TEST RE-TEST RELIABILITY OF BRACHIAL ARTERY FLOW-MEDIATED
DILATION IN HEALTHY ADULTS

BY

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LIST OF ABBREVIATIONS

BL - baseline
BMI – body mass index
CA⁺ - calcium
CAD – coronary artery disease
cm/s – centimeters per second
COX-2 – cyclooxygenase
CVD – cardiovascular disease
D – diameter
dynes/cm² – dyne per square centimeter pressure unit
ECG - echocardiogram
EDRF – endothelium-derived relaxing factor
eNOS – endothelial nitric oxide synthase
FMD – flow mediated dilation
HDL – high density lipoprotein
HIV – human immunodeficiency virus
hrs/wk – hours per week
ICAM – intracellular adhesion molecule
ICC – Intraclass correlation coefficient
in - inch
IRB – institutional review board
kg·m² – kilograms per square meter
lb - pound
LDL – low density lipoprotein

MHz - megahertz

mm - millimeters

mm Hg – millimeters of mercury

NO – nitric oxide

O₂ - oxygen

PAF – platelet-activating factor

PGI₂ – prostacyclin

ROI – region of interest

ROS – reactive oxygen species

S.D. – standard deviation

S.E.M. – standard error of the mean

SS – shear stress

V – velocity

VCAM – vascular cell adhesion molecule

VEGF – vascular endothelial growth factor

vWF – von Willebrand factor

WHO – World Health Organization

yr - year
ABSTRACT

Ultrasonographic assessment of flow mediated dilation (FMD) is a valuable noninvasive measurement of endothelial function; it has been shown to be a useful prognostic tool for cardiovascular disease risk. However, reproducibility of FMD in the literature varies greatly (Coefficient of Variations (CV) =2-84%). **Purpose:** Therefore the aim of this study was to examine the test-retest reliability and day-to-day variability in measurements of FMD in 14 healthy young adults. **Methods:** FMD was analyzed on three different occasions within a 21 day period for each subject after a ≥ 8 hour overnight fast. Ultrasound derived diameter of the brachial artery was measured at baseline (resting condition) and immediately following reactive hyperemia. FMD was calculated as percent change in diameter from baseline. **Results:** FMD measurements for interday comparisons demonstrated moderate to strong reliability (intraclass correlation coefficient [ICC] = 0.73, P=0.004; CV = 24.7%). Additionally, reliability for average baseline diameter (CV = 4.5%; ICC = 0.95; p<0.001) and max hyperemic diameter (CV = 4.8%; ICC = 0.93, p<0.001) were very strong. **Conclusion:** These results suggest that FMD can be measured with adequate reproducibility in healthy adults when following a strict protocol. These finding may have practical implications for future studies in the Health and Exercise Science Department at Wake Forest University.
INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of mortality in the world, accounting for 30% of deaths. Atherosclerosis is a precursor to CVD and is characterized by the thickening and hardening of the arteries; often affecting arteries that supply blood to the heart or brain; leading to myocardial infarction or stroke. The vascular endothelium plays an important role in the development and progression of atherosclerosis. The vascular endothelium is a single layer of cells that line blood vessels, forming a barrier between muscle cells and circulating blood and is responsible for maintaining vascular homeostasis through several protective properties such as anti-inflammatory, antithrombotic, anticoagulant, antihypertrophic, and fibrinolytic.

Vascular health is maintained by a balance between the production of vasodilating and vasoconstricting substances acting on the endothelium. In particular, the vasodilator nitric oxide (NO) plays a principal role in preserving vascular homeostasis by opposing the effects of vasoconstrictors. However, an imbalance of these substances, termed endothelial dysfunction, occurs after exposure to CVD risk factors such as smoking, obesity, hypertension, diabetes mellitus, physical inactivity and dyslipidemia.

The link between cardiovascular risk factors and endothelial dysfunction has been widely studied and supported in literature demonstrating that patients with reduced endothelial function are at a much greater risk of experiencing adverse cardiac events. Therefore, the assessment of endothelial function and detection of dysfunction has important prognostic value for the determination of CVD risk.

Early detection of endothelial dysfunction can lead to risk factor modification and improvement in vascular function. Flow mediated dilation (FMD) using ultrasonography
has become a widely accepted noninvasive technique to access endothelial function. FMD measures the ability of an artery to respond to shear stress (release of vasodilators) and is a measurement of NO availability and function. The FMD test uses ultrasound to assess vessel dilation following an occlusion period. Shear stress is generated following release of occlusion creating a period of reactive hyperemia which acts as a stimulus for NO production and FMD.

There are several extraneous factors that can affect FMD including use of medications, tobacco, supplements, caffeine, menstrual phase, exercise and timing and content of meal consumption. When conducting repeated measurements, it is imperative that the environment and the subject’s physical conditions are as similar as possible to ensure reproducibility between exams. There is no standardized protocol for FMD testing and variations in factors such as cuff placement for occlusion (upper arm or forearm), timing of image capture post occlusion, and technician training differ across studies making cross comparisons between studies difficult. Additionally, the assessment of FMD is technically challenging and requires a great deal of practice to become skilled. There is a need for laboratories to standardize protocols, properly train technicians, and establish validity by conducting internal reproducibility studies to obtain valid data for the use in clinical trials.

Therefore, the primary purpose of this study was to establish FMD test-retest reproducibility in a single center in healthy adults. The establishment of a rigid protocol and valid measurement data will be beneficial for future clinical studies in the Wake Forest University Health and Exercise Science Department.
Cardiovascular Disease

Cardiovascular diseases (CVD) are a cluster of disorders that affect the heart and blood vessels and are the leading cause of mortality in both men and women worldwide.\textsuperscript{11} According to the World Health Organization (WHO), 17.5 million people died from CVD in 2012; accounting for 30\% of deaths.\textsuperscript{11,64} Seventeen percent of national health expenditures in the United States can be attributed to CVD. Adults >65 years of age are at a higher risk of developing CVD therefore, with the aging population and longer life expectancy, CVD related mortalities and health expenditures are expected to continue to rise.\textsuperscript{32}

Arteriosclerosis is a precursor to CVD and is characterized by the thickening and hardening of the arteries; often affecting arteries that supply blood to the heart or brain. Over time, plaque formation occurs, obstructing the lumen of arteries and may lead to thrombosis and upon rupture or occlusion, depending on location, can result in myocardial ischemia, infarction, or acute stroke.\textsuperscript{44} The development of atherosclerosis is associated with prolonged exposure to CVD risk factors such as smoking, obesity, hypertension, diabetes mellitus, physical inactivity and dyslipidemia.\textsuperscript{50} The vascular endothelium may become damaged following exposure to these risk factors resulting in vessels that are unable to respond appropriately to chemical signals and stimuli,\textsuperscript{21} termed endothelial dysfunction.\textsuperscript{50}

The Vascular Endothelium

The vascular endothelium plays an important role in the development and progression of atherosclerosis. The vascular endothelium is a single layer of cells that

\textsuperscript{3}
line blood vessels and form a barrier between muscle cells and circulating blood.

Arteries are composed of a tunica intima, tunica media and tunica adventitia as shown in Figure 1. The tunica adventitia is the outer most layer containing mainly connective tissue which is useful in preserving artery shape and controls expansion. Vascular smooth muscle cells make up the tunica media and are responsible for regulating blood flow through vasodilation and vasoconstriction. The tunica intima is the inner most layer of the artery composed of connective tissue and the endothelium which comes in direct contact with the blood.⁶³

**Figure 1.** Structure of an artery wall⁴

A healthy endothelium is responsible for maintaining vascular homeostasis and has several protective properties such as anti-inflammatory, antithrombotic, anticoagulant, antihypertrophic, and fibrinolytic. Vascular homeostasis is accomplished by the utilization of several signaling pathways such as the autocrine, paracrine and
endocrine systems; additionally in response to chemical and mechanical stimuli, the vascular endothelium produces and releases numerous pro-atherogenic and anti-atherogenic molecules in response to stimuli (Figure 2).\(^{35}\)

**Figure 2.** Molecules released by the vascular endothelium in response to stimuli

<table>
<thead>
<tr>
<th><strong>Anti-atherogenic</strong></th>
<th><strong>Pro-atherogenic</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Vasodilators:</strong></td>
<td><strong>Vasoconstrictors:</strong></td>
</tr>
<tr>
<td>Prostacyclin, bradykinin, NO/EDRF, serotonin, histamine, substance P</td>
<td>Angiotensin II, endothelin, free radicals</td>
</tr>
<tr>
<td><strong>Antithrombotics:</strong></td>
<td><strong>Prothrombotics:</strong></td>
</tr>
<tr>
<td>Thrombomodulin, prostacyclin, NO, antithrombin, heparin</td>
<td>Thromboxane, thrombin, vWF</td>
</tr>
<tr>
<td><strong>Anti-inflammatory:</strong></td>
<td><strong>Pro-inflammatory:</strong></td>
</tr>
<tr>
<td>NO</td>
<td>VCAMs, PAF, ICAM</td>
</tr>
</tbody>
</table>

NO = nitric oxide, vWF = von Willebrand factor, VCAM = vascular cell adhesion molecule, PAF = platelet-activating factor, ICAM = intracellular adhesion molecule

When the endothelium becomes damaged or dysfunctional, the artery wall becomes more permeable, allowing for the migration of lipoproteins and smooth muscle into the artery, as well as the activation of T-cells and the formation of foam cells.\(^{62}\) If this process continues, fatty streaks in the vessel enlarge and become susceptible to rupture.
Major vasoconstrictor substances produced by the endothelium include endothelin and angiotensin II. These vasoconstrictors contribute to plaque formation by promoting smooth muscle cell proliferation contributing to plaque formation. Nitric oxide is the prominent vasodilator released by the endothelium. NO plays a principal role in preserving vascular homeostasis by opposing the effects of the above mentioned vasoconstrictors and also plays an important role in platelet formation.

*Nitric Oxide*

In healthy individuals, NO is produced endogenously by endothelial cells through the L-arginine – Nitric Oxide Synthase pathway. NO is produced when the amino acid L-arginine is oxidized by the enzyme nitric oxide synthase (eNOS). eNOS is one of three isoforms of NO and is responsible for the majority of NO production in the endothelium. There are several factors that affect the activity of eNOS including shear stress, hormones (estrogen), hypoxia, and low-density lipoproteins. These stimuli can increase the production or activation of eNOS by the influx of calcium into cells, causing an increase in NO production. When released in the vascular lumen, NO inhibits the aggregation of platelets and the adhesion of leukocytes to endothelial cells. Caterina et al. observed the ability of NO to inhibit leukocyte adhesion to cell walls by treating cytokine-stimulated human endothelial cells (causing an increase in vascular cell adhesion protein-1 (VCAM-1), a protein that regulates the adhesion of leukocytes to the vascular endothelium) with NO donors. NO inhibited the expression of VCAM-1 by 35-55%, demonstrating that NO inhibits the activity of adhesion molecules and some proinflammatory cytokines to vessel walls. These protective effects of NO help to prevent fibrous plaque formation, a major stage in the development of atherosclerosis.
On the contrary, the deficiency and lack of production of eNOS has been linked to a variety of complications including diabetes, hypercholesterolemia and atherosclerosis. Blair et al., induced hypercholesterolemia and atherosclerosis in vitro by treating endothelial cells with either low-density lipoprotein (LDL) or high-density lipoprotein (HDL) that had been isolated from human plasma. LDL altered the ability of eNOS to associate with caveolae (the main binding site of eNOS) and therefore attenuating NO production by the endothelium; whereas when LDL was removed eNOS returned to caveolae. Kuhlencordt et al., further supported this idea by showing mice genetically deficient of eNOS and fed a high fat diet had a 93.6% increase in lesion area in the aorta compared to control mice. Deficiency or inability to activate eNOS plays an important pathogenic role in the development of hypercholesterolemia and atherosclerosis (Figure 3).

**Figure 3:** Factors influencing activating or inhibiting eNOS

<table>
<thead>
<tr>
<th>Activation</th>
<th>Factors inhibiting eNOS</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Ca⁺</td>
<td>• Oxidative stress</td>
</tr>
<tr>
<td>• Shear stress</td>
<td>• Low density lipoproteins</td>
</tr>
<tr>
<td>• VEGF</td>
<td>• Reduced estrogen</td>
</tr>
<tr>
<td>• Hypoxia</td>
<td>• Smoking</td>
</tr>
<tr>
<td>• Estrogen</td>
<td>• Chronic inflammation</td>
</tr>
<tr>
<td>• Bradykinn</td>
<td></td>
</tr>
<tr>
<td>• Thrombin</td>
<td></td>
</tr>
<tr>
<td>• Acetylcholine</td>
<td></td>
</tr>
</tbody>
</table>

**eNOS regulation**

- Inhibition of platelet aggregation
- Inhibition of leukocyte adhesion
- NO-mediated vasodilation
- Vascular smooth muscle cell mitogenesis
- Blood pressure
- Endothelial cell proliferation

**Reduced eNOS expression**

- Hypercholesterimia
- Diabetes
- Atherosclerosis
- Hypertension

VEGF: vascular endothelial growth factor
Shear stress

Red blood cells cause a velocity gradient as they travel through a vessel; velocity is highest through the middle of the vessel and the slowest near the wall of the vessel. This blood flow causes shear stress, which is a frictional drag force that acts parallel to the vessel wall and causes adaptation to flow and possibly vascular remodeling. Maintaining a laminar shear stress is necessary for normal vascular function. Nonlaminar flow stimulates cell wall inflammation, leukocyte adhesion as well as plaque formation.18 Shear stress is estimated by the equation:

\[ \text{Shear stress} = \frac{\text{viscosity} \times \text{velocity}}{\text{diameter}} \]

Therefore, shear stress is highly dependent on the velocity of blood flow as well as the diameter of the artery. In order to maintain appropriate physiologic levels of shear stress, mechanoreceptors on the wall of the vessel detect deformation of the cell membrane caused by shear stress and adjust vascular tone via vasodilation which will result in a reduction of shear stress by increasing vessel diameter. Mechanoreceptors trigger a signaling cascade resulting in the production and release of vasodilators such as NO, prostacyclin (PGI\(_2\)), and endothelium-derived relaxing factor (EDRF) causing vasodilation to maintain vascular tone.46 The vasodilators enter smooth muscle cells and begin a cascade, decreasing calcium concentrations and resulting in endothelium-dependent vasodilation (flow-mediated vasodilation (FMD)) due to the relaxation of smooth muscle cells (Figure 4).
Figure 4. Endothelial shear stress and mechanisms of vasodilation

(a) Arrows correspond with blood flow velocity with the highest velocity at the center of the lumen and decreasing on the periphery of the vessel. The movement of red blood cells through the artery creates shear stress or a frictional force parallel to the wall surface causing deformation of the endothelial cells lining the vessel. (b) Mechanoreceptors on the cell membrane of endothelial cells detect shear stress and (c) a cascade begins; activating eNOS and COX-2 in order to produce the vasodilators NO and PGI\(_2\) that diffuse into vascular smooth muscle cells. (d) These vasodilators cause a decrease in Ca\(^{2+}\) concentration in smooth muscle cells allowing for the smooth muscle to relax and vasodilation to occur.

\(\text{eNOS} = \text{endothelial nitric oxide synthase; } \text{COX-2} = \text{cyclooxygenase; } \text{NO} = \text{nitric oxide; } \text{PGI}_2 = \text{prostaglandins; } \text{Ca}^{2+} = \text{calcium.}\)

**Endothelial Dysfunction**

Endothelial dysfunction can be defined as an imbalance between the synthesis of vasoconstrictors and vasodilators.\(^{20}\) Oxidative stress or the imbalance between the
production of reactive oxygen species (ROS) and antioxidants, a key mechanism involved in the development of endothelial dysfunction, is enhanced by cardiovascular risk factors such as hypertension, diabetes, and hypercholesterolemia. In healthy humans, there is an adequate supply of antioxidants to neutralize free radicals, however, factors such as obesity, smoking, high glucose consumption, and air pollutants can alter the balance between ROS and antioxidants. ROS alter the function of eNOS by promoting the production of oxygen \((\text{O}_2)\) instead of NO (referred to as eNOS uncoupling); transforming eNOS from a protective enzyme to a contributor to oxidative stress. Additionally, ROS inhibit and degrade NO, therefore excess ROS limit NO bioavailability, thus limiting the protective role of eNOS and NO to maintain vascular tone, regulate blood clotting and regulate the inflammatory process. Additionally, oxidative stress often increases the permeability of the endothelial barrier, allowing small particles such as low density proteins into the intima of arteries resulting in inflammation, cellular proliferation, and leukocyte adhesiveness.

**Endothelial Dysfunction and Cardiovascular Disease**

The link between cardiovascular risk factors and endothelial dysfunction has been widely studied and supported in literature. For instance, Perticone et al., examined vascular function in 225 patients never treated for hypertension by measuring forearm blood flow after intra-arterial infusion of acetylcholine. Patients who had the smallest percent increase (30-184%) in flow (greatest endothelial dysfunction) after acetylcholine infusion had a four times greater adverse cardiac event rate per 100 patient years (8.17) over 31.5 month follow-up than subjects with the greatest increase in flow (339-760%). Using the same technique to measure endothelial function, Schachinger et al., analyzed
patients with coronary artery disease. Patients who experienced adverse cardiovascular events after a 7.7 year follow up period had a significantly reduced forearm blood flow (6.5 ± 0.3 mL/min per 100 mL) compared to patients who experienced no cardiovascular events (9.1 ± 0.3 ml/min per 100 mL). Anderson et al., also studied endothelial function via vasoconstriction response to acetylcholine in coronary artery disease patients. Coronary artery disease (CAD) patients had a significantly impaired vasodilation response of the brachial artery than subjects with normal endothelial function (4.8 ± 5.5% vs 10.8 ± 7.6%). The reduced brachial dilation was most strongly linked to the coronary risk factors of endothelial dysfunction, presence of CAD and cigarette smoking. In a study comparing patients with normal, mild, and severe coronary dysfunction, patients with severe dysfunction had 14% higher incidence of cardiac events than those with mild or normal dysfunction over a 28 month follow up period.

Endothelial dysfunction is a strong predictor of vascular disease and strongly associated with cardiovascular complications. Therefore, the assessment of endothelial function and detection of dysfunction has important prognostic value for the development of CVD.

Clinical assessment of endothelial function

Endothelial dysfunction can be revealed by measuring plasma levels of cellular adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1). Rohde et al., demonstrated an association between carotid intimal-medial thickness and ICAM-1 and VCAM-1 plasma concentrations.

Additionally, plethysmography is an invasive technique to evaluate endothelial dysfunction in which blood flow is measured by inserting an arterial catheter into the
brachial artery while the subject is under local anesthesia. Using a transducer, arterial blood pressure and forearm blood velocity are measured. Next, vasoactive substances such as acetylcholine or nitroglycerine are inserted through the catheter and changes in blood flow. The change in baseline blood flow to maximum response of blood flow to acetylcholine are measured to evaluate endothelial response. For symptomatic individuals, direct and invasive techniques such as plethysmography may be the best option to evaluate endothelial function, however an invasive method may not be the preferred option for asymptomatic individuals.

Flow Mediated Dilation

Flow mediated dilation (FMD) using ultrasonography has become a widely accepted and utilized noninvasive technique to assess endothelial function. FMD was first developed in 1992 by Celermajer and colleagues. FMD can be calculated as a percent change from peak diameter following reactive hyperemia to the baseline diameter:

\[
FMD (\%) = \left( \frac{\text{peak diameter} - \text{baseline diameter}}{\text{baseline diameter}} \right) \times 100
\]

Physiology of FMD

FMD measures the ability of an artery to respond to shear stress (through the release of vasodilators) and is a measurement of NO availability. Koller and Sun et al, demonstrated that vasodilation increases proportionally to increases in shear stress. A baseline diameter is measured followed by a measurement after reactive hyperemia and the percent change is calculated. Reactive hyperemia is often created by inflating a blood pressure cuff distal to the measuring location on the artery to 50 mmHg above resting systolic blood pressure and the artery is occluded for 5-10 minutes. When the cuff is
released there is a sudden increase in blood flow to the proximal muscles causing an increase in shear stress that induces endothelial dilation as a consequence of NO release by the endothelium. This can be observed using high-frequency ultrasonography shown in Figure 5.59

**Figure 5.** Change in artery diameter during and following reactive hyperemia

Dashed line (---) represents time during occlusion

*FMD to predict cardiovascular events*

Although FMD does not directly measure coronary blood flow it is an indirect measure of NO production and has been shown to be a predictor of cardiovascular events in clinical and asymptomatic patients.62 It has also been shown to correlate with invasive techniques monitoring endothelial function in the coronary arteries.71 The mean value of FMD in healthy individuals is generally 5-15% however, studies have reported FMD as low as 3.0%28 and as high as 19.2%.9 Despite this wide range of values, FMD has been shown to be absent or greatly diminished in individuals with cardiovascular disease.52,67 Shechter et al., determined brachial artery FMD in 618 healthy adults using ultrasound of the brachial artery. Subjects were divided into groups; FMD ≤ 11.3% and >11.3%. Individuals with an FMD ≤ 11.3% at baseline had a significantly greater incidence of
adverse cardiovascular events than subjects with an FMD >11.3% (15.2% vs 1.2%, p=0.0001) after a 4.6 ± 1.8 year follow up. Likewise, in the MESA study, Yeboah and colleagues assessed how brachial artery FMD predicted incident CVD events in 3,026 adults free of CVD at baseline. After a five year follow up and adjusting for the Framingham risk score, FMD was significantly associated with incident of CVD (hazard ratio, 0.80; 95% confidence interval, 0.62 to 0.97; P=0.025). Additionally, adding FMD to Framingham risk calculations, accurately reclassified 29% of subjects in the low, intermediate, and high cardiovascular disease risk categories. A recent meta-analysis incorporating 14 prospective studies showed an inverse relationship between brachial FMD and CVD risk; concluding that for every 1% increase in FMD there is a 13% lower risk of a cardiovascular event.

However, not all studies have found FMD to be a significant predictor of CVD risk. The population being studied may have an influence on the ability of FMD to predict long term cardiovascular events. For instance, Frick et al., examined the prognostic value of FMD on CAD patients and found no difference in FMD values between individuals who experience cardiovascular events and those that did not after a 2.8 year follow up. Likewise, Ulriksen et al., found that a single measurement of FMD was not predictive of future cardiac events in patients with ischemic heart disease. Neunteufl et al., found that patients experiencing chest pain with FMD >10% had fewer cardiac events than patients with FMD below 10%; however after accounting for the presence of CAD the relationship was no longer significant. Thus, the usefulness of FMD to predict long term cardiovascular events may differ based on the specific population being tested.
Ultrasoundography for flow mediated dilation

Medical ultrasound is an imaging technique that uses sound waves with high frequencies that send pulses into bodily tissues and echo back to produce an image. Frequencies generally range from 1-18 megahertz. Lower frequencies have the ability to image deep into the body but yield a poorer resolution, whereas high frequencies produce high resolution images but the depth of images is limited.

There are several advantages of ultrasound imaging such that it is a painless, safe and non-invasive technique. Unlike x-rays there is no use of ionizing radiation and it is safe to take repeated measures on an individual. Additionally, ultrasound is measured in real time allowing for the technician to observe the movement of structures inside the body instead of simply viewing a snapshot at one moment in time. One potential limitation with ultrasound testing is that it is technically challenging and if the technician is not properly trained, inexperienced, or if proper procedures are not followed there is potential for measurement error with resulting variability.\textsuperscript{70}

For the purpose of FMD measurements, ultrasound systems must have vascular imaging software with two dimensional imaging and Doppler capabilities. The transducer must have a minimum frequency of 7 MHz to obtain images with adequate resolution. It is recommended that an ECG monitor is used to time images with the cardiac cycle.\textsuperscript{16,42}

Subject Preparation

There are several factors that can affect FMD as seen in Table 1. If conducting repeated measurements, it is imperative that the environment and the subject’s physical conditions are as similar as possible to ensure reproducibility between exams.
Table 1. Extraneous variables that can affect flow mediated dilation

<table>
<thead>
<tr>
<th>Variable</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medications</td>
<td>Many medications directly or indirectly effect the vascular system. When possible, subjects should refrain from taking any vasoactive medications at least four half-lives before testing.</td>
</tr>
<tr>
<td>Tobacco</td>
<td>Smoking and second hand smoke have been shown to reduce endothelial function. Subjects should refrain from exposure to smoking at least 12 hours before FMD testing.</td>
</tr>
<tr>
<td>Supplements</td>
<td>The ROS and antioxidant balance is an important factor in endothelial health. Several supplements have been documented to enhance FMD. Therefore, subjects should not take any supplements at least 72 hours before testing and diet should be controlled for.</td>
</tr>
<tr>
<td>Caffeine</td>
<td>Caffeinated beverages have been shown to increase FMD. Ingestion of caffeine should be avoided for 12 hours before FMD measurements.</td>
</tr>
<tr>
<td>Menstrual Phase</td>
<td>There is a parallel increase in eNOS activity and the production of estrogen and progesterone and therefore is likely to influence FMD response. Repeated measurements on women should be taken at the same time during the menstrual cycle.</td>
</tr>
<tr>
<td>Exercise</td>
<td>Acute bouts of exercise has been shown to augment FMD response. Subjects should avoid exercise for 12 hours before FMD testing.</td>
</tr>
<tr>
<td>Fasted</td>
<td>Consumption of a high fat meal has been shown to negatively affect FMD measurements. Subjects should be in a fasted state prior to FMD measurements.</td>
</tr>
<tr>
<td>Acclimatization</td>
<td>Before each FMD testing session, subjects should be in a comfortable position and have rested for 10-20 minutes in a quiet, temperature controlled room.</td>
</tr>
</tbody>
</table>
Standardization/Technique

Despite the growing popularity of FMD to assess endothelial function, there is still a need for standardization across studies with a defined protocol for measurement techniques. Factors such as variations in cuff placement for occlusion (upper arm or forearm), timing of image capturing post occlusion, and technician training differ across studies making cross comparisons difficult.\(^{70}\)

Studies have used both upper arm (proximal) and forearm (distal) cuff occlusion to induce reactive hyperemia in the brachial artery with varying results. The literature tends to support a greater increase in FMD and a prolonged increase in blood flow following upper arm occlusion.\(^{1,9,17,57,73}\) Peretz et al., found maximal FMD was greater using upper arm occlusion compared to forearm (16.2% and 7.3% respectively). However, repeatability of FMD was better when the cuff was placed on the forearm than upper arm placement lending a statistical advantage.\(^{57}\) Similarly, Vogel et al., found upper arm occlusion to display a significantly greater FMD response in both healthy (13.4% vs 5.6%) and CAD patients (11.3% vs. 1.6%).\(^{73}\)

Despite the greater FMD response with upper arm occlusion, Betik et al., suggests that the mechanisms contributing to FMD may differ depending if forearm or upper arm occlusion is utilized.\(^{6}\) The maximal increase in vessel diameter is suggested to occur between 45-60 seconds after release of occlusion. During this period a NOS inhibitor will eliminate any increase in diameter demonstrating that FMD is an endothelium-dependent process reliant on NO during the first 45-60 seconds post occlusion.\(^{37}\) When upper arm occlusion is utilized, maximal FMD generally occurs after 60 seconds indicating that other physiological factors might be involved and therefore may not be a
true representation of endothelium-dependent vasodilation. Whereas maximal FMD after forearm occlusion occurs sooner and may be a better representation of a true endothelium-dependent vasodilation and the recommended standard for measuring endothelial function.\textsuperscript{6}

FMD assessment of the brachial artery is technically challenging and requires a great deal of practice to become skilled. Following a step by step protocol and making note of anatomical landmarks are helpful to decrease intraobserver error. Welsch et al., observed the variability between technicians when measuring FMD in which two ultrasonographers performed scans on the same subjects on two separate days. There was a significant difference between technicians (P=0.0215). Additionally, two individuals analyzed the resulting images to determine reader variability. There was no significant difference in FMD when analyzed by different readers (P=0.5660).\textsuperscript{74} It is advised that clinical studies utilize a single technician to collect all ultrasound images.

\textit{Flow mediated dilation reproducibility}

The reproducibility and validity of brachial artery FMD has been reported. Pala et al., compared the precision and accuracy of ultrasonographic FMD to angiographic FMD in 40 adults.\textsuperscript{55} The mean FMD using ultrasound and angiographic measures were 14.1\% and 14.7\% respectively; giving a high correlation (r=0.93) between techniques. Harris et al., evaluated the reproducibility of FMD response to exercise in overweight men. On two separate days, FMD was measured before and three hours after walking on a treadmill for 45 minutes. There were no differences between trials in brachial artery diameter at baseline, immediate post exercise or at 1, 2, and 3 hours post exercise.\textsuperscript{29}
De Roos et al., evaluated the literature on within-subject variability in FMD and found that in the literature coefficients of variation range from 1.2%-13.6%. However, reproducibility studies vary widely with the number of times the measure is completed. Table 2 depicts the variability found in FMD in studies that presented CV data.

**Table 2. Measures of FMD reproducibility as reported in various studies**

<table>
<thead>
<tr>
<th>Author, year</th>
<th>Sample size</th>
<th>No. of measurements</th>
<th>CV FMD(%)</th>
<th>Baseline diameter (%)</th>
<th>Peak diameter (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>De Roos et al., 2003</td>
<td>13</td>
<td>6</td>
<td>50.3</td>
<td>4.8</td>
<td>5.2</td>
</tr>
<tr>
<td>Brook et al., 2005</td>
<td>33</td>
<td>4</td>
<td>---</td>
<td>4.2-5.2</td>
<td>---</td>
</tr>
<tr>
<td>Sorenson et al., 1994</td>
<td>40</td>
<td>4</td>
<td>1.8</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Harris et al., 2007</td>
<td>9</td>
<td>2</td>
<td>25.2</td>
<td>2.3</td>
<td>2.0</td>
</tr>
<tr>
<td>Herrington et al., 2001</td>
<td>127</td>
<td>2</td>
<td>45.3</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Malik et al., 2004</td>
<td>10</td>
<td>2</td>
<td>41.0</td>
<td>---</td>
<td>13.8</td>
</tr>
<tr>
<td>West et al., 2004</td>
<td>18</td>
<td>3</td>
<td>29.7</td>
<td>2.7</td>
<td>2.5</td>
</tr>
<tr>
<td>Liang et al., 1998</td>
<td>30</td>
<td>2</td>
<td>10.7</td>
<td>---</td>
<td>10.3</td>
</tr>
</tbody>
</table>

Dashed line (---) indicates information was not reported

**Summary**

In summary, the vascular endothelium plays a significant role in the onset of cardiovascular diseases. Endothelial dysfunction limits the protective role of eNOS and NO to maintain vascular tone, regulate blood clotting and maintaining a pro-inflammatory state, and is an important indication of future CVD risk. FMD is a widely used and important technique to assess endothelial function and has important
implications for early detection of endothelial dysfunction in diseased populations. However, there is a need for laboratories to standardize protocols, properly train technicians, and establish validity by conducting internal reproducibility studies to obtain valid data for the use in clinical trials.

Purpose

Therefore, the primary purpose of this study was to examine the test-retest reliability and day-to-day variability in measurements of brachial artery FMD in healthy young adults. The establishment of a rigid protocol and valid measurement data will be beneficial for future research studies at Wake Forest University in the Health and Exercise Science laboratory.
METHODS

Subjects

Fourteen healthy young volunteers were recruited for the study. The Institutional Review Board (IRB) of Wake Forest University approved the study protocol. The aim, design, and protocol of the study were explained to all of the volunteers, who all signed informed consent forms. Recruitment of subjects was conducted using IRB-approved flyers. Those interested completed a health questionnaire to determine eligibility.

Inclusion criteria for the study were as follows: 1) normal BMI (18.5-25 kg/m²), 2) resting blood pressure <140/90 mmHg. Exclusion criteria included: 1) history of or presence of chronic disease such as diabetes, cardiac failure, HIV, cancer, life threatening diseases, or peripheral artery disease, 2) presence of modifiable cardiovascular risk factors, 3) tobacco user. All 14 volunteers were eligible to be enrolled in the study (two men and twelve women) and underwent three examinations. The participants ranged in age from 22-25 years and all successfully completed the study.

Protocol

FMD was performed following previously published guidelines on each subject on three separate days, within a 21 day period, in the Pulmonary Function Lab at Wake Forest University. Twenty-four hours prior to each visit participants were instructed to refrain from taking caffeine, alcohol, supplements, medications, foods high in nitrate (chocolate, wine, beets) and engaging in intense exercise. Additionally, subjects were required to arrive to their appointment after having fasted for a minimum of eight hours. All examinations were performed in a quiet, temperature controlled room (71°F) and were scheduled between 6:30 A.M. to 10:00 A.M. All appointment times
were kept consistent for each of the three appointments to reduce diurnal effects. The same ultrasound technician performed all tests.

Upon arrival, participants’ height and weight were recorded and body mass index was derived. At each visit subjects completed a 24 hour food, medication, and exercise recall. Subjects were familiarized with the feel of the rapid cuff inflator by demonstrating the initiation of the cuff inflation and deflation for five seconds on the right forearm (non-testing arm).

Subjects were instructed to rest in the supine position for five minutes. Resting blood pressure was then recorded using a sphygmomanometer. Subjects continued to rest for another five minutes after blood pressure measurements were taken. After a total of 10 minutes of rest, a 6 x 83cm segmental pressure cuff (D.E. Hokanson, Bellevue, WA, USA) was wrapped loosely three inches below the antecubital crease. The subject’s left arm was comfortably immobilized in a U-shaped brace in the horizontal position with the palm facing up. Ultrasound transmission gel was applied directly to the arm.

Brachial artery diameter and blood flow velocity measurements were obtained with a 10-MHz linear-array transducer and the M-Turbo Ultrasound System (Sonosite Inc., Bothell, Washington, USA) 3-6 inches above the antecubital crease. All images were recorded in the longitudinal plane. Once an optimal image was observed with clear arterial wall interfaces (Appendix F – a), the transducer was positioned so the artery was at a ± 60° angle, and the gain optimized, the transducer was locked in place using a stereotactic adjustable probe holder. The technician took a photograph of the set up for each subject and made note of the distance from the antecubital crease, probe angle, and
artery landmarks so subsequent examinations of the brachial artery could be accurately replicated (Figure 6).

**Figure 6.** Example of arm, transducer, and inflator cuff position.

The transducer was held in place by a stereotactic clamp and the arm was stabilized in a plastic brace to limit movement.

Using the Doppler mode, a minimum of three baseline blood velocity measurements were imaged and saved. Next, a 60 second video of baseline brachial artery diameter was recorded and saved using the 2D high resolution mode. The patient was then asked to remain as still as possible and the forearm occlusion cuff was rapidly inflated using compressed air (E-20 rapid cuff inflator, D.E. Hokanson, Bellevue, WA, USA) to 50 mmHg above the subject’s resting systolic blood pressure to induce occlusion and ensuring reactive hyperemia of the brachial artery. Forearm occlusion was sustained for five minutes during which time the diameter was not recorded. Upon rapid deflation of the forearm cuff, hyperemic blood flow velocity was measured during the first 5-30
seconds at a ± 60° angle before immediately returning to 2D ultrasound mode to record 120 seconds of brachial artery diameter. All images and videos were recorded and exported to a USB device for further analysis. (Step by step protocol instructions can be found in Appendix G).

Ultrasound Image Analysis

Diameter

All ultrasound images were viewed and analyzed by a single technician using Brachial Analyzer for Research software (Medical Imaging Applications, Iowa City, Iowa, USA). This edge-detection, wall tracking software analyzed 450 frames per minute, yielding 450 baseline diameter measurements and 900 post-deflation frames (Appendix F - e and f). After uploading all frozen images and video clips the software was calibrated (10 mm marker distance = 0.053 mm/pixel). The analyzer then defined the Region of Interest (ROI) box to the segment of the vessel that had the most clear intima interface (Figure 7). The ROI remained in the same position, size and at the same angle when analyzing baseline and post occlusion diameters. The wall tracking software was launched. After the entire 60 second frame was analyzed a 3rd order polynomial curve was fit to the diameter output. Quality control was regulated by setting the trend threshold to ± two standard deviations from the 3rd order polynomial line of fit. This was necessary, as any outside noise or movement may have been picked up by the wall-tracking software (Figure 8, Appendix F – e and f). Average, minimum, and maximum diameter measurements for baseline and post-occlusion, defined as the distance in millimeters from the media to the intima interface, were recorded in an excel spreadsheet.
Figure 7. Example of Diameter ROI box for wall detection and diameter measurements

The ROI box was fitted to the area of the vessel with the clearest intima interface.

Size and position of ROI box remained constant for baseline and hyperemic diameter measurements.
Figure 8. Example of quality control in Brachial Analyzer Software

(a) Arrows indicate outside noise or movement detected by wall tracking software. (b) Corrected average, minimum, and maximum diameters after setting quality control threshold to ±2 standard deviations of 3rd order polynomial fit line.

Velocity

Baseline and post-occlusion velocity flow frozen images were uploaded to Brachial Analyzer for Research Software. The software was calibrated with velocity in cm/s on the y-axis and time in seconds on the x-axis. The ROI box encompassed three flow waves representing three full cardiac cycles. The automatic edge-detection software analyzed max and average flow for each of the three cardiac cycles. The velocity wave was manually outlined to better fit the wave form if necessary (Figure 9).
Figure 9. Example of Velocity ROI and measurement in Brachial Analyzer Software

The ROI box was fitted around the flow velocity waves, encompassing at least 3 full cardiac cycles. The yellow lines indicate the cardiac cycle currently being measured. The max and average flow velocity for each complete cardiac cycle was analyzed using the automatic edge-detection software.

Statistical Analysis

All statistical analyses were performed using SPSS software, version 22 (SPSS Inc., Chicago, Ill., USA) and Microsoft Excel 2013 (Microsoft, Seattle WA, USA). Descriptive statistics were used to describe the sample characteristics and are reported as mean ± standard deviation (S.D.). FMD was expressed as a percent change in diameter using the equation: FMD(%) = {((peak hyperemia diameter – baseline diameter)/baseline diameter) x 100. Flow velocity was converted to shear stress using the equation: \( SS_x = 8 \times \mu \times V_x/D_{BL} \) (\( SS = \) shear stress, subscript \( x \) indicated either baseline or hyperemia, 8 is a constant, \( \mu \) was viscosity of blood which was assumed to be 0.035 dynes/cm², and \( D_{BL} = \) diameter at baseline).
Reproducibility of FMD, baseline diameter, peak hyperemic diameter, baseline blood flow velocity, peak hyperemic blood flow velocity, baseline shear stress and peak hyperemic shear stress between three trials was assessed using an Intraclass correlation coefficient (ICC) and coefficient of variation (CV=S.D./mean). ICC values of <0.40, 0.40 to 0.75 and >0.75 represent poor, fair to good and excellent agreements, respectively. Results are reported as mean ± standard error of the mean (S.E.M.). Statistical significance was accepted at p < 0.05.
RESULTS

Subjects

Subject characteristics are presented in Table 3. Fourteen healthy adult subjects were enrolled into the study (12 women and 2 men). The mean age of the subjects was 23.2 years (range 22-25yrs). All subjects met the inclusion criteria, were normotensive (systolic 109.9 ± 5.5mm Hg; diastolic: 67.1 ± 5.1mm Hg), within the healthy BMI classification (22.2 ± 1.6 kg/m²), and physically active (one subject was injured and therefore reported < 1hr of physical activity per week however was normally physically active). All patients completed all three testing visits. A total of 42 ultrasound examinations of the brachial artery were conducted, of which 41 were analyzed and followed by assessment of endothelium-dependent flow mediated vasodilation. One measurement from the first testing session was not analyzed due to poor image quality such that the analyzing software could not detect the wall of the artery.
Table 3. Baseline characteristics of study participants

<table>
<thead>
<tr>
<th>Variable</th>
<th>All patients (n=14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (year)</td>
<td>23.2 ± 1.1</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>2/12</td>
</tr>
<tr>
<td>Ethnicity (% white)</td>
<td>100%</td>
</tr>
<tr>
<td>Height (in)</td>
<td>67.3 ± 4.3</td>
</tr>
<tr>
<td>Weight (lbs)</td>
<td>143.1 ± 18.4</td>
</tr>
<tr>
<td>BMI (kg·m(^{-2}))</td>
<td>22.2 ± 1.6</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>109.9 ± 5.5</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>67.1 ± 5.1</td>
</tr>
<tr>
<td>Physical activity (n (%))</td>
<td></td>
</tr>
<tr>
<td>&lt;1 (hrs/wk)</td>
<td>1 (7%)</td>
</tr>
<tr>
<td>2-3 (hrs/wk)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>4-5 (hrs/wk)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>5-6 (hrs/wk)</td>
<td>6 (43%)</td>
</tr>
<tr>
<td>7-8 (hrs/wk)</td>
<td>4 (29%)</td>
</tr>
<tr>
<td>9-10 (hrs/wk)</td>
<td>1 (7%)</td>
</tr>
<tr>
<td>&gt;10 (hrs/wk)</td>
<td>2 (14%)</td>
</tr>
</tbody>
</table>

Values are means ± S.D. and frequency (% of cohort), in = inches, lbs = pounds, kg·m\(^{-2}\) = kilograms per meter squared, mmHg = millimeters of mercury, hrs/wk = hours per week

Measures of Reliability

Baseline and hyperemic means and standard error of the means (S.E.M) for each visit and average CV and ICC across all three visits for FMD, average artery diameter, and peak artery diameter are displayed in Table 4. Baseline and hyperemic velocity and shear stress are displayed in Table 5. ICC reliability measures were found to be significant (p<0.05) for all variables. Mean FMD±SEM (expressed as a percent change from baseline to hyperemic condition) across all 14 participants for visits 1, 2, and 3 were
7.1±0.94, 6.6±0.67, and 7.3±0.65 respectively (Figures 10). CV across all three visits for FMD was 24% and ICC = 0.73 (p=0.004).

The overall mean baseline brachial artery diameter was 3.8 ± 0.8 mm (visit 1: 3.8±0.13, visit 2: 3.9±0.15 and visit 3: 3.7±0.12). Reliability statistics for average baseline diameter (CV = 4.5%; ICC = 0.95; p<0.001) and max hyperemic diameter (CV = 4.8%; ICC = 0.93, p<0.001) were the strongest of all variables (Figure 11 and 12).

**Table 4.** Vascular parameters for FMD measurements across three testing sessions and the combined average CV and ICC

<table>
<thead>
<tr>
<th>Variable</th>
<th>Visit 1</th>
<th>Visit 2</th>
<th>Visit 3</th>
<th>Overall Mean CV (%)</th>
<th>ICC</th>
</tr>
</thead>
<tbody>
<tr>
<td>FMD (% change)</td>
<td>7.1 ± 0.94</td>
<td>6.6 ± 0.67</td>
<td>7.3 ± 0.65</td>
<td>24.7</td>
<td>0.73*</td>
</tr>
<tr>
<td>Baseline artery diameter (mm)</td>
<td>3.8 ± 0.13</td>
<td>3.9 ± 0.15</td>
<td>3.7 ± 0.12</td>
<td>4.5</td>
<td>0.95*</td>
</tr>
<tr>
<td>Post occlusion max artery diameter (mm)</td>
<td>4.1 ± 0.14</td>
<td>4.1 ± 0.15</td>
<td>4.0 ± 0.12</td>
<td>4.8</td>
<td>0.93*</td>
</tr>
</tbody>
</table>

Values are means ± S.E.M. unless otherwise indicated.

All measures collected after an 8-h fast.

Overall Mean CV was calculated by taking each individual subject’s CV = [(SD/mean) x 100] across the three testing sessions and then determining the mean across these values to determine overall CV

*p<0.05
Table 5. Baseline and hyperemic vascular parameters for measurements across three testing sessions and the combined average CV and ICC

<table>
<thead>
<tr>
<th>Variable</th>
<th>Visit 1</th>
<th>Visit 2</th>
<th>Visit 3</th>
<th>Overall Mean CV (%)</th>
<th>ICC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Baseline measures</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Velocity (cm/s)</td>
<td>14.8 ±0.97</td>
<td>15.8 ± 1.28</td>
<td>14.2 ± 0.74</td>
<td>10.2</td>
<td>0.84*</td>
</tr>
<tr>
<td>Max velocity (cm/s)</td>
<td>81.9 ± 3.50</td>
<td>86.7 ± 4.22</td>
<td>82.0 ± 2.83</td>
<td>6.9</td>
<td>0.90*</td>
</tr>
<tr>
<td>Shear stress (dynes/cm²)</td>
<td>1.1 ± 0.09</td>
<td>1.2 ± 0.12</td>
<td>1.1 ± 0.07</td>
<td>10.4</td>
<td>0.90*</td>
</tr>
<tr>
<td><strong>Hyperemic Response</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Velocity (cm/s)</td>
<td>69.7 ±4.78</td>
<td>71.1 ± 4.54</td>
<td>59.2 ± 4.42</td>
<td>15.3</td>
<td>0.76*</td>
</tr>
<tr>
<td>Max velocity (cm/s)</td>
<td>115.1 ± 6.37</td>
<td>133.1 ± 6.54</td>
<td>134.9 ± 5.85</td>
<td>10.6</td>
<td>0.80*</td>
</tr>
<tr>
<td>Shear stress (dynes/cm²)</td>
<td>4.2 ± 0.36</td>
<td>4.9 ±0.40</td>
<td>5.1 ± 0.37</td>
<td>15.4</td>
<td>0.84*</td>
</tr>
</tbody>
</table>

Values are means ± S.E.M. unless otherwise indicated.

All measures collected after an 8-h fast.

Overall Mean CV was calculated by taking each individual subject’s CV = \([(SD/mean) \times 100]\) across the three testing sessions and then determining the mean across these values to determine overall CV.

*p<0.05
Figure 10. Individual subject variation in FMD (%) across trials

Each black line represents a female subject; blue lines represent male subjects. Y-axes represent FMD% for tests 1, 2, and 3 respectively, with the individual lines displaying day-to-day variation in FMD.
**Figure 11.** Individual subject variation in baseline artery diameter across trials.

Each black line represents a female subject; blue lines represent male subjects. Y-axes represent artery diameter for tests 1, 2, and 3 respectively, with the individual lines displaying day-to-day variation in brachial artery baseline diameter.
Figure 12. Individual subject variation in artery hyperemic maximal diameter across trials

Each black line represents a female subject; blue lines represent male subjects. Y-axes represent tests 1, 2, and 3 respectively, with the individual lines displaying day-to-day variation in hyperemic maximal brachial artery diameter.

24 hour food, medication, and exercise recall

All subjects had been fasted for at least eight hour prior to each testing visit. An email reminder was sent to remind participants (Appendix C) to avoid rigorous exercise, citrus foods (vitamin C), chocolate, caffeine, alcohol, and high fat meals 24 hours prior to each visit. There were five reports of the consumption of citrus foods, two reports of chocolate consumption, and 16 reports of coffee consumption in the 24 hour period prior
to measurements. Subjects participated in light to moderate intensity physical activities such as walking, resistance training, elliptical machine, yoga, cycling, and running. One subject reported playing in a vigorous intensity basketball game the night prior to their visit. Additionally, 7 of the 12 women were taking oral contraceptives (OC) (Table 6).
Table 6. Individual subject CV for FMD% over the 3 trials, and values at each trial for FMD %; confounding variables as reported on 24 hour recall logs and use of oral contraception listed for each subject.

<table>
<thead>
<tr>
<th>Gender</th>
<th>Oral Contraception Use</th>
<th>CV</th>
<th>Test 1 FMD % Factor</th>
<th>Test 2 FMD % Factor</th>
<th>Test 3 FMD % Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>F</td>
<td>Yes</td>
<td>0.05</td>
<td>n/a</td>
<td>7.8</td>
<td>7.2</td>
</tr>
<tr>
<td>F</td>
<td>Yes</td>
<td>0.08</td>
<td>7.3 Coffee</td>
<td>8.7 Coffee</td>
<td>8.2 Coffee</td>
</tr>
<tr>
<td>F</td>
<td>No</td>
<td>0.09</td>
<td>10.8 Coffee</td>
<td>9.5 Coffee</td>
<td>9.2 Coffee</td>
</tr>
<tr>
<td>F</td>
<td>Yes</td>
<td>0.10</td>
<td>9.2 Orange</td>
<td>11.2 2 Oranges</td>
<td>11.0 Coffee Orange</td>
</tr>
<tr>
<td>F</td>
<td>No</td>
<td>0.15</td>
<td>5.0 1tbs dark chocolate</td>
<td>5.0</td>
<td>6.5 5 pieces dark chocolate</td>
</tr>
<tr>
<td>F</td>
<td>Yes</td>
<td>0.19</td>
<td>4.7 Coffee</td>
<td>5.1 Coffee</td>
<td>6.6</td>
</tr>
<tr>
<td>F</td>
<td>Yes</td>
<td>0.20</td>
<td>8.5 Coffee</td>
<td>9.1 Coffee</td>
<td>6.1 Coffee</td>
</tr>
<tr>
<td>F</td>
<td>No</td>
<td>0.25</td>
<td>7.7 Coffee</td>
<td>6.1 Coffee</td>
<td>10.0 Coffee High fat meal</td>
</tr>
<tr>
<td>M</td>
<td>-</td>
<td>0.25</td>
<td>3.7 Coffee Beets</td>
<td>3.8 Coffee Beets</td>
<td>2.4 Coffee</td>
</tr>
<tr>
<td>F</td>
<td>Yes</td>
<td>0.25</td>
<td>7.3 Vit. D Calcium</td>
<td>4.7</td>
<td>5.1 Orange</td>
</tr>
<tr>
<td>M</td>
<td>-</td>
<td>0.38</td>
<td>6.2 Orange juice</td>
<td>2.7</td>
<td>5.4</td>
</tr>
<tr>
<td>F</td>
<td>Yes</td>
<td>0.40</td>
<td>3.0 Intense exercise</td>
<td>7.1</td>
<td>5.4</td>
</tr>
<tr>
<td>F</td>
<td>No</td>
<td>0.40</td>
<td>15.2</td>
<td>7.6</td>
<td>8.3</td>
</tr>
<tr>
<td>F</td>
<td>Yes</td>
<td>0.66</td>
<td>3.7</td>
<td>4.0</td>
<td>11.0 Coffee</td>
</tr>
</tbody>
</table>
CV = [(SD/mean) x 100] across the three testing sessions. FMD % = [(peak diameter – baseline diameter) / baseline diameter] x 100.
DISCUSSION

The main goal of this study was to investigate the day-to-day reliability of the measurement of FMD in the brachial artery of healthy young adults. In this group of 14, measurements of FMD presented acceptable reproducibility when controlling for extraneous factors and assessed by one ultrasound technician. FMD measurements on three separate days were reproducible and comparable to other reports in the literature (CV=24.7%, ICC=0.73; p=0.004) when following a rigid protocol. Additionally, measurements of baseline and hyperemic brachial artery diameter, average velocity, max velocity, and shear stress were reproducible displaying very strong reliability statistics (CVs=4.5-24.7%; ICCs = 0.73-0.95, p<0.05).

Reproducibility Statistics

There is no definite statistical threshold for the determination of FMD reproducibility. Since FMD is very sensitive to small changes in artery diameter the CV deemed acceptable for FMD reproducibility is greater than that of biological analyses. Based on ultrasound assessment guidelines, a change in the value of FMD of 2% to 3% over time is deemed acceptable. Harris et al., suggests that, based on an average brachial artery FMD of 10%, CV’s less than 35% meet reproducibility criterion. Additionally, ICC values of <0.40, 0.4 to 0.75, and >0.75 represent poor, fair to good and excellent agreements, respectively. In the present study the CV for FMD was 24.7% and ICC was 0.73 which meet the criteria for reproducibility.
Reproducibility of flow mediated dilation

Studies conducted to evaluate the reproducibility of FMD in the brachial artery have reported similar results as the present study.\textsuperscript{29,33,75} For example, Harris and colleagues examined the reproducibility of FMD response to acute exercise in nine, middle-aged, overweight men on two separate days after moderate intensity acute exercise. No significant differences in FMD were found between trials; CV was 25.2\%.\textsuperscript{29} West and colleagues found comparable results when measuring FMD on 18 adults with type 2 diabetes on three separate occasions, separated by a week, reporting a CV of 29.7\%. The study by West et al., was similar in nature to the present study as participants were asked to avoid alcohol, maintain a similar diet and physical activity patterns throughout the study, and discontinue the use of medications or supplements.\textsuperscript{75} Herrington et al., 2001 measured within subject reproducibility of brachial FMD response in 30 subjects on two separate occasions one week apart and reported a CV=26.3\%.\textsuperscript{33} All of the above studies were similar in that they closely followed established guidelines for ultrasound assessed FMD.\textsuperscript{16} Furthermore, all the above mentioned studies, including the present study, occluded the proximal forearm for 4-5 minutes below the antecubital fossa, tests were administered by a single technician, and CV was reported as CV = (S.D./mean) X 100.

Studies have reported weaker reproducibility of ultrasound assessment of FMD than the present study.\textsuperscript{28,48,61} Similarly, Malik et al., examined FMD in 20 healthy adults twice within two days resulting in a CV of 41\%.\textsuperscript{48} However, peak hyperemic diameter was determined within the interval of 60-80 seconds of post cuff release and peak diameter has been reported to occur anywhere between 45-90 seconds\textsuperscript{5,47,71} following
induced hyperemia; therefore it is possible that the true maximum diameter was not measured. Moreover, precautions to control for diurnal variability were not clearly described. De Roos and colleagues also reported a large within-subject variability of FMD with a CV of 50.3%. However, one limitation of De Roos was baseline and post-occlusion brachial artery diameters were manually derived with images only frozen every 15 seconds instead of using a video and wall tracking software that is capable of analyzing and averaging the diameter of hundreds of frames over a 60 second period which is likely to provide a more representative analysis of vasodilation over time.

In contrast, there have been reports of higher FMD reproducibility in the literature, as determined by ICC, than that found in the present study. Welsch et al., assessed FMD in 15 healthy adults on three occasions and reported a very strong ICC of 0.92. Welch et al., controlled for diurnal variations and additionally took blood samples to examine changes in lipids, protein, fibrinogen, glucose, electrolytes and viscosity factors between testing days that may affect FMD results. However CV was not reported making it difficult to compare results to other studies that did not report ICC. Interestingly, despite not controlling for many factors know to influence FMD, Sorenson et al., reported the best reproducibility (CV=1.8%). In that study, 40 healthy adult subjects who were scanned on 4 occasions were not instructed to fast nor were they scanned at the same time of day. Unfortunately, the exact method used to calculate variability was not thoroughly described in the study. Many studies have reported CV as CV’ for FMD (%) which is calculated as CV’ = (100 X SD) / (mean + 100). Calculating CV with this method drastically decreases CV values; for instance a CV of 29.7 = CV’ of 1.2. This may be the cause of literature reporting such notably low CV values.
Reproducibility of diameter measures

As found in the present study, baseline diameter has been shown to have relatively small variability (CV ranging from 1-13%). The day-to-day variation for baseline diameter in this study was CV=4.5%. The results from West et al., are very comparable to those of De Roos et al., (2002) who assessed within-subject variability of baseline diameter finding a CV of 4.8%. Harris et al., also reported comparable results, finding an ICC of 0.96 for baseline diameter (ICC=0.95 in the current analysis). A very strong level of reproducibility for peak hyperemic diameter was found in the current study (ICC=0.93; CV=4.8%); also similar to those of Harris et al., (ICC=0.93) and De Roos et al., (CV=4.8%).

Reproducibility of velocity measures

Reproducibility of velocity measurements among trials was strong for both baseline and post occlusion measurements (baseline average velocity: CV=10.2%, ICC=0.84; baseline max velocity: CV=6.9%, ICC=0.9; hyperemic average velocity: CV=15.3%, ICC=0.76; hyperemic max velocity: CV=10.6, ICC=0.8). These results are similar to those of West et al., who reported a CV of 10.8% for mean baseline velocity and a CV of 16.6% for peak hyperemic blood flow velocity.

Reproducibility of shear stress

The reproducibility of shear stress (dynes/cm²) was found to be strong at baseline and during reactive hyperemia (CV=10.4%, ICC=0.9; CV=15.4%, ICC=0.84, respectively). This was expected based on the high reproducibility found for diameter and velocity measurements as Shear stress = (viscosity x velocity)/diameter. The results
of this study were slightly lower but comparable to those found by Dammers et al. reporting a CV of 24.7% for baseline shear stress and 16.2% for peak hyperemic shear stress.¹⁹

Sources of variability

The reported CVs and ICCs for FMD in this study were satisfactory, however reproducibility was found to be on the upper range of what is deemed acceptable. Day-to-day variation in FMD in different populations have been reported with CVs ranging from 2-84%.³⁸,⁶¹,⁶⁸,⁷¹ Within any artery diameter measurement there is a wide array of physiological, biological, and technical (equipment or operator related) factors that may introduce confounding variables leading to variability over repeated testing sessions. Given that other variables such as diameter, velocity, and shear stress were found to have very strong levels of reproducibility, it is likely that individual biologic variability was a substantial source of variability in this study. However, physiological and technical factors must be noted as well.

This study was designed to control and document extraneous factors that may affect FMD. All testing sessions were performed in a quiet, temperature controlled room and all three tests were performed at the same time of day after ten minutes of rest and all sessions were within a three week period. A single sonographer performed all FMD assessments. Participants were instructed to arrive to each testing session after fasting for a minimum of eight hours and were asked to avoid taking supplements, limit participation in vigorous physical activity, and were asked to refrain from consuming specific foods and drinks that may affect vascular function 24 hours prior to each scheduled exam. Additionally, subjects completed an exercise, food, and medication log pertaining to the
day before each testing visit so the study investigator could evaluate compliance. Despite these attempts to reduce extraneous factors, out of the 42 total visits there were five reports of the! consumption of citrus foods, two reports of chocolate consumption, 16 reports of coffee consumption, and one report of engaging in vigorous activity 24 hours prior to testing sessions (Table 6).

It appears that reproducibility was greater when individuals kept diet consistent prior to each testing visit. For instance, the subject with the lowest CV (0.05) did not consume anything that has been shown to alter FMD; however, due to a poor recording, they only had 2 tests, instead of 3. Two other subjects with CVs < 0.10 were consistent with their diet; although they did not avoid coffee as requested, they were consistent in their consumption of the beverage across the 3 trials. When subjects consumed coffee prior to one or two visits but not all three, the CV was greater (CV = 0.19-0.66). One subject consumed 5 pieces of Hershey dark chocolate the evening prior to their testing visit and FMD was higher at that testing session. Another subject reported playing an intense game of basketball the night prior to their second testing visit; interestingly, their FMD% was highest after the intense exercise. This finding supports results found in the literature that acute exercise can augment FMD. However, one subject had no reports of consuming any foods, drinks, or medications known to influence FMD and had one of the poorest CV (CV = 0.4). Another unusual finding was an increase in FMD after a subject reported consuming a high fat breakfast (McDonalds); this is in contrast to the literature that acute consumption of a high fat meal decrease postprandial FMD. However, this subject consumed also consumed coffee and the high fat meal was consumed nearly 24 hours prior to the test, therefore the meal may not have affected the
FMD results. Thus, several factors reported in the participants food, supplement, and exercise log that may have been the cause of variability between test results. In general, individuals who maintained similar diets prior to each testing visit displayed the highest reproducibility (with one exception). Future studies of reproducibility should emphasize the importance of participants to maintain a consistent diet, supplement, and physical activity pattern 24 hours prior to testing visits.

One biological factor of note in this study is the gender of subjects. Studies have shown sex related differences in FMD response.\textsuperscript{31,76} For example, Hashimoto et al., evaluated FMD modulation based on sex and menstrual cycle in which FMD was assessed in 17 females a total of three times; once in each of the three phases of the menstrual cycle. Female FMD results were compared to those of 17 males. FMD varied according to menstrual cycle due to fluctuations in estradiol concentration, resulting in a greater FMD variability over time than that found in men. Given that the 12 women in the present study were examined at random stages of their menstrual cycle, this may have contributed to the variability observed. Additionally, seven of the 12 women in the study were taking oral contraceptives (OC). The type of OC taken may also influence FMD results. Since monophasic birth control pills deliver constant estrogen and progestin doses through the 21 day cycle, there should have little influence on FMD results if measurements are taken during the 21 day active phase. If data are collected during both the 21 days active and 7 days inactive pills, then this potentially may impact the measures. However, biphasic and triphasic OC contain different levels of progestin and estrogen causing fluctuation in hormones throughout the cycle (similar to the natural
menstrual cycle) and may have introduced variability in FMD response between trials. It is not known which type of oral contraceptives were being taken by women in this study.

Although the concept of ultrasonographic assessment to assess artery reactivity is relatively simple, it is technically difficult to master. It is crucial that the technician acquires hands on experience and is involved in multiple practice sessions to establish valid, consistent and high quality data. In the present study variability was reduced by having a single ultrasonographer who had months of practice conduct all tests. A strict study protocol was designed prior to the study and was followed closely for all testing sessions. Reproducibility between testing sessions was ensured by making note of anatomical landmarks, taking photos of the patients arm and probe set up, as well as making precise measurements for cuff and transducer placement. Acceptable image and video quality (with clear intima interfaces that could be detected by the wall tracking software) were obtained for all but one test (41 of the 42). In addition, human measurement error was reduced by the use of Brachial Analyzer for Research software automated edge-detection software (Medical Imaging Applications, Iowa City, Iowa, USA). Utilization of this software meant values for average, maximum, and minimum diameters were based on dozens of measurements across a segment of the brachial artery, reducing the influence of a single atypical or outlying diameter measurement to estimate FMD.

Limitations

The present study is not without limitations. First, a single technician conducted both the FMD assessments and the analyses and was therefore not blinded to the subject number or subject visit. This could have been resolved by having a blinded independent
investigator randomize the subject’s files and present the files in random order to the technician conducting the analyses. Also, current guidelines recommend ECG gating to isolate end diastolic diameters for FMD assessment.40 ECG gating was not used in the present study. However, it has been reported that artery diameters are not significantly different from ECG gated measures when averaged over an entire cardiac cycle as was the case in this study.70 The study design could have taken greater precautions to rule out the gender effect by controlling for hormone fluctuations as a result of the menstrual cycle. Lastly, subject adherence to diet restrictions and avoidance of intense exercise was not 100%.

Future Research

Ultrasonographic assessment of FMD is a valuable noninvasive measurement of endothelial function. Progressively more evidence is establishing FMD as a useful prognostic tool for cardiovascular disease risk. However there is still a great deal of variance between sites including factors such as subject preparation, cuff position, probe angle, analysis methods and software, ECG gating, as well as statistical reporting, therefore limiting its validity and comparability between studies. In order for brachial artery FMD to become a valid and reliable assessment tool a standardized protocol needs to be established across centers. Thijssen and colleagues have recently published a methodological and physiological guideline to try to standardize the assessment of FMD in humans7. If more investigations implement this rigorous protocol and control extraneous variables that have been reported to influence FMD, the reproducibility of brachial artery FMD can be greatly improved.
Conclusion

In conclusion, these results suggest that FMD can be measured with adequate reproducibility in healthy adults when following a strict protocol and when extraneous factors are controlled. These findings may have practical implications for future studies in the Health and Exercise Department at Wake Forest University; although the use of this technique for the clinical assessment of endothelial function in a clinical population is not clear. The data from this study should be further supported by larger scale studies.
REFERENCES


5. Berry KL, Skyrme-jones RAP, Meredith IT. Occlusion cuff position is an important determinant of the time course and magnitude of human brachial artery flow-mediated dilation. 2000;267:261-267.


8. Blair A, Shaul PW, Yuhanna IS, Conrad P a., Smart EJ. Oxidized low density...


doi:10.1161/01.CIR.0000131515.03336.f8.


doi:10.1161/CIRCULATIONAHA.106.652859.


48. Malik J, Wichterle D, Haas T, Melenovsky V, Simek J, Stulc T. Repeatability of


70. Thijssen DHJ, Black MA, Pyke KE, et al. Assessment of flow-mediated dilation in


74. Welsch MA, Allen JD, Geaghan JP. flow-mediated dilation. 960-965.


doi:10.1161/CIRCULATIONAHA.109.864801.
APPENDIX A

TEST RE-TEST RELIABILITY OF BRACHIAL ARTERY FLOW-MEDIATED DILATION IN HEALTHY ADULTS

Informed Consent Form to Participate in Research

INTRODUCTION
You are invited to be in a research study. Research studies are designed to gain scientific knowledge that may help other people in the future. You are being asked to take part in this study because you are a healthy adult. Your participation is voluntary. Please take your time in making your decision as to whether or not you wish to participate. Ask your study staff to explain any words or information contained in this informed consent document that you do not understand. You may also discuss the study with your friends and family.

WHY IS THIS STUDY BEING DONE?
The purpose of this research study is to take repeated measurements of your brachial artery on separate days and evaluate how close the results are to one another. These results will be helpful for future studies using ultrasound to evaluate the health of blood vessels.

HOW MANY PEOPLE WILL TAKE PART IN THE STUDY?
16 people at one research site will take part in this study.

WHAT IS INVOLVED IN THE STUDY?
You will be asked to come to the Wake Forest Health and Exercise Science Department on three separate days. Each visit will be separated by at least two days. You will come in at the same time of day for each visit and you will not be allowed to have consumed any food or drinks for 8 hours before each visit. You will also be asked to avoid alcohol for 48 hours before each visit, discontinue medications the day before your schedule visits and maintain similar exercise and diet habits throughout the study.

Assessment Visits
You will be required to attend a total of three testing visits. Testing days will be separated by at least two days and will take place at Wake Forest University Reynolda campus in the Health and Exercise Science Human Performance Laboratory in Reynolds Gymnasium. The procedure for all three visits is shown in the schedule of events below.

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- A picture of only your will be taken to replicate arm position for future testing sessions
- Gel will be placed above the elbow of your arm and a hand held device will be guided over the area and images of your artery will be captured
- A rapid inflator blood pressure cuff will be inflated tightly around your lower arm for five minutes
- The cuff will be released and more images will be captured

7. You will be scheduled for visit number 2 at the same time of day

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<th>Visit 2</th>
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<td>1.</td>
<td>You will lie on your back for 10 minutes</td>
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<td>2.</td>
<td>Your resting blood pressure will be measured</td>
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<td>3.</td>
<td>Ultrasound of arm</td>
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<td></td>
<td>• Your arm will be placed in a brace to limit movement</td>
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<td></td>
<td>• A picture of only your arm will be taken to replicate arm position for future testing sessions</td>
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<td></td>
<td>• Gel will be placed above the elbow of your arm and a hand held device will be guided over the area and images of your artery will be captured</td>
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<td>• A rapid inflator blood pressure cuff will be inflated tightly around your lower arm for five minutes</td>
</tr>
<tr>
<td></td>
<td>• The cuff will be released and more images will be captured</td>
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<tr>
<td>4.</td>
<td>You will be scheduled for your next visit at the same time of day</td>
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<td>1.</td>
<td>You will lie on your back for 10 minutes</td>
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<td>2.</td>
<td>Your resting blood pressure will be measured</td>
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<td>3.</td>
<td>Ultrasound of arm</td>
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<td></td>
<td>• A rapid inflator blood pressure cuff will be inflated tightly around your lower arm for five minutes</td>
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<tr>
<td></td>
<td>• The cuff will be released and more images will be captured</td>
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*Study Interventions*

If you are eligible and sign the consent form you will be scheduled for your first testing visit. Prior to each testing day you will be asked to refrain from any food, drink, or exercise at least 8 hours before the visit.

The study will take place on WFU Reynolda Campus in the Human Performance Laboratory in Reynolds Gymnasium. After the study is fully described to you and you have signed an informed consent you will fill out a 24 hour dietary recall and exercise log. Next, your weight, height, and age will be recorded and you will be asked questions about your age, race/ethnicity, and. You will then on your back for 10 minutes in a quiet room where your blood pressure will be measured. Then, you will be asked to stay as still as possible and your arm will be placed in a strap to decrease movement. The technician will feel your arm right above your elbow to find your brachial artery and mark your arm for the location of the artery. A picture of only your arm
(from 6-8 inches above your elbow to your hand hand) will be taken to replicate arm position for future testing sessions.

An ultrasound machine will be used to view your brachial artery. This is a painless scan in which a special gel will be applied directly to your skin and a small hand held device (transducer) will slowly be guided over your skin. This will produce pictures and video clips of your artery that will appear on the screen of the ultrasound machine and will be used for further study at a later time.

Once the images are obtained, a rapid inflation blood pressure cuff will be placed around your forearm. This cuff works just like a regular blood pressure cuff but will inflate and become tight around your forearm very quickly. The cuff will remain on your arm for five minutes and will cut off the blood flow to your lower arm during this time. After five minutes, the technician will push a button that will deflate the cuff very quickly, allowing blood to flow back to your lower arm. Following deflation, the ultrasound machine will be used again to take pictures and video clips of your artery. You will then schedule your next visit for at least two days later and at the same time of day as your first visit. Each testing visit should take 30-40 minutes.

HOW LONG WILL I BE IN THE STUDY?
You will be in the study until you have completed a total of 3 visits. This is a pilot study to determine the variability of ultrasound measurements in the Health and Exercise Science Department and therefore you will not be required to come back for long-term follow up visits.

You can stop participating at any time. If you decide to stop participating in the study we encourage you to talk to the investigators or study staff first.

WHAT ARE THE RISKS OF THE STUDY?
Being in this study involves minimal risk. You should discuss the risk of being in this study with the study staff.

**Ultrasound**
Ultrasound is a safe procedure that uses low-power sound waves, with no known risks.

**Rapid Cuff Inflator**
The rapid cuff inflator will cut off the blood flow to your lower arm for five minutes. This will likely cause feelings of mild discomfort, tingling, and may cause your hand to become pale, cold/warm, or clammy.

**Other factors**
There also may be other side effects that we cannot predict. You should tell the research staff about all the medications, vitamins and supplements you take and any medical conditions you have. You are expected to report any changes in your medications, vitamins, or supplements to the study team.

In addition, there is a slight risk of a breach of confidentiality. We will do our best to protect your confidential information. Taking part in this research study may involve providing information that you consider confidential or private. Efforts, such as coding research records,
keeping research records secure and allowing only authorized people to have access to research records, will be made to keep your information safe.

ARE THERE BENEFITS TO TAKING PART IN THE STUDY?
You are not expected to receive any benefit from participating in this research study. We hope the information learned from this study will benefit other people in the future as it relates to heart disease and diet.

WHAT OTHER CHOICES ARE THERE?
This is not a treatment study. Your alternative is not to participate.

What About My Health Information?
In this research study, any new information we collect from you is considered Protected Health Information. The information we will collect for this research study includes: brachial artery diameter, blood pressure, 24 hour food and exercise recall, body weight, height, age, race, and gender.

We will make every effort to keep your Protected Health Information private. We will store records of your Protected Health Information in a cabinet in a locked office or on a password protected computer.

Only the following people or organizations will be granted access to your Protected Health Information:
1) The study investigator and his/her staff, or others at Wake Forest University who oversee research.
2) Other people or laboratories providing services for this research project on behalf of Wake Forest University.

If required by law or court order, we might also have to share your Protected Health Information with a judge, law enforcement officer, government agencies, or others. If your Protected Health Information is shared with any of these groups it may no longer be protected by federal or state law.

Any Protected Health Information collected from you in this study that is maintained in the research records will be kept for an indeterminate period of time. This authorization does not expire and any research information entered into your medical record will be kept for as long as your medical record is kept by the Medical Center. You will not be able to obtain a copy of your Protected Health Information in the research records until all activities in the study are completely finished.

You can tell Gary Miller, PhD that you want to take away your permission to use and share your Protected Health Information at any time by sending a letter to this address:
Gary Miller, PhD Associate Professor, Wake Forest University Department of Health and Exercise Science Box 7868, Winston-Salem, NC 27109.

However, if you take away permission to use your Protected Health Information you will not be able to be in the study any longer. We will stop collecting any more information about you, but
any information we have already collected can still be used for the purposes of the research study.

By signing this form you give us permission to use your Protected Health Information for this study.

WHAT ARE THE COSTS?
There are no costs to you for taking part in this study. All study costs, including any study medications and procedures related directly to the study, will be paid for by the study.

WILL YOU BE PAID FOR PARTICIPATING?
You will receive no payment or other compensation for taking part in this study.

WHO IS SPONSORING THIS STUDY?
This study is partially sponsored by the Wake Forest University Translational Science Center and the Department of Health and Exercise Science.

WHAT ARE MY RIGHTS AS A RESEARCH STUDY PARTICIPANT?
Taking part in this study is voluntary. You may choose not to take part or you may leave the study at any time. Refusing to participate or leaving the study will not result in any penalty or loss of benefits to which you are entitled. If you decide to stop participating in the study we encourage you to talk to the investigators or study staff first. The investigators also have the right to stop your participation in the study at any time. This could be because it is in your best medical interest regarding any adverse events or because the entire study has been stopped. You will be given any new information we become aware of that would affect your willingness to continue to participate in the study.

WHOM DO I CALL IF I HAVE QUESTIONS OR PROBLEMS?
For questions about the study or in the event of a research-related injury, contact the study investigator, Gary Miller, PhD at (336) 758-1901.

The Institutional Review Board (IRB) is a group of people who review the research to protect your rights. If you have a question about your rights as a research participant, or you would like to discuss problems or concerns, have questions or want to offer input, or you want to obtain additional information, you should contact the Chairman of the IRB at (336) 716-4542.

You will be given a copy of this signed consent form.

Signatures
I agree to take part in this study. I authorize the use and disclosure of my health information as described in this consent and authorization form. If I have not already received a copy of the Privacy Notice, I may request one or one will be made available to me. I have had a chance to ask questions about being in this study and have those questions answered. By signing this consent and authorization form, I am not releasing or agreeing to release the investigator, the sponsor, the institution or its agents from liability for negligence.

Subject Name (Printed): __________________________________________
APPENDIX B
Screening Questionnaire

Name ________________________________  Gender: Male / Female

Phone ___________________  Email ____________________________

Date of Birth ______________  Age ______  Height __________  Weight __________

What is your ethnicity?
☐ Asian  ☐ Hispanic  ☐ Black  ☐ White  ☐ Hawaiian Native/Pac Island  ☐ Other

Have you had any of the following? (please circle)
Diabetes  Heart problems  High/Low Blood Pressure  Stroke  Cancer  HIV

Peripheral artery disease  Dyslipidemia  Other __________________________

Do you currently or have you smoked in the past?  Yes / No

Medications
Do you take any pills or medication?  Yes / No
If yes, please describe ______________________________________________________

Women: Date of last menstrual cycle: ________________

Exercise
In a normal week how many hours of moderate/vigorous physical activity do you accumulate? (please circle)

<1 hour  1-2 hours  3-4 hours  5-6 hours  7-8 hours  9-10 hours  >10 hours
APPENDIX C
Email Reminder

Dear,

Thank you for volunteering to be a part of my research study. There are a few things I would like to remind you of prior to your first testing session. On your first visit you will be asked to fill out a short questionnaire to determine if you are eligible to participate in the study. Once you are deemed eligible, you will be given time to read through an informed consent form and will be able to ask any questions you may have.

Next, you will be asked to fill out a 24 hour dietary and exercise recall form. *It is very important that you fill this out to the best of your ability. This is a reproducibility study therefore I ask that you try to maintain a similar diet and exercise pattern 24 hours prior to each study visit. Please avoid rigorous exercise, citrus foods (vitamin C), chocolate, caffeine, alcohol, and high fat meals. I will give you a copy of your dietary recall so you can try to consume similar foods the day before your next visit. Additionally, please refrain from taking any vitamins or supplements 24 hours prior to your testing session. If it is necessary to take any medications please do so at the same time prior to each testing session and note the name of the medication on your 24 hour dietary recall log.

Lastly, you will need to arrive to each session in a fasted state. Please avoid any food or liquids for at least 8 hours before your testing session. If possible try to maintain a similar pattern of activity (wake up at the same time) the morning of each test.

Thanks again and please let me know if you have any questions or concerns.
# APPENDIX D

**FOOD/PHYSICAL ACTIVITY LOG**

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## APPENDIX E

Data Recording Form

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APPENDIX F

Example brachial artery images

a. Example of high quality image with clear intima interface
b. Example of baseline velocity flow with artery at a 60° angle and calipers positioned inside of artery

c. Example of hyperaemic velocity flow with artery at a 60° angle and calipers positioned inside of artery
d. Example of Brachial Analyze for Research wall tracking software. 60 second video displaying baseline diameter analysis results

e. Example of Brachial Analyze for Research wall tracking software. 20-80 seconds post cuff release. Hyperemic diameter analysis results
f. Example of vascular landmarks used to identify location of vessel measurements for the same subject on their 3 separate visits. The red rectangular box showing an identifiable dark area above the brachial artery in all images. The oval showing an identifiable bring structure below the brachial artery in all visits.

Visit 1:
APPENDIX G

Step by Step testing procedure

1. Two days prior to each subject’s visit sent e-mail reminder (see Appendix A).

Visit 1 Only:
2. Subject arrived to Pulmonary Function lab and filled out the screening questionnaire (see Appendix B).
   a. The questionnaire was reviewed and the participant was deemed eligible or ineligible.
3. If eligible, the subject was asked to read through and sign an informed consent form. The technician verbally explained the procedure, purpose, and risks involved with the study to each participant. The expected feeling of the rapid cuff inflator cuff was explained in detail to reduce the surprise of the sudden inflation or deflation of the cuff.
4. The subject’s height and weight were measured and recorded.

Visits 1, 2, and 3
Subject Preparation
5. The subject completed a 24 hour dietary, exercise, and medication log (see Appendix C).
6. The subject was asked to quietly rest in a temperature controlled, quiet room, on a lab bench in the supine position for 5 minutes.
   a. After 5 minutes the technician took resting blood pressure using a mercury sphygmomanometer.
   b. The subject continued to quietly rest in for an additional 5 minutes.
7. The M-Turbo Ultrasound System (Sonosite Inc., Bothell, Washington, USA) was turned on and exam type was set to “Vascular”
   a. Pressing the “patient” key, the subject’s ID, blood pressure, and visit code were recorded. Then “done” was pressed.
8. Using a ruler, a small pen mark was drawn on the subject’s arm 3 inches below the antecubital crease.
   a. A 6 x 83cm rapid inflator segmental pressure cuff (D.E. Hokanson, Bellevue, WA, USA) was loosely wrapped around the subject’s left forearm (the top of the cuff where the mark was made).
9. The subject’s left arm was comfortably immobilized in a U-shaped brace in the horizontal position with the palm facing up, at an angle ~60 degrees from the torso.
10. Ultrasound transmission gel was applied directly to the arm.
11. The ultrasound was set to “Color” mode and the technician held the probe by hand to locate the brachial artery 3-6 inches above the antecubital crease in the longitudinal plane.
   a. Once the artery was located the probe was held in place and a marker was used to mark the location of the probe on the subject’s arm.
12. The probe was placed into an adjustable sterotactic clamp and positioned to the marked area on the subject’s arm. (The subject was asked to remain as still as possible)
   a. The probe was adjusted as needed until an optimal image was observed with clear arterial wall interfaces and the artery positioned at a +/- 60°
   b. The image was zoomed in one time
13. The technician took a photograph of the subject’s arm and probe set up for future replication.
   a. Additionally, the angle and position of the arm and probe, and vascular anatomical landmarks were noted.

*Baseline Velocity Flow Measurement*
14. The ultrasound machine was put in “2D” mode for grayscale imaging.
15. The “Doppler” key was pressed to measure velocity flow
   a. Using the touch pad and arrow keys, the sample line was placed in the center of the artery at a +/- 60 degree angle
   b. The calipers were adjusted to fit as closely to the walls of the artery while still remaining with in the artery
   c. The “Doppler” key was pressed a second time to activate Doppler Spectral Trace
   d. At least three baseline velocity images were saved by pressing “freeze” followed by “save”

*Baseline Diameter Measurements*
16. The ultrasound machine was placed in “2D” mode for grayscale imaging.
17. The “video” button was pressed and set to 60 seconds prospective video
18. The “clip” key was pressed – initiating the video
19. Once a “beep” was heard the “save” button was pressed

*Rapid Cuff Inflator*
20. The rapid cuff inflator machine (E-20 rapid cuff inflator, D.E. Hokanson, Bellevue, WA, USA) was turned on. (Turn on AG101 first followed by E20).
   a. The pressure was set to 50mmHg above the subject’s resting blood pressure by turning the crank on the front of the machine and the lock pressure knob was secured by turning the thumb nut counterclockwise (located behind the crank)
21. The subject was asked to remain as still as possible and the forearm occlusion cuff was rapidly inflated by pressing the “mode” button
   a. A stopwatch was started
   b. During the occlusion period the ultrasound machine was set to “Doppler” mode and using the touch pad and arrow keys, the sample line was placed in the center of the artery at a +/- 60 degree angle
   c. The calipers were adjusted to fit as closely to the walls of the artery while still remaining with in the artery
22. After 5 minutes of occlusion, the cuff was deflated by pressing the “mode” button
**Post-Occlusion Velocity Flow Measurements**

a. Immediately after deflation, the “Doppler” key was pressed again and 2-3 post-occlusion images were saved by pressing “freeze” followed by “save”

b. All velocity flow measurements were frozen and saved within 5-25 seconds after cuff deflation

**Post-Occlusion Diameter Measurements**

23. In no more than 25 seconds post-deflation, the ultrasound was switched to “2D” mode and the “clip” button was pressed, initiating a prospective 60 second video clip.

a. Once a beep occurred the video was “saved” and the “clip” was immediately pressed again to begin another 60 second video clip

b. Once a subsequent beep was heard “save” was pressed

24. The “patient” button was pressed followed by “end”

a. “Review” was pressed and the box next to the subject’s name was checked and their report was exported to a USB for future analysis.

**Test Completion**

25. The visit was then completed

a. The rapid inflator cuff was removed from the subject’s arm and the subject was allowed to sit up.

b. The subject was asked to try not to wash off the mark on the arm where the probe was located until their next visit
CIRRICULUM VITAE

Tara B. Richardson
richtb@wfu.edu

EDUCATION

• Wake Forest University, Winston-Salem NC, to be completed: May 2016
  o Master of Science in Health and Exercise Science
  o Thesis: “Test Re-test Reliability of Brachial Artery Flow-Mediated Dilation in Healthy Adults”
  o Advisor: Dr. Gary Miller, Ph.D.

• Western State Colorado University, Gunnison, CO, completed May 2014
  o Bachelor of Arts in Exercise and Sports Science (clinical)
  o Bachelor of Arts in Biology (pre-allied health)
  o Overall GPA: 3.94
    ▪ Exercise and Sports Science Major GPA: 3.98
    ▪ Biology Major GPA: 3.9

PROFESSIONAL EXPERIENCE

• Health Assessments Graduate Assistant, Wake Forest University, Department of Health & Exercise Science, 5/2015-present
  o Teach students fitness assessment techniques
  o Set up, clean up, and conduction of labs

• Graduate Teaching Assistant, Wake Forest University, Department of Health & Exercise Science, 8/2014-present
  o Teach “Exercise for Health: HES 101”
  o Lecture on various components of health
  o Teach methods to assess fitness

• Graduate Student Intern, Wake Forest University, Healthy Exercise and Lifestyle Programs, 8/2015-present
  o Interact and supervise adults engaging in exercise
  o Measure resting and exercise blood pressure
  o Assist in graded exercise stress testing
  o Monitored heart rate and rhythm strips
  o Assessment measures – lipid profile, glucose, pulmonary function, anthropometric measurements
  o Lead weekly weight and flexibility group exercise sessions

• Research Associate, Department of Health and Exercise Science, Wake Forest University, 5/2015-12/2-15, Research Advisor: Gary Miller, Ph.D.
  o Parents and Children Together Preventing Diabetes (PACT PD)
  o Responsible for downloading, configuring, and transporting accelerometers
• Research Associate, Gramercy Research Group, Winston-Salem, NC, 1/2015-4/2015
  o Healthy Campus Project
  o Meeting Inter-rater reliability standards
  o Conducting dining audits on various restaurants, stores and vending machines on and around campus

• High Altitude Lab Assistant, Western State Colorado University, 8/2013-8/2014
  o Max and sub-max metabolic testing, blood pressure reading, exercise prescription, lactate testing, flexibility, body composition, health/history screening
  o Organized lab materials
  o Helped with conduction of Exercise Physiology labs

• Physical Fitness Trainer, Western State Colorado University – 8/2012-12/2012
  o Constructed a 12-week exercise program incorporating resistance and aerobic training
  o Trained an adult woman 2-3 days a week
  o Completed a beginning and end of program exercise testing assessment that displayed significant improvements in strength and fitness

• Crazy Running Coach – 8/2015 - present
  o After school running program for elementary school students
  o Coach proper running form and sportsmanship

PRESENTATIONS
• Rocky Mountain ACSM, Western State Colorado University – 2014
  o “A community-based exercise intervention transitions metabolically abnormal obese adults to a metabolically healthy obese phenotype.”
  o 4th place award for poster presentation

• Aging Conference, Wake Forest University – 2016
  o “Body composition changes in older adults following beetroot juice supplementation, protein supplementation, and a 10 week resistance training intervention.”

• Speaker and Coordinator for a Nutrition Conference, Western State Colorado University - 2013
  o Gave a power point presentation on the importance of iron for endurance athletes
  o Organized location, advertisement, schedule, guest speakers, travel arrangements, and refreshments for conference

CERTIFICATIONS / SKILLS
• American Heart Association - CPR, First Aid, AED certified
• Wake Forest University – Collaborative Institutional Training Initiative (CITI)
• American College of Sports Medicine – Graduate student member
• Microsoft Word, Excel, PowerPoint, SPSS, Medical Graphics Ultima series cardiorespiratory metabolic system, Parvo Medics Metabolic system, Cholestech LDX lipid profile system, Anthropometric measurements – skin fold, height, weight, pulmonary function, body circumferences measurements, SonoSite M-turbo ultrasound system

PUBLICATIONS
