdot c \cdot L$$

The law is then corrected to account for scattering that occurs in biological mediums by a factor known as the dimensionless pathlength factor (DPF). The sum of absorbance between the two variants of hemoglobin provide the total blood volume in the tissue, including both arterial and venous blood. Unfortunately, there are limitations with this method as NIRS cannot distinguish between oxygen carried in hemoglobin versus myoglobin. The wavelength that would be able to adequately distinguish, does not penetrate deeply enough into the tissue (Kuebler et al., 1998).

In addition to using NIRS-ICG to examine hemoglobin oxygenation, it is used to measure blood flow through arterial concentration. Using this variation of the technique, ICG is injected into the antecubital vein and then drawn from the radial artery to enable measurement of ICG arterial concentration. A data analysis technique different than the Beer-Lambert law can then be used. Habazetti et al. calculated blood flow using Fick's Principle:

$$CO = \frac{VO_2}{C_a - C_v}$$

They then compared this calculation to muscle blood flow (MBF) for the quadriceps and intercostal muscles (Habazettl et al., 2010).

Fortunately, NIRS-ICG can be used to measure blood flow without the complex math of Fick's Principle of the Beer-Lambert Law. And, without the additional invasive practice of obtaining the arterial concentration of ICG. Several previous studies utilize blood flow index (BFI) to analyze NIRS-ICG data. BFI is calculated as the maximum change in absorbance divided by the rise time, or the amount of time it takes for this

change to occur. Rise time is limited to between 10% and 90% of maximum absorbance. BFI leads to a relative, rather than absolute, measure of blood flow. This calculation was developed to compare fluorescein flowmetry and laser Doppler flowmetry in the intestine (Perbeck, Lindquist, Proano, & Liljeqvist, 1990). It has been validated by several studies, one designed to evaluate the feasibility and reproducibility of BFI to measure cerebral blood flow (Wagner, Gertsch, Ammann, & Pfenninger, 2003). A second used BFI to calculate cerebral blood flow, and compared NIRS-ICG measurements to cerebral and galeal blood flow values obtained through radioactive microsphere sampling (Kuebler et al., 1998). Based on validation of this method by these and other research groups, this study will employ similar methods for analysis of NIRS-ICG absorbance. This will allow results found here to be comparable to results from similar studies. Additionally, this technique eliminates subject to subject variation by transforming blood flow into a proportional measure, rather than an absolute value. BFI can be thought of as interchangeable with slope.

### **Comparing Methodologies**

One way to assess the differences between NIRS-ICG and ultrasound is to compare their abilities in regards to spatial and temporal resolution. Spatial resolution refers to the ability to differentiate between the position of two objects. Temporal resolution refers to the precision of a measurement with respect to time. Ultrasound has good temporal resolution because measurements can be made at many different time points and the time between measurements is not prescribed. NIRS-ICG has limited temporal resolution as it requires a washout period between infusions to ensure the liver has time to metabolize the ICG (Kuebler et al., 1998). Ultrasound has poor spatial

resolution because it measures bulk blood flow. In other words, it does not measure blood flow with great specificity because it cannot differentiate between the skin or many muscle groups that the femoral artery supplies (Wagner et al., 2003). This is especially problematic because blood flow to one area could increase while blood flow to the other decreases, and the ultrasound would interpret this as no change in blood flow. Comparatively, NIRS-ICG provides good spatial resolution as it can isolate muscle from skin tissue and from fat in the lower extremity. Additionally, it allows measurements to be much more regionally specific based on the placement of the NIRS sensor.

Beyond the differences in temporal and spatial resolution, the comparison of NIRS-ICG and ultrasound presents further costs and benefits. Ultrasound is non-invasive, while NIRS-ICG requires the placement of an intravenous catheter to allow for the introduction of ICG into the bloodstream at controlled time points. Performing ultrasound measurements requires a skilled, trained individual. The quality of ultrasound images can vary dramatically based on the researcher and their level of experience with the technology. Contrastingly, beyond catheter placement, NIRS-ICG measurements do not rely on the technique of the user. The cost of NIRS-ICG is also much lower than ultrasound, especially when considering a setting where an ultrasound machine is not already available for use. 100mg of ICG may cost a couple hundred dollars, but the investment required to purchase an ultrasound machine, which is thousands and thousands of dollars, is much more cost prohibitive. A final consideration is the risk of allergic reaction that has been identified for ICG, especially in individuals

who have prior allergic reactions to shellfish. Luckily, this risk is quite limited with the incidence of allergic reactions “reported as 1:250,000” (Wagner et al., 2003).

### **Hypothesis**

It was hypothesized that NIRS-ICG will be a more feasible and reliable method, when compared to ultrasound, to measure skeletal muscle blood flow in the lower extremity after passive limb movement.



## Methods

### Experimental Outline

The study included in this thesis was approved by the Institutional Review Board (IRB) at the University of Oregon, Protocol # 02172011.029. The ultimate questions to be answered with this protocol were twofold. First, to what extent is NIRS-ICG a reliable methodology for measuring blood flow after passive limb movement? Second, how do NIRS-ICG and ultrasound compare as methodologies for this purpose? These questions were addressed through a protocol that includes 60 minutes of passive limb movement (PLM). However, before initiating this experimental protocol it was necessary to perform a positive control to ensure that NIRS-ICG and ultrasound could measure changes in femoral blood flow. To do so, a graduate student volunteer underwent a protocol similar to the one described below with dynamic knee extension (DKE) performed in place of PLM. Since this did produce a measurable change in blood flow, the planned protocol involving PLM was carried out.

Three subjects participated in the experiment, 2 males and 1 female. The experiment included two visits to the EEP lab that lasted approximately four hours in total; an initial prescreen and the study day. Subjects were compensated \$15 per hour for their participation, totaling \$75.

### *Prescreen Methods*

Prescreens were performed by a graduate student in the lab, Dylan Sieck, to determine subject eligibility. Subjects were eligible if they met the following criteria: healthy, non-smoking, aged 18-40, sedentary or recreationally active. Subjects were

excluded for the following criteria: if they were taking medications other than oral contraceptives, taking herbal remedies or dietary supplements, pregnant or breastfeeding, using illegal or recreational drugs, or engaging in more than five hours of exercise per week. Subjects were also asked about known allergies to drugs, medications, or foods. Due to the potential for increased incidence of allergic reaction to ICG, subjects with prior allergic reactions to shellfish were excluded. Once eligibility was determined, subjects were informed of the experimental methods, risks, and benefits of participation. Written informed consent was obtained, and subjects were made aware they could withdraw from the study at any time. At this point the subject was considered enrolled in the study, and given a 3-digit code to be used in place of identifying information to protect their privacy for the duration of the study. Subjects were instructed to abstain from exercise, alcohol, and caffeine for 24 hours prior to the start of their study session. They were also asked to arrive to the study having fasted for at least two hours prior.

### *Study Day Methods*

Prior to the subject's arrival, ICG was mixed and the Baxter Flo-Gard 6201 Volumetric Infusion Pump prepared. The NIRS machine was calibrated and attached to OxiplexTS software to be used for data collection. The ultrasound machine, 9Mhz linear-array vascular probe model iE33, Philips Diagnostic Ultrasound System (Bothell, WA), was also set up. Ambient temperature, humidity and barometric pressure were recorded. If a subject was female, a pregnancy test was administered upon arrival and participation in the study continued once a negative result was obtained. After weight and height of the subject were taken, they were instrumented with a three lead ECG and

blood pressure cuff. The SunTech TangoM2 machine was used to obtain blood pressure and the DatexOhmeda Cardiocap/5 ECG used for heart rate monitoring throughout the study.

The subject was instructed to lie supine (on their back, face up) on a padded table and baseline heart rate and blood pressure readings were taken. An intravenous catheter (IV) was then placed in the antecubital fossa on the subject’s arm. Next, one NIRS sensor was placed on each leg over the vastus lateralis and attached using ACE bandage loosely wrapped. The orientation of the sensor was marked on the subject’s leg using permanent marker in case adjustment or replacement was required. An effort was made to place the NIRS sensor away from areas that had visible vasculature as this could interfere with the quality of readings. Occlusion cuffs were placed below the knee on each of the subject’s legs to later be inflated to occlude blood flow to the lower half of the leg. With the subject instrumented, they were instructed to lay passively in a supine position for five minutes before the timer for the study was started.

The study started after this 5 minute rest. Figure 3 outlines the protocol.

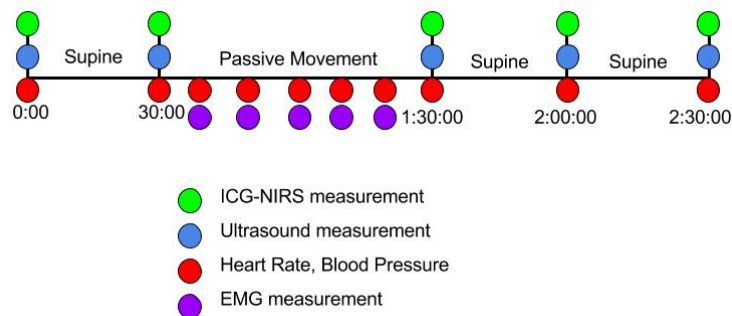


Figure 3: Schematic of Study Timeline

At the start of the study, the subject’s blood pressure and heart rate were taken. Then the first ICG injection was performed; a 5mg bolus of ICG was pushed through

the IV followed by a 5 mL flush of .9% saline. Simultaneous to the ICG push, a marker was placed using the OxiplexTS Software, which was recording absorbance via the NIRS sensors, so that this data could be analyzed relative to each ICG infusion after completion of the study. Two minutes after ICG infusion, ultrasound was used to obtain an 80 second recording of blood flow through the femoral artery on both legs. The ultrasound was also used to produce a still image of the femoral artery on each leg so depth and diameter of the vessel could be recorded. To obtain these ultrasound images and recordings, the occlusion cuffs were inflated to 250mmHg on the leg that was being viewed. The subject continued to rest in supine until 30 minutes after the beginning of the study, at which point the same process (blood pressure, heart rate, ICG-NIRS, ultrasound measurement) was repeated.

The subject was then instructed to remain passive as they were moved from supine position to an upright seated position. To do so, the occlusion cuffs were removed and the subject was fitted on an ergometer so their right leg could be moved from 90 degrees of leg flexion (hanging down) to 45 degrees of flexion for 60 minutes. At the same time the subject's resting (left leg) hung down at 90 degrees flexion for the duration of passive movement. Before movement began the subject's moving (right) leg was instrumented with an electromyogram electrode (EMG) that was placed on the skin over the vastus lateralis. This monitored the electrical activity of the muscle to help ensure the muscle was not activated during movement. The subject's foot on their moving leg was attached to the pad of the ergometer with ACE bandage to prevent slipping and blisters. Throughout the 60 minutes of PLM, blood pressure and heart rate

were recorded at 10 minute intervals. Figure 4 shows an image of a subject during PLM.



Figure 4: Subject performing passive limb movement.

Following movement, the subject was returned to a supine position for 60 minutes. The EMG was removed and occlusion cuffs replaced. The process of blood pressure, heart rate, ICG-NIRS, and ultrasound measurement was repeated three more times: immediately following movement, 30 minutes after movement, and 60 minutes after movement. With data collection complete, all remaining instruments including ECG leads, blood pressure cuffs, leg cuffs, NIRS sensor and IV were removed.

## **Data Analysis and Processing**

### *Ultrasound Capture, Processing and Analysis*

During the study day protocol, ultrasound measurements of the femoral artery in both the movement and resting legs were taken after each injection of ICG. So, at five time points throughout the duration of the study. After the study day was complete, the 80 second recording of blood passing through the vessel was processed using two computer programs. First, the Doppler Ultrasound Capture and Calibrate software calculated the mean blood velocity and peak blood velocity. Second, the Brachial Analyzer for Research Properties software was used to calculate the average diameter of the femoral artery during that recording. Diameters picked up by the analyzer that were below a 50 percent confidence interval were removed and the average calculated.

Once the raw data of the ultrasound recordings were transformed into average diameter and blood velocity, Excel was utilized to calculate femoral blood flow (FBF). Importantly, previous research has found that ultrasound measurement can lead to an "overestimate of mean blood velocity by up to 33%" (Buck et al., 2014). This overestimation occurs because the ultrasound beam is not narrow enough to be assumed to be infinitely narrow. This prevents a standard correction factor from being able to compensate for this error (Buck et al., 2014). However, since the blood vessel and beam widths are known, this overestimation can be accounted for during data analysis. Specifically, beam width ratio (BWR), the ratio of the ultrasound beam to the diameter of the vessel calculated by the brachial analyzer, was calculated. Based on the BWR value, a correction factor can be looked up. This correction factor was then incorporated into the calculation of FBF.

### *NIRS-ICG Analysis*

The software used to record absorbance from the NIRS sensors produces four absorbance measures every second, two for each leg at two different wavelengths - 782 nm and 828 nm. Theoretically, once ICG is injected into the venous blood, moves through the heart, and is pumped out through the arterial blood it begins to circulate systemically. In other words, the amount of ICG in the blood moving through the vastus lateralis goes from the minimum amount (0) to the maximum amount (100). Since the amount of ICG injected with each bolus is a constant 5mg, it is important to determine the amount of time it takes to go from 0% ICG to 100% ICG after each injection. Importantly, the minimum value for each subject and between legs can vary based on the volume of the blood in the tissue, the tissue's oxygenation state and many other factors.

To perform this analysis an Excel sheet was used, with code written by Dr. John Halliwill. The raw data from the Oxiplex Software was split based on the marker placed when each ICG infusion was performed. Absorbance values from 10 seconds after the marker to 60 seconds after the marker were used. In this range, minimum and maximum absorbance values were found and a moving average performed to limit the impact of potential outliers. Then, a regression calculation was used to determine the slope between the minimum and maximum absorbance values; this was limited from between 10% and 90% of the full range. The correlation between the slopes of the two wavelengths for data from each leg was also calculated.

The final values that were reported from this analysis were the slopes and correlations at each marker. The correlation showed the relationship between the

absorption measure and time to indicate how linear versus scattered the data was. Low correlation values of below .6 were used as a baseline to exclude data. The slope was transformed to be reported as rise time, by taking the inverse of the slope. This shows the time it takes to go from the minimum to maximum ICG expressed in seconds.

Although it may be useful in the future, statistical analysis to determine significant difference between rise time and correlations was not the focus of this analysis. With a low number of subjects and high variation between them, observing patterns in the data through visual representation was the main mode of data interpretation.

#### *Ultrasound and NIRS-ICG Comparison*

Two techniques were utilized to compare the FBF values produced by the ultrasound and the slope, rise time and correlation produced through NIRS-ICG measurement. First, a standard correlation graph was produced plotting FBF against slope at each marker for both the movement and resting leg. As shown in analysis performed by other research groups, FBF and slope (often referred to as BFI) act as proportional indicators of blood flow (Kuebler et al., 1998). This graph produces a correlation coefficient, which acts as a statistical measure for how close the data are to the line of regression. While this gives a general estimation of the extent to which these two techniques agree, this value is somewhat problematic. As pointed out by Bland-Altman, when two methods are used assess the same measure, they are very likely to be highly correlated (Bland & Altman, 1986). However, this correlation does not specify if these two methods have a high level of agreement. In other words, perfect correlation occurs when points lie perfectly along any straight line. However, perfect agreement only occurs when points fall along the line of equality (Bland & Altman, 1986). To



address this gap, a Bland-Altman graph will be used to determine to what extent NIRS-ICG and ultrasound are in agreement. A Bland-Altman graph can be thought of as a mean-difference plot, where the mean of two measurements is plotted on the x-axis and the difference between the two values is plotted on the y-axis.

## **Results**

### **Subjects**

All three subjects successfully completed the protocol. For subject 001, data at the third ICG injection (post00) for the control leg has been excluded; the NIRS sensor slipped out of the bandage wrapping and caused the absorbance values to overload. For subject 002, no data was obtained at the first marker (pre30) as data recording was mistakenly not started until the second infusion. Additionally, rise time and slope data were excluded for the movement leg at the third marker (post00) as correlation was extremely low and no clear start or end point to the upslope could be visually identified. For the same reason, slopes for the movement leg at the first (pre30), second (pre00), fourth (post30) and fifth marker (post60), and second marker (pre00) on the rest leg, were excluded for subject 003.

### **Heart Rate and Mean Arterial Pressure**

Throughout the duration of the study only minimal variation in heart rate was seen. Additionally, there was minimal difference in heart rate between the three different subjects. Mean arterial pressure followed the same pattern. Mean arterial pressure is the average pressure in the patient's arteries during one cardiac cycle (Hall, 2010).

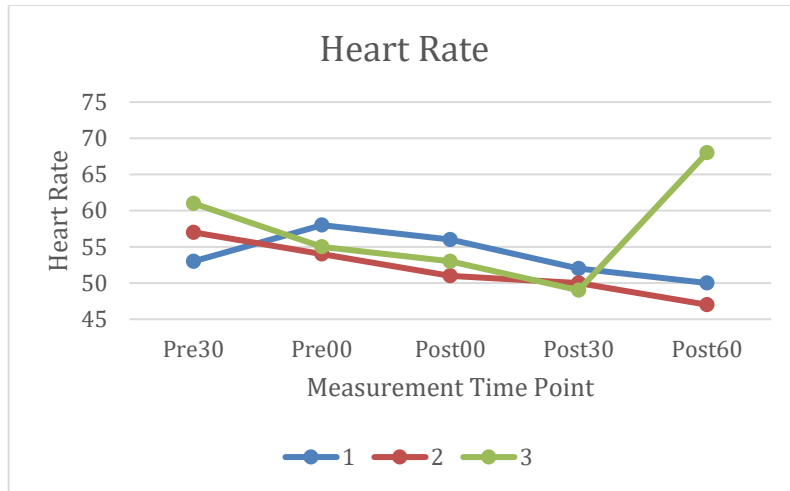


Figure 5: Heart rate of subjects 1, 2, and 3 throughout study duration.

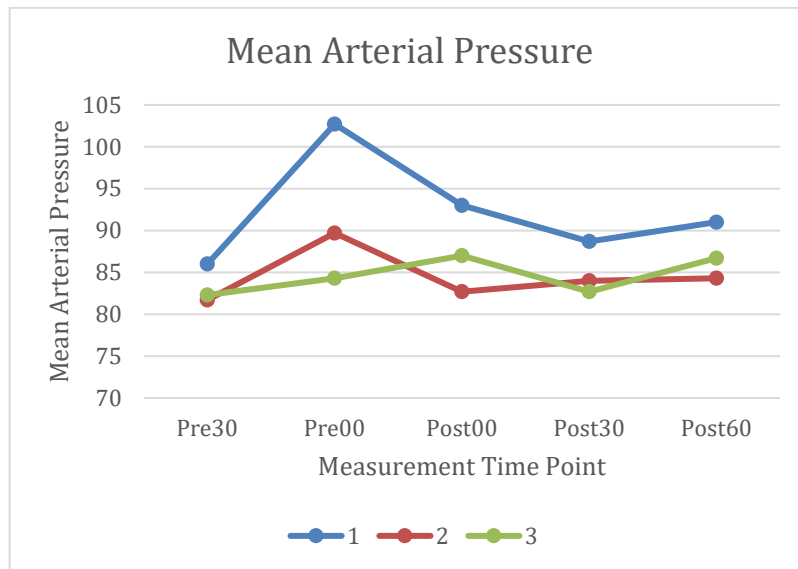


Figure 6: Heart rate of subjects 1, 2, and 3 throughout study duration.

## Ultrasound Measurements

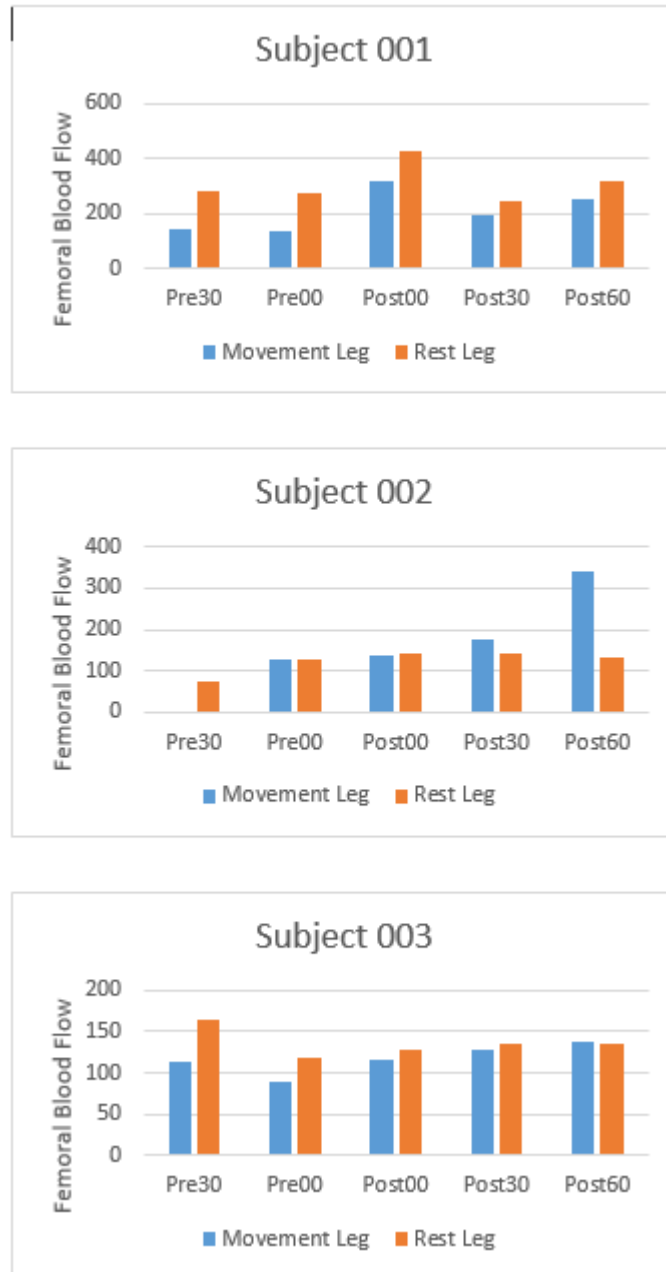


Figure 7: Femoral blood flow after each ICG injection.

## NIRS-ICG Measurements



Figure 8: Rise time after each ICG injection.

## Ultrasound and NIRS-ICG Measurements

In Figure 7 and 8, above, the blue bars represent the subject's movement leg and the orange bars represent the subject's resting leg. The measurements are separated into five time points, each representing when ICG was injected. "Pre" refers to before movement and "post" refers to after moment. The numerical markers refer to the number of minutes before or after movement. For instance, "Pre00" would indicate zero minutes, or immediately, before the start of movement. If a blue or orange bar are not present at a time point, this represents excluded data – as explained previously. For example, there is no data for subject 003 at the "pre00" time point because the start and end points of upslope could not be distinguished and had a very low correlation.

Looking at the ultrasound measurements, femoral blood flow (FBF) in the resting leg remains mostly stable across the duration of the study for all subjects. The movement leg displays more fluctuation. For the first subject, FBF in the movement leg is lower before movement than after. In the second subject, the movement leg's FBF is mostly stable until it increases dramatically at the last time point - 60 minutes after the termination of movement. In the third subject the pattern is harder to distinguish due to a large amount of excluded data. However, FBF in the movement leg appears to peak 30 minutes after the termination of movement.

The NIRS-ICG data is presented as rise time, which is the inverse of slope. This indicates the number of seconds it takes to go from 0% to 100% ICG. The first subject shows a clear decline in rise time from before to after movement, on the moving the leg. The resting leg also shows a decline in rise time after movement but to a lesser extent. Subject 2 displays the same pattern, but with less clarity since the first marker before

movement was not recorded. This pattern in the third subject is very unclear as hardly any moving leg data was usable, and the resting leg shows an increase in rise time after movement.

### Aggregated Data

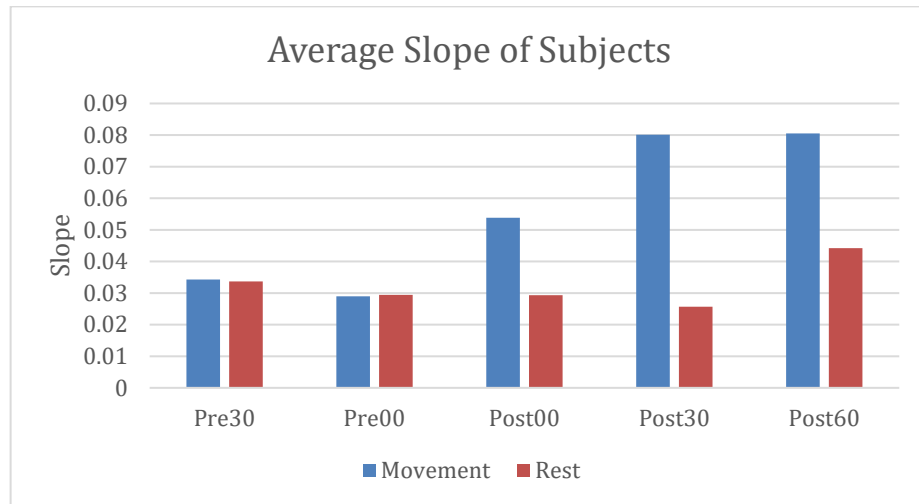


Figure 9: Average slope for all subjects.

Figure 9 shows the average slope for all three subjects after each ICG injection. Slope, which is proportional to FBF, remains stable in both legs before movement. After movement, slope in the movement leg increased while slope for the resting leg remains relatively constant. A slight increase in slope occurs 60 minutes after the end of movement.

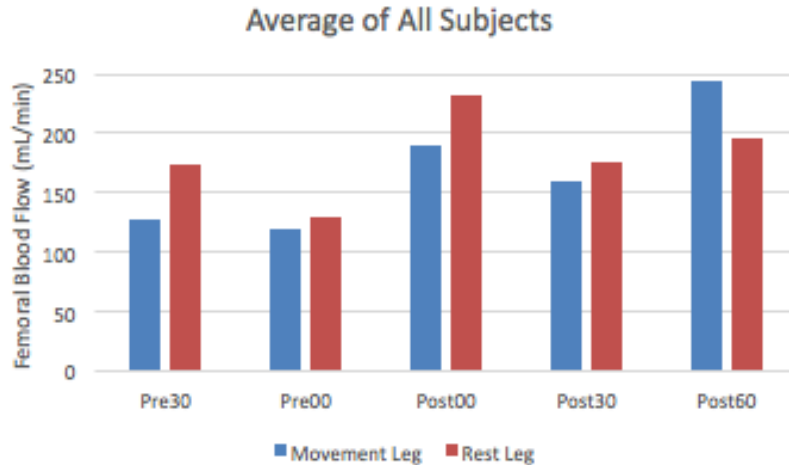


Figure 10: Average femoral blood flow for all subjects.

Figure 10 shows the average FBF for all three subjects after each ICG injection. FBF would be expected to increase after blood flow increases. This expectation is upheld by the graph above, as FBF in the movement and resting legs remain relatively stable before movement. After movement, FBF in the moving leg increases. This is true in the resting leg as well, but to a lesser degree.

### Correlation

Correlation describes the extent to which two variables change together; it considers the strength and direction of that relationship. The graphs below show the correlation between slope, obtained from NIRS-ICG measurements, and FBF, obtained from ultrasound measurements. Figure 11 represents this correlation after the raw data was transformed into a ratio between the movement leg values to resting leg values. The ratios before movement were close to 1, and after movement as skeletal muscle blood flow increased, these ratio values became larger. The Pearson's correlation coefficient for this data set = .238. Pearson describes the linear relationship between two variables, evaluating how closely a change in one variable is associated with a proportional



change in the other. Correlation coefficients range from -1 to +1. The more random a relationship, the closer the coefficients are to 0. While the correlation coefficient for this graph is quite low, it still indicates a correlated relationship. Furthermore, considering only data from three subjects were used to perform this correlation, it is difficult to make an extremely substantive claim based on comparing that limited number of data points.

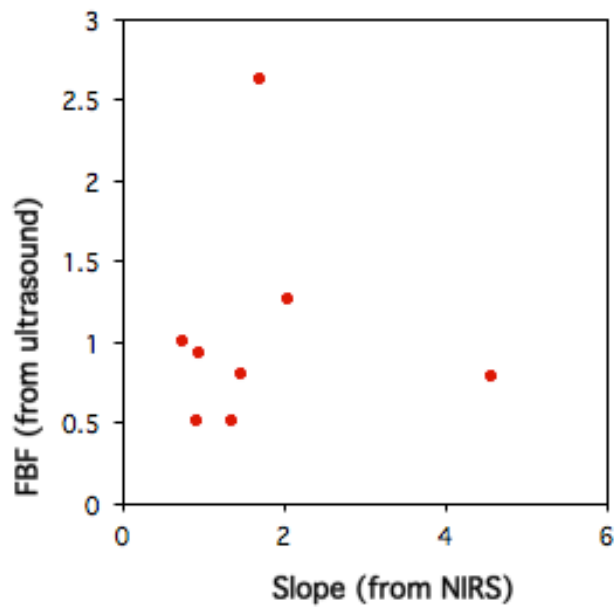


Figure 11: Correlation of ratios between methodologies.

On the following page, Figure 12 shows a similar correlation. However, instead of representing a ratio, this represents correlation after the raw data was transformed into percent difference. The two pre-movement values for each subject and methodology were averaged and the percent change was calculated comparing this average to each of the three post-movement values. The Pearson's correlation coefficient for this graph = .278.

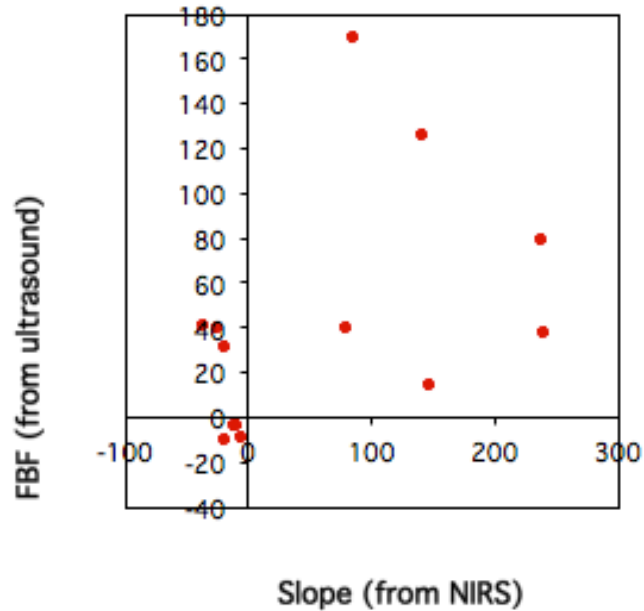


Figure 12: Correlation of percent difference between methodologies.

### Agreement

As described previously, especially when measuring two methods intended to evaluate the same measurement, describing relationships through correlation alone is not sufficient. The Bland-Altman graphs below displays the mean of the two methodologies on the x-axis and the difference between the two measurements on the y-axis. The mean represents the best guess of the true value for FBF. The dashed lines represent two standard deviations below and above that mean. Figure 13 represents the agreement between methodologies after the data has been processed into percent difference, as explained for Figure 12. In Figure 14, the agreement between methodologies is represented after the data was transformed into a ratio between movement and resting leg, as explained for Figure 11. It is best to observe trends in these two graphs by reading them from left to right, then noticing any changes in the

vertical distributions of the data points. This illuminates several trends. For instance, Figure 13 includes all the data points within the dashed lines while Figure 14 does not. This suggests that the data input into Figure 13 is more normally distributed. For both Bland-Altman graphs a clear trend line is not present and the data points are very scattered rather than clustered. This suggests that the limits of agreement are quite wide. However, it is important to note, the Bland-Altman graph only defines limits of agreement, it does not make a judgement as to if those limits are acceptable based on biological or clinical considerations (Giavarina, 2015). Again, based on the limited amount of data points utilized for this analysis so far, it is difficult to make final claims about agreement or correlation between the two methodologies. Both the correlation and Bland-Altman graphs should serve as representations of how to best process this data once more subjects have been moved through the protocol. For now, it is best to rely more heavily on the individual and averaged raw data as seen in Figures 7, 8, 9, and 10 when drawing conclusions about either method's ability to effectively measure regional skeletal muscle blood flow.

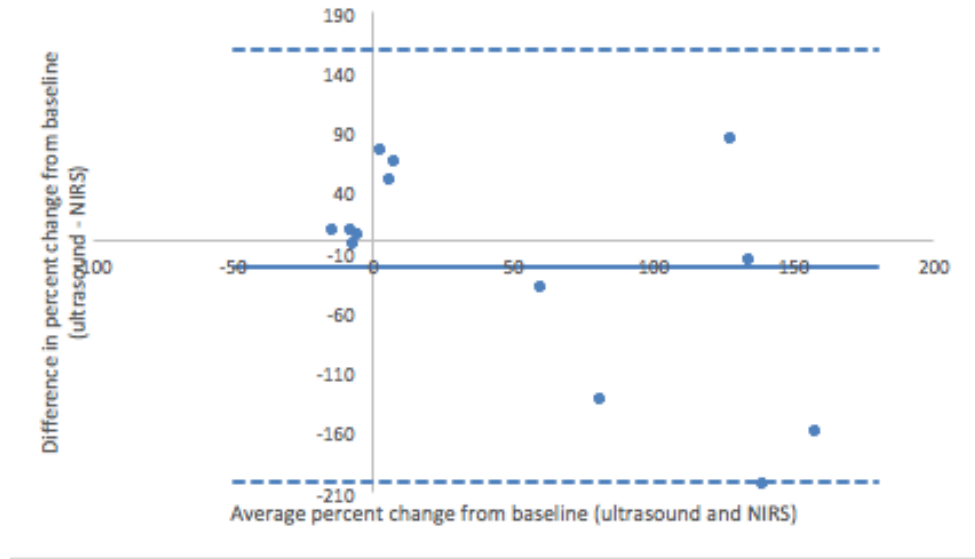


Figure 13. Agreement between percent difference from baseline.

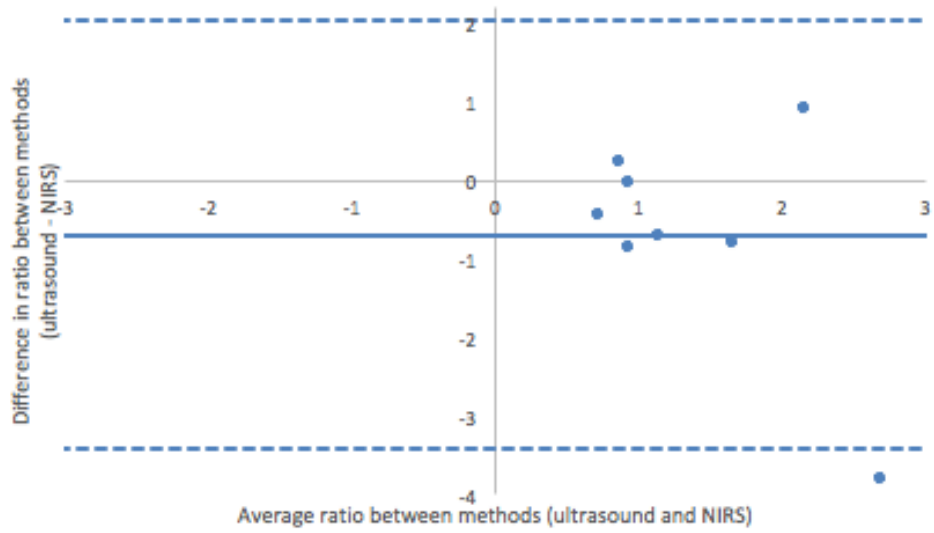


Figure 14. Agreement between movement to rest ratio.

## Discussion

### Ultrasound

Previous research has shown that passive limb movement causes an increase in blood flow (Trinity et al., 2010). For this reason, FBF should be higher after movement in areas where blood flow increases due to PLM. Subjects 001 and 002 did show this pattern, but subject 003 did not show a difference in FBF in the movement leg after movement. The peak increase in FBF occurred immediately after movement in subject 001, but did not occur until 60 minutes after movement for subject 002. With minimal data points, it is difficult to discern if this difference is due to physiological differences between the subjects, such as anatomical differences or varying activity level of the subjects. Or, if these discrepancies are more strongly related to flaws in ultrasound measurement. The methodology is vulnerable to a high amount of subjectivity from both the technique of the ultrasound operator and through processing of ultrasound images and recordings.

FBF in the resting leg should theoretically remain stable across the duration of the study, since PLM was not working to increase blood flow there. This was mostly true across all three subjects, with slight variations. For instance, in subject 001, FBF in the resting leg increased immediately after movement and then returned to resting levels by 30 minutes after movement. This could be indicative of a slight systemic increase in blood flow occurring as a result of PLM in a specific skeletal muscle. Looking at individual subjects, the expected FBF pattern was upheld in subjects 001. Subjects 002 and 003 showed barely any change throughout the duration of the study or between legs.

## **NIRS-ICG**

In the graphs above, the appearance of ICG is presented as rise time and slope. An inverse relationship between rise time and FBF is expected. As blood flow increases, the time it takes for blood, and thus ICG, to travel to that area should sharply decrease. This expected outcome was upheld by subject 001 and 002, since rise time decreased sharply after movement in the moving leg in both cases. Subject 003 cannot be said to uphold this pattern because the slope at all but one time point had to be excluded. Slope in the resting leg should theoretically remain stable throughout the duration of the study. Rise time in the resting leg followed a similar pattern to the moving leg in subject 001, but to a lesser extent. Again, this could be indicative of systemic changes in blood flow occurring as a result of PLM in a focused area. Another potential explanation for these fluctuations might be that ICG is not completely removed from blood circulation between injections. However, previous studies have indicated that ICG is completely excreted by the liver and "is cleared from the human circulation with a half-time of 3.2 to 3.4 minutes" (Kuebler et al., 1998). Rise time in the resting leg remained quite stable in subject 002, but fluctuated quite a bit in subject 003. Looking at individual subjects, the expected slope pattern was upheld in subjects 001 and 002. The pattern was not upheld by subject 003, but this was due to an inability to draw conclusions thanks to excluded data, not from an observed pattern.

When considering NIRS-ICG results as an average of slope across all three subjects, the visual patterns are much stronger. Since slope has an inverse relationship to rise time, it would be expected to increase as blood flow increases. In other words, we would expect slope in both legs to be similar and remain constant during the two

time points before movement. After movement, we would expect slope in the movement leg to increase and slope in the resting leg to remain stable. Both of these outcomes occurred when considering the three subjects together across the duration of the study. This indicates that NIRS-ICG is a feasible method to measure changes in femoral blood flow after PLM.

### **Comparison**

The correlation coefficients produced from comparing slope and FBF were both low ( $r_{ratio} = .238$ ,  $r_{percent\ difference} = .278$ ). With a p value of .0456 and a significance level of .05, the p value is smaller than the significance level. This suggests statistical significance in the case of both correlation methods. Additionally, the similarity between the two correlation values is promising as it indicates that outliers are not strongly skewing the data. The agreement between methodologies seen in the Bland-Altman graph enhances this story. The observation that lower measurements of blood flow are clustered more closely to the mean than higher blood flow measurements suggests that these methods may be more effective at accurately reflecting low blood flow than high blood flow. Considering the low number of subjects that went through the protocol to produce this data, NIRS-ICG is comparable to ultrasound for measurement of FBF.

## Conclusions

This study endeavored to develop knowledge about methodologies for measuring changes in skeletal muscle blood flow after 60 minutes of passive limb movement. In the context of previous research, the purpose of this study was to assess the ability for NIRS-ICG to reliably make this measurement and compare differences in results between the methods of ultrasound and NIRS-ICG. The experimental methods utilized were successful in confirming that both NIRS-ICG and ultrasound produce similar trends in blood flow changes after passive limb movement. While comparisons between the two methods were made, due to a low number of subjects that have completed the protocol thus far, final conclusions about their comparison will be more reliable after data from several more subjects can be incorporated.

With the understanding that both methods can successfully measure changes in skeletal muscle blood flow after passive movement, and that changes are indeed seen, we are one step closer to determining the factor that mediates histamine release and vasodilation. The next phase of this study will likely incorporate histamine blockade into a protocol similar to the one described throughout this thesis. This will determine if physical stimuli, independent of exercise related changes, leads to histamine release. Regardless of if this factor is shown as a mediator of this response, it will add to our pursuit of understanding the mechanisms of hemodynamic responses to exercise and movement. At a broader level, it contributes to the ultimate goal of working towards the ability to manipulate our understanding of these responses to improve the health of diseased, injured and aging populations.



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