Date: 1/13/2022

Title: Mechanisms of Hypertension in Women With Polycystic Ovary Syndrome

ID: NCT04327934

Document(s): Protocol w SAP

YALE UNIVERSITY
HUMAN INVESTIGATION COMMITTEE

Application to Involve Human Subjects in Biomedical Research
100 FR1 (2016-1)

SECTION I: ADMINISTRATIVE INFORMATION

Title of Research Project: Mechanisms of hypertension in women with polycystic ovary syndrome			
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Faculty Advisor:(required if PI is a student, resident, fellow or other trainee) ☐ **NA**		**Yale Academic Appointment:**	
Campus Address:			
Campus Phone:	**Fax:**	**Pager:**	**E-mail:**

Investigator Interests:

Does the principal investigator, or do any research personnel who are responsible for the design, conduct or reporting of this project or any of their family members (spouse or dependent child) have an incentive or interest, financial or otherwise, that may affect the protection of the human subjects involved in this project, the scientific objectivity of the research or its integrity? Note: The Principal Investigator (Project Director), upon consideration of the individual's role and degree of independence in carrying out the work, will determine who is responsible for the design, conduct, or reporting of the research.

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See Disclosures and Management of Personal Interests in Human Research
http://www.yale.edu/hrpp/policies/index.html#COI
☐ Yes x No

Do you or does anyone on the research team who is determined by you to be responsible for the design, conduct or reporting of this research have any patent (sole right to make, use or sell an invention) or copyright (exclusive rights to an original work) interests related to this research protocol?
☐ Yes x No

If yes to either question above, list names of the investigator or responsible person:

The Yale University Principal Investigator, all Yale University co-investigators, and all Yale University individuals who are responsible for the design, conduct or reporting of research must have a current financial disclosure form on file with the University's Conflict of Interest Office. Yale New Haven Hospital personnel who are listed as co-investigators on a protocol with a Yale University Principal Investigator must also have a current financial disclosure form on file with the University's Conflict of Interest Office. If this has not been done, the individual(s) should follow this link to the COI Office Website to complete the form: http://www.yale.edu/coi/

NOTE: The requirement for maintaining a current disclosure form on file with the University's Conflict of Interest Office extends primarily to Yale University and Yale-New Haven Hospital personnel. **Whether or not they are required to maintain a disclosure form with the University's Conflict of Interest Office, all investigators and individuals deemed otherwise responsible by the PI who are listed on the protocol are required to disclose to the PI any interests that are specific to this protocol.**

SECTION II: GENERAL INFORMATION

1. **Performing Organizations:** Identify the hospital, in-patient or outpatient facility, school or other agency that will serve as the location of the research. Choose all that apply:

a. Internal Location[s] of the Study:

☐ Magnetic Resonance Research Center (MR-TAC)
☐ Yale Cancer Center/Clinical Trials Office (CTO)
☐ Yale Cancer Center/Smilow
☐ Yale-New Haven Hospital
☐ Cancer Data Repository/Tumor Registry
☐ Specify Other Yale Location:
☐ Yale University PET Center
☐ YCCI/Church Street Research Unit (CSRU)
☐ YCCI/Hospital Research Unit (HRU)
☐ YCCI/Keck Laboratories
☐ Yale-New Haven Hospital—Saint Raphael Campus

b. External Location[s]:

☐ APT Foundation, Inc.
☐ Haskins Laboratories

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☐ Connecticut Mental Health Center
☐ Clinical Neuroscience Research Unit (CNRU)
☐ Other Locations, Specify:

☒ John B. Pierce Laboratory, Inc.
☐ Veterans Affairs Hospital, West Haven
☐ International Research Site
(Specify location(s)):

c. Additional Required Documents *(check all that apply)*: ☐ N/A

☐ *YCCI-Scientific and Safety Committee (YCCI-SSC)	Approval Date:
☐ *Pediatric Protocol Review Committee (PPRC)	Approval Date:
☐ *YCC Protocol Review Committee (YRC-PRC)	Approval Date:
☐ *Dept. of Veterans Affairs, West Haven VA HSS	Approval Date:
☐ *Radioactive Drug Research Committee (RDRC)	Approval Date:
☐ YNHH-Radiation Safety Committee (YNHH-RSC)	Approval Date:
☐ Yale University RSC (YU-RSC)	Approval Date:
☐ Magnetic Resonance Research Center PRC (MRRC-PRC)	Approval Date:
☐ *Nursing Research Committee	Approval Date:
☐ YSM/YNHH Cancer Data Repository (CaDR)	Approval Date:

☐ Dept. of Lab Medicine request for services or specimens form
☐ Imaging on YNHH Diagnostic Radiology equipment request form (YDRCTO request) found at http://radiology.yale.edu/research/ClinTrials.aspx)

Approval from these committees is required before final HIC approval is granted. See instructions for documents required for initial submission and approval of the protocol. Allow sufficient time for these requests. Check with the oversight body for their time requirements.

2. **Probable Duration of Project:** State the expected duration of the project, including all follow-up and data analysis activities.
   Six years
3. **Research Type/Phase: (Check all that apply)**
   a. **Study Type**
   ☒ Single Center Study
   ☐ Multi-Center Study
   Does the Yale PI serve as the PI of the multi-site study? Yes ☐ No ☒
   ☐ Coordinating Center/Data Management
   ☐ Other:

   b. **Study Phase** ☒ **N/A**
   ☐ Pilot ☐ Phase I ☐ Phase II ☐ Phase III ☐ Phase IV
   ☐ Other (*Specify)*

4. **Area of Research: (Check all that apply)** Note that these are overlapping definitions and more than one category may apply to your research protocol. Definitions for the following can be found in the instructions section 4c:

☒ Clinical Research: Patient-Oriented
☐ Clinical Research: Epidemiologic and Behavioral
☐ Clinical Research: Outcomes and Health Services

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☐ Translational Research #1 ("Bench-to-Bedside") ☐ Interdisciplinary Research
☐ Translational Research #2 ("Bedside-to-Community") ☐ Community-Based Research

5. Is this study a clinical trial? Yes ☐ No ☒

6. Does the Clinical Trials Agreement (CTA) require compliance with ICH GCP (E6)?
Yes ☐ No☐

7. Will this study have a billable service? *A billable service is defined as any service rendered to a study subject that, if he/she was not on a study, would normally generate a bill from either Yale-New Haven Hospital or Yale Medical Group to the patient or the patient's insurer. The service may or may not be performed by the research staff on your study, but may be provided by professionals within either Yale-New Haven Hospital or Yale Medical Group (examples include x-rays, MRIs, CT scans, specimens sent to central labs, or specimens sent to pathology). Notes: 1. There is no distinction made whether the service is paid for by the subject or their insurance (Standard of Care) or by the study's funding mechanism (Research Sponsored). 2. This generally includes new services or orders placed in EPIC for research subjects*.

Yes ☐ No☒

If answered, "yes", this study will need to be set up in OnCore, Yale's clinical research management system, for Epic to appropriately route research related charges. Please contact oncore.support@yale.edu

8.. Are there any procedures involved in this protocol that will be performed at YNHH or one of its affiliated entities? Yes ___ No _X__ *If Yes, please answer questions a through c and note instructions below. If No, proceed to Section III.*

a. Does your YNHH privilege delineation currently include the **specific procedure** that you will perform?

b. Will you be using any new equipment or equipment that you have not used in the past for this procedure?

c. Will a novel approach using existing equipment be applied?

If you answered "no" to question 8a, or "yes" to question 8b or c, please contact the YNHH Department of Physician Services (688-2615) for prior approval before commencing with your research protocol.

Please note that if this protocol includes Yale-New Haven Hospital patients, including patients at the HRU, the Principal Investigator and any co-investigators who are physicians or mid-level practitioners (includes PAs, APRNs, psychologists and speech pathologists) who may have direct patient contact with patients on YNHH premises must have medical staff appointment and appropriate clinical privileges at YNHH. If you are uncertain whether the study personnel meet the criteria, please telephone the Physician Services Department at 203-688-2615. ***By signing this protocol as a PI, you attest that you and any co-investigator who may have patient contact has a medical staff appointment and appropriate clinical privileges at YNHH****.*

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SECTION V: RESEARCH PLAN

1. **Statement of Purpose:** State the scientific aim(s) of the study, or the hypotheses to be tested.

Aim 1. To test the hypothesis that androgen excess drives sympathetic activation leading to increased BP in AE-PCOS. ***We hypothesize*** that in women with AE-PCOS: **1A.** pharmacological androgen suppression reduces sympathetic outflow, α-adrenergic vasoconstriction, improves baroreflex sensitivity (BRS), and BP; **1B.** androgen supplementation increases sympathetic outflow, impairs BRS, reduces BP, and decreases β-adrenergic vasodilation.

Aim 2. To test the hypothesis that androgen-driven sympathetic activation increases RAS activity and BP in AE-PCOS. ***We hypothesize*** that in women with AE-PCOS: **2A**. pharmacological androgen suppression reduces sympathetic activation of the RAS and lowers BP; **2B.** androgen administration increases sympathetic activation of RAS and increases BP.

Aim 3. To test the hypothesis that androgen excess alters autonomic regulation and endothelial function in women with PCOS. ***We hypothesize*** that in women with AE-PCOS: pharmacological androgen suppression reduces baroreflex sensitivity (BRS) and improves BP and endothelial function

Aim 4. To determine the effectiveness of the orally active gonadotropin releasing hormone (GnRH) antagonist, *elagolix* in suppressing testosterone. ***We hypothesize*** that taking *elagolix tablets*, 400 mg/day oral, 2X200 mg tablets/day for 7 days will be as effective at suppressing testosterone as the GnRH antagonist ganirelix acetate.

Aim 5: To test the hypothesis that sympathetic outflow, α-adrenergic vasoconstriction, baroreflex sensitivity, and blood pressure are increased in black women with AE-PCOS compared to white women with AE-PCOS.

2. **Background:** Describe the background information that led to the plan for this project. Provide references to support the expectation of obtaining useful scientific data.

Our scientific premise *is that in AE-PCOS women, the androgen-dominant hormonal milieu causes BP increases via sympathetic activation, vasoconstriction and renal sympathetic nervous system activation. Moreover, this androgen-dominant milieu increases BP via activation of the renin-angiotensin system. Aim 3 will address the impact of androgens on baroreflex sensitivity (BRS) and endothelial function, and determine the interaction of these on the control of BP.*

While AE-PCOS often gets unmasked in the process of evaluation for infertility for many affected women, the long-term health risks for this population often remain underappreciated; in particular, attention to cardiovascular and metabolic risk factors should be integral to their care. Common symptoms for which young AE-PCOS women initially seek assessment include menstrual irregularities (absent, infrequent or erratic periods), and symptoms related to hyperandrogenism, including hirsutism, bothersome acne, and even androgenic alopecia (4). Metabolic abnormalities are also common with AE-PCOS, including android type obesity, dyslipidemia, and insulin resistance (IR), with hyperinsulinemia (4, 30). One third of AE-PCOS women develop metabolic syndrome (MetS) by 49 years of age, with many developing MetS before they reach 40 yrs. This is in stark contrast to the 6.7% prevalence of MetS in U.S. women between ages 20-30 yrs, and the 15% prevalence in women between ages 30-40 yrs (27). In AE-PCOS, hyperinsulinemia

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exacerbates hyperandrogenism by inducing both ovarian and adrenal androgen production. These women manifest a spectrum of covert markers for increased CVD risk (such as higher BP, an atherogenic lipid profile, and a proinflammatory milieu), at younger ages compared to women without PCOS. Important for the present investigation is the proposed relationship between androgens and sympathetic regulation of BP that has not been previously examined in obese AE-PCOS women. Treatment of AE-PCOS women is also complicated since the most commonly utilized therapy in managing PCOS symptoms, such as oral contraceptive pills, can worsen BP, and many standard BP lowering drugs, such as RAS blockers, are contraindicated in women who want to become pregnant.

In the present proposal we will use physiological approaches in combination with cellular and molecular mechanistic techniques to address fundamental questions concerning the contribution of impaired autonomic and RAS function to BP regulation in AE-PCOS women and a model of hyperandrogenemia in female (HAF) rats to specifically understand if androgen signaling is relevant to these processes. Determining the mechanisms responsible for androgen-mediated increases in BP will not only allow successful intervention in young AE-PCOS women, but also break the cycle of increased androgens leading to early CVD, thus improving their long-term health and reducing health care costs. Should we demonstrate a relationship between androgens, SNSA, and the RAS, modulation of SNA could be a valuable treatment to inhibit the underlying causes of AE-PCOS and improve cardiovascular outcomes when other antihypertensive medications, such as RAS blockers or antiandrogens, are contraindicated.

Novel pathways or therapies: Mild hypertension (HT) common to AE-PCOS women usually goes untreated since the level of BP doesn't reach clinical guidelines for treatment. However, elevated BP is the most important risk factor for later CVD. Our studies that include human subjects, will determine if therapies aimed at addressing elevated SNA or plasma aldosterone ($P_{[ALD]}$) *will reduce BP in women with AE-PCOS and thus overall cardiovascular disease (CVD) risk.* While the pathogenesis of CVD and HT is multifaceted in AE-PCOS, SNS and renal dysfunction likely underlie the development of critical cardiovascular dysfunction. The mechanisms responsible for SNA are unknown, but we have preliminary evidence in the HAF rat model that androgens increase hypothalamic MC4R expression and intracerebroventricular blockade reduces their BP (60). In contrast, in other HT rat models, only males respond to MC4R blockade with a reduction in BP (16, 61), suggesting that hyperandrogenemia in the HAF rats may mediate the increased SNA. In addition, even mild physiological increases in $P_{[ALD]}$ predispose women to development of HT (101). Aldosterone can also cause CVD and endothelial dysfunction independent of its BP effect (83), since the vascular wall is an aldosterone target tissue, and vascular cells can produce local aldosterone (94), thus increasing vascular tone and structure directly *while at physiological concentrations* (80). Moreover, a direct correlation between aldosterone and intima-media thickness (IMT) has been shown in AE-PCOS women (12), consistent with the adverse role of the RAS in vascular remodeling and in the progression of atherosclerosis associated with high IMT (81).

Aim 3. Background. Endothelial function. Women with androgen AE-PCOS often develop endothelial dysfunction (ED) in their early twenties (46, 92). Endothelial dysfunction is a known early marker of (and probable causative factor in) CVD (50, 84, 86). Although young women with AE-PCOS commonly manifest a spectrum of covert CVD risk markers, including obesity, insulin resistance and hypertension, we believe that androgen excess in young women is a major driver of ED. We have generated preliminary endothelial-dependent vasodilation data in lean, insulin-sensitive AE-PCOS subjects, which support our androgen-dependent vascular pathology hypothesis. A major feature of the normally functional endothelium is the production of NO in response to a variety of agonists. ***Our scientific premise is*** *that this is a key causative link between infertility and CVD, and is even more broadly applicable to the sharp rise in CVD incidence at and beyond the menopausal transition.*

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Our proposal is high impact and can impact treatment of AE-PCOS women for the following reasons:

1. Mechanisms of HT are less well understood, and thus more challenging to treat, in women in whom complications including heart failure with preserved ejection fraction are more common, and the incidence of resistant HT is higher (40, 62, 63). 2. Even mild HT in AE-PCOS women is complex to treat because many women are trying to conceive, and many antihypertensives are teratogenic or unsafe for the fetus.

3. High free androgen index (FAI) in AE-PCOS women is associated with high BP independent of IR, body mass index (BMI), and dyslipidemia (13), but antiandrogens risk feminization of external genitalia of male fetuses if given during pregnancy and are thus contraindicated.

4. AE-PCOS women develop pre-HT in their early twenties, predisposing them to later frank HT and CVD (5, 46). However, these young women are not typically treated because their BP has not reached standard clinical treatment guidelines. Thus, an opportunity to prevent or reduce the long-term CVD health risk associated with AE-PCOS is missed.

5. The cause(s) of the mild BP increase in AE-PCOS is unknown, but SNS activation (82, 93) and renal sympathetic nerve activation (RSNA) may contribute to BP elevation.

6. According to the NIH and Rotterdam criteria, PCOS prevalence is 8-10% (85), that is approximately seven million U.S. women and more than 100 million women worldwide (72). *Using 2004 statistics, the cost of screening and treating PCOS women exceeds $4 billion annually, excluding CVD and other comorbidities*.

7. The AE-PCOS phenotype accounts for 75% of PCOS cases in the US. AE is also associated with endothelial dysfunction and HT, the most common risk factor for later CVD, so AE-PCOS treatment includes identification and treatment of these comorbidities in addition to patients' immediate concerns (85).

8. For Aim 4: the GnRH antagonist, ganirelix acetate is effective at suppressing testosterone in women with AE-PCOS but requires daily injections. With this Aim, we would like to determine if this new orally active GnRH antagonist is as effective so will spare the subjects the injections, thereby making participation in the studies less stressful.

Aim 5 Background:

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality in women. Chronic increases in sympathetic nervous system activity (SNSA) are associated with CVD and hypertension. The arterial baroreflex is a key autonomic homeostatic mechanism involved in beat-to-beat regulation of blood pressure whereby the sympathetic nervous system acts to maintain and control blood pressure. Young healthy black women have lower baroreflex sensitivity and higher sympathetic modulations at rest compared to their age matched white counterparts. These findings indicate differences in blood pressure regulatory mechanisms between racial groups are present in women less than 25 years of age (54). Further, the changes to the American Heart Association guidelines for diagnosis of stage 1 hypertension, defined as systolic blood pressure (SBP) between 130 and 139 mmHg or diastolic blood pressure (DBP) between 80 and 89 mmHg, have underlined the importance of understanding the effects of moderate increases in blood pressure in younger women, as these predict future cardiovascular morbidity (104).

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Blacks or African Americans are the largest racial minority group in the United States, making up approximately 13% of the population (41). Hypertension is more prevalent in the black versus the white population, and racial differences in blood pressure regulation have been documented (48). Moreover, hypertension manifests at a younger age in black compared with white people (28, 77) and contributes to their higher mortality (6). In the U.S., black women have increased obesity, dyslipidemia, hyperglycemia, and vascular disease (79). Race is a strong predictor of arterial stiffness in black and white adolescents (97) and there are a number of studies indicating lower compliance in the cardiovascular system of black versus white adults (98, 99), as well as attenuated vascular nitric oxide (NO) bioavailability in black relative to white adults (71). This is of particular interest because early changes in endothelial function, arterial stiffness, and intima wall thickness in young, normotensive individuals is associated with a greater risk of hypertension in later years (98-100). Attenuated vascular responsiveness in black compared to white subjects has been noted in more than one vascular bed, including conduit arteries (9, 74), whole limb microcirculation (10, 19, 35), and the cutaneous microvasculature (43, 75).

There have been studies addressing blood pressure regulation in black men (26), but few studies have addressed blood pressure regulation in black women or racial differences within women (73). Some investigations have suggested greater oxidative stress in blacks may contribute to the racial differences in blood pressure regulation (17, 24), but these were conducted in men (17). A recent study demonstrated attenuated cutaneous microvascular vasodilation in response to endothelium mediated local heating in healthy, young black adults relative to age- and sex-matched white adults. Interestingly, this study also demonstrated sex-based divergent mechanisms of microvascular dysfunction between black men and black women, indicating that within black men NADPH oxidase and xanthine oxidase contribute to impairments in cutaneous microvascular function, but these mechanisms played no role in the regulatory deficits in black women (73). **This study highlights the importance in sex specific studies to understand racial differences in BP regulation.**

We have shown an important role for norepinephrine, which can reflect sympathetic activity, in racial differences controlling blood pressure response to orthostasis (36) (Table 1). The studies in the Parent Grant and this supplement include measures of whole-body sympathetic outflow using the ***gold standard measurement*** microneurography so will provide a precise picture of sympathetic activity and function in women with AE-PCOS. The proposed studies in this supplement allow us expand our population sufficiently to make racial comparisons using microneurography.

Sympathetic Responses to Lower Body Negative Pressure Stimulus

Hormone	Black			White		
	Baseline	Presynope	Change	Baseline	Presynope	Syncope
$P_{[NE]}$, pg/ml	197 ± 144	365 ± 174*	167 ± 123	146 ± 96	232 ± 101	86 ± 64
$P_{[EPI]}$, pg/ml	20 ± 18	64 ± 62	49 ± 63*	23 ± 19	30 ± 8	7 ± 20

Table 1. Plasma norepinephrine ($P_{[NE]}$) is higher in black compared to white women in response to lower body negative pressure (n= 12/group) (36).

Although young women with AE-PCOS commonly manifest a spectrum of covert CVD risk markers, we have demonstrated that androgen excess in young women is a major driver of increased blood pressure, and that testosterone suppression decreases blood pressure and catecholamines. The preponderance of evidence indicates that hyperandrogenism is associated with increased sympathetic tone, impaired baroreflex function, and increased blood pressure in AE-PCOS (90, 93, 102). Despite this interesting work, studies have not assessed baroreflex function using direct measures of SNSA in the most common phenotype: obese, insulin resistant AE-PCOS women (52).

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Black women have the highest risks of developing PCOS (18), and black women with PCOS have high risk cardiovascular profiles, including higher LDL levels compared to white women with PCOS (2, 11, 15, 18, 45, 59). A recent review focused on the prevalence of PCOS in women living in the U.S. found the greatest prevalence in black women relative to other races and ethnicities examined (white, Asians, middle eastern, Indian) (18). This study showed the upper bound of the 95% CI of PCOS prevalence for white women overlapped with the lower bound of the 95% CI for black women, indicating that black women have substantially greater risk of developing PCOS compared to white women. This disparity was noted despite considerable variation within and across ethnic groups of phenotypes and women with different presentations of clinical hyperandrogenism such as hirsutism. A recent study suggested that Hispanic women with PCOS generally present the most severe phenotype both in terms of hyperandrogenism and metabolic features, while non-Hispanic black women had lower prevalence of metabolic syndrome (22). However, CVD and metabolic syndrome risk was increased in both groups compared to white women with PCOS, and black and white Hispanic women were not analyzed separately (22). The racial background of women with PCOS is an important consideration for studies in women with PCOS (56). However, race has not yet been addressed with regard to autonomic function and blood pressure regulation in studies directed at CVD in women with PCOS.

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B. INNOVATION

Our studies will unmask unique pathways for BP dysregulation in AE-PCOS previously unidentified, and determine therapies likely to be most successful for treating early HT. For example, spironolactone, an antiandrogen, used currently to treat acne in women with AE-PCOS also blocks the mineralocorticoid receptor; if our studies demonstrate that the primary pathophysiology mediating HT in AE-PCOS women is related to dysfunction in aldosterone, this (and related classes of) drug may serve a dual purpose of symptom control, as well as confer primary prevention against CVD for AE-PCOS women. Furthermore, should the SNS prove to be the primary pathophysiology behind BP dysregulation, beta blockers or drugs that target the SNS should be therapeutic options of choice.

Metabolic abnormalities may contribute to the sympathetic overactivity and insulin resistance in PCOS, and lead to end-organ damage (e.g. kidney). However, it remains unclear whether elevated sympathetic tone or reactivity is associated with metabolic disturbance in PCOS**.** Indeed, androgens, insulin resistance, and obesity likely interact to increase sympathetic tone in AE-PCOS women. We use a unique approach, that of GnRH suppression in humans, to examine women with and without AE-PCOS. Therefore, our studies will isolate the components of this complex syndrome, as well as their interactions, and thus provide the basis for alternative treatment options not associated with teratogenicity or adverse fetal outcomes for AE-PCOS women.

4. Previous investigations examining the androgen effects on BP control have confined their focus to either neural or renal mechanisms, mainly in males. This proposal creates a new paradigm that focuses on the integration of androgens with BP control mechanisms in females.

6. The naturally high, chronic androgenic environment in AE-PCOS women provides a unique opportunity to study the impact of chronic androgens on women's cardiovascular systems leading to HT and cardiovascular dysfunction. This is especially important because the elevated androgens in AE-PCOS women remain throughout their lifetime continuing even after menopause.

7. While previous studies have used microneurography, the gold standard technique, to determine resting SNA in lean AE-PCOS, this is the first study to fully examine SNA in the more common, insulin resistant, obese, AE-PCOS phenotype using microneurography, thus producing the first definitive data on this important BP control mechanism in women with AE-PCOS.

8. These are the first studies to use elagolix in women with AE-PCOS to suppress testosterone.

BACKGROUND

What is AE-PCOS? The complete etiology of AE-PCOS remains unknown, but the underlying mechanism responsible for AE-PCOS is hormonal imbalance. The pathophysiology involves dysregulation of a number of endocrine signals including: 1) altered pulsatility of hypothalamic GnRH from the arcuate nucleus, resulting in altered secretion of the pituitary gonadotropins: an exaggerated frequency and amplitude of pituitary luteinizing hormone (LH) release which contrasts with the follicle stimulating hormone (FSH) dominant milieu of normal reproductive physiology; 2) excess production of androgens under the influence of both elevated LH and excess insulin as well as disordered insulin action in target tissues; 3) failure of ovarian folliculogenesis, resulting from the altered gonadotropin signaling and hyperandrogenemic milieu. While ovaries of AE-PCOS women are replete with oocytes containing immature follicles, mechanisms that underlie dynamics of normal follicular growth and egg maturation are

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disordered; thus the "cysts" of AE-PCOS ovaries represent arrested follicles that contain immature eggs; 4) in addition to androgen excess, AE-PCOS is a state of progesterone deficiency, a consequence of failure to achieve ovulation. Disordered ovarian estradiol, disrupted ovarian progesterone and excessive ovarian androgen secretion, in addition to compensatory hyperinsulinemia, summarize the endocrine aberrations of AE-PCOS. Each hormonal aberration, through feedback at the hypothalamopituitary axis, contributes to the pathogenesis of the disorder.

Sympathetic activity and baroreflex function in insulin resistance and AE-PCOS. In lean AE-PCOS women, microneurography has confirmed elevated SNSA (90, 93) which is an important component of AE-PCOS etiology (31). Poor baroreflex function may contribute to high vasoconstriction observed with HT in AE-PCOS women. AE-PCOS is also associated with impaired vagal and cardiac baroreflex sensitivity (34), insulin resistance (IR), contributing to sympathovagal dysfunction during standing (47), and impaired autonomic control (53). In support of these findings, data from animals (78) and humans (80) indicate that testosterone is an independent predictor of muscle (M) SNA in AE-PCOS, and is the strongest independent factor explaining their augmented central SNA. Testosterone depletion attenuates, while testosterone supplements restore baroreflex-mediated bradycardia (102). Neonatally androgenized mice develop increased sympathetic tone and HT concomitant with other features similar to those of AE-PCOS. Interestingly, the testosterone-MSNA relationship may not be as powerful in obese women with AE-PCOS (52), as in lean AE-PCOS women (93), possibly due to an independent effect of obesity or the small range of testosterone and free androgen index (FAI). Nevertheless, taken together, the preponderance of evidence supports our hypothesis that *hyperandrogenism is associated with increased sympathetic tone, impaired baroreflex function, and increased BP in AE-PCOS women*. Despite this interesting work, studies have not assessed baroreflex function using direct measures of MSNA in the most common phenotype: *obese, insulin resistant AE-PCOS women* (52). As shown in **(Fig 1),** AE-PCOS women have increased systolic BP relative to IR controls; suppressing ovarian testosterone with a GnRH antagonist lowers systolic BP; and testosterone administration (2.5 mg/day) increases SBP over IR controls. This chronic BP rise to 130-140 mm Hg in SBP, or to 88 mm Hg in MAP, increases the risk of future organ damage and is a harbinger for future CVD, especially in the context of the metabolic and hormonal milieu of AE-PCOS.

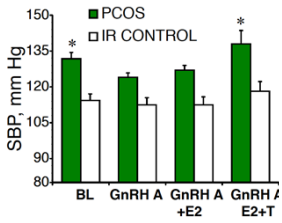

Figure 1. Systolic blood pressure (SBP) is higher in IR women with AE-PCOS (n=7) and

While the current JNC8 guidelines define HT as 140/90mmHg (1), new SPRINT trial data suggest that maintenance of BP at 120/80 in men and women reduces all-cause mortality (32). Thus any BP above 120/80 would contribute to later CVD.

Renin-angiotensin-aldosterone system (RAAS) contributes to increased BP in AE-PCOS women. Earlier investigations (12), including those in our laboratory (89), found greater serum aldosterone ($S_{[ALD]}$) increases during both exercise (60% VO_{2max} @ 35°C) and mild **lower body**

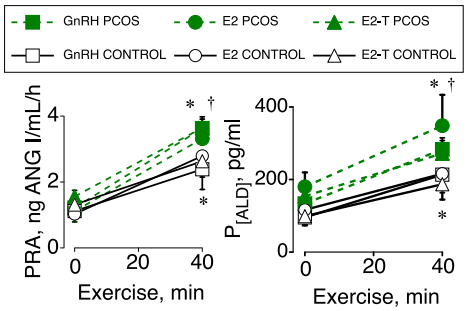

Figure 2. $S_{[ALD]}$ and PRA increases are greater in AE-PCOS compared to IR Controls during mild cycle

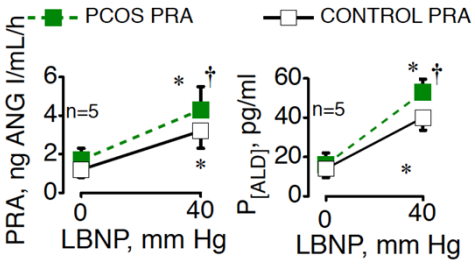

Figure 3. $S_{[ALD]}$ and PRA increases are greater in response to RAS stimulation with LBNP in women with AE-PCOS

negative pressure (LBNP to -40 mm Hg) in AE-PCOS women (89), and greater PRA response in with lower body negative pressure (LBNP) in AE-PCOS women **(Figs 2-3)**. The fact that LBNP does not have a differential effect on AE-PCOS women despite their higher BP suggests that their RAAS is dysfunctional, since one would expect that the elevated BP would cause an attenuated PRA response.

Hormonal effects on SNS and baroreflex function. Our preliminary data (n=4) indicate increased resting SNS activity in obese women with AE-PCOS compared to obese, IR controls **(Fig 4), although** and baroreflex sensitivity (BRS) appears similar or only slightly greater in AE-PCOS women. Importantly, while BP remained unchanged, estrogen administration reduced resting MSNA **(Fig. 5, left)**, ***while testosterone administration increased BRS gain (derived from the modified Oxford)*** in 3 of 4 IR women with PCOS **(Fig. 5, right)**. Thus o*ur studies showed that testosterone effects baroreflex control of HR, BP, and MSNA.* These findings support our hypothesis that *testosterone exposure increases sympathetic outflow, as well as cardiovagal and sympathetic baroreflex gain in AE-PCOS women.*

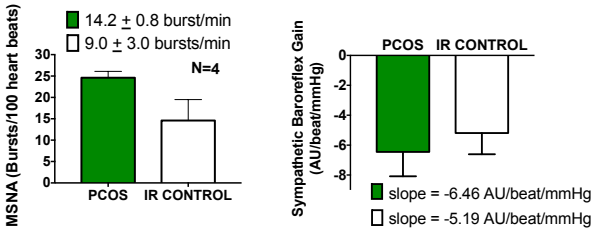

Figure 4. Resting sympathetic nerve activity (MSNA) and baroreflex slope in is greater in obese PCOS and IR

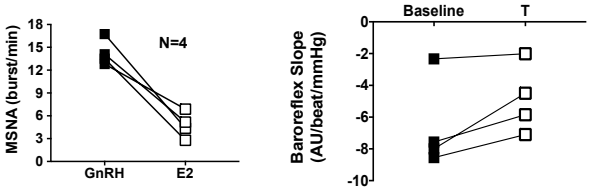

Figure 5. Estrogen reduced resting sympathetic nerve activity (MSNA) in obese IR AE-PCOS (left). Testosterone

***Summary of human studies*.** Our resting, exercise and LBNP findings in control and AE-PCOS women are consistent with our hypothesis that *the RAAS contributes to the pathogenesis of hypertension in obese IR women with AE-PCOS*. Moreover, our studies show that resting SNSA and BRS are greater in AE-PCOS, and that estrogen reduces and testosterone increases SNSA in AE-PCOS women. Findings from our proposed studies will have an immediate impact on treatment because mild BP and $S_{[ALD]}$ increases in AE-PCOS are not currently treated because they do not reach standard treatment guidelines (as noted above). However, these mild increases in BP are chronic, and can induce organ damage leading to future frank HT and CVD. We propose to explore how the chronically high androgen milieu in AE-PCOS promotes excess SNSA (44, 82, 93), poor baroreflex function (34), sympathetic and RAAS activation leading to elevated BP in AE-PCOS women. **Our findings will provide the basis for novel treatment options to manage the underlying causes of AE-PCOS and the primary cardiovascular co-morbidities, HT and vascular dysfunction.**

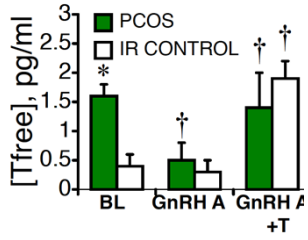

Figure 6. Serum free testosterone is suppressed with a GnRH antagonist, and increased testosterone administration in

Controlling hormone exposure in our human studies. The study of female reproductive hormones on body systems is difficult in young women because the plasma levels of reproductive hormones fluctuate throughout the menstrual cycle. Thus, we use temporary menstrual cycle suppression to isolate individual effects of these hormones on baroreflex and renal function. Ganirelix acetate is a synthetic decapeptide that when given continuously in therapeutic doses competitively blocks the GnRH receptors on the pituitary gonadotroph inducing a rapid and reversible suppression of gonadotropin secretion (69, 70), with suppression of estrogen and progesterone to postmenopausal levels in cycling women. As mentioned above, in AE-PCOS women, progesterone is chronically

low, androgens are elevated, and estradiol is variable; GnRH suppression prevents ovarian androgen production, although the adrenal glands continue to produce small amounts of testosterone. In both control and AE-PCOS women, GnRH suppression is achieved after two days of antagonist administration **(Fig 6)**, and this suppression is reversed upon cessation of drug therapy. When reproductive function is temporarily suppressed, we study the effects of testosterone on BP control systems in control and AE-PCOS young women by adding testosterone back in controlled doses **(Fig 7)**. We chose the GnRH antagonist in these studies as our "gold standard" to ensure that androgens are suppressed with minimal impact on other physiological systems. The androgen receptor antagonist, spirolactone, for example, blocks the mineralocorticoid receptor; and flutamide, an androgen antagonist, may increase aldosterone (39), so would interfere with our questions regarding androgen effects on CV and renal systems.

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] (14) 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 (91). In eumenorrheic women, elagolix administration leads to suppression of estrogens to postmenopausal levels after 24 hours of administration (91). Importantly, elagolix is safe and well tolerated. The suppression of the hypothalamic-pituitary-ovarian axis is reversed upon cessation of drug therapy.

3. **Research Plan:** Summarize the study design and research procedures using non-technical language that can be readily understood by someone outside the discipline. **Be sure to distinguish between standard of care vs. research procedures when applicable, and include any flowcharts of visits specifying their individual times and lengths**. Describe the setting in which the research will take place.

PARTICIPANTS: For Aims 1, 1a,2,2a, two groups (n=36 each) of women will be recruited to complete this study: 1) IR, obese young women between the ages of 18 and 40 years (Controls); 2) IR, obese young women between the ages of 18 and 40 years with AE-PCOS. **For Aim 3,** two groups (n=16 each) of women will be recruited to complete this study: 1) IR, obese young women between the ages of 18 and 40 years (Controls); 2) IR, obese young women between the ages of 18 and 40 years with AE-PCOS. As women sign up for the study, they will complete medical histories, including the **Berlin questionnaire (www.sleepmedicine.com)** to screen for Obstructive Sleep Apnea (See Appendix 1). They will also fill out a hair growth chart. Subjects who smoke, have diabetes, sleep apnea or BP>140/90 will be excluded. Subjects will not be taking medications during the study, including any insulin sensitizing, CV medications, or hormonal contraception 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. All women (early proliferative phase in Controls) will undergo **3-hr oral glucose tolerance test (OGTT;** 75 gram glucose load**)** to determine IR (8). We define our subjects in both groups as having IR with AUC of glucose 140-200 mmol/L and AUC of insulin 40-90 μU (57, 64). Sexually active participants will use barrier contraception for the duration of the study, and take pregnancy tests within 24 hours of participation. Subjects are excluded if they have lost > 5 kg of weight within the past 6 months or exercise more than three times/week. Attheir initial consent visit, after signing the consent form, subjects will be randomly assigned to take a GnRH antagonist with or without methyl testosterone (see "Study Design" below). They will be told immediately of the results of the randomization.

AE-PCOS subjects: Diagnosis of AE-PCOS will be based on modified NIH criteria requiring the presence of *both* **clinical hyperandrogenism** (hirsutism based on Ferriman Gallwey score of >8 and/or acne), *and* **biochemical hyperandrogenemia** (elevated serum total testosterone; ELISA,

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see Authentication). **Anovulation** (defined as a total of ≤ 8 menses per year) *and* presence of **polycystic ovarian morphology** on transvaginal ultrasound as determined by a trained obstetrician/gynecologist (Dr. Pal), **obesity and IR** will be additional enrollment criteria for the AE-PCOS population. **Sex as a biological variable:** Only females will be used for these studies since AE-PCOS is a female-specific disorder.
Control subjects will be **obese, IR** women, with regular menses every 26-34 days. Subjects will be excluded if they have any symptoms of AE-PCOS subjects, including polycystic appearing ovaries (as determined by transvaginal ultrasound (Dr. Pal).

For Aim 1, 2 and 3: All subjects will be given urine collection containers to collect their overnight urine prior to visits 4,5 and 6.

For Aim 1, 2 and 3: All subjects will self administer Antagon injections for a total of 10 days. Subjects may be kept on Antagon for as long as 18 days if there is a challenge with experiment scheduling. Subjects will also ingest a testosterone pill for seven days. Subjects may also be kept on testosterone for up to 11 days with regard to scheduling issues.

For Aim 5: Subjects will participate in only one baseline study (Fig 7). We will recruit black and white women to participate in this study with AE-PCOS.

EXPERIMENTAL PROTOCOLS:

Aims 1 & 2. Study design (Fig 7) and Hormone administration (Fig 8): IR subjects with and without AE-PCOS will be randomly (MiniTab, © cleverbridge, Inc.) divided into two **Protocol** groups (4 groups total, n=18 each) to be tested 3 times each: **Protocol 1:** prior to GnRH antagonist (Day 1); GnRH antagonist (250 μg/day in 0.5 ml normal saline, Days 3 and 10). **Protocol 2:** prior to GnRH antagonist (Day 1), GnRH antagonist alone (Day 3), GnRH antagonist + methyltestosterone (Day 10). In both Protocols, Day 1 in Controls occurs in the proliferative phase of the menstrual cycle (days 1-7, low hormone phase); GnRH suppression begins on days 26-28 of that cycle. Non-cycling women with AE-PCOS begin testing at their convenience.

*Women may take the Antagon pills for as long as 18 days. We allow this flexibility because of challenges with scheduling subjects. There is no increase in risk, and we do everything possible to avoid the rare instance where taking this drug needs to be extended. If the drug is extended, “day 10” procedures occur on the final day of “GnRH antagonist + methyltestosterone” administration.

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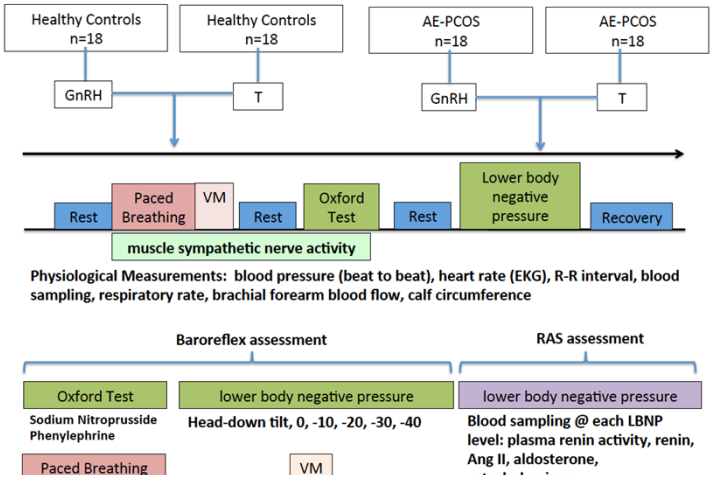
Figure 7. The <u>Overall Plan for Human Studies</u> proposed in Aims 1 and 2. Studies to assess the baroreflex and renin angiotensin system (RAS) will be performed in the same protocol. Overnight (12 hour) urine will be collected on all subjects prior to these protocols.



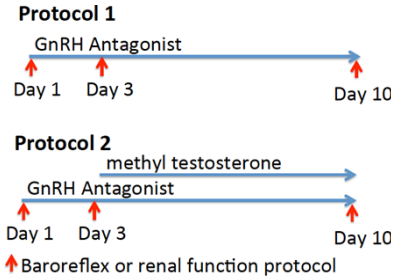


Figure 8. Hormone

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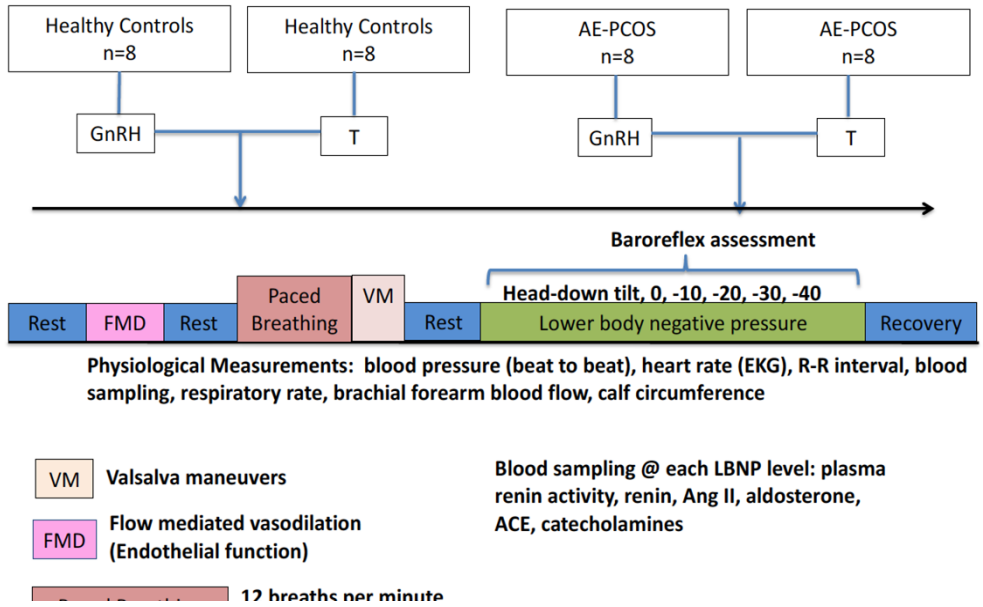
Aim 3. Study design (Fig 7a) and Hormone administration (Fig 8): IR subjects with and without AE-PCOS will be randomly (MiniTab, © cleverbridge, Inc.) divided into two Protocol groups (4 groups total, n=8 each) to be tested 3 times each: Protocol 1: prior to GnRH antagonist (Day 1); GnRH antagonist (250 µg/day in 0.5 ml normal saline, Days 3 and 10). Protocol 2: prior to GnRH antagonist (Day 1), GnRH antagonist alone (Day 3), GnRH antagonist + methyltestosterone (Day 10). In both Protocols, Day 1 in Controls occurs in the proliferative phase of the menstrual cycle (days 1-7, low hormone phase); GnRH suppression begins on days 26-28 of that cycle. Non-cycling women with AE-PCOS begin testing at their convenience.

Figure 7a. The protocol Studies proposed in Aim 3. Studies to assess the baroreflex and renin angiotensin system (RAS) will be performed in the same protocol. Overnight (12 hour) urine will be collected on all subjects prior to these protocols.



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). On all days that subjects visit the laboratory, they will take a rapid Covid 19 test according to the directions of the test. The subject will take this test in a separate chamber. This test will involve putting a “q-tip” like swab in each nostril and rubbing in a circular motion ten times and then placing the swab into analysis fluid from the test kit. A member of our research team will observe the swabbing procedure and read the results of the test. If the test is positive, the subject will not proceed further with the experiment, and will be asked to leave the laboratory immediately and suggest contacting their personal physician. If the test is negative, we will proceed with the experiment.

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Preliminary Testing. Because naive volunteers may be used, all subjects are brought to the laboratory prior to the experimental days, thoroughly trained in all maneuvers and familiarized with all routines to ensure that they are as comfortable as possible on the first experimental day. Each subject is individually trained on how to do the GnRH antagonist injection, either by the PI, the Post Doc or a nurse at the Reproductive Endocrinology clinic. During the training, the subject is shown how to clean the area first with an alcohol pad, how to open the package with the syringe to avoid sticking their fingers and to maintain sterility. The subjects are taught to use either their leg or abdomen, and how to insert the needle and press down to inject the fluid. They are also taught to use pressure should there be bleeding after the injection. In addition, the subjects are all provided with detailed instructions provided by the manufacturer, alcohol pads and sterile gauze pads.

Research Design:
Group 1 (Fig 8):

1) prior to GnRH suppression;
2) during GnRH suppression (Ganirelix acetate alone, 250 µg/day in 0.5 ml normal saline, 2 days);
3) Ganirelix + methyltestosterone ((oral, 5.0 mg/day, 7 days (on day 10));

Group 2 (Fig 8):

1) prior to GnRH suppression;
2) during GnRH suppression (Ganirelix acetate alone, 250 µg/day in 0.5 ml normal saline, 2 days);
3) during GnRH suppression (Ganirelix acetate alone, 250 µg/day in 0.5 ml normal saline, 2 days) and again at 10 days; Control subjects will first be tested in the proliferative phase of the menstrual cycle (days 8-12) because estrogen status at this time is similar to non-cycling PCOS subjects.

Women with normal menstrual cycles will begin GnRH suppression on days 26-28.

Women may take the Antagon pills for as long as 18 days. We allow this flexibility because of challenges with scheduling subjects. There is no increase in risk, and we do everything possible to avoid the rare instance where taking this drug needs to be extended. If the drug is extended, "day 10" procedure occur on the final day of "GnRH antagonist + methyltestosterone" administration.

Research Protocol (Fig 8):
Visit 1: **Screening, informed consent, orientation.**
Visit 2: **Ultrasound (YMG)**
Visit 3: **Oral glucose tolerance test (OGTT).**
Visit 4: (BL): **Baroreflex and RAS testing**
Visit 5: (GnRH): **Baroreflex and RAS testing**
Visit 6: (GnRH +T): **Baroreflex and RAS testing**
OR
Visit 5: GnRH): **Baroreflex and RAS testing**
Visit 6: GnRH): **Baroreflex and RAS testing**

Visit 1: Screening, informed consent, orientation:

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We will describe the study in detail. Subjects will fill out a medical history form (including medications) so we can make sure it is safe for the participant to be in this study. They will have a pregnancy test at no cost and if eligible for the study and decide to participate, we will teach them how to give a self-injection of Antagon, a drug that will be used in this study. We will also orient the participant to where the research studies will be done and demonstrate the equipment and procedures that will be used for the study.

Visit 2. Transvaginal ultrasound. The subjects will undergo evaluation using transvaginal ultrasound. We will perform transvaginal ultrasound using a linear probe on a Phillips 3-D ultrasound on all subjects. There are no known risks associated with this procedure. The procedure is painless and will require approximately twenty minutes to perform. The ultrasound examination will be performed in the Yale Center for Reproductive Medicine by a gynecologist on the staff of the Yale New Haven Hospital and who is accredited by the American Institute for Ultrasound in Medicine. The ultrasound will be a focused exam looking only at the ovaries. With the subject's permission, any incidental pathology noted at the time of the exam will be sent to the subject's private gynecologist for possible further evaluation. All follicles and/or cystic structures are measured. Ovarian volume is calculated as the measure of a prolate ellipsoid. Ovarian morphology is determined by the methods described in the literature for identifying polycystic ovaries in women with PCOS (3, 4).

Visit 3. Oral glucose tolerance test. Subjects will undergo an **OGTT at The John B. Pierce Laboratory** to quantify glucose tolerance and insulin resistance. These tests are done in the early proliferative phase of the menstrual cycle in cycling women. A standard (3 hour) OGTT will be conducted to determine glucose tolerance in all subjects within 2 weeks of the start of the study. One hour will be added for postural acclimation and insertion of the IV for a total of a 4 hour visit. Blood samples for serum concentration of glucose and insulin will be drawn at baseline prior to ingestion of a 75g glucose beverage (Glucose Drink; Azer Scientific, Morgantown, PA) and at every 30-minute time point following drinking for three hours. A total of 45ml (3 tablespoons) will be drawn.

Visits 4-6 (Visits 5 and 6 are identical to Visit 4):

Protocols 1A-B: Autonomic function: **Baroreflex testing (Fig 9).** We will examine **autonomic function** in our human subjects at **rest** (cardiovagal), during **Valsalva maneuver** (sympathetic), during the **modified Oxford protocol** (sympathetic, **Fig 9**) (20, 23, 33, 42) and with **integrated baroreflex** response during **LBNP** (23, 103). Our primary measure of SNSA is peripheral nerve activity, as measured by **muscle sympathetic nerve activity (MSNA)**. We will also determine HR, BP (EKG and beat-to-beat), and a catheter will be inserted into a forearm vein for blood sampling. Following the instrumentation period, resting HR, pulse (or R-R) interval (PI), BP, and MSNA will be recorded for 10 minutes, and a blood sample drawn for analysis of $S_{[E_2]}$, progesterone ($S_{[P_4]}$), sex hormone binding globulin, ($P_{[SHBG]}$), albumin, free ($P_{[Tfree]}$) and bound ($P_{[T]}$) testosterone, $S_{[ALD]}$, serum angiotensin converting enzyme ($S_{[ACE]}$), angiotensinogen ($S_{[ANG]}$), renin, ($P_{[Renin]}$), PRA, $P_{[ANG\ II]}$, norepinephrine ($P_{[NE]}$), epinephrine ($P_{[EPI]}$), electrolytes, creatinine (Cr) and osmolality (P_{Osm}). After the first blood sample and **a 30-min rest phase**, subjects will begin a seven-minute period of **rest with paced breathing** (12 breaths per minute) while HR, BP and MSNA are measured (**Fig 9**).

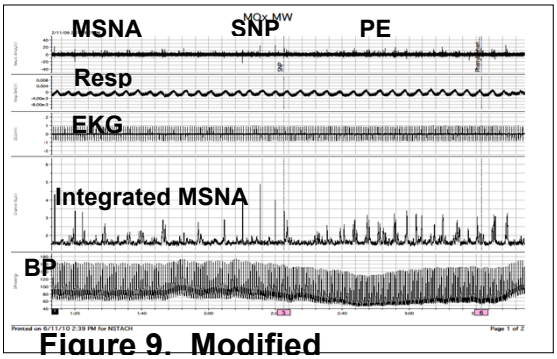

Figure 9. Modified

The subjects will synchronize breathing to a metronome set to a cadence of ~12 breaths per minute (0.2 Hz). Following baseline measurements, subjects will perform two **Valsalva maneuvers**. *Methods are described in* ***Procedures***.

Sympathetic baroreflex sensitivity assessment: modified Oxford technique ***(Aim 1)***. To assess baroreflex control of sympathetic outflow, HR, BP, MSNA and PI will be measured during arterial pressure changes induced by infusion of sodium nitroprusside (SNP, endothelium-independent vasodilator) and phenylephrine (PE, an α-receptor agonist) (20, 23, 42). After 10-minute baseline period, SNP (100 µg bolus) will be given followed 1 minute later by PE-HCL (150 µg). This protocol decreases BP~15 mm Hg below baseline levels and then increases BP~15 mm Hg above baseline levels over a short period of time (See bottom tracing, **Fig 9**). Infusions are sequential, repeated twice in each subject with at least 15 minutes between each cycle of infusions. **Fig 9** shows change in BP and increase in (integrated) MSNA that follows SNP infusion, and the decreased MSNA following PE infusion in a typical subject. Sympathetic baroreflex sensitivity is defined as the slope relating MSNA and DBP during the sequential infusions of SNP and PE (20). Cardiovagal baroreflex sensitivity is defined as the slope relating PI and SBP (20, 23, 42). The modified **modified Oxford technique** has been approved under HIC #0512000875. The PE and SNP will be infused by Chris Moore (Emergency Medicine).

Integrated baroreflex assessment (LBNP) ***(Aim 1)*****.** After the modified Oxford protocol, we will assess integrated baroreflex function during incremental LBNP. Although low-level LBNP has been used to isolate cardiopulmonary baroreceptor input, arterial baroreceptors appear to be involved even at very low levels of LBNP (51, 96) so we use the term "integrated" to acknowledge the limitations of distinguishing cardiopulmonary versus arterial baroreceptor functions (23, 88, 103), and it is not the goal of these studies to distinguish the contributions of these respective baroreceptor inputs. **LBNP protocol:** During a 30-minute rest period after the SNP and PE infusions, microneurography electrode is removed, and subjects are instrumented in the left arm for the measurement of forearm blood flow (FBF, 87). We will measure baseline variables, tilt the subjects to 7° head down (HDT), return the subject to supine, then apply LBNP at –10, –20, –30 and –40 mm Hg (for 4 min each) in descending order. Heart rate, BP (beat-to-beat), PI and SkBF are measured continuously, and calf volume (circumference, to determine the extent of lower level volume shift and ensure relaxed muscles during LBNP). Blood sample is taken prior to and at the end of each stage of LBNP for the measurement of $S_{[ALD]}$, $S_{[ACE]}$, $S_{[ANG]}$, $P_{[Renin]}$, PRA, $P_{[ANG\ II]}$, $P_{[NE]}$, $P_{[EPI]}$. FBF is measured at 30-sec intervals, at rest and during the entire LBNP protocol. The total blood taken is 160 ml per visit. With FBF and BP data, we compute forearm vascular resistance (FVR) and conductance (FVC). If subjects experience presyncopal symptoms or if BP falls by 20% of baseline, we discontinue the study. If the test is stopped early we use the slopes generated from the completed levels of LBNP, but exclude data collected during symptoms or exaggerated BP falls. To determine peripheral vascular responses we analyze FVR and FVC as functions of LBNP. We will also determine changes in peripheral vascular responses by examining changes in HR as functions of SBP and LBNP (i.e. Δ HR/Δ SBP or Δ LBNP). **LBNP** has been approved under HIC #0512000875.

Flow Mediated Vasodilation (FMD): ***(Aim 3)*** 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

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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 in order 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 (25, 76) and differs in the upper and lower extremities (68).

Exercise. ***(Aim 3)*** We also evaluate the blood flow and vasodilatory response after occlusion with hand grip exercise. In this experiment, subjects perform static handgrip exercise at 20% maximal voluntary contraction (MVC) during the arterial occlusion by inflating a pneumatic upper arm cuff to 200 mm Hg for 2 to 5 minutes. We measure the vessel diameter and blood velocity, skin blood flow and blood pressure for 5 minutes after the cuff release. The order of rest vs. exercise experiments is randomized.

Aim 4. In this proposal, we will recruit women with AE-PCOS who have already participated in prior Aims and taken ganirelix. At least one month after they have completed the study, we will recruit these women to take elagolix for seven days. We will collect blood samples to compare serum concentrations of total and free testosterone, E_2 and SHBG prior to and following the 7-day elagolix treatment and compare these blood variables with the changes due to ganirelix found while participating in Aims 1-3.

Study Design: Humans. All subjects will receive (elagolix, 400 mg/day oral, 2X200 mg tablets/day for 7 days) (91). All women will begin taking elagolix in the first week of their menstrual cycle,.

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 21 statistical software, that includes calculations to compensate for missing data, and Prism (GraphPad) curve fitting software where appropriate. ***Data analysis.*** The slopes of the linear regressions for PI-SBP (cardiovagal baroreflex gain), PI-LBNP (integrated baroreflex gain), resting MSNA, slopes for MSNA-DBP (sympathetic baroreflex gain during Oxford and Valsalva), will be compared between treatment conditions (GnRH antagonist vs. GnRH antangonist+T and between groups (Control vs AE-PCOS) using a 2-way repeated measures ANOVA with supplemental *post hoc* testing with Tukey's HSD test. **Sample size.** The desired statistical test is two-sided, and we assume an alpha level of =0.01 to account for multiple comparisons (37). In our laboratory, we observed effect sizes (SD) of PI-SBP intercept (with n=4) of 0.39 (0.16), our most stringent variable. A sample size of 15 women per group will allow us ≥ 80% statistical power ($1-\beta > 0.9$) to test within group comparisons, and this same sample size will allow $[1-\beta]=0.80$ to distinguish any between-group differences from chance alone (29). Due to the technical challenges of performing microneurography on multiple occasions in obese women, we overestimate and account for a ~ 20% failure rate, so we will recruit 18 subjects per group. **R&R: 1)** ***GnRH antagonist suppression/hormone add-back*** is the gold standard and most reliable method to control sex hormones in young women. **2) Microneurography** is the gold standard and most sensitive and reliable available measure for MSNA. **3) Lower body negative pressure** and the **Modified Oxford Technique** are the gold standards and most sensitive and reliable available measures of autonomic function in humans. We will report our methods and findings in detail and **full transparency** to permit others to reproduce and extend our findings. **Sex as a biological variable:** Only women are included in these studies because AE-PCOS is a female-specific disorder.

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Characterization of the RAS during LBNP in humans *(Aim 2)*. We will use the protocol described in **Fig 7** with the hormone intervention described in **Fig 8**. Before beginning the protocols, during 7° HDT, at 0 (supine rest) and in the final minute of each level of LBNP we will draw blood to determine $S_{[ALD]}$, $S_{[ACE]}$, $S_{[ANG]}$, $P_{[Renin]}$, PRA, $P_{[ANG\ II]}$, $P_{[NE]}$, $P_{(38)}$, electrolytes, Cr) and Osm. Urine samples will be collected just prior and after baroreflex/RAS LBNP protocol for electrolytes.

Statistical analyses: We use ANOVA as described in Protocols 1. **Sample Size** is based on calculations in Aim 1, as the Aim 1 variables are the more stringent.
Expected results, interpretation of data, limitations and anticipated problems*:** Based on our hypothesis, ***we expect that women with AE-PCOS will have greater RAS hormonal responses to LBNP, reflecting greater RAS activation. ***We expect*** that testosterone suppression will reduce PRA, $S_{[ALD]}$, $S_{[ANG\ II]}$, PRA and $P_{[Renin]}$ response to LBNP, suggesting lower RAS activation in both Control and AE-PCOS. ***We expect*** that increasing $P_{[Tfree]}$ and FAI will increase RAS hormone response to LBNP, reflecting greater RAS activation responses to LBNP in AE-PCOS. Since the methods to be used are in place in our laboratory, we don't anticipate any problems in performing the studies in this Aim.

Expected results, interpretation of data, limitations and anticipated problems*: *We expect AE-PCOS women will have greater resting MSNA compared to Controls. ***We anticipate*** that slopes for MSNA-DBP will be greater in AE-PCOS, indicating increased sympathetic BRS compared to Control. ***We expect*** that testosterone suppression will decrease slopes for MSNA-DBP in AE-PCOS, and testosterone administration will increase slopes for MSNA-DBP in both Control and AE-PCOS. In contrast, ***we expect*** reduced slopes of PI-SBP with modified Oxford and paced breathing, indicating decreased sympathetic and cardiovagal BRS in AE-PCOS women; ***we expect*** a steeper PI-SBP slope with androgen suppression, and a steeper PI-SBP slope with modified Oxford, as well as paced breathing and Valsalva in both groups. Since all methods to be used are in place in our laboratory, we don't anticipate any problems in performing the studies in this Aim.

Statistical analyses: We use ANOVA as described in Protocols 1 & 2 to test changes in blood pressure, FMD and baroceptor sensitivity . **Sample Size** is based on calculations in Aim 1, as the Aim 1 variables are the more stringent.

Aim 3: ***We expect*** AE-PCOS women will have greater resting blood pressure compared to Controls. ***We expect*** that women with AE-PCOS will have greater RAS hormonal responses to LBNP, reflecting greater RAS activation. ***We expect*** that testosterone suppression will reduce PRA, $S_{[ALD]}$, $S_{[ANG\ II]}$, PRA and $P_{[Renin]}$ response to LBNP, suggesting lower RAS activation in both Control and AE-PCOS. ***We expect*** that increasing $P_{[Tfree]}$ and FAI will increase RAS hormone response to LBNP, reflecting greater RAS activation responses to LBNP in AE-PCOS. **We expect** Flow Mediated Vasodilation to be lower in the the women with AE-PCOS compared to control women, and we expect the suppression of testosterone to eliminate this difference. ***We expect*** that increasing $P_{[Tfree]}$ and FAI will increase FMD in women with AE-PCOS, with little impact on Controls. ***We anticipate*** that women with AE-PCOS will have lower baroreceptor sensitivity, compared to Controls, and that this will be improved with testosterone suppression. Finally, we expect increasing $P_{[Tfree]}$ and FAI will increase baroreflex sensitivity in women with AE-PCOS, with little impact on Controls. Since the methods to be used are in place in our laboratory, we don't anticipate any problems in performing the studies in this Aim.

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Aim 4. ***We expect*** that testosterone will be suppressed similarly during elagolix and ganirelix treatments. We will use Cronbach's alpha as a measure of internal consistency or reliability (95).

Aim 5. We expect black women with AE-PCOS will have greater resting blood pressure compared to white women with AE-PCOS. ***We anticipate*** that slopes for MSNA-DBP will be greater in black women with AE-PCOS, indicating increased sympathetic BRS compared to white women with PCOS.

PROCEDURES

Muscle sympathetic nerve activity will be quantified by identification and measurement of sympathetic bursts in the integrated neurogram, and expressed as burst frequency (number of bursts per unit time). ECG surface electrodes are applied to the chest, an elastic band incorporating a strain gauge element is strapped around the thorax for recording respiratory movements, and a sensor cuff of a servopulse-plethysmography arterial pressure monitor (FINAPRES, Ohmeda) is placed on the middle phalanx of the long finger (held at heart level). The experiments are carried out in an environmentally controlled chamber at an ambient temperature of 27.0 ± 0.1 °C. The experiment will begin with a 60-minute control period. During such time the subjects rest in the reclined position. This procedure has been approved under HIC #0512000875.

Microneurography Recording of multiunit postganglionic **muscle sympathetic nerve activity** (MSNA) will be made from the common peroneal nerve as it winds around the fibular head with the subject in the supine position with her thigh comfortably supported. This procedure requires us to first trace the course of the nerve using small electrical stimuli applied to the surface of the skin over the nerve. This allows the microneurographer to find and focus the recording electrode tip on a nerve fascicle associated with muscle sympathetic activity. The time required to focus the electrode on the nerve averages about 30 min and will not exceed 60 min. In optimal conditions the track of the nerve from superficial stimulation is accurate and the nerve remains in position during insertion. An uninsulated subcutaneous electrode will serve as the reference. Mechanoreceptor afferent activity can be recorded during light tapping on the muscle belly or passive muscle stretch. A suitable intrafascicular recording site for MSNA consists of regular bursts and synchronous with the cardiac cycle. The neural activity is amplified ($2x10^4$), filtered (0.5-5.0 kHz), rectified, and integrated. The amplified and filtered nerve signal is also led to an audio monitor and through a resistance capacitance circuit (time constant 100 ms). The integrated nerve signal is digitized at 400 Hz along with the ECG signal while respiratory and arterial pressure signals are also sampled at 400 Hz (MacLab 8s). Data for analysis is selected from periods of quiet breathing in which subjects were relaxed and not talking. Microneurography was approved under HIC # 8079, **#**0512000875 and HIC #1609018353

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Cardiovascular variables. Recorded with a 16-channel computerized data-acquisition system at a sampling Speed of 400 Hz (ADI Instruments PowerLab 9, Castle Hill, Australia). Beat-to-beat DBP, SBP and pulse interval (PI) are determined using peak detection algorithms (44). Brachial artery BP and HR are measured with an automated sphygmomanometer (Colin Medical Instruments Corp, Komaki, Japan). Heart rate and pulse interval will be determined beat-to-beat from lead II of the ECG recording.

Cardiovagal baroreflex sensitivity, as we previously described (88), will be determined by slope of linear relationship between PI and SBP at rest, during Valsalva maneuver (67, 87), and during the modified Oxford technique (21, 23, 65, 66). **R&R**: At rest, baroreflex sequence requires three

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or more consecutive beat-to-beat increases in PI with simultaneous increase or decrease in SBP (7). Only those sequences with a correlation greater than 0.85 are included in the analysis. Average of individual slopes in each condition will be taken as a measure of resting cardiovagal BRS. Incidence of baroreflex sequences will be reported as sequences per 100 pulses to normalize for differences in HR among subjects and across experimental days (55). We recognize that this method for evaluating BRS has limitations (49), but it is a widely accepted method, is predictive of cardiovascular morbidity (58), and is an indicator of autonomic control (21). **Sympathetic baroreflex sensitivity** will be evaluated by determining slope of the linear relationship between MSNA and DBP at rest, during Valsalva maneuver and during the modified Oxford technique (23, 65, 66). **R&R**: MSNA can be distinguished from other sources of nerve activity by the following criteria: 1) presence of spontaneous pulse synchronous bursts; 2) increased activity during Valsalva; 3) muscle afferent activity with muscle stretch; 4) no change of activity during light stroking of skin or startling the subject with loud noises, indicating a lack of skin nerve activity. **Valsalva maneuver:** Subjects will perform Valsalva maneuver following deep inspiration: expiratory mouth pressure (measured with a pressure transducer; 40 mm Hg, 20 seconds) by blowing through a 1-inch diameter tube against an open glottis. **Cardiovagal baroreflex** is assessed during phase IV (overshoot phase) of the Valsalva maneuver, the phase occurring right after the release of the strain (67, 87). Consecutive SBP values during phase IV will be linearly regressed against corresponding PI from the SBP time point where PI begins to lengthen and continued to the point of maximal BP increase. **Lower body negative pressure** and **Forearm blood flow (FBF)**, as previously described by us (88).

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 60ml of blood (4 tablespoons) will be removed for analysis.
Plasma hormones. Aldosterone, free and total testosterone, ACE, PRA, renin and AII are measured with ELISA. Albumin and Cr are measured using spectrophotometry methods. Catecholamines are measured using HPLC methods.
Urine analysis. Subjects are instructed to completely empty their bladder when voiding into a collection vessel and urine collection times are recorded. Urine osmolality and concentrations of sodium and potassium are measured immediately; an aliquot is frozen for creatinine analysis.

4. Genetic Testing N/A ☒

5. **Subject Population:** Provide a detailed description of the types of human subjects who will be recruited into this study.

Subjects: Two groups (n=36 each) of women will be recruited to complete this study: 1) IR, obese young women between the ages of 18 and 40 years (Controls); 2) IR, obese young women between the ages of 18 and 40 years with AE-PCOS. A third group of subjects with the same characteristics [between ages of 18 and 40 years (Controls); 2) IR, obese young women between the ages of 18 and 40 years with AE-PCOS], n=16 in each group will be recruited for **Aim 3.** For Aim 4, n=15 women from each of the previous groups will be recruited to participate in this study. African American women that consent to participate in Aims 1, 2 and 3 will be included for Aim 5 (now CLOSED).

6. **Subject classification:** Check off all classifications of subjects that will be specifically recruited for enrollment in the research project. Will subjects who may require additional

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safeguards or other considerations be enrolled in the study? If so, identify the population of subjects requiring special safeguards and provide a justification for their involvement.

☐ Children	☒ Healthy	☐ Fetal material, placenta, or dead fetus
☐ Non-English Speaking	☐ Prisoners	☐ Economically disadvantaged persons
☐ Decisionally Impaired	☐ Employees	☐ Pregnant women and/or fetuses
☒ Yale Students	☒ Females of childbearing potential	

NOTE: Is this research proposal designed to enroll children who are wards of the state as potential subjects? ☐ Yes ☒ No (If yes, see Instructions section VII #4 for further requirements)

7. **Inclusion/Exclusion Criteria:** What are the criteria used to determine subject inclusion or exclusion?

AE-PCOS subjects: Diagnosis of AE-PCOS will be based on modified NIH criteria requiring the presence of *both* **clinical hyperandrogenism** (hirsutism based on Ferriman Gallwey score of >8 and/or acne), *and* **biochemical hyperandrogenemia** (elevated serum total testosterone; ELISA, see Authentication). **Anovulation** (defined as a total of ≤ 8 menses per year) *and* presence of **polycystic ovarian morphology** on transvaginal ultrasound as determined by a trained obstetrician/gynecologist (Dr. Pal), **obesity and IR** will be additional enrollment criteria for the AE-PCOS population. **Sex as a biological variable:** Only females will be used for these studies since AE-PCOS is a female-specific disorder.
Control subjects will be **obese, IR** women, with regular menses every 26-34 days. Subjects will be excluded if they have any symptoms of AE-PCOS subjects, including polycystic appearing ovaries (as determined by transvaginal ultrasound (Dr. Pal).

Exclusion criteria
All subjects will be excluded from the study if they are taking medication that interact with the study medication. Subjects may not take medications during the study except for Tylenol and other medications approved by the PI. The subjects fill out a medical questionnaire in which we ask them if they are taking any medication, and we ask them for a history of medications in this same form.

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. partial or complete hysterectomy.
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.
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).
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.

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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.
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.

8. How will **eligibility** be determined, and by whom?

Eligibility will be determined by the P.I. (Dr. Stachenfeld) and by the Co-I (Dr. Pal) 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 PCOS or insulin resistance, 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.

9. **Risks:** Describe the reasonably foreseeable risks, including risks to subject privacy, discomforts, or inconveniences associated with subjects participating in the research.

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, in itself, presents little danger to the subject's welfare.

Transvaginal ultrasound. There is a small risk of infection with transvaginal ultrasound if proper disinfection procedures are not followed. The procedure is painless and will require approximately twenty minutes to perform. There is some slight pressure during this procedure, which some women may find uncomfortable. This is performed in the Obstetrics/Gynecology Yale Medical Group clinic.

Microneurography: The risk of microneurography is a peroneal paresthesia for 2-10 days following the experiment. The incidence of parethesias is reported to be less than 10%, in less than 1% of all subjects paresthesias can persist for up to two months. These paresthesias resolve spontaneously.

The Valsalva maneuver is a common stimulus used in human respiratory physiology. The Valsalva maneuver is also common in daily life (e.g. when lifting heavy objects). Both stimuli may result in a feeling of breathlessness that may induce some anxiety. However, changes are reversed immediately upon resumption of volitional breathing of room air. Subjects will be able to return to room air breathing immediately based on the termination criteria. These criteria have

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been set based on human blood oxygen dissociation computations. Below 80% blood oxygen saturation, the slope of the oxygen dissociation curve is steep, resulting in increased oxygen dissociation (from hemoglobin) given a step decrease in the partial pressure of inspired oxygen. **The Valsalva maneuver** differs from a voluntary apnea in that it is associated with well-documented, acute changes in blood pressure. This maneuver has been used across a wide range of individuals, including clinical populations.

GnRH antagonists (Antagon and Cetrotide) 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 tolerate Antagon quite well, some may experience headaches, breast tenderness, transient mood changes, some temporary bloating of the abdomen and moderate irregular vaginal bleeding. In our studies, the subjects begin the Antagon/Cetrotide treatments on days 25-27 of their menstrual cycle (or ~11-12 days after ovulation) to avoid discomfort to irregular bleeding, so this has not been a reported side effect in our experience. There are no documented long-term side effects of Antagon. Subjects in our studies have reported minimal side effects from using Antagon over a period of 16 days. In one of our studies, one of 15 subjects reported occasional vasomotor symptoms during Antagon treatment. In our >10 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.

Elagolix (Aim 4) In our studies, the subjects with menstrual cycles begin the elagolix 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. 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 elagolixin in our studies before, but subjects in our studies have reported minimal side effects from using Antagon over a period of 16 days. There will be at least one month between the end of taking GnRH Antagonist ganirelix acetate for the first part of this study and beginning this part to ensure washout of the ganirelix acetate. Subcutaneous injections of ganirelix acetate is associated with a mean half-life of 12.8 hours are almost completely (69, 70) Thus providing a month "washout" between finishing ganirelix acetate and elagolix, both well tolerated drugs, should cause no danger to the subjects.

Short-term methyl testosterone treatment is not usually associated with any major health risks. Methyl testosterone was chosen in this study because it is a component of Estratest, a widely used hormone therapy for menopausal women. It is therefore FDA approved for use in women at this dose and has few side effects. **Long term** use of methyl-testosterone can cause abnormal hair growth, abnormal skin sensations, anxiety, hair loss on the scalp, breast growth, changes in sexual desire, general body discomfort, headache, mood changes and acne. Testosterone treatment in women for longer than 6 months has not been studied.

We have used the combination of GnRH antagonist and methyl testosterone in HIC #1508016301 in women with and without PCOS with no ill effects or side effects.

The Oxford techniques using the **phenylephrine and sodium nitroprusside** doses proposed here have been performed safely in humans in many laboratories (20, 23, 33, 42, 65, 66). Risks associated with infusions these drugs are small changes in blood pressure that are not even noticed by the subjects. Possible side effects may include flushing or a sense of warmth, nausea, headache and nervousness. Blood pressure can potentially fall by 20-30mm during this technique. Rare risks to phenylephrine and sodium nitroprusside infusion may include severe bradycardia or low heartrate, sulphite allergic reaction, methemoglobinemia (high

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methemoglobin) or thiocyanate toxicity. The drug infusions are done under the supervision of a doctor (Christopher Moore).

10. **Minimizing Risks:** Describe the manner in which the above-mentioned risks will be minimized.

Potential risks associated with blood sampling, by implantation of catheter needles and microdialysis probes. We reduce the risks because only experienced individuals place needles or probes by using proper sterile technique.
Nina Stachenfeld will closely supervise all experiments. Thus, trained and experienced personnel will supervise subjects during every phase of participation.

Transvaginal ultrasound. There is a small risk of infection with transvaginal ultrasound if proper disinfection procedures are not followed. We reduce the risk by having only Dr. Pal perform the ultrasounds, who is an experienced Ob/Gyn (Department Chair). This is performed in the Obstetrics/Gynecology Yale Medical Group clinic.

Microneurography. The likelihood of the occurrence of symptoms following microneurography will be minimized by the following certain procedures. First, few problems or symptoms are reported in laboratories whose microneurographers have received training from individuals with a documented record of success and safety. Dr. Usselman or Dr. Stachenfeld will perform all microneurography studies. Dr. Usselman is a highly qualified microneurographer in the laboratory with over six years' experience and this will reduce the risk of occurrence of symptoms following the experiment. The time spent searching for a nerve fascicle within the peroneal nerve will be limited to < 60 min. No nerve site will be sampled more than once. The recording electrodes are inspected under a dissecting microscope to insure electrode tip integrity (those electrodes found with damaged tips are not used) and each recording electrode will be used only once for intraneural recording. Placement of the electrodes and positioning in the common peroneal nerve are performed under sterile conditions. Metal microelectrodes are steam sterilized at Yale New Haven Hospital before use. The site of electrode placement is cleaned with alcohol and only investigators wearing sterile gloves handle the sterile microelectrodes. Following these simple guidelines, the risk of occurrence of symptoms following microneurography will be small. Microneurography studies were conducted at the Pierce Laboratory under HIC **#**0512000875.

Antagon, Cetrotide, elagolix and reproductive hormone administration. To ensure the safety of the women participating in this study, we will carefully interview each subject about her medical history. Throughout the study, if the subjects are sexually active, they use barrier contraception to prevent pregnancy. Subjects will be removed from the study if they become pregnant during the research period, do not follow the prescribed Antagon or hormonal administration regimen, or if the investigators and/or medical supervisors feel their safety is at risk. All drugs except elagolix have been approved under HIC #0512000875.

Flow mediated Vasodilation does not carry any risks. However, there is temporary discomfort towards the end of the occlusion period. We take care to warn the subjects about this when they consent to the study and remind them of this when we begin the FMD procedure.

The Oxford technique is done under the supervision of Chris Moore, MD (Emergency Department).

11. **Data and Safety Monitoring Plan:** Include an appropriate Data and Safety Monitoring Plan (DSMP) based on the investigator's risk assessment stated below. (Note: the HIC will make

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the final determination of the risk to subjects.) For more information, see the Instructions, page 24.

1. Personnel responsible for the safety review and its frequency:

The principal investigator will be responsible for monitoring the data, assuring protocol compliance, and conducting the safety reviews at the specified frequency which must be conducted at a minimum of every 6 months (including when reapproval of the protocol is sought). During the review process, the principal investigator (monitor) will evaluate whether the study should continue unchanged, require modification/amendment, continue or close to enrollment. Either the principal investigator or the HIC have the authority to stop or suspend the study or require modifications.

2. The risks associated with the current study are deemed moderate for the following reasons: (choose those that apply)

1. We do not view the risks associated with the protocol as minimal. **YES** In this study we use I.V. catheters to draw blood for hormone analysis. We also insert microneurography needles for neural measurement.
2. Given the now established safety and validity of the current protocol in our prior work, we do not view the proposed studies as high risk. **YES**. The investigators involved in these protocols all have extensive experience with these protocols. Although we have assessed the proposed study as one of moderate risk, the potential exists for anticipated and/or unanticipated adverse events, serious or otherwise, to occur since it is not possible to predict with certainty the absolute risk in any given individual or in advance of first- hand experience with the proposed study methods. Therefore, we provide a plan for monitoring the data and safety of the proposed study as follows:

3. 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 principal investigator (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).

4. 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

5. Plan for Determining Seriousness of Adverse

Events: Serious Adverse Events:

In addition to grading the adverse event, the PI 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 may require medical or surgical intervention to prevent one of the other outcomes listed in this definition,
   or
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 PI to consider the grade of the event as well as its —seriousnessll when determining whether reporting to the HIC is necessary.

6. 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.

The investigator will report the following types of adverse events to the HIC; 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.

These adverse events or unanticipated problems involving risks to subjects or others will be reported to the HIC within 48 hours of it becoming known to the investigator, using the appropriate HIC forms found on the website.

7. Plan for reporting adverse events to co-investigators on the study, as appropriate the protocol's research monitor(s), e.g., industrial sponsor, Yale Cancer Center monitors, the Hospital Research Unit's (HRU) Science and Safety Committee (SSC), Cancer Center's Quality Assurance, Compliance and Safety Committee (QUACS) Protocol Review Committee (PRC), DSMBs, study sponsors, funding and regulatory agencies, and regulatory and decision-making bodies.

d. For multi-site studies for which the Yale PI serves as the lead investigator: **N/A**

i. How will adverse events and unanticipated problems involving risks to subjects or others be reported, reviewed and managed?
ii. What provisions are in place for management of interim results?
iii. What will the multi-site process be for protocol modifications?

d. For multi-site studies for which the Yale PI serves as the lead investigator: **N/A**
i. How will adverse events and unanticipated problems involving risks to subjects or others be reported, reviewed and managed?
ii. What provisions are in place for management of interim results?
iii. What will the multi-site process be for protocol modifications?

a. What is the investigator's assessment of the overall risk level for subjects participating in this study?
b. If children are involved, what is the investigator's assessment of the overall risk level for the children participating in this study?
c. Include an appropriate Data and Safety Monitoring Plan. Examples of DSMPs are available here http://www.yale.edu/hrpp/forms-templates/biomedical.html for
    i. Minimal risk
    ii. Greater than minimal

d. For multi-site studies for which the Yale PI serves as the lead investigator: **N/A**

i. How will adverse events and unanticipated problems involving risks to subjects or others be reported, reviewed and managed?
ii. What provisions are in place for management of interim results?
iii. What will the multi-site process be for protocol modifications?

12. **Statistical Considerations:** Describe the statistical analyses that support the study design.

Aim 1: 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 21 statistical software that includes calculations to compensate for missing data, and Prism (GraphPad) curve fitting software where appropriate. ***Data analysis.*** The slopes of the linear regressions for PI-SBP (cardiovagal baroreflex gain), PI-LBNP (integrated baroreflex gain), resting MSNA, slopes for MSNA-DBP (sympathetic baroreflex gain during Oxford and Valsalva), will be compared between treatment conditions (GnRH antagonist vs. GnRH antangonist+T and between groups (Control vs AE-PCOS) using a 2-way repeated measures ANOVA with supplemental *post hoc* testing with Tukey's HSD test. **Sample size.** The desired statistical test is two-sided, and we assume an alpha level of =0.01 to account for multiple comparisons (37). In our laboratory, we observed effect sizes (SD) of PI-SBP intercept (with n=4) of 0.39 (0.16), our most stringent variable. A sample size of 15 women per group will allow us ≥ 80% statistical power (1-β >0.9) to test within group comparisons, and this same sample size will allow [1-β]=0.80 to distinguish any between-group differences from chance alone (29). Due to the technical challenges of performing microneurography on multiple occasions in obese women, we overestimate and account for a ~ 20% failure rate, so we will recruit 18 subjects per group. **R&R: 1) *GnRH antagonist suppression/hormone add-back*** is the gold standard and most reliable method to control sex hormones in young women. **2) Microneurography** is the gold standard and most sensitive and reliable available measure for MSNA. **3) Lower body negative pressure** and the **Modified Oxford Technique** are the gold standards and most sensitive and reliable

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available measures of autonomic function in humans. We will report our methods and findings in detail and **full transparency** to permit others to reproduce and extend our findings. **Sex as a biological variable:** Only women are included in these studies because AE-PCOS is a female-specific disorder.

Aim 2: Statistical analyses: We use ANOVA as described in Protocols 1. **Sample Size** is based on calculations in Aim 1, as the Aim 1 variables are the more stringent.

Expected results, interpretation of data, limitations and anticipated problems*:** Based on our hypothesis, ***we expect that women with AE-PCOS will have greater RAS hormonal responses to LBNP, reflecting greater RAS activation. ***We expect*** that testosterone suppression will reduce PRA, $S_{[ALD]}$, $S_{[ANG\ II]}$, PRA and $P_{[Renin]}$ response to LBNP, suggesting lower RAS activation in both Control and AE-PCOS. ***We expect*** that increasing $P_{[Tfree]}$ and FAI will increase RAS hormone response to LBNP, reflecting greater RAS activation responses to LBNP in AE-PCOS. Since the methods to be used are in place in our laboratory, we don't anticipate any problems in performing the studies in this Aim.

Expected results, interpretation of data, limitations and anticipated problems*: *We expect AE-PCOS women will have greater resting MSNA compared to Controls. ***We anticipate*** that slopes for MSNA-DBP will be greater in AE-PCOS, indicating increased sympathetic BRS compared to Control. ***We expect*** that testosterone suppression will decrease slopes for MSNA-DBP in AE-PCOS, and testosterone administration will increase slopes for MSNA-DBP in both Control and AE-PCOS. In contrast, ***we expect*** reduced slopes of PI-SBP with modified Oxford and paced breathing, indicating decreased sympathetic and cardiovagal BRS in AE-PCOS women; ***we expect*** a steeper PI-SBP slope with androgen suppression, and a steeper PI-SBP slope with modified Oxford, as well as paced breathing and Valsalva in both groups. Since all methods to be used are in place in our laboratory, we don't anticipate any problems in performing the studies in this Aim.

Aim 3: Statistical analyses: We use ANOVA as described in Protocols 1. **Sample Size** is based on calculations in Aim 1, as the Aim 1 variables are the more stringent.

Expected results, interpretation of data, limitations and anticipated problems*: *We expect AE-PCOS women will have greater resting blood pressure compared to Controls. ***We expect*** that women with AE-PCOS will have greater RAS hormonal responses to LBNP, reflecting greater RAS activation. ***We expect*** that testosterone suppression will reduce PRA, $S_{[ALD]}$, $S_{[ANG\ II]}$, PRA and $P_{[Renin]}$ response to LBNP, suggesting lower RAS activation in both Control and AE-PCOS. ***We expect*** that increasing $P_{[Tfree]}$ and FAI will increase RAS hormone response to LBNP, reflecting greater RAS activation responses to LBNP in AE-PCOS. **We expect** Flow Mediated Vasodilation to be lower in the the women with AE-PCOS compared to control women, and we expect the suppression of testosterone to eliminate this difference. ***We expect*** that increasing $P_{[Tfree]}$ and FAI will increase FMD in women with AE-PCOS, with little impact on Controls. ***We anticipate*** that women with AE-PCOS will have lower baroreceptor sensitivity, compared to Controls, and that this will be improved with testosterone suppression. Finally, we expect increasing $P_{[Tfree]}$ and FAI will increase baroreflex sensitivity in women with AE-PCOS, with little impact on Controls. Since the methods to be used are in place in our laboratory, we don't anticipate any problems in performing the studies in this Aim.

Aim 4. ***We expect*** that testosterone will be suppressed similarly during elagolix and ganirelix treatments. We will use Cronbach's alpha as a measure of internal consistency or reliability (95).

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SECTION VI: RESEARCH INVOLVING DRUGS, BIOLOGICS, RADIOTRACERS, PLACEBOS AND DEVICES

If this section (or one of its parts, A or B) is not applicable, state N/A and delete the rest of the section.

A. DRUGS, BIOLOGICS and RADIOTRACERS

1. **Identification of Drug, Biologic or Radiotracer:** What is (are) the **name(s)** of the drug(s) biologic(s) or radiotracer(s) being used? Identify whether FDA approval has been granted and for what indication(s).

Antagon and Cetrotide are FDA-approved for women undergoing in vitro fertilization and have been approved for use in HIC protocols 10294, 12676, 25890 and 26534 (women with PCOS). **Elagolix** is FDA-approved for women undergoing in vitro fertilization.

Methyl testosterone is FDA approved for use in women at a higher dose (100 mg) to treat acquired, generalized hypoactive sexual desire disorder (HSDD) in premenopausal women.
.

All protocols which utilize a drug, biologic or radiotracer **not** approved by, but regulated by, the FDA, or a radiotracer regulated by the RDRC, must provide the following information:

a. What is the Investigational New Drug (IND) **number** assigned by the FDA?
b. Who holds the IND?
c. All protocols which utilize a radiotracer not approved by, but regulated by the FDA must provide the IND number: _____
Alternatively, use of the investigational radiotracer may be under RDRC/RSC oversight: (check if appropriate)_____

For all investigational radiotracers, attach a copy of the RDRC/RSC application (for radioisotopes used in the PET Center, PET Center personnel may complete this step)
Go to http://rsc.med.yale.edu/login.asp?url=myApps.asp. When you have logged in, complete the application and attach a copy to this submission.

Alternatively, an **exemption from IND filing requirements** may be sought for a clinical investigation of a drug product that is lawfully marketed in the United States. If there is no IND and an exemption is being sought, review the following categories and complete the category that applies (*and delete the inapplicable categories)*:

Exempt Category 1
The clinical investigation of a drug product that is lawfully marketed in the United States can be exempt from IND regulations if all of the following are yes:

i. The intention of the investigation is NOT to report to the FDA as a well-controlled study in support of a new indication for use or to be used to support any other significant change in the labeling for the drug. ☒ Yes ☐ No
ii. The drug that is undergoing investigation is lawfully marketed as a prescription drug product, and

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the intention of the investigation is NOT to support a significant change in the advertising for the product. ☒ Yes ☐ No

iii. The investigation does NOT involve a route of administration or dosage level or use in populations or other factor that significantly increases the risks (or decreases the acceptability of the risks) associated with the use of the drug product. ☒ Yes ☐ No

iv. The investigation will be conducted in compliance with the requirements for institutional (HIC) review and with the requirements for informed consent of the FDA regulations (21 CFR Part 50 and 21 CFR Part 56). ☒ Yes ☐ No

v. The investigation will be conducted in compliance with the requirements regarding promotion and charging for investigational drugs. ☒ Yes ☐ No

Exempt Category 2 (all items i, ii, and iii must be checked to grant a category 2 exemption)

☐ i. The clinical investigation is for an *in vitro* diagnostic biological product that involves one or more of the following (check all that apply):

☐ Blood grouping serum
☐ Reagent red blood cells
☐ Anti-human globulin

☐ ii. The diagnostic test is intended to be used in a diagnostic procedure that confirms the diagnosis made by another, medically established, diagnostic product or procedure; and

☐ iii. The diagnostic test is shipped in compliance with 21 CFR §312.160.

Exempt Category 3

☐ The drug is intended solely for tests in vitro or in laboratory research animals if shipped in accordance with 21 CFR 312.60

Exempt Category 4

☐ A clinical investigation involving use of a placebo if the investigation does not otherwise require submission of an IND.

2. **Background Information:** Provide a description of previous human use, known risks, and data addressing dosage(s), interval(s), route(s) of administration, and any other factors that might influence risks. If this is the first time this drug is being administered to humans, include relevant data on animal models.

NONE

3. **Source:** a) Identify the source of the drug or biologic to be used.

b) Is the drug provided free of charge to subjects? ☐ Yes ☐ No
If yes, by whom?

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4. **Storage, Preparation and Use:** Describe the method of storage, preparation, stability information, and for parenteral products, method of sterilization and method of testing sterility and pyrogenicity.

   Check applicable Investigational Drug Service utilized:

   ☐ **YNHH IDS**
   ☐ **CMHC Pharmacy**
   ☐ **PET Center**
   ☐ **Other:**
   ☐ **Yale Cancer Center**
   ☐ **West Haven VA**
   ☒ **None**

   Note: If the YNHH IDS (or comparable service at CMHC or WHVA) will not be utilized, explain in detail how the PI will oversee these aspects of drug accountability, storage, and preparation.

   We will purchase the GnRH antagonist through the reproductive endocrinology clinic at YNHH through Dr. Lubna Pal . We will purchase the methyl testosterone via Compounded Solutions. Dr. Pal will provide the prescription.

5. **Use of Placebo: ☒ Not applicable to this research project**

   If use of a placebo is planned, provide a justification which addresses the following:

   1. Describe the safety and efficacy of other available therapies. If there are no other available therapies, state this.
   b. State the maximum total length of time a participant may receive placebo while on the study.
   c. Address the greatest potential harm that may come to a participant as a result of receiving placebo.
   d. Describe the procedures that are in place to safeguard participants receiving placebo.

6. **Use of Controlled Substances:**

   Will this research project involve the use of controlled substances in human subjects?
   ☐ Yes ☒ No *See HIC Application Instructions to view controlled substance listings*.

   If yes, is the use of the controlled substance considered:
   ☐ Therapeutic: The use of the controlled substance, within the context of the research, has the potential to benefit the research participant.
   ☐ Non-Therapeutic: *Note, the use of a controlled substance in a non-therapeutic research study involving human subjects may require that the investigator obtain a Laboratory Research License. Examples include controlled substances used for basic imaging, observation or biochemical studies or other non-therapeutic purposes. See Instructions for further information.*

7. **Continuation of Drug Therapy After Study Closure ☒ Not applicable to this project**

   Are subjects provided the opportunity to continue to receive the study drug(s) after the study has ended?
   ☐ Yes If yes, describe the conditions under which continued access to study drug(s) may apply as well as conditions for termination of such access.

   ☐ No If no, explain why this is acceptable.

B. DEVICES

NONE

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1. Are there any investigational devices used or investigational procedures performed at Yale-New Haven Hospital (YNHH) (e.g., in the YNHH Operating Room or YNHH Heart and Vascular Center)? ☐Yes **X** No *If Yes, please be aware of the following requirements*:

a. A YNHH New Product/Trial Request Form must be completed via EPIC: **Pull down the Tools tab in the EPIC Banner, Click on Lawson, Click on "Add new" under the New Technology Request Summary and fill out the forms requested including the "Initial Request Form," "Clinical Evidence Summary, " and attach any other pertinent documents. Then select "save and submit" to submit your request**; and

b. Your request must be reviewed and approved **in writing** by the appropriate YNHH committee before patients/subjects may be scheduled to receive the investigational device or investigational procedure.

2. What is the name of the device to be studied in this protocol?

Has this device been FDA approved? ☐ Yes ☐ No
If yes, state for what indication.

3. **Background Information:** Provide a description of previous human use, known risks, and any other factors that might influence risks. If this is the first time this device is being used in humans, include relevant data on animal models.

4. **Source:**
   a) Identify the source of the device to be used.

   b) Is the device provided free of charge to subjects? ☐ Yes ☐ No

5. What is the PI's assessment of risk level (significant or non-significant) associated with the use of the device?

☐ **Significant Risk (SR) Device Study:** A study of a device that presents a potential for serious risk to the health, safety, or welfare of a participant and 1) is intended as an implant; 2) is used in supporting or sustaining human life; or otherwise prevents impairment of human health; 3) is of substantial importance in diagnosing, curing, mitigating or treating disease, or otherwise prevents impairment of human health; or 4) otherwise presents a potential for serious risk to the health, safety, or welfare of a participant.

Significant Risk Devices require an Investigational Device Exemption (IDE) issued by the FDA.

What is the **IDE number** assigned by the FDA?

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Did the FDA approve this IDE as **Category A** (experimental/investigational) or as **Category B** (non-experimental/investigational)?

Who holds the IDE?

☐ **Non-Significant Risk (NSR) Device Study:** A study of a device that does not meet the definition for a significant risk device and does not present a potential for serious risk to the health, safety, or welfare of participants. Note that if the HIC concurs with this determination, an IDE is not required.

6. **Abbreviated IDE or Exempt IDE:** There are abbreviated requirements for an IDE and there also are exemptions to the requirement for an IDE. *See the criteria in the HIC Application Instructions,* Section VI.B.4 at http://www.yale.edu/hrpp/resources/docs/100FR1aHICProtocol_Application_Instructions5-25-11.pdf *to determine if these pertain to this study.*

☐ **Abbreviated IDE or Exempt IDE** – *If criteria set forth in the HIC Application Instructions are met, copy and paste the completed relevant section from the Instructions into this application.*

7. **Investigational device accountability**:
   a. State how the PI, or named designee, ensures that an investigational device is used only in accordance with the research protocol approved by the HIC, and maintains control of the investigational device as follows:

      Maintains appropriate records, including receipt of shipment, inventory at the site, dispensation or use by each participant, and final disposition and/or the return of the investigational device (or other disposal if applicable):

Documents pertinent information assigned to the investigational device (e.g., date, quantity, batch or serial number, expiration date if applicable, and unique code number):

Stores the investigational device according to the manufacturer's recommendations with respect to temperature, humidity, lighting, and other environmental considerations:

Ensures that the device is stored in a secure area with limited access in accordance with applicable regulatory requirements:

Distributes the investigational device to subjects enrolled in the IRB-approved protocol:

SECTION VII: RECRUITMENT/CONSENT AND ASSENT PROCEDURES

1. **Targeted Enrollment: Give the number of subjects:**
   a. targeted for enrollment at Yale for this protocol_124__
   b. If this is a multi-site study, give the total number of subjects targeted across all sites_N/A__

2. **Indicate recruitment methods below.** Attach copies of any recruitment materials that will be used.

☒ Flyers	☒ Internet/Web Postings	☐ Radio
☒ Posters	☐ Mass E-mail Solicitation	☐ Telephone

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☐ Letter ☒ Departmental/Center Website ☐ Television
☒ Medical Record Review* ☒ Departmental/Center Research Boards ☐ Newspaper
☒ Departmental/Center Newsletters ☐ Web-Based Clinical Trial Registries
☒ YCCI Recruitment database ☒ Clinicaltrials.gov Registry (do not send materials to HIC)
☒ Other (describe): MyChart/PCOSChallenge

***Requests for medical records should be made through JDAT as described at**
http://medicine.yale.edu/ycci/oncore/availableservices/datarequests/datarequests.aspx
We will use the YCCI to help us connect with their with database of volunteers who have indicated willingness to be contacted for research studies. YCCI uses OnCore as the database but pulls clinical data from Epic. We are interested in using this database to help us recruit overweight/obese insulin resistant women with and without PCOS. Both of these groups are challenging to recruit with only flyers posted in standard places around the medical school and Yale University campus.

For recruitment, we will utilize the Joint Data Analytics Team (JDAT) to identify potential subjects with insulin resistance and PCOS. 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 blood pressure regulation 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, and brief information about the study. See below for the information potential subjects will receive.

We have applied through JDAT.
The information potential subjects will receive:

- You are receiving this MyChart message because you are a woman between the ages of 18-40 years of age and may interested in participating in research projects. One project, for which you may or may not be eligible investigates the connection between insulin and blood pressure control. One part of the research investigates how women who are insulin resistant (but not diabetic), with or without Polycystic Ovary Syndrome (PCOS) regulate their blood pressure. Approximately 60 subjects with insulin resistance will be enrolled in this study and half will have PCOS. We are recruiting two groups of women: 1) insulin resistant, overweight/obese women (Controls); 2) insulin resistant, overweight/obese young women PCOS. Subjects should have BMI between 27-35 kg/m^2. There will be four data collection visits over a six- week period, in addition to screening.

- You cannot participate in the protocol if you are diabetic.

- Possible Benefits: This research is not designed to benefit you directly. However, you will receive payment for your participation and free parking. In addition, knowledge gained from the results may help us to better understand hypertension in women with insulin resistance or Polycystic Ovary Syndrome (PCOS).

- Confidentiality and Privacy: Any personal health data gathered will remain confidential and will be stored in a locked file cabinet, only accessed by study personnel. Your personal information will not be entered onto a computer and you will only be identified by subject number on any of our electronic devices. When the results of the research are published or discussed, no information will be included that would reveal your identity. We understand that information about you obtained in connection with your health is

personal, and we are committed to protecting the privacy of that information. If you decide to participate, please contact the study coordinator at 203-562-9901 x219.

- Participation in this study is completely voluntary. You are free to decline to participate, to end participation at any time for any reason, or to refuse to answer any individual question at any time. Refusing to participate will involve no penalty or loss of benefits to which you are otherwise entitled (such as your health care outside the study, the payment for your health care, and your health care benefits).

- Questions: If you have any further questions about this study, you may contact the investigator, Nina Stachenfeld, at 203-562-9901 x219. If you would like to talk with someone other than the researchers to discuss problems, concerns, and questions you may have concerning this research, or to discuss your rights as a research subject, you may contact the Yale Human Investigation Committee at (203) 785-4688.

  PI: Nina Stachenfeld, PhD.
  What the study involves:
  Study participants will be asked to participate in physiological studies in our laboratory with our research team.
  There are some medications administered in this study.
  Your answers and all personal information will be protected by assigning you a study number which will be kept in a locked file cabinet.
  If you are interested in learning more about this study, please call 203-562-9901 x219 to speak with a study coordinator. You could also contact us by email at nina.stachenfeld@yale.edu
  Future research opportunities:
  You may also create a volunteer profile through the Research Tab in MyChart.

PCOS Challenge: The National Polycystic Ovary Syndrome Association is a leading 501(c)(3) nonprofit patient support an advocacy organization globally that is advancing the cause for women and girls with PCOS serving over 50,000 members.
https://www.pcoschallenge.org/

We would like to use PCOS Challenge recruitment program to help recruit subjects for our studies.

1) Key Points

- PCOS Challenge study recruitment campaigns offer the most targeted and cost-effective ways to reach the largest number of women with PCOS available today. We offer the ability to geo-target recruitment efforts in any state.

- PCOS Challenge is the largest nonprofit support organization globally for women with PCOS serving **over 55,000 members**.

- 92% of PCOS Challenge members have been diagnosed with PCOS.

- PCOS Challenge **has close to 1,000 members in Connecticut and more followers on social media.**

- **Here are quotes from study partners about the effectiveness of PCOS Challenge Campaigns**

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- **Single-State Campaigns -** "We had 233 contacts in the last month, which outpaces our normal number...This proves to be an effective method of reaching out to people we wouldn't normally be able to reach. I would like to discuss expanding our campaign to more places."

- **Campaign Example See** - https://pcoschallenge.org/apple-womens-health-study/

2) Study Recruitment Campaigns Details
Current rates through December 31, 2021 for a one-month recruitment campaign are:

- **1 State (Connecticut)** - $2,500

- **The campaign above includes one month of:**

  - Bi-weekly emails in the state(s) you are targeting (Sent every other week). *We will include IRB approved flyers.*
  - Study promotional page on PCOSChallenge.org with additional information about the study (i.e. Title, Description, Goals, Eligibility Criteria, Exclusions, Locations, and Compensation) as well as relevant videos, articles, or audios such as a *PCOS Challenge* interview with the PI. *We will include IRB approved flyers.*
  - 160x600 banner ad (provided by study organization) promoting the study on every page of PCOSChallenge.com (Over 50,000 pages). *We will use IRB approved flyers.*

Promotion through PCOS Challenge Social Media Profiles (Over 120,000 Followers)

3. Recruitment Procedures:

a. Describe how potential subjects will be identified. Through phone screening by the PI.

b. Describe how potential subjects are contacted. They respond to advertisements. We will also work with the YCCI. The YCCI has a database of volunteers willing to be contacted for studies. The Clinical Trials Resources dept. within YCCI will assist in identifying subjects who have indicated willingness to be contacted and fit the criteria for our study, and we will contact them by phone.

c. Who is recruiting potential subjects? The PI

4. Screening Procedures

a. Will email or telephone correspondence be used to screen potential subjects for eligibility prior to the potential subject coming to the research office? ☒ Yes ☐ No

b. If yes, identify below all health information to be collected as part of screening and check off any of the following HIPAA identifiers to be collected and retained by the research team during this screening process.

We ask height, weight and if they have ever been diagnosed with PCOS or insulin resistance. We ask if they have regular periods, if they smoke or are taking medications.

HEALTH INFORMATION TO BE COLLECTED

HIPAA identifiers:

☒ Names

☐ All geographic subdivisions smaller than a State, including: street address, city, county, precinct, zip codes and their equivalent geocodes, except for the initial three digits of a zip code if, according to the current publicly-available data from the Bureau of the Census: (1) the geographic unit formed by combining all zip codes with the same three initial digits contains more than 20,000 people, and (2) the initial three digits of a zip code for all such geographic units containing 20,000 or fewer people is changed to 000.

☒ Telephone numbers

☐ Fax numbers

☒ E-mail addresses

☐ Social Security numbers

☐ Medical record numbers

☐ Health plan beneficiary numbers

☐ Account numbers

☐ All elements of dates (except year) for dates related to an individual, including: birth date, admission date, discharge date, date of death, all ages over 89 and all elements of dates (including year) indicative of such age, except that such ages and elements may be aggregated into a single category of age 90 or older

☐ Certificate/license numbers

☐ Vehicle identifiers and serial numbers, including license plate numbers

☐ Device identifiers and serial numbers

☐ Web Universal Resource Locators (URLs)

☐ Internet Protocol (IP) address numbers

☐ Biometric identifiers, including finger and voice prints

☐ Full face photographic images and any comparable images

☐ Any other unique identifying numbers, characteristics, or codes

5. Assessment of Current Health Provider Relationship for HIPAA Consideration:

Does the Investigator or any member of the research team have a direct existing clinical relationship with any potential subject?

☐ Yes, all subjects

☒ Yes, some of the subjects

☐ No

If yes, describe the nature of this relationship. It is possible that some of the subjects could be patients of Dr. Pal.

6. Request for waiver of HIPAA authorization: (When requesting a waiver of HIPAA Authorization for either the entire study, or for recruitment purposes only. Note: if you are collecting PHI as part of a phone or email screen, you must request a HIPAA waiver for recruitment purposes.)

Choose one:

☐ For entire study

☒ For recruitment purposes only

☐ For inclusion of non-English speaking subject if short form is being used **and a translated HIPAA research authorization form is not available on the University's HIPAA website**

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i. Describe why it would be impracticable to obtain the subject's authorization for use/disclosure of this data;
ii. If requesting a waiver of **signed** authorization, describe why it would be impracticable to obtain the subject's signed authorization for use/disclosure of this data; While recruiting over the phone, we ask the individual's name, sex and phone number. We also ask height and weight. This information is not written down, but is used to determine if she will qualify for the study.

By signing this protocol application, the investigator assures that the protected health information for which a Waiver of Authorization has been requested will not be reused or disclosed to any person or entity other than those listed in this application, except as required by law, for authorized oversight of this research study, or as specifically approved for use in another study by an IRB.

Researchers are reminded that unauthorized disclosures of PHI to individuals outside of the Yale HIPAA-Covered entity must be accounted for in the "accounting for disclosures log", by subject name, purpose, date, recipients, and a description of information provided. Logs are to be forwarded to the Deputy HIPAA Privacy Officer.

7. **Required HIPAA Authorization:** If the research involves the creation, use or disclosure of protected health information (PHI), separate subject authorization is required under the HIPAA Privacy Rule. Indicate which of the following forms are being provided:
☒ Compound Consent and Authorization form
☐ HIPAA Research Authorization Form**.**

8. **Consent Personnel:** List the names of all members of the research team who will be obtaining consent/assent. Nina Stachenfeld

9. **Process of Consent/Assent:** Describe the setting and conditions under which consent/assent will be obtained, including parental permission or surrogate permission and the steps taken to ensure subjects' independent decision-making.

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. When the subject arrives for the consent process, we review the protocol with her and indicate that she should ask any questions or express concerns before signing the consent form. We stress that she is under no obligation to sign the consent form or participate in the study if she is 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.

As described we will also utilize the Joint Data Analytics Team (JDAT) to identify potential subjects with insulin resistance and PCOS. Please refer to Section VII #2 for information interested subjects will receive.

10. **Evaluation of Subject(s) Capacity to Provide Informed Consent/Assent:** Indicate how the personnel obtaining consent will assess the potential subject's ability and capacity to consent to the research being proposed.

During the initial phone conversation, we have a give and take conversation enabling the potential subject to ask questions and express concerns about participation. If the subject cannot speak English well enough to understand the protocol, we do not allow her to continue the consent process. The same conversations take place at the time of consent at the laboratory.

11. Documentation of Consent/Assent: Specify the documents that will be used during the consent/assent process. Copies of all documents should be appended to the protocol, in the same format that they will be given to subjects.

Consent Forms

12. Non-English Speaking Subjects: Explain provisions in place to ensure comprehension for research involving non-English speaking subjects. If enrollment of these subjects is anticipated, translated copies of all consent materials must be submitted for approval prior to use. N/A

12(a) As a limited alternative to the above requirement, will you use the short form* for consenting process if you unexpectedly encounter a non-English speaking individual interested in study participation and the translation of the long form is not possible prior to intended enrollment?
YES ☐ NO ☒

Note* If more than 2 study participants are enrolled using a short form translated into the same language, then the full consent form should be translated into that language for use the next time a subject speaking that language is to be enrolled.

Several translated short form templates are found on our website at: http://www.yale.edu/hrpp/forms-templates/biomedical.html. If the translation of the short form is not available on our website, then the translated short form needs to be submitted to the IRB office for approval via amendment prior to enrolling the subject. ***Please review the guidance and presentation on use of the short form available on the HRPP website.***

If using a short form without a translated HIPAA Research Authorization Form, please request a HIPAA waiver in the section above.

13. Consent Waiver: In certain circumstances, the HIC may grant a waiver of signed consent, or a full waiver of consent, depending on the study. If you will request either a waiver of consent, or a waiver of signed consent for this study, complete the appropriate section below.
☒ **Not Requesting a consent waiver**
☐ **Requesting a waiver of signed consent**
☐ **Requesting a full waiver of consent**

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SECTION VIII: PROTECTION OF RESEARCH SUBJECTS

Confidentiality & Security of Data:

a. What protected health information (medical information along with the HIPAA identifiers) about subjects will be collected and used for the research?

The subjects will provide private information on a medical history form 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), Dr. Pal,and Ms. Leone will have access to this information.

b. How will the research data be collected, recorded and stored?

We collect non digital data in notebooks that do not identify the subject by name (only by subject initials, 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, date and number

c. How will the digital data be stored? ☐ CD ☐ DVD ☒ Flash Drive ☐ Portable Hard Drive ☒ Secured Server ☒ Laptop Computer ☒ Desktop Computer ☐ Other

d. What methods and procedures will be used to safeguard the confidentiality and security of the identifiable study data and the storage media indicated above during and after the subject's participation in the study?

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 are stored in a locked filing cabinet within Dr. Stachenfeld's laboratory.

All portable devices must contain encryption software, per University Policy 5100. *If there is a technical reason a device cannot be encrypted please submit an exception request to the Information Security, Policy and Compliance Office by clicking on url* http://its.yale.edu/egrc or email it.compliance@yale.edu

e. What will be done with the data when the research is completed? Are there plans to destroy the identifiable data? If yes, describe how, by whom and when identifiers will be destroyed. If no, describe how the data and/or identifiers will be secured.

The individual subjects' files are stored in the John B. Pierce Laboratory, either in the office of the Principal Investigator or in locked storage areas in the laboratory. Access to these files is limited to the Principal Investigator, Dr.Pal and Ms. Leone.

f. Who will have access to the protected health information (such as the research sponsor, the investigator, the research staff, all research monitors, FDA, Yale Cancer Center Data and Safety Monitoring Committee (DSMC), SSC, etc.)? (please distinguish between PHI and de-identified data)

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The Principal Investigator, Dr. Pal and Ms. Leone listed in this protocol will have access to PHI.

g. If appropriate, has a Certificate of Confidentiality been obtained? N/A For NIH funded research, HHS automatically issues Certificates of Confidentiality (CoC) to persons engaged in biomedical, behavioral, clinical or other research, in which identifiable, sensitive information is collected. These Certificates protect the privacy of subjects by limiting the disclosure of identifiable, sensitive information. Thus, this we do not have to apply for a CoC for this research.
https://grants.nih.gov/grants/guide/notice-files/NOT-OD-17-109.html

h. Are any of the study procedures likely to yield information subject to mandatory reporting requirements? (e.g., HIV testing – reporting of communicable diseases; parent interview -incidents of child abuse, elderly abuse, etc.). Please verify to whom such instances will need to be reported.
N/A (no)

SECTION IX: POTENTIAL BENEFITS

Potential Benefits: Identify any benefits that may be reasonably expected to result from the research, either to the subject(s) or to society at large. (Payment of subjects is not considered a benefit in this context of the risk benefit assessment.)

The primary benefits to the volunteers will be the satisfaction derived from participating in a research program designed to assess blood pressure changes in women with AE-PCOS.

SECTION X: RESEARCH ALTERNATIVES AND ECONOMIC CONSIDERATIONS

1. **Alternatives:** What other alternatives are available to the study subjects outside of the research?
   The alternative is not to participate.

2. **Payments for Participation (Economic Considerations):** Describe any payments that will be made to subjects, the amount and schedule of payments, and the conditions for receiving this compensation.
   Aims 1 & 2. Subjects will receive up to $800.00 for participating in all aspects of the study. Subjects will not be charged for any of the tests. They will be paid $40.00 for the orientation procedure (including ultrasound), $60 for the oral glucose tolerance test, $150.00 for each blood pressure research study, $15.00 for each day they are taking Antagon, and a $100.00 finishing bonus. If they do not complete all parts of the study, they will be paid for the parts completed. Payment will be in check form following completion of the study.

   Aim 3. Subjects will receive up to $770.00 for participating in all aspects of the study. Subjects will not be charged for any of the tests. They will be paid $40.00 for the orientation procedure (including ultrasound), $60 for the oral glucose tolerance test, $100.00 for each blood pressure research study, $15.00 for each day they are taking Antagon, and a $100.00 finishing bonus. If they do not complete all parts of the study, they will be paid for the parts completed. Payment will be in check form following completion of the study.

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Aim 4. Subjects will receive up to $200 for participating in all aspects of the study. They will be paid $50 for each blood draw, $10 for each day taking Elagolix, and a $30 finishing bonus. If they do not complete all parts of the study, they will be paid for the parts completed. Payment will be in check form following completion of the study.

Aim 5. Subjects will receive up to $350.00 for participating in all aspects of the study. Subjects will not be charged for any of the tests. They will be paid $40.00 for the orientation procedure (including ultrasound), $60 for the oral glucose tolerance test, $150.00 for the blood pressure research study, and a $100.00 finishing bonus. If they do not complete all parts of the study, they will be paid for the parts completed. Payment will be in check form following completion of the study.

3. **Costs for Participation (Economic Considerations):** Clearly describe the subject's costs associated with participation in the research, and the interventions or procedures of the study that will be provided at no cost to subjects.
There will be no costs to subjects. If transportation is required the laboratory will reimburse up to $20. According to the rules of the Internal Revenue Service (IRS), payments that are made to the subject as a result of their participation in a study may be considered taxable income.

4. **In Case of Injury:** This section is required for any research involving more than minimal risk, and for minimal risk research that presents the potential for physical harm (e.g., research involving blood draws).
Medical therapy will be offered for any physical injuries sustained as a consequence of the subject's participation in this research. The subject or the subjects' insurance company will be responsible for the cost of such therapy.

   a. Will medical treatment be available if research-related injury occurs? yes
   b. Where and from whom may treatment be obtained? Subject's personal physicians
   c. Are there any limits to the treatment being provided? Based on insurance limitations
   d. Who will pay for this treatment? The subject or the subjects' insurance company will be responsible for the cost of such therapy.
   e. How will the medical treatment be accessed by subjects? Appointment with physician

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APPROVED BY THE YALE UNIVERSITY IRB 1/13/2022