Endothelial Function across the Menstrual Cycle as Assessed in Naturally Cycling Women by Three Relevant Techniques

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A THESIS

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

March 2014

An Abstract of the Thesis of

Erin Madison for the degree of Bachelor of Arts
in the Department of Human physiology to be taken March 2014

Title: Endothelial function across the menstrual cycle as assessed in naturally cycling women by three relevant techniques

Approved: [redacted]

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This project is the first attempt to determine whether the menstrual cycle hormones have an effect on female blood vessel function as measured by two recently proposed tests of vessel reactivity. The larger implications of this study will impact future clinical research, in terms of subject preparation. Specifically, if it is found that young, healthy, naturally menstruating female subjects' blood vessel reactivity is affected by the cyclical fluctuation of the hormones estrogen and progesterone, then future research using these two new techniques should study female subjects according to the findings of this project. Namely, subjects should be studied during the menstrual cycle phase that elicits the lowest overall response when compared to the two other menstrual cycle phases observed, as this is indicative of the lowest augmentation of the results by the effect of the hormones. This is important because it is necessary to control for as many variables as possible in order to ensure confidence in the results of a study.

Blood vessel function in this study will be assessed using three techniques that monitor changes in blood flow and diameter by way of ultrasound imaging. Using ultrasound, a blood vessel's resting diameter can be quantified, and then a test can be performed in order to quantify the effect of that test of the size of the blood vessel. The amount a blood vessel "dilates," or increases in size, is a measure of the health of the blood vessel; in general, the larger the percent change in size, the healthier the blood vessel is. The female hormones have been shown to improve blood vessel dilation when assessed by a long-used technique known as "flow-mediated dilation," but it has not yet been determined whether other tests of vessel function, such as a handgrip model or a passive limb movement model, are similarly affected. Thus, this study seeks to determine the relationship between these hormones and these three tests of vessel function, using flow-mediated dilation as a barometer for the results observed.

Acknowledgements

This thesis would not have been possible without the tireless efforts of the Minson lab. I would like to give special thanks to Doctor Christopher Minson and graduate student Brett Ely. Doctor Minson provided endless support and infectious energy during a project that proved to be quite intensive and challenging. I know I could not have completed this project without Dr. Minson, or without the tireless efforts of Brett Ely, who received more messages about girls menstruating than she ever thought possible. She was an excellent mentor and friend to me during this process, and I am indebted to her for every question she answered, and every problem she solved.

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Introduction

The number one cause of death in the United States is cardiovascular disease, a condition that is responsible for one in every three American deaths. Cardiovascular disease (CVD) is a term that signifies pathological function of the heart, the blood vessels, or both, and includes dysfunction such as Coronary Artery Disease, Cardiomyopathy, Arrhythmia, and Heart Failure. Significantly, many forms of CVD have a connection to the process of atherosclerosis, or the narrowing of blood vessels by the accumulation of fatty plaques within large and intermediate sized arteries. Atherosclerosis is often preceded in these arteries by the onset of damage to the vascular endothelium by risk factors such as hypertension[1], hyperlipidemia[2], physical inactivity and obesity, diabetes mellitus[3], and cigarette smoking[4]. As such, the assessment of endothelial function in blood vessels has become an important means for evaluating cardiovascular risk factors, based on its linkage to the initial stages of hypertension and cardiovascular disease[5,6,7].

Vascular endothelium

The endothelium constitutes the lining of many internal systems including the circulatory and lymphatic systems, and it serves many important functions

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[6] Dakak N, Husain S, Mulcahy D, Andrews NP, Panza JA, Waclawiw M, Schenke W & Quyyumi AA. Contribution of nitric oxide to reactive hyperemia: impact of endothelial dysfunction. Hypertension 32, 9–15, 1998.

[7] Takase B, Uehata A, Akima T, Nagai T, Nishioka T, Hamabe A, Satomura K, Ohsuzu F & Kurita A. Endothelium-dependent flow-mediated vasodilation in coronary and brachial arteries in suspected coronary artery disease. Am J Cardiol 82, 1535–1539, A1537–1538, 1998.

based on its location. Most notably, vascular endothelial cells are capable of modulating vessel tone by releasing various relaxing or constricting factors, which diffuse through the endothelium and act on the smooth muscle that surrounds arteries. Importantly, the endothelium regulates vessel tone in a manner that is dependent on the rate of flow through the vessel by reacting to shear stress along the vessel wall. Due to the properties of laminar flow, blood drags against the endothelium and distorts the cells in the direction of the flow, activating endothelial nitric oxide synthase (eNOS). Nitric oxide (NO) is a lipophilic gas that is arguably the strongest relaxing factor released by the endothelium in response to shear stress[8]. In 1980, Robert F. Furchgott performed Nobel-prize winning experiments[9] that showed that the endothelium of vessels was capable of producing a vasodilating substance (endothelial derived relaxing factor, or EDRF) and in 1988 Rubyani, Vanhoutte and colleagues demonstrated that the vasodilator suggested by Furchgott was in fact nitric oxide (NO)[10]. It is now well accepted that the endothelium produces and releases a large number of both vasodilating and vasoconstricting agents. Their importance to maintaining vessel tone and vascular health is paramount, and thus these vasodilating substances have been a longstanding area of study.

Important to the study of endothelial function is the manner by which it is quantified, whether by invasive or by non-invasive means. The coronary arteries are the most dangerous site of endothelial dysfunction due to their proximity to

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[8] Hall E., Jon and M. Guyton. Textbook of Medical Physiology. Elsevier. 12th ed, 2011. Pp 195-7.
[9] Furchgott, Robert F. Endothelium-derived relaxing factor: Discovery, early studies and identification as nitric oxide (Nobel Lecture). *Angew. Chem. Int.* Ed., 38: 1870–1880.
[10] Rubanyi GM, Romero JC, Vanhoutte PM. Flow-induced release of endothelium-derived relaxing factor. *Am J Physiol Heart Circ Physiol* 250: H1145–H1149, 1986.

the heart, and as such they are the most important vessels to assess. However, the ability to assess the function of the coronary arteries is complicated by the fact that their study requires an invasive process or very expensive tests. Thus, it is important to assess the function of these arteries by a manner that is relatively inexpensive and minimally or non-invasive, particularly in at-risk populations that are more vulnerable to invasive procedures. Fortunately, studies have shown a positive correlation between non-invasive conduit vessel function and invasively measured endothelial function of the coronary arteries[11,12].

Non-invasive assessment of endothelial function is made more important by the fact that endothelial dysfunction precedes gross morphological signs and clinical symptoms of atherosclerosis[13]. In fact, atherosclerosis is a disease that can remain asymptomatic for decades[14], Atherosclerosis is characterized by the gradual deposit and accumulation of fatty cells such as cholesterol along the luminal walls of arteries, decreasing endothelial function and causing decreased blood flow distal to the stenosis. Specifically, when vascular endothelial cells become damaged they increase the expression of adhesion molecules, which decreases their ability to produce nitric oxide and other substances that are produced in order to prevent such adhesion[15]. Thus, arteries affected by atherosclerosis eventually lose distensibility, and their ability to vasodilate in

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[11] Anderson et al 1995

[12] Uehata A, Gerhard MD, Meredith IT, *et al.* Close relationship of endothelial dysfunction in coronary artery and brachial artery. *Circulation.* 1993; 88: I-618 (abstr).

[13] Dick H. J. Thijssen, Mark A. Black, Kyra E. Pyke, Jaume Padilla, Greg Atkinson, Ryan A. Harris, Beth Parker, Michael E. Widlansky, Michael E. Tschakovsky, and Daniel J. Green. Assessment of flow-mediated dilation in humans: a methodological and physiological guideline. *Am J Physiol Heart Circ Physiol* 300: H2-H12, 2011.

[14] Ross R. 1999. Atherosclerosis – an inflammatory disease. N Engl J Med, 340:115-26.

[15] Hall E., Jon and M. Guyton. Textbook of Medical Physiology. Elsevier. 12th ed, 2011. Pp 195-7.

response to shear stress is lessened[16]. This further necessitates a non-invasive test of endothelial function in order to properly diagnose and mitigate the symptoms of atherosclerosis before damage to the endothelium causes vascular injury and damage to vulnerable tissues such as the heart, kidneys, and brain[17].

Flow mediated dilation

Finally, in 1992, a non-invasive test of endothelial function was developed, and with this came the advent of the use of flow-mediated dilation in clinical research. Based on work in the early 1990s by Celermajer and his colleagues, a technique was developed for use as a noninvasive, proxy assessment of vascular health termed flow mediated dilation (FMD)[18]. The vascular reactivity of the vessels to this technique is described generally as any vasodilation of an artery following an increase in luminal blood flow and internal-wall shear stress[19]. FMD was found to be dependent on a functional endothelial lining in arteries, which was furthered by work that illuminated the existence of ion channels within endothelial cells capable of sensing shear stress through a vessel. FMD has thus been termed a test of endothelium-dependent vasodilation (EDVD). These ion channels were discovered when FMD decreased in situ with the use of eNOS inhibitors. The action of these ion channels has been extensively studied[20], and the physiological process

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[16] Cox DA, Vita JA, Treasure CB, *et al.* Atherosclerosis impairs flow-mediated dilation of coronary arteries in humans. *Circulation.* 1989;80:458-465.

[17] Hall E., Jon and M. Guyton. Textbook of Medical Physiology. Elsevier. 12th ed, 2011. Pp 195-7.

[18] *Celermajer DS, Sorensen KE, Gooch VM, Spiegelhalter DJ, Miller OI, Sullivan ID, Lloyd JK, Deanfield JE. Non-invasive detection of endothelial dysfunction in children and adults at risk of atherosclerosis. Lancet. 1992; 340: 1111–1115.*

[19] Thijssen et al, Assessment of flow-mediated dilation in humans, 2011.

[20] Davies P.F. Hemodynamic shear stressand the endothelium in cardiovascular pathophysiology. *Nat Clin Pract* 6:16-26, 2009.

in response to FMD within an artery has been documented[21]. Specifically, following acute occlusion of an artery with externally applied suprasystolic pressure, an increase in blood flow through the vessel is induced upon release of the occlusion. This hyperemia increases the laminar shear stress in a parallel direction across the arterial lumen,[22] and this signal is propagated to the endothelial cells by luminal machanoreceptors which are deformed by the shear stress at the level of the cell membrane.[23] This mechanotransduction activates a signal transduction pathway in which G-protein expression of Phosphokinase A (PKA) is increased, which in turn signals for an increase in eNOS activity, catalyzing the conversion of L-arginine to NO by eNOS.[24] eNOS acts as the enzymatic catalyst that converts L-arginine (an amino acid), into citrulline and NO. Once NO is produced by eNOS, it must diffuse through the tunica media in order to act on the smooth muscle surrounding the vessel. Following diffusion, NO activates soluble guanylate cyclase, an enzyme that converts GTP to GMP, which induces the relaxation of smooth muscle and the subsequent vasodilation of the artery being tested.[25] FMD has thus been used as an accepted clinical research test of endothelial function; however there is a current debate on the validity and reliability of FMD as a bioassay of nitric oxide availability.

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[21] Dick H. J. Thijssen, Mark A. Black, Kyra E. Pyke, Jaume Padilla, Greg Atkinson, Ryan A. Harris, Beth Parker, Michael E. Widlansky, Michael E. Tschakovsky, and Daniel J. Green. Assessment of flow-mediated dilation in humans: a methodological and physiological guideline. *Am J Physiol Heart Circ Physiol* 300: H2–H12, 2011.

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[25] AH, Ryan, Steven K. Nishiyama, D. Walter Wray and Russel S. Richardson. *Hypertension.* 2010; 55:1075-1085.

Nitroglycerin administration

The administration of nitroglyercine (NTG) is common in studies of FMD, for the reason that it elicits a maximal vasodilatory response from the vasculature by way of production of NO inderpendent of the endothelium. When NTG is introduced *in vivo* it produces free radical NO, which then activates guanylate cyclase, which increases cGMP levels. This cascade of events eventually leads to the dephosphorylation of myosin light chains, which are responsible for maintaining tone in the smooth muscle. This dephosphorylation leads to relaxation of the smooth vessel and subsequent global vasodilation of large arteries and veins[26]. The purpose of this being used in the study of the vasculature is to compare the endothelium's capability to vasodilate naturally (in response to shear stress), with its maximal capacity to vasodilate independent of the endothelium. This comparison not only acts as a measure of the function of the blood vessels, but also allows for comparison between subjects since dividing percent dilation following FMD by the maximal percent dilation following GTN administration normalized each subject's response.

Endogenous female sex hormones and endothelial function

Of interest to this study, research has shown that EDVD as assessed by FMD can be transiently affected by sex hormone fluctuations as observed during the MC. As cross-sectional demonstration of this hormonal action, there is evidence of a gender discrepancy in the incidence of CVD in which its prevalence is much higher

[26] Chen, Zhiqiang, Matthew W. Foster, Jian Zhang, Lan Mao, Howard A. Rockman, Toshihiro Kawamoto, Kyoto Kitagawa, Keiichi I. Kayayama, Douglas T. Hess and Jonathon S. Stamler. An essential role for mitochondrial aldehyde dehydrogenase in nitroglycerin bioactivation. Proc Natl Acad Sci U S A. 2005 August 23; 102(34): 12159-12164.

in males than in age-matched females, up until the onset of menopause[27]. Coinciding with the onset of menopause when the levels of estrogen and progesterone are chronically low, the risk for CVD in women surpasses that of age-matched men, indicating that these ovarian hormones are cardioprotective in some way[28]. In fact, estrogen receptors and progesterone receptors have been identified in human vascular endothelium[29].

In support of the positive effect that the sex steroids may have on the vasculature, it has been found that endothelial function fluctuates along with estrogen and progesterone across the menstrual cycle, as evidenced by changes in the vessel's reactivity to flow mediated dilation (FMD), a hallmark of endothelium-dependent vasodilation[30]. In this study, it was shown that FMD was sensitive to changes in endothelial function associated with the fluctuation of hormones across the menstrual cycle. Specifically, endothelium-dependent vasodilation (EDVD) increases when circulating levels of estrogen increase, such as during ovulation; and decrease when estrogen levels decrease, such as during menstruation [31,32]. The effect of estrogen on the vasculature occurs by both short and long term mechanisms. It would appear that estrogen acting in a short term vasodilatory

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[27] Tunstall, P.H., K. Kuulasmaa, P Amouyel et al. Myocardial infarction and coronary deaths in the World Health Organization MONICA Project. Registration procedures, event rates, and case-fatality rates in 38 populations from 21 countries in four continents. *Circulation.* 1994;90:583-612.

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[31] Meendering JR, Torgrimson BN, Miller NP, Kaplan PF, Minson CT. Ethinyl estradiol-to-desogestrel ratio impacts endothelial function in young women. Contraception 2009; 79:41-9.

[32] Williams et al 2001

capacity may occur via mediation by G-protein coupled estrogen receptors,[33] in which the introduction of exogenous estrogen can increase vessel diameter transiently. In a long term capacity, such as across each monthly hormone cycle, estrogen also appears to promote vasodilation by increasing the expression and action of eNOS, which would seek to explain the increase in the FMD response during ovulation when circulating estrogen levels are high. The action of estrogen on the vasculature is further understood by studying its effect on postmenopausal women, in which short and long term estrogen administration has been shown to enhance FMD[34,35]. Furthermore, the use of oral contraceptives (combination oral contraceptives, COCs) containing estrogen (ethinyl estradiol, EE) acted to increase NO bioavailabity as measured by FMD in premenopausal women, as well[36]. When attempting to compare sex differences with minimal influence from the female sex steroids, it is recommended to study the vasculature of any premenopausal population during the first seven days of the menstrual cycle, when hormone levels are low and thus would not augment the results of a test such as FMD[37].

However, it should also be noted that the action of these hormones is complicated, because as estrogen appears to enhance endothelial function, the effect of progesterone seems to antagonize the effect of estrogen, as shown by a

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[33] Meyer MR, Prossnitz ER, Barton M. The G protein-coupled receptor GPER/GPR30 as a regulator of cardiovascular function. *Vascular Pharmacology*. 2011; 55: 17-25.

[34] Lieberman EH, Gerhard MD, Uehata A, et al. Estrogen improves endothelium-dependent, flow-mediated dilation in postmenopausal woman. *Ann Intern Med.* 1994;121:946-941.

[35] Sherwood A, Bower JK, McFetridge-Durdle J, Blumenthal JA, Newby LK, Hinderliter AL. Age moderates the short term effects of transdermal 17beta-estradiol on endothelium-dependent vascular function in postmenopausal women. *Arterioscler Thromb Vasc Biol.* 2007; 27:1782-7.

[36] John S. Jacobi, Schlaich MP, Delles C, Schmieder RE. Effects of oral contraceptives on vascular endothelium in premenopausal woman. *Am J Obstet Gynecol.* 2000;183:28-33.

[37] Dick H. J. Thijssen, Mark A. Black, Kyra E. Pyke, Jaume Padilla, Greg Atkinson, Ryan A. Harris, Beth Parker, Michael E. Widlansky, Michael E. Tschakovsky, and Daniel J. Green. Assessment of flow-mediated dilation in humans: a methodological and physiological guideline. *Am J Physiol Heart Circ Physiol* 300: H2–H12, 2011.

study by *Miner et al* (2011)[38]. In this study, FMD testing was performed when the endogenous hormones of naturally cycling women were suppressed by a gonadotropin-releasing hormone antagonist (GnRHa), and then again when they were supplemented with oral progesterone alone, transdermal estrogen alone, or a combination of the two together. The results of the study revealed that progesterone antagonized the beneficial effects seen with supplementation of estrogen alone, as evidence by a decrease in the response to FMD. This purported affect of progesterone is also suggested endogenously by the observation that FMD is higher in the OV phase and lower in the ML phase, when progesterone is at its highest.

Alternative tests to FMD

Other such tests of EDVD and nitric oxide (NO) bioavailability that have been proposed in recent years include progressive handgrip exercise[39] and passive limb movement[40]. Progressive handgrip (HG) exercise has been shown to induce vasodilation via the creation of shear stress across the vessel during an exercise bout, leading to smooth muscle relaxation and vessel enlargement. This response was shown to be mediated by NO based on the reduction in blood flow during the same exercise following the introduction of *N* -monomethyl-L-arginine, (L-NMMA) an NO antagonist. L-NMMA is a methylated derivative of L-arginine, a precursor to

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[38] Miner, Jennifer A., Emily R. Martini, Michael M. Smith, Vienna E. Brunt, Paul F. Kaplan, John R. Haliwill, and Christopher T. Minson. Short term oral progesterone administration antagonizes the effect of transdermal estradiol on endothelium-dependent vasodilation in young healthy women. *Am J Physiol Heart Circ Physiol.* 301: H1716-H172, 2011.

[39] Wray, Walter D., Melissa A. H. Witman, Stephen J. Ives, John McDaniel, Anette S. Fjeldstad, Joel D. Trinity, Jamie D. Conklin, Mark A. Supiano and Russell Richardson. Progressive handgrip exercise: evidence of nitrc oxide-dependent vasodilation and blood flow regulation in humans. *American Journal of Physiology – Heart and Circulatory Physiology.* 300:H1101-H1107, 2011.

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NO. L-NMMA acts as a blockade to NO production at the level of eNOS by acting as a competitive inhibitor at the site of the enzyme.

Similarly, passive limb movement (PLM) has been shown to induce a robust increase in blood flow to the moving limb via the creation of rhythmic shear stress across the vessel, inducing vasodilation that is also NO mediated, as determined by infusion of L-NMMA[41]. However, these tests have not yet been assessed in comparison with FMD or throughout the menstrual cycle.

It has recently been suggested that FMD may not be a reliable *in vivo* bioassay of nitric oxide bioavailability, whose results are made incomparable due to a multitude of measurement and analysis techniques that are unique to individual research groups[42]. In the last three years, alternative techniques such as the handgrip and the passive limb movement have been proposed as more reliable bioassays for endothelium-derived NO bioavailability in humans; however, they have received relatively little attention in comparison to FMD. In order to determine the usefulness of these newer techniques as a replacement for FMD, it is necessary to better understand them in the context of the field of study that FMD has encompassed.

Objective

The purpose of this study was to exploit the sex steroid changes across the menstrual cycle to look at three different approaches used to assess endothelial function. We tested the hypothesis that subject responses to FMD, HG, and PLM

[41] Richardson et al, Progressive handgrip, 2011
[42] Tschakovsky ME & Pyke KE. Counterpoint: Flow-mediated dilation does not reflect nitric oxide-mediated endothelial function. *Journal of Applied Physiology*. 99: 1235-1237, 2005.

would be positively affected by the presence of estrogen during the OV phase, while GTN, a test independent of endothelial function, would not.

Methods

Subjects. Seven healthy, active females (exercise limited to <8 hours per week), between the ages of 18 and 30 participated in this study. All subjects were nonsmokers with a BMI of <25, were not taking any prescription medication, and were not using any hormonal form of contraceptive. All subjects had not been taking birth control for at least 2 months prior to being studied. All subjects were required to take a urine pregnancy test and confirm a negative result prior to each study. Exclusion criteria were hypertension, smoking, hypercholesterolemia, cardiovascular disease, metabolic disorders, menstrual disorders, or a personal or family history of blood clots. Subjects abstained from alcohol, exercise, vitamins, and over-the-counter medications for 24 h prior to each trial, as well as caffeine and food 12 h prior. Approval for this study was granted by the Institutional Review Board at the University of Oregon, and each subject provided written and oral consent for the protocol prior to participation.

Study Design. After a successful screening interview in which a health and activity history were taken, subjects were scheduled for three study days across the menstrual cycle within a two month period: once during the early follicular (EF) phase, once during ovulation (OV), and once during the mid-luteal (ML) phase.

In order to monitor the menstrual cycle of each subject and ensure the proper hormone profiles for each study day, frequent communication and the use of hormone sensitive tests were necessary. The EF phase was scheduled based on

subject indication of the onset of menses, with all EF trials occurring within the first three days of the menstrual cycle. The OV phase was determined by the use of commercially available testing strips (SunMark One Step Ovulation Predictor), which detect changes in the urine output of Luteinizing Hormone (LH), a small glycoprotein that is secreted by the anterior pituitary in response to Gonadotropin-Releasing Hormone (GnRH) levels. Roughly 48 hours before ovulation the rate of secretion of GnRH is increased and thus LH secretion is also increased, signaling the onset of ovulation, which is characterized by high levels of estrogen. The increased serum concentration of estrogen during the OV phase is attributed to the growing ovarian follicle, which secretes estrogen. As cycle length is a function of variation between the EF and OV period (while the period between OV and EF of the next cycle is fairly set), catching OV presents a challenge. Thus, subjects were given 7 ovulation tests in the period of time that previous cycle lengths indicated that the OV phase may fall. Subjects were then schedule within 48 hours of that LH surge. The ML phase, characterized by increased levels of serum progesterone, was scheduled within roughly 7 days of a positive OV test, in order to catch peak progesterone levels. Progesterone levels peak during the ML phase as a result of the LH surge of the OV phase inducing the granulosa and theca cells of the developing follicle to convert into mainly progesterone secreting cells. Thus, the levels of estrogen begin to fall following OV.

These three study days were counterbalanced across subjects, so that roughly equal numbers of women started the study during different phases, in order to minimize any order effects associated with the scheduling of study days

across the menstrual cycle. Upon arrival at the lab, height, weight, and waist-to-hip measurements were taken, followed by a fasted blood draw by a trained phlebotomist. These venous blood samples were usually collected from an antecubital vein for analysis of estrogen and progesterone serum concentrations, The samples were then transferred (BD Vacutainer, Franklin, NJ, USA) and centrifuged for 15 minutes at 1300 g centrifuge force at 4°C. Serum was then collected from the Vacutainers and stored at -70°C until the end of the entire study, when the samples were shipped to Oregon Clinical and Translational Research Institute for analysis (OCTRI, Portland, OR, USA).

Subjects were then instrumented with a three-lead electrocardiogram (ECG) and a blood pressure cuff on the left brachium (CardioCap 5, Datex-Ohemda, Louisville, CO), just distal to the cubital fossa. Heart rate was measured continuously throughout each protocol via EKG, and was recorded along with blood pressure prior to and after each protocol. Blood pressure was also continuously measured for subsequent analysis using a Nexfin automated blood pressure cuff placed on the left, third digit (Nexfin Monitor, Model 1, BMEYE B.V., Amsterdam, The Netherlands).

Subjects then rested for 20 minutes in a supine or seated upright position, depending on the first protocol to be performed. On each study day the subject had an FMD, an HG, a PLM, and a GTN performed on them, in a randomized order that was fixed for each subject prior to the first study day. All ultrasound data were collected using a high-resolution Doppler ultrasound machine (Terason t3000cv, Teratech, Burlington, MA) instrumented to a 10.0-MHz linear array transducer

probe. Doppler ultrasound data were collected at 20 frames/s (Camtasia Studio, TechSmith, Okemos, MI). Following each study day, these video files were transferred to a computer that was outfitted with vessel edge-detection analysis software (DICOM, Perth, Australia).

Flow-mediated Dilation

FMD was performed as has been previously described by members of our research group[43]. Namely, the ultrasound transducer probe was placed on the brachial artery (using an insonation angle of 60°) between 3 and 10 cm proximal to the antecubital fossa and after one minute of baseline diameter and velocity readings, a blood pressure cuff (Zimmer, Dover, OH) that was positioned on the right arm just distal to the antecubital fossa was inflated to 250 mmHg (E20 Rapid Cuff Inflator, D. E. Hokanson, Bellevue, WA). The occlusion was maintained for a continuous 5 min, and recording continued for 3 min post-release to capture the vasodilatory effect of the shear stress that followed cuff release. FMD was assessed as the percent change in brachial artery diameter from baseline to peak dilation, using the following equation:

$$\text{FMD\%} = \frac{[\text{peak diameter (in mm)} - \text{baseline diameter (in mm)}]}{[\text{baseline diameter (in mm)}]} \times 100$$

[43] Miner, Jennifer A., Emily R. Martini, Michael M. Smith, Vienna E. Brunt, Paul F. Kaplan, John R. Haliwill, and Christopher T. Minson. Short term oral progesterone administration antagonizes the effect of transdermal estradiol on endothelium-dependent vasodilation in young healthy women. *Am J Physiol Heart Circ Physiol.* 301: H1716-H172, 2011.

Handgrip

The subject lay supine with their right arm outstretched to 90 degrees, rested on a cushioned, retractable platform. After a 20 min rest period, the ultrasound probe was placed 3-10 cm proximal to the antecubital fossa, for longitudinal imaging and blood velocity tracing. The probe was placed in a clamp to maintain the same position and angle over the brachial artery for the duration of the scan. After 1 min of baseline imaging, the subject gripped a commercial hand grip dynamometer at a force of 4 kg, 8 kg, and 12 kg, each for a duration of 3 min with a 1 min rest between each workload. Subjects gripped at a frequency of 1 Hz to the cadence of a metronome, and were given verbal feedback as to the accuracy of their cadence and force for each contraction. Subjects were encouraged to perform rapid contractions, with the goal of limiting the contraction time per second to .25 seconds. The main outcome variables for this test were the change in vessel diameter, expressed as a percent change from baseline to the rest period after each workload and characterized by slope, as well as the change in flow from baseline to each rest period, also characterized by slope.

Passive limb movement

The passive limb movement was performed with the subject seated in a cushioned phlebotomy chair, at an approximate angle of 110 degrees between their torso and legs. In this posture, the subject was outfitted with a blood pressure cuff (Zimmer, Dover, OH) just below the patellar tendon of their right leg, for the use of limiting the gravitational effects the PLM itself can have on blood flow. The

leg was stabilized with the use of a suctioned bag placed around the subjects' distal thigh. Both legs rested on a wheeled cart until the beginning of the test. After a 20 min rest period, the right femoral artery of the subject was imaged, and the cuff was inflated to 250 mmHg (E20 Rapid Cuff Inflator, D. E. Hokanson, Bellevue, WA). After 1 min of normalization, the cart was pushed aside and the right leg was supported in full extension for a period of 1 min while baseline measurements for velocity and diameter were taken. After baseline, a technician passively flexed and then extended the right leg of the subject, at a cadence of 1 Hz, along with a metronome. After a period of 2 min of passive limb movement, the technician once again held the limb in extension, as 1 min of post-movement data were collected. The cuff was then deflated. The main outcome variable for this test was the change in femoral blood flow, as calculated by using arterial diameter and Vmean, with the equation blood flow = V mean × π × (vessel diameter/2)^2 × 60, where blood flow is in milliliters per minute. The reactive hyperemia for this test was expressed as area under the curve, comparing the baseline readings to the two minutes of PLM.

Nitroglycerine administration

NTG is used in research for its action as a nitric oxide donor, which explains its use in clinical practice for patients suffering from angina pectoris, a painful constriction of the coronary blood vessels, and congestive heart disease[44,45,46]. NTG (1,2,3-trinitroxypropane) acts through activation of guanylate cyclase, thus

[44] Bennett BM, McDonald BJ, Nigam R, Simon WC. Review Biotransformation of organic nitrates and vascular smooth muscle cell function. Trends Pharmacol Sci. 1994 Jul; 15(7):245-9.
[45] Thatcher GR, Nicolescu AC, Bennett BM, Toader V. Review Nitrates and NO release: contemporary aspects in biological and medicinal chemistry. Free Radic Biol Med. 2004 Oct 15; 37(8):1122-43.
[46] Fung HL. Biochemical mechanism of nitroglycerin action and tolerance: is this old mystery solved? Annu Rev Pharmacol Toxicol. 2004;44:67-85.

production of cGMP and *in vivo* vasodilation[47]. As such, NTG administration represents endothelium-independent vasodilation (EIVD), as opposed to FMD, which represents endothelium-dependent vasodilation (EDVD). The administration of NTG was always the last test performed on each study day to ensure that the NTG did not affect the results of the other three tests. After 20 min of rest following the third test of each test day, new baseline images were recorded for 1 min followed by a sublingual administration of 0.4 mg NTG. The brachial artery was continuously imaged for 10 min following NTG administration. Subjects then remained supine for an additional 10 min before leaving the testing area to ensure stable blood pressure. The main outcome variable for this test was the percent change in brachial artery diameter as determined by the comparison of peak vessel diameter to baseline diameter, expressed as a percent change.

Results

Subject characteristics.

Baseline characteristics for the subject population studied are displayed in Table 1.

	Age (years)	Height (m)	Body Mass (kg)	BMI	SBP (mmHg)	DBP (mmHg)	HR (bpm)
Mean ± Stdev	21 ± 2	1.67 ± 0.13	61.7 ± 10.97	21.8 ± 1.61	107 ± 6	66 ± 4	58 ± 8
Range	19-25	1.56-1.85	48.8 - 79.1	19.8-23.1	99-117	62-74	47-67

Table 1. Values are means ± standard deviation. All subjects were normotensive.

[47] Chen, Zhiqiang, Matthew W. Foster, Jian Zhang, Lan Mao, Howard A. Rockman, Toshihiro Kawamoto, Kyoto Kitagawa, Keiichi I. Kayayama, Douglas T. Hess and Jonathon S. Stamler. An essential role for mitochondrial aldehyde dehydrogenase in nitroglycerin bioactivation. Proc Natl Acad Sci U S A. 2005 August 23; 102(34): 12159–12164.

FMD

A statistical comparison of the three phases of the menstrual cycle (MC) in relation to the FMD data collected revealed no significant effect of the MC on this measure of endothelial-dependent vasodilation (EDVD). The average FMD percent change in vessel diameter for EF was 8.26 ± 3.41%, for OV 7.97 ± 1.56%, and for ML 8.29 ± 2.10%, thus exhibiting an average trend that was highest in the ML phase, and the lowest in the EF phase. When FMD is expressed relative to maximal endothelium-independent vasodilation (EIVD) as measured by GTN on each test day (by diving the %FMD by the %GTN), the results yielded do show a trend in which the highest response to FMD is present in the OV phase (EF 0.42±0.19, OV 0.48±0.16, and ML 0.41±0.07), though these results are not statistically significant by one-way repeated measures ANOVA (see Fig. 1). When FMD is corrected for shear stress (a description of blood flow through a vessel determined by the velocity and size of the vessel), the results yielded show the highest response to FMD during the ML phase, with the lowest response in the EF phase (EF 0.014±0.001, OV 0.014±0.002, ML 0.019±.006).

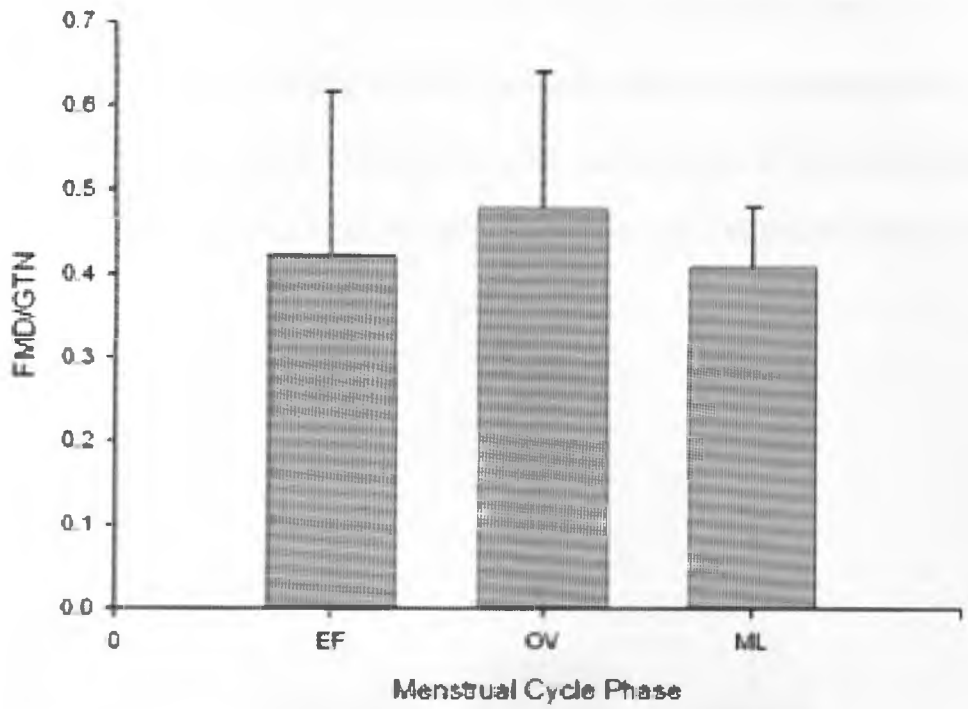


Figure 1. *The effect of the MC phase on FMD corrected for maximal vasodilatory capacity. Results presented are averages for each phase with error bars showing standard deviation.*

GTN

EIVD did not exhibit statistical significance when tested across the MC. The administration of sodium nitroprusside yielded 21.1%±7.64 for EF, 17.98±5.36 for OV, and 20.3±4.34 for ML, thus demonstrating the highest percent vasodilation in the EF phase and the lowest percent vasodilation in the OV phase.

PLM

The blood flow (BF) elicited by the PLM across the three phases in each subject did not produce a statistically significant trend. The highest change in blood flow from rest to maximal (highest blood flow observed – baseline blood flow) was seen during ML, with the lowest ΔBF seen during OV (average delta blood flow to the femoral artery: EF 290.3±197.6 mL/min, OV 238.9±170.3

mL/min, ML 327.0±125.4 mL/min). When the change in BF from rest to maximal is expressed as a percent change from baseline ([Max flow – baseline flow]/ baseline flow x 100%), the highest response seen occurs during OV, with the lowest phase being ML (EF 236.7±177.7%, OV 277.3±242.7%, ML 234.8±194.3%) (see Fig. 2).

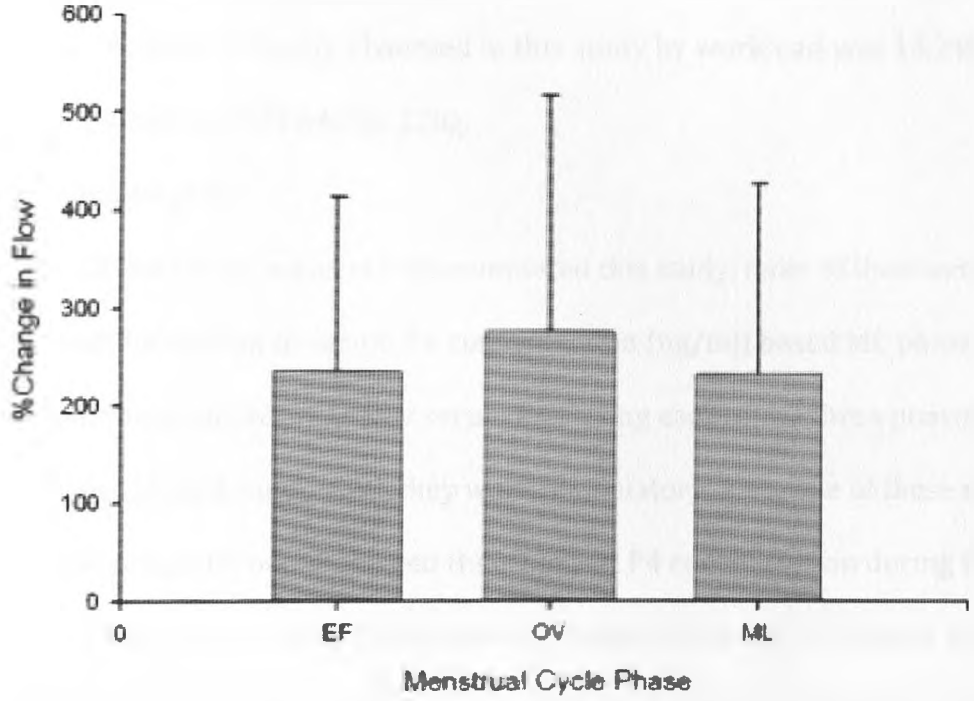


Figure 2. *The effect of menstrual cycle phase on the percent change in flow elicited by the PLM. Results presented are averages for each phase with error bars showing standard deviation.*

HG

The results for this study of active hyperemia have been evaluated in terms of the slope of the line produced when the percent change in vessel diameter from baseline was graphed for each subject across the three workloads. The dilation slope produced by this study did not reveal a statistically significant trend when evaluated across the MC phases. The highest response (slope, in units of %change in diameter/kgWorkload) to the HG was seen during EF, and the lowest during OV

(EF 1.08±0.61, OV 0.69±0.28, ML 0.97±0.56). The range of difficulty experienced by subjects in this study during the HG can be determined roughly by comparing each subject's MVC (maximal voluntary contraction, collected at the initial screening visit) to each workload (for example, a subject with an MVC of 27 kg during the 4kg workload was contracting at 4kg/27kg=14.8% of her maximal capacity). The average relative difficulty observed in this study by workload was 13.2% for 4kg, 26.4% for 8kg, and 39.6% for 12kg.

Progesterone (P4)

Of the seven subjects who completed this study, most of them exhibited the expected fluctuation in serum P4 concentration (ng/ml) based MC phase. However, two subjects had low serum P4 during each of the three phases of the MC that were studied, suggesting they were anovulatory. Also, one of these subjects with low progesterone displayed their highest P4 concentration during the OV phase, rather than the ML phase, which is unusual (see Fig. 3). Thus, it should be noted that these tests were not compared to the hormone levels themselves (due to time constraint), they were rather compared only to the MC phases and their presumed hormonal profiles. See Table 2 for actual hormone data.

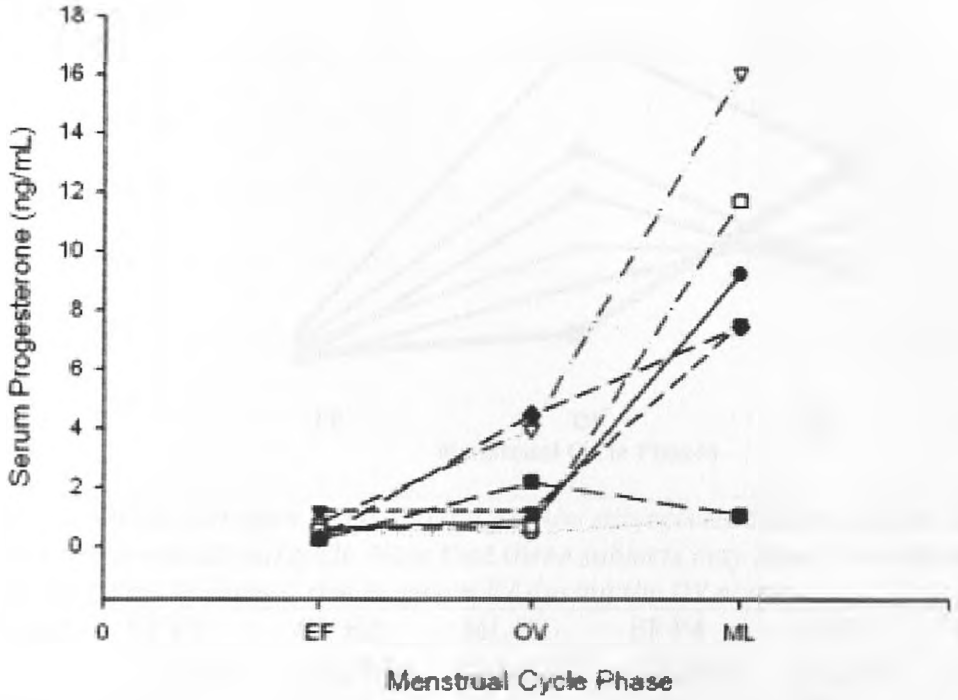


Figure 3. Serum progesterone levels (ng/mL) in the subjects studied as observed across the three phases of the menstrual cycle. Note that two subjects exhibited low P4 levels in all three phases of the menstrual cycle.

Estrogen (E2)

The estrogen data collected reveals that three subjects may have been anovulatory during this study, as evidenced by their lack of rise in serum E2 during the OV phase (see Fig. 4). This includes the two subjects who had low P4 levels throughout the MC, as well as one other subject. See Table 2 for actual hormone data.

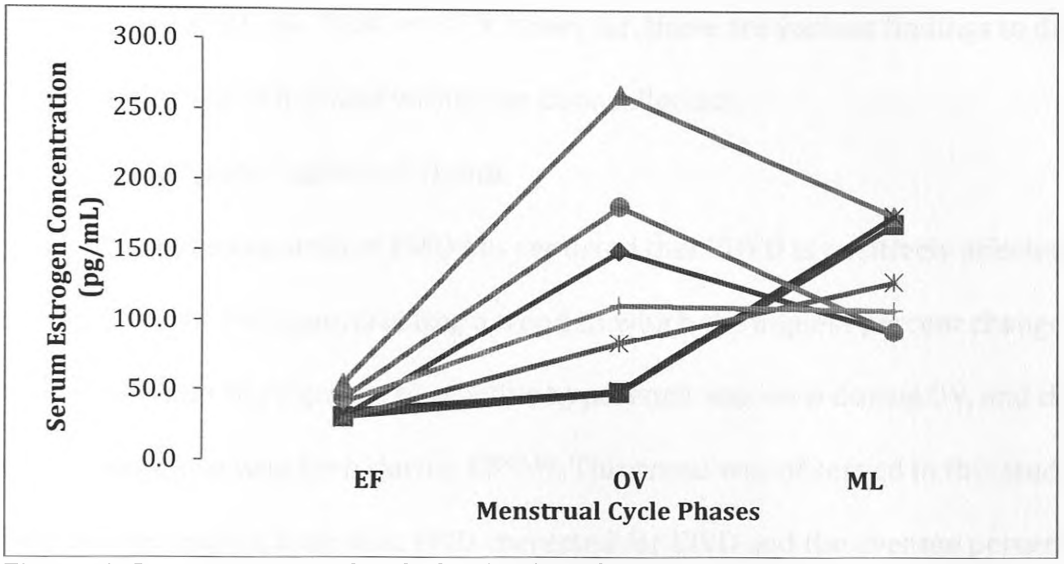


Figure 4. Serum estrogen levels (pg/mL) in the subjects studied across the three phases of the menstrual cycle. Note that three subjects may have been anovulatory, while four clearly show a rise in serum E2 during the OV phase.

Subject	EF E2 (pg/mL)	OV E2 (pg/mL)	ML E2 (pg/mL)	EF P4 (ng/mL)	OV P4 (ng/mL)	ML P4 (ng/mL)
3	30.3	149.0	91.6	0.60	0.87	9.30
5	30.4	47.7	169.2	0.45	0.49	1.00
9	54.2	260.8	175.0	1.20	1.20	7.60
12	34.4	48.8	175.0	0.81	4.00	16.20
13	29.2	83.1	127.8	0.26	2.20	1.10
14	43.6	180.9	92.0	0.57	0.65	11.80
15	40.6	110.5	107.6	0.31	4.50	7.50

Table 2. E2 and P4 data across the menstrual cycle phases for all seven subjects that were studied.

Discussion

This is the first study to examine not only the relationship between these three tests of nitric oxide bioavailability and blood vessel function, but also the first to assess the potential effect of the hormones estrogen and progesterone on the rhythmic handgrip exercise and passive limb movement responses. It should be noted that one-way repeated measures ANOVA statistics did not reveal any significant effect of menstrual cycle phase (EF, OV, or ML) on the major outcome

variables for FMD, HG, PLM, or GTN. However, there are various findings to discuss in terms of trends observed within the data collected.

Data displaying the "expected" trends

Previous research of FMD has reported that EDVD is positively affected by serum levels of estrogen, creating a trend in which the highest percent change in vessel diameter in response to reactive hyperemia was seen during OV, and the lowest response was seen during EF[48,49]. This trend was observed in this study for only two measures, including FMD corrected for EIVD and the average percent change in flow during the PLM.

FMD corrected for maximal dilatory capacity

The significance of looking at the relationship between EDVD and EIVD is that it offers a way to normalize the response a subject has to EDVD in terms of their maximal vasodilatory capacity (see Fig. 1). It should be noted here that although the FMD results were not significant, the effect of baseline diameter on FMD was also not significant, thus the baseline diameter for any subject did not affect their response to EDVD. This is important because baseline arterial diameter has been determined as a point of difficulty in the accurate reflection of vessel response when using Doppler ultrasound to assess vessel function as there is evidence to suggest that a systemic difference in vascular endothelial function is

[48] Hashimoto, Masayoshi, MD; Masahiro Akishita, MD; Masato Eto, MD; Michiro Ishikawa, MD; Koichi Kozaki, MD; Kenji Toba, MD, PhD; Yoko Sagara, MD; Yuji Taketani, MD, PhD; Hajime Orimo, MD, PhD; Yasuyoshi Ouchi, MD, PhD. Modulation of endothelium-dependent flow-mediates dilation of the brachial artery by sex and menstrual cycle. *Circulation:* 92: 3431-3435, 1995.

[49] Williams, Maro R. I, Roderick A. Westerman, Bronwyn A. Kingwell, Jason Paige, Peter A. Blombery, Krishnankutty Sudhir, and Paul A. Komesaroff. Variations in endothelial function and arterial compliance during the menstrual cycle. *The Journal of Clinical Endocrinology & Metabolism.* 86(11): 5389-5395, 2001

associated with the initial size of the artery[50], as well as the fact that a larger resting vessel diameter will yield a lower percent change in diameter (the main outcome variable of FMD or GTN) compared to a vessel with a lower resting diameter even when both vessels increase in diameter by the same amount (in mm).

Percent change in BF for PLM

The average percent change in flow in response to the PLM also displayed the same trend as FMD in previous studies (see Fig. 2). Both of these measures are reported measures of nitric oxide bioavailability, though the PLM has not yet been studied in relation to the female sex hormones estrogen and progesterone. Thus, it is a novel and interesting finding that the percent change in flow elicited by the PLM seems to exhibit the expected trend, albeit without statistical significance. Per the Richardson study (2012), the increase in blood flow elicited by the PLM is largely NO-dependent, thus, it stands to reason that estrogen may have an effect on the response to this test owing to the data that shows that estrogen increases NO synthase activity[51] as well as antioxidant capacity[52], thus has the potential to increase the vasodilatory and BF response observed. The PLM is proposed to elicit NO production by the premise of vascular deformation caused by the rhythmic shortening and lengthening of the leg muscles during the PLM. This change in muscle length may stimulate a mechanosensitive mechanism that induces the

[50] Thijssen D. H., E. A. Dawson, M. A. Black, M. T. Black, N. T. Cable, D. J. Green. Heterogeneity in conduit artery function in humans: impact of arterial size. American Journal of Physiology. 2008;295:H1927-H1934.
[51] Hayashi T., K. Yamada, T. Esaki, M. Kuzuya, S. Satake, T. Ishikawa, H. Hidaka, A. Iguchi. Estrogen increases endothelial nitric oxide by a receptor-mediated system. *Biochem Biophys Res Commun.* 1995;214:847-855.
[52] Mendelsohn M. E. and R.H. Karas. The protective effects of estrogen on the cardiovascular system. *N Engl J Med.* 1999;340:1801-1811.

reported increase in blood flow and subsequent downstream vasodilation[53,54,55,56,57]. Specifically, according to the 2012 Richardson study, the onset of the PLM stimulates group III mechanosensitive skeletal afferent (sensory) fibers[58]. The concept of vascular deformation eliciting vasodilation has been previously described by *Clifford et al* (2006)[59] using isolated rat feed arteries. In this study, mechanical compression of these isolated arteries caused immediate vasodilation, with increasing numbers of compressions causing a greater extent of vasodilation. To determine the origin of the observed dilation, the research group removed the endothelium of these arteries and found that the compression-induced dilation was abated, but not completely abolished, indicating both an endothelium-dependent, and an endothelium-independent mechanism of vasodilation.

However, though it would appear that the PLM is ~80% NO mediated (based on 80% abolishment of BF following infusion of L-NMMA), 20% of this test of endothelial function is as of yet unknown. Potential mechanisms that have been proposed include the hyperpolarization of smooth muscle by muscle activation and the immediate release of potassium (K+) to the interstitium and the plasma, as hyperemia is inhibited in contracting muscle when K+ channels are inhibited in

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[53] Mohrman DE & Sparks HV (1974a). Myogenic hyperemia following brief tetanus of canine skeletal muscle. Am J Physiol 227, 531–535.
[54] Mohrman DE & Sparks HV (1974b). Role of potassium ions in the vascular response to a brief tetanus. Circ Res 35, 384–390.
[55] Hamann JJ, Buckwalter JB & Clifford PS (2004). Vasodilatation is obligatory for contraction-induced hyperaemia in canine skeletal muscle. J Physiol 557, 1013–1020.
[56] Clifford PS, Kluess HA, Hamann JJ, Buckwalter JB & Jasperse JL (2006). Mechanical compression elicits vasodilatation in rat skeletal muscle feed arteries. J Physiol 572, 561–567.
[57] Kirby BS, Carlson RE, Markwald RR, Voyles WF & Dinenno FA (2007). Mechanical influences on skeletal muscle vascular tone in humans: insight into contraction-induced rapid vasodilatation. J Physiol 583, 861–874.
[58] Trinity, Joel D., H. Jonathan Groot, Gwenael Layec, Matthew J. Rossman, Stephen J. Ives, Sean Runnels, Ben Gmelch, Amber Bledsoe, and Russell S. Richardson. Nitric oxide and passive limb movement: a new approach to assess vascular function. *The Journal of Physiology.* 590(6): 1413-1425, 2012.
[59] Clifford et al., Mechanical compression, J. Physiol 572, 561-567.

canines[60]. Thus, the Richardson lab proposes that further research is warranted to determine the mechanism behind passive movement-induced vasodilation.

GTN

It is not a novel finding that GTN was not affected by the menstrual cycle phases, as it has been previously shown that this measure of EIVD is not affected by the natural fluctuation of MC hormones[61]. This is logical in that EIVD by definition should occur independently of the function of the endothelium, and thus independently of the effects of the hormones on the availability of the NO that is produced by the endothelium.

HG

HG represents small muscle mass, dynamic exercise that functions on the property of active hyperemia, in which vasodilation is triggered by the release of metabolites from the muscle tissue as metabolism increases and oxygen is depleted in response to a given workload. The mechanism for the increase in blood flow that is elicited by exercise is still debated, as it seems to be a combination of neural vasoconstrictor activity as well as the release and action of multiple vasodilator substances[62]. In a study by Shoemaker et al. (1997), it was found that by the second minute of rhythmic handgrip contractions, brachial artery diameter of the active arm was significantly greater than at rest ($P<.05$), demonstrating vasodilation in response to small muscle exercise. *Richardson et el.* (2012) then

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[60] Hamann J.J., J.B. Buckwalter and P.S. Clifford. Vasodilation is obligatory for contraction-induced hyperaemia in canine skeletal muscle. *J Physiol* 557;(2004):1013-1020.

[61] Williams, Maro R. I, Roderick A. Westerman, Bronwyn A. Kingwell, Jason Paige, Peter A. Blombery, Krishnankutty Sudhir, and Paul A. Komesaroff. Variations in endothelial function and arterial compliance during the menstrual cycle. *The Journal of Clinical Endocrinology & Metabolism.* 86(11): 5389-5395, 2001.

[62] Clifford, Philip S., and Ylva Hellsten. Vasodilatory mechanisms in contracting skeletal muscle. *J Appl Physiol.* 97;(2004):393-403.

showed that the increase in FMD as a result of HG was largely NO-dependent by way of NO blockade by L-NMMA. Exercise appears to increase the shear rate across the vessel, acting in a similar fashion as the shear stress produced by FMD.

However, there is some debate as to the mechanism of effect of HG, as well as a difficulty in comparing studies in the current literature due to the lack of a universal measurement technique. For example, previous studies of HG (using workloads of 9.5% of MVC) have demonstrated a combined contribution of both nitric oxide and acetylcholine to HG-induced vasodilation of the brachial artery[63], as well as previous studies citing a potential contribution of prostagladins[64,65], adenosine[66,67], and ATP-sensitive potassium channels[68]. Also, *Takeshita et al* suggested that the decrease in forearm blood flow observed following L-LNMMA infusion before the onset of HG exercise was due to a decrease in resting blood flow, rather than from decreasing the change in blood flow elicited by the exercise itself[69]. The Richardson HG study cites a significant difference in the change in blood flow following L-NMMA infusion, thus illuminating the difficulty in comparing HG studies owing to the myriad of experimental techniques being utilized (for workload, frequency of contraction, arm position etc). In this light, the

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[63] Shoemaker J.K., J.R. Halliwill, R.L. Hughson and M.J. Joyner. Contributions of acetycholine and nitric oxide to forearm blood flow at exercise onset and recovery. H2388-H2395. 1997.
[64] Kilborn, A., A Wennmalm. Endogenous prostaglandins as local regulators of blood flow in man: effect of indomethacin on reactive and functional hyperemia. *J Physiol.* 1976;257:109-121.
[65] Wilson, J.R., S.C. Kapoor. Contribution of prostaglandins to exercise-induced vasodilation in humans. *Am J Physiol.* 1993;265:H171-H175.
[66] Goonewardene I.P., F. Karim. Attenuation of exercise vasodilation by adenosine deaminase in anesthetized dogs. *J Physiol.* 1991;442:62-79.
[67] Persson M.G., A. Ohlen, L. Lindbom, P. Hedqvist, and L.E. Gustaffson. Role of adenosine in functional hyperemia in skeletal muscle as indicated by pharmacological tools. *Naunyn Shmiedebergs Arch Pharmacol.* 1991;343:52-57.
[68] Duncker, D.J., N.S. Van Zon, J.D. Altman, T.J. Pavek, and R.J. Bache. Role of K+ ATP channels in coronary vasodilation during exercise. *Circulation.* 1993;88:1245-1253.
[69] Endo, Toyonari, M.D., Tsutomu Imaizumi, Tatsuya Tagawa, M.D., Masanari Shiramoto, M.D., Shin-ichi Ando, M.D., Akira Takeshita, M.D. Role of nitric oxide in exercise-induced vasodilation of the forearm. *Circulation.* 90 (6);(1994): 2886-2889.

Takeshita study does posit that more strenuous exercise may have more of an NO component[70].

Confounding variables

It is most likely that no significant results amounted from this study as a consequence of the variability displayed within each subject response to the four measures studied, as well as the low n value and the anovulatory menstrual cycle of three of the seven subjects studied.

Variation

A statistical analysis was performed to quantify this variation known as the coefficient of variation (CV), which is a ratio of the standard deviation to the mean for each outcome variable. The CV offers a description of the dispersion of data in a series around the mean such that multiple measures, even those with differing outcome variables and units, can be compared. For this study, the average CV for FMD was the lowest, followed by GTN, delta flow for the PLM, and HG percent dilation slope. This data suggests that of these four measures, FMD was the least variable and HG was the most variable. The average CV for FMD was found to be 14.46, which is comparable to previous studies of FMD variability reporting a CV of 13.9[71]. This finding may lend support to the use of FMD and GTN over the use of the PLM or the HG, which were found to be highly variable (2 and 3 times that of FMD in the present study). It should be noted that previous research on the repeatability of HG has yielded CV values between sessions in the same subject of

[70] Endo, Toyonari M.D. et al, 1994

[71] Hijmering, M.L., E.S.G. Stroes, G. Pasterkamp, M. Sierevogel, J.D. Banga, T.J. Rabelink. Variability of flow mediated dilation: consequences for clinical application. Atherosclerosis 157 (2001) 369–373.

as low as 4.08 +/- 0.7% at rest and ranging from 2.90±0.4% to 3.96±0.5% during exercise[72]. However, in this study the relative workload for each subject only ranged from 8-12% of their MVC, while in our study the relative workload ranged from 10.8-44%. Thus, the studies are not equivalent in terms of the difficulty experienced by the subject, which may also indicate a more intense stimulus to the vasculature owing to the intensity-dependent vasodilation increases demonstrated by Richardson et al (2012)[73] as well as others[74].

Low n value

Furthermore, only 7 subjects completed this study in its entirety, thus the effect of the variability observed for all four protocols that were utilized is likely significant. Had a larger sample population size been tested, it is possible that the effect of this variability could have been minimized.

Estrogen and Progesterone

The hormone results collected in this study would suggest that three subjects were anovulatory. This finding could account, at least in part, for the lack of effect of the menstrual cycle phases that were observed. Without a significant rise in E2 during the OV phase (in the case of three subjects), or a significant rise in P4 during the ML phase (in the case of two subjects) the hormone profiles for a portion of our subject population become indistinct from one another, thus

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[72] Shoemaker JK, Pozeg ZI, Hughson RL. Forearm blood flow by Doppler ultrasound during test and exercise: tests of day-to-day repeatability. Medicine and Science in Sports and Exercise [1996, 28(9):1144-1149]

[73] Wray, Walter D., Melissa A. H. Witman, Stephen J. Ives, John McDaniel, Anette S. Fjeldstad, Joel D. Trinity, Jamie D. Conklin, Mark A. Supiano and Russell Richardson. Progressive handgrip exercise: evidence of nitrc oxide-dependent vasodilation and blood flow regulation in humans. *American Journal of Physiology – Heart and Circulatory Physiology.* 300:H1101-H1107, 2011.

[74] Tschakovsky M.E., A. M. Rogers, K. E. Pyke, N. R. Saunders, N. Glenn, S. J. Lee, T. Weissgerber, and E. M. Dwyer. Immediate exercise hyperemia in humans is contraction intensity dependent: evidence for rapid vasodilation. *J Appl Physiol* 96;(2004): 693-644.

potentially accounting for the lack of significant difference seen in the responses elicited by these three tests across the MC.

Conclusion

In conclusion, this study failed to find support for the hypothesis that these three tests of endothelial function would be positively affected by the increase in serum estrogen during OV. Though no statistically significant results were yielded from the present study in regards to their respective sensitivity to the fluctuation of the MC hormones estrogen and progesterone, trends were observed in assessment of the tests themselves, most clearly illuminated by the high proportion of variation between study days within subjects. Specifically, in reference to the finding of CV, the variability was found to be highest for HG, and lowest for FMD. This finding tends to say that of these three tests of endothelial function, FMD may be the most reliable. Of course, without statistically significant results, this is merely an observation.

The hormone data collected from this study would suggest that up to three subjects were anovulatory. This could potentially diminish the effect of estrogen on the trends observed, though the low n value for this study and the high amount of variability seems to be more of a confounding factor. Specifically, it would appear that the variability observed in this study was of greater magnitude than the potential effects of the hormones themselves.

Thus, further study in this area is necessary in order to better understand the effects of the endogenous fluctuation of estrogen and progesterone on these tests of endothelium-dependent vasodilation. It is especially important that new,

proposed tests of endothelial function such as the handgrip and the passive limb movement be measured in relation to the menstrual cycle hormones if they are to be widely accepted into research practices. Most importantly, the lack of standardization of any test thus used in this field of study greatly minimizes the ability to compare the results of studies between lab groups, which depreciates our collective knowledge base. Therefore, it is important not only that methodologies be standardized, but also that subject preparation be so as well. Most significant to this study is whether the menstrual cycle of female subjects should be monitored when performing tests of hand grip and passive limb movement, and, if so, this would mean future studies would need to perform these tests of endothelial function during the early follicular phase, when estrogen and progesterone are at their lowest serum concentrations. Thus, future study must better assess the effects of these hormones, especially on the hand grip and passive limb movement tests, which are as yet untested in this regard.

Appendix I

Glossary of Terms

Afferent (sensory) fibers are nerves that take information from different types of receptors in the body and transmit it to the central nervous system and the brain

Coronary arteries are the arteries that supply blood directly to the heart muscle in order to oxygenate the heart muscle as it works to pump blood to the rest of the body

Diabetes mellitus is a term that includes both Type I and Type 2 diabetes. Type 1 diabetes is the condition in which the body is not able to produce insulin, a hormone that allows the body to bring sugar from the blood stream into cells. People with Type I diabetes thus have to take insulin in order to regulate their blood sugar. Type 2 diabetes is the condition in which the cells in the body cannot respond to insulin, though the body is capable of producing insulin (unlike Type 1 diabetes).

Endogenous refers to processes that occur within the body. Endogenous substances are those that originate from within an organism, including the menstrual cycle hormones that are produced naturally by the female reproductive organs

Endothelium is a thin layer of specialized cells that line the inside of a blood and lymphatic vessels, providing an interface between the circulating fluids and the vessel wall. This layer of cells contain many ion channels and other proteins that allow passage of molecules from the circulating fluids to the vessel, as well as the release of different chemicals in reaction to changes within the vessel

Exogenous substances are those that are introduced to an organism from the outside. These can be substances that the body already produces, or completely foreign substances

G-protein coupled receptors can respond to many different stimuli including pressure, temperature, or chemicals. Once stimulated, they elicit a cascade of events that amplify the response to the signal, to speed and spread the response

Hyperlipidemia is the condition in which the body has an elevated level of "lipids," more commonly known as fats

Hypertension is a term that signifies "high blood pressure," it is a common medical condition that puts stress on the blood vessels, as the high pressure of flow through the vessels over time can cause damage and stretching. If untreated, it can lead to cardiovascular disease such as coronary artery disease

Insonation angle is the angle at which the ultrasound probe meets the blood vessel. It must be maintained within a strict range in order to accurately represent the flow through the blood vessel

Mechanoreceptors are specialized receptors that can respond to mechanical pressure or deformation. These receptors, like the ones in the skin that feel "touch," are physically altered in their shape by pressure, and this physical change elicits a cascade of events such that the brain interprets the signal and the body reacts accordingly

Pathological is a medical term meaning illness or dysfunction of a certain part of system

Vasoconstriction is a term that means the narrowing of a blood vessel by the contraction of the smooth muscle lining the vessel. This is also an important function of the vessel, as it increases the pressure in the vessel as needed for postural changes and during exercise in order to redirect blood flow to tissues that need nutrients

Vasodilate means the increase in the diameter of a blood vessel when the smooth muscle that surrounds the vessel relaxes, allowing for expansion of the blood vessel. Vasodilation can occur in response to many different stimuli, and is an important vessel function that protects the lining of the vessel from dangerous increases in blood or lymphatic flow through the vessel

Appendix ii

Institutional Review Board approved Consent Form document

INFORMED CONSENT FORM

TITLE: "A Means for Assessing Vascular Health in Women: A Critical Analysis of Three Relevant Techniques" **Protocol # 02012013.001**

INVESTIGATOR: Erin Madison, Dr. C.T. Minson and Colleagues

APPROVED BY INSTITUTIONAL REVIEW BOARD:

This is an important form. Please read it carefully. It tells you what you need to know about this study. If you agree to take part in this research study, you need to sign the form. Your signature means that you have been told about the study and what the risks are. Your signature on this form also means that you want to take part in this study.

You are invited to participate in a research study conducted by Erin Madison, an undergraduate student supervised by Dr. Christopher Minson from the University of Oregon, Department of Human Physiology. We will use this data to develop an honors thesis. You were selected as a possible participant in this study because you are a healthy young woman who meets the specific criteria for investigating effects of hormones on blood vessel function.

Why is this study being done?

The number one cause of death in the United States is cardiovascular disease (CVD). Thus, the aim of the study is to assess three different techniques that are currently used to measure vascular health, the results of which could be supportive of implementing one of these techniques in the clinical setting in order to better assess cardiovascular risk.

This project seeks to compare three early CVD detection techniques in women that could be used clinically. These tests (a handgrip exercise, passive leg tilt, and flow-mediated dilation) are all proposed to assess the ability of blood vessels to relax (or dilate), termed "endothelial function". The goal of this study is compare and contrast the sensitivity of each test, as we already know that endothelial function changes across the menstrual cycle. We hope to determine if these tests could be used interchangeably.

What will happen in the study?

1. If you meet all the initial subject criteria (based on the initial screening form) and are interested in participating in the study we will schedule an appointment with you to meet with one of the investigators of the study to discuss the project, to see the laboratory, and to read this form. The visit should last about 30 minutes. Your height and weight and resting blood pressure will be measured, and you will be asked questions about your health history.

2. You will then return to Dr. Minson's laboratory to participate in the experimental protocol on three separate testing days across the course of a month. Each day of testing will take approximately 3 hours. You will be asked to refrain from alcohol, caffeine, exercise, food, and vitamin C for 10 hours prior to the start of each study day. In addition you will be asked to refrain from all over-the-counter medications (such as aspirin, ibuprofen, or allergy medication) for 24 hours prior to the start of the study. If you were unable to refrain from these substances/activities you will not be able to participate in the study.

3. Each study day you will be asked to wear a loose-fitting T-shirt and athletic shorts with an elastic waistband, such that the investigators have easy access to the two arteries that will be studied each study day

4. Prior to starting the study each day, you will need to have a negative pregnancy test (meaning that you are not pregnant). This will require you to collect a sample of urine and have it tested by one of the female investigators. If the test is positive (meaning that you may be pregnant), you will not be allowed to participate in the study.

5. After a negative pregnancy test is observed we will prep you for the study by placing 5 sticky electrodes on your skin and attaching a small wire or lead to each electrode. These leads will be attached to a monitor that will allow us to measure your heart rate and heart rhythm. These electrodes will be placed on your skin by female lab personnel. The electrodes will be placed on your body in the following locations: 2 electrodes are placed on your upper chest close to your shoulder (about where your bra strap goes, one on the left and one on the right); 2 electrodes will be placed on your stomach just above your hip bones (about where your pants line is) on the left and right side; and one will be placed just below your left breast, approximately where the bottom of your bra is.

6. Prior to each study day, you will have 1 small flexible needle (these are called "intravenous catheters", and are smaller than the lead of a pencil) placed into a vein near your elbow. The skin will be sterilized before this procedure. This will remain in your skin throughout the study. We will take about 2 teaspoons of blood (about 1 oz) from your vein so that we can measure your hormone levels. The vials in which we collect the blood will be coded such that only the investigators can determine that the samples came from you and the time each sample was taken. No one else will be able to determine your identity from the sample. Once the study is completed and all samples are analyzed, any remaining or extra sample will be destroyed. Blood samples are not being collected for diagnostic purposes. The results will not be reviewed by a physician. However, if results fall outside of the normal range, you will be informed that you should consult your primary care physician for an additional medical evaluation, and you will receive a non-interpretive, paper copy of said hormone levels for use by your personal physician.

7. During the study time, we will periodically inflate a small cuff that is placed on your middle and ring fingers of one of your hands to measure your blood pressure (Nexfin device). We will only inflate this cuff on one finger for about 30 minutes at a time. If the cuff becomes uncomfortable, let the investigator know and they will turn it off for a few minutes.

8. Handgrip: Laying on the padded exam table, with your right arm at heart-level, you will perform a maximal voluntary contraction. The maximum contraction will be performed three times, lasting for no more than one second each, with a ten second break between each contraction. You will then begin a handgrip exercise at a resistance of 3 kg, squeezing at one voluntary contraction every two seconds. You will do this for three minutes. After three minutes, you will rest for one minute before beginning the next workload. During that minute of rest, we will use an ultrasound transducer probe to image your brachial artery to measure blood flow and artery diameter. You will then repeat the same exercise for a 6 kg and 9kg workload. There is a possible risk of discomfort during this protocol due to the repetitive contraction of small muscles, but this should not exceed that which can be experienced by a weight lifting exercise at the gym that focuses on the arms and leads to fatigue. This discomfort should subside shortly after completion of this protocol.

9. Passive Leg Tilt: Laying with your body on the padded exam table, and your legs supported on a rolling cart, we will inflate a cuff just under your right knee to restrict blood flow to the lower portion of your leg. There is possible risk of discomfort due to the continued inflation of this blood pressure cuff, which will occlude the vessels in your lower leg. This discomfort should be mild, such as that would be experienced if your foot fell asleep. If this discomfort becomes too great, please alert an investigator and the cuff will be deflated. We will be

using the ultrasound transducer probe to gain an image of your femoral artery (in your thigh area) to measure blood flow and artery diameter for one minute. We will then passively move your leg off of the table, until your knee is flexed at 90 degrees. We do not want you to assist us with your muscles at all. We will tilt your leg back and forth (from straight to bent, and back again) every two seconds for a period of two minutes. We will then have you rest, while we continue to use the ultrasound transducer probe to collect changes in your artery for two minutes. After this final minute of scanning, the cuff will be deflated, totaling a period of 5 minutes of lower-leg occlusion. If you assist us with the movement, or contract your muscles, we will stop the test, have you rest for 10 minutes, and begin the protocol again.

10. Flow-mediated Dilation: During the study you will have a blood pressure cuff placed around your forearm. We will position an ultrasound transducer probe on your upper arm (above your elbow) at the brachial artery. We will occlude blood flow to your arm by inflating the blood pressure cuff on your lower arm to 250 mmHg. There is possible risk of discomfort due to the occlusion of the forearm muscles. This discomfort should be mild, and only comparable to the lower portion of the arm falling asleep. However, this discomfort should be immediately alleviated by deflation of the cuff. If this discomfort exceeds a tolerable level, please alert the investigator so that the cuff can be deflated early. We will stop blood flow to your arm for a period of 5 minutes with a 20-minute recovery period between each blood flow occlusion. We will use the ultrasound transducer probe to image your brachial artery before and after the blood pressure cuff is inflated and released. This test may be repeated 1-2 times.

11. Nitroglycerine Administration: Lastly, we will administer nitroglycerin below your tongue and continue to image your brachial artery for 10 more minutes. You will continue to lie supine for 15 minutes and wait for you to return to baseline. This will cause your blood vessels to relax, and will slightly lower your blood pressure. This test causes the smooth muscle of your brachial artery blood vessel to relax and dilate allowing an increase in blood flow, and we call this test 'endothelium-independent vasodilation'.

12. You should notify the investigator immediately if you feel any significant discomfort or concern about your well being at any time during or after the study.

How long will I be in the study?

You will participate in this study over the course of one month. You will come into the lab for studies on three days, based around your menstrual cycle. One day will be within one to three days of when you begin menstruation. Another day will be during ovulation. To test for ovulation, we will need you to collect a sample of urine and use an ovulation prediction kit to test for an increase in luteinizing hormone. You will be given several ovulation prediction kits to use. Approximately 10-15 days after you begin menstruating (depending on the normal length of your menstrual cycle), you will begin taking the tests daily, until you get a positive result (indicating you are having a surge in luteinizing hormone, that corresponds with ovulation). You will then notify the subject contact person that you are ready for that study day (to occur within 1-2 days of a positive test). A third visit will be approximately 8-10 days after you ovulated.

Each study day will last approximately three hours, for a total of approximately 10 hours of participation (including the screening appointment).

What are the risks of the study?

1. Intravenous catheters: There may be some discomfort during the blood draw. Once the needle is in place, the pain should subside. After the blood draw, the needle will be withdrawn and a sterile dressing will be applied. Any swelling or redness after the study should be gone a few hours after completion of the study. Although the needles are sterile, there is a slight risk of infection at the site where the needle was placed in your skin. You will be instructed how to keep the areas clean for a day or two following the study. The most common complications of inserting a small needle into a vein is a small bruise and pain at the site of the needle location which may last several days after removal of the needle. A small amount of bleeding may occur directly after removal of the catheter. Application of pressure and a gauze dressing will alleviate the bleeding.

2. Finger blood pressure: In some people, this blood pressure cuff causes their finger to become red and uncomfortable after a long period (over 40 minutes). We will only inflate the cuff for 15 minutes at a time and then give your finger a rest. If your finger becomes uncomfortable during the 15 minutes the cuff is inflated, let the investigator know and they will turn it off for a few minutes. There are no major risks associated with this device.

3. Occlusion by the blood pressure cuff: During both the Passive leg tilt and the Flow-mediated dilation procedures a blood pressure cuff will be inflated to super-arterial pressures, such that the arteries below the cuff will be occluded. The discomfort associated with arterial occlusion should be mild, at a level comparable to the lower leg or forearm falling asleep. This discomfort should be relieved nearly instantly following the deflation of the blood pressure cuff. However, if this discomfort exceeds a tolerable level, the subject will be instructed to inform the investigators such that the cuff can be prematurely deflated in order to relieve this discomfort.

4. Handgrip Exercise: Exercising with a handgrip dynamometer several minutes may cause discomfort in the forearm of the exercising arm. The sensations experienced during the handgrip exercise is relieved immediately upon letting go of the handgrip dynamonometer with no known persisting side effects beyond the discomfort experienced while contracting the muscle. Handgrip exercise is popularly used within research protocols with no reported adverse side effects. The risk level is similar to that of a light weight lifting exercise performed at a gym.

5. Nitroglycerin administration: We will administer the standard dose ~~a low dose~~ of nitroglycerin under your tongue. This drug has minimal risk when used in low doses. Nitroglycerin is commonly prescribed to prevent and treat angina pectoris (chest pain), a condition occurring from constriction in the arteries of the heart. Nitroglycerin is a potent blood vessel dilator, causing the artery to open up and increase blood flow. You may feel slight light-headedness or dizziness, but this symptom should not last more than a few minutes. Please inform your investigator if these feelings persist during the study and do not simply come and go. You may feel your heart rate slowing as this drug causes a drop in blood pressure (transient hypotension), but this will also last only a few minutes. After nitroglycerin administration, we will take ultrasound images for several minutes to monitor changes in your blood flow and vessel size. You will remain laying down on your back for an additional 15 minutes to insure that your blood pressure and heart rate are back to their normal values before we allow you to leave the study area. There is a risk that you may experience a headache after this treatment, and if you are predisposed, you may experience the onset of a migraine headache.

6. Emergencies: In the event of an emergency, you will be transported by ambulance to a local emergency facility.

May I participate if I am pregnant or breast-feeding?

This study is based off studying women when they are normally menstruating. Therefore, pregnant and/or breast-feeding mothers are not eligible for participation at this time. You must have a negative pregnancy test no more than 24 hours before each study day. If the pregnancy test is positive (meaning that you are pregnant), you will not be able to take part in the study. There is no cost for the pregnancy test.

Are there benefits to taking part in this study?

This study will not make your health better.

What other choices do I have if I don't take part in this study?

This study is only being done to gather information. You may choose not to take part in this study.

What are the costs of tests and procedures?

You will not need to pay for any tests or procedures that are done just for this research study. You will receive $10/hour each study day. This money is for the inconvenience and time you spent in this study. If you start the study but stop before the study has ended, you will receive money for the part of the study you have participated in.

With full participation, we anticipate that you will receive $100 total for your participation.

Who can answer my questions?

You may talk to Dr. Christopher Minson at any time about any question you have on this study. You may contact Dr. Minson by calling (541) 346-4105. You may also contact Erin Madison by calling (541) 346-4507.

What are my rights if I take part in this study?

Taking part in this research study is your decision. You do not have to take part in this study, but if you do, you can stop at any time. Your decision whether or not to participate will not affect your relationship with The University of Oregon.

You do not waive any liability rights for personal injury by signing this form. All forms of medical diagnosis and treatment whether routine or experimental, involve some risk of injury. In spite of all precautions, you might develop medical complications from participating in this study.

The University of Oregon is not able to offer financial compensation nor absorb the costs of medical treatment should you be injured as a result of participation in this research. If such complications arise, the researchers will assist you in obtaining appropriate medical treatment that will be provided at the usual charge.

The investigators may stop you from taking part in this study at any time if it is in your best interest, if you do not follow the study rules, or if the study is stopped. If you are physically injured because of the project, you and your insurance company will have to pay your doctor bills. If you are a UO student or employee and are covered by a UO medical plan, that plan might have terms that apply to your injury.

If you experience harm because of the project, you can ask the state of Oregon to pay you. If you have been harmed, there are two University representatives you need to contact. Here are their addresses and phone numbers: General Counsel, Office of the President, 1226 University of Oregon, Eugene, OR 97403-1226, (541) 346-3082, and Research Compliance Services, 5237 University of Oregon, Eugene, OR 97403-5237, (541) 346-2510. A law called the Oregon Tort Claims Act limits the amount of money you can receive from the State of Oregon if you are harmed.

Following completion of the study, we will invite all participants in the study to return to the lab at a specified date to discuss any new findings as a result of this study. If you are not able to attend on that date but are interested in our findings, we will send you a letter describing the results and new findings from this study.

What about confidentiality?

Any information that is obtained in connection with this study and that can be identified with you will remain confidential and will be disclosed only with your permission. Subject identities will be kept confidential by assigning you a "subject identification number". The names associated with each subject identification number will be kept in a locked file cabinet in Dr. Minson's office.

I have had an opportunity to have my questions answered. I have been given a copy of this form. I agree to take part in this study.

If you have questions regarding your rights as a research subject, contact Research Compliance Services, 5237 University of Oregon, Eugene, OR 97403-5237, (541) 346-2510.

Your signature indicates that you have read and understand the information provided above, that you willingly agree to participate, that you may withdraw your consent at any time and discontinue participation without penalty, that you will receive a copy of this form, and that you are not waiving any legal claims, rights or remedies.

_____ _____ Please print: _____

(Date) (Signature of Participant)

_____ _____

(Date) (Signature of Individual Obtaining Consent)

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