Cardiovascular disease risk in women with endometriosis

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Confidentiality Statement:

Confidentiality of all information in the study will be maintained by identifying subjects by code numbers, which are subsequently linked to their data files. All electronic data will be stored on a password protected computer. No individuals, other than the professionals directly involved in the study will be allowed to read data forms. Investigators will be required to respect the confidentiality code. No subjects are identified by name in any of the published literature and only by code in major data storage areas. Only initials are used in the experimental laboratory notebook. All books are kept in the laboratory. We have never had a violation of confidentiality.

Synopsis

Purpose: To systematically interrogate the mechanisms underlying elevated cardiovascular disease risk in women with endometriosis and to measure the effectiveness of emerging endometriosis treatments on endothelial function and cardiovascular disease (CVD) risk. Our overarching goal is to (1) determine the role of estrogen suppression in mediating endothelial dysfunction.

Specific Aims:

To test the hypothesis that estrogen suppression impairs endothelial dysfunction increase inflammatory cytokines and negatively impact quality of life (QoL) in endometriosis. ***We hypothesize*** that in women with endometriosis:

1. endothelium mediated dilation and peripheral microvasodilation are impaired relative to control;

2. estrogen suppression will attenuate flow-mediated dilation and peripheral microvasodilation.

3. estrogen suppression in women with endometriosis increases inflammatory cytokines.

4. estrogen suppression in women with endometriosis will change scores on a QoL survey indicating greater physical pain and emotional challenges.

We further hypothesize that in healthy control women:

1. endothelium mediated dilation and peripheral microvasodilation are impaired during estrogen suppression compared to the ovulatory phase when estradiol is elevated;
2. endothelium mediated dilation and peripheral microvasodilation are impaired relative to control, and this impairment is exaggerated when healthy control women are compared in the ovulatory phase.

Primary Objectives of this study is to determine whether estrogen suppression reduces endothelial function.

Secondary Objective

To determine changes in sera levels of HDL, LDL cholesterol and triglycerides as well as immune function in response to estrogen suppression in women with endometriosis.

To determine changes in scores on a QoL survey in response to estrogen suppression in women with endometriosis.

Study Design

This is a prospective study using established physiological techniques used in human research. We will be using pre-post intervention to determine the effect of our intervention.

Study Date Range and

We expect this study to take up to eight years from its start, including analysis.

Number of Study Sites

There are two study sites, the Yale School of Medicine and YNNH (if needed for screening subjects).

Primary Outcome Variables

Flow mediated vasodilation, skin blood flow. These variables are the precise variables needed to determine changes in endothelial function and lipid profile affected by estradiol suppression (Elagolix).

Secondary and Exploratory Outcome Variables (if applicable)

Sera levels of HDL, LDL cholesterol and triglycerides. Sera levels of ET-1, IL-6, TNFα, CRP and IL-1 β. Scores of QoL survey.

Study Population

Two groups (n=47) of women will be recruited to complete this study: 1) healthy women between the ages of 18 and 45 years (Controls); 2) women between the ages of 18 and 45 years with endometriosis. Subjects who smoke, have diabetes, or BP>140/90 will be excluded. Subjects will not be taking any insulin sensitizing or CV medications, or hormonal contraception or other hormones (estrogens, progestins, androgens) for at least four weeks prior to participating in these protocols. We do make exceptions for Tylenol in the event the subject has acute pain. Sexually active participants will use barrier contraception for the duration of the study and take pregnancy tests within 24 hours of participation. We require a Control group to compare the response to the GnRH antagonist in the endometriosis group. We are also comparing a baseline or pre-testing vs. GnRH antagonist in a pre-post intervention study design. We require a Control group to document dysfunctional responses to the antagonist and the estrogen suppression treatments. We have no similar data in our previous work.

Endometriosis subjects: The subjects will have a prior diagnosis by laparoscopy by their own physician and provide a report to Dr. Taylor.

Control subjects will be healthy women, with regular menses every 26-34 days. Subjects will be excluded if they have a surgical diagnosis of endometriosis, any symptoms of endometriosis, including severe dysmenorrhea or pelvic pain.

Number of Participants

47 (this amendment increases the number potentially by 12 new control subjects).

Study Schedule

All subjects will undergo microvascular skin blood flow analysis two times, one time while taking the GnRH antagonist and one time with prior to taking Elagolix. Thus, the research will involve 3 visits to the lab over approximately 45 days.

In a ***pre-post study*** design all subjects participate in the first study within the first 7 days of their menstrual cycle and followed by a GnRH antagonist (Elagolix, 400 mg/day oral, 2X200 mg tablets/day for 4-7 days) (33). Scheduling issues may extend Elagolix ingestion up to 7 days. All women will begin taking Elagolix in the first week of their menstrual cycle. If scheduling permits, women can complete both tests within the same menstrual cycle, if not, we will begin the Elagolix testing during the first 7 days of the next menstrual cycle. Elagolix reduces E2 concentrations within 24 hours (33). This timeframe should allow for a thorough interrogation of vascular effects without being overly burdensome on the subjects.

Research Design:
Physiological Studies are conducted:
1) during the first 7 days of the menstrual cycle.
2) during GnRH suppression (Elagolix), 400 mg/day oral, 2X200 mg tablets/day for 4-7 days).

Research Protocol: Aim 1
Visit 1: Screening, informed consent, orientation.
Visit 2: [during the first 7 days of the menstrual cycle.]: Physiological studies, QoL survey*
Visit 3: [while taking Elagolix]: Physiological studies, QoL survey*
*only endometriosis subjects take the QoL survey

For the amendment: Healthy control women are recruited for this study:
Research Design: Aim 1a Healthy Controls
Physiological Studies are conducted:
1) during the first 7 days of the menstrual cycle.
2) during the ovulatory phase of the menstrual cycle, or between 14-18 days into the menstrual cycle
3) during GnRH suppression (Elagolix), 400 mg/day oral, 2X200 mg tablets/day for 4-7 days);

Research Protocol:

Visit 1: Screening, informed consent, orientation.
Visit 2: [during the first 7 days of the menstrual cycle.]: Physiological studies
Visit 3: [between 14-18 days into the menstrual cycle.]: Physiological studies
Visit 4: [while taking Elagolix]: Physiological studies

Women with Endometriosis can choose to participate in the protocol described in Aim 1, or in Aim 1a described below.

Physiological Studies are conducted: Aim 1a Endometriosis Research

1) during the first 7 days of the menstrual cycle.
2) during the ovulatory phase of the menstrual cycle, or between 14-18 days into the menstrual cycle
3) during GnRH suppression (Elagolix), 400 mg/day oral, 2X200 mg tablets/day for 4-7 days);

Research Protocol:
Visit 1: Screening, informed consent, orientation.
Visit 2: [during the first 7 days of the menstrual cycle.]: Physiological studies, QoL survey*
Visit 3: [between 14-18 days into the menstrual cycle.]: Physiological studies, QoL survey*
Visit 4: [while taking Elagolix]: Physiological studies, QoL survey*

Research Design: Aim 1b Healthy Controls
Physiological Studies are conducted:

1) during the ovulatory phase of the menstrual cycle, or between 14-18 days into the menstrual cycle

Research Protocol:
Visit 1: Informed consent.
Visit 2: [between 14-18 days into the menstrual cycle.]: Physiological studies

Protocol Revision History

Version Date	Summary of Substantial Changes
August 25, 2021	We have revised this protocol to the updated IRB format, including a new consent form and modified the study from its original version (which did not accrue subjects).
March 15, 2022	We have revised this protocol: 1. Corrected Table 1. 2. Removed text in red. 3. Pages 2,3, 17,20,22: Changed from randomized double blind to crossover design randomized single blind crossover design 4. Pages 14,15,18,20,21,24 survey for subjects with endometriosis.
March 29	1. Confirm that no subjects have been enrolled to date. **No subjects have been enrolled.** 2. What's the status of the funding for the project? Is the NIH still funding the project, and if so, did the NIH accept the change in study design from double-blind to single-blind design? **NIH is funding this project. We decided to stay with the double blinded design, so these changes have been removed from the protocol.** 3. Provide a rationale for the change in study design. **We decided to stay with the double blinded design, so these changes have been removed from the protocol.** 4. Please let me know which of the following recruitment materials will be used in the study: - Endo flyer.docx(0.01) 5. We are only using Endo flyer.docx(0.01) 6. The primary objective from the synopsis in the protocol doesn't match the primary objective (erased) in the Study purpose and objectives paragraph. Please make the information consistent. **This is corrected (See Synopsis)** 7. The protocol indicated that there are 5 appendixes. I was not able to find the following information; therefore, please provide: **NIH told us we do not need a DSMB Plan, so these have been removed.** Appendix 1 Recruiting Flyer: **added** Appendix 2 Recruiting Script: **added** Appendix 3: Medical History: added Appendix 4: Written Instructions (Consent Form): **added** 8. Please remove double-blind and replace it with single-blind on page 20 of the document: **We are keeping the double blind design.** 9. Please confirm that each subject (healthy control or subjects with endometriosis) will receive a placebo at one of the visits and Elagolix at another. **This dosing protocol is described clearly on page 21 of the protocol.** The order of the assignments of placebo/drug will be randomized. If so, please make that information clear in the consent. **Done, page 3** 10. Replace the word treatment/pretreatment in the consent with the word dosing, schedule etc. Treatment implies a therapeutic/clinical effect. **done** 11 Consent page 3 Elagolix paragraph - point 3 information is repeated in point 4:You may take Elagolix longer should scheduling deem it necessary. You will

	not take Elagolix longer than 7 days. " please erase the repeated information. **done** 12. Visit 2, you note that "We will give you printed and verbal instructions listing what to do before you arrive at the lab.". Provide the printed instructions given to the subjects. **This is removed because the instructions are in the consent form. We remind subjects of these instructions, either by phone, text according to the subject's preference, but there is no specific script. We do not provide a script because each subject is different, so communications are geared to each subject. We do provide instructions for taking the pills Uploaded to IRES** 13. Visit 2 point 2d. Did you make the typo in converting 30 ml (30ml= 2 tablespoons) to tablespoons, or 60 ml is collected, and 4 tablespoons conversions is correct? **Corrected to 30 ml=2 tablespoons** 14. Consent describes the following blood draw schedule V1=30ml, V2= 30 ml, V3 and V4 also 30ml. = 120 ml. However, the protocol on page 23 indicated 60 ml. Please make the documents consistent. **Corrected** **AMENDMENT:** **We have removed the Baseline study. Including this in the protocol was an error and not consistent with the NIH protocol. Removing this reduces visits to the laboratory, and thus risk to the subjects. We have changed the number of visits, and subject payment throughout the protocol and consent form.** Pages: 3, 21, 27 Consent form to reflect these changes: Pages: 2, 6, 9, 13 AMENDMENT We added a new method to recruit subjects using the YCCI email list. Pg 26, 27

June 30, 2022	We have removed Dr. Valerie Flores from the protocol who is leaving Yale
March 1,2023	We are adding the ability to use JDAT for specifically recruiting women with endometriosis. Pg 27
May 9, 2023	AMENDMENT We are adding one more laser Doppler probe for heating to examine heating induced endothelial mediated vasodilation. This will also include application of a Nitroglycerin patch.
Jun 12	AMENDMENTS 1) Changing the research design from randomized control trial to "pre-post" for the baseline vs Elagolix treatment: Changed from randomized double-blind design to pre-post design on protocol pages: 18,19, 21, 23. There will be no placebo. Subject will perform the first (baseline) study in the first 7 days of the menstrual cycle. 2) Changes to medical history form: added social security number (page 1), added oophorectomy to Hospitalization History section (page 2), removed Nutritional History section (page 3), revised COVID-19 History section to ask about symptoms experienced in the last week instead of the last year (page 4), and added questions for women with endometriosis to the Gynecological History section (page 5).

3) Revised exclusion criteria to include total oophorectomy instead of hysterectomy (page 20). Partial oophorectomies are not excluded.
4) Changes to consent form: one statement was revised for clarity (page 2), two statements were changed to reflect updated pre-post research design (page 2, 3), and one statement was revised to correct an error (page 5).
5) For this new amendment: we have added a new consent form specific for subjects recruited for patients recruited through the Yale infertility clinic. The three consent forms labeled Consent Aim 1 Endo CLINIC, Consent Aim 1 Endo RESEARCH, Consent Aim 1 Endo CONTROL, to make clear to staff the correct consent to used for each subject. In addition, we have added descriptions within each consent form to describe the subject population.

Sept 5 Amendment

1) Increase the subject recruitment number and increase in duration of study by two years.

Sept 27 Amendment

1) Remove partial oophorectomies from the exclusion criteria.

January 2024 Amendment

Adding a protocol that will test (physiological studies) healthy control women during the ovulatory phase of the menstrual cycle.

Adding a protocol that will test (physiological studies) women with endometriosis during the ovulatory phase of the menstrual cycle (for subjects recruited only via our Research mechanism). *We are not planning to increase the number of women we recruit with endometriosis. The additional consent form provided is for women with endometriosis who are interested in participating in an additional test during ovulation. Thus, the comparison would be pilot studies for this group. When entering the study, women with endometriosis will be given the option of participating in the protocol that includes either the 3 or the 4 physiological visits. We are setting the study up in this way because of the challenges recruiting this patient group.*

January 2025 Amendment

The John B. Pierce Laboratory is being removed as the supporting research institution. It is being replaced by the Yale School of Medicine. The protocol and all consent forms have been modified.

Statement of Compliance

This document is a protocol for a human research study. The purpose of this protocol is to ensure that this study is to be conducted according to the Common Rule at 45CFR46 (human subjects) and other applicable government regulations and Institutional research policies and procedures.

Abbreviations

Abbreviation	Explanation
CVD	Cardiovascular disease
GnRH	Gonadotropin releasing hormone
FMD	Flow mediated vasodilation
LDL-C	Low density lipoprotein cholesterol
HDL-C	High density lipoprotein cholesterol
TG	triglycerides
IL-6	Interleukin 6
IL-1β	Interleukin 1 beta
TNFα	Tumor necrosis factor alpha
CRP	C-reactive protein
NO	nitric oxide
ACh	Acetylcholine
E2	17-β estradiol
SNP	Sodium nitroprusside
IR	Insulin resistance
ET-1	Endothelin-1
HR	Heart rate
PI	Pulse interval
BP	Blood pressure
$S_{[E_2]}$	17 β-estradiol
$P_{[SHBG]}$	sex hormone binding globulin
$S_{[P_4]}$	progesterone
P_{Osm}	Plasma osmolality
IE	ischemic exercise
RH	Reactive hyperemia

Glossary of Terms

Glossary	Explanation
dyslipidemia	A disorder of lipoprotein metabolism, including lipoprotein overproduction or deficiency
endothelial dysfunction	diminished production/availability of nitric oxide and/or an imbalance in the relative contribution of endothelium-derived relaxing and contracting factors
flow mediated dilation	A method used to determine endothelial function in which the dilation of an artery when blood flow increases in that artery is measured following occlusion.
endothelial dysfunction	diminished production/availability of nitric oxide and/or an imbalance in the relative contribution of endothelium-derived relaxing and contracting factors
flow mediated dilation	A method used to determine endothelial function in which the dilation of an artery when blood flow increases in that artery is measured following occlusion.
skin microdialysis	Skin microdialysis consists of the implantation of semi-permeable probes into the upper dermis, and perfusion with a physiological buffer.
laser Doppler flowmetry	Non-invasive method to measure blood cell perfusion in the microvasculature of tissues and organs.
Ovulatory phase	Ovulation is when the ovary releases a mature egg and is associated with an increase in estrogen. This is approximately 14 days into a 28-day menstrual cycle.

Table of Contents

Synopsis 2

Purpose.....2
Primary Objective.....2

Secondary Objective.....2

Study Design.....2

Study Date Range.....2

Number of Study Sites.....2

Primary Outcome Variables.....2

Secondary and Exploratory Outcome Variables (if applicable).....2

Study Population.....3

Number of Participants 3

Study schedule..... 3
Research design.....3
Research Protocol.....4

Protocol Revision History.....5-7

Statement of Compliance.....8

Abbreviations 9

Glossary of Terms.....10

1 Background/Literature Review.....14

1.1 Background 14-15

1.2 Prior Experience (if applicable).....15

2 Rationale/Significance 16

2.1 Rationale and Study Significance.....16

2.2 Purpose of Study/Potential Impact 16

2.3 Potential Risks and Benefits.....16

2.3.1 Potential Risks 16-17

2.3.2 Potential Benefits 18

3 Study Purpose and Objectives 18

3.1 Hypothesis.....18

3.2 Primary Objectives 18

3.3 Secondary Objective 18

3.4 Tertiary Objective.....18

4 Study Design 20

4.1.1 General Design Description 20-21

4.1.2 Study Date Range and Duration 21
4.1.3 Number of Study Sites 21
4.2 Outcome Variables 21
4.2.1 Primary Outcome Variables 21
Secondary and Exploratory Outcome Variables (if applicable) 21
4.3 Study Population 21
4.3.1 Number of Participants 22
4.3.2 Eligibility Criteria/Vulnerable Populations 22
5 Study Methods/Procedures 24
5.1 Study Procedures 24
5.1.1 Data Collection 29
5.1.2 Adverse Events Definition and Reporting 29-30
5.2 Informed Consent 30
5.2.1 Screening (if applicable) 31
5.2.2 Recruitment, Enrollment and Retention (if applicable) 31
5.2.3 Study Visits (is applicable) 32
5.2.4 Statistical Design 33
5.2.5 Statistical Analysis 33
5.2.6 Sample Size Calculation 33
5.2.7 Planned Analysis 33
5.2.8 Analysis of Subject Characteristics (if applicable) 33
5.2.9 Interim Analysis (if applicable) 33
5.2.10 Handling of Missing Data 33
6 Trial Administration 34
6.1 Ethical Considerations: Informed Consent/Assent and HIPAA Authorization 34
6.2 Institutional Review Board (IRB) Review 35
6.3 Subject Confidentiality 35
6.4 Deviations/Unanticipated Problems 36
6.5 Data Quality Assurance 36
6.6 Study Records 37
6.7 Access to Source 37
6.8 Data or Specimen Storage/Security 37
6.9 Retention of Records 38
6.10 Study Monitoring 38
6.11 Study Modification 38

6.12 Study Completion 38
6.13 Funding Source 38
6.14 Conflict of Interest Policy 38
6.15 Publication Plan 38
Appendices 39
Table 1. Microdialysis studies Aim 1. 39
References 39-41

1 Background/Literature Review

1.1 Background

Endometriosis is an estrogen dependent gynecological disorder associated with considerable chronic pelvic pain, pain during intercourse and is a major cause of infertility (5, 9). This disorder affects 6% - 10% of reproductive age women and can be as high as 35-50% in women experiencing pain or infertility (6, 11, 18, 21), and ~ 50% of infertile women (6). *Endometriosis is a major cause of hysterectomy and hospitalization in the US and was associated with annual societal costs estimated at $69.4 billion in 2009 (6, 27)*. Despite its prevalence, endometriosis is underdiagnosed and associated with a up to a 7-yr delay from the onset of symptoms to definitive diagnosis (6). Moreover, endometriosis is a debilitating condition, leading to loss of work time and has a significant impact on quality-of-life.

The primary clinical features of endometriosis are chronic pelvic pain, pain during intercourse, and infertility (5, 9) resulting from inflammatory endometriotic lesions. Although the pathophysiology of endometriosis is not completely understood, the earliest and still most widely accepted theory for the etiology of endometriosis is retrograde menstruation in which endometrial cells are transported into the peritoneal cavity (24, 25). Retrograde menstruation is required but not sufficient for the development of endometriosis. After adhesion, endometrial cells proliferate and gradually invade the peritoneal tissue (29). These endometrial cell implants are then vascularized and further developed via inflammatory processes (9). The pathophysiology generally revolves around genetic predisposition, estrogen dependence, progesterone resistance and inflammation (6).

Endometriosis and cardiovascular disease. Cardiovascular disease is the leading cause of death in women. Emerging epidemiologic data demonstrate a clear association between endometriosis, reproductive risk factors and accelerated cardiovascular risk (1, 17, 21, 22). The chronic, systemic inflammation associated with endometriosis may impact cardiovascular function though several mechanisms, including LDL metabolism and through direct actions on adhesion molecules (3) or endothelial cells (16). Endothelial dysfunction is a first manifestation of cardiovascular disease predictive of long-term disease morbidity and mortality (8).

As described above, endometriosis derives from the presence of endometrium like tissue in sites outside the uterine cavity. While endometriosis is a local inflammatory syndrome, the inflammatory process is systemic (2). Endometriosis is associated with higher risk of hypercholesterolemia and hypertension (22). Emerging epidemiologic data demonstrate a clear association between endometriosis, reproductive risk factors, inflammation and accelerated cardiovascular risk (1, 17, 21, 22, 34).

In healthy women, estradiol increases nitric oxide (NO) bioavailability and improves or protects endothelial function. In women not planning to become pregnant, the most common treatment is for endometriosis is the suppression of ovulation and menstruation, and reduction of estradiol to postmenopausal levels through hormone modulating therapy. A common treatment is gonadotropin releasing hormone (GnRH) agonists combined with estrogens and progestins (add-back therapy). While GnRH agonists are effective at treating the symptoms of endometriosis, suppressing estrogens in young women carries draw backs, such as vasomotor symptoms and bone loss, and the potential for exacerbating the cardiovascular comorbidities. Thus, suppressing estrogens to treat symptoms in women with endometriosis may increase risk for endothelial dysfunction and cardiovascular dysfunction through other mechanisms.

Thus, the association between cardiovascular risk and endometriosis creates a conundrum: while estrogen exposure is generally considered beneficial for women from a cardiovascular standpoint, the standard of practice for endometriosis treatment includes estrogen suppression, which may exacerbate long-term cardiovascular risk via an independent mechanism. Thus, future treatments may lie in partial estrogen suppression in an orally active GnRH antagonist that we can now test within this protocol.

Our scientific premise is that in women with endometriosis, elevated cardiovascular disease risk is the result of endothelial dysfunction and chronic systemic inflammation through LOX-1 receptor activation. This cardiovascular disease risk is exacerbated by standard estrogen suppression treatments.

We use a unique approach, that of gonadotropin releasing hormone (GnRH) suppression with a GnRH antagonist (Elagolix, the first orally active GnRH antagonist), so we can examine women with endometriosis during periods of controlled low estrogens. This protocol will enable us to isolate the impact of estrogen exposure on the cardiovascular system in women with endometriosis. Our hypothesis is that infertility-associated elevated cardiovascular disease risk in women with endometriosis is the result of chronic systemic inflammation mediated through LOX-1 receptor mechanisms. The studies proposed here specifically addresses endothelial dysfunction in endometriosis.

Feasibility and Preliminary Data.

Estrogen and peripheral microvascular function. In healthy women, E2 mediates release and consequent vasodilation in skin microvasculature s in a dose-response manner (Fig 1). That NO mediates the cholinergic vasodilatory response in the skin is well known (4, 14), this system has not been studied in women with endometriosis, and neither the effect of E2.

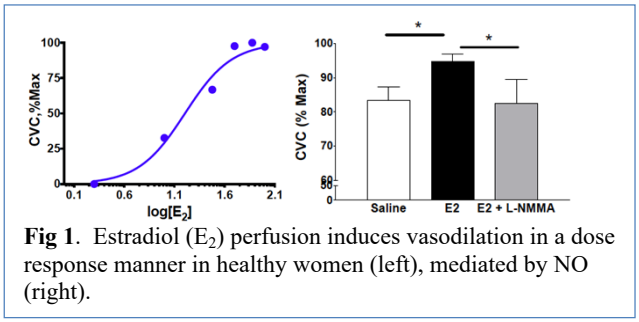


Fig 1. Estradiol (E_2) perfusion induces vasodilation in a dose response manner in healthy women (left), mediated by NO (right).

NO the

but

has

1.2 Prior Experience (if applicable)

We have used microdialysis in many of our prior studies over the last 10 years, in a number of populations including women with PCOS and older women. This is the first time we will use this technique in women with endometriosis, but we do not anticipate that microdialysis will impact these women differently. Finally, we are using Elagolix in our current study in women with PCOS, and have used GnRH suppression in our studies for many years (30, 31, 36-38).

2 Rationale/Significance

2.1 Rationale and Study Significance

Our scientific premise is that women with endometriosis have elevated CVD risk resulting from chronic systemic inflammation induced endothelial dysfunction. In these women, CVD risk may be further exacerbated by standard estrogen suppression treatments**. Previous studies have lacked rigor** in examining this estrogen inflammation paradox and subsequent activation of vascular specific mechanisms in women with endometriosis. Despite, strong preclinical data, these studies have not systematically investigated this link between elevated inflammation and atherosclerotic risk in women with this common and devastating reproductive disease. Additionally, endometriosis treatments to date have focused on reducing pain and sparing fertility status but have not considered their impact on cardiovascular function or health. We will examine two emerging interventions that have shown efficacy for reducing endometriosis symptoms and long-term CVD risk.

2.2 Purpose of Study/Potential Impact

Overall Impact. This series of studies in women with endometriosis will delineate the roles of estrogen and systemic inflammation in endometriosis-associated accelerated CVD risk. These studies will evaluate novel signaling mechanisms including the linkage common to both cardiovascular disease and endometriosis through the downstream activation of the ubiquitous scavenger receptor LOX-1. Thus, our studies have the potential to identify clinically relevant therapeutic targets and interventions thus decreasing CVD burden in women with endometriosis.

Our proposal is also high impact for the following reasons:

1. Endometriosis is a major cause of hysterectomy and hospitalization in the US and was associated with annual societal costs estimated at $85 billion in 2012 (6, 27) .
2. The common practice of progestin-based therapies in the management of endometriosis-associated pain is variable and is associated with greater incidence of depression(10).
3. The standard of practice for endometriosis treatment includes estrogen suppression, which may exacerbate long-term CVD risk via independent mechanisms. Recently approved treatments may lie in partial estrogen suppression with orally active gonadotropin releasing hormone (GnRH) antagonist, *Elagolix*, but its effects on cardiovascular function are unknown.
4. Systemic inflammation is increased in endometriosis (2, 23, 26) and serves as a putative mechanistic link between this gynecological disorder and elevated CVD risk in women.

2.3 Potential Risks and Benefits

2.3.1 Potential Risks

Potential risks associated with blood sampling, include infection from failure to observe proper sterile conditions, and hematoma (bruise) from careless technique. The latter may be associated with some discomfort but presents little danger to the subject's welfare.

All substances (ACh, E2, SNP) added to the lactated Ringer's for perfusing the microdialysis probes have been used previously clinically and/or during research in humans:

Microdialysis delivers small amounts of the substances to a nickel-sized area of the skin. The small quantities used and the extremely localized administration during microdialysis is unlikely to produce systemic effects. To our knowledge, there are no reports of long side effects of these substances administered through microdialysis. The chance of adverse reactions to these substances is extremely small given the minute amount delivered to a very small area of skin, the lack of adverse reactions to similar amounts delivered via microdialysis in many other studies, and lack of adverse effects in human cell cultures. There is a slight chance of a sensitivity or allergic reaction to these substances that could produce redness, itching, rash, and/or swelling. A severe reaction (anaphylactic shock) could also cause fever, difficulty in breathing, changes in pulse, convulsions, and/or loss of consciousness. Although rare, it is possible that a subject could experience lightheadedness, flushing, nausea, "pounding heart" sensation, and/or vomiting.

GnRH antagonist (Elagolix) put the subjects into a temporary menopausal state, so they may experience vasomotor symptoms (i.e. "hot flashes") during the initial period of administration. Additionally, although most women (controls or endometriosis) tolerate Elagolix quite well, some may experience headaches, breast tenderness, transient mood changes, some temporary bloating of the abdomen and moderate irregular vaginal bleeding. The subjects will begin the Elagolix/baseline treatment on days 1-7 of their menstrual cycle (or 1-7 days after the beginning of menstruation) to avoid discomfort due to irregular bleeding, though this has not been a reported side effect of GnRH suppression in our experience. Long-term side effects of Elagolix include the potential for bone loss however this risk is not relevant after short exposures (<6 months of continuous use). We have not used Elagolix in our studies before, but (control or PCOS) subjects in our studies have reported minimal side effects from using Antagon (another GnRH antagonist) over a period of 16 days. In one of our studies, one of 15 subjects reported occasional vasomotor symptoms during Antagon treatment. In our ~12 years of using Antagon, two subjects reported transient changes in mood during Antagon treatment. The subjects reported no other adverse effects due to the Antagon or hormone administration, and the side effects did not cause any of the subjects to leave the study. We have used GnRH antagonist in HIC #1508016301 in women with and without Polycystic Ovary Syndrome with no ill effects or side effects. GnRH antagonist is used in treatment for endometriosis.

The risk of **flow mediated vasodilation** is discomfort during the ischemia portion of the test.

Estrogens may increase the chance of getting blood clots or stroke.

Placebo pills will be made using our compounding pharmacy with microcrystalline cellulose 101 powder. These placebos carry no risk.
With this new amendment, we have eliminated placebo pills.

Acetylcholine serves as a neurotransmitter at peripheral ganglia and as a mediator of the parasympathetic actions of the autonomic nervous system. These drugs will not have systemic effects because microdialysis methods distribute the agonists only in the local area of perfusion. **Sodium nitroprusside** (**SNP**) is a medication is intravenously infused in cases of acute hypertension to rapidly lower blood pressure in much higher doses than those proposed in these protocols (e.g. 1.5 ug/kgBW/min). *We expect no such BP lowering effects in our low dose of* 28 mM. Further, we do not infuse SNP directly into the vein, but perfuse the skin and micro vessels with this low dose. For these reasons, and we have not seen any effect on BP in our prior studies (35, 38-40).

Nitroglycerin is a medication applied subcutaneously to prevent angina attacks (chest pain) but when applied chronically can lower blood pressure because nitroglycerin induces vasodilation**.** We expect no such BP lowering effects in our brief application of this patch. If a subject experiences hypotension during the patch administration, we will immediately remove the patch, clean the arm with soap and water and lie the subject head down and elevate their legs (Trendelenburg position) until they feel better. We return the subject to a seated position and offer fluids. We will discontinue the study.

Quality of Life/Endometriosis Health Survey: There may be mild psychological discomfort associated with answering some of the questions; participants are told that they have the right to refuse to answer any interview or survey questions. Only authorized personnel may access completed forms.

Ovulation test strips do not pose risks.

2.3.2 Potential Benefits

Short term: The primary benefits to the volunteers will be the satisfaction derived from participating in a research program designed to assess estrogen suppression on skin blood flow in women with and without endometriosis.

Long term: The results could benefit medical science, as we hope to gain an improved understanding of the effects of estrogen on blood pressure regulation in women with endometriosis. Endometriosis impacts 5-10% of women in the US, and its cardiovascular effects are usually left untreated.

3 Study Purpose and Objectives

3.1 Hypothesis

Hyp1A. endothelium-dependent vasodilation in brachial artery and the peripheral microvasculature are impaired in women with endometriosis.

Hyp1B. estrogen suppression (GnRH antagonist) will impair vasodilation in brachial artery and peripheral microvasculature.

Hyp 1C. estrogen suppression (GnRH antagonist) will impair vasodilation in brachial artery and peripheral microvasculature induced by local heating.

Hyp 1D. in healthy control women endothelium mediated dilation and peripheral microvasodilation are impaired during estrogen suppression compared to the ovulatory phase when estradiol is elevated;

Hyp 1E. endothelium mediated dilation and peripheral microvasodilation are impaired in women with endometriosis relative to control, impairment is exaggerated when healthy control women are compared in the ovulatory phase;

Hyp 1F. in women with endometriosis endothelium mediated dilation and peripheral microvasodilation are impaired during estrogen suppression compared to the ovulatory phase when estradiol is elevated.

3.2 Primary Objectives

Primary Objectives of this study is to determine whether estrogen suppression reduces endothelial function.

3.3 Secondary Objective

To determine changes in sera levels of HDL, LDL cholesterol and triglycerides as well as immune function in response to estrogen suppression in women with endometriosis.

3.4 Tertiary Objective

Hyp1C. estrogen suppression (GnRH antagonist) will improve Quality of Life in women with endometriosis.

CLINICAL STUDY PROTOCOL

4 Study Design

4.1.1 General Design Description

PARTICIPANTS: Two groups (N=47) number increased due to withdrawals, and is increased again here because we will recruit a new group of control subjects to participate in amendment described here) of women will be recruited to complete this study: 1) healthy women between the ages of 18 and 45 years (Controls, n-35); 2) women between the ages of 18 and 45 years with endometriosis (n=12). Subjects who smoke, have diabetes, or BP>140/90 will be excluded. Subjects will not be any insulin sensitizing, CV medications, or hormonal contraception, or other hormones (estrogens, progestins, androgens) for at least four weeks prior to participating in these protocols. We do make exceptions for Tylenol in the event the subject has acute pain. Sexually active participants will use barrier contraception for the duration of the study and take pregnancy tests within 24 hours of participation. We require a Control group to compare the response to the GnRH antagonist in the endometriosis group. We are also comparing a with and with GnRH antagonist in a pre-post design. We will test subjects before and after taking 4-7 days of GnRH antagonist pills. We require a Control group to document dysfunctional responses to the antagonist and the estrogen treatments.

Endometriosis subjects: The subjects will have a prior diagnosis by laparoscopy by their own physician and provide a report to Dr. Taylor.
Control subjects will be healthy women, with regular menses every 26-34 days. Subjects will be excluded if they have a surgical diagnosis of endometriosis, any symptoms of endometriosis, including severe dysmenorrhea or pelvic pain.
This is a prospective study using established physiological techniques used in human research. We will be using a pre-post design to determine the effect of our intervention for Aim 1.

To determine the effects of estrogen suppression on endothelial function in women with endometriosis. Rationale: Estrogens are cardio protective in women, especially in women with compromised cardiovascular systems. Suppressing estrogens will induce endothelial dysfunction in women with endometriosis.

With this new amendment, we will also test whether suppressing estrogens will induce endothelial dysfunction in healthy control women, and whether differences between groups are exaggerated when estrogens are elevated in the health control group in the luteal phase.

We have also included a consent form for women with endometriosis who are ovulating and want to participate in a test during ovulation. These would be pilot studies as we do not expect all women in this group to participate in this test and do not plan to recruit a separate, additional group of subjects for it.

We will test subjects in the follicular phase, the ovulation phase and then after taking taking 4-7 days of GnRH antagonist pills.

Study Design:
All subjects will undergo microvascular skin blood flow analysis two times, one time while taking the GnRH antagonist and one time with prior to beginning the pills. Thus, the research will involve 3 visits to the lab over approximately 45 days.

In a **pre-post** design all subjects will receive participate in a baseline study first followed by a GnRH antagonist (Elagolix, 400 mg/day oral, 2X200 mg tablets/day for 4-7 days) (33). Scheduling issues may extend Elagolix ingestion up to 7 days. All women will begin taking

Elagolix in the first week of their menstrual cycle. Elagolix reduces E2 concentrations within 24 hours (33). This timeframe should allow for a thorough interrogation of vascular effects without being overly burdensome on the subjects. We will recruit subject both through advertising and the other means described in this protocol, as well as throughout Dr. Taylor's practice and the infertility clinic at Yale. The baseline is first because women who are recruited through Dr. Taylor's practice will begin taking Elagolix and continue for as long as 30 days prior to beginning fertility treatment. If they sign up for our study, they will not be taking other drugs that interfere with their treatment or our study so the baseline must be done first. After they begin Elagolix, they will do our protocol within the first 4-7 days of treatment. We may ask these latter subjects to delay beginning Elagolix for as long as one menstrual cycle to participate in our study depending on when they had their last menstrual period.

In the amendment, the women will use ovulation prediction test strips to participate in a third day of physiological studies that will take place during ovulation when estrogens are predicted to be elevated. This is a urine test using urine prediction strips provided by the laboratory that the subject can do at home. We will test subjects in the follicular phase, the ovulation phase and then after taking 4-7 days of GnRH antagonist pills.

4.1.2 Study Date Range and Duration

We expect this study to take up to eight years, including analysis. April 2022-December 2029.

4.1.3 Number of Study Sites

There are two study sites, the Yale School of Medicine and YNNH for screening subjects.

4.2 Outcome Variables

4.2.1 Primary Outcome Variables

Flow mediated vasodilation, skin blood flow. These variables are the precise variables needed to determine changes in, endothelial function and lipid profile.

Secondary and Exploratory Outcome Variables (if applicable)

Sera levels of HDL, LDL cholesterol and triglycerides. Sera levels of ET-1, IL-6, TNFα, CRP and IL-1 β. We will also examine answers to questions from the Endometriosis Health Profile Questionnaire to determine changes in Quality of Life (QoL).

4.3 Study Population

Aim 1, 1a, 1b: Two groups (N=47 of women will be recruited to complete this study: 1) healthy women between the ages of 18 and 45 years (Controls, n=35) Aims 1,1a,1b; 2) women between the ages of 18 and 45 years with endometriosis (n=12) Aims 1,1a. Subjects who smoke, have diabetes, or BP>140/90 will be excluded. Subjects will not take any insulin sensitizing, CV medications, or hormonal contraception or other hormones (estrogens, progestins, androgens) for at least four weeks prior to participating in these protocols. We do make exceptions for Tylenol in the event the subject has acute pain. Sexually active participants will use barrier contraception for the duration of the study and take pregnancy tests within 24 hours of participation. We require a Control group to compare the response to the GnRH antagonist in the endometriosis group. We are also comparing GnRH antagonist to baseline in pre-post intervention design. We require a Control group to document dysfunctional responses to the antagonist and the estrogen treatments. We have no similar data in our previous work.

Endometriosis subjects: The subjects will have a prior diagnosis by laparoscopy by their own physician and provide a report to Dr. Taylor.

Control subjects will be healthy women, with regular menses every 26-34 days. Subjects will be excluded if they have a surgical diagnosis of endometriosis, any symptoms of endometriosis, including severe dysmenorrhea or pelvic pain.

Aim 1a: We are recruiting a new healthy control group to participate in the amendment described here in which we test the hypothesis that endothelial function diminished during Elagolix treatment compared to the ovulatory phase as well as compared to women with endometriosis regardless of their level of estrogen.

Aim 1b: We have also provided a consent form for this amendment to provide the opportunity for any healthy control woman who has already participated in our study to participate in physiological studies in the luteal phase. This would mean returning to our laboratory for only one test. We can use these data to compare with the earlier testing data after a full interview to ensure that there have been no changes in her medication or health status.

Aim 1a: We have also included a consent form for women with endometriosis who are ovulating and want to participate in a test during ovulation. These would be pilot studies as we do not expect all women in this group to participate in this test and do not plan to recruit a separate, addition group of subjects for it.

4.3.1 Number of Participants

47

4.3.2 Eligibility Criteria/Vulnerable Populations

Subjects: **Aim 1,1a,1b.** Two groups (N=47) of women will be recruited to complete this study: 1) young women between the ages of 18 and 45 years (Controls) (N=35); 2) young women between the ages of 18 and 45 years with endometriosis (N=12). Subjects who smoke, will be excluded. Subjects will not be taking medications during the study, including any insulin sensitizing, CV medications, or hormonal contraception or other hormones (estrogens, progestins, androgens) for at least four weeks prior to participating in these protocols. We do make exceptions for Tylenol in the event the subject has acute pain. Sexually active participants will use barrier contraception for the duration of the study and take pregnancy tests within 24 hours of participation.

For Aim 1a: we will recruit an additional 12 healthy control women. 1) young women between the ages of 18 and 45 years (Controls); Subjects who smoke, will be excluded. Subjects will not be taking any insulin sensitizing or CV medications, or hormonal contraception or other hormones (estrogens, progestins, androgens) for at least four weeks prior to participating in these protocols. We do make exceptions for Tylenol in the event the subject has acute pain. Sexually active participants will use barrier contraception for the duration of the study and take pregnancy tests within 24 hours of participation. Participants that have already completed Aim 1 or are in progress of completing Aim 1 will be contacted to participate in Aim 1b as well.

Inclusion Criteria:
Endometriosis subjects: Endometriosis will be diagnosed by history of the disease seen at the time of prior surgery or will be diagnosed by classic clinical symptoms of the disease (cyclic progressive pelvic pain) using prior surgical report reviewed by Dr. Taylor. Women with endometriosis and severe acute pain requiring immediate treatment will be excluded. Only women meeting inclusion criteria for endometriosis will be included in the new pre-post design. **Control subjects** will be healthy women, with regular menses every 26-34 days. **Control subjects** will also be evaluated by Dr. Taylor and will be excluded from the study if they have any of the symptoms or characteristics described above for endometriosis patients. All

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subjects will be excluded if they are taking any medications contraindicated with the drugs/biologics used in the protocol.

Subjects with the following histories or conditions will be excluded from the study:

Gynecologic: a. current or past estrogen-dependent neoplasia, b. unexplained vaginal bleeding, c. history of uterine fibroids, d. current pregnancy, e. known or suspected breast or uterine cancer, f. complete oophorectomy, g. PCOS.
Cardiac: a. myocardial infarction, ventricular tachycardia or fibrillation, b. angina, c. valvular disease (mitral insufficiency or stenosis, aortic insufficiency or stenosis), d. congestive heart failure, orthopnea, paroxysmal nocturnal dyspnea, e. current arrhythmias, f. prosthetic valves, g. BP>140/90.
Pulmonary: a. current cigarette smokers, or pipe or cigar smokers, b. chronic obstructive pulmonary disease, c. adult asthma, d. dyspnea on exertion, e. current bronchitis, pneumonia, or tuberculosis, f. lung carcinoma, g. pulmonary embolus, recent (less than 1 year), g. sleep apnea.
Vascular: a. claudications or history of peripheral vascular disease, b. abdominal or thoracic aortic aneurysm, or repair of same, c. cerebral aneurysm, vascular malformations, d. hypertension, systolic or diastolic.
Gastrointestinal: a. GI malignancy, b. hepatitis, current, c. splenomegaly from any cause, d. Cholecystitis, e. current diverticulosis or diverticulitis, inflammatory bowel disease, ulcerative colitis, Crohn's disease.
Infectious Disease: any ongoing intercurrent infection.
Hematologic/Oncologic: a. receiving chemotherapy or radiation therapy, b. any metastatic malignancy, c. anemia (hematocrit < 35), d. thrombocytopenia or thrombocytosis, e. neutropenia, f. hematologic malignancy, g. bleeding dyscrasia.
Neurologic: a. history of cerebral vascular accident with any neurologic sequels, b. uncontrolled seizures (e.g., more than 1 seizure/year), c. transient ischemic attacks, d. dementia, e.
neurologic conditions producing dyscoordination, peripheral neuropathy, or myopathy.
Endocrine: a. diabetes mellitus, b. any untreated endocrinopathy, c. hyperlipidemia: LDL > 130 mg/dl, total cholesterol level > 240 mg/dl, and triglycerides > 170 mg/ml.
Renal: a. chronic renal diseases, b. any history of renal disease or impairment, c. current urinary tract infection.
Musculoskeletal: a. inflammatory arthritis history (e.g., rheumatoid, psoriatic, Reiters), b. any history of pathologic fractures, including vertebral compression fractures.
Pharmacologic: a. any illegal drug use (self-report), b. alcohol use greater than an average of 4 oz/day over 30 days.
Allergic reaction: We ask subjects if they are aware of any allergies to nitroglycerin or if they are aware of any allergies to adhesives used to administer skin patches and exclude them from participation accordingly.

Eligibility will be determined by the P.I. (Dr. Stachenfeld) and by the Co-I (Dr. Taylor) by interview with potential subjects. We will conduct an initial interview by phone and the subject will fill out a medical history questionnaire during the orientation session. In this interview, we will ask the subject if she has been diagnosed with endometriosis, we will ask her approximate height and weight. We will ask her name and if she has any other health issues that would preclude her from participating in the study.

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5 Study Methods/Procedures

5.1 Study Procedures

Standard Preliminary Procedures for all Protocols. The subjects maintain their normal diet and activity for three days prior to any experimental protocol and avoid alcohol and caffeine for 12 hrs prior to arriving at the laboratory. The subjects will arrive at the laboratory at 0800 having fasted for 8 hrs prior to the cardiovascular experiments. They will eat their normal diet for three days prior (see Procedures) and drink water based on their body weight. On the morning of the experiment, we assess hydration from urine specific gravity (S.G.) of the initial urine sample. If S.G.>1.020, the subjects will drink 5 ml/kg of water, followed by a 30 min control period until hydration is deemed sufficient (S.G. <1.020). Subjects may not take Advil or other anti-inflammatories while taking Elagolix or the four days leading up to the testing days. We are testing small changes in vessel size, so want the subjects to avoid drugs that may cause vasodilation.
Preliminary Testing. Because naive volunteers may be used, all subjects are shown the room where testing will take place and, thoroughly trained in all maneuvers and familiarized with all routines to ensure that they are as comfortable as possible on the first experimental day.

To determine the effects of estrogen suppression on endothelial function in women with endometriosis. Rationale: Estrogens are cardio protective in women, especially in women with compromised cardiovascular systems. Suppressing estrogens will induce endothelial dysfunction in women with endometriosis.

Study Design:
All subjects will undergo microvascular skin blood flow analysis three times, once before and one time while taking the GnRH antagonist. Thus, the research will involve 3 visits to the lab over approximately 45 days.

In a **pre-post** design all subjects will tested prior to beginning the GnRH antagonist (Elagolix, 400 mg/day oral, 2X200 mg tablets/day for 4-7 days) (33). All women will begin taking Elagolix in the first week of their menstrual cycle. Elagolix reduces E2 concentrations within 24 hours(33). This timeframe should allow for a thorough interrogation of vascular effects without being overly burdensome on the subjects. Baseline (or pre-testing) will also take place in the first 7 days of the menstrual cycle.

Research Design:
Physiological studies
1 Prior to beginning GnRH suppression (baseline)
2) during GnRH suppression (Elagolix), 400 mg/day oral, 2X200 mg tablets/day for 4-7 days;

Research Protocol: Aim 1
Visit 1: Screening, informed consent, orientation.
Visit 2: prior to beginning Elagolix (baseline): Physiological studies, Health Profile Questionnaire* (QoL survey*)
Visit 3: while taking Elagolix: Physiological studies, Health Profile Questionnaire* (QoL survey*)
*Only endometriosis subjects will take the Questionnaire

For the amendment: Healthy control women are recruited for this study:
Research Design: Aim 1a Healthy Controls
Physiological Studies are conducted:
2) during the first 7 days of the menstrual cycle.

3) during the ovulatory phase of the menstrual cycle, or between 14-18 days into the menstrual cycle
4) during GnRH suppression (Elagolix), 400 mg/day oral, 2X200 mg tablets/day for 4-7 days);

Research Protocol:
Visit 1: Screening, informed consent, orientation.
Visit 2: [during the first 7 days of the menstrual cycle.]: Physiological studies
Visit 3: [between 14-18 days into the menstrual cycle.]: Physiological studies
Visit 4: [while taking Elagolix]: Physiological studies

Women with Endometriosis can choose to participate in the protocol described in Aim 1, or in Aim 1a described below.
Physiological Studies are conducted: Aim 1a Endometriosis Research
4) during the first 7 days of the menstrual cycle.
5) during the ovulatory phase of the menstrual cycle, or between 14-18 days into the menstrual cycle
6) during GnRH suppression (Elagolix), 400 mg/day oral, 2X200 mg tablets/day for 4-7 days);

Research Protocol:
Visit 1: Screening, informed consent, orientation.
Visit 2: [during the first 7 days of the menstrual cycle.]: Physiological studies, QoL survey*
Visit 3: [between 14-18 days into the menstrual cycle.]: Physiological studies, QoL survey*
Visit 4: [while taking Elagolix]: Physiological studies QoL survey*
Research Design: Aim 1b Healthy Controls
Physiological Studies are conducted:
1) during the ovulatory phase of the menstrual cycle, or between 14-18 days into the menstrual cycle
Research Protocol:
Visit 1: Informed consent.
Visit 2: [between 14-18 days into the menstrual cycle.]: Physiological studies

Procedures:
Flow Mediated Vasodilation (Conduit Artery Endothelial function). Continuous measures of brachial artery diameter and blood flow velocity will be simultaneously measured using the Sonoscope S2 ultrasound imaging system. After 15 minutes of supine rest, a longitudinal image of the brachial artery is recorded, after which forearm ischemia is induced by rapidly inflating a blood pressure cuff (placed immediately distal to the olecranon process) to 200 mmHg for 5 minutes. Upon cuff release, blood flow increases to the ischemic tissue (reactive hyperemia). The increase in shear stress associated with this rapid blood flow increase stimulates NO release from endothelial cells, causing vasodilation. We record the change in brachial artery diameter 15 seconds prior to cuff deflation continuously thereafter for 2 minutes. Simultaneous recordings of blood flow velocity will be measured with a Doppler probe to quantify shear rate. Quantifying shear stress is important in determining endothelium-dependent dilation since the magnitude and duration of this stimulus varies among individuals and differs in the upper and lower extremities. Participants will also perform a hand grip exercise at 20% of their maximal handgrip strength for 3 minutes while the forearm cuff is inflated to 200 mmHg. We will again record the change in brachial artery diameter 15 seconds prior to cuff deflation continuously thereafter for 2 minutes.

Local heating and microdialysis perfusions. Local heating, 17 β-estradiol (E2) and acetylcholine (ACh) microdialysis perfusions will be conducted in an environmental chamber set at 27ºC. Subjects will eat a diet controlled for water and sodium the night before and the morning of the drug perfusion under each experimental condition (see Procedures). The

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subject will rest in the semi-recumbent position throughout the protocol. During a 60-min resting period, the subjects will be instrumented for the measurement of plasma variables (intravenous catheter will be inserted into a forearm), heart rate (ECG), and arterial blood pressure (Finometer, see Procedures). During the control period, we will insert three microdialysis probes through the forearm skin (see Procedures). After the probe insertions, the subjects will rest for at least 90 minutes to allow for recovery from the microdialysis probe placement (37). During this resting period, we will administer the Endometriosis Health Profile Questionnaire to the endometriosis subjects (15). To determine peripheral vascular responses to the drug perfusions, we will measure changes in SkBF at the site of the perfusions using heated Doppler flowmetry (see Procedures). The Doppler probes both control and measure local skin temperature at the microdialysis sites (see Procedures).

Following recovery from the microdialysis probe insertions we will measure resting SkBF for 15 min at all sites with the local heating devices off. Following resting measurements, in the first probe we will perfuse lactated Ringers, in the second probe we will perfuse incremental doses of ACh (1 x 10^{-10} 1 x 10^{-1} M) (32), in the third probe incremental doses of E_2 (10, 30, 50, 75, 100 nM) (39). (Table 1) The perfusion rate for all probes will be 5.0 µl/min. Following each series of perfusions, the temperature of the local heaters will be increased by 0.1°C/5 sec to a local skin temperature of 43°C, where it will be clamped ~30 min and 28 mM of the NO donor sodium nitroprusside will be perfused through all microdialysis sites to maximal endothelium-independent vasodilation. Skin blood flow will be normalized based on percent of the maximal response induced by the SNP perfusion/heating to account for the anatomical differences in the number of blood vessels under each probe.

J.B. Pierce Lab Human Microdialysis Studies Table 1

Microdialysis Probe	Site-specific protocol	28 mM SNP Maximal Vasodilation
(1) Ringers (control site)	Local Heating to 42° eNOS-dependent vasodilation	
(2) Ach dose response	1 x 10^{-10} - 10^{-1} M	
(3) E2 dose response	10, 30, 50, 75, 100 x10^{-9} M	

During the microdialysis experiments we will also place a fourth laser Doppler probe equipped with a local heating device with no microdialysis probe. We will heat this probe to 40°C to induce endothelium mediated vasodilation. When we see a plateau in the heating response (~ 30-40 min), we will briefly remove the local heating device and place a 0.6 mg/hr Nitroglycerin transdermal patch (24 m^2, Mylan Pharmaceuticals, Morgantown, WV) in the skin area and quickly replace the laser Doppler probe. We will continue the measurement until we reach a new plateau. We are using the nitroglycerin transdermal patch in place of the NO donor sodium nitroprusside to induce maximal endothelium independent vasodilation. so we can normalize skin blood flow based on percent of the maximal response induced by the nitroglycerin/heating to account for the anatomical differences in the number of blood vessels under each probe. The nitroglycerin patch serves as a less invasive alternative to the sodium nitroprusside that is used to achieve maximal vasodilation at the other microdialysis sites because using microdialysis probe does not need to be inserted through the forearm skin. Cutaneous vascular conductance (CVC=LDF/MAP) will be calculated and normalized to %CVCmax based on published procedures. CVCmax will be determined by sodium nitroprusside in the 3 microdialysis probe sites and by the nitroglycerin patch in the local heating site. Comparisons between CVC values will allow us to evaluate the effect of estrogen suppression on vasodilation. The nitroglycerin will be wiped off the skin once the test is completed. If participants blood pressure decreases the subject will be given enough time to return to normal and be supported per standard of care procedures. (12, 13, 19, 20, 28, 35, 36, 38).

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We will collect blood samples for the measurement of estradiol, progesterone, endothelin-1, and sera concentrations of inflammatory markers (IL-6, TNFα, CRP, IL-1β) at the end of the recovery period just prior to beginning the perfusions.

To determine ovulation, we will provide the subjects with ovulation prediction strips and instructions on how to use the strips. From approximately the tenth day through the eighteenth day of the menstrual cycle prior to the research studies, subjects will test a sample of their urine at home to identify ovulation. We will use these test results to schedule the timing of the research studies the following month. The subject repeats tests on the few days prior to scheduled Visit 3 test day to confirm ovulation on the specific test days. The days for starting and beginning the ovulation tests are adjusted depending on the length of the cycle as the subject describes this to us.

Data analysis

Flow mediated vasodilation. Machine-learning software (Brachial Analyzer for Research, Medical Imaging Applications, USA) will be used to analyze ultrasound video files. After vessel walls are manually defined in a region of interest in a single frame of the file, edge-detection software is used to calculate vessel diameter throughout the rest of the video. Each video is manually reviewed to ensure that the artery walls are detected correctly, and the same region of interest is used consistently throughout each trial to minimize artificial intra-subject variation. Average baseline diameter was measured using one minute of longitudinal images of the brachial artery during the initial resting period. To determine the maximal reactive hyperemia diameter, measurements will be obtained continuously for the 90-s period following cuff release. Diameters will be compiled into three-second average time bins; the peak diameter and time-to-peak were recorded. The following equation is used to calculate the FMD response:

$$\%FMD = \frac{Max\ RH\ diam - Average\ BSL\ diam}{Average\ BSL\ diam} \times 100\%$$

PROCEDURES

GnRH antagonist. In a ***pre-post*** design all subjects will assigned to participate in baseline testing (pre testing, without Elagolix within the first 7 days of the menstrual cycle) first followed by Elagolix (400 mg/day oral, 2X200 mg tablets/day for 4-7 days); (33). Scheduling issues may extend Elagolix ingestion up to 7 days. All women will begin taking Elagolix in the first week of their menstrual cycle. The research will involve 3 visits to the lab over approximately 45 days. With this method of administration, the GnRH antagonist is easily stopped in the unlikely event of uncomfortable side effects. Administration of the GnRH antagonist suppresses steroidogenesis, leading to low or undetectable plasma estrogen and progesterone concentrations, which occurs within two days of initiation of administration (33). The suppression of the hypothalamic-pituitary-ovarian axis is reversed upon cessation of drug therapy. Administration of a similar GnRH antagonist. In these studies, Elagolix will be given as a treatment for experimental purposes or as standard of care in infertility patients. If scheduling permits, we will test the women in the same cycle, if not we will do the Elagolix testing during the next menstrual cycle.

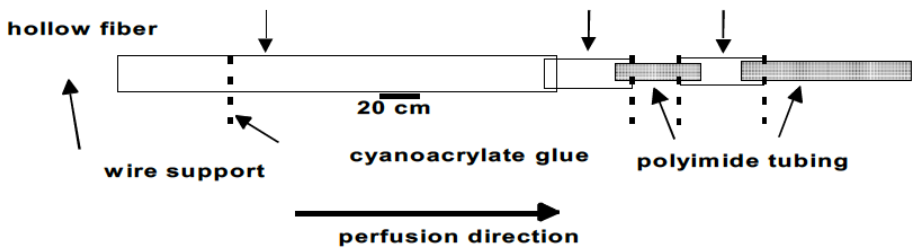
For subjects participating in the amendment (Aim 1a): In a ***pre-post*** design all subjects will be assigned to participate in baseline testing (pre testing, without Elagolix within the first 7 days of the menstrual cycle) first followed by their ovulation test (test 2), followed by Elagolix (400 mg/day oral, 2X200 mg tablets/day for 4-7 days) (33). Scheduling issues may extend Elagolix ingestion up to 7 days. All women will begin taking Elagolix in the first week of their menstrual cycle. The research will involve 4 visits to the lab over approximately 45-90 days, depending on scheduling.

Gonadotropin releasing hormone antagonist (Elagolix). Elagolix [*R*-(+)-4-[2-[5-(2-fluoro-3-methoxy-phenyl)-3-(2-fluoro-6-trifluoromethyl-benzyl)-4-methyl-2,6-dioxo-3,6-dihydro-2*H*-pyrimidin-1-yl]-1-phenyl-ethylamino]-butyrate] (7) has high antagonistic activity against naturally occurring GnRH. Elagolix in therapeutic doses (50-400 mg/day) acts by competitively blocking the GnRH receptors on the pituitary gonadotroph and subsequent transductions pathway, inducing a rapid, reversible suppression of gonadotropin secretion (33). In eumenorrheic women, Elagolix administration leads to suppression of estrogens to post-menopausal levels after 24 hours of administration (33). Importantly, Elagolix is safe and well tolerated. In this proposal, we will use Elagolix to suppress estrogens in both women with endometriosis and in healthy control women. The suppression of the hypothalamic-pituitary-ovarian axis is reversed upon cessation of drug therapy.

Cardiovascular variables. For all SkBF studies, arterial blood pressure will be continuously recorded from the middle digit of the left hand positioned at heart level by the Penaz method (Finometer, Finapres Medical Systems, Affligem, Belgium). The literature supports the use of the finger blood pressure in tracing beat-to-beat changes in central SBP (31). Heart rate will be determined beat-to-beat from lead II of an ECG recording (ADInstruments). Systolic, diastolic and mean blood pressures are measured in the right arm positioned at heart level with an automated sphygmomanometer device (Colin STBP model 780B, Aichi, Japan) during the microdialysis studies.

Skin blood flow (SkBF) and local skin heating or the response to topical drugs, respectively is measured using four separate probes at rest and during microdialysis with a Laser Doppler Monitor, PF 5020 LDPM Unit (Perimed AB, Stockholm, Sweden). The probe can maintain any temperature between 27°C and 43°C and heated by 0.1°C increments. **Cutaneous vascular conductance (CVC)** will be calculated as mean SkBF/mean arterial pressure.

Intradermal microdialysis: The microdialysis probes used in these proposed studies are constructed by CMA Microdialysis (North Chelmsford, MA). The microdialysis probe consists of an 8 cm piece of polyimide tubing, 4.5 cm of hollow fiber dialysis tubing, 6 cm of polyimide tubing, 20 cm of PE 50 tubing, and 13 cm of PE 10 tubing. The polyimide tubing is inserted 1 cm into the hollow fiber tubing and fixed on each end leaving 2 cm of exposed membrane. The hollow fiber microdialysis membrane is a regenerate cellulose membrane with an approximate 13,000 Dalton molecular weight cut-off.



Probe placement: Under sterile conditions the probe is placed in the intradermal space with a 27- gauge needle used as a guide cannula. Our use of this small gauge needle minimizes tissue irritation and eliminates the need for local anesthetic. Local anesthetic will not be used during probe placement and thus, the potential interference of lidocaine is eliminated. We use ice on the site locally to reduce blood flow and



Microdialysis set-up (from laboratory) with and without laser Doppler probes.

minimize release of vasoactive substances. The entrance and exit site on the skin are separated by at least 2 cm. The guide cannula is inserted horizontally in the dermis and the microdialysis probe is fed through the guide cannula. The needle is removed with the probe left in place at a depth of ≈ 2-5 mm. After placement the probe is perfused with lactate ringers at a rate of 10 µl/min with a Harvard infusion pump to flush out the glycerol packing solution. A 90-120 min recovery period after probe insertion is required to allow local SkBF to return to baseline before data measurements can be made.

Blood sampling: Blood sampling is done via 20-gauge catheter placed in an arm vein. Subjects will be in the posture they will assume for at least 30 min prior to sampling to ensure a steady state in plasma volume and constituents. Blood sampling is done from free-flowing blood. Approximately 45ml of blood (3 tablespoons) will be removed for analysis for Aim 1. Total removal for Aim 1a will be 60ml and for Aim 1b will be 15ml.

Plasma hormones: Progesterone, E2, ET-1, IL-6, TNFα, CRP and IL-1 β are measured with ELISA.

Urine analysis: Subjects are instructed to completely empty their bladder when voiding into a collection vessel and urine collection times are recorded. Urine osmolality is measured immediately.

The Endometriosis Health Profile-30 questionnaire is a 30-item questionnaire developed to determine quality of life (QoL) of women with endometriosis in both clinical and research settings. In our study, we also use this to determine changes in QoL while the women are undergoing our interventions. The questionnaire consists of 30 questions asking about health and symptoms of our subjects (15)

5.1.1 Data Collection

We collect non-digital data in notebooks that do not identify the subject by name (only by subject initials, codes, date and number). These notebooks are stored in the laboratory or office areas. We also store data in excel spreadsheets on PCs associated with our laboratory. Again, the subjects are only identified by subject initials, codes, date and number. These are separate from health histories and consent forms, which are stored in locked file cabinets and kept in the lab or in offices associated with the PI.

All papers with identifying information will be shredded after no longer needed or after 25 years. Until then, they are kept in locked cabinets in the PI's laboratory in or offices associated with the PI.

Confidentiality of all information in the study will be maintained by identifying subjects by code numbers. No individuals, other than the professionals directly involved in the study, will be allowed to read data forms. Investigators will be required to respect the confidentiality code. No subjects are identified by name in any of the published literature and only by code in data storage areas, to which access is limited to study personnel. The individual subject files are the only place where names are noted and all files are kept in the office of the PI. Access to the PI's office is limited to study personnel. To date, we have never had a violation of confidentiality. The Yale Human Investigation Committee may inspect all study records. The files with the subject's names are stored in a locked filing cabinet within Dr. Stachenfeld's laboratory.

5.1.2 Adverse Events Definition and Reporting

1. Attribution of Adverse Events:

Adverse events will be monitored for each subject participating in the study and attributed to the study procedures design by the PI (Nina Stachenfeld) according to the following categories:

a.) Definite: Adverse event is clearly related to investigational procedures(s)/agent(s). b.) Probable: Adverse event is likely related to investigational procedures(s)/agent(s). c.) Possible: Adverse event may be related to investigational procedures(s)/agent(s). d.) Unlikely: Adverse event is likely not to be related to the investigational procedures(s)/ agent(s). e.) Unrelated: Adverse event is clearly not related to investigational procedures(s)/ agent(s).

2. Plan for Grading Adverse Events:
The following scale will be used in grading the severity of adverse events noted during the study:

1. Mild adverse event
2. Moderate adverse event
3. Severe

3. Plan for Determining Seriousness of Adverse Events: Serious Adverse Events:
In addition to grading the adverse event, the DSMB will determine whether the adverse event meets the criteria for a Serious Adverse Event (SAE). An adverse event is considered serious if it:

1. is life-threatening
2. results in in-patient hospitalization or prolongation of existing hospitalization
3. results in persistent or significant disability or incapacity
4. results in a congenital anomaly or birth defect OR
5. results in death
6. based upon appropriate medical judgment, may jeopardize the subject's health and require medical or surgical intervention to prevent one of the other outcomes listed in this definition
7. adversely affects the risk/benefit ratio of the study

An adverse event may be graded as severe but still not meet the criteria for a Serious Adverse Event. Similarly, an adverse event may be graded as moderate but still meet the criteria for an SAE. It is important for the DSMB to consider the grade of the event as well as its seriousness when determining whether reporting to the HIC is necessary.

4. Plan for reporting serious AND unanticipated AND related adverse events, anticipated adverse events occurring at a greater frequency than expected, and other unanticipated problems involving risks to subjects or others to the HIC/IRB.

The investigator and DSMB will report the following types of adverse events to the site specific HRPP/IRB; a) serious AND unanticipated AND possibly, probably or definitely related events; b) anticipated adverse events occurring with a greater frequency than expected; and c) other unanticipated problems involving risks to subjects or others.

5.2 Informed Consent

Consent forms describing in detail the study intervention, study procedures, and risks are given to the participant and written documentation of informed consent is required prior to starting intervention/administering study intervention. The following consent materials are submitted with this protocol:

- Consent Form

5.2.1 Screening (if applicable)

The screening process is done via the recruiting process described below and assisted by Dr. Taylor. We ask potential subjects if they are women with or without endometriosis when signing up. The PI or postdoc asks questions related to exclusion and inclusion during the initial phone interview (See Appendix 2).

5.2.2 Recruitment, Enrollment and Retention (if applicable)

Subjects will be recruited by advertisement from The Yale School of Medicine, Yale University, the Yale Fertility Clinic and surrounding communities. We will use Departmental/Center Newsletters and Web-Based Clinical Trial Registries, in addition to social media. We will also use the YCCI email database for recruitment (See below). Interested study subjects can call or email the PI or Postdoctoral Associate to gain more information and to undergo a telephone screening. We will advertise in the common eating establishments and mailrooms on campus, as well as on bulletin boards throughout campus buildings. We will note the age group we are recruiting for on the flyers (Appendix 1). While recruiting over the phone, we ask the individual's first name, gender and phone number. We also ask height, weight and if they have been diagnosed with endometriosis. This information is not written down but is used to determine if subjects may qualify for the study. In addition, Dr. Taylor may refer patients directly to the study.

We have applied through JDAT to contact women with endometriosis (not controls).
For recruitment, we will utilize the Joint Data Analytics Team (JDAT) to identify potential subjects with endometriosis. This is a behind-the-scenes search run by JDAT that will not identify the potential participants to the researchers, and therefore the researchers will receive no protected information. Once identified, potential subjects will be sent a message via MyChart that provides information about our endometriosis cardiovascular research study as well as information on how to contact us, and brief information about the study. Potential subjects who do not use MyChart will receive a paper mailing including information about the study as well as information on how to contact us.

The MyChart language is provided in a document including the message patients receive via JDAT describing the study and inviting them to participate.

YCCI Add:
Healthy Women Volunteers Needed
If you are a **woman who is 18 to 45 years** you may be eligible to participate in a free and confidential study designed to look at how estrogen may affect cardiovascular health.

Minority women are encouraged to participate as it is important to study different groups of people to understand what treatments work best for most women.
Compensation up to $380 for Aim 1, $490 for Aims 1a Healthy Control and 1a Endo research, $200 for Aim 1b.

To learn more or to see if you are eligible to participate, please email Nina Stachenfeld at nina.stachenfeld@yale.edu.

Sponsored by the National Institutes of Health, National Heart Lung, and Blood Institute;
HIC#: 2000022193

The protocols will be thoroughly described during the initial phone conversation before the orientation. (See Appendix 2for telephone script). Subjects are identified only by first names during this initial conversation. We also write down the caller's phone number in case we need to cancel or change the appointment. When the subject arrives for the consent process, we review the protocol and indicate that she should ask any questions or express concerns before

signing the consent form. We stress that subjects are under no obligation to sign the consent form or participate in the study if they are uncomfortable with any aspects of it. We also stress that she will not be penalized if she decides not to participate. In addition, we tell the subjects that they are allowed to withdraw their consent at any time without prejudice and are advised of this fact prior to participating. These explanations are in the consent form as well.

Implementation of the Consent Process
Project staff will implement the written informed consent process during the lab visit that follows initial screening. The researcher will provide the participant with two copies of the consent form signed and dated by the researcher and allow them sufficient time to read the document. The researcher will then review the document with participants, answering any questions from the participant, and querying comprehension periodically. Participants will indicate their consent by signing both copies of the consent form. One copy will be given to the participant and the other will be retained in the project records.

Participant Retention and Adherence Strategies
A variety of strategies will be used to support participant retention, including calls from the PI or Postdoctoral Associate to check in and assess any issues with study medications. Research staff will assess protocol compliance for maintaining engagement, and financial compensation for completing study milestones (e.g., lab visits, outcome assessments). We have successfully applied similar methods in previous intervention studies much longer than the proposed study.

5.2.3 Study Visits (is applicable)

Research Protocol: Aim 1 Healthy Controls and Endometriosis
Visit 1:
- Screening,
- informed consent,
- orientation.

Visit 2: [pre-testing or baseline)]:
- Physiological studies, QoL survey (endometriosis subjects)

Visit 3: [while taking Elagolix)]:
- Physiological studies, QoL survey (endometriosis subjects)

Aim 1a Healthy Controls and Endometriosis:
Research Protocol:
Visit 1:
- Screening,
- informed consent,
- orientation.

Visit 2: [pre-testing or baseline]:
- Physiological studies, QoL survey (endometriosis subjects)

Visit 3 [ovulatory phase]:
- Physiological studies, QoL survey (endometriosis subjects)

Visit 4: [while taking Elagolix]:
- Physiological studies, QoL survey (endometriosis subjects)

Adds an additional visit for subjects who have completed our initial studies and wish to participate in this new amendment.

Aim 1b Healthy Controls:

Visit 4 [ovulatory phase]:

- Physiological studies,

Statistical Method

5.2.4 Statistical Design

5.2.5 Statistical analyses: Univariate statistics [mean (SD)] will be generated on all study variables first, which will determine the normality or the heteroscedacity of the data. Data will be analyzed using SPSS statistical software, that includes calculations to compensate for missing data, and Prism (GraphPad) curve fitting software where appropriate. Data analysis. Data will be analyzed within each group [Control and GnRH ant and among each of the pharmacologic conditions (Ringers, E2, ACh). Analyses will be performed separately within each group (with or without endometriosis). Skin blood flow and CVC for each of the three conditions (Ringers, E2, ACh) will be determined. Dose-response curves will be determined for each of the pharmacological conditions. D-R curves will be determined and compared using Prism software. While we will lack statistical power to draw specific conclusions for interactions in our data, we will look for them and interpret the data accordingly. Sample Size Considerations

5.2.6 Sample size calculation. Sample size calculations are based on our primary outcome variable of interest: CVC. The desired statistical test is two-sided, and we will assume an alpha level of =0.01 to account for multiple comparisons (26). Given 12 women per group and alpha=0.01, this effect size would allow us >80% power to differentiate these changes from chance (26).

5.2.7 Planned Analyses. Data will be analyzed within each group [Control and GnRH ant and among each of the pharmacologic conditions (Ringers, E2, ACh). Analyses will be performed separately within each group (with or without endometriosis). Skin blood flow and CVC for each of the three conditions (Ringers, E2, ACh) will be determined. Dose-response curves will be determined for each of the pharmacological conditions. D-R curves will be determined and compared using Prism software. While we will lack statistical power to draw specific conclusions for interactions in our data, we will look for them and interpret the data accordingly.

5.2.8 Analysis of Subject Characteristics (if applicable)

We will use t-tests to analyze differences between groups in BMI, hormone levels, height, weight, and age.

5.2.9 Interim Analysis (if applicable)

N/A

5.2.10 Handling of Missing Data

We will use SPSS programs for handling of missing data.

6 Trial Administration

6.1 Ethical Considerations: Informed Consent/Assent and HIPAA Authorization

Consent forms will be Institutional Review Board (IRB)-approved and the participant will be asked to read and review the document. The PI or Post Doc will explain the research study to the participant and answer any questions that may arise. This conversation will take place in a private room.

APPROVED BY THE YALE UNIVERSITY IRB 5/29/2025

Staff Training
All researchers, project staff, and research assistants working on this project will complete online training on Responsible Conduct of Research and Human Subjects Research offered by the Collaborative Institutional Training Initiative (CITI) program as well as Good Clinical Practice in Social and Behavioral Science, the NIH-approved module hosted by the Society for Behavioral Medicine. Completion of the module quizzes in the courses result in the certificate of completion for the training which is submitted to the Yale University IRB. Refresher trainings will be completed three years after the initial training, and some training is updated via Yale University annually.

Given our protocols, it is highly unlikely that a previously unknown condition will be discovered. If this occurs, we will let the subject know that this possibility exists and refer him/they to their physician.

The protocols will be thoroughly described during the initial phone conversation before the orientation. Subjects are identified only by first names during this initial conversation. We also write down the caller's phone number in case we need to cancel or change the appointment. When the subject arrives for the consent process, we review the protocol and indicate that she should ask any questions or express concerns before signing the consent form. We stress that subjects are under no obligation to sign the consent form or participate in the study if they are uncomfortable with any aspects of it. We also stress that she will not be penalized if she decides not to participate. In addition, we tell the subjects that they are allowed to withdraw their consent at any time without prejudice and are advised of this fact prior to participating. These explanations are in the consent form as well.

Implementation of the Consent Process
Consent forms will be Institutional Review Board (IRB)-approved. Project staff will implement the written informed consent process during the lab visit that follows initial screening. The researcher will provide the participant with two copies of the consent form signed and dated by the researcher and allow them sufficient time to read the document. The researcher will then review the document with participants, answering any questions from the participant, and querying comprehension periodically. Participants will indicate their consent by signing both copies of the consent form. One copy will be given to the participant and the other will be retained in the project records.

Participant Retention and Adherence Strategies
A variety of strategies will be used to support participant retention, including calls from the PI or Postdoctoral Associate to check in and assess any issues with study medications. Research staff will assess protocol compliance for maintaining engagement, and financial compensation for completing study milestones (e.g., lab visits, outcome assessments). We have successfully applied similar methods in previous intervention studies much longer than the proposed study.

Subject Understanding
A verbal explanation will be provided in terms suited to the participant's comprehension of the purposes, procedures, and potential risks of the study and of their rights as research participants. We will ask questions about the more complex procedures and have the subjects explain the procedures back to us. For example, we will ask the subjects to tell us what kind of instruments will be used for microdialysis and where we will make the measurements, and how long they will be here for the studies. We will also ask them to explain to us what the Elagolix pill might do to them. In addition, we review these prior to each visit in case the subject has forgotten. Finally, prior to beginning the study, and after the consent is signed,

we provide a short orientation where we show the subjects the room and probes for microdialysis.

Safeguards for Vulnerable Populations (Children, Pregnant Women)
Children under 18, pregnant women, prisoners, and non-English speaking participants will be excluded from this study. Participants with any medical contraindications will be excluded. Compensation will be structured so it is not coercive for members of economically disadvantaged groups. The consent discussion will be presented at a level suitable for each participant.

6.2 Institutional Review Board (IRB) Review

The protocol will be submitted to the IRB for review and approval. Approval of the protocol must be obtained before initiating any research activity. Any change to the protocol or study team will require an approved IRB amendment before implementation. The IRB will determine whether informed consent and HIPAA authorization are required.

A study closure report will be submitted to the IRB after all research activities have been completed.

6.3 Subject Confidentiality

Participant confidentiality and privacy is strictly held in trust by the participating investigators, their staff, and the sponsor(s) and their interventions. Therefore, the study protocol, documentation, data, and all other information generated will be held in strict confidence. No information concerning the study or the data will be released to any unauthorized third party without prior written approval of the sponsor.

All research activities will be conducted in as private a setting as possible.

The study monitor, representatives of the Institutional Review Board (IRB), or regulatory agencies may inspect all documents and records required to be maintained by the investigator, including but not limited to, medical records (clinic or Yale School of Medicine) for the participants in this study. The clinical study site will permit access to such records.

The study participant's contact information will be securely stored at each clinical site for internal use during the study. At the end of the study, all records will continue to be kept in a secure location for as long a period as dictated by the reviewing IRB, Institutional policies, or, if applicable, sponsor requirements.

Study participant research data, which is for purposes of statistical analysis and scientific reporting, will be transmitted to and stored at the Yale School of Medicine. This will not include the participant's contact or identifying information. Rather, individual participants and their research data will be identified by a unique study identification number. The study data entry and study management systems used will be secured and password protected. At the end of the study, all study databases will be de-identified and archived at the Yale School of Medicine.

6.4 Deviations/Unanticipated Problems

A protocol deviation is any noncompliance with the study protocol. The noncompliance may be either on the part of the participant, the investigator, or the study site staff. As a result of deviations, corrective actions are to be developed by the site and implemented promptly.

It is the responsibility of the site investigator to identify and report deviations within 7 working days of identification of the protocol deviation. All deviations must be addressed in study source documents, reported to the study sponsor, and the reviewing Institutional Review Board (IRB) per their policies.

Unanticipated problems involving risks to participants or others include, in general, any incident, experience, or outcome that meets all of the following criteria:
Unexpected in terms of nature, severity, or frequency given (a) the research procedures that are described in the protocol-related documents, such as the Institutional Review Board (IRB)-approved research protocol and informed consent document; and (b) the characteristics of the participant population being studied;
Related or possibly related to participation in the research ("possibly related" means there is a reasonable possibility that the incident, experience, or outcome may have been caused by the procedures involved in the research); and
Suggests that the research places participants or others at a greater risk of harm (including physical, psychological, economic, or social harm) than was previously known or recognized.

The investigator will report unanticipated problems (UPs) to the reviewing Institutional Review Board (IRB) and to the study sponsor. The UP report will include the following information:
Protocol identifying information: protocol title and number, PI's name, and the IRB project number;
A detailed description of the event, incident, experience, or outcome;
An explanation of the basis for determining that the event, incident, experience, or outcome represents an UP;
A description of any changes to the protocol or other corrective actions that have been taken or are proposed in response to the UP.

To satisfy the requirement for prompt reporting, UPs will be reported using the following timeline:
UPs that are serious adverse events (SAEs) will be reported to the IRB and study sponsor, if applicable within 2 days of the PI becoming aware of the event.
Any other UP will be reported to the IRB and study sponsor within 7 days of the PI becoming aware of the event of the investigator becoming aware of the problem.
All UPs should be reported to appropriate institutional officials within 48 hours, the supporting agency head (or designee), and the Office for Human Research Protections (OHRP) within & 2 weeks of the IRB's receipt of the report of the problem from the investigator.

6.5 Data Quality Assurance

The Principal Investigator has extensive experience with biomedical research. Her project staff also has long experience on best practices for screening and consenting potential participants. Any new trainees participating in these studies will also be trained on best practices for screening and consenting potential participants. The project team will include an experienced post-doctoral associate who has previously conducted biomedical research.

All researchers, project staff, and research assistants working on this project will complete online training on Responsible Conduct of Research and Human Subjects Research offered by the Collaborative Institutional Training Initiative (CITI) program as well as Good Clinical Practice in Social and Behavioral Science, the NIH-approved module hosted by the Society for Behavioral Medicine. Completion of the module quizzes in the courses result in the certificate of completion for the training which is submitted to the Yale University IRB.

Refresher trainings will be completed three years after the initial training, and some training is updated via Yale University annually.

6.6 Study Records

Lab Group Meetings: Data safety and management procedures, interim data analyses, and quality control issues are discussed at weekly meetings involving pertinent laboratory personnel. These meetings are led by Dr. Stachenfeld or the Postdoctoral associate.

We collect non-digital data in notebooks during the experiments that do not identify the subject by full name and rather identify subjects by their initials, date and assigned number. These notebooks are stored in the laboratory or office areas. We also store data in excel spreadsheets on computers associated with our laboratory. Again, the subjects are only identified by subject initials, date and number.
The subjects do not keep study diaries, etc. They receive a copy of their consent forms.
The subjects will provide private information on a medical history form (Appendix 3) to indicate any reason to exclude them from the study. This information includes details on their race/ethnicity, medical history, allergies to medication, current medication use, hospitalization, exercise habits, alcohol, smoking and menstrual periods. Only the Principal Investigator (Dr. Stachenfeld), the Post-doctoral associate and Ms. Leone will have access to this information. These records are stored in locked cabinets in Dr. Stachenfeld's laboratory space.

6.7 Access to Source

Source data will be maintained per Medical Records policy in a password protected, secure, Health Insurance Portability and Accountability Act (HIPAA) compliant, web-based electronic database with a built-in audit trail.

Only Institutional Review Board (IRB) approved research team members who have current HIPAA and Collaborative Institutional Training Initiative (CITI) Good Clinical Practice (GCP) and human subjects' protection training will be authorized to access records.

6.8 Data or Specimen Storage/Security

We collect non-digital data in notebooks that do not identify the subject by full name and rather identify subjects by their initials, date and assigned number. These notebooks are stored in the laboratory or office areas. We also store data in excel spreadsheets on computers associated with our laboratory. Again, the subjects are only identified by subject initials, date and number. Stored specimens are identified only by subject number and cannot be connect to the subjects' identity. These are stored either in the -20 freezers in Dr. Stachenfeld's laboratory or in the -80 freezers at the Yale School of Medicine. Lab notebooks are stored in Dr. Stachenfeld's laboratory.

6.9 Retention of Records

25 years or less.

6.10 Study Monitoring

The NIH indicated that a DSMB is not required for this study because of the small number of subjects.

6.11 Study Modification

Any change to the study will be submitted to the HIC for review as an amendment.

6.12 Study Completion

We are unsure as funding is pending.

6.13 Funding Source

NIH 1R01HL161000. Salary support is from the Yale School of Medicine via NIH funding.

6.14 Conflict of Interest Policy

The independence of this study from any actual or perceived influence, such as by the pharmaceutical industry, is critical. Therefore, any actual conflict of interest of persons who have a role in the design, conduct, analysis, publication, or any aspect of this trial will be disclosed and managed. Furthermore, persons who have a perceived conflict of interest will be required to have such conflicts managed in a way that is appropriate to their participation in the trial. The study leadership in conjunction with the appropriate conflict of interest review committee has established policies and procedures for all study group members to disclose all conflicts of interest and will establish a mechanism for the management of all reported dualities of interest.

Any investigator who has a conflict of interest with this study (patent ownership, royalties, or financial gain greater than the minimum allowable by their institution, etc.) must have the conflict reviewed by the Yale Provost's Committee on COI with a Committee-sanctioned conflict management plan that has been reviewed and approved by the study sponsor prior to participation in this study. All investigators will follow the applicable conflict of interest policies.

6.15 Publication Plan

We hope to submit manuscripts relating to Aim 1 after the third and fourth years of this project. The PI is responsible for all publications. Should these studies be funded by NIH, we will follow the following **dissemination plan**

I. Disseminating the Results

1. **Presentation at Scientific Meetings.** The Post-doctoral fellow and investigator will present at the major scientific meetings, such as Experimental Biology, and the American Heart Association, etc.
2. **Publication in High Impact Scientific Journals** – we will submit results for publication as soon as possible after data collection, analysis and writing. An electronic version of final, accepted, peer-reviewed manuscripts will be submitted to the National Library of Medicine's PubMed Central within 12 months after the initial publication date as directed by the NIH Public Access Policy.

Appendices

Appendix #	Title	Section	Topic
1	Flyers	5.2.2	Study Procedures
2	Recruiting Script	5.2.1 & 5.2.2	Study Records
3	Medical History	6.6	Study Records

List of Tables

Table 1. Microdialysis studies Aim 1.

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