Protocol Full Title: Vascular mechanisms for the effects of loss of ovarian hormone function on cognition

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Specific Aims

Alzheimer's Disease (AD) is the leading cause of dementia in the elderly and the 6th leading cause of death in the U.S.[1] Almost 2/3 of individuals with AD in the US are women.[1] There is no cure for AD, and presently no interventions to prevent or delay neuropathologic progression of the disease. Cerebrovascular damage is a well-recognized component of AD and there is strong evidence that traditional cardiovascular risk factors significantly increase the risk of AD.[2] The loss of estrogen with menopause appears to augment the age-associated increase in the incidence of cardiovascular risk factors[3-6] which could also explain the additional increased risk of AD in postmenopausal women.

Estrogen, specifically 17β-estradiol (E_2), helps maintain neuronal integrity and cognitive function.[7-9] A major site of E_2 action is the prefrontal cortex (PFC) which supports executive cognitive function and working memory[10] and is also affected in AD.[11] Cognitive deficits and altered patterns of brain activation have been demonstrated with both surgical[12] and pharmacologic suppression of ovarian function,[13-15] and reversal of these deficits with E_2 replacement or recovery of ovarian function strongly suggests an important role for E_2 in supporting cognition.[15,16] Although numerous epidemiological studies have shown beneficial effects of E_2 on cognition,[17] the pivotal Women's Health Initiative Memory Study (WHIMS) found an increased risk of cognitive decline and dementia with conjugated equine estrogen (CEE) plus progesterone in postmenopausal women ≥65 years of age.[18-21] The specific formulation of hormone therapy (HT) as well as the timing of administration relative to menopause may help to explain the conflicting results from clinical trials,[22] and further clinical intervention studies of the cognitive effects of E_2 in women near menopause are needed.

Among its many important physiological roles, E_2 is a key regulator of peripheral vascular function and is thought to protect against cardiovascular disease by stimulating nitric oxide (NO**)** production and promoting a vasodilatory state.[23-25] E_2-deficient postmenopausal women show arterial stiffening and impaired endothelial-dependent vasodilation (endothelial dysfunction) compared to age-matched premenopausal women, and E_2-based hormonal therapy appears to attenuate these impairments in treated compared to untreated women.[26-31] Arterial stiffening and endothelial dysfunction have been associated with a higher burden of small vessel cerebrovascular disease,[32] and have been linked to cognitive impairment and dementia.[33-36]

The mechanisms for the putative effects of E_2 on cognition are not known. However, detectable impairments in cognition after just 8 weeks of ovarian hormone suppression[13,14] imply a relatively acute time frame. The ability of E_2 to acutely affect vascular function[37,38] thus makes this a highly plausible mechanism for the effects of E_2 on cognitive function. Elucidating these mechanisms may help in the development of new interventions to delay, prevent or attenuate cognitive decline in the aging population.

The <u>overall goal</u> of this application is for the candidate to build on her work to date and develop the skills and experience to become a leader in the area of cognitive impairment and cardiovascular risk. The <u>specific goal</u> of the project is to investigate vascular dysfunction as a mechanism for the effects of E_2 on cognitive function. To do so, we will measure vascular and cognitive function in 51 healthy women nearing menopause (40-60 yrs) who will be enrolled in Dr. Wendy Kohrt's NIH-funded <u>F</u>emales, <u>A</u>ging, <u>M</u>etabolism and <u>E</u>xercise (FAME) study before and after 6 mo of ovarian hormone suppression with gonadotropin releasing hormone agonist (GnRHa), plus or minus exercise, versus placebo. In addition, this sub-study proposes an E_2 add-back portion (separate from the parent FAME study) in the GnRHa no exercise group. These participants will complete and additional 3 mo of GnRHa plus transdermal E_2 add-back (see Methods, 2.c.2, Fig 2. Study Design) with vascular and cognitive measures at the end of the add-back period.

This study provides critical additions to previous work by: 1) conducting a novel investigation of arterial stiffness and endothelial dysfunction as possible mechanisms accounting for the observed effects of GnRHa on cognition; 2) studying younger women to better distinguish the effects of E_2 deficiency from those of aging; 3) incorporating a true E_2 add-back condition to distinguish between E_2 and progesterone effects and 4) extending the duration of GnRHa treatment to help clarify cognitive effects.

Specific Aim 1. Determine the effects of 6 mo of ovarian hormone suppression with GnRHa on:

1) Patterns of activation in the PFC during tasks of executive cognitive function using functional magnetic resonance imaging (fMRI).
2) Arterial stiffness determined by ultrasound measures of carotid artery compliance and pulse wave velocity, and endothelial function measured by brachial artery flow-mediated dilation (FMD).

Hypothesis 1a. GnRHa treatment will significantly decrease PFC activation, increase arterial stiffness and decrease endothelial function compared to placebo.
Hypothesis 1b. Changes in PFC activation with GnRHa will be inversely associated with expected increases in arterial stiffness and positively associated with expected declines in endothelial function.

Specific Aim 2. Determine the effects of 3 mo of exogenous E_2 add-back on:
1) Patterns of brain activation in the PFC during tasks of executive cognitive function using fMRI.
2) Arterial stiffness and endothelial function as measured in Specific Aim 1 (2).

Hypothesis 2a. E_2 add-back will restore PFC activation, arterial stiffness and endothelial function to baseline (pre-GnRHa) levels.

Sub-study Exploratory Aim: A sub-study will enroll late-perimenopausal and postmenopausal women who have recently completed vascular testing under another protocol. The protocol for participants in this Sub-study is limited to cognitive testing and fMRI scanning at a single timepoint. The aim of this Sub-study is to compare vascular and cognitive measures in the above intervention population (premenopausal women before and after placebo or GnRHag with or without exercise) with late-perimenopausal and postmenopausal women. These comparisons will generate preliminary data for an anticipated R01-level application.

Research Strategy

1. Significance.

1.a. Importance of the problem. Alzheimer's Disease (AD) is the leading cause of dementia in the elderly and the 6th leading cause of death in the U.S.[1] Costs of caring for individuals with dementia is expected to reach $1.1 trillion by 2050, with a 500% increase in combined Medicare and Medicaid spending[1]. Almost 2/3 of individuals with AD in the US are women.[1] There is no cure for AD. Currently approved therapies modestly treat symptoms but do not alter the underlying course of the disease. Although delaying the onset of clinical symptoms by 5 years could reduce prevalence by 50% in 10 years,[39] there are presently no available treatments to prevent or delay neuropathologic progression of AD. Vascular damage is recognized as a component of AD and there is compelling evidence that traditional cardiovascular risk factors significantly increase the risk of AD.[2] The loss of estrogen with menopause appears to augment the age-associated increases in cardiovascular risk factors,[3-6] which may further increase the risk of AD in women. Understanding the potentially modifiable vascular contributions to the disease is critical to inform the development of effective new therapeutic interventions.

1.b. Knowledge to be gained. The goal of this study is to add to the scientific knowledge of the potential vascular contributions to cognitive impairment due to estrogen deficiency, specifically the role of arterial stiffening and endothelial dysfunction that may underlie the negative effects of estrogen deficiency on cognitive function in women.

Estrogen and cognitive function

Numerous studies have reported beneficial effects of HT on cognitive function and risk of dementia in postmenopausal women.[17] However, the pivotal WHIMS study reported an increased risk of dementia and cognitive decline with CEE plus medroxyprogesterone acetate (MPA), although not with CEE alone.[18-21] It has been suggested that the WHIMS results should be considered with respect to the "critical timing hypothesis", which holds that HT initiated near the time of menopause may be beneficial, but when started after a prolonged period of E_2 deprivation may be ineffective or potentially harmful.[22] The women in the WHIMS study were on average 20 years post menopause[21] in contrast to the women in more positive observational studies, who tended to be younger and closer to menopause.[40,41] Data from the Study of Women's Health Across the Nation (SWAN) study, designed to assess cognitive performance and the effect of HT across the stages of menopause, provides initial support for the critical timing hypothesis. Investigators reported HT initiated prior to the final menstrual period had beneficial effects on verbal memory (subserved in part by the PFC) and processing speed, while HT initiated after menopause was detrimental.[42] More recently, an ancillary study to WHIMS in younger women (WHIMSY) reported no effect – beneficial or harmful - of CEE or CEE+MPA compared to placebo on multiple cognitive domains.[43] Although participants in WHIMSY were younger (50-55), they were still on average 9 years postmenopause. The Kronos Early Estrogen Prevention Study[44] (KEEPS) recently reported findings from the ancillary Cognitive and Affective Study in 662 women within 3 years of menopause randomized to 4 years of oral CEE, transdermal E_2 or placebo and also found no beneficial or detrimental effects of HT on cognitive function.[45] In addition to timing of HT relative to menopause, the specific

formulation of HT may be important in determining effects on cognition,[46] for example, HT with with MPA has been shown to have detrimental effects on verbal memory even in early postmenopausal women.[47,48]

Ample evidence documents a role for E_2 in supporting cognitive function. Much of the research to date has focused on the hippocampus which supports memory and is the brain region in which the neuropathology of AD first appears. In animal studies, E_2 has been shown to increase synaptic density and plasticity in the hippocampus[7,8] and to enhance the formation and release of acetylcholine,[9] a key neurotransmitter responsible for learning and memory, and a major population of neurons that is lost in AD.[49] Other animal studies have demonstrated E_2 reduces the formation and accumulation of beta-amyloid[50] and phosphorylated tau protein,[51] precursors of the amyloid plaques and neurofibrillary tangles that define AD. Although the hippocampus appears to be a key E_2-sensitive structure, examination of other brain regions that may be vulnerable to changes in E_2 with aging is needed. The PFC, located at the anterior frontal lobes is a primary site of E_2 action in the brain, and a region also vulnerable to AD pathology. The PFC has abundant E_2 receptors,[52] and E_2 concentrations 7-fold higher than the hippocampus.[53] The PFC supports executive cognitive function,[11] which encompasses a complex set of higher level cognitive tasks that serve to regulate purposeful, goal-directed activities. These include attention, reasoning, planning, initiating actions, inhibiting inappropriate responses, coordinating tasks, cognitive set-switching and monitoring task performance.[54] Executive function is critical for problem solving and activities of daily living, and also serves to enhance learning and memory.[55] Working memory – the temporary storage and manipulation of new information - is another aspect executive function supported by the PFC.[55] Declines in executive function, including attention and working memory, are commonly reported by menopausal women, however, few studies of HT on cognition have rigorously evaluated executive function.[56]

Experimental studies in humans provide evidence for beneficial effects of E_2 on PFC-mediated cognitive functions.[15,57-60] Joffe et al demonstrated improved working memory in perimenopausal and early (<5 years) postmenopausal women after 12 weeks of transdermal E_2 treatment.[58] E_2 treatment also increased PFC activation by fMRI in a subset (n=11) of women. A similar increase in PFC activation during working memory tasks was shown in postmenopausal women treated with 21 days of CEE although performance did not change,[57] emphasizing the importance of neuroimaging in detecting effects that may precede clinically significant impairment. Recent work by Craig et al. in premenopausal women receiving GnRHa treatment for uterine fibroids demonstrated reduced PFC activation during a verbal encoding task and decreased verbal recognition after 8 weeks of treatment compared to wait-listed controls.[14] These changes were reversed after restoration of ovarian function and resumption of menses postoperatively.[16]

This proposed study will extend previous work by providing the first investigation of potential mechanisms underlying the effects of E_2 on executive function. We address important limitations of previous work by: 1) including an E_2 add-back group to isolate the effects of E_2 versus other ovarian hormones or direct effects of GnRHa; and 2) extending the treatment duration to 6 months, which is likely to produce even more profound and consistent effects on cognition.[61] Although there are multiple possible mechanisms by which E_2 might affect cognition, Craig's work suggests effect occur within a relatively short time frame (8 weeks), making mechanisms such as E_2-related changes in body composition and subsequent insulin sensitivity less likely; indeed these parameters showed no change after 8 weeks of GnRHa treatment in healthy premenopausal women.[62,63] In contrast, our preliminary data indicate that changes in vascular function are detectable after 3 days of ovarian hormone suppression. Furthermore, administration of E_2 has been shown to acutely improve arterial stiffness[26] and endothelial function,[38] making these highly plausible mechanisms for the observed negative effects of E_2 withdrawal on cognitive function.

Vascular function and cognitive impairment

The coexistence of vascular lesions and neurodegenerative pathology in most patients with AD has led to a reevaluation of the notion of vascular dementia and AD as distinct entities.[64] It is now widely believed that these diseases exist on a spectrum, with the majority of patients having features of both. Brain imaging studies have associated both arterial stiffness and endothelial dysfunction with white matter hyperintensities that reflect small vessel cerebrovascular disease, supporting a link between peripheral and cerebral vascular health.[32] As in the peripheral circulation, nitric oxide (NO) appears to play a key role in cerebral vascular function. NO synthase (NOS) inhibition has been shown to reduce cerebral blood flow and impair cerebral autoregulation in humans.[65,66] Numerous studies have implicated large artery stiffness in the development of cognitive impairment and dementia.[33] Endothelial dysfunction has also been linked to cognitive decline, specifically executive function, in adults with established cardiovascular disease.[34,35] A more recent study found a significant association between reduced endothelial function, measured by brachial artery FMD, and reduced working memory-related brain activation by fMRI in healthy middle-aged adults.[67]

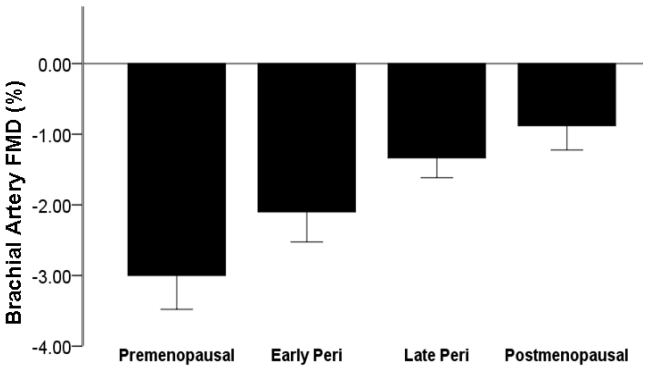
Estrogen and vascular function

E_2 is thought to have protective effects on the peripheral vasculature, as evidenced by the low incidence of cardiovascular disease in premenopausal women compared to men, whereas this difference rapidly narrows after menopause.[68,69] Peripheral endothelial dysfunction, characterized by impaired endothelial-dependent vasodilation, is an independent predictor of cardiovascular events and precedes the development of clinical atherosclerotic disease, making it an excellent early indicator of overall vascular health.[70] Arterial stiffness, measured by ultrasound measurement of carotid artery compliance, is also a major risk factor for CVD.[71] E_2 receptors are present on vascular smooth muscle and endothelial cells.[23] E_2 is known to increase NO bioavailability by stimulating both the expression and activity of endothelial (e)NOS[24] and to inhibit the production of harmful reactive oxygen species that can scavenge NO.[25,72] E_2 action thus promotes relaxation of vascular smooth muscles cells and a vasodilatory state. Importantly, activation of non-genomic mechanisms, such as induction of eNOS activation through the Akt signaling pathways,[73] implies vascular function can respond rapidly to changes in E_2 levels. We[26] and others[27] have previously demonstrated that E_2-deficient postmenopausal women have reduced endothelial function and greater arterial stiffness compared to age-matched premenopausal women, and several studies, including ours,[29] show that arterial stiffening is attenuated in postmenopausal women on E_2-based HT compared to non-HT users.[28,30,31] We have recently shown that endothelial function is reduced[74] and arterial stiffness is increased (Hildreth et al, in review) across the stages of the menopause transition in healthy women. Moreover, these preliminary data demonstrate that acute ovarian hormone suppression increases arterial stiffness and reduces endothelial-dependent vasodilation in premenopausal women (Figure 1). Much less is known about the effects of E_2 on the intracranial vasculature, although E_2 does appear to enhance cerebral blood flow.[75,76]

2. Innovation. This study proposes an innovative interventional paradigm to study vascular mechanisms underlying the effects of E_2 on cognitive function. In addition to providing new mechanistic insights, this study addresses several shortcomings of previous work that used fMRI to assess changes in brain activation with ovarian hormone suppression. First, we will be better able to determine the effects of E_2 deficiency from those of aging by examining the effects of GnRHa in younger women. Second, we will have a true E_2 add-back condition to isolate the effects of E_2 as opposed to other ovarian hormones or effects of GnRHa itself on cognition. Third, women will be studied after 6 months of ovarian hormone suppression. This longer duration is likely to produce even more pronounced/consistent effects on brain activation. The use of fMRI in this study is resource-intensive. However, fMRI is widely available, requires no ionizing radiation, and is sensitive to early brain changes occurring prior to clinically obvious cognitive impairment. This approach is thus consistent with identifying subtle early changes in cognitive function that would precede clinical symptoms and functional impairment when interventions are most likely to be effective. Further, this study design allows us to glean information from shorter-term studies, rather than studying women over years of E_2 deficiency. This project would not be possible solely within the K23 funding mechanism without Dr Kohrt's collaboration. Enrolling participants from Dr Kohrt's FAME study investigating the bioenergetic and metabolic consequences of the loss of ovarian function in women saves on recruitment and screening costs, as well as the substantial cost of GnRHa treatment. This permits funds from this K23 award to support the fMRI imaging and analysis.

3. Approach

Figure 1. Changes in FMD in response to GnRH antagonist in pre-, early peri-, late peri- and postmenopausal women.

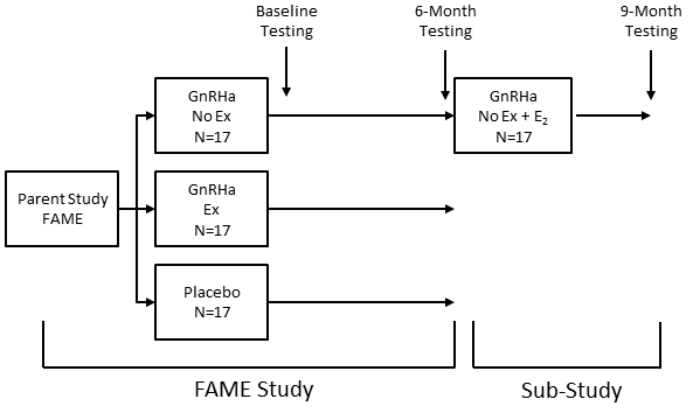


3.a. Preliminary work. Through my work as a lead investigator on Dr Schwartz's R01-supported Pioglitazone Or Exercise to Treat MCI (POEM) study, I have gained substantial experience in running a large complex clinical intervention trial, including recruiting, screening and enrolling a carefully characterized population, and experience with the IRB approval process. From work on the POEM study I have also become comfortable with the assessment of cognitive function in the research setting. I have worked closely with Dr Grigsby on the POEM study, which included several of the cognitive tests to be used in the proposed work. I have 3 first-authored publications on cognitive function (see Biosketch). As part of my previous T32 support, I added vascular measures, including carotid artery compliance and endothelial function, to the POEM study. I have collaborated with Dr Moreau on this aspect of the POEM study, as well as serving as co-investigator on her R01-supported study, Biological mechanisms of arterial stiffening with age and estrogen deficiency. Dr. Moreau and I have co-authored 2 papers on the results of this study (see Biosketch) on endothelial function[74] and arterial stiffening (in review; first author) across the stages of the menopause

transition. We have two additional manuscripts in preparation to be submitted this summer on 1) the role of telomeres; and 2) oxidative stress on vascular function in women with aging and E_2 deficiency. With Drs. Schwartz and Moreau I have published the primary manuscript from the R01-funded Testosterone and Exercise in Aging Men study (PI Dr. Schwartz), and with Dr. Moreau have begun analyzing the vascular data performed in that study. This has included analysis of ultrasound data for the determination of carotid artery compliance that will be used in the proposed study. Developing proficiency in performing and analyzing these studies in addition to brachial-artery mediated FMD is a major goal of this career development award. Although preliminary data is not required for this award, data from Dr. Moreau's work demonstrates significant reductions in brachial-artery FMD in response to 3 days of ovarian hormone suppression with GnRH antagonist (Fig 1, manuscript in preparation; candidate is co-author).

3.b. Methods

Figure 2. Study design.



Subjects. Volunteers will be participants who have been recruited, screened and randomized to participation in the FAME study. Women will be nearing menopause (40-60 yrs) with normal menstrual cycles. Subjects will have had a medical history, physical examination, depression assessment, and clinical laboratory evaluation (complete blood count, comprehensive metabolic panel and TSH). Participants will be excluded from FAME for the following: irregular menstrual cycles, serum FSH>25mIU/mL measured during the first 5 days of the menstrual cycle, pregnant or lactating, on hormonal therapy, known hypersensitivity to leuprolide, score of ≥18 on the Beck Depression Inventory, proximal femur or lumbar spine T-score <-2.0, abnormal vaginal bleeding, thyroid dysfunction, uncontrolled hypertension (>150/90), currently exercising ≥30 min/d >4 d/wk or BMI≥35kg/m^2. Eligible FAME volunteers will be randomized to receive monthly injections of placebo (saline) or GnRHa (leuprolide acetate, 3.75 mg) for 6 mos. GnRHa-treated women are further randomized to endurance exercise training, or no exercise. Prior to beginning the intervention treatment for FAME, volunteers will also be invited to participate in this study of vascular mechanisms for the cognitive effects of ovarian suppression. Participants will be excluded for: Mini Mental State Examination[77] score ≤27, history of neurologic disease or major psychiatric illness, major depressive episode within the past 12 months, history of learning disability, or < high-school education. In order to protect the health and safety of the participants, exclusion criteria for this study will include any contraindication to magnetic resonance scanning (e.g., metal in body, claustrophobia, pregnant). These exclusions are specific to MRI and are consistent with most studies involving MRI. Potential participants will be screened for the presence of any of these exclusion criteria prior to participating in this MRI study.

Volunteers will undergo vascular and cognitive/fMRI testing at baseline and 6 months, corresponding to the beginning and end of the FAME study. Baseline testing will be performed during the early follicular phase (i.e, days 2-6) of the menstrual cycle.

Separate from the parent FAME study, women randomized to GnRHa and no exercise will complete an additional 3 months of GnRHa + transdermal E_2 add-back, with vascular and cognitive/fMRI testing at 9 months (Fig 2).

Sub-study. An additional group of up to 30 late-perimenopausal or postmenopausal women who have had carotid artery compliance and brachial artery FMD performed within the last 6 months will also be enrolled. There is no intervention performed in this group, rather, they will serve as a comparison to the other premenopausal intervention groups. Similar to premenopausal women, late perimenopausal and postmenopausal women will be excluded for: Mini Mental State Examination[77] score ≤27, history of neurologic disease or major psychiatric illness, major depressive episode within the past 12 months, history of learning disability, < high-school education, or contraindications to MRI. Experimental procedures in this group of participants will be limited to cognitive testing, fMRI and apolipoprotein ε4 genotyping.

Experimental procedures

Drug intervention. Volunteers will receive monthly intramuscular injections of placebo (saline) or leuprolide acetate (3.75mg depot suspension; Abbott Laboratories; Abbott Park, IL) for 6 mo. A subset (N=10) of women receiving GnRHa will continue for an additional 3 mo. Absence of pregnancy will be confirmed before each dose. A single injection of leuprolide produces an initial stimulation (~2 wk) followed by a prolonged

suppression of pituitary gonadotropins and ovarian hormone secretion; repeated monthly dosing maintains suppression of ovarian function. Leuprolide has been used clinically for up to 1 yr without serious adverse events, although there may be a decrease in bone mineral density that recovers slowly following cessation of treatment.[78,79] Side effects are those typical of E_2 deficiency including hot flashes, headaches, nausea, emotional lability, decreased libido, acne, myalgias, sleep disorder, reduction in breast size, and vaginal dryness. There may be a development or worsening of depression and the occurrence of memory disorders. In studies of women with uterine fibroids, GnRHa 3.75 mg/mo induced amenorrhea in 61%, 86%, and 90% of women after 1, 2, and 3 mo of treatment, respectively.[80,81] Return of menses typically occurred within 2-3 mo of cessation of therapy. Injections will be administered in a blinded manner, but participants on GnRHa may know their assignment because of the induction of amenorrhea and menopausal symptoms. Subjects will be instructed not to discuss their assignment with the outcome assessors. Subjects receiving an additional 3 mos of GnRHa in this sub-study will also receive weekly transdermal 17β-E_2 add-back therapy (0.075 mg/d) and 1 cycle of MPA (5mg/d x 12 d at week 6 of E_2 add-back). The rationale for the MPA is to prevent break-through bleeding, and thus enhance participant retention, and to prevent potential endometrial hyperplasia, although this is unlikely to occur during the 3-mo intervention. Additionally, we are interested in isolating the effects of E_2 alone and not in combination with MPA because of the potential antagonizing effects of MPA on E_2 action[56] and because MPA has been shown to have negative effects on cognition.[19,47,48] Thus, although technically a combination HT intervention, the add-back is E_2-based, with minimal progesterone exposure. The first 6 months of drug intervention will be done as part of the FAME study, thus there will be no cost to this proposed sub-study during this period. Study drug for the 3-month E_2 add-back portion unique to this sub-study will be paid for by the sub-study (Figure 3).

Brachial artery FMD. Brachial artery FMD and endothelium-independent dilation will be performed as previously described,[82-84] and will conform strictly to published guidelines for assessing FMD in human subjects.[85] The dilation of the brachial artery in response to the stimulus of forearm ischemia has been shown to be dependent on the release of NO from the vascular endothelium.[86] Brachial artery diameter and flow velocity will be acquired by ultrasound (GE Vivid i) and analyzed using commercial software (Vascular Analysis Tools 5.5.1, MIA). Endothelial-independent dilation will be determined by measuring the dilation response to sublingual nitroglycerine (0.4 mg). Blood pressure will be measured prior to the FMD. All images will be coded by number and blinded to group assignment. Placement of the ultrasound probe will be measured with a tape measure and landmarks identified (e.g., branch-points and veins) to ensure that the same location of the brachial artery is analyzed. In Dr. Moreau's laboratory, the coefficient of variation **(CV)** and intra-class correlation coefficient **(ICC)** for trial-to trial reliability measured in 10 individuals for baseline brachial artery diameter, peak diameter and FMD (%) were 2% and 0.97, 1.5% and 0.99, and 2.2% and 0.99, respectively.

Carotid artery compliance. Common carotid artery diameter will be measured from the images derived from a GE Vivid i ultrasound machine equipped with a linear array transducer as previously described. Carotid diameter images will be analyzed using software (Carotid Analyzer) as previously described.[87] A longitudinal image of the cephalic portion of the carotid artery will be acquired ~1 cm distal to the carotid bulb. Time points that correspond with systolic expansion (within 60ms of the ECG T-wave) and basal diastolic relaxation (onset of the ECG R-wave) will be selected. The distance (diameter) between the vessel far-wall boundary (lumen/intima interface), and a near-wall boundary (adventitia/media interface), will be measured. Similarly, far wall intimal-medial thickness will be measured at end diastole as previously described.[88] The pressure waveform and amplitude will be obtained from the common carotid artery using arterial applanation tonometry, a high-fidelity strain gauge transducer (Millar Instruments), as previously described. This tonometer has been shown to register a pressure wave with harmonic content that does not differ from that of an intra-arterially recorded wave, and the use of the tonometer on an exposed artery records a waveform identical to that recorded intra-arterially. The combination of ultrasound imaging of the common carotid artery with simultaneous applanation tonometric-obtained arterial pressure waveforms from the contralateral artery permits noninvasive determination of carotid artery compliance. The CV and ICC for trial-to trial reliability measured in 13 individuals for carotid artery diameter, carotid artery distention, pulse pressure and carotid artery compliance were 0.7% and 0.99, 4.2% and 0.99, 3.7% and 0.97, and 3.1% and 0.99, respectively.

Pulse wave velocity. Arterial stiffness will be measured using the combination of high-resolution ultrasonography and applanation tonometry in a large elastic artery in the central circulation (carotid) [87]. Indirect measures of central arterial stiffness (pulse-wave velocity [PWV], pulse wave analyses [PWA]) will be performed using the SphygmoCor XCEL (Atcor) system [89]. PWV is measured using a blood pressure cuff placed on the upper thigh and applanation tonometry of the carotid artery in the neck[89]. PWA provides a measurement of central aortic blood pressure and augmentation index by placing a blood pressure cuff over the brachial artery in the upper arm [89]. The

brachial artery waveform is analyzed by the SphygmoCor to provide a central aortic waveform, and central blood pressure measurements and augmentation index. Although PWV is an indirect measure of arterial stiffness, it has been a gold standard in the literature, including studies of arterial stiffness and cognitive function.

fMRI measurements. The scanning portion of the study will take place at the Intermountain Neuroimaging Consortium at the Center for Innovation and Creativity at 1777 Exposition Dr., Boulder, CO 80301. The MRI device for these scans is FDA approved for research with human subjects and has all the safety inherent in a clinical MRI scanner. The radio frequency fields conform to guidelines determined by the FDA and the FDA has designated MRI scanners to be a non-significant risk device. MR techniques non-invasively produce images and measurements from tissues in the intact, living human.

Following the informed consent process, but prior to going into the MRI scanner, the MRI technologist on duty will ask participants to remove all jewelry and metal objects from their pockets. Participants will be required to change into scrubs to prevent any possible risk from metallic objects or decorations in their clothing.

In an MRI scan the subject lies down on a table and is placed into a long donut-shaped magnet. A specially designed coil will be placed around the head to provide better images (as is done with standard clinical examinations). As the MRI scan is performed, the subject will hear loud rapping and knocking noises that are normal for a MRI scan.

Because the cognitive challenge of a working memory task has been shown to reveal differential patterns of activation for young versus older individuals [90] and sensitivity to acute ovarian suppression,[91] such a task will be employed in the current study. In addition, as differences between groups may only become apparent when load is varied,[90,91] 3 different size memory loads will be employed.

During the MRI scan a standard N-back test (Perlstein, 2003) will be administered to participants through a projector screen viewable from inside the scanner bore and using button boxes to respond. Participants will use the index fingers of each hand to indicate either a “target” or “non-target” response. In the 0-back condition, the target is any letter that matches the pre-specified “target” letter (i.e., “c”). In the 1-back condition, the target is any letter identical to the letter immediately preceding it (i.e., the letter presented one trial back). In the 2-back condition, the target is any letter that was identical to the one presented two trials back. Stimuli are pseudorandom sequences of letters presented in a fixed central location using E-Prime presentation software (Psychology Software Distribution, York, U.K.). Stimuli are presented for a 920-ms duration with a 1380-ms interstimulus interval. Participants complete 12 trials in each block and complete a total of 9 blocks (three blocks of each of the three conditions). A short break (5–20 s) between every 3 blocks is provided to allow participants to rest and collect baseline brain activity. Prior to the start of the actual task, participants are trained on each of the three conditions. Participants are practice with three types of feedback on their performance (passive viewing, immediate feedback, and feedback at the end of block) per condition, until they demonstrate that they understand the task and their performance stabilizes. Reaction times and accuracy measures are obtained for each trial. The total task will be divided into three fMRI runs of around 10 minutes. In addition, a high-resolution structural scan will be obtained (6 min) as well as a resting-state scan that will allow for investigation of functional connectivity (6 min).

Images will be acquired on a Siemens 3 Tesla Tim Trio MRI system. Functional images will be acquired with BOLD contrast using a standard T2-weighted gradient-echo echo-planar imaging (EPI) technique angled parallel to the AC-PC line, while high resolution anatomical images will be obtained with a T1-weighted full head 3 dimensional MPRAGE 5 echo sequence (IPAT built-in) in the sagittal plane. The rationale for using fMRI is that this neuroimaging modality avoids ionizing radiation (e.g. with PET or SPECT) yet allows localization of activity (vs. EEG). fMRI is widely available and has been used in earlier studies, facilitating comparison with previous work.

Cognitive function. Standard cognitive tests that have demonstrated sensitivity to E_2[15,60,92,93] will be administered by a trained psychometrician in a private setting. The battery takes approximately 1 hour to complete and consists of the following tests (see also Appendix A):

1) Rey Auditory Verbal Learning Test[94]
2) Wide Range Achievement Test 4-reading[96]
3) Trail Making Test A and B[98]
4) Digit Span Forward and Backward[95]
5) Controlled Oral Word Association Test[97]
6) Stroop Color Word Interference Test[99]

This battery is not sufficient for comprehensive cognitive evaluation or clinical diagnosis. Nonetheless, any subject that meets pre-specified cut-points for potentially significant cognitive impairment will have her results reviewed by Dr. Grigsby and be referred to her healthcare provider for further evaluation.

Laboratory Measures. Fasting plasma concentrations of glucose, insulin, total-, HDL- and LDL-cholesterol, triglycerides, estrone, estradiol, total testosterone, progesterone, DHEAS, albumin, SHBG and hemoglobin A1c will be measured at baseline and 6 months as part of the FAME study. These measures will be repeated at 9 months in the E_2 add-back group with costs for those 9-month assays covered by the sub-study. All assays will be performed by the University of Colorado CCTSI CTRC core laboratory. Genotyping for the apolipoprotein Cys112Arg polymorphism (i.e., APOEε-4 allele) will be performed by Dr. Christina Aquilante's laboratory at the University of Colorado using polymerase chain reaction (PCR) amplification and pyrosequencing. Investigators will remain blinded to genotype and results will not be revealed to participants as this does not provide clinically relevant information at this time. The genotyping will be done as APOE genotype is an important determinant of risk for cognitive impairment. This test is not part of the parent FAME study and will be covered by the sub-study. Figure 3 shows which interventions, tests and procedures will be done as part of the FAME study and which will be done as part of the sub-study.

Figure 3. Interventions, tests and procedures

Intervention	Baseline to 6-months	6-months to 9-months
GnRHa	FAME	Sub-study
Estradiol	N/A	Sub-study
MPA	N/A	Sub-study

Test/Procedure	Baseline	6-months	9-months
APOE Genotype	Sub-study	N/A	N/A
Hormone levels	FAME	FAME	Sub-study
Metabolic measures	FAME	FAME	Sub-study
Vascular measures	Sub-study	Sub-study	Sub-study
Cognitive measures	Sub-study	Sub-study	Sub-study
fMRI	Sub-study	Sub-study	Sub-study

Questionnaires. The following questionnaires will be administered at each of the 3 monthly Lupron injection visits for those subjects participating in the estrogen add-back portion of the study:

1. Menopausal Symptom List[100]. This questionnaire records the frequency and severity of menopause-related symptoms, including vasomotor, psychological and general somatic symptoms.
2. Pittsburgh Sleep Quality Index[101]. This questionnaire monitors sleeping behavior.

We will also obtain a reproductive/pregnancy history using a questionnaire. Because pregnancy complications have been associated with increased cardiovascular risk, data from this questionnaire will support further investigation into whether these factors contribute to vascular aging in women.

3.c. Analysis Plan and Sample Size Justification (prepared by Pamela Wolfe, MS and Kim McFann, PhD)

fMRI analysis: Standard image pre-processing will be conducted with the FMRIB Software Library (FSL; http://www.fmrib.ox.ac.uk/fsl/index.html), including motion correction with MCFLIRT, brain tissue extraction with BET, spatially smoothing with a Gaussian kernel (FWHM = 8 mm), mean-based intensity normalization, and high-pass temporal filtered to remove low-frequency noise. Data from any individual with more than 2mm motion in any direction will be discarded. Statistical analyses will be conducted with FMRIB's improved linear model convolved using a double-gamma hemodynamic response function. For comparisons across individuals, parameter and variance estimates for each participant's data will be registered to Montreal Neurological Institute standard stereotaxic space (MNI152) with the two-stage registration procedure implemented in FMRIB's Linear Image Registration Tool. The FMRIB's Local Analysis of Mixed Effects (FLAME 1+2) will be used to model the mixed-effects variance for each contrast of interest, taking into account both fixed effects (e.g., hormonal status) and random effects. Statistically-defined clusters will be considered significant, if they exceeded a specific voxel-wise threshold (e.g., $p < 0.0025$, $Z=3.02$) and consisted of at least the required number of contiguous voxels to correct for a whole-brain error rate at $p < 0.05$ as determined by the AlphaSim algorithm within Analysis of Functional NeuroImages (AFNI; Cox, 1996). Based on group differences in the relevant studies described above we expect that our sample of 17 individuals per group will be adequate. Specifically, Desmond and Glover[102] found that with a liberal threshold of 0.05, about 12 subjects are required to achieve 80% power at the single voxel level for typical activations. Our sample size will be 42% larger.

Vascular measures: Two-sample *t* tests with an alpha = 0.05 will be used to compare change from baseline in FMD and carotid artery compliance at 6 months between GnRHa and placebo-treated women. Previous work from Dr. Moreau's laboratory measuring FMD before and after ovarian hormone suppression in 22 premenopausal women found a change of 2.8±1.9% (unpublished data). For FMD, 17 subjects/group give 80% power to test the null hypothesis of no difference against the alternative of a meaningful 2 ± 2% change[26,29,103]. For carotid artery compliance, 17 subjects/group also give 80% power to test the null hypothesis of no difference against the alternative of a meaningful 0.25 ± 0.25 unit change[26,29,103]. We will also perform ANOVA,

using baseline measures as a covariate and comparing the means of the two groups. The effect of E_2 add back will be evaluated with a paired *t* test using data from 17 volunteers receiving an additional 3 months of GnRHa with E_2 add-back; at an alpha =0.05 level, there will be >80% power to detect a meaningful 1 SD change in either FMD or compliance.

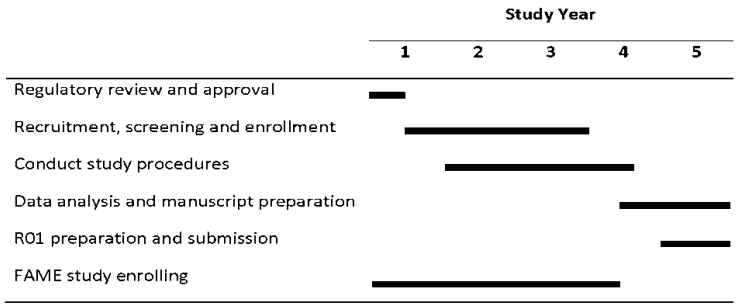
Correlation among changes: To address hypothesis 1b, we will estimate the correlations of changes in PFC activation with changes in arterial stiffness and changes in endothelial function over the first 6 months of intervention. With N=17 (assuming the placebo data will add little to an evaluation of change), a Pearson's correlation coefficient of 0.5 will be just significant; that is, power is just over 50%.

Laboratory measures: The hormone levels will be used to demonstrate ovarian suppression with GnRHa, and subsequent increase in E_2 levels with the add-back, and to adjust for day-to-day variability in E_2 levels between vascular and cognitive/fMRI testing. Lipids and other metabolic measures are viewed as potential covariates affected by E_2; this will be evaluated by backward elimination, regressing the change in PFC activation, arterial stiffness, or endothelial function on an indicator for treatment and the potential covariates.

Measures of cognitive function: Although we are not powered to detect clinically meaningful changes in these measures, we are interested in any changes in performance with ovarian suppression or E_2 add-back. Suggestive trends will inform hypothesis generation and design of future studies.

3.d. Proposed Timeline. See Fig 4.

Figure 4. Proposed Timeline



3.e. Mentors. Mentors' research expertise and responsibilities under this proposed career development award are described below.

Dr. Robert S. Schwartz , Senior faculty mentor. Dr. Schwartz will supervise the proposed career development and research. As Division Head of Geriatrics, he will ensure Dr. Hildreth can devote at least 75% of her time to activities directly related to this proposed career development application. He will assist her development into an independent investigator by mentoring her in clinical investigation, supporting her in obtaining needed resources, assisting in the development of new collaborations, and supporting her academic advancement and promotion.

Dr. Kerrie L. Moreau, Co-mentor. Dr. Moreau will mentor Dr. Hildreth in the acquisition and analysis of the vascular measures to be used in this study, so that she is able to perform these measures independently. Dr. Moreau will assist in the analysis and reporting of findings from the proposed study and provide guidance on future grant applications. With Dr. Schwartz, she will also provide mentorship in professional development to promote the candidate's successful transition to an independent research career.

Dr Marie T. Banich, Consulting mentor. Dr. Banich will supervise the neuroimaging and fMRI analysis at her laboratory in Boulder, and oversee the candidate's training in neuroimaging and assessment of executive function in clinical research. Dr. Banich will assist Dr. Hildreth in data interpretation and preparation of manuscripts, and provide guidance as she prepares her R01 application toward the end of the award period.

Dr Jim Grigsby, Consulting mentor. Dr. Grigsby will oversee the administration and interpretation of the group cognitive testing data. He will review any profiles that meet pre-specified criteria for potentially significant impairment, and discuss these results with participants and their health care providers. He will assist the candidate in understanding the attributes and uses of specific cognitive tests, such that the candidate will be able to effectively work with neuropsychologists to develop appropriate cognitive testing batteries for future research.

Dr Wendy M. Kohrt, Consulting mentor. Dr. Kohrt will provide scientific advice and infrastructural support (e.g., access to study population) to leverage the limited research funds available through the K23 Career Development Award. In addition to specific scientific mentorship, Dr. Kohrt will provide guidance on general clinical research issues (e.g., study design, randomization, recruitment and adherence, unbiased outcomes assessment, data and safety monitoring) and on career advancement.

3.f. Limitations. Enrolling participants from the FAME study is a major strength that permits the majority of the K23 resources to support the cost of fMRI imaging and analysis, yet it imposes additional subject burden. To

minimize subject burden, we have limited testing to 1 hr each of fMRI, cognitive and vascular testing. We acknowledge the challenges of enrolling sufficient numbers of participants from FAME to complete the proposed work. Dr. Kohrt has a long history of successfully recruiting and retaining participants in clinical intervention trials. Her ongoing NIH-funded POWER study has randomized 80 women to 5 months of ovarian hormone suppression or placebo. During this study of similar intensity and time commitment to FAME, 31 of 40 women invited agreed to participate in an ancillary study requiring a fat biopsy, and 17 of 23 participated in a study that required a blood draw and pelvic examination. Of 80 women randomized, 9 have dropped and only one drop was due to side effects of GnRHa. The FAME study is early in its recruitment. Target enrollment is 24 in each of the 3 arms (placebo, GnRHa and GnRHa + exercise) and there are currently no other ancillary studies. We are thus confident we can enroll sufficient numbers from the parent study. The candidate will apply for additional pilot funding through the SCOR pilot, CCTSI Co-Pilot, and Center for Women's Health award mechanisms to support the enrollment of additional subjects if needed. In this event, she would be able to utilize the existing recruiting mechanisms and resources from the SCOR grant.

This model of ovarian hormone suppression does not mimic the gradual process of natural menopause. However, menopause occurs at a time when women are also beginning to experience other age-related physiological changes, making it difficult to isolate the effects of aging from those of ovarian failure. Because this is a mechanistic study, this model strengthens the design to allow us to effectively study how changes in arterial stiffness and endothelial function due to loss of ovarian function may mediate changes in cognition. Ideally we would have a placebo group during the add-back portion of the study; due to resource constraints and subject burden this was not included. To preserve blinding, cognitive testing and fMRI analysis will be performed by personnel blinded to subject group. Ideally we would measure changes in cerebral arterial compliance in response to ovarian hormone suppression, but these methods have not been developed. The measures of peripheral vascular function proposed for this study are non-invasive, well- validated, and have been correlated with white matter changes in the brain.[32]

Participant burden, location and scheduling of equipment preclude performing the vascular and fMRI testing on the same day. Although this raises the possibility of day-to-day variability affecting our results, this will be minimized by testing all participants during the early follicular phase (i.e., days 2-6) of the menstrual cycle at baseline. Testing for visits 2 and 3 (for subset receiving add-back) will be done while participants are receiving GnRHa. We will measure E_2 concentrations at each visit to allow us to adjust for differences in hormone levels and tests will be scheduled at the same time of day. Data have shown moderate to strong reliability of fMRI BOLD signal changes and cardiovascular reactivity when tested simultaneously on two occasions 3 months apart.[104]

Changes in patterns of brain activation may not translate into detectable declines in performance on cognitive tests in this young healthy population, and the study would be underpowered to detect such changes. However, data from the cognitive test battery will be informative and any trends observed with GnRHa treatment and E_2 add-back will help inform future studies. Due to resource constraints and subject burden we have limited our assessment of cognitive function to examination of the PFC and tests of executive function/working memory. This approach is based on the existing literature supporting the PFC as the site of E_2 mediation of executive function however, other brain regions and cognitive domains may also be affected by the intervention and could be explored in future studies. It has been suggested that the BOLD response may not accurately reflect brain activity in individuals with existing cerebrovascular pathology;[105] this is unlikely to be problematic in our relatively young, healthy volunteers. The use of fMRI is a new direction for the candidate; key mentorship in this area will be provided by Dr. Banich, a recognized expert in the use of fMRI in clinical studies, and by incorporation of formal training in neuroimaging into the career development plan. As research increasingly moves toward detection of preclinical changes in brain activity and cognition, the candidate's ability to incorporate neuroimaging into future research is a major strength of this application.

We are proposing to study one possible mechanism for the effects of E_2 on cognition. It is plausible that E_2 acts through other mechanisms, particularly with the longer duration proposed in this study. Additional mechanisms may include direct effects on beta-amyloid accumulation and deposition, tau protein hyperphosphorylation, mitochondrial actions on brain activation, antioxidant properties, and effects on insulin sensitivity, body composition and lipids. It is also possible that other ovarian hormones (e.g. progesterone, testosterone, DHEA) may have important effects on cognitive function. Likewise, other aspects of GnRHa treatment, including hot flashes, and effects on sleep and mood may affect cognition. Data on menopausal symptoms, sleep quality and depression as well as hormone levels, lipids, insulin sensitivity and body composition will be collected as part of the FAME study allowing us to account for these potential covariates.

3.g. Interpretation and future directions

Specific Aim 1 will determine the effects of ovarian hormone suppression on PFC activation, arterial stiffness and endothelial function. Previously published and preliminary data suggest we will observe the hypothesized negative effects on brain activation and vascular function in response to GnRHa treatment. If changes in PFC activation and vascular function are correlated as we hypothesize, this would lead us to investigate whether other therapeutic or lifestyle interventions that target vascular function (i.e. exercise) could counteract the negative effects of ovarian suppression. Future studies could also measure intra-cranial blood flow and compliance, structural brain changes or brain expression of E_2 receptors to more closely examine effects on the brain. If changes in PFC activation are not correlated with changes in vascular function, this would lead us to investigate other possible mechanisms for the negative effects of ovarian hormone suppression on cognition (e.g. inflammation, oxidative stress, amyloid accumulation, tau hyperphosphorylation).

Specific Aim 2 will isolate the effects of E_2 on PFC activation, arterial stiffness and endothelial function. If E_2 add-back restores PFC activation and vascular function as hypothesized, this would support a role for E_2 and further investigations into mechanisms and interventions to counteract the effects of E_2 deficiency. If PFC activation and vascular function are not restored, or only partially restored, this would lead us to investigate the contributions of other ovarian hormones or effects of GnRHa.

Protection of Human Subjects
The proposed study meets the definition of a clinical trial in that it is a prospective biomedical research study of human subjects that involves random assignment to pharmacologic and behavior intervention groups. However, we consider it to be clinical research on a small number of subjects, rather than a 'clinical trial' because it is designed to reveal mechanistic consequences of the loss of ovarian function. It is not a Phase III clinical trial.

1. Risks to Human Subjects

a. Human Subjects Involvement and Characteristics.
We will enroll a total of 51 subjects in this study. Subjects will be healthy women 40-60 years of age who are enrolled in the Females, Aging, Metabolism and Exercise (FAME) study (Dr. Wendy Kohrt, PI). For detailed subject characteristics, inclusion and exclusion criteria, please see the Research Strategy, section 2.c.2.

b. Sources of Materials.
The research materials that will be collected from human subjects include:
- medical records when necessary to follow up on health status
- blood and tissue samples for screening and for study-specific outcomes, as described in the research strategy
- data from all the study procedures, as described in the research strategy

The identity of subjects and the data acquired as a result of this study will be treated with professional standards of confidentiality. All HIPAA guidelines for the protection of health information will be followed. Only members of the research team will have access to protected health information (PHI) collected from the volunteers. Confidentiality of electronic data will be maintained by using only patient identification numbers for data entry. The master document linking patient names with identification numbers will be maintained in a separate password-protected file with restricted access. Paper records will be kept in individual study charts that are stored either in a locked cabinet or in a locked office. Data records will be maintained for at least 7 years after the publication of results. At that time, the data records may be destroyed by the PI by deleting them from electronic media and by shredding paper documents, but some of the data records may be retained indefinitely. All members of the research group have individual computers that are part of the institution network with institutional oversight of security. Files that contain data that could jeopardize blinding to treatment code are password-protected to restrict access. Sharing of data with investigators or monitoring personnel outside the institution is typically facilitated by electronic transfer, in a manner that is compliant with HIPAA regulations.

c. Potential Risks.
- Vascular measures (brachial artery FMD, carotid artery compliance and pulse wave velocity). There are no known risks to participants associated with these procedures. Some participants may have discomfort in the hand (pins and needles) during the cuff occlusion portion of the FMD measurement.
- Functional magnetic resonance imaging (fMRI) measurements. The magnetic field of the MR environment has the potential to cause burns or bodily injury if ferrous metal objects are implanted in the body, or if personal articles containing ferrous material are brought into the environment.The risk of MRI to pregnant women and fetuses is currently unknown. The MRI may cause discomfort due to scanner noise. There may be some discomfort from lying still and in one position for a long time. At sufficient exposure levels, peripheral nerve stimulation is perceptible as "tingling" or "tapping" sensations. PNS symptoms will usually subside shortly after the scan is completed. Participants may feel nervousness or feelings of claustrophobia.There is a risk that the image will reveal an observation concerning an individual research participant that has potential clinical importance but is beyond the aims of this protocol. In the event of the confirmation of a significant anomaly in a participant's brain image, this information will likely be distressing to the participant.
- Cognitive testing. Subjects may experience frustration or anxiety during the testing session. It is possible that this testing could reveal previously unrecognized significant cognitive impairment that could be distressing to the subject.
- Study drugs. As participants in the FAME study, all participants in this proposed research will be randomized to receive placebo (saline) or GnRHa (leuprolide acetate) 3.75 mg/mo by intramuscular injection every month for 6 months (6 doses), plus or minus exercise. Women randomized to GnRHa and no exercise will receive 3 additional monthly doses after completion of the FAME study, for a total of 9 doses. During these 3 additional months, these women will also receive weekly transdermal 17β-estradiol 0.075 mg/d (12 doses), and oral medroxyprogesterone acetate 5mg/day for 12 days (12 doses) at the beginning of week 6 of estradiol therapy to minimize breakthrough bleeding and thereby enhance

participant retention.

Leuprolide acetate

Contraindications for use include: hypersensitivity to GnRH, GnRH agonist analogs or any of the excipients in Lupron Depot; undiagnosed vaginal bleeding; known or suspected pregnancy; lactation.

In women who are pregnant, use of leuprolide acetate may cause fetal abnormalities.

Leuprolide acetate may cause an anaphylactic reaction in volunteers with hypersensitivity to GnRH.

Regular menstruation should stop during leuprolide acetate therapy but spotting or breakthrough bleeding may occur. Cessation of menses does not ensure that pregnancy will not occur. Normal menstrual function is usually restored within 2-3 months after therapy is discontinued.

Leuprolide acetate may cause symptoms related to hypoestrogenism including hot flashes, headaches, nausea, emotional lability, decreased libido, acne, myalgia, sleep disorder, reduction in breast size, and vaginal dryness. Bone loss may occur, although the amount lost over the 24-week intervention should not be clinically significant and should be recovered slowly after discontinuation of leuprolide acetate treatment. There may be a development or worsening of depression and the occurrence of memory disorders.

Leuprolide acetate therapy for 24 weeks may cause an increase in fat mass (1-2 kg), a decrease in fat-free mass (1-1.5 kg), an increase in total, LDL, and HDL cholesterol (5-10%), and an increase in triglycerides (15-20%).

Local injection site reactions including induration and abscess may occur.

Transdermal 17β-estradiol

Contraindications to use include: hypersensitivity to estradiol or any of the components in the transdermal formulation; arterial thromboembolic disease; breast cancer; deep vein thrombosis; pulmonary embolism; estrogen-dependent neoplasia; undiagnosed vaginal bleeding; liver disease; pregnancy; or thrombophilic disorder. Transdermal estradiol may cause irritation at the application site, edema, fluid retention and weight gain, bloating, nausea, vomiting, headache, depression, breast tenderness and withdrawal bleeding. Rare but serious side effects include heart disease, myocardial infarction, breast cancer, hypercalcemia, gallbladder disease, venous thromboembolism, anaphylaxis, stroke, endometrial cancer, ovarian cancer, pulmonary embolism and breast cancer.

Medroxyprogesterone acetate

Contraindications to use include: hypersensitivity to progesterone or any of the ingredients, breast cancer, liver disease, estrogen or progesterone-dependent neoplasia, pregnancy, thromboembolic disorders, cerebral vascular disease, and undiagnosed vaginal bleeding. Oral progesterone may cause weight gain, abdominal pain, dizziness, headache, nervousness, amenorrhea, menstrual spotting, reduced libido and fatigue. Rare but serious side effects include anaphylaxis, decreased bone mineral density and fracture of bone.

- Venipuncture. There is a small risk of local hematoma, infection, and thrombosis associated with intravenous blood sampling.
- Confidentiality and privacy. The use of questionnaires, interviews, and collection of personal medical information poses a risk to confidentiality and privacy and may cause embarrassment.

2. Adequacy of Protection Against Risks

a. Recruitment and Informed Consent.

Subjects will be recruited from the FAME study. After volunteers complete screening and are determined to be eligible for the FAME study, they will be invited to participate in this sub-study. Interested participants will meet individually with a member of the research team to review all aspects of the study and consent form in a private, quiet environment. Volunteers will be encouraged to ask any questions they may have regarding the study and what is expected of them should they choose to participate. A description of the MRI procedures is included in the consent form, and all participants will be given a verbal description of the MRI screening process and potential risks of MRI studies.The member of the study team obtaining consent will stress that: 1) all research participation is voluntary, 2) subjects may withdraw at any time, and 3) the decision to participate or not participate will not affect the subject's medical care or any benefits to which the subject is entitled. They will further be asked to explain the consent form in their own words to ensure understanding of the study and what is expected of them. The consent form details the experimental protocol and potential risks, and must be signed prior to any participation in the study. The protocol and consent forms will be approved by the Colorado Multiple Institution Review Board (COMIRB) and the CTRC. The CTSA at the University of Colorado AMC has a designated officer for review of human subjects protections for research utilizing CTSA resources. Each volunteer will be given a copy of the signed consent form; the original will be kept in the subject's study file. In compliance with HIPAA, each participant will sign the institutional COMIRB/HIPAA form: Authorization to Use or Release Health Information for Research Purposes. This form describes all information that will be obtained

for research purposes, and who will have access to this information. All subjects must have the capacity to understand the nature of the study and potential risks involved in the study. The purpose and procedures for each study test will be reviewed with the subject prior to its administration to ensure subjects understand what is being asked of them, and agree to proceed.

b. Protections Against Risks.

- Vascular measurements (brachial artery FMD, carotid artery compliance, and pulse wave velocity). There are no known risks to participants associated with these procedures. If a participant has significant discomfort or pain with the cuff occlusion for the FMD measurement, the procedure will be stopped.
- fMRI measurements. This protocol will be performed using an MR scanner employing pulse sequences and hardware that have been approved by the FDA for human clinical use. The field strength is 3 Tesla and all relevant operating characteristics (RF power deposition, rate of change of the field gradients, coil design) fall within the limits of FDA guidelines for NMR exposure. Participants will be carefully screened to exclude those who may have metal in or on their bodies that cannot be removed (e g., bullets, metal filings, body piercings, etc.). MR Facility rules strictly forbid staff from entering the magnet room carrying metal objects. The risk of claustrophobia is minimized by screening subjects for self-reported claustrophobia and making sure the subject is lying comfortably with head and neck supported and providing ear protection with headphones, a mirror to see out, a button to signal distress, and an intercom. Scan time will be kept to a minimum. If they are unsure about whether or not they may be pregnant, female participants will be given the opportunity to complete a urine pregnancy test immediately before the scanning period, and those with a positive result will not be scanned. With regard to PNS, participants are given a squeeze ball to use in case of an emergency. They are informed that if they experience PNS related sensations or are otherwise uncomfortable, they can alert the MRI technologist via the squeeze ball and the technologist will stop the scan immediately.

  In the case of an anomalous finding in a brain image, the following procedure is followed:

  1. The technologist and/or research personnel flag potential abnormalities.
  2. The MRI technologist notifies the INC Director of Operations, the MRN Director of Research and Clinical Operations, and the P.I.
  3. The scan gets queued to the radiologist worklist in COINS. All cases of suspected incidental findings are sent for formal neuroradiologic review at MRN.
  4. The radiology review contains a written summary of the findings and classifies the referral status into one of these categories:
     - There is not enough information from the MRI scan to complete a full review. No obvious abnormalities found.
     - MRI shows nothing obvious that needs medical attention.
     - MRI shows something that may or may not be of medical concern. Participants should consider discussing the enclosed report with their doctor.
     - MRI shows something that needs to be brought to the attention of your doctor. Participants may also be contacted by the study team and/or MRN Medical Director about this report.
  5. The PI will get an electronic copy of the radiology review (coded via URSI) as soon as the review is completed. If an urgent referral is recommended, the PI should discuss the review with the Medical Director prior to contacting the participant.
  6. If a referral is recommended, the PI will contact the participant and explain that an unusual feature was observed in their scan. The PI provides the contact information for the Medical Director who reviewed the image (this information is in the letter mailed to the participant as well). Routine referrals are handled on a case by case basis and up to the PI/Medical Director to determine if the participant should receive a call in advance.
  7. All cases reviewed will generate a formal radiology report, which is printed on letterhead and a copy of which is mailed to the participant. In the case of an urgent referral, someone from the study team or the Medical Director will contact the participant prior to the letter being mailed.
- Cognitive testing. Testing will be conducted in a dedicated, private, quiet room by an experienced psychometrician. Any subject who expresses distress or discomfort with the testing may elect to take a break, or stop the testing. Subjects meeting pre-specified cut-points on any of the cognitive tests that may indicate clinically significant cognitive impairment will be flagged for review by Dr. Grigsby. Dr. Grigsby would discuss the results with the volunteer and her health care provider to ensure appropriate follow up and clinical evaluation. We have obtained a Certificate of Confidentiality from the NIH to provide an additional level of protection for the cognitive tests.

- Study drugs. The risks associated with use of the study drugs will be minimized by enrolling participants who do not have contraindications for their use. All participants will have been screened for contraindications to GnRHa through the FAME study. Participants will be observed for 30 min after drug injection to monitor for new hypersensitivity reactions. Absence of pregnancy will be confirmed before injections are administered. Women will be instructed that they should not become pregnant while taking study drugs because of risks to the fetus. They will be instructed that cessation of menses may or may not occur during the study and that cessation of menses does not provide protection against pregnancy; contraception (not hormonal) must be used.
- Venipuncture. The risks of hematoma and infection are minimized by having trained personnel perform the procedures using sterile techniques**.**
- Confidentiality and privacy. These risks will be minimized by not including personal identifying information on the forms, when possible, and by conducting interviews and collection of personal information in a private setting.

Evaluation of study-related events
Participants will meet with a study clinician every 4 weeks to review a Health Status Questionnaire that the participants will complete. The questionnaire queries about changes over the past 4 weeks in medications, health status and concerns with study medications. The clinician will specifically note on the form whether there are any concerns that are possibly, probably, or definitely related to the study. Participants will also be instructed to report concerns that may be study related when they occur to Dr. Hildreth. Dr. Hildreth will initiate event reports for both the programmed and spontaneous complaints.

3. Potential Benefits of the Proposed Research to Human Subjects and Others

The potential benefits to an individual participant in the study are not known. All participants will gain information about their general health status and vascular function, although the results of the vascular testing and imaging have limited relevance outside of the research setting. Cognitive test results likewise are for research purposes only and no clinical interpretation will be provided unless significant impairment is suspected. The potential benefits of the study to women in general could be significant, including information about the loss of ovarian hormones on vascular function and cognition. This knowledge could lead to new interventions to prevent or delay cognitive decline.

4. Importance of the Knowledge to be Gained

Results from this study will add to the scientific knowledge of vascular contributions to cognitive impairment, specifically possible vascular mechanisms – arterial stiffness and endothelial dysfunction – that may underlie the effects of estrogen on cognitive function in women. This information will be important in informing future studies of therapeutic or lifestyle interventions to prevent or delay cognitive decline in the aging population.

5. Data and safety monitoring plan

The NIH requirements for a Data and Safety Monitoring Board (DSMB) are as follows (from the SF424 guide): “NIH specifically requires the establishment of Data and Safety Monitoring Boards (DSMBs) for multi-site clinical trials involving interventions that entail potential risk to the participants, and generally for Phase III clinical trials. Although Phase I and Phase II clinical trials may also need DSMBs, smaller clinical trials may not require this oversight format, and alternative monitoring plans may be appropriate.” As discussed above, the proposed study is viewed as mechanistically driven clinical research but not a ‘clinical trial.’ In this context and based on the NIH DSMB recommendations, we propose that safety monitoring can be performed by the research team, with oversight by an independent local Safety Officer. During the first 6 months of this study which coincide with subjects’ participation in the FAME study, the Safety Officer will be monitoring the safety of the vascular studies, fMRI studies and cognitive testing only, as any GnRHa-related safety issues will be monitored by the FAME Safety Officer or DSMB.

Roles and responsibilities of the Safety Officer
The Safety Officer will: 1) monitor recruitment, enrollment, and adherence of study participants; 2) approve criteria for modifying or discontinuing the drug interventions for individual subjects; and 3) review serious adverse events (SAEs). Any data sets that the Safety Officer may ask to review will be prepared by a biostatistician. The Safety Officer will remain blinded to treatment status unless, for safety reasons, it is decided that knowledge of the treatment code is important.

The objectives of the Safety Officer will be to assess the safety of the interventions and to assure the highest

degree of subject safety. The Safety Officer will: 1) review the protocol as funded and make suggestions for any changes (especially safety related); 2) determine appropriate adverse effect endpoints to be monitored and generate individual- and study-stopping rules; 3) review study progress and data quality; 4) determine formatting for data reports; 5) review endpoints for safety and efficacy; 6) submit reports and suggestions to the PI and the NIH; and 7) add to or modify this list of objectives.

Because of the nature of the study, the Safety Officer will need to monitor safety only and not efficacy. In the event the Safety Officer determines that the study or a phase of the study should be stopped for reasons of safety, this will be communicated to the PI and the NIH; the PI will then inform the UC-AMC IRB.
Safety Officer meetings will address issues of protocol design (including any proposed changes in design, data management or analysis), recruitment, retention, data management, and data quality. The Safety Officer will summarize any information to be noted or acted on by the PI.

Defining and reporting serious adverse events (SAEs)
We will promptly notify the IRB (within 5 days of the occurrence) when unexpected SAEs (unanticipated problems) occur, defined as death, life threatening illness, hospitalization or prolongation of hospitalization, congenital anomaly/birth defects, and persistent/significant disability. Any SAE that is unexpected and related or possibly related to the drug or other research intervention will be reported. SAEs that are unrelated to the research intervention will not be reported to the IRB (however, we will report these to the Safety Officer and NIH if they wish to be informed). Risks that are described in the protocol and consent will not be reported promptly to the IRB unless they occur more frequently or are more serious than expected. One exception to this rule is in the case of a death. All deaths must be reported, whether or not the death was related to the research.

In addition to following the requirements above, we anticipate that the Safety Officer will define study-specific SAEs that trigger the cessation of the intervention for an individual. Because the study interventions include a drug intervention that is used clinically (i.e., risks are known), we do not believe study-stopping rules are necessary.

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