HIGH ANTIOXIDANT AND ANTI-INFLAMMATORY LOAD IMPROVES
NON-NITRIC OXIDE-MEDIATED CUTANEOUS MICROVASCULAR FUNCTION
IN REPRODUCTIVE-AGED HEALTHY WOMEN

A Thesis in
Kinesiology
by
Virginia G. Content

© 2023 Virginia G. Content

Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Master of Science

December 2023
The thesis of Virginia G. Content was reviewed and approved by the following:

Lacy M. Alexander  
Professor of Kinesiology  
Thesis Advisor  

W. Larry Kenney  
Professor of Physiology and Kinesiology  

Alison D. Gernand  
Associate Professor of Nutritional Sciences  

Jonathan B. Dingwell  
Professor of Kinesiology  
Kinesiology Graduate Program Director
ABSTRACT

The human cutaneous circulation is utilized as an in vivo bioassay to examine mechanisms of systemic vascular function and dysfunction in health and disease. Local drug infusions (e.g., atorvastatin) and systemic interventions [e.g., inhibition of transcriptional regulators (NF-κB)], are approaches to investigate mechanisms underlying microvascular dysfunction that mediate accelerated cardiovascular disease risk. However, in the absence of disease, a high antioxidant and/or anti-inflammatory load can impact redox mechanisms, resulting in altered endothelial function as measured by reduced nitric oxide (NO)-dependent vasodilation.

The purpose of this thesis was to measure endothelial microvascular function during high antioxidant and anti-inflammatory load in healthy reproductive-aged women. In a randomized, single-blind, placebo-controlled design, eleven women were randomly assigned a placebo and a 5-day oral salsalate (1500mg, twice daily) intervention. Participants underwent graded intradermal microdialysis with the endothelium-dependent agonist acetylcholine (ACh: $10^{-10}$ – $10^{-1}$ M, 33 °C) alone and in combination with the following: 15 mM $N^G$-nitro-L-arginine methyl ester [L-NAME; non-selective NO synthase (NOS) inhibitor], 0.02 mM Atorvastatin [statin; lectin-like oxidized low-density lipoprotein receptor 1 (LOX-1) inhibitor], and L-NAME + statin (combo). Laser-doppler flux was measured over each microdialysis site, cutaneous vascular conductance was calculated as flux divided by mean arterial pressure, and normalized to site-specific maximum (CVC$_{\%}$max: 28 mM sodium nitroprusside, 43 °C). Compared to placebo, salsalate treatment increased CVC$_{\%}$max at both the control and NOS-inhibited sites. NO-dependent vasodilation was not different between placebo and salsalate trials ($p=0.70$). During placebo, localized statin did not alter the CVC$_{\%}$max response ($p=0.66$); however, during salsalate trials, statin treatment mitigated the increase in CVC$_{\%}$max ($p=0.004$), but improved NO-dependent vasodilation ($p=0.02$). These data suggest that NF-κB-inhibition with salsalate improved
endothelial function in healthy women through non-NO-dependent mechanisms. Statin administration did not alter endothelial function during placebo conditions but mitigated the increase in non-NO-dependent vasodilation induced by salsalate. These data demonstrate that anti-inflammatory treatments, such as inhibition of NF-κB activation, can improve cutaneous microvascular function in reproductive-aged healthy women via non-NO-dependent mechanisms.
# TABLE OF CONTENTS

LIST OF FIGURES .................................................................................................................. vi

LIST OF TABLES .................................................................................................................. vii

ACKNOWLEDGEMENTS ................................................................................................. viii

Chapter 1  INTRODUCTION ............................................................................................... 1

  Background and Significance ......................................................................................... 1
  Evaluation of Endothelial Function in Humans ............................................................. 3
  Antioxidants and Endothelial Function ......................................................................... 4
  Nuclear factor-κB and Endothelial Function ................................................................. 5
  Previous Findings ......................................................................................................... 5
  Summary ....................................................................................................................... 7

Chapter 2  METHODS ........................................................................................................ 8

  Participants ................................................................................................................... 8
  Microvascular Function Assessment .......................................................................... 9
  Systemic Salsalate Intervention ............................................................................... 10
  Data and Statistical Analysis .................................................................................. 10

Chapter 3  RESULTS ......................................................................................................... 12

Chapter 4  DISCUSSION ................................................................................................... 15

  Systemic Salsalate .................................................................................................... 15
  Localized Statins .................................................................................................... 17
  Further Considerations .......................................................................................... 17
  Summary and Conclusions .................................................................................... 18

BIBLIOGRAPHY ............................................................................................................... 19

Appendix: Informed Consent .................................................................................... 26
LIST OF FIGURES

Figure 1-1: Signaling pathway of Nitric Oxide (NO)-cyclic guanosine monophosphate (cGMP) in the vascular smooth muscle. BH4, tetrahydrobiopterin; eNOS, endothelial nitric oxide synthase; L-arg, L-arginine; NO, nitric oxide; ONOO-, peroxynitrite; L-orn, L-ornithine; GTP, guanosine triphosphate; sGC, soluble guanylyl cyclase; PKG, protein kinase G. Created by Biorender.com.

Figure 3-1: Acetylcholine (ACh)-induced vasodilation during co-infusion of Ringer's (Control, circles) or NOS-inhibition (L-NAME, squares) in healthy women (n = 11) before (Pl, closed symbols) and after (Sal, open circles) five days of oral salsalate treatment (A). Although not significant, nitric oxide (NO)-contribution to ACh-induced vasodilation increased after salsalate treatment (B). Data are mean ± standard error. *p < 0.001 Placebo-Control vs. Placebo-L-NAME; *p < 0.05 Salsalate-Control vs. Salsalate-L-NAME.

Figure 3-2: Acetylcholine (ACh)-induced vasodilation during co-infusion of Ringer's (Control, circles), atorvastatin (statin, up triangles), or L-NAME + atorvastatin (Combo, down triangles) in healthy women (n = 11) before (Pl, closed symbols, A) and after (Sal, open symbols, B) five days of oral salsalate treatment. Local statin treatment mitigated the salsalate-induced increase in endothelial function, but improved NO-dependent vasodilation (n = 10, C). The dotted and dashed lines indicate average NO-contribution without statin treatment in salsalate and placebo (derived from Figure 3-1B), respectively. Data are mean ± standard error. *p < 0.05 vs. Placebo-Control; *p < 0.05 vs. Salsalate-Control.
LIST OF TABLES

Table 1-1: Summary of human vascular physiology studies that have utilized antioxidant/anti-inflammatory interventions. Up arrows (↑) indicate improvement and down arrows (↓) indicate impairment in the mechanism of interest following the intervention.................................................................6

Table 3-1: BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; CHO, cholesterol; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol; IUD, intrauterine device; SSRIs, selective serotonin reuptake inhibitors. Data are mean (standard deviation)........................................................................................................12
ACKNOWLEDGEMENTS

To Dr. Lacy Alexander, thank you for being a guiding light as I made my way through my thesis work. Being in your presence has continued to shape my critical thinking, and through this, I know I will thrive as a future independent researcher as I trust the process.

To Dr. Larry Kenney, thank you for the remarkable guidance and mentorship you have provided me. You inspire me to seek nothing short of exceptional. I know that will serve me well as I progress in my education, career, and life.

To Dr. Alison Gernand, thank you for your feedback and comments that have allowed further improvement of my thesis. I am grateful for the time and efforts you have put forth in assisting me to complete my thesis.

To Sue Slimak, thank you for all the time that you took to help and make the lab run smoothly. I truly appreciate it!

To Auni Williams, thank you for being a great lab mate and friend. The transition from my postbaccalaureate program to graduate school was seamless because of you. Thank you for the laughs we shared in the lab and for helping me grasp things from items on the top shelf to physiology.

Finally, thank you to my family, friends, and community who have continuously believed in me in all my endeavors. From the random phone calls and texts that tell me I can and will go far, I am forever grateful for you all.
This work was supported by NIH Grant R01 HL161000 (LMA). The opinions or assertions contained within this thesis are the private views of the authors and should not be construed as official or reflecting the views of the Pennsylvania State University or the National Institutes of Health.
Chapter 1

INTRODUCTION

Background and Significance

Reactive oxygen species (ROS) are the product of molecular oxygen being reduced (i.e., gain of electrons), containing a minimum of one oxygen atom and one unpaired electron. Common examples of ROS include superoxide anion (O$_2^-$) and hydroxyl radical (HO-) (1, 2). The sources of ROS include peroxisomes, cytoplasm, and mitochondria. Specifically, the mitochondria produces the largest percent of ROS due to oxidative metabolism (3, 4). ROS are critical for cell signaling, proliferation, and survival, but an imbalance of ROS production without effective antioxidant responses leads to pathological oxidative stress (1, 5). Oxidative stress has been implicated in the development of endothelial dysfunction, which involves the impairment of vascular function and potentially the development of cardiovascular diseases (CVDs) (6).

The vascular endothelium is composed of endothelial cells that serve several functions that regulate vascular tone, growth, cell proliferation, and angiogenesis (7). The endothelium produces a variety of autocrine and paracrine signaling mediators (8), including nitric oxide (NO). NO is a vasoprotective gasotransmitter that plays an integral role in mediating the relaxation of the vascular smooth muscle. Using L-arginine as a substrate, endothelial NO synthase (eNOS) synthesizes NO in endothelial cells, where it requires co-factors such as tetrahydrobiopterin (BH$_4$). Further, NO synthesis rates are amplified after Ca$^{2+}$/calmodulin binding (8–11). NO then diffuses into the vascular smooth muscle where it activates soluble guanylyl cyclase (sGC) and promotes the formation of cyclic guanosine monophosphate (cGMP) from guanosine triphosphate (GTP). As shown in Figure 1-1, the presence of cGMP induces
relaxation of the vascular smooth muscle (i.e., vasodilation) due to activation of protein kinase G (PKG) and reduced concentration of intracellular calcium (12).

Figure 1-1. Signaling pathway of Nitric Oxide (NO)-cyclic guanosine monophosphate (cGMP) in the vascular smooth muscle. BH4, tetrahydrobiopterin; eNOS, endothelial nitric oxide synthase; L-arg, L-arginine; NO, nitric oxide; ONOO-, peroxynitrite; L-orn, L-ornithine; GTP, guanosine triphosphate; sGC, soluble guanylyl cyclase; PKG, protein kinase G. Created by Biorender.com.

Endothelial dysfunction is characterized by pro-inflammatory/coagulant properties and dysregulation of vascular tone resulting from reduced NO bioavailability and increased production of both vasoconstrictors and reactive oxygen species (8). Endothelial dysfunction predates the onset of overt CVD and occurs early in the pathological vascular remodeling that occurs in hypertension and diabetes (13–17).
Evaluation of Endothelial Function in Humans

Flow-mediated dilation (FMD) is a non-invasive method used to examine endothelial function in humans (18–22). This technique is often completed in large peripheral conduit arteries (e.g., brachial artery) to examine changes in diameter in response to blood flow-associated shear stress (e.g., cuff inflation) (23). Following cuff inflation, shear stress initiates the release of various vasodilators, such as NO, prostaglandins (PGI₂), and endothelium-derived hyperpolarizing factors (EDHFs) (24–27). FMD is predictive of future cardiovascular (CV) events, as each percent point increase in brachial artery FMD is an 8-13% lower risk of CV events (28, 29). In addition, FMD has been utilized to test the efficacy of therapies (e.g., exercise) on endothelial function (30, 31). To reduce variability, guidelines have been published and utilized to promote comparability between studies (32, 33).

Impairments in microcirculatory function can predate detectable endothelial dysfunction in the conduit circulation. Thus, there is considerable interest in examining mechanisms underlying microcirculatory dysfunction associated with traditional and non-traditional cardiovascular disease risk factors prior to the onset of overt organ damage (34). Direct infusion of pharmacological agents, such as acetylcholine (i.e., endothelium-dependent agonist), are used to delineate mechanisms mediating endothelial dysfunction. However, arterial infusion studies are invasive and costly. They also require several experimental trials if multiple signaling pathways are being tested and appropriate blinding and washout are often not feasible.

Intradermal microdialysis is a minimally invasive technique that is also used to assess mechanisms underlying endothelial dysfunction in cohorts of patients with accelerated CVD risk (35). Intradermal microdialysis has several technological and experimental advantages including, 1) the ability to perfuse low concentrations of pharmacological agents targeted to specific vascular signaling pathways without having a systemic impact, 2) several microdialysis probes can be inserted for a single experimental trial and multiple pharmacological agents can be
perfused simultaneously, 3) skin blood flow can be measured in real-time using laser-Doppler flowmetry in line with localized pharmacological agent perfusion, and 4) the interstitial milieu can be sampled through analysis of the dialysate.

**Antioxidants and Endothelial Function**

Acute antioxidant administration has been used to examine NO metabolism and mechanisms underlying endothelial dysfunction. For example, the powerful antioxidant ascorbic acid improves endothelial function, and more specifically, NO-dependent vasodilation (36, 37). Ascorbic acid quenches the free radical of molecular oxygen that readily reacts with NO. Additionally, like other powerful antioxidants, they serve as molecular stabilizers for the essential co-factors (e.g., BH₄) for NO production (38). In human vascular physiology studies, administered high doses of ascorbic acid improves endothelial function in cohorts with traditional and non-traditional cardiovascular disease risk factors (36, 39).

In addition to ascorbic acid, 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors (statins) also have potent anti-inflammatory and anti-oxidative properties. Statins bind to HMG-CoA reductase and alter the conformation of the enzyme, rendering the enzyme non-functional. This prevents the conversion of HMG-CoA to mevalonic acid, which has a key role in the synthesis of cholesterol (40). When used systemically, this class of drugs inhibit the last phase of cholesterol synthesis in the liver. However, the cardiovascular benefits of statins cannot be completely explained solely by the reduction in low-density lipoproteins. The pleotropic properties of statins include 1) their abilities to directly inhibit the uptake of cholesterol by the low-density lipoprotein receptor on vascular cells, 2) inhibition of the production of free radicals by cytosolic and mitochondrial sources, and 3) direct stabilization of eNOS (40–42). In vascular studies, statin interventions improve endothelial function in a variety of clinical cohorts with elevated CVD risk (43–45).
**Nuclear factor-κB and Endothelial Function**

Nuclear factor-κB (NF-κB), a transcription factor, is a ubiquitous intracellular signaling mediator involved in inflammation. NF-κB is bound to IκB protein when it is inactive. In the presence of cytokines and reactive oxygen species, the IκB kinase (IKK) complex is activated, which enables a liberated NF-κB to enter the nucleus and target gene expression (46–48). Oral salsalate (i.e., salicylate) given at high doses over a short period of time (5 days) has been utilized experimentally to inhibit the NF-κB pathway. Salsalate specifically binds to the subunit of the IKK complex known to phosphorylate IκB, IKK-β. This inhibits the IKK-β activity and thus prevents the translocation of NF-κB to the nuclear domain and remains in the cytosol (49, 50). In a variety of studies, this approach has been used to reduce inflammation of the vascular endothelium. Importantly, this drug does not inhibit the synthesis of other endothelium-dependent vasodilators including prostacyclins through the cyclooxygenase pathway (51, 52). Systemic administration of oral salsalate improved vascular endothelial function in middle-aged and older humans with obesity/overweight (53). Microvascular function also improved with salsalate treatment in young adults with non-traditional cardiovascular disease risk factors (54–56).

**Previous Findings**

Most studies examining endothelial function focus predominantly on the vascular disease pathology. However, several studies have documented attenuated endothelial function in healthy control subjects following interventions targeting cytosolic and transcriptional regulators of oxidant stress. In both the conduit arteries and microcirculation, ascorbate supplementation attenuated endothelium-dependent vasodilation in healthy control subjects (36, 37). Similarly, oral salsalate modestly reduces NO-dependent vasodilation in the microcirculation in healthy young adults. **Table 1-1** summarizes the human vascular physiology studies that have utilized
administration of various interventions to interrogate mechanisms underlying vascular
dysfunction. Across the vascular tree, from large elastic arteries to the microcirculation, there is a
consistent finding of improved endothelial function in clinical cohorts following intervention that
reduce oxidant and/or inflammatory processes. However, following similar interventions,
endothelial function is impaired in healthy control groups.

<table>
<thead>
<tr>
<th>Citation</th>
<th>Clinical Cohort</th>
<th>Intervention</th>
<th>Mechanism of Interest</th>
<th>Cohort</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moreau et al.,</td>
<td>Postmenopausal Status</td>
<td>Ascorbic acid (Systemic)</td>
<td>Large elastic artery compliance</td>
<td>↑</td>
</tr>
<tr>
<td>2005</td>
<td></td>
<td></td>
<td></td>
<td>↓</td>
</tr>
<tr>
<td>Moreau et al.,</td>
<td>Sedentary Postmenopausal Status</td>
<td>Ascorbic acid (Systemic)</td>
<td>Arterial compliance, Beta-stiffness index</td>
<td>↑</td>
</tr>
<tr>
<td>2006</td>
<td></td>
<td></td>
<td></td>
<td>↓</td>
</tr>
<tr>
<td>Moreau et al.,</td>
<td>Postmenopausal Status</td>
<td>Ascorbic acid (Systemic)</td>
<td>Femoral blood flow, vascular conductance</td>
<td>↑</td>
</tr>
<tr>
<td>2007</td>
<td></td>
<td></td>
<td></td>
<td>↓</td>
</tr>
<tr>
<td>Holowatz, L. A., &amp; Kenney, W. L., 2007</td>
<td>Hypertension</td>
<td>L-ascorbate (Local)</td>
<td>Endothelium-dependent dilation</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↓</td>
</tr>
<tr>
<td>Greaney et al.,</td>
<td>Major Depressive Disorder</td>
<td>Salicylate (Systemic)</td>
<td>Endothelium-dependent dilation</td>
<td>↑</td>
</tr>
<tr>
<td>2022</td>
<td></td>
<td></td>
<td></td>
<td>↓</td>
</tr>
<tr>
<td>Dillon et al.,</td>
<td>Endometriosis</td>
<td>Atorvastatin (Local and Systemic)</td>
<td>Endothelium-dependent dilation</td>
<td>↑</td>
</tr>
<tr>
<td>2022</td>
<td></td>
<td></td>
<td></td>
<td>↓</td>
</tr>
</tbody>
</table>

Table 1-1. Summary of human vascular physiology studies that have utilized antioxidant/anti-inflammatory interventions. Up arrows (↑) indicate improvement and down arrows (↓) indicate impairment in the mechanism of interest following the intervention.
Summary

Elucidating the mechanisms underlying endothelial dysfunction is important for the development of therapeutic strategies to reduce cardiovascular morbidity and mortality. Appropriately controlled studies in human vascular physiology also include a healthy cohort for comparative purposes. Interventions including administration of high doses of antioxidants and inhibition of transcriptional regulatory factors may have deleterious effects in healthy control subjects. The study included within this thesis aimed to examine the microvascular function during high antioxidant and anti-inflammatory load resulting from combined systemic NF-κB inhibition and localized atorvastatin treatment in healthy women. We hypothesized that combined systemic NF-κB inhibition and localized atorvastatin treatments would attenuate cutaneous microvascular function through NO-mediated cutaneous microvascular function in reproductive-aged healthy women.
Chapter 2

METHODS

Participants

This was a randomized, single-blind, placebo-controlled design study and as a component of a larger clinical trial, *Mechanisms and interventions addressing accelerated cardiovascular disease risk in women with endometriosis* (R01 HL161000-01, NCT05069740). All experimental procedures were approved in advance by the Institutional Review Board at the Pennsylvania State University. A Food and Drug Administration Investigational Drug Number was obtained for all protocols (IND 78,954). Oral and written consents were obtained voluntarily from all subjects before participation and in accordance with the guidelines set forth by the Declaration of Helsinki. All testing was conducted in Noll Laboratory at the Pennsylvania State University.

Eleven healthy women were screened by clinical staff for neurological, cardiovascular, metabolic, or dermatological diseases. Screening included a physical examination, medical health history questionnaire, and a blood chemistry analysis (Chem 24, Quest Diagnostics, Pittsburgh, PA). Inclusion criteria included: 1) 18-45 years of age, 2) body mass index < 40, 3) non-hypertensive (<140/<90 mmHg), 4) non-diabetic (HbA1c < 6.5%), 5) Total cholesterol (CHO) < 200 mg/dL, 6) high-density lipoprotein (HDL) ≥ 50 mg/dL, 7) low-density lipoprotein (LDL) < 100 mg/dL, and 8) Triglycerides < 150 mg/dL. Racial identification was self-reported and participants identified themselves and both their biological parents. Participants were non-smokers and were not taking prescription medications that could alter peripheral vascular control. None of the participants were pregnant or breastfeeding at the time of participation. All women
were premenopausal. Women were tested without regard to menstrual cycle or oral contraceptive pill phase.

**Microvascular Function Assessment**

To assess microvascular function, all participants underwent a cutaneous microdialysis experiment. Participants were instructed to refrain from alcohol and caffeine for 12 hours and strenuous exercise for 24 hours before the experiment. Four intradermal microdialysis fibers (10 mm, 55 kDa, CMA Linear 31 probe, Harvard Apparatus, Holliston, MA) were inserted into the intradermal layer of the ventral aspect of the left forearm for local delivery of pharmacological agents, as previously described in detail (57–59). Fibers were randomized with one of the following interventions: lactated Ringer’s (control), 15 mM N\textsuperscript{G}-nitro-L-arginine methyl ester [L-NAME; non-selective NO synthase (NOS) inhibitor; Calbiochem, EMD Millipore, Billerica, MA], 0.02 mM Atorvastatin [statin; lectin-like oxidized low-density lipoprotein receptor 1 (LOX-1) inhibitor; USP, Rockville, MD], and L-NAME + statin (combo). All agents were perfused through microdialysis fibers at a rate of 2 µL/min (Bee Hive controller and Baby Bee microinfusion pumps; Bioanalytical Systems, West Lafayette, IN).

After the placement of microdialysis fibers, 60-90 min was allowed for hyperemia resolution. During the hyperemia resolution phase, site-specific pharmacological agents were perfused to allow for a drug wash-in phase. Laser-Doppler flowmeter probes, used to measure local red blood cell flux, were placed in local heating units (VP12 and VHP2; Moor Instruments, Wilmington, DE) set to a thermoneutral 33 °C directly over each microdialysis site. Following baseline measurements, increasing concentrations of acetylcholine (ACh; endothelium-dependent vasodilator; USP, Rockville, MD) from $10^{-10}$ to $10^{-1}$ M were co-perfused with the control,
L-NAME, statin, and combo agents sequentially for 5 min each. At the conclusion of the dose-response protocol, maximal cutaneous vasodilation was induced by increasing local skin temperature to 43 °C and perfusing 28 mM sodium nitroprusside (USP, Rockville, MD). Automated blood pressure (Connex Spot Monitor, Welch Allyn, Skaneateles Falls, NY) was measured every 5 min throughout the protocol.

**Systemic Salsalate Intervention**

Participants were randomly assigned oral salsalate (1500mg, twice daily) or placebo treatments for 5 days, in a randomized, single-blind, crossover design. Participants began treatment with the first dose on the evening of day 1 and the last dose the morning of the experimental visit (day 5). There was a minimum of a 14-day washout period to minimize any carryover effect. This regimen increased serum salicylate concentrations to the therapeutic range (10–30 mg/100 mL) and reduces total and NF-κB in vascular endothelial cells (53). Serum salicylate concentrations were measured on days 3 and 5 of the salsalate treatment. Participants were monitored for any signs or symptoms of toxicity (e.g., tinnitus, nausea). One subject had serum salicylate concentrations in the toxic range on day 5. Data from the eleven healthy women who completed both pre- and post-treatment experimental visits are included.

**Data and Statistical Analysis**

Red blood cell flux (perfusion units) data were recorded at 1000 Hz and stored for offline analysis (Powerlab and LabChart, ADInstruments, Bella Vista, NSW, Australia). Average values for red blood cell flux were obtained during 5 min of baseline, during the last minute of each dose, and at maximum. Cutaneous vascular conductance (CVC) was calculated as red blood cell
flux divided by mean arterial pressure, normalized as a percentage of the site-specific maximum (CVC-%max). Area under the dose-response curve (AUC) was calculated using the trapezoid rule (Prism v10.0, GraphPad Software, La Jolla, CA). The NO-dependent contribution was calculated as the difference between the AUC of the control and the L-NAME sites. The NO-dependent contribution, following statin treatment, was calculated as the difference between the AUC of the statin and combo sites. All data were analyzed using two- or three-way, mixed-model, repeated-measures analysis of covariance (SAS, v. 9.4; Cary, NC), with post-hoc Tukey-Kramer corrections applied to account for multiple corrections. Text and table results are presented as mean (SD). Significance was set at α < 0.05. Figure results are presented as mean ± SE for visual clarity of main effects.

An a priori power analysis indicated that a minimum of 10 subjects would be sufficient to detect a meaningful physiological difference of 15% in CVC-%max between treatments (α = 0.05, β = 0.80). The repeated measures design provided additional power in determining the impact of local statin and systemic NF-κB-inhibition on microvascular function. Outliers were defined using the Grubbs’ Test for Outlier (Prism v10.0, GraphPad Software, La Jolla, CA).
Chapter 3

RESULTS

Participant characteristics are presented in Table 3-1. Oral salusalte increased plasma salicylate to therapeutic concentrations in the healthy women. Plasma salicylate was in the therapeutic range on day 3 [16 (6) mg/dL] and day of experimental visit during salusalte treatment [19 (5) mg/dL, \( p=0.024 \)], except in one participant that exceeded therapeutic range on day 5. High-sensitivity C-reactive protein (hs-CRP) concentrations were [1.8 (1.1) mg/L] during placebo and [1.5 (1.1) mg/L] during salusalte (\( p=0.052 \)).

Table 3-1. Participant Characteristics.

<table>
<thead>
<tr>
<th>Mean (SD)</th>
<th>Oral Contraceptives</th>
<th>IUD/Implant</th>
<th>SSRIs</th>
<th>Regular Menstrual Cycles</th>
<th>Irregular Menstrual Cycles</th>
<th>With Children</th>
<th>Complicated Pregnancy</th>
</tr>
</thead>
<tbody>
<tr>
<td>( n ) (Caucasian/Latin)</td>
<td>11 (10/1)</td>
<td>4</td>
<td>3</td>
<td>1</td>
<td>8</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>34 (7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.6 (0.1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>63 (10)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m(^2))</td>
<td>24 (3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>100 (11)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>64 (7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>76 (8)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.0 (0.2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total CHO (mg/dL)</td>
<td>175 (26)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDL (mg/dL)</td>
<td>62 (11)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDL (mg/dL)</td>
<td>95 (25)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>87 (25)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; CHO, cholesterol; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol; IUD, intrauterine device; SSRIs, selective serotonin reuptake inhibitors. Data are mean (standard deviation).
**Figure 3-1** shows cutaneous vasodilatory (CVC\%\text{max}) responses to ACh in control and the NOS inhibited sites (L-NAME) following placebo and salsalate treatments (**Figure 3-1A**). There was an overall main effect of salsalate treatment ($p < 0.001$), with both the control and L-NAME sites shifted upwards (both $p < 0.05$). There was no change in the NO contribution following salsalate treatment (**Figure 3-1B**, $p=0.7028$).

![Graph](image)

**Figure 3-1.** Acetylcholine (ACh)-induced vasodilation during co-infusion of Ringer's (Control, circles) or NOS-inhibition (L-NAME, squares) in healthy women ($n = 11$) before (Pl, closed symbols) and after (Sal, open circles) five days of oral salsalate treatment (A). Although not significant, nitric oxide (NO)-contribution to ACh-induced vasodilation increased after salsalate treatment (B). Data are mean ± standard error. *$p < 0.001$ Placebo-Control vs. Placebo-L-NAME; *$p < 0.05$ Salsalate-Control vs. Salsalate-L-NAME.

**Figure 3-2** shows cutaneous vasodilatory (CVC\%\text{max}) responses to ACh in control, statin-treated and combo sites following placebo and salsalate treatments. There were no differences between the control vs statin sites during placebo (**Figure 3-2A**, $p=0.664$). There was a main effect of salsalate treatment in the statin site ($p < 0.001$). However, following the salsalate treatment, the statin-treated site was attenuated compared to the control site (**Figure 3-2B**, $p=0.0043$). When NOS was inhibited in combination with statin treatment (combo), there was no
effect of salsalate treatment of CVC\%_{\text{max}} (p=0.09). However, localized statin treatment did improve NO contribution (area under the curve) following salsalate treatment (Figure 3-2C, p=0.0245).

**Figure 3-2.** Acetylcholine (ACh)-induced vasodilation during co-infusion of Ringer's (Control, circles), atorvastatin (statin, up triangles), or L-NAME + atorvastatin (Combo, down triangles) in healthy women (n = 11) before (Pl, closed symbols, A) and after (Sal, open symbols, B) five days of oral salsalate treatment. Local statin treatment mitigated the salsalate-induced increase in endothelial function, but improved NO-dependent vasodilation (n = 10, C). The dotted and dashed lines indicate average NO-contribution without statin treatment in salsalate and placebo (derived from Figure 3-1B), respectively. Data are mean ± standard error. *p < 0.05 vs. Placebo-Control; #p < 0.05 vs. Salsalate-Control.
Chapter 4

DISCUSSION

This thesis aimed to measure endothelial function during high antioxidant and anti-inflammatory load in healthy reproductive-aged women. The major findings of this study include: 1) endothelial function was improved following systemic salsalate treatment and was not mediated by an increase in NO-dependent vasodilation, 2) during placebo conditions, local atorvastatin treatment did not alter endothelial function, 3) statin treatment mitigated the salsalate-induced increase in endothelial function, but improved NO-dependent vasodilation. This data suggests that NF-κB inhibition improved endothelial function in healthy women, most likely through non-NO-dependent mechanisms. Further, antioxidant exposure (statin) following salsalate treatment mitigated these improvements but enhanced NO-dependent vasodilation.

Systemic Salsalate

Oral salsalate (i.e., salicylate) was utilized to understand the role of systemic inflammation in endothelium-dependent dilation. This approach was pioneered by Seals and colleagues and has been adopted by other labs (53–55, 60). In the present study, 91% of the women reached therapeutic salsalate concentrations by day 3 and 91% of the women had plasma concentrations in the therapeutic range by day 5, as one subject exceeded therapeutic concentrations on day 5. In clinical studies examining cohorts with evidence of microcirculatory dysfunction, systemic inhibition of NF-κB improves NO-dependent vasodilation (54, 55). Contrary to our hypothesis, salsalate administration improved cutaneous microcirculatory function in a group of apparently healthy-reproductive aged women. This is evidenced by the overall upward shift in both the control site and the NOS-inhibited site following salsalate treatment.
There are several potential non-NO-dependent mechanisms that may have contributed to improvement in microcirculatory function following the salsalate intervention including (but not limited to) changes in the endothelin-1 system, cyclooxygenase/prostacyclin, and/or endothelium-dependent hyperpolarizing mechanisms. Endothelin-1 (ET-1) is a potent vasoconstrictor and known to be altered by reproductive hormones in young women (61). ET-1 mediates vasoconstriction through the ETA/B receptors on the vascular smooth muscle, but vasodilation through the ETB receptor on the endothelium. In the present data, because there was no difference in NO-dependent vasodilation, this would suggest that salsalate may have reduced the ET-1 vasoconstrictor balance versus changing ETB receptor mediated function in the endothelium. There is evidence in individuals with type 2 diabetes that one of the off-target effects of salsalate is enhancing insulin sensitivity (62). ET-1 has demonstrated to be involved in insulin resistance, where salsalate may have impacted ET-1 signaling and altered ETA/B receptor expression (63).

Of the other non-NO derived endothelium-dependent signaling pathways, it is unlikely that systemic salsalate treatment impacted the cyclooxygenase/prostacyclin system. Salsalate is a weak COX-1 inhibitor and this dosing regimen does not significantly inhibit COX-1 in endothelial cells (53). Further, had COX-derived signaling been inhibited, we would have likely observed an attenuation in non-NO-dependent vasodilation, not an increase.

Systemic NF-κB inhibition with salsalate may have increased endothelium-derived hyperpolarizing factors, including, but not limited to, hydrogen sulfide (H₂S) and hydrogen peroxide. H₂S is a third endogenous gasotransmitter that has vasoactive properties critical for vascular function (64). H₂S is produced enzymatically from the substrate cysteine by cystathionine-β-synthase (CBS), cystathionine-γ-lyase (CSE), 3-mercaptopuruvate sulfur transferase (3-MST) and cysteine aminotransferase (CAT) (65). The H₂S system is dynamic and responds to changes in inflammatory and oxidant stress mediated through NF-κB (66). H₂S -
mediated vasodilation is upregulated in habitually active older adults (67), thus it is possible that H$_2$S may account for the improved microcirculatory function in healthy women treated with salsalate.

**Localized Statins**

Statins are well known to have potent anti-oxidant and anti-inflammatory effects in the vasculature (41, 42, 68). To reduce its systemic effect as a cholesterol-reducing treatment, atorvastatin was perfused locally using intradermal microdialysis. Local perfusion of atorvastatin allows direct examination of the anti-oxidant properties of the drug on vascular function (68, 69). In our previous studies, we have found that local atorvastatin treatment improved endothelial function in women with endometriosis (43). Interestingly, in this small group of patients, when statins were given systemically for one week and then acutely through intradermal microdialysis, vascular function was reduced. Based on these prior data, we hypothesized that acute localized administration of statins to a healthy group of women would attenuate vasodilatory function through their potent antioxidant properties. Our findings indicate that localized statin administration did not impact endothelial function in healthy women.

Following systemic salsalate treatment, CVC$_{\text{max}}$ was increased in the statin treated site compared to the placebo condition. However, the magnitude of the increase in CVC$_{\text{max}}$ was reduced in comparison to the control site from the salsalate conditions. There was also an increase in NO-dependent vasodilation with the statin and concurrent salsalate treatment.

**Further Considerations**

NO-mediated endothelial-dependent vasodilation was similar in our healthy controls compared to healthy groups in previous studies using the same approach (43, 55). This shows the repeatability of our findings in regard to NO contribution in this group. In comparison to prior
studies, the current subject characteristics are more heterogeneous because the group was recruited as a matched control for the larger clinical study.

There are several limitations that warrant discussion. First, habitual physical activity can improve microcirculatory function (70). The participants in the present study reported their engagement in physical activities (e.g., walking, running, biking) in a typical week, but detailed measures of physical activity were not assessed. Second, one participant reported regular medication use for mental health conditions (e.g., anxiety) and ~65% (7/11) reported using some form of hormonal contraception. Several of the participants were either taking oral contraceptives or had low-dose progestin releasing implants [intrauterine devices (IUDs)]. There is considerable debate about the impact of these exogenous hormones and the level of control necessary in laboratory-based experiments (71). Synthetic progestins can alter non-NO-dependent vasodilation, but this has not been systematically explored in women with locally secreted low dose hormonal implants like IUDs (72). Finally, COVID status was not considered. The literature remains equivocal about the long-term impact of COVID infection on endothelial function (73, 74). At the time of participation in the current study, subjects did not have an active infection, but any potential residual effects of COVID-19 infection were not considered.

**Summary and Conclusions**

In conclusion, these data suggest that endothelial function was improved following systemic salicylate treatment and local atorvastatin did not alter endothelial function in healthy reproductive-aged women. Systemic inhibition on NF-κB improved endothelial function, through non-NO-dependent mechanisms that could be due to changes in the ET-1 system and/or an increase in endothelium-derived hyperpolarizing factors.


37. Holowatz LA, Kenney WL. Local ascorbate administration augments NO- and non-NO-dependent reflex cutaneous vasodilation in hypertensive humans.


Appendix

Informed Consent

CONSENT FOR RESEARCH
The Pennsylvania State University

Title of Project: Endometriosis and Microvascular Dysfunction (IRB#18347)
Principal Investigator: Lacy M. Alexander, Ph.D
Address: 113 Noll Laboratory
Telephone Number: 814-867-1781

Subject’s Printed Name: _____________________________

We are asking you to be in a research study. This form gives you information about the research. Whether or not you take part is up to you. You can choose not to take part. You can agree to take part and later change your mind. Your decision will not be held against you and there will be no penalty or loss of benefits to which you are entitled. Please ask questions about anything that is unclear to you and take your time to make your choice.

KEY INFORMATION

The following is a short summary of this study to help you decide whether or not to be a part of this research. More detailed information is listed later in this form. If you have any questions, be sure to ask the study team.

Why am I being invited to take part in a research study?
We invite you to take part in a research study because you have endometriosis.

What is the purpose of this research study?
The purpose of this voluntary research study is to learn how endometriosis impairs the lining of blood vessels and increases the risk for heart disease.
How long will the research study last?

- Screening (1 Visit)  
  less than 1.5 hour/visit
- Pill Pick up (2 Visits)  
  less than 0.25 hour/visit
- Experiments (2 Visits)  
  6 hours/visit
- Total: ~14 Hours over the course of 4 months

What will you need to do?

For this study, you will be asked to participate in 1 screening visit, 2 pill pick up visits, and 2 experimental visits. You will be asked to take the placebo and assigned drug treatment for 30 days each prior to each experimental visit.

What are the main risks of taking part in the study?

For this study, the main risks to know about are: the potential for infection with any of our materials used in this study. There are also potential side effects with the drug treatment.

What are the possible benefits to you that may reasonably be expected from being in the research?

We cannot promise any benefits to you from your taking part in this study. However, possible benefits include a medical screening. You will also feel good knowing you are helping to identify the reasons for the increased risk for CVD in women with endometriosis. Results of the study may benefit other people in the future by helping us learn more about endometriosis.

What happens if you do not want to be in this research?

Participation in research is completely voluntary. You can decide to participate or not to participate.

DETAILED INFORMATION

The following is more detailed information about this study in addition to the information provided above.

1. Why is this research study being done?

Endometriosis, a disorder that occurs in women, is when tissue normally found inside the womb is also found outside of the womb. This disorder impairs the function of the endothelium, the cells that line the body’s blood vessels (endothelium). The endothelium helps to control blood flow in healthy vessels. Women with this disorder not only have an increased risk for high blood pressure and high cholesterol, but they also have an increased risk for cardiovascular disease.

With this study, we will learn how endometriosis impairs the lining of blood vessels and increases the risk for heart disease.

Approximately 25 people will take part in this research study at Noll Laboratory/ Clinical Research Center at Hershey Medical Center.

2. What will happen in this research study?

Only women with endometriosis will complete this study. You will be randomly assigned to take 30 days of one of the drug treatments (simvastatin (statin) or Duavee™) and 30 days of a harmless inactive (placebo) pill. This means that whichever study treatment you receive will be determined purely by chance, like flipping a coin. You will have a 1 out of 2 chance of
receiving any one of the study treatments. The order in which you complete the drug treatment or placebo will be random and will be separated by 30 days between the drug treatment which you are assigned and the placebo.

Depending on the drug treatment group you are assigned to you will participate in only the circled days or procedures.

Please read the descriptions of the circled items, then write your initials by the circled days or procedures.

We may ask you to repeat a trial, procedure, or test. This could happen for many reasons such as equipment failure, power outage, inconclusive test results, etc. You do not have to repeat a trial, procedure, and/or test if you do not wish to do so.

Note: This study involves the use of drugs that are not approved by the FDA to treat disease. All of the drugs used in the microdialysis procedure have been previously used in humans research volunteer. The FDA approved the use of the drugs for this study and how we deliver them to a local area of skin with the microdialysis procedure. For the microdialysis procedure we dilute the drugs in Lactated Ringer’s, a type of saline fluid like that found throughout your body. The drugs are:

**Acetylcholine (ACH)** – like a substance made by your body; causes blood vessels to dilate

**Bazedoxefine** – a selective estrogen receptor modulator (SERM); this substance acts like estrogen in certain parts of your body like the blood vessels but blocks the effects of estrogen in other parts of the body like in the uterus “womb”.

**L-NAME** – blocks the body from making nitric oxide; causes blood vessels to get smaller.

**Sodium nitroprusside (SNP)** – supplies nitric oxide; causes blood vessels to dilate

_______ initial A. Screening Visit

1. You are to not eat, and drink only water, for 12 hours before the screening.
2. The research nurse and/or Clinical Research Center (CRC) staff will perform the screening. The staff measures your height and weight, blood pressure (BP), heart rate (HR) and waist circumference. The staff reviews your medical history. Women of childbearing age must provide a urine sample for a pregnancy test. You will take an Endometriosis Health Survey.
3. The staff will draw 30 ml (2 Tbsp) of blood from a vein in your arm. We will send some of the blood to a lab to see if the proteins, blood cells, electrolytes, etc. are within normal range. We may test the blood for other substances of interest.
4. The researchers do not perform genetic tests on the blood nor look for disease (e.g. HIV).

**Oral Drug Pretreatments**

1. We assign you one of the oral drugs or to a harmless inactive pill (placebo) to take for 30 days. The oral drug treatments are either Simvastatin (statin) or Duavee™. Duavee™ is a combination of a selective estrogen receptor modulator (SERM) call bazedoxefine with conjugated estrogen. The assignment to the oral drug treatment is random and the order if you take the drug treatment or the placebo pill first is also random. You will not be aware of which treatment you will be receiving.
a. The simvastatin (statin) dose is 10 mg/day taken as one pill; Duavee is 0.45mg of conjugated estrogen and 20mg/day of the SERM bazedoxefine combined into one pill; and the placebo is 1 tablet a day.

b. You will come to the lab to pick up the pre-treatment pills. During that time you must provide a urine sample for a pregnancy test. If the test is positive we will not give you the pills and you will be withdrawn from the study.

2. After 30 days of taking the first pretreatment, you will come to the lab for the experiments described below.

3. Following the experiments, you will then take no pretreatments (washout) for at least 30 days.

4. You repeat this one more time with the oral drug or placebo depending on what one you received first.

**initial B. Statin (Simvastatin) Therapy**

1. You will take one 10 mg tablet of statin each morning for 30 days.

2. We will give you the bottle of tablets to take home with you.

3. We will give you verbal instructions about the therapy, as well as written instructions and information sheets to take home with you.

4. If you have side effects from the Simvastatin, such as dark urine or muscle soreness, etc.
   a. Call:
      ii. Study head, Lacy M. Alexander, Ph.D. (W: 814-867-1781)
   b. You will return to the lab for the nurse to draw a 10 ml (<1 Tbsp) blood sample to check wellness and liver function markers.
      i. If the tests show that your liver function markers are too high, you will stop the Simvastatin and withdraw from the study.
      ii. We check your liver function markers at prescribed intervals until they return to normal. This will require extra 10-ml (<1 Tbsp) blood draws.
   c. See Section 3 below and the separate Simvastatin handout for more information.

**initial C. SERM (CE+BZE) Therapy**

1. You will take one 0.45mg/20mg tablet of SERM each morning for 30 days.

2. We will give you the bottle of tablets to take home with you.

3. We will give you verbal instructions about the therapy, as well as written instructions and information sheets to take home with you.

4. If you have side effects from the SERM
   a. Call:
      ii. Study head, Lacy M. Alexander, Ph.D. (W: 814-867-1781)
   b. See Section 3 below and the separate SERM handout for more information.

**initial D. Placebo Therapy**

1. You will take one tablet of Placebo each morning for 30 days.

2. We will give you the bottle of tablets to take home with you.
3. We will give you verbal and written instructions about the therapy.
4. If you have side effects from the Placebo
   a. Call:
      ii. Study head, Lacy M. Alexander, Ph.D. (W: 814-867-1781)
   b. See Section 3 below and the separate SERM handout for more information.

       initial E. Preparation for microdialysis (MD) and flow mediated dilation (FMD) experiments

1. We will give you printed and verbal instructions listing what to do before you arrive at the lab. Please follow the instructions with care. If you have questions, please contact us right away.
2. Do not drink alcohol in the 12 hours leading up to the experiment.
3. Do not drink caffeine (ex. coffee, tea, Coca Cola, chocolate) in the 12 hours leading up to the experiment.
4. On the day of the experiment
   a. Refrain from hard exercise, physical labor, and other tasks in which you might exert yourself more than you would on an easy walk.
   b. We will measure your blood pressure, heart rate, and oral temperature.
   c. All women of childbearing age who, in the previous two weeks, have not completed a pregnancy test, must provide a urine sample for a pregnancy test.
   d. The nurse draws 30 ml of blood to analyze for relevant substances of interest.
   e. You will take the Endometriosis Health Survey.
   f. We will insert the microdialysis (MD) probes:
      i. After you wash the skin on your forearm, we will place a tight band around the forearm to ensure your veins are easily visible.
      ii. For each MD site, we make pairs of pen-marks on the arm 2.5 cm (1 inch) apart and away from any veins. The marks serve as entry and exit points for the MD tubing. We then remove the tight band.
      iii. We will clean the arm with an orange fluid called “povidone iodine”, and alcohol, after which, an ice bag will be placed on the site for 5 minutes to numb the skin.
      iv. We will then we insert a thin needle into the skin near each entry mark. The needle’s tip travels between the layers of skin for 2.5 cm (1 inch). The needle exits the skin near the matching exit-mark.
      v. We will thread the MD tubing through the needle and then withdraw the needle leaving the tubing in the skin.
      vi. We will prepare 4 MD sites.
      vii. Any skin redness caused by the insertion fades in about 60 minutes. During this time, Lactated Ringer’s, a saline fluid similar to that found throughout your body, flows through the MD-tubing.
   g. Skin Blood Flow (SkBF):
      i. We tape a thin fiber optic laser Doppler flowmeter probe and its holder over each MD site.
ii. The thin probe measures skin blood flow with a weak laser light. We measure skin blood flow throughout the experiment.

iii. We control the temperature of the holders. The holders start at 34°C (93°F).

h. **Heart Rate:** We place 3 ECG tabs on your chest and connect them to an ECG machine.

i. **Blood pressure:** We may use two methods to measure blood pressure. Both methods use a cuff that inflates on your upper arm. In one, we listen with a stethoscope at the inside of your elbow. Another method, a critical care machine, makes the measure. The critical care machine also measures heart rate. During the experiment, we record blood pressure and heart rate every 5 to 7 minutes.

---

**F. MD Experiment**

**Acetylcholine (ACh) Dose Response**

\[
\begin{align*}
\text{initial} & \quad \text{Probe 1. Lactated Ringer’s only (control)} \\
\text{initial} & \quad \text{Probe 2. Lactated Ringer’s + LNAME} \\
\text{initial} & \quad \text{Probe 3. Lactated Ringer’s + BZE}
\end{align*}
\]

**Sodium Nitroprusside (SNP) Dose Response**

\[
\begin{align*}
\text{initial} & \quad \text{Probe 4. Lactated Ringer’s only (control)}
\end{align*}
\]

1. First we will add the Lactated Ringer’s, LNAME, and BZE to the MD sites as stated above.
2. After 30 minutes we will add ACh to probes 1, 2, and 3, and SNP to probe 4.
3. Then every 5 minutes we will increase the dosage of ACh and SNP until each site receives 10 total doses.
4. Once the final dose of ACh and SNP have been delivered, all test substances will be removed from all sites, with the exception of Lactated Ringer’s. This leaves only Lactated Ringer’s flowing through the tubing.
5. With Lactated Ringer’s continuing to flow, we will then warm the probe holders at all sites to 43°C (108°F).
6. Once the probes reach 43°C (108°F), we will wait 30 minutes and then add SNP to all sites. While ensuring the temperature remains at 43°C (108°F), adding SNP will cause the blood vessels at those sites to dilate as much possible.
7. 10 minutes after receiving SNP, we will remove the MD tubing from your skin. Sterile bandages will be placed over the sites, and we can provide an ice bag to reduce the chance of bruising, if you wish.
8. Lastly, we will measure blood pressure and heart rate before you depart.

---

**G. Flow Mediated Dilation (FMD):** FMD measures the health of blood vessels using doppler ultrasound.

1. First, a blood pressure cuff will be placed around your forearm.
2. We will then place ultrasound gel on your upper arm, just above the elbow. This facilitates the movement of sound waves from the ultrasound probe into your body, allowing us to measure the size of your blood vessels and the speed of your blood.
3. We will first take a “resting” measurement, then inflate the cuff for 5 minutes to stop blood flow to and from the forearm.
4. After 5 minutes, we will deflate the cuff and obtain a second measurement for 3 minutes.
5. We will then repeat steps 1-4, but this time you will also be performing a handgripping exercise at 20% of your maximal handgrip strength for 2 or 3 minutes while the cuff is inflated (step 3).

**Initial H. Biopsy Experiment**
1. We will take three small pieces (1 at each experiment visit) of skin from your arm (skin biopsy) using standard techniques.
2. First, you wash the site with soap and warm water, then sit in a recliner.
3. We clean your skin and the top of the lidocaine-vial with alcohol. An approved clinician injects lidocaine into the skin to ensure the biopsy sites are numb. We then wait a few minutes after injecting the lidocaine to give the drug time to work.
4. We clean the biopsy site 3 times with an alcohol pad.
5. We gently touch the site with the tip of a needle to see if you can feel anything. You may feel the slight pain of the pin-prick or only pressure. If you can feel pain, we wait a little longer. If needed, the approved clinician may add more lidocaine into the skin.
6. We use a punch-tool that looks like a screwdriver that has a round, hollow tip. The tip is 3mm (0.12 in) in diameter. The hollow tip acts like a cookie cutter. We place the tip of the punch against the skin at the biopsy site and apply mild pressure. You will feel the pressure. The tip of the punch goes about 3 mm (0.12 in) into the skin. The punch collects a small piece of skin about 3mm x 2mm (0.12 in x 0.08 in).
7. Following the biopsy, we apply pressure with a sterile dressing to the site to stop any bleeding.
8. The piece of skin will be placed into a small container.
9. You will be given instructions about caring for the biopsy site.

3. **What are the risks and possible discomforts from being in this research study?**

   - **There is a risk of loss of confidentiality.** If your information or your identity is obtained by someone other than the investigators, but precautions will be taken to prevent this from happening. The confidentiality of your electronic data created by you or by the researchers will be maintained to the degree permitted by the technology used. Absolute confidentiality cannot be guaranteed.

   - **Microdialysis:** The risks are less than that for a blood draw because microdialysis uses only a small, local area of skin. In contrast, a blood draw involves not only skin, but also large blood vessels and blood.
     - **Pain and bruising:** In an effort to lessen any small amount of pain and bruising you might experience, similar to a blood draw, ice will be used to numb your arm when we insert the tubing. In addition, the small needle reduces any pain when we insert the tubing. You are not likely to have pain after the tubing is in place. You may feel a little pain when we remove the tubing from your skin.
     - **Feeling lightheaded:** Needles make some people feel lightheaded or cause them to faint.
• **Break in the tubing:** Although rare, should the tubing break as we remove it from the skin, the remaining tubing will be removed by pulling on the other end. This presents no added risk for you. While even more rare, there is a possibility the tubing may break, leaving a piece under your skin; should this happen, we will remove it similar to how you would a splinter.

• **Bleeding:** We stop any bleeding with mild pressure and sterile gauze.

• **Infection:** Though infection is possible, the risk is very small because we use sterile techniques and supplies, like those used with blood draws. We apply a sterile bandage to the site after the experiment. You will be given instructions on how to care for the site.

• **Fluid flowing through the tubing:** The substances flowing through the tubing only go to a 2.5 cm² (0.4 inch²) area of skin at each tubing site.
  - **ACh, Simvastatin, LNAME, SNP:** These substances stop or mimic the action of your body’s natural chemicals upon the blood vessels in the skin. A small amount of these substances enter the skin around the tubing. This only affects the blood flow in the vessels in a nickel-sized area of skin. The effect of these substances is gone within an hour after the experiment.
  - **Reaction to the substances:** The amount that enters the skin is very small; however, there is a chance of having a bad reaction to the substances. A mild reaction might produce redness, itching, rash, and/or swelling, while a worse reaction could cause fever, breathing problems, changes in pulse, convulsions, and/or fainting.
  - **Feeling lightheaded.** Although rare, you could feel lightheaded. You could feel sick to your stomach or vomit, and you may become flushed or feel like your heart is pounding. We end the MD procedure if you have any of these signs and symptoms. We and other researchers have used these substances with microdialysis in skin, and there have been no reports of lasting, bad reactions. If a bad reaction should occur, we summon medical help.

• **Lactated Ringer’s Solution:** This fluid, containing salt, potassium, lactate and chloride, is similar to the natural fluids in your skin. The acid content is like that of your body’s natural fluids. A bad reaction to this fluid is highly unlikely.

• **Laser Doppler Flowmetry:**
  - **Temporary vision impairment:** Weak lasers can hurt your eye if you stare into the light for a long time. We do not turn on the laser until the probes are in place.
  - **Tape:** The tape may irritate your skin.

• **Blood Pressure** (manual, critical care monitor): The researchers measure blood pressure with the method used in a doctor’s office and/or they can use a machine. The cuff will first inflate on your upper arm, then as the cuff slowly deflates, the researchers listen with a stethoscope at the bend in the elbow. In like manner, the critical care monitor takes a reading.
  - **Arm feeling numb:** During the short time the researchers inflate the cuff, your arm may feel numb or tingly.
  - **Bruising:** The cuff could cause mild bruising.
• **Povidone Iodine:** Researchers and hospitals use this orange-colored fluid to clean the skin. You should inform us if you are allergic to iodine or to shellfish, as this could cause you to have a bad reaction to this fluid. In this case, we use only alcohol instead.
  o **Allergic reaction:** A bad reaction could cause redness, itching, rash, and/or swelling. A worse reaction could also cause fever, breathing problems, changes in pulse, convulsions, and/or fainting.

• **Blood Draw:** Blood draws often cause mild pain, bruising, swelling, or bleeding. There is also a slight chance of infection or a small clot. If you are nervous about needles, your blood pressure and heart rate may increase for a little while.
  o **Feeling lightheaded:** You may also feel lightheaded, sick to your stomach, or may faint. By using the same techniques employed in hospitals, we keep the chance of infection minimal. Do not exercise hard for 24 hours before a blood draw.

• **Tape and sticky disks:**
  o **Rash:** The tape or sticky disks could cause a rash. During screening, you tell us if you are sensitive to tape.
  o **Mild abrasion.** If a disk sticks very strongly, removing the disk could cause an abrasion, like a rug-burn on your skin. An abrasion can feel tender or slightly painful, and can increase risk of infection. If you are sensitive to tape, you may have an increased chance for abrasion. An abrasion has occurred only twice during the years that the disks have been used in similar studies in our lab. We may use an adhesive remover like that used in a doctor’s office to remove the disks. If you get an abrasion a nurse will check the site. Antibiotic ointment and a sterile bandage are applied. We tell you how to take care of the site.
  o **Allergic reaction to adhesive remover:** You could have an allergic reaction to the adhesive remover which could include rash, itching, fever, or breathing problems. Also, it could include changes in pulse, and/or blood pressure, convulsions, shock, and/or fainting. If a bad reaction should occur, we summon medical help right away.

• **Medical Screening:** You may feel shy about giving health information. The staff collects the information in a private and professional manner. You may feel shy about being measured. You may request someone of the same sex to conduct the screening.

• **Initial screening form:** Only members of our lab group use this form. We use the form to help decide whether you are a good candidate for the study. You may feel shy about answering questions. You may request someone of the same sex to ask you the questions. We collect the information in a private and professional manner. We keep the completed information confidential and secure.

• **Endometriosis Symptom Survey:** Only members of our lab group use this form. You may feel shy about answering questions. There may be some mild psychological discomfort associated with answering some of the questions. You have the right to refuse to answer any questions that you find uncomfortable. In the event that we learn that you are experiencing psychological distress, we will offer a referral to mental health resources in your geographic
area. You may also call a national hotline that can provide further assistance SAMHSA’s 1-800-662-HELP (4357). We collect the information in a private and professional manner. We keep the completed information confidential and secure.

- **Local heating**: We measure the temperature of your skin under the holders. During heating, the skin feels very warm but will not hurt.
  
  - **Skin redness**: The heating makes the skin under the holder red like when you take a hot bath. The redness goes away within several hours. Some people may be more sensitive to heating. If your arm feels too hot, tell us, and we reduce or stop the heating.

- **ECG**: This machine measures the electrical activity of your heart. You have 3 wires from the machine taped to spots on your chest. There have been no adverse effects. The tape may irritate your skin.

- **Latex**: Some gloves and medical materials are made of latex rubber.
  
  - **Allergic reaction**: You must inform us if you are allergic to latex and decline to participate in the study.

- **Statin Therapy**: We will give you verbal and written information about Simvastatin. See the handout for more details about Simvastatin, its use, and side effects. Simvastatin is a drug often prescribed by doctors for people who have high blood cholesterol. Simvastatin lowers blood cholesterol. In this study, the approved clinician directs the Simvastatin therapy.
  
  - **Headache**: Simvastatin could cause you to have a headache. You will stop taking Simvastatin if you get blurred vision or decreased or rust-colored urine.
  
  - **Allergic reaction**: You will stop taking the drug if you have an allergic reaction. This could include problems with breathing, closing of your throat or a rash. Also an allergic reaction could include swelling of the lips, tongue, or face.
  
  - **Muscle or liver problems.** There have been rare cases of muscle or liver problems with Simvastatin use. We will check your liver function with a blood draw before you start Simvastatin. We also check your liver function if you have symptoms of liver problems while taking the Simvastatin therapy. If the blood test shows a rise in certain liver markers, we will tell you to stop taking Simvastatin. Then we would take more blood to test for the markers’ to return to normal. Early symptoms of muscle or liver problems include muscle pain, soreness, or weakness.
  
  - **Flu-like symptoms**: You could also have fever or flu-like symptoms. You may have yellowing of your skin or eyes, stomach pain, or feel tired. You may have dark colored urine or pale colored stools. If you have any of these symptoms, stop taking Simvastatin. Then contact the approved clinician right away.
  
  - **Harm developing fetus**: Simvastatin could harm a developing fetus. If you are a woman who becomes pregnant while taking Simvastatin, you must stop taking Simvastatin right away. Then tell the researcher and your health care provider right away that you became pregnant while on Simvastatin.
o **Alcohol use.** While taking Simvastatin, you should not drink more than two servings of alcoholic beverages (i.e. beer, wine) per day.

o **Reaction with grapefruit juice:** Grapefruit and grapefruit juice may interact with this drug. Do not eat grapefruit nor drink grapefruit juice while taking Simvastatin.

- **SERM (Bazedoxifene +Conjugated Estrogen, Duavee®) Oral Intervention:** This SERM is a drug approved by the FDA and used by postmenopausal women to treat hot flashes and other menopausal changes.

  o **Ovarian cysts:** Because you are not in menopause, taking Duavee™ may increase the risk of ovarian cysts and/or affect the thickness of the lining in your uterus. We will reduce these risks by drawing blood to monitor changes in your reproductive hormones [estrogen, progesterone and luteinizing hormone (LH), or follicle stimulating hormone (FSH)].

  o **Abnormal uterine bleeding and bleeding outside of menses.** You may experience some spotting or bleeding outside your normal menstrual period.

  o **Allergic reaction:** A very serious allergic reaction to this product is rare. However, get medical help right away if you notice any symptoms of a serious allergic reaction, including: rash, itching/swelling (especially of the face/tongue/throat), severe dizziness, trouble breathing.

- **Skin Biopsy:** You may stop the procedure at any time. Trained staff performs the biopsy. If you wish, you may lie back in the reclining chair during the biopsy. We make sure that you are informed and ready.

  o **Feeling nervous:** You may still be nervous about needles or the procedure, if so, your blood pressure and heart rate may increase for a little while.

  o **Feeling lightheaded:** You may also feel lightheaded, sick to your stomach, or may faint. The lidocaine numbs the site so that you feel very little or no pain during the biopsy. You feel the pressure of the biopsy tool on your skin. As with any event that breaks the skin, you could get an infection.

  o **Infection:** Trained staff use sterile techniques to keep the risk of infection very small. The skin biopsy may cause some pain, swelling, bleeding, and bruising.

  o **Bleeding/bruising:** Gauze will be pressed onto the site to stop any bleeding. We will place a sterile bandage on the site and give you instructions about caring for the biopsy site.

  o **Scar:** The biopsy is likely to leave a small scar. If you have skin that is known to overreact to injury, you may produce a scar that is larger and easier to see.

  o **Pain:** There may be some minor pain for a couple of days when the lidocaine wears off, and may be like that felt after some blood draws.

- **Lidocaine:** You may feel brief pain and burning from the needle when we first inject the lidocaine into the skin.

  o **Allergic reaction:** Although unlikely you could have a bad reaction to the lidocaine. You may feel a ringing in your ears or a metallic taste in your mouth. An allergic reaction could produce redness, itching, rash, and/or swelling. A worse reaction could also cause fever, breathing problems, changes in pulse, convulsions, and/or fainting. If a bad reaction should occur, we summon medical help right away.
• **FMD Test / Doppler Ultrasound:** There is a small chance the probe could irritate the skin. Although temporary, minor redness may occur where the researchers place the probe against the arm. The gel is the same as that used with medical ultrasound tests, and may feel cool or cold on the skin. A bad reaction to the gel is highly unlikely.
  - **Numbness/tingling/bruising:** While the researchers inflate the cuffs, the arms and feet may feel numb or tingly, and the color of the skin may change slightly. The cuffs may cause mild bruising.

4. **What are the possible benefits from being in this research study?**

4a. **What are the possible benefits to you?**
You will receive a medical screening that could inform you about your health. You will learn the cholesterol level in your blood and your blood pressure. This knowledge is important because high blood pressure and blood cholesterol can lead to many serious health problems. If you have high blood pressure or blood cholesterol, we suggest you to follow-up with a health care provider. You will also feel good knowing you are helping to identify the reasons for the increased risk for CVD in women with endometriosis.

4b. **What are the possible benefits to others?**
The results of the research may help scientists to better understand Endometriosis. Endometriosis causes chronic pelvic pain and pain during intercourse. It also can reduce or end a woman’s ability to bear children. This disorder affects 6% - 10% of women of childbearing age, and can occur in as many as 35-50% of women who have pain or cannot bear children. Despite these statistics, little research has been devoted to this disorder. This research can help to draw attention to the importance of this malady to women’s health.

In addition, endometriosis impairs the function of the “endothelium” or the lining of blood vessels. Impaired endothelium increases the risk for getting high blood pressure and high blood cholesterol, and is a hallmark of cardiovascular disease (CVD). CVD is the leading cause of death in women. Women with endometriosis have an increased risk for these diseases. This study will expand the knowledge base regarding the mechanisms by which endometriosis increases risk for these diseases in women. The data from this study serves as a foundation for further investigation.

Also, this study provides experience, education and degree-work for students of The Pennsylvania State University.

5. **What other options are available instead of being in this research study?**
You may decide not to participate in this research.

6. **How long will you take part in this research study?**
   - Screening (1 Visit) less than 1.5 hour/visit
   - Pill Pick up (2 Visits) less than 0.25 hour/visit
   - MD/FMD Experiments (2 Visits) 6 hours/visit
   - **Total:** ~14 Hours
7. How will your privacy and confidentiality be protected if you decide to take part in this research study?

7a. What happens to the information collected for the research?

Efforts will be made to limit the use and sharing of your personal research information to people who have a need to review this information. Reasonable efforts will be made to keep the personal information in your research record private. However, absolute confidentiality cannot be guaranteed.

- A list that matches your name with your code number will be kept in a locked file or password protected file in a room that is locked when unoccupied. Only authorized members of the lab have access to the list.
- Your research records will be labeled with your code number and will be kept in a safe area in a locked file or password protected computer in a room that is locked when unoccupied.
- Your research samples will be labeled with your code number. We keep some samples in a dedicated ultralow freezer in Noll Lab/Clinical Research Center at Hershey Medical Center until analysis. We send some samples to Quest Labs for analysis. For specimens sent to Quest Labs, you will be identified by your code number.

This research is covered by a Certificate of Confidentiality from the National Institutes of Health. This means that the researchers cannot disclose information that identifies you to anyone not connected with the research. This protection also prevents this information from being used or disclosed for legal proceedings, such as being accessed through a court order. The Certificate of Confidentiality however does not prevent disclosures required by law, such as information about child abuse or neglect and harm to yourself or others. Also, your information may be disclosed in accordance with any consent you provide, including for your medical treatment or use in other research. Additionally, the Certificate of Confidentiality does not prevent your information from being disclosed to the National Institutes of Health in order for it to evaluate or audit the research, or prevent disclosures required to meet FDA requirements. For additional information ask the principal investigator or a member of the study team or contact the Office for Research Protections at (814) 865-1775.

In the event of any publication or presentation resulting from the research, no personally identifiable information will be shared.

We will do our best to keep your participation in this research study confidential to the extent permitted by law. However, it is possible that other people may find out about your participation in this research study. For example, the following people/groups may check and copy records about this research.

- The Office for Human Research Protections in the U. S. Department of Health and Human Services
- The Food and Drug Administration
- The Institutional Review Board (a committee that reviews and approves research studies) and Penn State’s Office for Research Protections.
7b. What will happen to my research information and/or samples after the study is completed?

We may use your research information and your biological samples for future research studies or may share your information and your biological samples with other investigators here or at other institutions for future research without your additional informed consent. Future research may be similar to this study or completely different. Before we use or share your information or samples we will remove any information that shows your identity.

8. What happens if you are injured as a result of taking part in this research study?

In the unlikely event you become injured as a result of your participation in this study, medical care is available. It is the policy of this institution to provide neither financial compensation nor free medical treatment for research-related injury. By signing this document, you are not waiving any rights that you have against The Pennsylvania State University for injury resulting from negligence of the University or its investigators.

9. Will you be paid or receive credit to take part in this research study?

You will receive:

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Amount</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>MD Experiment</td>
<td>$160.00</td>
<td>($15.00 / MD probe inserted + $20.00 completing experiment) (4 MD probes/experiment; 2 total experiments)</td>
</tr>
<tr>
<td>Biopsy</td>
<td>$100.00</td>
<td>($50.00 each: 2 total experiments)</td>
</tr>
<tr>
<td>FMD Experiment</td>
<td>$100.00</td>
<td>($50.00 each: 2 total experiments)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>$360.00</strong></td>
<td></td>
</tr>
</tbody>
</table>

For each experiment, we pay you the amount of money equal to the part of the trial that you complete. For instance, if you complete half of a MD experiment, we pay you for each probe that we insert plus $10.00 for that trial. This is because $10.00 is one-half of $20.00. We may ask you to repeat a trial. If you agree to repeat a trial, we pay you for the repeated trial as stated above. We reimburse you for gasoline if you live more than 20 miles from Noll Lab or the Clinical Research Center at Hershey Medical Center.

You will be paid in the form of a check. You will need to provide your social security number and address to receive a check for payment and for tax reporting purposes.

If you do not complete the study for any reason, you will be paid for the visits you have completed.

10. Who is paying for this research study?

The institution and investigators are receiving a grant from the National Institutes of Health to support this research.

11. What are your rights if you take part in this research study?
Taking part in this research study is voluntary.

- You do not have to be in this research.
- If you choose to be in this research, you have the right to stop at any time.
- If you decide not to be in this research or if you decide to stop at a later date, there will be no penalty or loss of benefits to which you are entitled.

The person in charge of the research study can remove you from the research study without your approval. Possible reasons for removal include adverse reaction to the statin or SERM. Other possible reasons for removal from the study include if the researcher deems that your health or behavior adversely affects the study or increases risks to you beyond those approved by the Institutional Review Board and agreed upon by you in this document. You may decline to answer certain questions. You may decide not to comply with certain procedures. However, your being in the study may be contingent upon answering these questions or complying with the procedures.

During the course of the research you will be provided with any new information that may affect your health, welfare or your decision to continue participating in this research.

12. If you have questions or concerns about this research study, whom should you call?

Please call:

- Study head, Lacy M. Alexander, Ph.D. (W: 814-867-1781)
- The research nurse, Susan Slimak RN (W: 814-863-8556, H: 814-880-4396)

If you:

- Have questions, complaints or concerns about the research, including questions about compensation.
- Believe you may have been harmed by being in the research study.

You may also contact the Office for Research Protections at (814) 865-1775, IRB-ORP@psu.edu if you:

- Have questions regarding your rights as a person in a research study or any privacy issues.
- Have concerns, complaints, or general questions about the research.
- You may also call this number if you cannot reach the research team or wish to offer input or to talk to someone else about any concerns related to the research.

You may visit the Office for Research Protections’ website at https://www.research.psu.edu/irb/participants for:

- Information about your rights when you are in a research study;
- Information about the Institutional Review Board (IRB), a group of people who review the research to protect your rights; and
- Links to the federal regulations and information about the protection of people who are in research studies. If you do not have access to the internet, copies of these federal regulations are available by calling the ORP at (814) 865-1775.
A description of this clinical trial will be available on http://www.ClinicalTrials.gov. This Web site will not include information that can identify you. At most, the Web site will include a summary of the results. You can search this Web site at any time.

**INFORMED CONSENT TO TAKE PART IN RESEARCH**

**Signature of Person Obtaining Informed Consent**

Your signature below means that you have explained the research to the subject or subject representative, provided the subject or subject representative an opportunity to discuss and consider whether or not to participate in the research, and have answered any questions the subject or subject representative has about the research.

______________________________
Signature of person who explained this research

________
Date

__________________
Printed Name

(Only approved investigators for this research may explain the research and obtain informed consent.)

**Signature of Person Giving Informed Consent**

Before making the decision about being in this research you should have:

- Discussed this research study with an investigator,
- Read the information in this form, and
- Had the opportunity to ask any questions you may have.

Your signature below means that you have received this information, have asked the questions you currently have about the research and those questions have been answered. You will receive a copy of the signed and dated form to keep for future reference.

**Signature of Subject**

By signing this consent form, you indicate that you voluntarily choose to be in this research and agree to allow your information to be used and shared as described above.

___________________________
Signature of Subject

________
Date

__________________
Printed Name