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Cardiovascular Disease Risk and Menopause: Effects of Cardiorespiratory Fitness, Exercise, and Follicle Stimulating Hormone

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CARDIOVASCULAR DISEASE RISK AND MENOPAUSE: EFFECTS OF CARDIORESPIRATORY FITNESS, EXERCISE, AND FOLLICLE STIMULATING HORMONE

A Dissertation Presented

By

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ABSTRACT

CARDIOVASCULAR DISEASE RISK AND MENOPAUSE: EFFECTS OF CARDIORESPIRATORY FITNESS, EXERCISE, AND FOLLICLE STIMULATING HORMONE

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Menopause is associated with adverse changes in cardiovascular disease risk factors. A reduction in estrogens is most commonly associated with changing cardiovascular disease risk; however, recent observations suggest that the increase in follicle stimulating hormone that accompanies menopause may also influence risk, potentially through its influence on lipid levels. The changes in cardiovascular disease risk factors may adversely affect endothelial cell function, a pre-clinical marker for cardiovascular disease. Whether cardiorespiratory fitness is protective of endothelial health in this population, thereby mitigating the changes in risk that accompany menopause, is unclear. This dissertation evaluated differences in endothelial health and endothelial responses to acute exercise in women in various menopausal stages and with different levels of cardiorespiratory fitness. Endothelial health was assessed using flow-mediated dilation and endothelial microparticles (EMPs). The project also evaluated whether follicle stimulating hormone (FSH) levels were related to lipid levels in a large

cohort of postmenopausal women. We found that: 1) endothelial function declines with menopause, independent of cardiorespiratory fitness, 2) EMPs are reduced with acute, moderate intensity exercise in midlife women, despite differences in menopausal status and cardiorespiratory fitness, 3) High FSH is related to dyslipidemia in postmenopausal women. Together, these data suggest that menopause and cardiorespiratory fitness differentially impact factors related to cardiovascular disease risk.

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CHAPTER 1

INTRODUCTION

Cardiovascular disease (CVD) risk increases with menopause, with adverse changes in traditional CVD risk factors, such as blood pressure and lipids, beginning during the transitional (i.e. perimenopausal) years and continuing into postmenopause (108, 109). These changes have been largely attributed to the reduction in estrogens that accompany menopause; however, recent evidence suggests that follicle stimulating hormone (FSH) may independently affect CVD risk (44), potentially through its influence on lipid metabolism (25). The effects of FSH on lipid metabolism have largely been uninvestigated, making FSH a novel target that may influence CVD risk with menopause. The changes in risk, either through the effects of estrogen, FSH, or both, may adversely affect endothelial cell function, a preclinical marker of CVD (123).

Endothelial cells line blood vessels throughout the vasculature and are involved in smooth muscle migration and proliferation, thrombolytic activity, inflammation, vasodilation, and vasoconstriction. Normal endothelial cell function is associated with a favorable balance of vasodilatory compared to vasoconstrictive factors, whereas in a dysfunctional state, this balance favors vasoconstrictive factors (36). Endothelial dysfunction is largely associated with a reduction in nitric oxide bioavailability, which can be assessed through flow-mediated dilation (FMD) (182). Changes in endothelial health are also associated with increased endothelial cell activation and apoptosis, which can be assessed through circulating endothelial microparticles (EMPs: $CD62E^{+}$ and $CD31^{+}/42b^{-}$, respectively) (208). Endothelial microparticles levels and FMD change with disease and each have been proposed as novel biomarkers for CVD (2, 14).

Endothelial dysfunction may worsen with menopause. When comparing pre- to post-menopausal women there is consistently lower endothelial function in postmenopausal women (70, 71, 104, 123, 139). A cross-sectional study showed a progressive decline in FMD from pre- to peri- to post-menopause, independent of age (123); however, other work has shown that there is no difference in FMD between peri- and post-menopausal women (158). Endothelial microparticle levels may also differ with menopause, but to date, only one study has assessed EMPs in women at different menopausal stages (158). Therefore, to what extent and at what point during menopause endothelial dysfunction occurs is unclear.

High cardiorespiratory fitness is generally associated with high levels of endothelial function; however, this benefit may be lost with menopause (139, 151). When comparing sedentary to active postmenopausal women there was no difference in FMD between groups (151). Further, the beneficial improvements in endothelial function with exercise training appear to be mediated, in part, by the effects of estrogen, as endothelial dysfunction may not improve with exercise training in postmenopausal women in the absence of estrogen therapy (124). Despite some evidence that there may be a resistance to the benefits of exercise training on endothelial function with menopause, there are conflicting results in the literature and most studies have only evaluated postmenopausal women. Therefore, future work needs to address if this resistance occurs and at what point it begins.

Exposure to an acute exercise stress may help to elucidate differences in endothelial function that may not be apparent at rest. For example, when comparing sedentary to active men, there was no difference in FMD at baseline, but following an

acute bout of exercise, sedentary men had worse endothelial function, reflecting the difference in CVD risk between the two groups (68). This has also been shown in smokers compared to non-smokers (54), in lean compared to obese premenopausal women (43), and recently in peri- compared to post-menopausal women (158). This paradigm has been largely unused in the menopause literature and may help to further characterize differences in endothelial function, and therefore CVD risk, that might not be apparent in the absence of the acute hemodynamic stress of exercise.

This dissertation aimed to address many of the gaps in the literature discussed above. This project evaluated differences in endothelial health, via FMD and EMPs, in pre-, peri-, and late post-menopausal women before and after an acute bout of exercise. Peri- and post-menopausal women were stratified by cardiorespiratory fitness to assess whether there is an endothelial resistance to the benefits of chronic exercise with menopause. Finally, the relationship between FSH and lipid levels was assessed in a cohort of postmenopausal women from the Kuopio Ischaemic Heart Disease Risk Factor Study. These questions were addressed through the following aims and hypotheses:

Aim 1: To determine the effects of menopause and cardiorespiratory fitness on markers of endothelial health.

Aim 1a: To determine whether there are differences in endothelial health, assessed via flow-mediated dilation and $CD31^{+}/42b^{-}$ and $CD62E^{+}$ endothelial microparticles, in women in progressive menopausal stages.

Hypothesis 1a: There will be a progressive decline in FMD and an increase in $CD31^+/42b^-$ and $CD62E^+$ EMPs in women in progressive menopausal stages.

Aim 1b: To determine whether cardiorespiratory fitness affects differences in endothelial health in women in progressive menopausal stages.

Hypothesis 1b: Perimenopausal women with higher cardiorespiratory fitness will have higher FMD and lower $CD31^+/42b^-$ and $CD62E^+$ EMPs compared to their low-fit counterparts, while FMD and $CD31^+/42b^-$ and $CD62E^+$ EMPs values will not differ in high vs. low-fit late postmenopausal women (Figure 1.1).

Aim 2: To determine the effects of menopause and cardiorespiratory fitness on endothelial responses to acute exercise.

Aim 2a: To determine whether there are differences in endothelial responses, assessed via flow-mediated dilation and $CD31^+/42b^-$ and $CD62E^+$ endothelial microparticles, to acute exercise in women in progressive menopausal stages.

Hypothesis 2a: In response to acute exercise, the change in FMD will progressively decrease and the change in $CD31^+/42b^-$ and $CD62E^+$ EMPs will progressively increase in premenopausal, perimenopausal, and late postmenopausal women, respectively.

Aim 2b: To determine whether cardiorespiratory fitness affects differences in endothelial responses to acute exercise in women in progressive menopausal stages.

Hypothesis 2b: Compared to their low-fit counterparts, high-fit perimenopausal and late postmenopausal women will have an increase in FMD and a decrease in $CD31^+/42b^-$ and

$CD62E^+$ EMPs in response to acute exercise. FMD responses will progressively decrease and $CD31^+/42b^-$ and $CD62E^+$ EMPs levels will progressively increase in high-fit premenopausal, and high- vs. low-fit perimenopausal, and late postmenopausal women respectively (Figure 1.2).

Aim 3: To evaluate the relationship between FSH and lipid levels in women in a large cohort of postmenopausal women.

Hypothesis 3: FSH levels will be positively related to total cholesterol, low-density lipoprotein cholesterol and triglycerides and negatively related to high-density lipoprotein cholesterol.

Figures

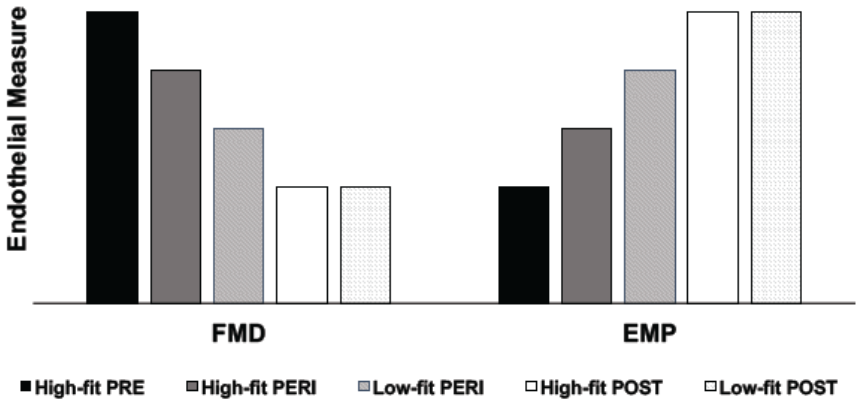


Figure 1.1. Proposed Hypotheses for Endothelial Changes with Menopause.
PRE: premenopausal; PERI: perimenopausal; POST: postmenopausal; EMP: endothelial microparticles; FMD: flow mediated dilation.

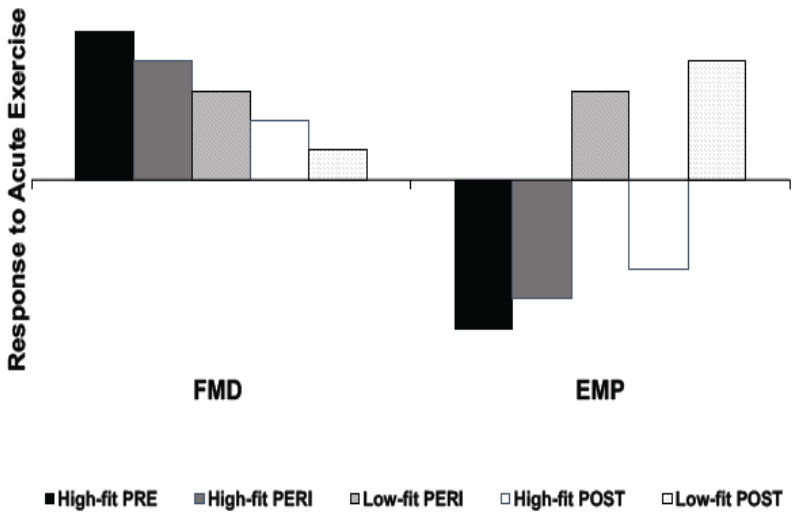


Figure 1.2. Proposed Hypotheses for Endothelial Responses to Acute Exercise.
Pre: premenopausal; PERI: perimenopausal; POST: postmenopausal; EMP: endothelial microparticles; FMD: flow mediated dilation.

CHAPTER 2

LITERATURE REVIEW

Introduction

Menopause, which is associated with the cessation of ovarian function, is an inevitable transition that women experience as they age. This transition is generally associated with changes in sex hormone levels, which adversely affects cardiovascular disease risk. Increases in cardiovascular disease (CVD) risk factors, such as blood pressure and low-density lipoprotein cholesterol occur around the final menstrual period (108). While reductions in estrogens have been the primary focus of research related to increasing CVD risk with menopause, there is an increase in follicle stimulating hormone (FSH) with menopause that appears to have independent effects on risk (45). This may be related to the effects of FSH on lipid metabolism (169), although this avenue remains largely unexplored.

The changes in CVD risk with menopause appear to adversely affect endothelial cell function (123). Endothelial dysfunction is a subclinical marker for cardiovascular disease and is involved in the initial stages of atherosclerosis (195). Endothelial dysfunction is prognostic of cardiovascular events in postmenopausal women (147) and has been proposed as a novel risk factor for cardiovascular disease (195).

In a non-menopausal (i.e. premenopausal women and men), healthy population, exercise improves endothelial function by decreasing inflammation and reactive oxygen species and increasing vascular tone and nitric oxide bioavailability (39). In a healthy, menopausal (i.e. perimenopausal and postmenopausal) population, the impact of exercise on endothelial function is not well established (Figure 2.3). Exercise-training studies

provide evidence that there may be resistance to the expected improvements in endothelial function across the menopausal transition (124, 139). Data from our laboratory show that peri- and postmenopausal women have different endothelial and inflammatory responses to an acute bout of exercise (158). Therefore, continued evaluation of the role of menopausal status and exercise is warranted. Evaluating the vascular changes in women across the menopausal transition with differing cardiorespiratory fitness levels will allow us to better understand whether exercise, other therapeutic approaches, or both in combination, may be beneficial in this population. This chapter will provide a general overview of menopause and changes in CVD risk with menopause. As a central feature of this dissertation, endothelial function, assessment of endothelial function, and the impact of exercise on endothelial function in a healthy, non-menopausal and menopausal populations will be reviewed.

Menopause

Thirty-four percent of women die from cardiovascular disease (58), which is the leading cause of death of women in developed countries (111). As women age, their risk factors for cardiovascular disease increase (108, 189) and 67% of sudden deaths occur in women with no history of the disease (126). Menopause marks a change in cardioprotection in women. This change is generally attributable to the increase in CVD risk factors surrounding the final menstrual period. Importantly, this increase in CVD risk is independent of age and begins during the menopausal transition, when hormone levels start to change (110) (Figure 2.4).

Menopausal Stages

Menopause occurs at approximately 51 years old in most women and is a multi-stage transition around the final menstrual period (205). The menopausal transition is composed of early and late perimenopause. During this time, estrogen levels decline (67). In early perimenopause, the menstrual cycle becomes variable in length. This variability is defined by a menstrual cycle that is more than 7 days different from what an individual considers normal and occurs at least twice within 10 menstrual cycles. Early perimenopause is also marked by variably elevated follicle stimulating hormone (FSH) levels. Late perimenopause is marked by intervals of amenorrhea, or a lack of menstrual cycle, that last for at least 60 days but less than one year. During this time, FSH values are variable but are typically greater than 25 IU/L (67).

After perimenopause, a woman transitions to early and then late postmenopause. Early postmenopause is defined as 1 to 5 years of amenorrhea. This is typically accompanied with an elevation in FSH, while estradiol decreases for the first 2 years of amenorrhea and then stabilizes. Late postmenopause is defined as more than 5 years of amenorrhea. Although estrogen levels may not change significantly during this time, FSH levels, while still elevated compared to premenopausal women, may decline (67).

Menopausal Changes in CVD Risk Factors

The changes in hormone levels with menopause negatively impact CVD risk. In a longitudinal study of women progressing from pre- to peri- to post-menopause there were increases in low-density lipoprotein cholesterol (LDL-C), triglycerides, body mass index (BMI), fasting plasma glucose, and pulse pressure, with the most dramatic changes in LDL-C, triglycerides and BMI occurring during perimenopause. Further, there was an

increase in intima media thickness, an independent predictor of CVD (110), which was corroborated in a separate longitudinal analysis (201). Many of these changes appear to be independent of aging (108). Women who go through menopause at a later age have better CVD outcomes (35) and increased longevity (159); women have 18% lower risk of developing CVD if they go through menopause at age 52 vs. 44 (35).

Compared to perimenopausal women, postmenopausal women have higher cardiovascular disease risk. This may be attributable to continued increases in lipids, body mass index, insulin resistance, and total cholesterol (110, 172). Postmenopausal women have higher rates of central adiposity (49), with up to a five times greater risk of developing central adiposity (i.e. visceral adiposity) compared to premenopausal women. This risk doubles during the menopausal transition (42). Following the final menstrual period, lipid levels are estimated to increase by 1.75%, insulin by 2.84% and systolic blood pressure by 0.26mmHg per year, independent of age and weight (108). While there is a shift in the cardiovascular profile at the initiation of, and throughout, the menopausal transition, the reasons behind this shift are still poorly understood. Possible explanations for this change are linked to changes in estrogen and FSH levels.

Effects of Menopausal Hormonal Changes on CVD Risk

Estrogens. A decline in estrogen is one of the most commonly associated changes with menopause. Although, estrogen levels are not stable in premenopausal women and vary with menstrual cycle (94, 146), diet, alcohol, and stress (107). Despite these fluctuations, premenopausal estrogen levels remain higher than in postmenopausal women (67). Estrogen is present in circulation as estrone, estradiol, or estriol (135) and is synthesized from cholesterol and carried in the blood via sex hormone binding globulin

(SHBG) (107). Sex hormone binding globulin levels may change with menopause. Some studies have reported relatively stable levels of SHBG with menopause (136, 166), while others have shown a significant reduction (18, 143). Therefore, not only estrogen levels, but also the delivery of estrogen may change with menopause.

The location of estrogen production varies in premenopausal compared to postmenopausal women. In premenopausal women, estrogen is primarily produced in the ovaries, but also in the liver and peripheral tissues (64). In postmenopausal women, the majority of estrogen is synthesized from circulating androstenedione, testosterone, and estrone in peripheral tissues, such as adipocytes (165). The difference in synthesis between premenopausal and postmenopausal women may be attributable to the cessation of ovarian function with menopause. Once produced, estrogen binds to estrogen receptors throughout the body and in the vasculature (112).

Estrogen receptors are pervasive throughout the body, including in the vasculature, and are present as nuclear and g-protein coupled receptors. There are two nuclear receptor types, ERα and ERβ. When estrogen binds to either receptor, the receptor translocates to the nucleus, activating gene transcription. The activation of ERα and ERβ lead to different responses. In general, ERα leads to cardioprotective effects, while ERβ is associated with an inflammatory response. The plasma bound estrogen receptor, GPER, was more recently discovered, and like ERα, appears to be cardioprotective and anti-inflammatory. The effects of estrogen through its receptors can be genomic or non-genomic, causing both short and long-term effects (4, 23). With menopause, there is an overall reduction in circulating estrogen levels and there may be a

shift in estrogen receptor phenotype, with an increase in the proportion of ERβ vs. ERα, potentially contributing to the increase in CVD risk (132).

While sex hormones levels and changes in receptor phenotype may affect CVD risk, the trajectory of sex hormone changes with menopause also impact cardiovascular outcomes. It has been demonstrated that women who have higher estrogen levels prior to their final menstrual period (FMP) and lower levels immediately after the FMP have lower risk of developing carotid plaque (i.e. atherosclerosis) than women who have higher estrogen levels after the FMP and 43% lower risk compared to those who have chronically low levels of estrogen pre-and post-FMP (45); however, further research needs to be done in this area. This seemingly contradictory effect of estrogen from pre- to post-menopause may be related to changes in the effects of estrogen. *In vivo* work suggests that estrogen has anti-inflammatory effects in early stages of menopause and pro-inflammatory effects in later stages of menopause, potentially due to changes in receptor phenotype, with a shift towards ERβ (132). Similarly, higher levels of estrogen after menopause are related to increased incidence of insulin resistance (59) and higher lipid levels (188)(188). The negative effects of changes in estrogen levels, and potentially of estrogen receptor phenotype, are relatively well established, but there is also evidence to suggest that changes in follicle stimulating hormone may impact CVD risk.

Follicle Stimulating Hormone. Unlike estrogen, follicle stimulating hormone (FSH) levels remain elevated following menopause. FSH is mainly produced in the anterior pituitary and in premenopausal women is involved in regulating the menstrual cycle by up-regulating estrogen production and stimulating follicle maturation. As estrogen levels decline with menopause, FSH levels rise. The effects of chronically high

FSH following menopause are still poorly understood. Women with lower FSH after menopause have been reported to have lower risk of developing CVD than those with higher FSH (45); however, a recent report suggests that higher FSH is associated with reduced CVD risk (198). Higher postmenopausal FSH has also been related to lower incidence of type 2 diabetes (12) and lower intima media thickness (11). Interestingly, the relationship between FSH and intima media thickness also appears to be influenced by age, with an inverse relationship between FSH and intima media thickness that was present in older (age 64-73), but not younger (age 53-62) postmenopausal women (11). This data suggests that the relationship between FSH and CVD risk may be influenced by a variety of factors including age and menopausal status, and the mechanisms behind these relationships remain unclear.

The influence of FSH on lipid metabolism is one potential mechanism by which FSH influences CVD risk (27, 100). High FSH levels reduce LDL-receptor number in hepatocytes, leading to a sharp increase in circulating LDL-C *in vitro*. *In vivo*, high levels of FSH are related to higher LDL-C and total cholesterol in postmenopausal women (169). FSH also stimulates lipid biosynthesis (32, 100), as well as the release of leptin in adipocytes (100), potentially contributing to postmenopausal weight gain and adverse changes in lipid levels (32). Based on this evidence, FSH may independently impact CVD risk through effects on lipid metabolism; however, this possibility has been largely uninvestigated.

Overall, there is evidence to suggest that estrogen and FSH independently affect CVD risk (45); however, the magnitude of and mechanisms behind these effects have yet to be fully elucidated, suggesting that more complex characteristics than absolute

hormonal levels are at play. For example, the trajectory of change in hormones through the FMP, age at menopause, and premenopausal hormonal levels also affect CVD risk after menopause (44, 45). The changes in cardiovascular disease risk with menopause, either through effects of estrogen. follicle stimulating hormone, or others factors, may lead to changes in endothelial cell function.

Vascular Endothelium

Endothelial cells are found lining blood vessels throughout the body. These cells have many dynamic properties that maintain vascular homeostasis (194). This includes, but is not limited to, the modulation of vaso-constriction and -dilation, blood viscosity, and the release of, and response to, inflammatory factors. Endothelial cells are also semi-permeable to fluids and proteins. These factors are transported either through endothelial membranes via vesicle-mediated transport mechanisms or between cells through paracellular junctions. The permeability of the cells is largely affected by intra- or extra-cellular calcium concentration (192). Endothelial cells act as a physical barrier between blood and the vessel wall (20), but also release substances that include vasodilators, vasoconstrictors, plasminogen activators and inhibitors, and platelet modulators. The most recognized of these substances are nitric oxide, prostacyclin, endothelin-1, Von Willebrand factor, and plasminogen activator. These factors are predominantly paracrine; they act locally on the blood vessel walls in which the cells are located and do not affect other vessels (162).

Vasodilators

Nitric oxide (NO) is one of the most extensively studied factors that endothelial cells release. Nitric oxide is formed when the amino acid l-arginine is converted to l-citrulline and NO through activation of nitric oxide synthase (NOS) (Figure 2.5). Nitric oxide synthase is present as endothelial NOS (eNOS), inducible NOS (iNOS), found in circulating immune cells, and neuronal NOS (nNOS), and is activated through its coupling with tetrahydrobiopterin (BH_4). Under ischemic conditions, NO may also be formed through the reduction of nitrite, although this pathway is less common (101). Nitric oxide is a powerful vasodilator that is released in response to factors such as shear stress, acetylcholine, substance P, alpha-adrenergic stimulation, bradykinin, serotonin, thrombin, and platelet-aggregation. Shear stress is a commonly used technique to study nitric oxide release. Shear stress triggers vasodilation when nitric oxide causes guanylate cyclase to form cyclic guanosine monophosphate (GMP), which then stimulates calcium activated potassium channels. The influx of potassium leads to hyperpolarization of the adjacent smooth muscle, causing smooth muscle relaxation. Beyond vasodilation, nitric oxide also inhibits superoxide generation, monocyte adhesion, smooth muscle proliferation, and platelet aggregation (129).

Nitric oxide is only one of several vasodilators released by endothelial cells. Prostacyclin also contributes to vasodilation through smooth muscle relaxation. Prostacylin is formed when phosholipase A is activated and is converted first to prostaglandin through the action of cyclo-oxygenase enzymes and then to prostacyclin via prostacyclin synthatase (116). Prostacylin inhibits platelet aggregation and smooth muscle proliferation (119), and decreases cholesterol accumulation in endothelial cells

(203). Like nitric oxide, prostacyclin is released in response to shear stress (53), bradykinin, and thrombin (193). Prostacyclin is up-regulated by leukocyte contact, arterial wall stretching, interleukin-1, and platelet-derived growth factor (52) and is down-regulated by glucocorticoids, age, diabetes mellitus, and coronary artery disease (31).

Vasoconstrictors

Endothelial cells do not exclusively release vasodilatory factors; they also release vasoconstrictive factors. Endothelin-1 (ET-1) is the most powerful of these substances and is 10 times more potent than the endothelial-released vasoconstrictor, angiotensin II (193). Endothelin-1 is formed in endothelial cells when the 39-amino acid peptide, big endothelin-1 is converted to ET-1, a 21-amino acid peptide via endothelin converting enzyme. Endothelin-1 can act on smooth muscle cells to stimulate calcium release, causing smooth muscle contraction. Endothelin-1 can also bind to receptors on endothelial cells, triggering the release of NO and can increase smooth muscle proliferation (148). Endothelin-1 is released in response to stimuli such as thrombin, epinephrine (207), insulin, hypoxia, and angiotensin II (187). Unlike nitric oxide and prostacyclin, endothelin-1 has a dual response to shear stress; it is up-regulated in response to low levels of shear stress and is down-regulated in response to high levels of shear stress (93). Endothelin-1 is inhibited in the presence of its antagonists, nitric oxide (15) and prostacyclin (37) through the suppression of the transcription factor nuclear factor kappa b, NFκB (133).

Other Factors

Endothein-1, nitric oxide, and prostacyclin are a few of the factors released by endothelial cells; however, they are a small number of many. Other factors are involved in smooth muscle migration and proliferation, platelet adhesion, and blood viscosity. A few examples of these are: plasminogen activator, which is anti-thrombolytic; and Von Willebrand factor, which mediates platelet adhesion in response to endothelial injury. Endothelial cells can also express adhesion molecules, which allow immune cells to interact with the endothelium (187).

Endothelial Dysfunction

Endothelial cells are key regulators of vascular health; therefore, anything that disrupts endothelial function can impact basic cellular function and change the balance of factors that the cells release. Factors such as coronary artery disease, hypertension, dyslipidemia, diabetes, aging, and other cardiovascular disease risk factors may also contribute to endothelial dysfunction. This dysfunction is characterized by symptoms such as vasospasm, exercise-induced hypertension, or unstable coronary syndrome, but can also go unrecognized (162). In the presence of endothelial dysfunction there is typically a shift in balance between vasoconstrictors and vasodilators (36). With normal endothelial cell function, the balance between vasodilators (e.g. nitric oxide and prostacyclin) and vasoconstrictors (e.g. endothelin-1) favors vasodilation. In a dysfunctional state, this balance favors vasoconstriction, thereby changing vascular tone.

The changes in vascular tone that occur with endothelial dysfunction may be related to changes in smooth muscle reactivity or endothelial cell function. For example, nitric oxide hyperpolarizes smooth muscle, leading to vasodilation. Endothelial

dysfunction, assessed via nitric oxide-mediated vasodilation, may therefore be due to impaired nitric oxide release or reduced smooth muscle reactivity to nitric oxide. Since the function of smooth muscle and endothelial cells are intimately tied together, due to shared signaling mechanisms and anatomical location, decreased arterial stiffness is impacted by and may impact endothelial function (130). Similar risk factors lead to the dysfunction of both tissues, making a causative relationship difficult to determine. Despite a lack of clarity as to the full nature of the interactions between the two, endothelial dysfunction is predictive of the progression of coronary intima media thickening and therefore coronary artery disease (66). This dysfunction can be assessed using flow-mediated dilation or by evaluating circulating endothelial microparticles or other circulating factors such as cellular adhesion molecules. Flow-mediated dilation is dependent on the function of endothelial cells as well as smooth muscle, while endothelial microparticles are found in circulation, allowing each to provide unique and complimentary information about endothelial cell health.

Flow-mediated Dilation

Flow-mediated dilation (FMD) is a relatively quick, non-invasive test that allows for an assessment of endothelial function in response to shear stress. Flow-mediated dilation is a peripheral test that provides insight into the disease process and is reflective of coronary artery function (3, 180). Low FMD has been related to a variety of disease states including hypertension (99), hyperlipidemia (171), diabetes (105), and smoking (22). Lower FMD is also present in sedentary compared to active older men (139) and is prognostic of cardiovascular events in postmenopausal women (147).

Flow-mediated dilation utilizes ultrasound and Doppler imaging of a peripheral artery before, during, and after blood vessel occlusion with the use of an inflatable cuff. The magnitude of dilation of the occluded vessel after the cuff has been released, relative to baseline, is a measure of endothelial function (Figure 2.6); dilation should occur in response to the increased shear stress caused by reactive hyperemia due to nitric oxide release. Despite the simplicity of the technique, FMD can be influenced by a variety of factors including cuff position (10), artery choice, the magnitude and duration of the stimulus (140), diet (10), exercise (34, 68), caffeine and alcohol consumption, and medications (68, 103). Given the many factors that can affect FMD, measurement technique is important.

To date, no organization has published universal guidelines on how to perform FMD. Various review articles by experts in the field have provided recommended guidelines (51, 63, 182), but until one unified guideline is accepted, discrepancies in practice between researchers can make data difficult to compare and interpret. Thijssen, et al. (2011) and Flammer, et al. (2012) published independent guidelines that aimed to minimize the potential sources of error that were discussed above and a recent article by Greyling, et al. (2016) identified factors and provided a grading system to evaluate if an FMD study is of high enough quality to be included in a systematic review. While these guidelines were published independently, the recommendations are consistent.

All groups recommend that the study be completed in a warm, quiet room to minimize participant distractions and sympathetic activation due to cold or excitement. The inflatable cuff should be placed 1 to 2 cm distal to the elbow crease and to the ultrasound probe, so that the area being measured is not responding to an ischemic

stimulus (51, 182). Occlusion should last for 5 minutes to elicit a predominately nitric oxide mediated response. The occlusion should take place either at the brachial or radial artery, which is associated with coronary artery endothelial function (180). When the technician is completing a flow-mediated dilation study, technical errors in flow velocity and imaging can be minimized by using a probe insonation angle of 60° and by measuring peak diameter continuously for 180 seconds after cuff release. In order to minimize vascular changes due to lifestyle habits, participants should be fasted, refrain from exercise, smoking, caffeine, alcohol, and medication for six hours before the study (51, 182). The assessment of endothelial function using FMD can help to provide insight into changes in endothelial function that can impact cardiovascular risk.

Endothelial Microparticles

Endothelial microparticles (EMPs) also provide insight into the state of the endothelium, but unlike FMD, these particles are assessed in circulation (Figure 2.7). High levels of EMPs have been related to a variety of disease states including end stage renal disease (2), acute coronary syndrome (9), diabetes (186), and cardiovascular disease (161). In fact, EMP levels have been proposed as a potential novel biomarker for cardiovascular disease (2). Endothelial microparticle levels are lower in groups with low cardiovascular disease risk, such as premenopausal women (92) and normal weight individuals (46).

Endothelial microparticles are small vesicles (100nm-1μm), which contain surface markers from their cell of origin and carry proteins and miRNA. The surface markers on EMPs give an indication of the state of their cell of origin. These markers can include cellular adhesion molecules or membrane receptors, such as nitric oxide synthase; however, many of these markers are non-specific to endothelial cells and therefore are difficult to isolate and interpret. The main markers that are specific to EMPs are markers of endothelial activation ($CD62E^{+}$) and endothelial apoptosis ($CD31^{+}/42b^{-}$)(41). There are small numbers of EMPs in circulation at all times, but levels may increase in response to both acute and chronic stimuli such as inflammation, exercise, or disease (41). Although, still not fully understood, EMPs may also contribute to cellular processes such as cell survival and angiogenic capacity (41).

Endothelial microparticles are able to act on other cells within the vascular network. They enhance inflammatory cascades either through recruitment of white blood cells, through their membrane markers, or by fusing with other nearby cells and releasing their content. Endothelial microparticles are also involved in angiogenesis, thrombosis, and cell regeneration (138). Endothelial microparticles can be taken up by endothelial cells and appear to influence cell function. For example, EMPs have been shown to be protective of endothelial apoptosis (79, 196). Despite these many roles, the effect that EMPs have on other cells is still not fully understood. Therefore, although they are generally considered markers of endothelial status, their role appears to be more complicated (33, 73, 154, 208).

Similar to FMD there are no universal guidelines for measuring EMPs and the use of this technique to assess endothelial health is still relatively new. Endothelial

microparticle levels are affected by factors such as inflammation, reactive oxygen species (41), centrifugation speed during preparation, duration of sample storage (38), storage temperature (160), lipid levels (190), and even vascular damage due to venipuncture (210). Endothelial microparticle levels also change in response to exercise (196) or to changes in physical activity patterns (16). The many factors that influence EMPs makes the interpretation and comparison of EMPs across studies challenging. Despite this, EMPs are related to a variety of disease states and may provide unique information on the overall state of endothelial cells that cannot be determined via FMD or can be used to complement physiological measures of endothelial function.

Endothelial Function and Menopause

Endothelial function appears to decline with menopause. A significant reduction in FMD has been seen even during the menopausal transition after accounting for other CVD risk factors and worsens with prolonged estrogen deficiency (123). Endothelial microparticle levels also appear to change with menopause. Compared to low-active postmenopausal women, perimenopausal women have lower levels of $CD62E^+$ EMPs (158) and when comparing postmenopausal women with high vs. low estrogen levels, women with high estrogen have lower levels of $CD62E^+$ EMPs (43). These findings suggest changes in endothelial function with menopause and that these changes may negatively impact CVD risk.

Effects of Estrogen

Estrogen deficiency is a unique factor that contributes to endothelial dysfunction and vascular changes in menopausal women. Throughout the menopausal transition,

estrogen levels fall. This decline has been correlated with endothelial dysfunction in postmenopausal women (84, 149, 179) and prolonged estrogen deficiency may hinder improvements in endothelial function (124). Lower estradiol levels can lead to structural changes such as a larger adventitial diameter (201) or an increase in intima media thickness (110). An increase in adventitial diameter has been associated with an adverse cardiovascular disease profile (30, 83) and is increased most between late perimenopause and postmenopause (201). A decline in estrogen can also increase vasoconstriction and shear stress levels, potentially leading to structural changes such as increased intima media thickness. This increase has been seen in women across the menopausal transition and is related to higher premenopausal levels of systolic blood pressure, pulse pressure, triglycerides, glucose, body mass index, and high-density lipoproteins (110). These changes combined can all adversely affect endothelial function.

Estrogen modulates endothelial function through a variety of roles and can cause vasodilation, by increasing nitric oxide (87, 113), endothelial nitric oxide synthase, and prostaglandin synthesis (74). Estrogen further contributes to vasodilation by inhibiting vasoconstriction and sympathetic activation (114). There is also evidence to suggest that not just estrogen, but also estrogen receptors modulate nitric oxide production (148). Estrogen also decreases low-density lipoprotein cholesterol oxidation, which is a precursor to atherosclerosis and endothelial dysfunction. Finally, estrogen is both anti-oxidative and anti-inflammatory, therefore indirectly effecting endothelial function (174).

Estrogen therapy consistently improves endothelial function in postmenopausal women (71, 104, 124, 175). Following 8 weeks of estrogen therapy, early postmenopausal women had improved basal NO release, with no change in endothelial

independent vasodilation (175). This suggests that estrogen deficiency reduces NO bioavailability, a finding that has been corroborated in animal studies (17, 50). While aging may compound many of the primary and secondary effects due to estrogen deficiency, there was reduced endothelial function in young women one week following oophorectomy (134). There is also reduced function in young women with premature ovarian failure (84), in amenorrhoeic athletes (145), and following ovariectomy in rodents (17, 50). The anti-inflammatory and anti-oxidative effects of estrogen deficiency can also impact endothelial function. When postmenopausal women were treated with an inhibitor for the inflammatory cytokine tumor necrosis factor-α, there was in improvement in FMD (121). Similarly, the use of ascorbic acid, an anti-oxidative therapy, improved FMD in postmenopausal women (124).

Effects of Follicle Stimulating Hormone

Unlike estrogen, follicle stimulating hormone (FSH) levels remain elevated following menopause. Although less studied, there is evidence to suggest that FSH may also impact endothelial function and CVD risk. FSH receptors are present on some endothelial cells (e.g. from tumors and the umbilicus) (142, 173), though they may not be present on all endothelial cells, and when stimulated, promote angiogenesis; this benefit is attenuated with supra-physiologic FSH levels (173). FSH may also affect endothelial progenitor cells, which are involved in endothelial cell repair; high levels of FSH promote bone marrow derived osteoclast activity (56), potentially disrupting endothelial progenitor cell formation. There is also evidence to suggest that FSH may adversely impact lipid levels (27, 100), and in a regression analysis, menopausal status, but not estradiol was related to the endothelial response to acute exercise (158). FSH may

independently affect endothelial function; however, this possibility has been largely uninvestigated.

Treatment

Endothelial function appears to decline with menopause, thereby negatively impacting CVD risk; however, clinicians have struggled to find an effective treatment method to improve cardiovascular outcomes in this population. In the past, hormone therapy was commonly used in menopausal women; estrogen supplementation improves factors such as blood pressure and basal nitric oxide release (175). Despite the positive effects of estrogen on the cardiovascular system, hormone therapy (HT) in postmenopausal women did not improve CVD risk (77, 206). In fact, the Women's Health Initiative study, a longitudinal study investigating the effects of HT in postmenopausal women, was terminated early following reports of adverse outcomes, such as increased incidence of stroke and breast cancer (206). Evidence suggests that the type (e.g. conjugated equine estrogen vs. oral estradiol) (164, 168), delivery method (e.g. transdermal vs. oral) (118, 152), and the timing of HT (e.g. early vs. late postmenopause) (7) may affect outcomes; however some of these hypotheses are still being tested. Currently, HT is most commonly used to acutely treat women who are in the early stages of menopause for conditions such as menopausal symptoms and is generally not recommended for women in later stages of menopause (5). Exercise shares similar signaling pathways to estrogen and improves cardiovascular outcomes (65, 106), making it a potential therapeutic candidate to improve endothelial function in this population.

Cardiovascular Risk and Exercise

Exercise is a potent stimulus to improve endothelial function through its multifaceted effects on vascular tone, inflammation, reactive oxygen species, thrombosis and platelet aggregation (39). These benefits are well-established in healthy men and non-menopausal women, but may not remain in a menopausal population (122)

Non-menopausal Population

Improved Vascular Tone. In a non-menopausal population, chronic exercise training increases secretion of vasodilatory factors in response to exercise-induced shear stress. This may be attributed to an increase in nitric oxide and endothelial nitric oxide synthase production (eNOS). This adaptation has been seen with aerobic training studies (62, 85) and is illustrated by a two times greater vasodilatory capacity in runners versus sedentary individuals (72). Increased endothelial production of nitric oxide is hypothesized to be due to shear stress as well as increased catecholamines, adenosine triphosphate, and eNOS circulation (129). Exercise training can also enhance vasodilation through increased prostacyclin release and high-density lipoprotein synthesis. High-density lipoproteins up-regulate the synthesis of prostaglandin-2 and contribute to increased vasodilation (53).

Although increases in vasodilatory factors are beneficial to endothelial function, in order for vasodilation to improve, a positive balance must exist between vasodilatory and vasoconstrictive factors like endothelin-1. Chronic exercise training decreases endothelin-1 levels, which may be inversely related to increases in nitric oxide production (39). This reduction is only seen with endurance (i.e. aerobic) exercise training and the adaptation is lost with training cessation (102). Beyond changes in

vasodilatory and vasoconstrictive factors, exercise can also affect inflammatory factors and reactive oxygen species.

Decreased Reactive Oxidative Species and Inflammation. Exercise acutely increases the production of reactive oxygen species (ROS), such as NADPH oxidase. Chronic elevation of ROS can damage endothelial cells and cause nitric oxide degradation. However, due to the transient increase in ROS after an acute bout of exercise, endothelial damage does not typically occur. With chronic exercise training, the body begins to adapt to the increased oxidative burden with increased expression of antioxidant enzymes such as superoxide dismutase and glutathione peroxidase (24, 76). This increase in antioxidant species can have a protective effect on the endothelium by increasing nitric oxide bioavailability. However, even with the protective effects from antioxidant species, ROS production continues through aerobic metabolism and the oxidation of pre-existing molecules such as nitric oxide synthase (19).

Much like ROS, acute exercise can lead to an increase in inflammation, although this response is reduced in trained individuals (1). This inflammatory response is typically attributed to muscle damage from either heavy exercise or novel activities. Inflammation disrupts endothelial function and increases the expression of vascular cell adhesion molecules, C-reactive protein, and intracellular adhesion molecules, all of which increase platelet adhesion. With chronic exercise training, these factors decrease, allowing for improved endothelial function (184).

Acute exercise can also lead to changes in circulating cytokine levels. Most studies investigating the response of cytokines to acute exercise have been completed

men, making findings difficult to extrapolate to women. In 20 young, healthy men, 30 minutes of treadmill exercise at 75% of VO_{2max} lead to increased levels of the inflammatory cytokines interleukin-8 and interleukin-6 and growth factors involved in angiogenesis, such as vascular endothelial growth factor, all of which may impact endothelial cell function. These increases were independent of exercise training status (96). Cytokine responses to acute exercise may be independent of intensity and training status (156, 157), but effected by body mass and gender. Two hours of acute exercise completed by sedentary and obese and/or overweight participants showed an increase in IL-8, but in female subjects only (26).

Decreased Thrombosis and Platelet Activity. Although vasomodulation and inflammatory responses are an important component of endothelial function, endothelial cells have other roles in promoting normal vascular function. Platelet aggregation and activation can lead to endothelial dysfunction and thrombus formation. Chronic exercise training decreases platelet aggregation (144), while acute exercise in sedentary individuals increases platelet activation (86). Conversely, chronic exercise training increases tissue plasminogen activator, allowing for improved clot lysis (144). This is thought to be in response to increased intracellular calcium levels, shear stress, and an upregulation of tissue plasminogen activator mRNA from exercise (40, 91). Tissue plasminogen activator typically declines with age; however, chronic aerobic exercise training has been shown to halt this decline (167).

Menopausal Population

The benefits of exercise on endothelial function in a non-menopausal population are well established; however, the data in healthy women across the menopausal

transition is limited. The majority of studies focus on a single stage during the menopausal transition, menopausal symptoms, or the impact of hormone therapy (71, 90, 98, 124). This makes the identification of when changes in vascular function occur, and therefore, when an intervention may be most effective difficult. Further, there has been a scarcity of research on the effects of a single bout of acute exercise on endothelial function. Overall, our understanding of changes in the vasculature across the menopausal transition is limited.

Endothelial Benefits of Exercise. Endothelial function may improve following exercise training. After 12 weeks of aerobic exercise, initially targeting 250 kcal/session and progressing to 350 kcal/session, FMD improved in previously sedentary postmenopausal women, but only in those with significant impairment (<4.5% or <50th percentile FMD) prior to the intervention (177). Similarly, FMD improved in postmenopausal women after 6 months of aerobic training at 50%, 100% or 150% of physical activity guidelines, with those with the greatest initial impairment experiencing the greatest improvement (178). In a separate trial, following 24 weeks of aerobic exercise, beginning at 30% of heart rate reserve (HRR) and progressing to 60% HRR, previously sedentary postmenopausal women improved FMD, but there were no changes in similarly aged-men. Importantly, women had lower function initially, again suggesting that significantly impaired endothelial function may be necessary for exercise-induced improvements (13). In a similar study, after 24 weeks of aerobic training (30-60% HRR), postmenopausal women had improved microvascular vasodilatory capacity (75). Further, when comparing overweight sedentary to active postmenopausal women, active women

had higher FMD (150), suggesting that chronic training attenuates declines in endothelial function following menopause.

Rodent studies also support the benefits of exercise training on endothelial function. Following 8 weeks of aerobic training, endothelial dependent vasodilation improved in the aortas of ovariectomized (OVX) rats. This improvement was accompanied by a reduction in reactive oxygen species production (17). Similarly following 8 weeks of swim training, there was an improvement in endothelial dependent vasodilation and an increase in antioxidant enzymes in OVX rats (28). OVX rats were also able to improve endothelial dependent vasodilation following 14 weeks of isometric strength training (50).

The improvements observed with exercise training may be due to the acute effects exercise has on endothelial function. Although less commonly employed than exercise training studies, acute exercise studies may provide additional insight into the adaptability of the endothelium in response to the hemodynamic challenge of an exercise bout. An acute exercise model may also elucidate differences in FMD in groups with differing CVD risk that are not apparent prior to exercise. For example, FMD was similar prior to acute exercise in sedentary vs. active middle-aged and older men; however, following an acute bout of exercise, active men had higher FMD than sedentary men, highlighting the differences in CVD risk between the groups (68). Two separate studies showed that following an acute bout of exercise; postmenopausal women improved FMD, while premenopausal women did not. However, like the previous studies, the postmenopausal group had significantly lower FMD at baseline (70, 71).

Overall, there is evidence that suggests that endothelial function may improve following aerobic exercise in healthy postmenopausal women; however, there are methodological limitations that make the interpretation of these results challenging. As discussed previously, FMD is influenced by a variety of factors, and when not done following published guidelines, may be capturing a non-NO mediated response. Many of the studies showing the beneficial effects of exercise used varying methodologies, with several studies using cuff placement proximal to the probe, initiating a non-NO mediated vasodilation (70, 71, 150). Further, subject restrictions prior to FMD were largely unreported or varied considerably across trials; for instance, one trial provided participants with a meal prior to measurement (70). In addition, not all studies controlled for the time of day that measurements were taken, ignoring the potential effects of diurnal variations on FMD. Further, inclusion criteria varied across studies, with at least one study including women who were on CVD-related medication (178) or were overweight/obese (150). Importantly, no study classified women into specific menopausal stages, potentially washing out differences between early and late postmenopausal women, and several studies included women who had undergone surgical menopause (70, 71). In the cross-sectional analysis showing higher FMD in active compared to sedentary women, physical activity status was based on self-report and VO_{2max} did not differ between groups (150), suggesting that cardiorespiratory fitness may not have been the sole contributor to the observed differences between groups. While some rodent studies have supported the benefits of training on endothelial function (17, 28, 50), most utilized ovariectomized (OVX) young animals, a model that has several limitations as a direct correlate for menopause in humans.

Endothelial Resistance to Exercise. There is also a strong body of evidence to suggest that following menopause, there is a reduction in endothelial benefits associated with exercise; although, some of the methodological issues discussed above are present in this literature as well. Improvements in FMD as a result of exercise may only occur when estrogen supplementation is concurrently delivered. In previously sedentary postmenopausal women, FMD only improved after 12 weeks of aerobic training (65-80% maximum heart rate, MHR) in those who were given estrogen (124). This has also been shown in vessels from OVX rats subjected to chronic high shear stress, as might occur with exercise training; only arteries from rats treated with estrogen improved NO-mediated vasodilation and showed functional remodeling, with no, or adverse changes in the non-estrogen treated group (181). Several studies that did not include estrogen supplementation found no effect of training on FMD. Following 8 weeks of brisk walking (70-75% MHR), there was no change in FMD in postmenopausal women, while there was an improvement in age-matched men (139). Similarly, there was no improvement in FMD following 18 weeks of aerobic training (60-85% MHR) in postmenopausal women (21). Preliminary data from a randomized controlled trial showed no improvement in FMD in postmenopausal women with 8 weeks of moderate intensity continuous training or high intensity interval training (209) or in a separate study, after 2 weeks of continuous or high-intensity interval training (89). In cross-sectional analyses comparing highly-active postmenopausal women to their sedentary age-matched peers, there was no difference in FMD (139, 151), while similarly-aged sedentary men had worse FMD compared to their active peers (139) (Figure 2.8). This suggests that long-term training confers no endothelial protection following menopause. The lack of benefit from training

on endothelial function in menopausal women has also been shown at the microvascular level (151), indicating that resistance may be pervasive throughout the vascular network.

The acute exercise literature also supports the hypothesis that there may be endothelial resistance to exercise with menopause. In early peri-, late peri- and early post-menopausal women there was increased vascular conductance (i.e. greater resistance) and reduced blood flow during leg extension exercise with progressive menopausal stages (120). Postmenopausal women did not exhibit a change in FMD in response to varying intensities of acute exercise (209). Similarly, we previously showed that following an acute bout of aerobic exercise, postmenopausal women demonstrated no change in FMD, while perimenopausal women had a trend for enhanced FMD, which was accompanied with lower levels of EMPs related to endothelial activation and apoptosis. The FMD response to acute exercise was related to menopausal status, LDL-C, DBP, and cardiorespiratory fitness, suggesting that menopause itself, as well as changes in CVD risk factors that may accompany menopause impact endothelial function (158). This acute response may provide insight into the adaptability of the endothelium and together, these studies support the concept that changes in the vasculature may be occurring early during menopause. While the argument can be made that the exercise stimulus in some studies was not enough to elicit improvements in endothelial function, almost all protocols improved VO_{2max}, suggesting an appropriate stimulus to improve cardiorespiratory fitness; further, the cross-sectional analyses of sedentary vs. active women, most of whom had been highly active for decades (139, 151), suggests that intensity and intervention duration are not a limiting factor.

Hormonal changes may contribute to the endothelial resistance to exercise seen with menopause. As discussed previously, estrogen acts directly on the vasculature to increase NO production. As estrogen levels decline, there may be an overall reduction in NO bioavailability, hindering the ability of the endothelium to respond to acute and chronic exercise. This is supported by the observation that NO production is reduced following OVX (17), that FMD is reduced with acute exercise and repeated FMD trials in postmenopausal women (158), and that FMD in postmenopausal women improves with BH_4 treatment (125), a cofactor involved in the coupling of eNOS, as well as with l-arginine treatment, a cofactor involved in the production of NO (88). Further, ERα expression is reduced with lower levels of estrogen (6, 55, 78), potentially perpetuating the negative effects of estrogen deficiency on the endothelium, as this receptor phenotype is generally associated with the cardioprotective effects of estrogen (23). The increase in inflammation (156, 157) and oxidative stress (141) with a single bout of exercise may also be exacerbated by estrogen deficiency, hindering the ability of the endothelium to adapt. Along with estrogen, other factors may also mediate the endothelial response to exercise. FSH stays consistently high following menopause (67). As discussed previously, FSH may affect endothelial cell function (142, 173), either directly through endothelial progenitor cells (56) or indirectly through effects on lipid metabolism (169). The decline in FMD (123) and the reduced conductance with acute exercise (120) following menopause were both related to high FSH levels, and high postmenopausal FSH levels have also been related to CVD risk (44, 45); therefore, FSH likely exerts an independent effect on endothelial function and responses to exercise, but this has largely been uninvestigated.

Overall, endothelial resistance to the benefits of exercise may occur following menopause; however, the literature is conflicting and difficult to interpret due to variability across studies in measurement technique, classification of menopausal stage, and subject population. Further, most studies have small sample sizes, making results difficult to generalize. Future research investigating this question with more controlled experimental designs are needed. Finally, if this resistance does occur, it is unclear when this resistance begins (e.g. during perimenopause or postmenopause) and how to treat it.

Summary

During the menopausal transition, women experience a decline in cardioprotection, leading to adverse cardiovascular profiles. This decline may be partially attributable to declines in endothelial function. In a healthy, non-menopausal population, exercise training and acute exercise improve endothelial function. In a healthy menopausal population, the response to acute and chronic exercise has not been well characterized. While there have been several studies evaluating responses to exercise training, there are limited data across the menopausal transition and acute exercise studies are sparse. This dissertation was designed to characterize changes in endothelial function and responses to exercise with menopause, and the modulatory role of cardiorespiratory fitness on these responses. These outcomes could have important clinical implications. Specifically, they may inform when an intervention is appropriate, and if there is a point at which some of these changes may be irreversible with exercise. Further, we aimed to determine whether FSH is related to lipid levels in postmenopausal women, evaluating another potential therapeutic target to improve CVD risk in this population.

Figures

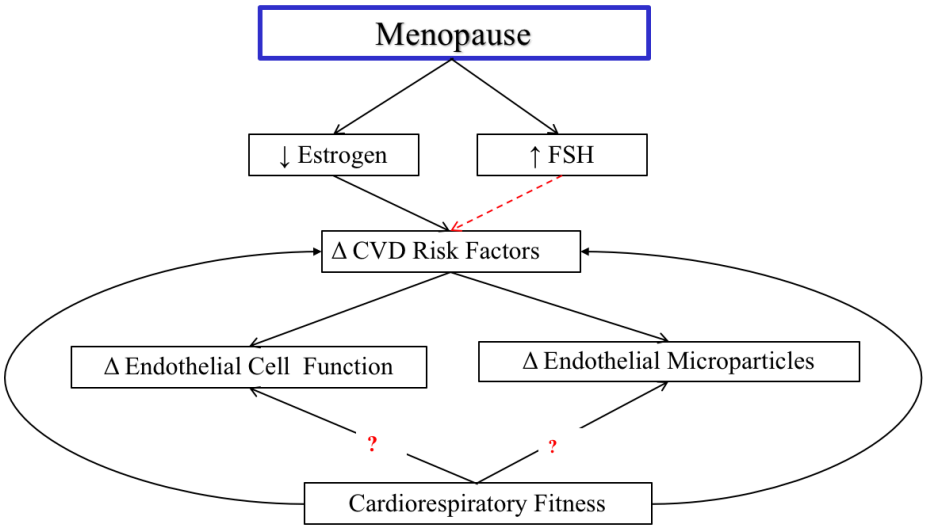


Figure 2.1. Conceptual Framework.
Menopause leads to a reduction in estrogen and an increase in follicle stimulating hormone (FSH), which adversely affect cardiovascular disease (CVD) risk factors; however, the mechanisms behind the effects of FSH on CVD risk factors are still unclear. Changes in risk factors for CVD adversely affect endothelial function and change endothelial microparticle levels. While cardiorespiratory fitness is generally considered protective of endothelial health, potentially through its influence on risk factors for CVD, whether this benefit remains in a menopausal population is unclear.

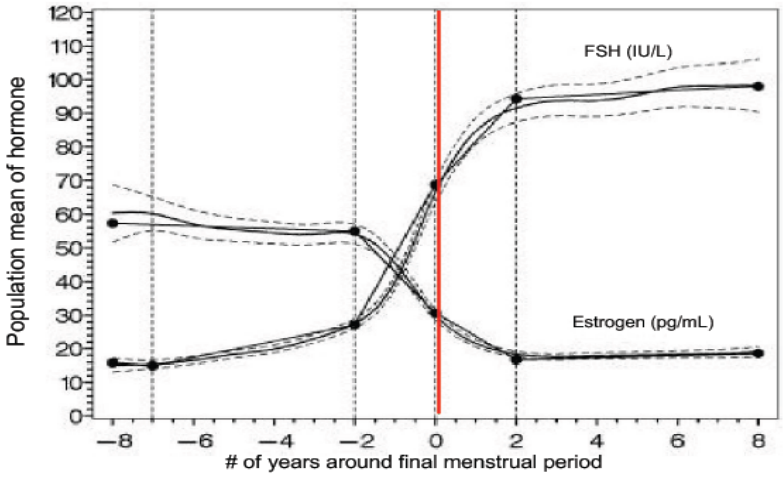


Figure 2.2. Changes in Estrogen and Follicle Stimulating Hormone.
Prior to a woman's final menstrual period, represented by the red line, estrogen levels begin to decline, while follicle stimulating hormone (FSH) levels rise. Estrogen remains low following the final menstrual period while FSH remains elevated (Adapted from Harlow et al., 2013).

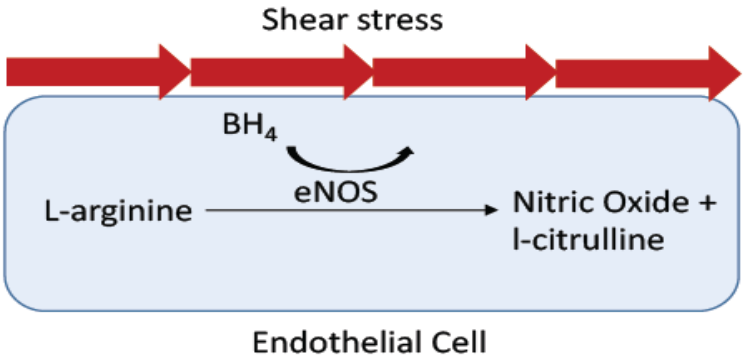


Figure 2.3. Nitric Oxide Formation.
In endothelial cells, l-arginine is converted to l-citrulline and nitric oxide when endothelial nitric oxide synthase (eNOS) is activated through its coupling with tetrahydrobiopterin (BH_4). This pathway is typically stimulated by shear stress on the endothelial cell surface.

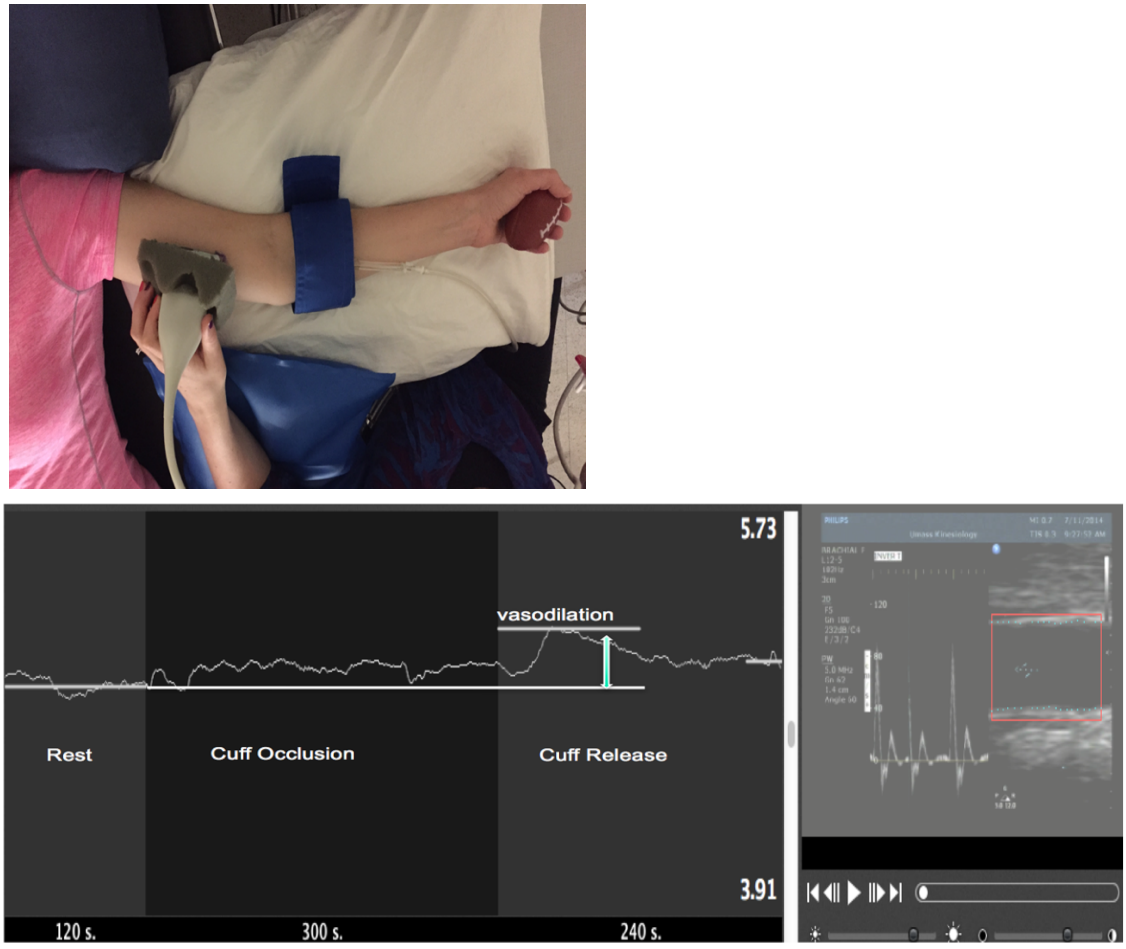


Figure 2.4. Flow-mediated Dilation Technique.
During each flow-mediated dilation study (FMD) an ultrasound and Doppler probe is used to image the brachial artery, with a small inflatable cuff placed around the participant's forearm (upper panel). Throughout the study artery diameter (bottom left) and flow rate (bottom right) are continuously tracked during 2 min of rest, 5 min of forearm blood flow occlusion, and 4 min following cuff release. When the cuff is released, vasodilation should occur and FMD is calculated as the change in artery diameter from baseline to maximum vasodilation (bottom panel).

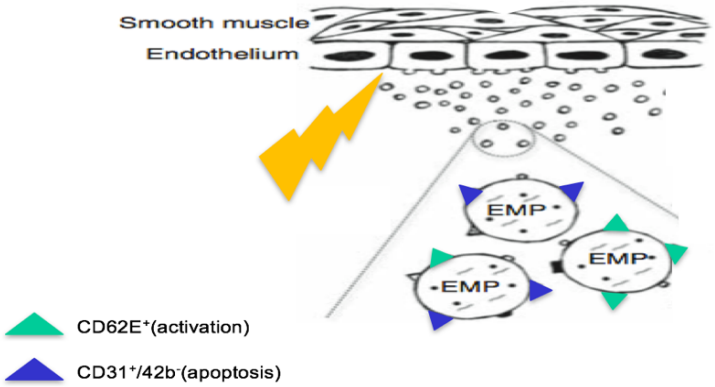


Figure 2.5. Endothelial Microparticle Formation.
Endothelial microparticles (EMPs) are released from the endothelium when it undergoes an acute stress. Microparticles are formed from the membrane of endothelial cells and contain markers that indicate the state of the endothelial cell of origin. These markers include markers of endothelial activation ($CD62E^{+}$) and of endothelial apoptosis ($CD31^{+/}42b^{-}$) (Adapted from Yong et al., 2013).

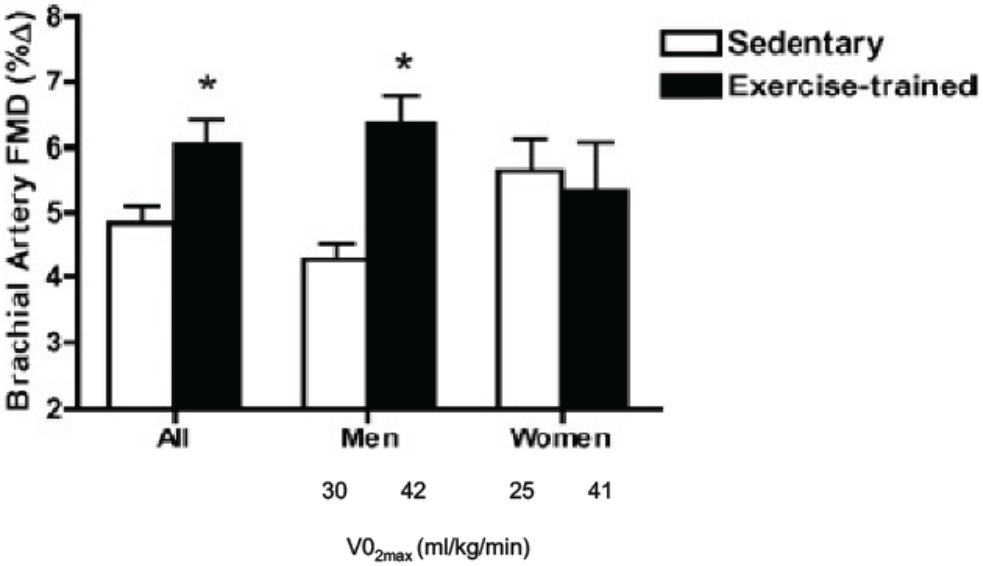


Figure 2.6. Sex Differences in the Relationship Between Cardiorespiratory Fitness and Endothelial Function.
In a cross-sectional analysis, endurance trained postmenopausal women and age-matched men were compared to their sedentary peers. While there was a benefit of cardiorespiratory fitness on endothelial function in men, this benefit was not present in postmenopausal women (Adapted from Pierce et al., 2011).

CHAPTER 3

METHODS

Introduction

The aims of this study were to determine 1) if there are differences in endothelial health in women in progressive menopausal stages before and after acute exercise; 2) if endothelial health and endothelial responses to acute exercise are affected by cardiorespiratory fitness; and 3) if follicle stimulating hormone (FSH) is related to lipid levels in a large cohort of postmenopausal women. These aims were addressed by: examining the magnitude of difference in brachial artery flow mediated dilation (FMD) and endothelial microparticles (EMPs) before and after an acute bout of exercise in women in progressive menopausal stages and with high and low cardiorespiratory fitness; and by determining whether FSH is associated with lipid levels in postmenopausal women participating in the Kuopio Ischaemic Heart Disease Risk Factor Study. These aims were evaluated using an experimental study (Aims 1 & 2) and an analysis of data from the Kuopio Ischaemic Heart Disease Risk Factor Study (Aim 3).

Experimental Study (Aims 1 and 2)

This study evaluated difference in endothelial function in women in progressive menopausal stages before and after acute exercise. The study included perimenopausal and late postmenopausal women with differing cardiorespiratory fitness levels and a high-fit premenopausal referent group.

Participant Characteristics:

Sample Size. The study was originally powered to include high-fit premenopausal, perimenopausal, and late postmenopausal women (n=12 per group). This number was based on sample size estimations using a power of 0.80 and an alpha of 0.05 and was powered to detect changes in the primary outcome, flow-mediated dilation. Flow-mediated dilation was calculated as both absolute (i.e. mm) and relative (i.e. percent relative to baseline) change in artery diameter. In order to detect changes in FMD in progressive menopausal stages, a sample size estimate was calculated based on FMD differences in women (mm change in brachial artery diameter) across the menopausal transition (mean ± SD; premenopause: 0.33 ± 0.05 mm; early perimenopause: 0.30 ± 0.08 mm; late perimenopause: 0.21 ± 0.08 mm; late postmenopause: 0.16 ± 0.04 mm) (123). Based on this estimate, 5 participants were needed per group to detect a difference between premenopausal and late perimenopausal women, while 31 were needed to detect a difference between premenopausal and early perimenopausal women; therefore, perimenopausal groups were combined. Twelve participants were needed per group to detect a difference between early and late perimenopausal women. Three participants were needed per group to detect a difference in premenopausal vs. late postmenopausal women and 6 participants were needed per group to detect a difference between late peri- and late post-menopausal women. A sample size estimate was also calculated from a study evaluating the FMD response to acute exercise in postmenopausal women, measured as percent change in brachial artery diameter (%FMD) from pre- (5.3 ± 1.3%) to post-exercise (9.9 ± 1.4%) (70). This estimate showed that 9 participants were needed per group. Finally, a sample size estimate was completed based on the FMD response to

acute exercise in 7 low-fit perimenopausal and 8 low-fit late postmenopausal women (141). To detect a difference in the FMD response to acute exercise in perimenopausal women (pre-exercise: 6.4 ± 1.6%; post-exercise: 8.5 ± 3.0%) 11 participants were needed per group, while 435 were needed to detect a difference in postmenopausal women. Given the lack of response to acute exercise in the postmenopausal group, this number was not included in the final sample size determination (pre-exercise: 6.5 ± 1.4%; post-exercise: 6.2 ± 2.9%). The final sample size was chosen based on the highest number of participants needed per group to detect either outcome. Calculations were completed with a one-tailed test, using means and standard deviations, as we hypothesized that FMD would decrease across the menopausal transition. Data collection was previously completed in low-fit women and included 7 perimenopausal and 8 late postmenopausal women.

Inclusion Criteria. Participants were stratified into menopausal stages based on STRAW+10 guidelines (67):

- Premenopause: regular menstrual cycles; women in the premenopausal group were at least 40 years old, to keep the average age similar to the perimenopausal group
- Early perimenopause: variable menstrual cycle length (more than 7 days different from normal and occurring at least twice within 10 consecutive cycles)
- Late perimenopause: 60 days or more, but less than 1 year of amenorrhea
- Late postmenopause: more than 5 years of amenorrhea

Early postmenopausal women were not included as a group based on work by Moreau et al. (2012) and Moore et al. (2012). Moreau et al. (2012) examined baseline

changes in flow-mediated dilation in women in progressive menopausal stages; the smallest difference in FMD between groups was between late perimenopausal and early postmenopausal women (123) (Figure 2.9). Moore et al. (2012) examined vascular responses to acute exercise and found that there was a blunted response beginning in late perimenopause and continuing into early postmenopause (120). There was no reason to believe that this blunting effect would not continue into late postmenopause. By selecting these groups, we were able to highlight changes in endothelial function that may occur during early menopausal stages, while also evaluating more prolonged trends that may occur over time with the inclusion of late postmenopausal women.

In addition to menstrual cycle criteria, participants also met the following inclusion criteria:

- 65 years old or younger
- Normal blood pressure (<140/90 mmHg)
- Non-diabetic (fasting plasma glucose <126 mg/dl)
- Participation in less than 150 min/week of moderate intensity physical activity or less than 75 min/week of vigorous intensity physical activity, accumulated in 10 minute bouts (i.e. low-fit) OR participation in at least 300 min/week of moderate intensity physical activity or at least 150 min/week of vigorous intensity physical activity, accumulated in 10 minute bouts, with a similar level of activity over the past 2 years (i.e. high-fit)
- Nonsmokers
- Normal lipid levels (LDL-C ≤159 mg/dl, HDL-C >40 mg/dl, TG<150 mg/dl)

- VO_{2peak} ≥80th percentile, if classified as high-fit

Exclusion Criteria.

- Hormone replacement therapy, menopausal symptom treatment, or oral contraceptive use in the past 6 months
- History of heart or blood vessel diseases (e.g. peripheral arterial disease, coronary artery disease)
- Previous heart attack or cardiovascular intervention (e.g. pacemaker implant)
- Hysterectomy prior to menopause or not have undergone natural menopause
- Self-reported long-term menstrual irregularities prior to menopause
- Use of certain vitamin/supplements, lipid lowering and/or anti-inflammatory medications in the past 4 weeks
- Current and/or history of breast cancer (with radiation or chemotherapy treatment), vaginal bleeding, abnormal uterine/ovary structure, blood clots, acute liver or gallbladder diseases
- Muscle, bone, or other condition that limits ability to exercise
- Contraindication to an exercise test
- Absolute or relative termination criteria during exercise test
- History of fainting with blood draws
- Pregnancy
- Participation in a research study that conflicted with this study's protocols

Protocol:

Study Overview:

All participants completed three study visits. The first visit involved testing of cardiovascular disease risk factors, a bone mineral density & body composition scan, and screening for other potential exclusion criteria. The second visit included a maximal exercise test and an FMD familiarization trial. The third visit was used for the majority of data collection on the primary outcome, FMD. The visit began with a blood draw and 2 FMD trials, followed by 30 minutes of moderate-intensity treadmill exercise, a period of rest and then a second blood draw and two more FMD trials. Study protocols are outlined in Table 3.1, along with potential exclusion time points (Table 3.2) and are discussed in detail below. There was no set time between visits.

Study Location:

Most experimental procedures were completed in the Molecular and Cardiovascular Physiology Lab, located in the Totman Building at the University of Massachusetts Amherst. A Lunar DEXA machine (Madison, WI) located at University Health Services on the Amherst campus was used for all DEXA scans. EMP analyses were completed in the Flow Cytometry Core on the Amherst campus.

Equipment:

Flow-mediated Dilation Equipment. A Philip's HDXE11 Ultrasound System (Bothell, WA) with an L-12-5 probe with an insonation angle set to 60° was used for all vascular imaging. All images were streamed directly to version 2.8.0 of the FMD Studio Software (Quipu, Pisa). The software collected continuous data on vessel diameter, shear

stress rate, and blood flow velocity. This software has been compared to the commonly used software, Brachial Analyzer, and was found to give measurements with a difference of less than 1% (47). Further, in a multicenter reproducibility study, the coefficient of variation for the software was 9.9 ± 8.4% for intra-session measurements and 12.9 ± 11.6% for inter-session measurements (57). The variability for inter-session measurements has been as low as 5.2% (153). This variability was minimized in this study by using the same imager for all FMD measurements, by using rapid cuff inflation/deflation (D.E. Hokanson, Bellevue, WA), and by monitoring blood pressure and ECG (G.E. Dash 2000, Milwaukee, WI) every minute during each study. The average variance for repeated pre- and post-exercise FMD trials in this study was 0.1%.

Blood Processing Equipment. Serum and plasma were isolated from blood samples using a Sorvall Legend X1R centrifuge (Osterode, Germany) and were stored in a Fisher Scientific Isotemp freezer (Asheville, NC) at -80°C until analysis. Estradiol and FSH were analyzed on a Multiskan MS Microplate Reader (Vaanta, Finland) using commercially available ELISA kits. Endothelial microparticles were analyzed using a BD Dual LSR Fortessa (San Jose, CA).

Exercise Equipment. A Cybex 550T treadmill (Medway, MA) was used for all graded exercise tests and exercise sessions. The Parvo Medics TrueOne 2400 (Sandy, UT) collected oxygen consumption data and was calibrated for gas analysis and flow volume before each test. Data output was computed using associated software. During the exercise test (Visit 2), 12-lead ECG was monitored using the Cardio-Card system (Brewerton, NY). During the exercise session (Visit 3), heart rate was monitored with a Polar FT1 Heart Rate Monitor (Kempelle, Finland).

Testing:

All study protocols were approved by the University of Massachusetts Amherst Institutional Review Board (protocol # 2014-2028).

Visit 1 (1 hour)

Orientation, Informed Consent, Questionnaires and Baseline Measurements. Participants were contacted by phone or email before the first visit and instructed to arrive fasted for 12 hours and to wear loose non-metallic clothing. Before beginning any testing protocols, participants signed an informed consent document (Appendix A). The document was explained to the participant and she was given time to review and ask any questions before signing. After signing the informed consent, the participant filled out a health history form (Appendix B), a quality of life questionnaire (Appendix C), and three physical activity questionnaires. The health history form was used to assess menopausal status and menopausal symptom history. The physical activity questionnaires included: International Physical Activity Questionnaire (Appendix D), to assess self-reported time spent in moderate and vigorous physical activity; Stanford Brief Activity Questionnaire (Appendix E), to assess occupational and leisure time physical activity; Physical Activity and Performance Questionnaire (Appendix F), to provide a detailed assessment of a participant's average weekly exercise routine. The physical activity questionnaires were used as an initial screening to verify the participant's physical activity level. Laboratory personnel explained the procedures for the day, and any questions participants had were answered. Baseline height, weight, and blood pressure were taken. Blood pressure was taken according to established guidelines (137), and after the participant had been seated and relaxing for several minutes. Blood pressure was taken 2-3 times and averaged.

Blood draw. The participant was instructed to sit in a reclining chair. A tourniquet was tied around the arm and trained laboratory personnel located the desired vein. Using sterile procedures, a needle was inserted into the target vein and ~5 ml of blood was extracted. The blood vial was labeled with de-identified participant information and brought to University Health Services for verification of inclusion/exclusion criteria for lipid levels and fasting plasma glucose.

DEXA. The participant was escorted to University Health Services Radiology Department to undergo a DEXA scan. This scan delivers a dose of radiation equivalent to approximately 1/20th that of a chest x-ray and is considered safe (131). Before each scan, a quality control check was performed to ensure that the machine could accurately identify the density of different tissues. The scan took approximately 5 minutes. The participant was asked to lie supine on the machine during the measurement. At the end of the scan, the participant was instructed to follow standard exercise testing restrictions, which included refraining from drinking caffeine, alcohol or eating for 3 hours and vigorous exercise for 24 hours prior to the second visit. The participant was also instructed to follow a 3-day low nitrate diet (Appendix G) to familiarize her with the diet protocol in preparation for the final visit. This helped to eliminate any nitrates in the diet that could affect measures of endothelial function.

Visit 2 (1.5 hours)

Flow-mediated Dilation Familiarization. When the participant arrived, she was informed of the procedures for the day and was given the opportunity to ask any questions. An FMD measurement was completed. This test was used as a familiarization

trial, to avoid potential heart rate and blood pressure changes due to the novelty of the FMD technique. The testing room was set to a comfortable temperature, with the lights dimmed. An automated blood pressure cuff was placed on the dominant arm and 3 electrodes were placed on the torso to monitor heart rate and blood pressure throughout the study. The test began with the participant relaxing on the bed in a supine position for ~10 minutes, with the non-dominant arm propped up with pillows to be level with the heart. A small ball was placed in the hand to maintain finger position and to avoid excessive movement that could have impacted results. A small inflatable cuff was placed around the widest part of the forearm and laboratory personnel took 3 resting blood pressure measurements and used an ultrasound and Doppler probe to image the brachial artery. After 2 minutes of baseline measurements, the cuff was inflated to 200 mmHg for 5 minutes and the technician continued to image the artery and take blood pressures every minute. After the 5 minute period, the cuff was deflated, and 4 minutes of measurements were recorded. Throughout the study, blood velocity, shear rate, and artery diameter were streamed digitally and captured with the FMD Studio Suite software.

The FMD technique can be influenced by a variety of factors including time of measurement, hormonal fluctuations, cuff placement, inflation duration, medication use, diet, and exercise. Multiple guidelines have been published to standardize the FMD technique (51, 63, 182), with a recent paper summarizing the necessary techniques for an FMD study to be of a high enough quality to be included in a systematic review (63). Using these guidelines, a study can score between 0-10, with 10 representing the best evidence. Our technique scored a 9.6 and we adhered to FMD measurement guidelines in the following ways:

1) Participants completed FMD studies in the morning.

2) Menstruating women (i.e. premenopausal and early perimenopausal) completed visit 3 during menstrual cycle days 1-6.

3) Participants refrained from eating for 6 hours, exercise, caffeine, smoking, and alcohol for 12 hours, and any vitamins/supplements for 72 hours prior to the third visit.

4) During each FMD study, imaging was completed on the non-dominant arm, with cuff placement distal to the imaging probe.

5) The forearm cuff was inflated to 200 mmHg during the five-minute occlusion period.

6) Continuous measurements of artery diameter and shear rate were taken throughout the study and using an insonation angle of 60°.

7) Each study was competed in a quiet, temperature-controlled room and did not begin until the participant had been resting supine for at least 10 minutes.

VO2 max. This test was utilized to determine maximal oxygen uptake, maximal heart rate, and if any electrocardiogram (ECG) or blood pressure abnormalities existed that would exclude the participant from the study. Maximal oxygen uptake and heart rate data were used to prescribe intensity during the acute exercise bout on visit 3. Prior to the visit, participants were stratified as low or moderate risk based on American College of Sports Medicine (ACSM) guidelines (Appendix H). A physician (Dr. Gregory Little) supervised exercise tests for moderate risk participants, while trained laboratory personnel supervised tests for low risk participants.

The testing protocol was explained to the participant and electrodes were placed on the chest in a modified 12-lead electrocardiogram (ECG) position (Appendix I). Resting blood pressure and heart rate/rhythm were recorded with the participant in a

supine, seated, and standing position. The Borg scale for rating of perceived exertion (RPE) was explained, as well as hand signals for communication during the test. The participant was fitted with a facemask and given several minutes to become accustomed to it, while being familiarized with the use of the treadmill. After this time, the testing protocol began. The participant began by self-selecting a brisk walking or comfortable jogging pace. The treadmill grade was increased by 2% every 2 minutes, and speed was increased as needed, until VO_{2max} was achieved, the participant indicated she did not want to continue, or an absolute or relative termination criterion occurred that necessitated an early termination of the test (Appendix J). Throughout the test, RPE and blood pressure were recorded every 2 minutes and heart rate was recorded every minute.

At the end of the test, the facemask was removed and the participant continued to walk on the treadmill at a comfortable speed for 2-4 minutes before sitting down. Measurements of heart rate and blood pressure continued every 2 minutes until heart rate and/or rhythm and blood pressure began to return to pre-test conditions, and for a minimum of 6 minutes. At this time, electrodes were removed. The participant was given a second low nitrate diet log and was instructed to refrain from eating for 6 hours, exercise, smoking, caffeine, and alcohol for 12 hours, and any vitamins/supplements for 72 hours before the final visit. If participants did not achieve a true VO_{2max}, according to standard criteria (Appendix J), VO_{2peak} values were recorded. VO_{2peak} was defined as the highest VO_2 value achieved during the exercise test. For all analyses, VO_{2max} and VO_{2peak} were treated as the same variable.

Visit 3 (3.5 hours)

The visit began with a blood draw and two baseline flow-mediated dilation studies (see visits 1 and 2 for protocol). Flow-mediated dilation and blood draws were also completed after acute exercise.

Acute Exercise Bout. The participant was asked to complete 30 minutes of exercise on the treadmill at the heart rate that was achieved during the VO_{2max} test corresponding to 60-64% of peak oxygen uptake. The participant began and ended the exercise session with a 5 minute warm up and cool down. Laboratory personnel used a heart rate monitor to verify that the participant maintained the desired intensity throughout the exercise session. Thirty minutes after the end of the cool down, a blood draw and two more flow-mediated dilation tests were performed.

Data Analysis:

Blood Analyses. Blood samples were centrifuged to separate serum or plasma, and then frozen at -80°C until analysis. Serum was analyzed for estradiol and follicle stimulating hormone (FSH) using 96-well colorimetric ELISA assays. All samples were run in duplicate. Plasma was analyzed for $CD31^{+}/42b^{-}$ and $CD62E^{+}$ endothelial microparticles using fluorescent activated cell sorting. Briefly, samples were collected in acid citrate dextrose tubes and spun to obtain platelet free plasma, and later, cell-free plasma. Cell free plasma was incubated with antibodies for CD62E (BV421), CD31 (APC), and CD42b (PE). CountBright beads (Life Technologies) were added prior to analysis, to allow for calculations of EMPs/µl plasma. Samples were fixed with 2% paraformaldehyde and analyzed using fluorescent activated cell sorting within 24 hours.

Data was analyzed using FACS DIVA software, with 900 nm calibration beads used to set the forward and side-scatter parameters.

Flow-mediated Dilation Analysis

Brachial artery diameter was analyzed using the FMD Studio software. Brachial artery data was digitized during data collection and streamed to the software from the Philip's HDXE11 Ultrasound System during the flow-mediated dilation studies. To determine the most accurate FMD values for each study, videos were re-analyzed until a region of interest was selected that allowed for a clear analysis of baseline and peak diameter. When this was not possible, separate analyses were completed with a video that optimized the region of interest for baseline diameter and a separate video for peak diameter. These analyses were reviewed and edited until two independent analyses determine the same artery diameter at each time point within 0.02 mm. In this case, the average of the two values was used. Percent change in brachial artery diameter was calculated for all studies.

Kuopio Ischaemic Heart Disease Risk Factor Study (Aim 3)

The Kuopio Ischaemic Heart Disease Study is a prospective cohort study including midlife and older men and postmenopausal women in Finland. This analysis only included postmenopausal women who completed a baseline visit and did not report hormone therapy use (n=588). Participants were classified as postmenopausal if they had experienced at least one year of amenorrhea. Women were recruited into four distinct age groups: 53-56 years old, 59-62 years old, 64-68 years old and 71-73 years old. All

participants underwent a comprehensive screening for biological variables including, but not limited to, blood pressure, lipid levels, menstrual status, and diabetes status.

Statistical Analyses:

All data are presented were analyzed using SPSS software with a significance level of alpha = 0.05. Data were evaluated for the adherence to assumptions for each statistical test proposed. Shapiro Wilk tests were used to assess normality and Levene's tests were used to assess equal variance. When necessary, data was transformed to meet the assumptions of the statistical tests. Differences between group baseline characteristics were analyzed with an ANOVA, chi-squared or independent t-tests. The specific aims were evaluated using the following approach:

Aim 1a: To determine whether there are differences in endothelial health, assessed via flow-mediated dilation and $CD31^{+}/42b^{-}$ and $CD62E^{+}$ endothelial microparticles, in women in progressive menopausal stages.

Aim 1b: To determine whether cardiorespiratory fitness affects differences in endothelial health in women in progressive menopausal stages.

Aim 2a: To determine whether there are differences in endothelial responses, assessed via flow-mediated dilation and $CD31^{+}/42b^{-}$ and $CD62E^{+}$ endothelial microparticles, to acute exercise in women in progressive menopausal stages.

Aim 2b: To determine whether cardiorespiratory fitness affects differences in endothelial responses to acute exercise in women in progressive menopausal stages.

Differences in FMD and EMPs were assessed using repeated measure ANOVAs. Differences by menopausal status were evaluated in high-fit women (exercise x menopausal status). Differences by menopausal status and fitness were also evaluated in perimenopausal and postmenopausal women combined (menopausal status x fitness x exercise). To evaluate differences at each time point between groups, one-way ANOVAs, independent t-tests, or an equivalent non-parametric test were used. To evaluate differences between time points within each group, paired t-tests or an equivalent non-parametric test were used.

Aim 3: To evaluate the relationship between FSH and lipid levels in women in a large cohort of postmenopausal women.

The relationship between FSH and lipid levels was evaluated in postmenopausal women participating in the Kuopio Ischaemic Heart Disease Risk Factor Study. Participants were stratified into quartiles based on baseline FSH levels and any differences in baseline characteristics between groups were assessed using an ANOVA or chi-squared tests. Linear regression was used to evaluate the relationship between FSH and lipid levels. Covariates included years since final menstrual period, length of estrogen exposure (e.g. age at menarche, oral contraceptive use, hormone therapy use, parity, hysterectomy, oophorectomy), estradiol, testosterone, sex hormone binding globulin, physical activity and cardiovascular disease risk factors, such as smoking history, blood pressure, body mass index (BMI) and waist-to-hip-ratio (WHR). All variables were checked for normality and transformed, if necessary. Model 1 evaluated

the relationship between lipid levels and FSH after adjusting for age, date of examination, and hormonal factors. Model 2 adjusted for cardiovascular disease risk factors. Model 3 adjusted for use of lipid lowering medications. Models were assessed to determine which variables were most related to lipid levels and FSH to form the final model. Logistic regression was used to evaluate odds ratios in women who had signs of dyslipidemia (i.e. medication use or values indicative of dyslipidemia) compared to those who did not, using the linear regression models to inform which covariates to include. Groups were also stratified by age and BMI to evaluate if any effect modification was present.

Summary

Data from these studies have the potential to enhance knowledge about factors that may influence CVD risk with menopause. Findings will allow for a characterization of the magnitude and timing of changes in endothelial health that may accompany menopause. These results will also identify whether cardiorespiratory fitness remains beneficial for endothelial health during and after the menopausal transition. Finally, the characterization of the relationship between FSH and lipid levels will help to enhance our understanding of the influence of FSH on CVD risk in postmenopausal women. Together with future research, these outcomes may allow for more targeted interventions to reduce the increase in CVD risk factors with menopause.

Tables

Visit Number	Tasks
Before Visit 1	• Pre-screening interview • Refrain from eating and drinking, except water for 12 hours • Wear loose fitting, nonmetallic clothing • If desired, fill out questionnaires and read informed consent

Visit 1	• Review:
	  ○ Informed consent
	  ○ Health History
	  ○ Physical Activity Questionnaires
	  ○ Quality of Life Questionnaire
	• Measure height, weight, blood pressure
	• Complete blood draw (lipids and glucose)
	• Complete DEXA scan

Before Visit 2	• Follow low nitrate diet for 3 days • Refrain from eating, drinking, and smoking 3 hours before, and vigorous exercise 24 hours before visit 2
Visit 2	• Familiarization FMD* • VO_2 max test
Before Visit 3	• Follow low nitrate diet for 3 days • Early perimenopausal and premenopausal women arrive for visit 3 between cycles days 1-6 • Refrain from eating for 6 hours prior to the visit • Refrain from exercise, smoking, caffeine, alcohol for 12 hours before the visit • Refrain from taking vitamins/supplements for 72 hours before the visit
Visit 3	• Baseline FMD and blood draw • Exercise session • Post-exercise FMD and blood draw

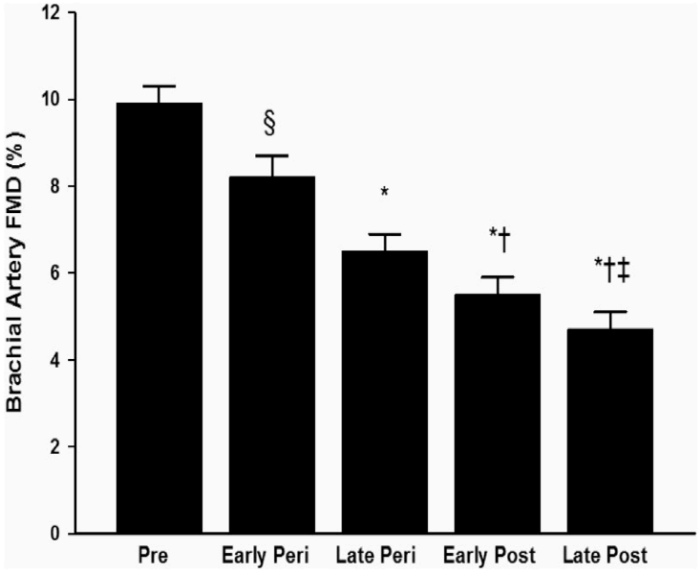
Table 3.1. Study Overview

*FMD: flow mediated dilation (vessel function study); DEXA: dual energy x-ray absorptiometry (body composition study); QOL: quality of life

Visit	Possible exclusion
Before visit 1	Age; does not fit criteria for menopausal groups; history of cardiovascular disease, diabetes or other exclusion criteria listed on pre-screening form; unable to exercise; participating in another research study that may confound results
Visit 1	Blood pressure; physical activity questionnaire suggests participant does not meet physical activity criteria; answers on health history that affect inclusion/exclusion criteria; pregnancy
Before Visit 2	Elevated plasma glucose levels or abnormal lipid profile
Visit 2	Absolute or relative contraindication during or before graded exercise test; VO_{2peak} below the 80th percentile for high-fit women

Table 3.2. Possible Exclusion Time Points.

Figures



*, P < 0.001 and §, P = 0.03 vs. premenopausal women; †,P < 0.001 vs. early perimenopausal; ‡, P < 0.001 vs. late perimenopausal.

Figure 3.1. Endothelial Function in Women in Progressive Menopausal Stages.
Data from a cross-sectional analysis showed that the smallest difference in FMD between groups in progressive menopausal stages is between early and late postmenopause (Moreau et al., 2012).

CHAPTER 4

CARDIORESPIRATORY FITNESS MAY NOT ATTENUATE THE DECLINE IN ENDOTHELIAL FUNCTION WITH MENOPAUSE

Introduction:

Cardiovascular disease (CVD) is the leading cause of death in women in developed countries and accounts for approximately 400,000 deaths per year in women in the United States (58). Menopause, or the cessation of ovarian function, represents a time during which risk factors for cardiovascular disease (CVD) increase dramatically. The increase in CVD risk has been attributed to the reduction in estrogen and increase in follicle stimulating hormone (FSH) that accompany menopause (44). These changes begin during the menopausal transition and continue into postmenopause (108, 109).

Endothelial dysfunction is a preclinical marker for CVD (3) and has been shown to be prognostic of cardiovascular events in postmenopausal women (147). Despite this, few studies have evaluated changes in endothelial function with menopause. Several studies have demonstrated reduced endothelial function in postmenopausal compared to premenopausal women (70, 71, 151); however, there is limited evidence on women undergoing the menopausal transition. Given that the years surrounding menopause are a time of dramatic physiological changes, midlife is a key time to evaluate CVD risk.

Evaluating endothelial responses to acute exercise may reveal differences in endothelial function in populations with differing CVD risk that are not otherwise apparent. We have previously shown lower endothelial function in post- compared to peri-menopausal women that was not detectable until after acute exercise (158). This

divergent response has also been shown in sedentary vs. active men, with lower function in the sedentary men that was only detectable after an acute bout of exercise (68).

High cardiorespiratory fitness (CRF) is generally considered protective of endothelial function and higher levels of endothelial dysfunction have been shown in sedentary compared to active men (139). However, the benefits of cardiorespiratory fitness on endothelial function in transitioning and postmenopausal women have yet to be established. Two cross-sectional analyses showed no differences in endothelial function in trained vs. sedentary postmenopausal women (139, 151), suggesting that the benefits of fitness on endothelial function may not remain after menopause. Whether this lack of benefit occurs in perimenopausal women is unknown.

Overall, there are limited data evaluating differences in endothelial function in women at different menopausal stages. Further, the effect of CRF on endothelial function in this population is also unclear. Therefore, the aim of this study was to evaluate differences in endothelial function in high- and low-fit perimenopausal and postmenopausal women before and after an acute bout of exercise.

Methods:

Healthy high-fit premenopausal (n=11), perimenopausal (n=12), and postmenopausal women (n=13) and low-fit perimenopausal (n=7) and postmenopausal women (n=8) were recruited for this study. FMD data on low-fit women has been previously published (158). All participants were between 40 and 65 years old and were non-smokers. Participants were excluded according to resting blood pressure (>140/>90 mmHg), fasting plasma lipids (LDL-C >160 mg/dl, HDL-C <40 mg/dl, TG> 150 mg/dl), and fasting plasma glucose (>126 mg/dl). Participants were also excluded if they reported

a history of cardiovascular diseases, long-term menstrual irregularities, breast cancer with radiation or chemotherapy treatment, vaginal bleeding, abnormal uterine/ovary anatomy that negatively impacted fertility, if they used medications and/or supplements that are known to influence endothelial function, or if they had any exercise limitations.

Menopausal status was defined using STRAW+10 (67) criteria based on self-reported menstrual cycle. Premenopausal women were experiencing regular menstrual cycles, perimenopausal women were experiencing irregular cycles or 2-11 months of amenorrhea, and postmenopausal women had experienced at least 5 years of amenorrhea. All participants were between 40 and 65 years old, non-smokers and had not taken hormone therapy within the 6 months prior to study enrollment. Postmenopausal women had to undergo natural menopause to qualify for the study. Follicle stimulating hormone (FSH, http://www.abcam.com/human-follicle-stimulating-hormone-elisa-kit-ab108641.html) and estrogen (17-beta–estradiol, https://www.thermofisher.com/order/catalog/product/KAQ0621) levels were assessed in all participants as a complement to menstrual cycle criteria. Estradiol and FSH were not evaluated in 2 low-fit perimenopausal women, as blood samples were not taken in those participants.

Participants were initially stratified by CRF based on self-reported physical activity levels assessed via the International Physical Activity Questionnaire (IPAQ). Low-fit women were not meeting physical activity guidelines (150 minutes/week of moderate intensity activity, 75 minutes/week of vigorous intensity activity, or an equivalent combination of the two, accumulated in 10 minute bouts). High-fit women were, on average, at least doubling guidelines and had been doing so for 2 years prior to

study enrollment. High-fit participants had to achieve a VO_{2peak} of at least the 80th percentile for their age, based on American College of Sports Medicine guidelines (183) to qualify for the study. After initial screenings, each participant completed three study visits. The University of Massachusetts Amherst Institutional Review Board approved all study protocols and participants signed an informed consent document before beginning any study visits.

Study Overview

On the first visit, participants underwent a dual energy x-ray absorptiometry scan (DEXA) to assess body composition, anthropometric measurements, a blood pressure screening, and measurements of fasting plasma glucose and cholesterol. During the second visit, participants underwent an FMD familiarization trial and a ramped treadmill VO_{2max} test (Parvo Medics TrueOne 2400, Sandy, UT) with 12 lead electrocardiogram. Heart rate and VO_2 data from the test was used to prescribe exercise intensity on the third visit.

On the third visit, participants underwent 2 initial FMD trials. Following the trials, they were fitted with a chest-worn heart rate monitor (Polar FT1, Polar Electro, Lake Success, NY) and then exercised on a treadmill for 30 min at the heart rate corresponding to 60-64% VO_{2peak}. Throughout the exercise session, heart rate, blood pressure and rate of perceived exertion were assessed every 2 min. Workload was adjusted as necessary to maintain the desired heart rate. Each session began and ended with a 5 min warm-up and cool-down.

Flow-mediated Dilation

Brachial artery flow-mediated dilation (FMD) was measured following established guidelines (182), and as previously published (158). Briefly, participants were instructed to arrive for the third visit having followed a three-day low nitrate diet and having refrained from taking any vitamins/supplements for 72 hours, from consuming any alcohol or caffeine or completing any exercise for 12 hours, and from consuming any foods or beverages, with the exception of water, for 6 hours prior to the visit. Menstruating women (i.e. early perimenopausal and premenopausal women) were measured between menstrual cycle days one and six.

All FMD measurements took place in a quiet temperature-controlled room and began in the morning. The study began following a 10 min supine rest. Measurements were taken on the non-dominant arm with an ultrasound and Doppler probe (Philip's HD11XE Ultrasound System, Bothell, WA) placed proximal to an inflatable cuff on the participant's forearm (D. E. Hokanson, Bellevue, WA) and with an insonation angle of 60°. Blood pressure was taken on the non-dominant arm every minute using an automatic cuff and heart rate was recorded throughout the study with a 3-lead electrocardiogram (GE Dash 2000, Friedurg, Germany). Throughout the study, brachial artery diameter was continuously tracked during 2 min baseline, 5 min forearm cuff occlusion (200 mmHg) and 4 min following cuff release.

Data Analysis:

All statistical analyses were completed in SPSS v24. Significance was set at an alpha level of 0.05. All data was evaluated for adherence to the assumptions of each statistical test. Some variables were not normally distributed due to a few extreme data

points (e.g. a constrictor response to FMD). Analyses were performed with and without the extreme values included. Given that the outcomes were the same, analyses performed in all participants are reported. Differences in baseline characteristics were assessed using one-way ANOVAs, t-tests, or an equivalent non-parametric test. Differences were evaluated in high- and low-fit women within the perimenopausal and postmenopausal groups and in high-fit premenopausal, perimenopausal, and postmenopausal women.

All FMD trials were analyzed using Cardiovascular Suite Software (FMD Studio, Quipu, Pisa, Italy). Each trial was evaluated to determine the average baseline and peak diameter achieved during the study. FMD was calculated as ($Diameter_{peak}$-$Diameter_{baseline}$)/ $Diameter_{baseline}$) * 100. To capture full vasodilatory capacity (i.e. max-FMD), studies were also evaluated for the minimum artery diameter following cuff release. Max-FMD was calculated as ($Diameter_{peak}$-$Diameter_{minimum}$)/ $Diameter_{baseline}$) * 100. Differences in FMD parameters were evaluated using repeated measure ANOVAs and post-hoc testing. Given that there was only a high-fit premenopausal group, the overall effect of fitness was assessed in perimenopausal and postmenopausal women only. To evaluate the influence of menopausal status, data was also assessed in the high-fit groups. FMD data was not obtained in one premenopausal participant due to equipment failure. In order to perform repeated-measure ANOVAs, max-FMD was natural log transformed. Non-transformed data are presented for ease of interpretation. Pearson correlations were evaluated in all participants to assess if age, FSH, or estradiol were related to FMD or max-FMD. Data are presented as mean ± SEM.

Results

Participant characteristics:

Participants were healthy, had few risk factors for cardiovascular disease, and had similar baseline characteristics across menopausal stage and fitness categories (Table 4.1). Within perimenopausal women, the only differences between groups were lower body fat percentage and body mass index (BMI) and higher time spent in moderate-to-vigorous physical activity (MVPA) and VO_{2peak} in high-fit women. Within postmenopausal women, the only differences were higher MVPA, $VO_{2peak,}$ and HDL-C and lower body weight and body fat percentage in high-fit women. Within high-fit women, there was a significant difference in age across all groups, though the overall age range was only 16 years. All other risk factors were similar across high fit-premenopausal, perimenopausal, and postmenopausal women, with the exception of lower FSH in premenopausal women, higher HDL-C in perimenopausal compared to premenopausal women, and lower estradiol in postmenopausal compared to premenopausal women.

Flow-mediated dilation

There was a main effect of menopausal status on FMD within high-fit women ($p=0.005$) and within perimenopausal and postmenopausal women combined ($p=0.047$). Within high-fit women, FMD was lower in the postmenopausal compared to the premenopausal group ($p=0.004$, Figure 4.1A). Within perimenopausal and postmenopausal women, FMD was lower in postmenopausal women ($p= 0.047$, Figure 4.1B). There was a main effect of fitness ($p=0.006$); within high- and low-fit

perimenopausal and postmenopausal women; FMD was lower in the high-fit women (high: 5.0±0.4%, low: 6.9±0.5%, Figure 4.2). In perimenopausal women, there was no difference in FMD before exercise (high: 6.1±1.3%, low: 6.4±0.6%, p=0.773); however, in response to acute exercise, the high-fit women had lower FMD compared to the low-fit women (high: 5.5±0.6%, low: 8.5±1.1%, p=0.021). Conversely, in postmenopausal women, there was a difference in FMD before (high: 4.2±0.7%, low: 6.5±0.5%, p=0.043), but not after acute exercise (high: 4.2±0.5 %, low: 6.2±1.0% p=0.08; Figure 4.3). There was no difference in baseline artery diameter within or between groups at any time point (Table 4.2).

In all participants combined, pre- and post-exercise FMD was negatively related to age (pre-exercise: r=-0.421, p=0.003; post-exercise: age: r=-0.455, p=0.001) and FSH (pre-exercise: r=-0.308, p=0.038; post-exercise, r=-0.372, p=0.009 Figure 4.4A), but not estradiol (pre-exercise: r=0.093, p=0.40; post-exercise: r=0.205, p=0.16, Figure 4.4B)

Max-FMD

There was no effect of menopausal status on max-FMD in high-fit women (p=0.285). However, there was an effect of menopausal status in perimenopausal and postmenopausal women combined (p=0.009, Fig 4.5), with lower max-FMD in perimenopausal vs. postmenopausal women (perimenopause: 6.9±0.7%, postmenopause: 9.8±0.7%) and no effect of fitness (p=0.422) or exercise (p=0.553). Within low-fit women, there was a main effect of menopausal status (p=0.005), with lower max-FMD in postmenopausal compared to perimenopausal women, both before (perimenopause: 9.8±1.0%, postmenopause: 6.6±0.9%, p=0.029) and after exercise (perimenopause: 12.7±3.9%, postmenopause: 6.4±0.7%, p=0.029, Figure 4.6).

Similar to FMD, in all participants combined, max-FMD was negatively related to age (pre-exercise: r=-0.292, p=0.04; post-exercise: r=-0.342, p=0.016). Max-FMD was related to FSH before, but not after exercise (pre-exercise: r=-0.315, p=0.029; post-exercise: r=-0.130, p=0.384). There was no relationship between max-FMD and estradiol (pre-exercise: r=0.157, p=0.287; post-exercise: r=0.039, p=0.794).

Discussion

The aim of this study was to evaluate differences in endothelial function in women at different menopausal stages and with differing levels of cardiorespiratory fitness (CRF). The primary findings were: 1) FMD was lower in postmenopausal compared with premenopausal women, 2) CRF did not have a beneficial effect on FMD in healthy perimenopausal or postmenopausal women, 3) CRF affected the FMD response to acute exercise in perimenopausal women, 4) follicle stimulating hormone (FSH) was negatively associated with FMD, 5) max-FMD was not influenced by menopausal status, but may have been influenced by cardiorespiratory fitness.

There is clear evidence to suggest that endothelial function declines in the postmenopausal years. Multiple cross-sectional analyses have shown reduced FMD in postmenopausal compared to premenopausal women (70, 71, 151). However, few studies have evaluated changes in endothelial function that may occur during the perimenopausal years- when important physiological changes are occurring. In a cross-sectional analysis of healthy, low-fit women at different menopausal stages, there was a stepwise reduction in FMD with progressive menopausal stage (123). Our data support and extend these findings. We show that in highly fit healthy women, endothelial function is lower in the postmenopausal years. We further demonstrate lower function in healthy postmenopausal

compared to perimenopausal women. Together, these data suggest that there is a decline in endothelial function with menopause that is not mitigated by high levels of cardiorespiratory fitness.

Endurance exercise training is considered a potent stimulus to improve endothelial function in most populations; however, the response to exercise training in postmenopausal women is controversial (204). Some studies have reported improvements in FMD with training (13, 176), while others have shown no benefit of training (89, 139). These conflicting results may be due to differences in menopausal classification, study populations, and FMD protocols across studies. When solely evaluating the effect of CRF on FMD, there is increasing evidence to suggest that it may not have an effect on FMD in a postmenopausal population. When comparing postmenopausal sedentary women to postmenopausal active women, there was no difference in FMD between the two (151). This lack of benefit was also shown in a separate study in which older active men had enhanced FMD compared to age-matched sedentary men, but there was no difference between active and sedentary age-matched postmenopausal women (139). Although the resistance to the benefits of fitness on endothelial function have been shown in postmenopausal women, whether this benefit remains in perimenopausal women had not been investigated to this point.

Our data suggest that there is no protective effect of fitness on endothelial function in perimenopausal or in postmenopausal women. This may indicate that the effects of a changing hormonal environment outweigh the effects of exercise. In support of this, Moreau et al. reported that postmenopausal women did not improve FMD with exercise training unless they were supplemented with estradiol (124). Interestingly, we

found no relationship between FMD and estradiol, but a significant negative relationship between FSH and FMD. During perimenopause, prior to the final menstrual period, FSH levels rise and then remain elevated in postmenopause (67). Increasing evidence suggests that FSH may have an effect on CVD risk that is independent of the effects of changing estrogen levels (11, 198). The relationship between FSH and endothelial function is unclear; however, Moore et al., reported a negative relationship between vascular conductance and FSH in women at different menopausal stages (120). Further, a negative relationship between FSH and FMD was also reported in women in progressive menopausal stages (123) and together with our data, suggests that FSH may influence endothelial function in aging women.

The endothelial response to acute exercise may provide further insight into differences in endothelial function that are not otherwise apparent. We have previously reported no difference in FMD in low-fit perimenopausal vs. postmenopausal women before exercise; however, perimenopausal women showed a trend for higher FMD after acute exercise (158). In the current analysis, we also found a divergent response to acute exercise in perimenopausal women, such that low-fit perimenopausal women had higher FMD than high-fit women following exercise. This divergent response may be driven by habituation to the exercise stimulus in the high-fit women. This hypothesis is supported by the lack of response to acute exercise in the high-fit premenopausal, perimenopausal, and postmenopausal groups. Interestingly, neither postmenopausal group responded to acute exercise. This may suggest that the lack of an effect of long-term exercise training in postmenopausal women may be due to the lack of an acute response to exercise.

Brachial artery FMD is a well-validated technique and is considered a gold standard for assessing nitric-oxide mediated vasodilatory capacity. FMD is traditionally measured by assessing the difference in baseline artery diameter compared to the peak vasodilatory diameter. However, there is increasing recognition that constriction may occur during the cuff occlusion period of an FMD trial (i.e., low-flow mediated constriction)(60, 69, 199). If artery diameter is lower than baseline following cuff release, this may lead to an underestimation of vasodilatory capacity, and therefore, endothelial function. Since we observed trials with diameters less than baseline following cuff release, we calculated the change in artery diameter from the minimum value achieved following cuff release to peak vasodilation (max-FMD). This analysis eliminated all differences by menopausal status that we observed in high-fit women when analyzing FMD in the traditional manner. However, in low-fit women an effect of menopausal status remained. Therefore, contrary to the results of the traditional FMD analysis, these data indicate that fitness is protective of the decline in endothelial function in postmenopause.

When assessing max-FMD, values across all groups were significantly enhanced compared to the traditional FMD calculations. Most notably, the average FMD in high-fit postmenopausal women was approximately 4.2%, which would indicate significant endothelial dysfunction. Our participants had a low cardiovascular disease risk profile and the FMD values we report are in line with what others have reported in high-fit postmenopausal women (151). Therefore, max-FMD may better represent endothelial function in this population. Taken together, the differing interpretation based on our FMD

data vs. max-FMD data suggest that further evaluation should be made regarding the contribution of total vessel dilatory capacity in FMD studies.

Limitations

While this study provides novel insight into changes in endothelial function with menopause and the role of cardiorespiratory fitness, it has several important limitations. Participants in this study were selected to have few risk factors for cardiovascular disease, to allow us to better evaluate the independent effects of menopause and cardiorespiratory fitness on FMD. While this may be considered a strength of the study, it is possible that women in both fitness categories were too healthy to see a significant difference in FMD between groups. Sample sizes in this study were small, particularly in the low-fit groups, therefore we may be underpowered for some analyses. Finally, given that this study was cross-sectional and designed to characterize differences in endothelial function across groups, we are unable to determine causation or evaluate what factors may be contributing to the observed responses. For examples, we may not have found a benefit of fitness due to remodeling of the vasculature that has occurred in high-fit women, leading to a larger lumen:wall ratio with no change in FMD or that high-fit women are relying on other vasodilatory factors; these warrant further investigation. Given that menopause and aging are intimately connected, it is possible that some of the differences that we observed between groups were due to differences in age; however, the range of ages across groups was as narrow as possible. Further, we separated participants into quartiles based on age and found that there was no effect of age when evaluating FMD in perimenopausal and postmenopausal women. There was an effect of age when evaluating high-fit women, with a significant difference in the youngest compared to the

oldest women (p=0.018). There was no difference in age within each menopausal stage by fitness, therefore we do not believe that age influenced these results.

Conclusion

Data from this study suggests that postmenopause is associated with a reduction in endothelial function and that fitness is not protective of endothelial function in perimenopausal or in postmenopausal women. This reduction may be associated with the increase in FSH that accompanies menopause. This may suggest that factors other than cardiorespiratory fitness may be important targets for improving CVD in aging women. It also highlights the need for further research to better understand changes in endothelial function and the role of cardiorespiratory fitness in perimenopausal and postmenopausal women.

Funding Sources: American College of Sports Medicine Foundation Doctoral Student Research Grant & University of Massachusetts Amherst Faculty Research Grant

Tables

	Premenopause	Perimenopause		Postmenopause	
	High-fit (n=11)	**Low-fit (n=7)^**	**High-fit (n=12)**	**Low-fit (n=8)^**	**High-fit (n=13)**
Age (yrs)	44.5 ± 1.0	47.3 ± 1.5	50.8 ± 1.0#	58.9 ± 1.4	60.5 ± 1.0#$
Height (cm)	163.5 ± 1.2	163.7 ± 3.1	165.4 ± 2.0	166.5 ± 3.1	161.6 ± 2.3
Weight (kg)	61.8 ± 2.2	72.9 ± 7.2	62.5 ± 3.0	69.0 ± 4.3	58.2 ± 2.5*
Estradiol (pg/ml)	58.7 ± 10.7	117.3 ± 27.1	75.2 ± 32.7	16.5 ± 9.0	6.7 ± 4.2#
FSH (mIU/mL)	8.5 ± 2.2	49.9 ± 14.0	66.6 ± 17.9#	104.2 ± 8.1	102.6 ± 9.5#
Body Fat (%)	29.6 ± 2.5	40.8 ± 2.6	26.9 ± 2.1*	41.9 ± 1.5	28.0 ± 2.1*
BMI (kg/m^2)	23.1 ± 0.7	27.0 ± 2.3	22.7 ± 0.7*	24.8 ± 1.3	22.2 ± 0.7
HDL-C (mg/dL)	71.2 ± 4.9	70.4 ± 9.7	82.0 ± 4.8	78.4 ± 4.8	94.7 ± 2.8*#
LDL-C (mg/dL)	101.9 ± 7.0	84.7 ± 8.4	98.8 ± 6.1	116.5 ± 9.5	117.1 ± 7.2
TG (mg/dL)	50.1 ± 5.8	52.0 ± 6.9	40.6 ± 7.1	46.4 ± 5.7	42.8 ± 3.6
FPG (mg/dL)	94.5 ± 2.0	92.4 ± 3.0	93.9 ± 1.8	99.5 ± 1.7	96.5 ± 1.9
SBP (mmHg)	102.7 ± 2.4	104.6 ± 5.6	106.0 ± 2.8	117.3 ± 5.2	106.3 ± 3.7
DBP (mmHg)	58.9 ± 1.5	64.6 ± 4.5	59.2 ± 1.8	64.6 ± 2.7	61.9 ± 2.3
MVPA (MET-min/wk)	3446.6 ± 429.5	293.1± 98.0	3695.9 ± 528.5*	100.3 ± 40.9	4107.1 ± 684.1*
VO_2 peak (ml/kg/min)	47.0 ± 2.4	30.1 ± 1.6	49.1 ± 2.4*	28.3 ±1.1	43.8 ± 1.8*

Table 4.1. Participant Characteristics.

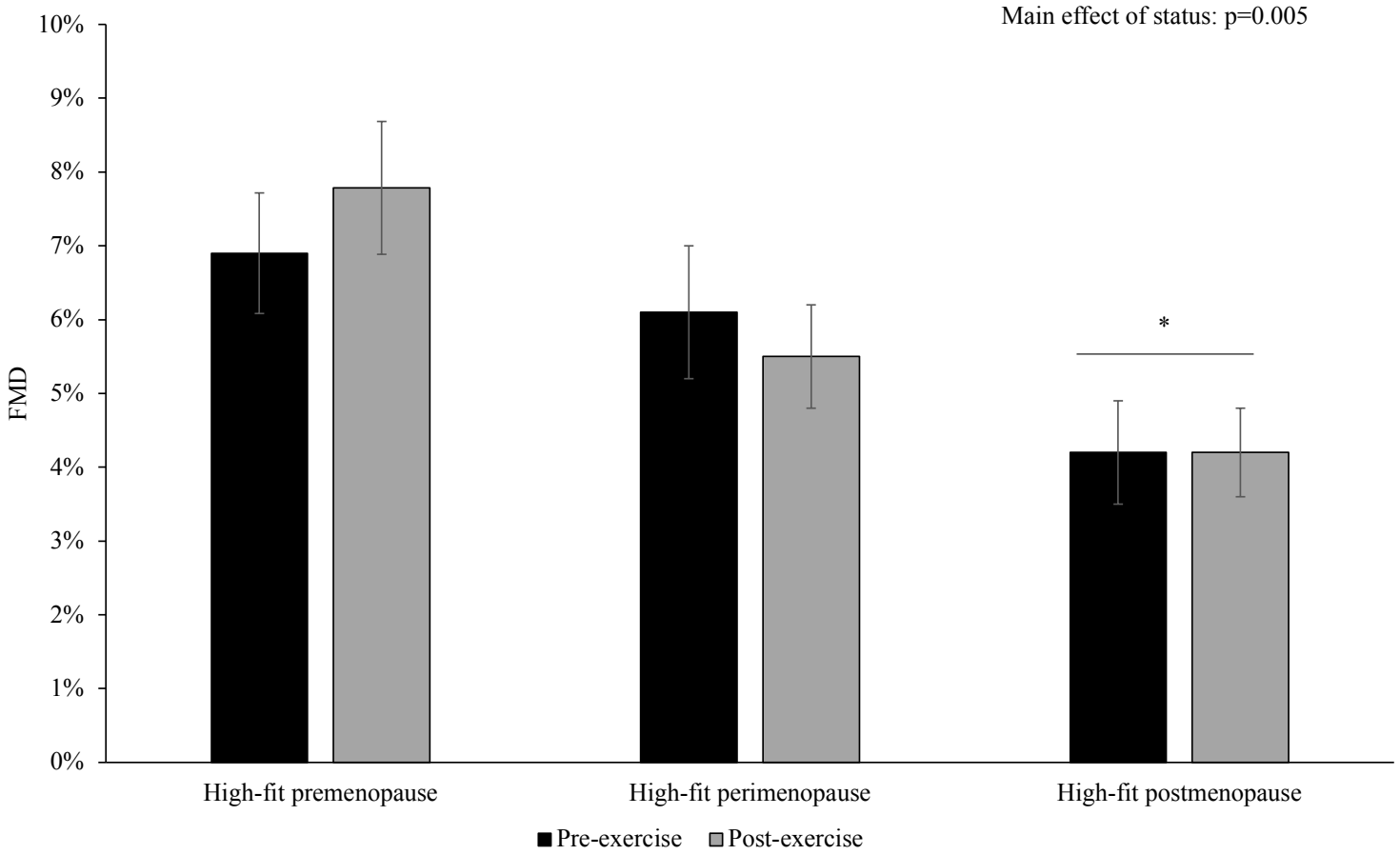
FSH: follicle stimulating hormone, BMI: body mass index, HDL-C: high-density lipoprotein cholesterol, LDL-C: low-density lipoprotein cholesterol, TG: triglycerides, FPG: fasting plasma glucose, SBP: systolic blood pressure, DBP: diastolic blood pressure; MVPA: moderate-to-vigorous physical activity *different than low-fit group; # different than high-fit premenopausal group; $ different than high-fit perimenopausal group, ^Serviente et al., 2016, data are presented as mean±SEM

	Premenopause	Perimenopause		Postmenopause	
	High-fit (n=11)	**Low-fit (n=7)**	**High-fit (n=12)**	**Low-fit (n=8)**	**High-fit (n=13)**
Average pre-exercise diameter (mm)	3.4 ± 0.1	3.2± 0.1	3.3 ± 0.1	3.3 ± 0.1	3.4 ± 0.1
Average post-exercise diameter (mm)	3.4 ± 0.1	3.2 ± 0.1	3.3 ± 0.1	3.3 ± 0.1	3.4 ± 0.1

Table 4.2. Baseline Artery Diameter.
Baseline artery diameter did not differ between groups, before or after exercise. data are presented as mean±SEM

Figures

A


Main effect of status: p=0.005
10%
9%
8%
7%
6%
5%
4%
3%
2%
1%
0%
FMD
*
High-fit premenopause
High-fit perimenopause
High-fit postmenopause
Pre-exercise
Post-exercise

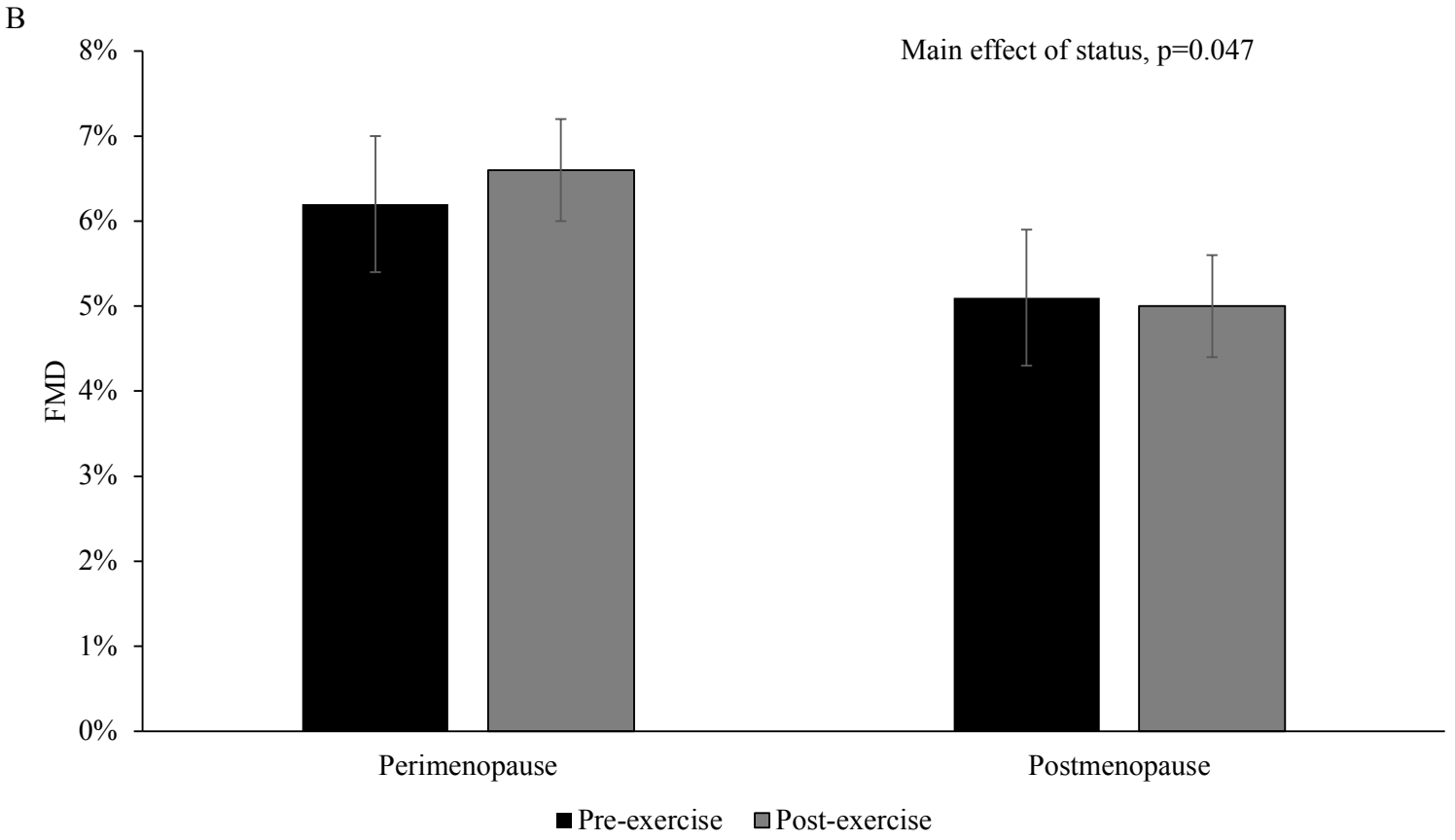


Figure 4.1. The Influence of Menopausal Status on FMD.
There was a main effect of menopausal status on FMD in high-fit women, with lower values in postmenopausal compared to premenopausal women (A). In perimenopausal and postmenopausal women, there was lower FMD in postmenopausal women (B). *compared to premenopausal group, data are presented as mean±SEM

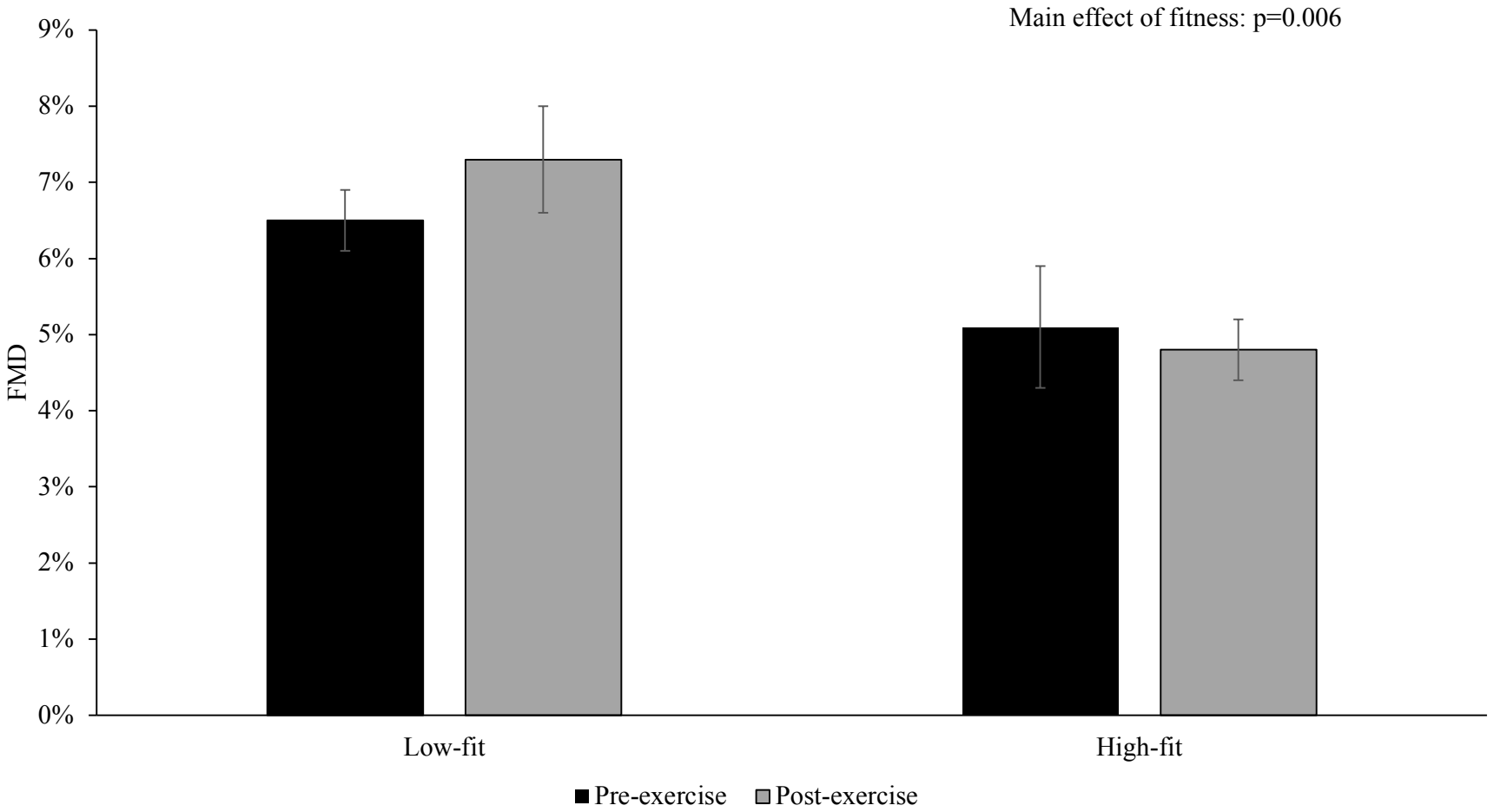


Figure 4.2. The Influence of Fitness on FMD.
There was a main effect of fitness on FMD, with lower values in high-fit compared to low-fit women, data are presented as mean±SEM

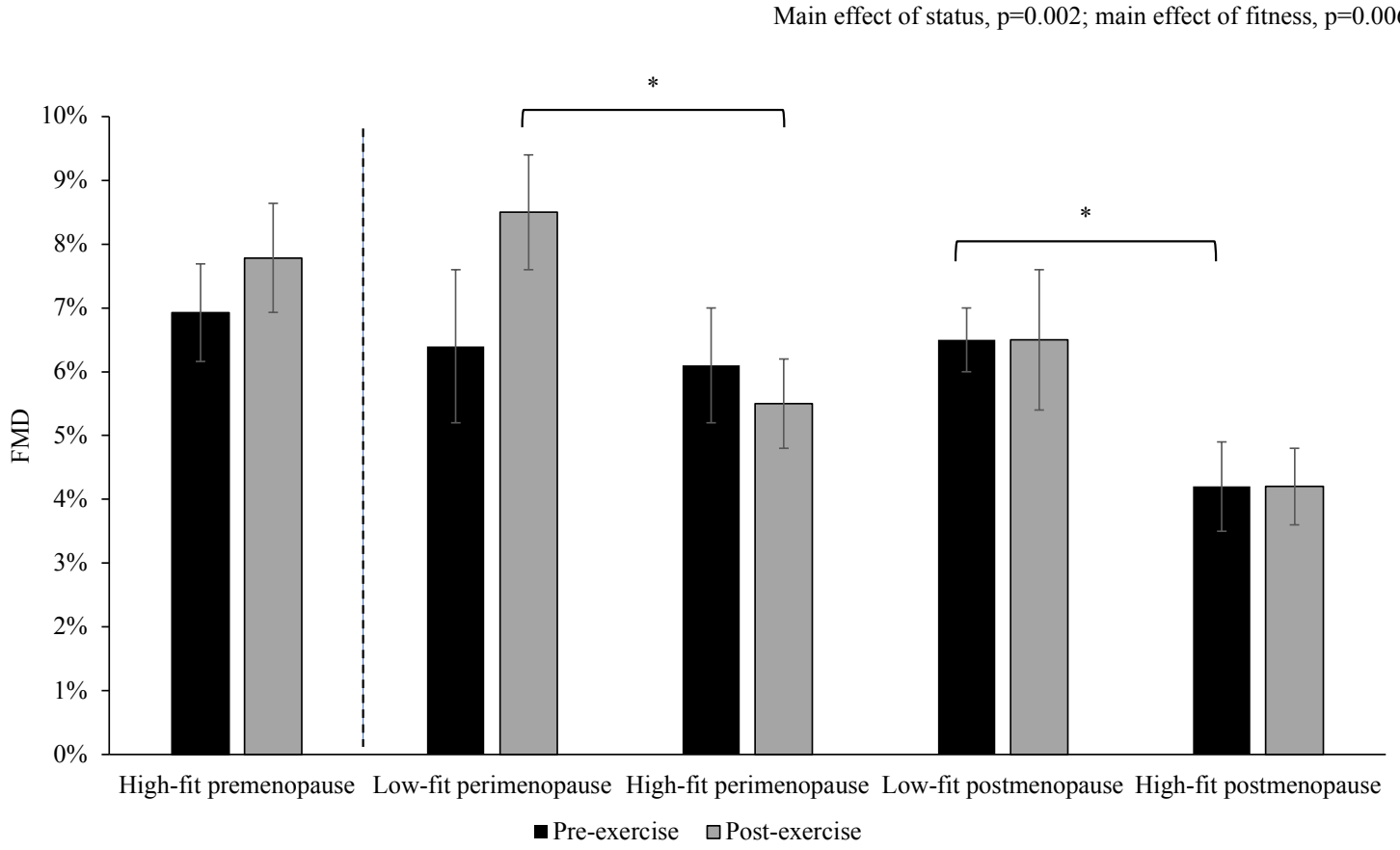
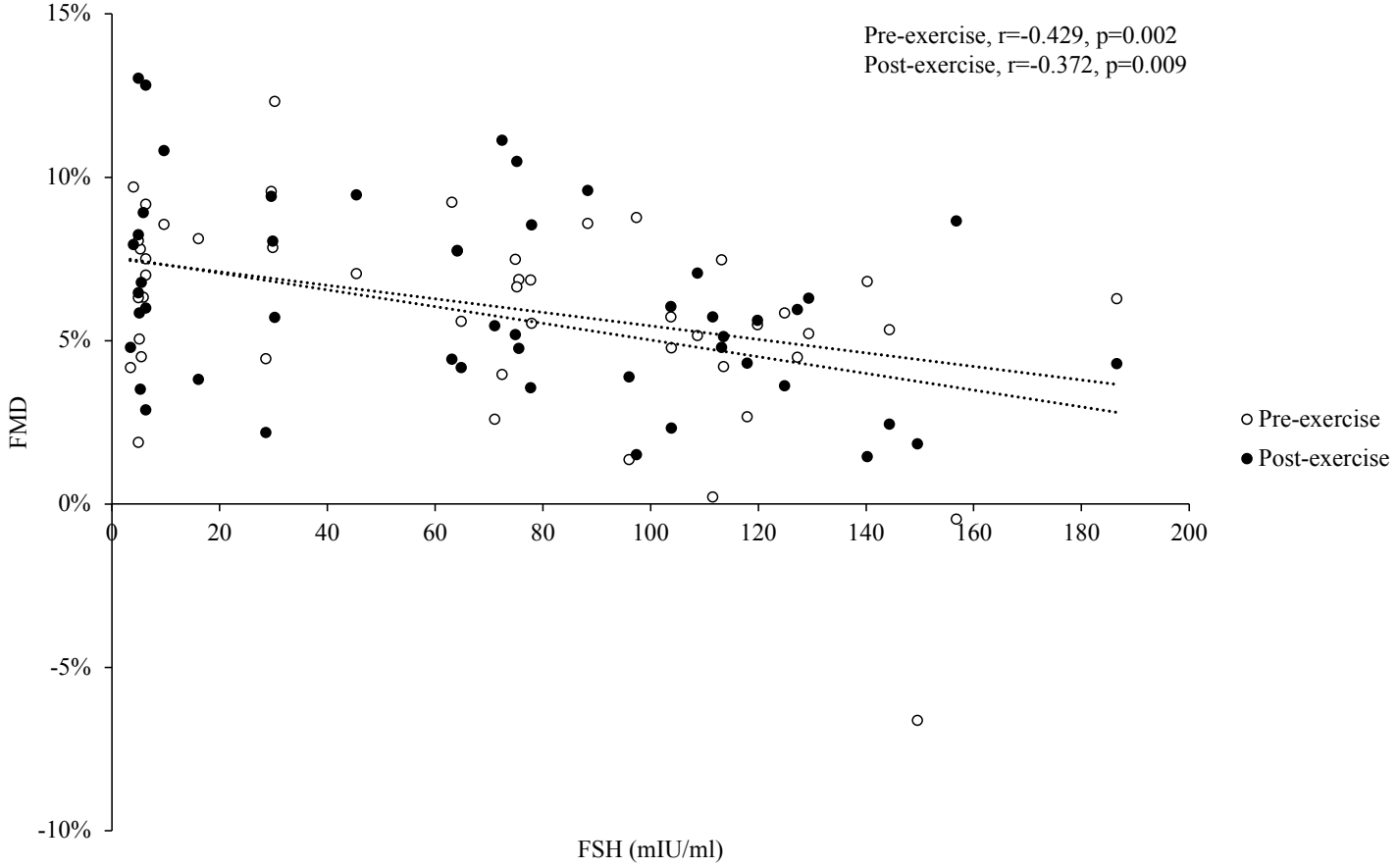


Figure 4.3. The Influence of Aerobic Fitness and Menopausal Status on FMD.
Overall, FMD was lower in postmenopausal compared to perimenopausal women and in high-fit compared to low-fit women. High-fit perimenopausal women had lower FMD following acute exercise compared to low-fit perimenopausal women. High-fit postmenopausal women had lower FMD compared to low-fit postmenopausal women before acute exercise. *p<0.05, data are presented as mean±SEM

A


Pre-exercise, r=-0.429, p=0.002
Post-exercise, r=-0.372, p=0.009
15%
10%
5%
0%
-5%
-10%
FMD
0
20
40
60
80
100
120
140
160
180
200
FSH (mIU/ml)
Pre-exercise
Post-exercise

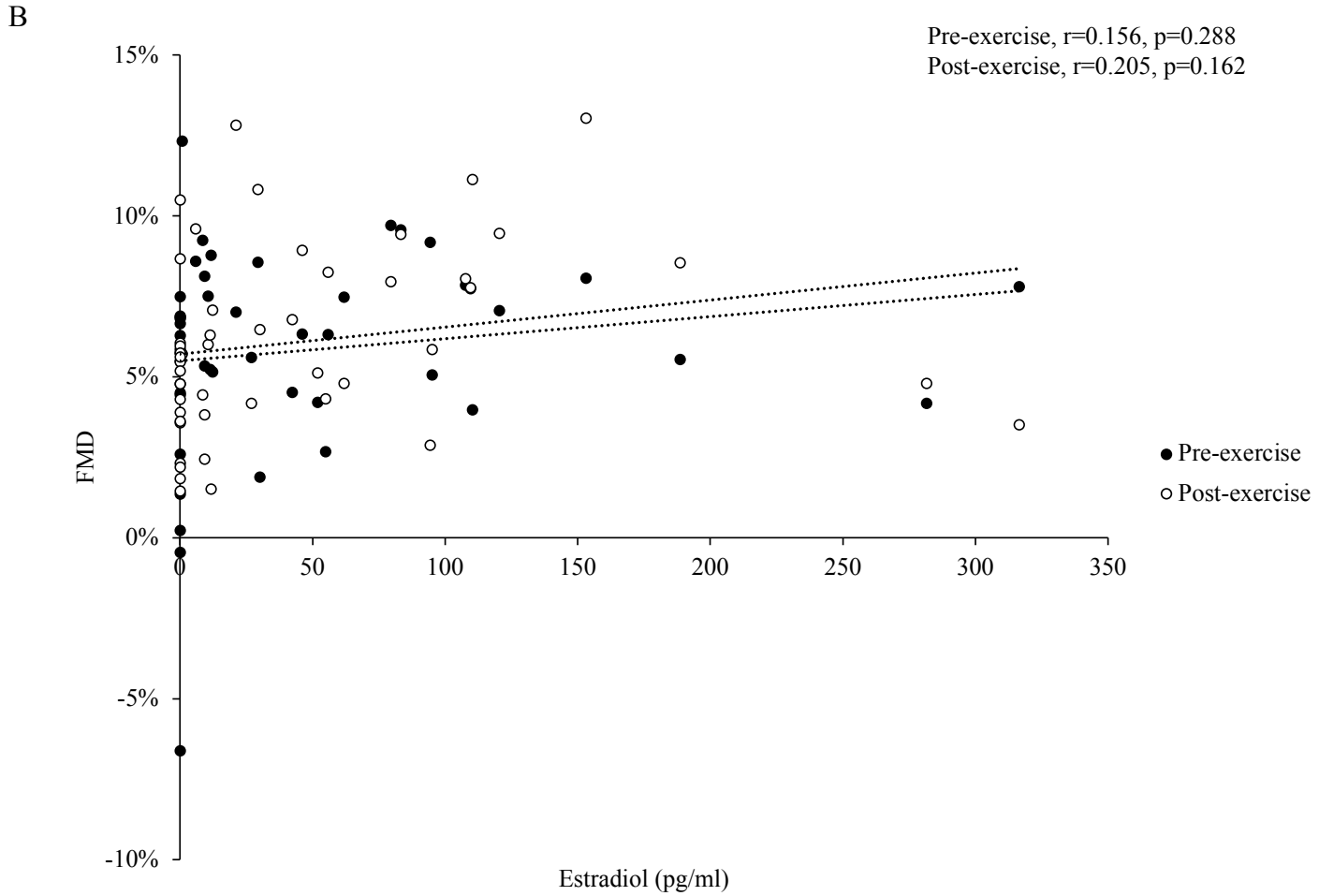


Figure 4.4. Associations between Sex Hormones and FMD.

Follicle stimulating hormone (FSH) was negatively related to flow-mediated dilation (FMD) before and after acute exercise (A). Estradiol was not related to FMD before or after acute exercise (B).

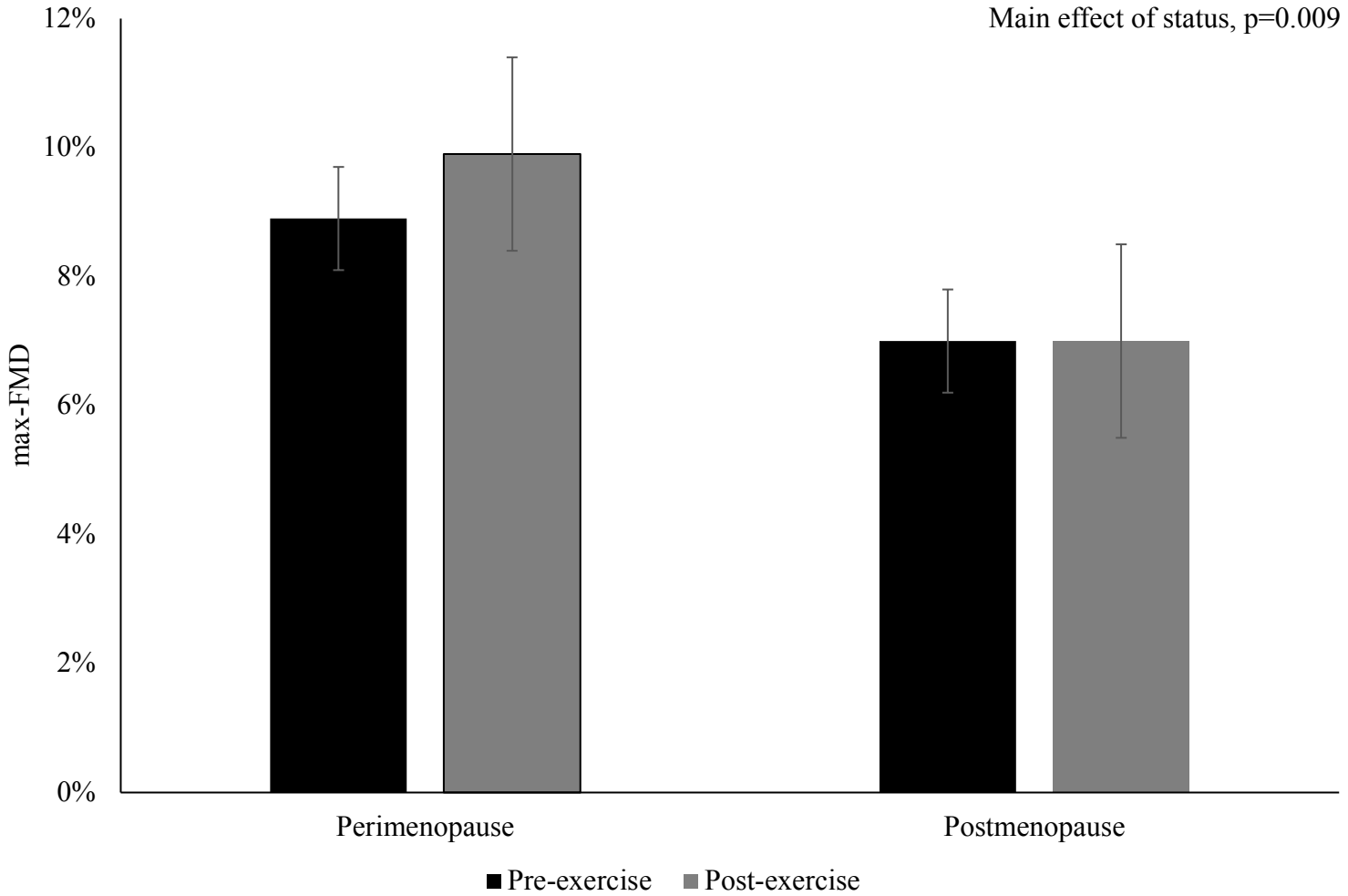


Figure 4.5. The Influence of Menopausal Status on Pre-dilation-FMD.
There was a main effect of menopausal status on max-FMD in perimenopausal and postmenopausal participants, with lower function in postmenopausal women, data are presented as mean±SEM

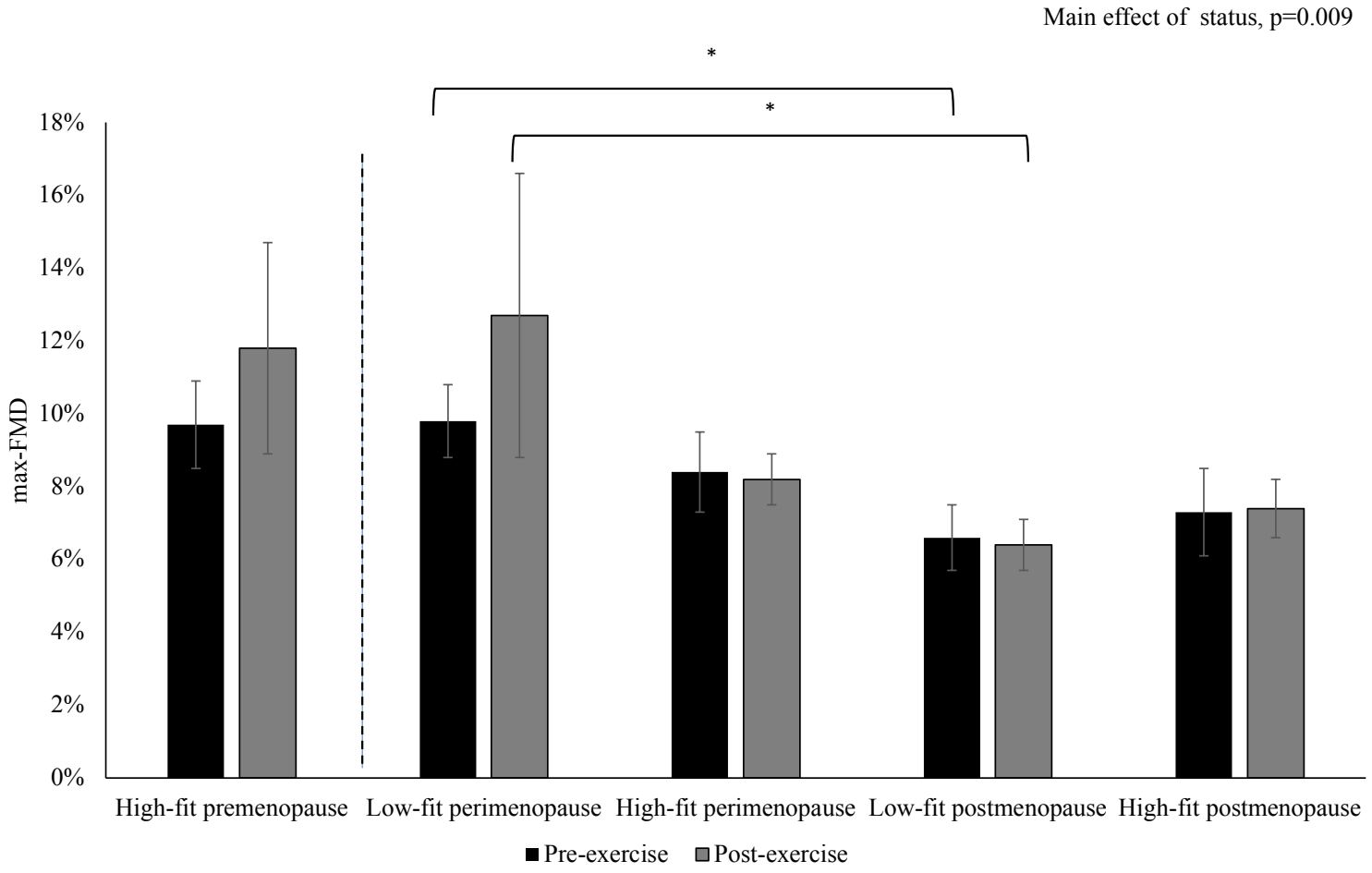


Figure 4.6. The Influence of Aerobic Fitness and Menopausal Status on Max-FMD.
While there was a main effect of menopausal status on max-FMD, there was no difference in max-FMD by menopausal status within high-fit women. Within low-fit women there was lower max-FMD before and after acute exercise in postmenopausal compared to perimenopausal women. *$p<0.05$, data are presented as mean±SEM

CHAPTER 5

MODERATE INTENSITY EXERCISE REDUCES ACTIVATED AND APOPTOTIC ENDOTHELIAL MICROPARTICLES IN HEALTHY MIDLIFE WOMEN

Introduction:

Endothelial microparticles (EMPs) serve as biomarkers of endothelial status, a key indicator of cardiovascular health. Endothelial microparticles are small vesicles (100nm-1μm) that are released from endothelial cells in response to stimuli such as inflammation, shear stress, or oxidative stress. High levels of EMPs related to endothelial activation ($CD62E^+$ EMPs) and apoptosis ($CD31^+/42b^-$ EMPs) have been found in individuals with disease or heightened disease risk (2, 9, 46, 161, 186).

Midlife represents a time during which there is accumulating CVD risk in women, in part due to menopause. Menopause is a female-specific condition associated with a dramatic increase in traditional risk factors for CVD, such as blood pressure, cholesterol and fasting plasma glucose (108). These changes are generally attributed to the reduction in estrogen that accompanies menopause. There is some evidence to suggest that the hormonal and cardiovascular-related changes associated with menopause influence EMP levels. In data from the Kronos Early Estrogen Prevention Study (KEEPS), in perimenopausal (i.e. transitioning) and early postmenopausal women at low risk for CVD, microparticles, including activated EMPs, explained 24% of the increase in carotid intima media thickness over 4 years (115). Further, estrogen status appears to directly influence EMP levels. For example, postmenopausal women with lower levels of

estrogen had higher levels of activated EMPs compared to postmenopausal women with higher levels of estrogen (80). Similarly, premenopausal women had higher levels of activated EMPs during the luteal compared to the follicular phase of the menstrual cycle (185). Together, this evidence suggests that EMP levels may be affected by menopausal stage; however, research is limited in this area.

High cardiorespiratory fitness and regular exercise are generally considered protective of cardiovascular health and would therefore be expected to influence EMP levels. Following a six-month training intervention there was a reduction in apoptotic ($CD31^+/42^-$) and activated ($CD62E^+$) EMPs in midlife and older men and women (48). Further, in a study directly evaluating the influence of fitness on EMPs, there was no difference in activated ($CD62E^+$) EMPs prior to exercise in trained vs. untrained men, but only trained men had an increase in EMPs following acute exercise (170). Finally, following an acute bout of exercise, low-fit postmenopausal women had higher levels of apoptotic ($CD31^+/42b^-$) EMPs in circulation compared with perimenopausal women (158). These data suggest that fitness and menopause may influence EMP levels and that an acute bout of exercise may be required to reveal these differences; however, further studies are needed to evaluate these relationships. Therefore, the purpose of this study was to investigate differences in $CD31^+/42b^-$ and $CD62E^+$ EMPs in women at different menopausal stages and with high and low levels of cardiorespiratory fitness before and after an acute bout of moderate intensity exercise.

Methods:

Protocols were approved by the University of Massachusetts Amherst Institutional Review Board and participants provided written informed consent before beginning any study protocol.

Study Overview:

Participants completed three study visits. Height, weight, and blood pressure were measured on the first visit, along with fasting plasma glucose, cholesterol, and body fat percentage, via a dual energy x-ray absorptiometry (DEXA) scan. To evaluate cardiorespiratory fitness and to prescribe acute exercise intensity, participants completed a graded treadmill VO_{2max} test on the second visit. On the third visit, venous blood was collected before and 30 minutes after an acute bout of treadmill exercise (30 min, 60-64% $VO_{2\ peak}$) to evaluate differences in EMP levels by menopausal status and CRF.

Participant Characteristics

Participants were recruited based on menopausal status and cardiorespiratory fitness. High-fit premenopausal women were recruited to serve as a reference group (n=11). High and low-fit perimenopausal (high: n=12; low: n=5) and postmenopausal women (high: n=13; low: n=8) were recruited to evaluate the influence of menopausal status and fitness on EMPs. Blood was not collected at pre-exercise for one premenopausal participant and at post-exercise for one high-fit perimenopausal participant. Data on low-fit participants were previously published (158). EMP analyses were performed in all participants for this study for batch analysis.

Participants were categorized by menopausal status using STRAW+10 guidelines (67). Premenopausal women were experiencing regular menstrual cycles. Perimenopausal women were experiencing irregular cycles (i.e., early perimenopausal) or 2-11 months of amenorrhea (i.e., late perimenopausal). Postmenopausal women were at least 5 years postmenopausal and had undergone natural menopause. High-fit participants achieved a VO_{2peak} of at least the 80th percentile for their age (137). Low-fit participants were not meeting physical activity guidelines (61) based on self-report (International Physical Activity Questionnaire) and had an average VO_{2peak} of approximately the 40th percentile.

Participants were healthy and had low risk for CVD. Participants were normotensive (<120/<80mmHg), non-diabetic (fasting plasma glucose<126mg/dl), had normal lipid levels (LDL-C <160 mg/dl, HDL-C >40 mg/dl, TG< 150 mg/dl), and were non-smokers. Participants were excluded if they reported long-term menstrual irregularities, abnormal uterine or ovary anatomy that affected fertility, acute liver or gallbladder disease, breast cancer with radiation or chemotherapy treatment or if they were taking medications, vitamins, or supplements known to influence endothelial function. Participants were also excluded if they reported hormone therapy use within six months prior to study enrollment or if they had any exercise limitations.

Blood Assessments

Fasting plasma glucose and cholesterol were measured using standard techniques. Follicle stimulating hormone (Abcam, Cambridge, Massachusetts) and 17-beta-estradiol (Life Technologies, Frederick, Maryland) were measured from stored (-80°C) serum samples using commercially available ELISA assays and standard procedures.

Endothelial microparticles were measured from samples taken before and 30 minutes after acute exercise. Whole blood was collected in acid citrate dextrose tubes, and after other blood collection tubes were drawn, to avoid potential effects due to the initial venipuncture. Whole blood was gently and continuously mixed until processing, spun at 1200 x g for 15 min to separate plasma, and then aliquoted and stored at -80°C until analysis.

For analysis, plasma was thawed at room temperature and then spun, initially at 4500 x g for 15 min (20°C) and then at 13,000 x g for 2 min (20°C). Aliquots of each sample (12.5 µl) were added to 96-well plates in duplicate and incubated with 5 µl of CD31 (APC, Biolegend, San Diego, California), 42b (PE, Biolegend, San Diego, California) and 62E (BV421, Becton Dickson and Company, San Jose, California) antibodies for 20 min and then fixed with 2% paraformaldehyde. Antibody and sample volumes were selected based on titration experiments (data not shown). Samples were stored overnight at 4°C and then analyzed by flow cytometry (BD Dual LSR Fortessa) the following morning. Prior to flow cytometric analysis, 12.5 µl of CountBright Absolute Counting Beads (Molecular Probes, Eugene, Oregon) were added to each well.

To identify microparticle populations, gates were set according to size using 900 nm NIST beads (Polysciences, Warrington, PA) to select events that were <900 nm (Figure 5.1). $CD31^{+}/42b^{-}$ and $CD62E^{+}$ EMPs were selected using fluorescent minus one gating strategies. Total events/µl of plasma was calculated using standard protocols for CountBright beads and the number of events/total microparticle events (EMP%) were calculated.

Statistical analyses:

All analyses were completed in SPSS v24, with an alpha level of 0.05 for statistical significance. Despite attempting multiple transformations, not all variables were normally distributed. Natural log transformation of all variables was closest to a normal distribution; therefore, data were natural log transformed for analysis. Non-transformed data are presented for interpretation. To account for the lack of normality for some variables, values on the extreme ends of the distribution were removed to achieve a normal distribution. Analyses with and without these values gave the same outcome; therefore, analyses run in all participants are reported. Repeated measure ANOVAs were used to evaluate differences in EMPs, EMP%, and total microparticle events across groups, before and after acute exercise. To evaluate the effect of menopausal status, differences were evaluated in high-fit women and in perimenopausal and postmenopausal combined. The influence of fitness was evaluated in perimenopausal and postmenopausal women only. Differences between groups were evaluated using one-way ANOVAs, independent t-tests, or an equivalent non-parametric test. Differences within groups were evaluated using paired t-tests or an equivalent non-parametric test. Data are presented as mean±SEM

Results:

Participants were healthy, had few risk factors for CVD and had similar risk factor profiles between groups (Table 5.1). Blood pressure, estradiol, follicle stimulating hormone, low-density lipoprotein cholesterol (LDL-C), fasting plasma glucose, and triglycerides did not differ by fitness within each menopausal stage. Within perimenopausal women, body fat percentage was lower, while high-density lipoprotein

cholesterol (HDL-C), time spent in moderate-to-vigorous physical activity (MVPA) and VO_{2peak} were higher in high-fit women. Within postmenopausal women, body mass and body fat percentage were lower, while HDL-C, MVPA, and VO_{2peak} were significantly higher in high-fit women.

Total Microparticles

All values for total microparticles and EMPs are presented in Table 5.2. In high-fit women, there was a main effect of exercise on total number of microparticles ($p<0.001$), with lower levels 30 min after acute exercise. There was no effect of menopausal status on total microparticles ($p=0.461$). Within perimenopausal and postmenopausal women there was also a main effect of exercise ($p<0.001$), but not menopausal status ($p=0.485$) or fitness ($p=0.772$, Figure 5.2).

$CD62E^+$ EMPs

Within high-fit women, there was a main effect of exercise ($p=0.003$), with lower EMPs/μl of plasma following acute exercise. There was no effect of menopausal status ($p=0.521$). Within perimenopausal and postmenopausal women, there was a main effect of exercise ($p=0.021$), but no effect of fitness ($p=0.912$) or menopausal status ($p=0.818$) on $CD62E^+$ EMPs/μl plasma (Figure 5.3A).

Within high-fit women, the percentage of microparticles that were $CD62E^+$ was greater after acute exercise ($p<0.001$), but this response was not influenced by menopausal status ($p=0.706$). Within perimenopausal and postmenopausal women there was a main effect of exercise ($p=0.001$) and no effect of menopausal status ($p=0.531$) or fitness ($p=0.804$) on the percentage of EMPs that were $CD62E^+$ (Figure 5.3B).

$CD31^+/42b^-$ EMPs

Within high-fit women, there was a main effect of exercise ($p<0.001$), with lower EMPs/μl of plasma following acute exercise. There was no effect of menopausal status ($p=0.401$). Within perimenopausal and postmenopausal women, there was a main effect of exercise ($p=0.001$) and no effect of menopausal status ($p=0.435$) or fitness ($p=0.777$, Figure 5.4A).

Within high-fit women, the percentage of EMPs that were $CD31^+/42b^-$ was not influenced by exercise ($p=0.528$) or menopausal status ($p=0.535$). Within perimenopausal and postmenopausal women, there was no effect of menopausal status ($p=0.317$), fitness ($p=0.672$) or exercise ($p=0.690$), on the percent of total events that were $CD31^+/42b^-$ (Figure 5.4B).

Discussion

The aim of this study was to evaluate the influence of menopausal status and cardiorespiratory fitness on two EMP populations before and after acute exercise. The results indicate that acute moderate intensity aerobic exercise reduces total circulating microparticles, $CD31^+/42b^-$ EMPs, and $CD62E^+$ EMPs in healthy midlife women. We further show that the percentage of $CD62E^+$ EMPs increased with acute exercise. These responses occurred across groups, despite difference in menopausal status and cardiorespiratory fitness.

Our results show a clear reduction of EMPs in response to acute moderate intensity exercise. This reduction may represent one of the mechanisms by which long-term training is beneficial for cardiovascular health. The effect of acute exercise on EMPs is equivocal in the literature. The differences in study results may be due to a variety of

factors including differences in participant characteristics (e.g. physical activity levels, age, sex), sampling time points, EMP population, and exercise mode (e.g. cycle ergometer, treadmill) across studies. In men, almost all studies have reported either an increase (43, 97) or no change in EMPs (117, 163, 202) in response to acute exercise. Although, one study did report an increase in activated EMPs in trained, but not untrained young men following acute moderate intensity exercise, suggesting a potential role of fitness (170). Studies in women have reported increases (43, 158), reductions (163), and no change in EMPs in response to acute exercise (43, 97, 158). For example, in overweight and obese inactive young women, there was an increase in activated EMPs and no change in apoptotic EMPs the morning following acute high-intensity cycle ergometer exercise (43). Conversely, in young aerobically fit women, there was no change in apoptotic or activated EMPs 5 minutes following an acute bout of moderate-to-vigorous cycle ergometer exercise (97). Given the many differences between study designs, it is difficult to evaluate which specific factor is leading to the variability in responses across studies. This study compared multiple groups across the same time point, allowing for a clearer evaluation of the effects of acute exercise on EMPs in different populations.

Beyond discrepancies in measurement time point and participant characteristics, most studies in women have evaluated a young population. High levels of EMPs are related to a variety of disease states including end stage renal disease (2), acute coronary syndrome (9), diabetes (186), and cardiovascular disease (161), among others. Menopause is associated with an increase in risk factors for cardiovascular disease (108); however, whether EMP levels change with menopause is still unclear. Higher levels of

$CD62E^+$ EMPs have been reported in postmenopausal women with lower compared to higher levels of estrogen (80). We also reported higher activated EMPs/μl plasma in postmenopausal compared to perimenopausal women and higher levels of apoptotic EMPs/μl plasma following acute exercise in postmenopausal women (158). This evidence suggests that menopause may have an adverse effect on EMPs and EMP responses to acute exercise. However, our current results suggest that acute exercise, but not menopausal status, influences EMP levels in healthy women. The discrepancy in results may be due to differences in the protocol to isolate and assess EMPs between the studies; however, when we evaluated the percent change in EMPs in response to acute exercise, rather than events/μl plasma, the direction of the response in this study compared to the prior evaluation were similar.

In addition to an effect of menopausal status, we also anticipated a beneficial effect of cardiorespiratory fitness on EMP levels, given the protective effects of fitness on cardiovascular health; however, we found no effect of cardiorespiratory fitness on EMPs in our population. This lack of effect may be due to the fact that both high- and low-fit women recruited for this study were healthy and had low CVD risk. Therefore, cardiovascular disease risk factors other than fitness may have a greater effect on EMP levels and responses to acute exercise. This is supported by findings from the KEEPs trial, which showed higher $CD62E^+$ EMPs in postmenopausal women with higher Framingham risk and coronary artery calcification scores (81).

Exercise intensity may also influence EMP levels. Moderate intensity exercise is generally associated with less of an inflammatory response and lower shear stress than high intensity exercise. Given that EMP release can be triggered by inflammation (29)

and shear stress (82), it is likely that different intensities of exercise may elicit a different EMP response. Similar to our results, Shill et al. reported a decrease in activated EMPs following moderate intensity treadmill exercise in young active women. Conversely, they found no change in EMP levels following high intensity (10 x 1 min intervals) treadmill exercise (163). Further, the high intensity, longer duration exercise of marathon running increased apoptotic EMPs in middle-aged men and women (155). Therefore, these data, in combination with that presented herein, suggest that acute moderate intensity exercise reduces EMP levels in healthy women of different ages.

Another consideration regarding the reduction in circulating EMPs in response to acute exercise is increased EMP clearance. EMP clearance is still poorly understood, but is at least partially explained through uptake by endothelial cells. Jansen et al. reported uptake of EMPs to endothelial cells (79). Further, Wahl et al. reported a reduction in $CD31^{+}/42b^{-}$ EMPs in highly trained men 60 minutes following varying intensities of acute cycle ergometer exercise. They also reported greater uptake of EMPs to endothelial cells (196). Together, these studies may suggest that the reduction in EMPs that we found with acute exercise may be due to enhanced clearance of EMPs to endothelial cells. Further, the increase in percentage of EMPs that were $CD62E^{+}$ might indicate faster clearance of apoptotic EMPs relative to activated EMPs or greater appearance of activated EMPs in response to exercise.

Endothelial microparticles are circulating markers of endothelial status but are also recognized as important signaling factors. Low levels of EMPs have been shown to promote angiogenesis, while high levels may inhibit it (127). EMPs have also been shown to be protective of endothelial apoptosis (79, 196) and $CD62E^{+}$ EMPs may have

positive effects on inflammation and neovascularization, among other effects (8). Acute exercise may alter EMP signaling mechanisms; however, it is challenging to separate the contribution of other microparticle populations, such as platelet-derived microparticles, from the effects of EMPs. Endothelial cells exposed to post-exercise serum, containing apoptotic EMPs, had reduced apoptosis compared to cells exposed to pre-exercise serum (196). Similarly, exposure to post-exercise microparticles increased proliferation, migration, tube formation, and wound closure in cultured endothelial cells (202). Procoagulant activity of microparticles has also been shown to increase following acute moderate intensity cycle ergometer exercise in young men (170). Finally, miRNAs of endothelial origin were increased after acute cycle ergometer exercise in young, trained men; these miRNAs have been found in EMPs and can act on smooth muscles cells, potentially changing endothelial-smooth muscle communication (197). Therefore, there may be a functional benefit of acute exercise on EMPs; however, this warrants further investigation.

Limitations

This study provides novel insight into the influence acute moderate intensity exercise on EMP levels; however, there are several limitations worth noting. While this study shows a clear effect of acute exercise on EMPs, the study is underpowered for the effects of menopausal status and fitness. Further, EMPs levels are sensitive to storage and centrifugation speed. Longer storage at -80°C has been shown to increase the number of $CD31^{+}/42b^{-}$ and $CD62E^{+}$ EMPs, while high speed centrifugation may lead to some EMP loss (191). Both of these factors could potentially influence our results, particularly since data collection for this study occurred over several years. However, given the consistency

in our findings across groups and time points, we do not believe these limitations had a large effect on our outcomes.

Conclusions

Overall, these data indicate that acute moderate intensity aerobic exercise leads to a reduction in total microparticles, as well as activated and apoptotic EMPs in healthy midlife women. This effect occurred despite differences in cardiorespiratory fitness and menopausal status across groups. The reduction in EMPs that we report may suggest that some of the beneficial effects of long term aerobic exercise on cardiovascular health in this population are due to the acute effects of exercise on EMP levels.

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Tables

	Premenopause	Perimenopause		Postmenopause	
	High-fit (n=11)	**Low-fit (n=5)[#]**	**High-fit (n=12)**	**Low-fit (n=8)[#]**	**High-fit (n=13)**
Age (yrs)	44.5 ± 1.0	48.4 ± 1.6	50.8 ± 1.0	58.9 ± 1.4	60.5 ± 1.0
Height (cm)	163.5 ± 1.2	164.1 ± 3.9	165.4 ± 2.0	166.5 ± 3.1	161.6 ± 2.3
Weight (kg)	61.8 ± 2.2	65.2 ± 5.7	62.5 ± 3.0	69.0 ± 4.3	58.2 ± 2.5*
Estradiol (pg/ml)	58.7 ± 10.7	117.3 ± 27.1	75.2 ± 32.7	16.5 ± 9.0	6.7 ± 4.2
FSH (mIU/mL)	8.5 ± 2.2	49.9 ± 14.0	66.6 ± 17.9	104.2 ± 8.1	102.6 ± 9.5
Body Fat (%)	29.6 ± 2.5	37.4 ±1.8	26.9 ± 2.1*	41.9 ± 1.5	28.0 ± 2.1*
BMI (kg/m^2)	23.1 ± 0.7	24.1 ± 1.6	22.7 ± 0.7	24.8 ± 1.3	22.2 ± 0.7
HDL-C (mg/dL)	71.2 ± 4.9	62.4 ± 8.0	82.0 ± 4.8*	78.4 ± 4.8	94.7 ± 2.8*
LDL-C (mg/dL)	101.9 ± 7.0	93.0 ± 8.4	98.8 ± 6.1	116.5 ± 9.5	117.1 ± 7.2
TG (mg/dL)	50.1 ± 5.8	51.8 ± 8.1	40.6 ± 7.1	46.4 ± 5.7	42.8 ± 3.6
FPG (mg/dL)	94.5 ± 2.0	95.4 ± 2.6	93.9 ± 1.8	99.5 ± 1.7	96.5 ± 1.9
SBP (mmHg)	102.7 ± 2.4	101.0 ± 6.3	106.0 ± 2.8	117.3 ±5.2	106.3 ± 3.7
DBP (mmHg)	58.9 ± 1.5	61.6 ± 4.7	59.2 ± 1.8	64.6 ± 2.7	61.9 ± 2.3
MVPA (MET-min/wk)	3446.6 ± 429.5	354.7 ± 122.2	3695.9 ± 528.5*	100.3 ± 40.9	4107.1 ± 684.1*
VO_2 peak (ml/kg/min)	47.0 ± 2.4	30.4 ± 2.1	49.1 ± 2.4*	28.3 ± 1.1	43.8 ± 1.8*

Table 5.1. Participant Characteristics.

FSH: follicle stimulating hormone; BMI: body mass index; HDL-C: high-density lipoprotein cholesterol; LDL-C low-density lipoprotein cholesterol; TG: triglycerides; FPG: fasting plasma glucose; SBP: systolic blood pressure; DBP; diastolic blood pressure; MVPA: moderate-to-vigorous physical activity. *different than low-fit, [#]Serviente et al., 2016, data are presented as mean±SEM

	Total MP events		$CD31^+/42b^-$ Events/ul plasma		$CD31^+42b^-$ Events/Total EMP events		$CD62E^+$ Events/ul plasma		$CD62E^+$ Events/Total EMP events	
	Pre-exercise	**Post-exercise**	**Pre-exercise**	**Post-exercise**	**Pre-exercise**	**Post-exercise**	**Pre-exercise**	**Post-exercise**	**Pre-exercise**	**Post-exercise**
Premenopause	12003.0±3972.7 (10)	5911.8±1277.8* (11)	101.0±33.9 (10)	50.1±10.3* (11)	17.5±0.1% (10)	17.6±0.2% (11)	52.8±12.1 (10)	39.2±5.8* (11)	10.6±0.7% (10)	14.9±1.1%* (11)
Low-fit perimenopause	15703.9±10426.9 (5)	7115.5±1733.1 (5)	128.2±85.8 (5)	59.1 ± 14.2 (5)	17.1 ± 0.2% (5)	17.6±0.4% (5)	65.7 ± 36.8 (5)	43.5 ± 10.8 (5)	10.4 ± 1.1 % (5)	13.4 ± 1.0% (5)
High-fit perimenopause	13155.8±2655.7 (12)	7966.6±1561.9* (11)	107.5±20.5 (12)	66.5±12.8* (11)	17.4±0.2% (12)	17.3±0.2% (11)	65.0 ±14.6 (12)	51.9± 12.5* (11)	10.7±0.8% (12)	13.1±1.1%* (11)
Low-fit postmenopause	10547.3±2975.5 (7)	6047.1±669.3 (8)	84.8±24.7 (7)	51.4±5.4 (8)	17.0±0.4% (7)	17.2±0.2% (8)	84.7±31.0 (7)	38.7±4.6 (8)	15.5±2.5% (7)	13.2±1.3% (8)
High-fit postmenopause	9904.8±1474.2 (13)	5752.7±933.9* (13)	79.4±13.1 (13)	47.2±7.7* (13)	17.3±0.4% (13)	17.1±0.1% (13)	47.8±5.9 (13)	38.6±5.8* (13)	10.8±0.4% (13)	15.2±1.4%* (13)

Table 5.2. Microparticle Variables.

Microparticles were measured before and 30 minutes following acute exercise in an all participants. Data are presented as Mean±SEM (n). MP: microparticles; *$p<0.05$ compared to pre-exercise

Figures

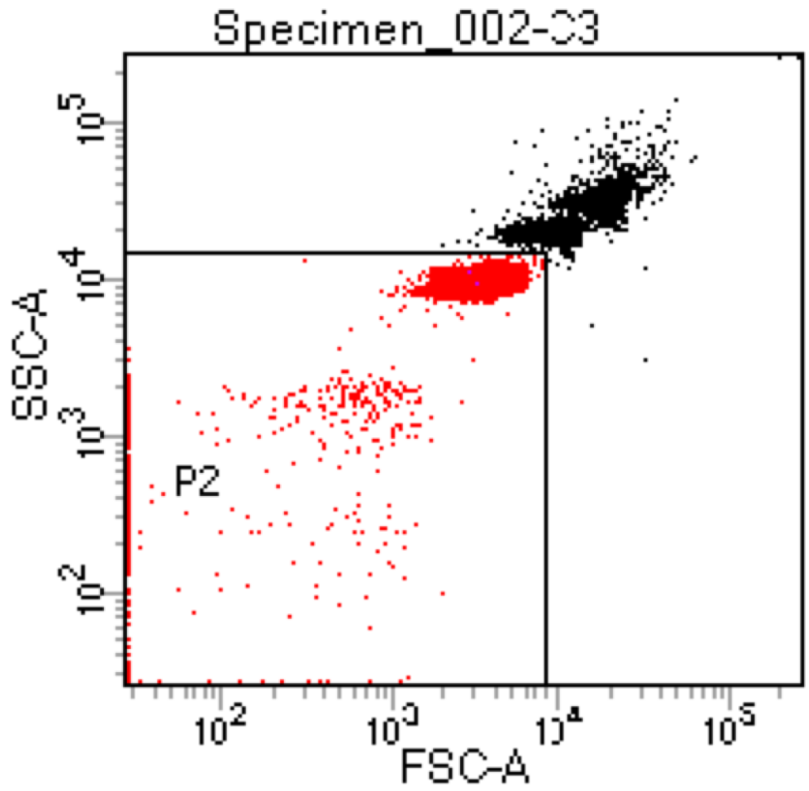


Figure 5.1. Size Gating.
Microparticle events selected using 900nm NIST beads. P2 represents microparticle events.

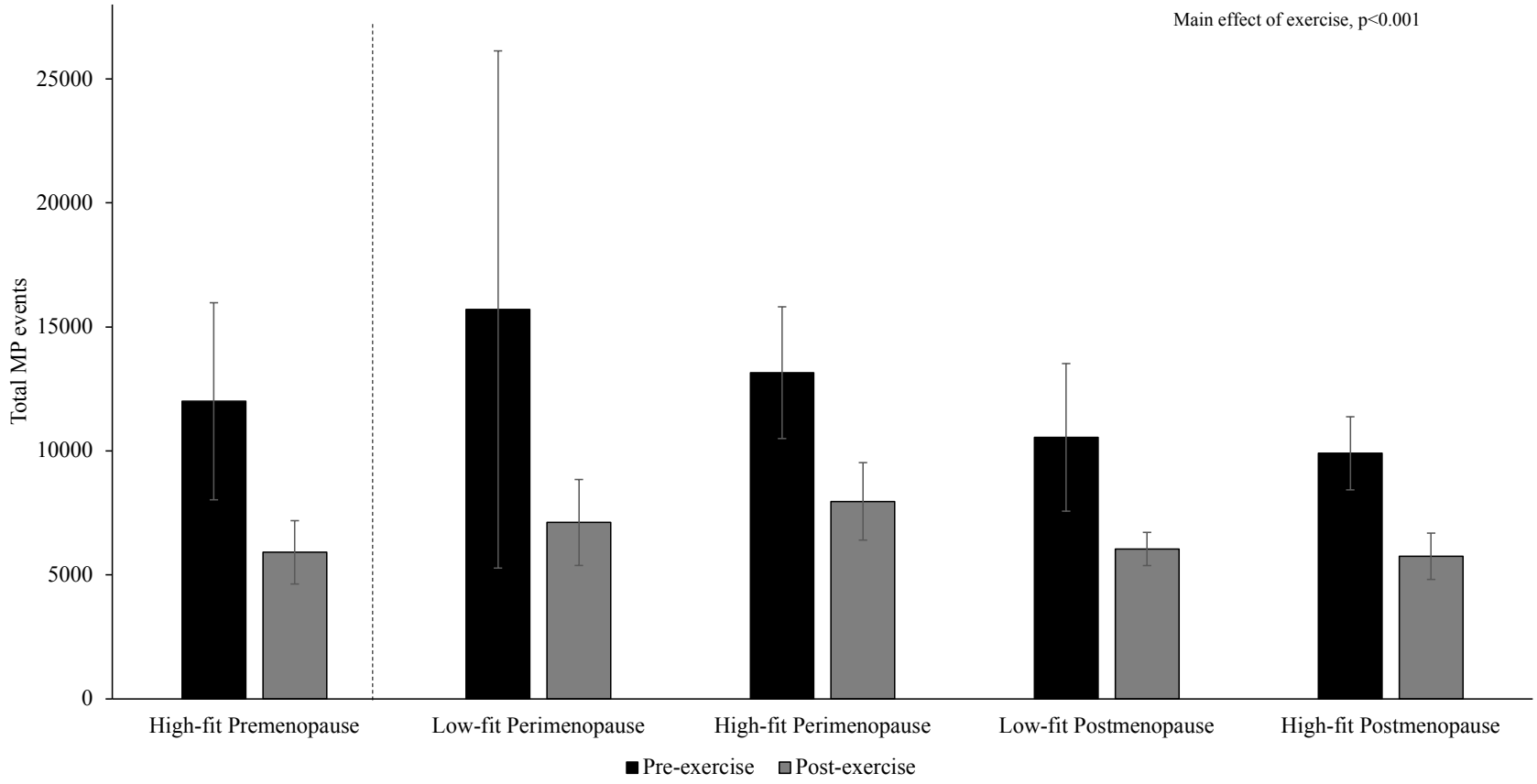
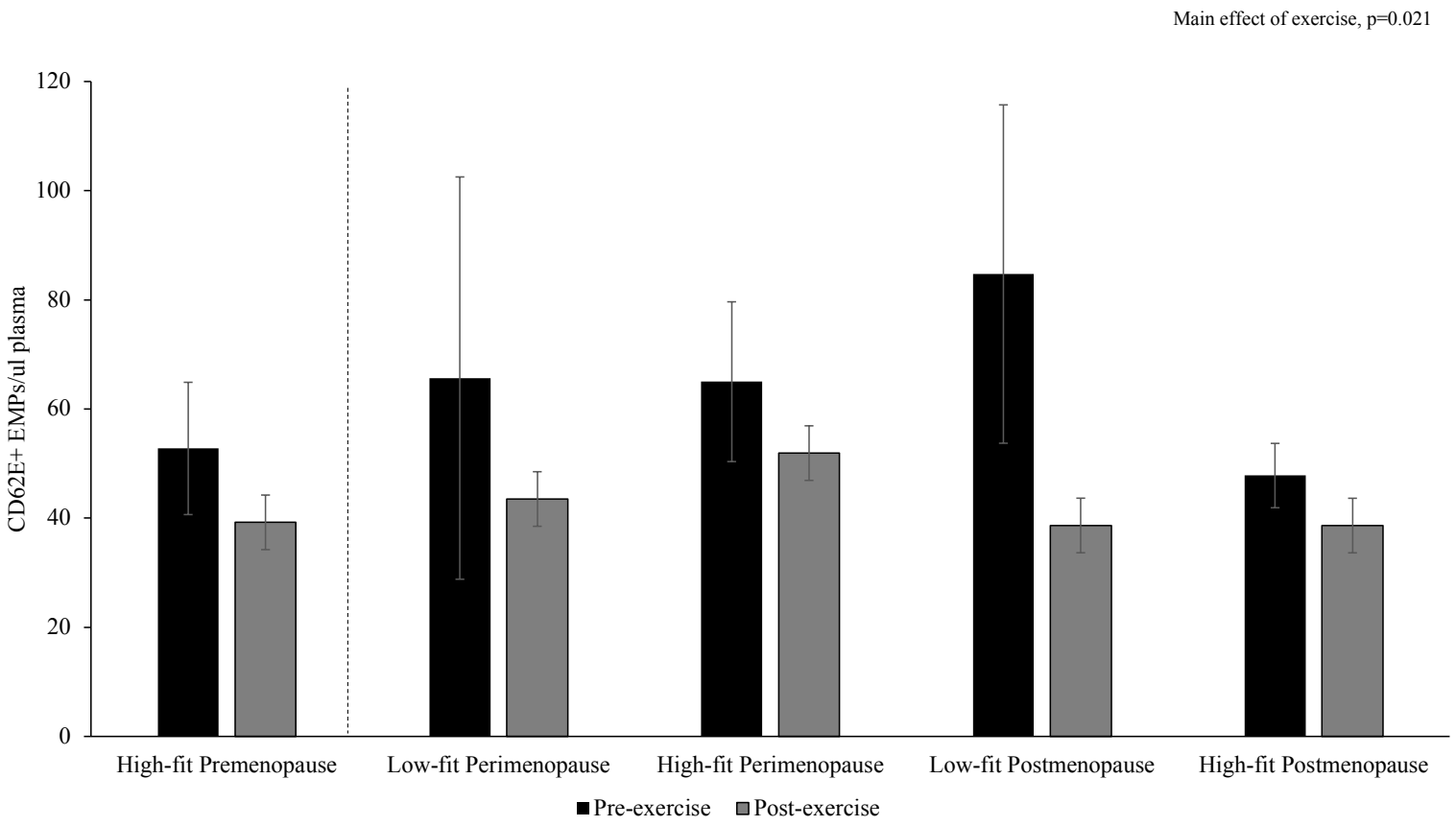


Figure 5.2. Total Microparticle Events.
Total microparticle events were significantly reduced 30 minutes following acute exercise, independent of menopausal status or fitness. MP: microparticles, data are presented as mean±SEM

A


Main effect of exercise, p=0.021
CD62E+ EMPs/ul plasma
120
100
80
60
40
20
0
High-fit Premenopause
Low-fit Perimenopause
High-fit Perimenopause
Low-fit Postmenopause
High-fit Postmenopause
Pre-exercise
Post-exercise

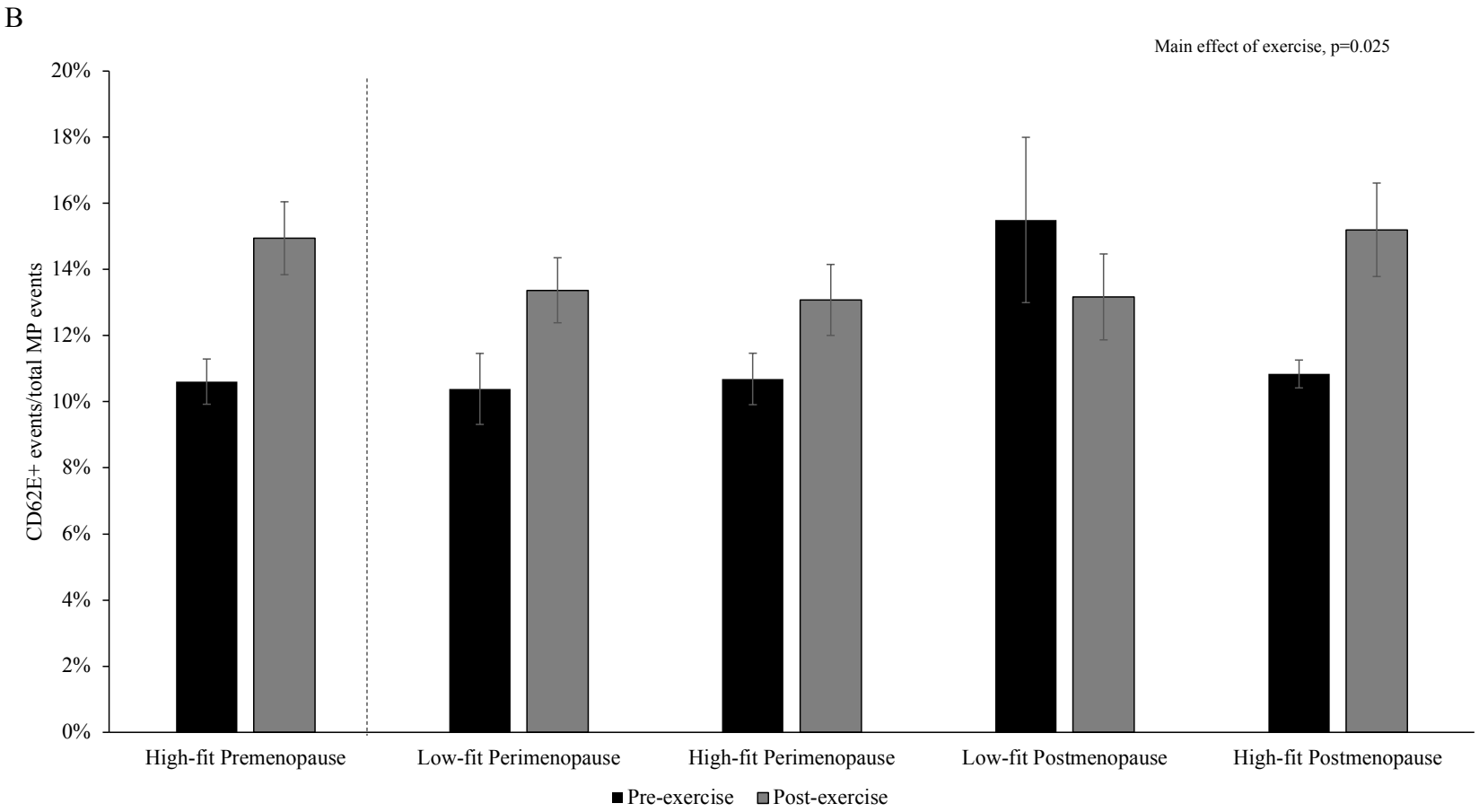
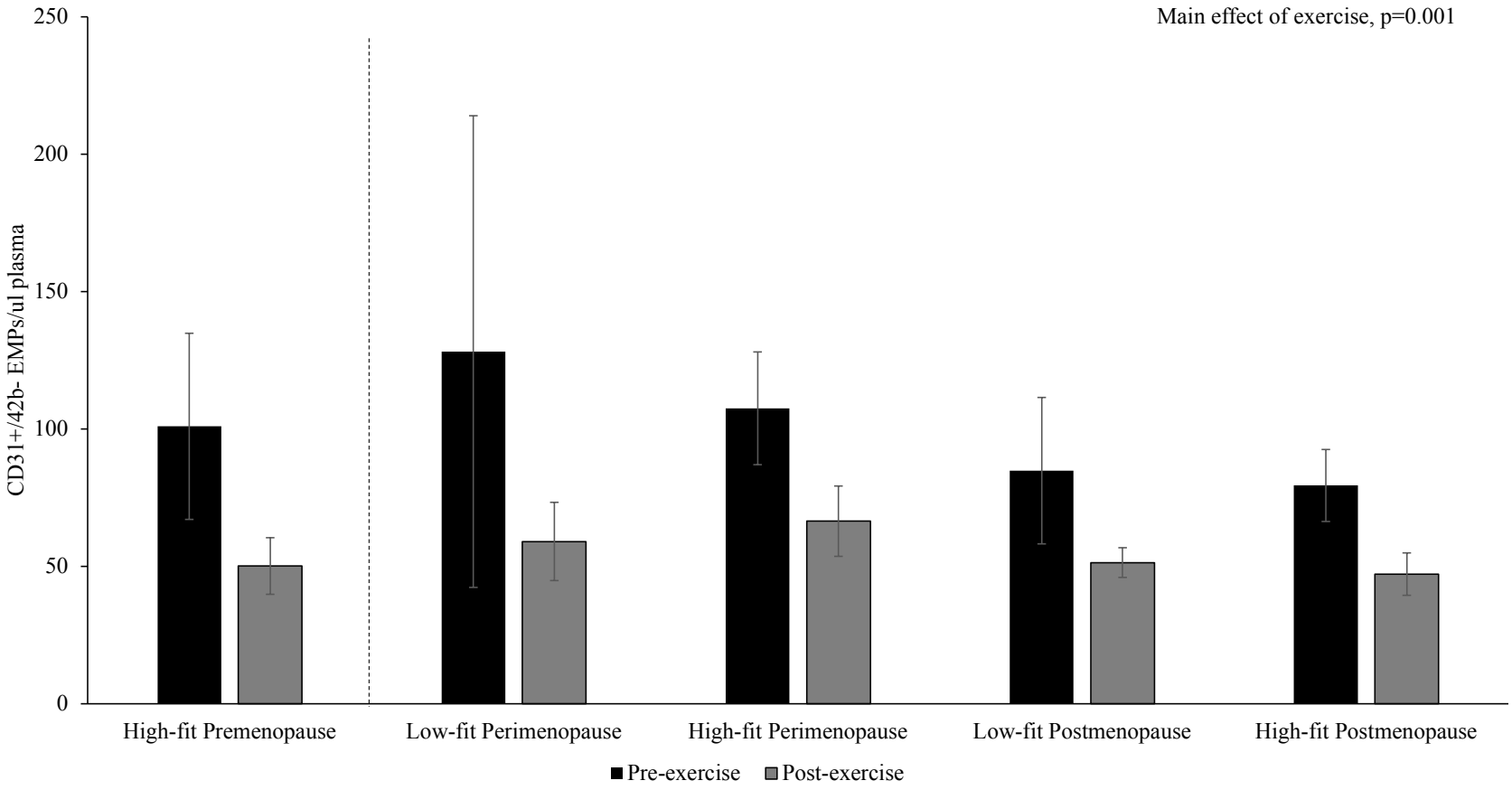


Figure 5.3. Activated Endothelial Microparticles.

The number of activated microparticles/μl plasma was reduced with acute exercise (A), while the percentage of activated microparticles was increased with acute exercise (B). EMPS: endothelial microparticles, MPs: microparticles, data are presented as mean±SEM

A


Main effect of exercise, p=0.001
CD31+/42b- EMPs/ul plasma
250
200
150
100
50
0
High-fit Premenopause
Low-fit Perimenopause
High-fit Perimenopause
Low-fit Postmenopause
High-fit Postmenopause
Pre-exercise
Post-exercise

B

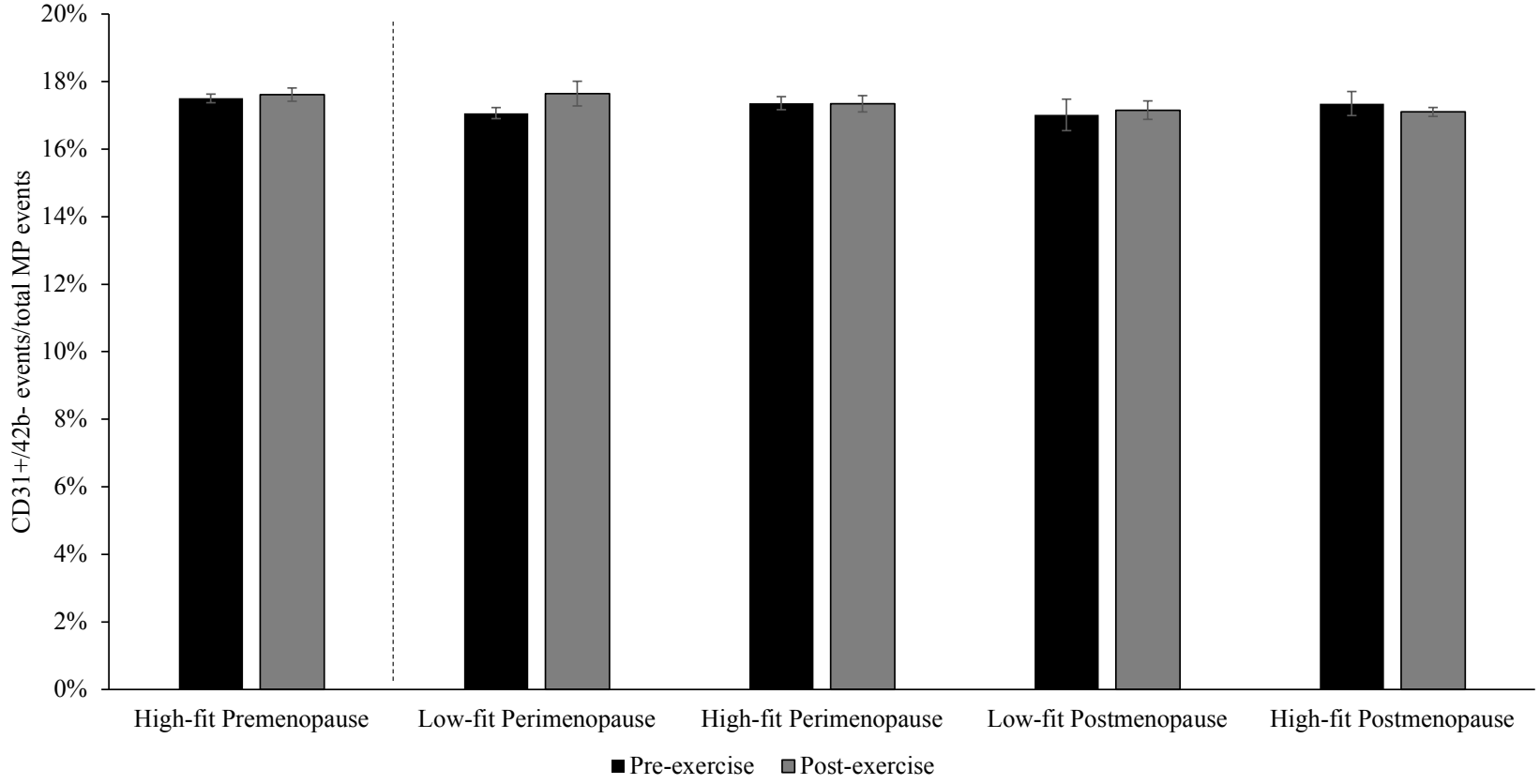


Figure 5.4. Apoptotic Endothelial Microparticles.

The number of apoptotic microparticles/μl plasma was reduced with acute exercise (A), while the percentage of apoptotic microparticles did not change with acute exercise (B). EMPs: endothelial microparticles, MP: microparticles, data are presented as mean±SEM

CHAPTER 6

FOLLICLE STIMULATING HORMONE IS ASSOCIATED WITH LIPIDS IN POSTMENOPAUSAL WOMEN

Introduction

Menopause is associated with changes in sex hormone levels. Although many of the physiological changes with menopause have been attributed to changes in estrogens, there is increasing evidence to suggest that follicle stimulating hormone (FSH) may have an independent effect on cardiovascular disease risk (45, 198). FSH levels being to rise during the perimenopausal years. FSH levels remain elevated postmenopause, although there is considerable variability in postmenopausal FSH levels. The influence of the rise in FSH on cardiovascular disease risk is controversial. Some studies have suggested that high FSH levels may negatively influence cardiovascular disease risk (44, 45), while others have suggested that it is beneficial (11, 12, 198). Regardless of the direction of the influence of FSH on CVD risk, there is compelling evidence to suggest that FSH may exert an effect on CVD risk through its influence on lipid levels.

Animal research has shown that FSH can act on low-density lipoprotein cholesterol receptors (169), demonstrating a direct effect of FSH on lipids. Furthermore, an increase in FSH, independent of estrogen levels, led to an increase in both total cholesterol and low-density lipoprotein cholesterol (169). While an association between lipids and FSH has been shown in several human studies (27, 169), there is a scarcity of evidence in this area, especially regarding the relation in older, postmenopausal women. Therefore, the aim of this study was to evaluate whether FSH levels are related to lipid levels in postmenopausal women.

Methods

Study Population

Participants were members of the Kuopio Ischaemic Heart Disease Risk Factor Study (KIHD). This is a prospective cohort study in men and women in Eastern Finland designed to identify risk factors for metabolic and cardiovascular diseases. Female participants were enrolled in the KIHD study between March 1998 and February 2001. Eligible women were a random sample of 1,173 postmenopausal women living in Kuopio or the surrounding area. Women were recruited into four age categories: 53 to 56 years, 59 to 62 years, 64 to 68 years, and 71 to 73 years. Ultimately, 920 women (87.4% of those invited) completed the baseline clinical assessments and were enrolled in the cohort.

For the present analysis, women were excluded if they reported current hormone therapy use or if FSH or lipid values were missing. The final sample size for this analysis was 588. The Research Ethics Committee at the University of Kuopio approved study procedures and participants provided written informed consent before beginning any study procedures.

Blood Collection and Biochemical Measurements

Blood samples were collected from participants at a clinic visit between 8 and 10 AM, while participants were fasted and had abstained from smoking cigarettes for 12 hours, and alcohol consumption for 3 days. Plasma and serum was separated and then stored, within 1 hour of venipuncture, at -20°C or -80°C until analysis. Biochemical measures, including hormonal factors such as FSH, 17-beta estradiol, sex-hormone

binding globulin (SHBG), and testosterone were assessed between June 2001 and February 2002.

Follicle stimulating hormone, 17-beta estradiol, and testosterone were measured using commercially available immunoradiometric assays. Serum FSH was determined with sandwich technique, applying an immunoradiometric assay manufactured by Diagnostic Product Corporation (Coat-A-Count FSH IRMA). Serum 17-beta-estradiol was assayed between 1999 and 2001 with a radioimmunoassay manufactured by DiaSorin. Serum testosterone (17b-hydroxy-4-androsten-3-one) was determined with Spectria Testosterone (125I) radioimmunoassay kit (Orion Diagnostica Espoo, Finland). 125I label measurements for FSH, E2, and testosterone were carried out by gamma counter (Wallac 1261 MultiGamma) using a RiaCalc LM Evaluation Program. Coefficients of variation were 5%, 7.6-12%, and 7.9-12.2% respectively. Sex hormone binding globulin was measured with a fluoroimmunoassay (AutoDELFIA SHBG, Wallac Co., Turku, Finland). Total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C) and triglycerides (TG) were assessed enzymatically (CHOD-PAP method, Boehringer Mannheim, Mannheim, FRG). Coefficients of variation were 2.3%, 5.2%, 9.2% and 1.9% respectively.

Clinical Measures and Questionnaire Assessments

Clinical and reproductive outcomes such as time since the final menstrual period, oral contraceptive use, history of hormone therapy, hysterectomy and oophorectomy were evaluated based on self-report. Participants were asked about behavioral factors such as smoking and physical activity on validated questionnaires (95). A trained interviewer then reviewed all questionnaire responses. History of cardiovascular and metabolic

diseases, along with medication use, were assessed during physician administered interviews.

Resting blood pressure was measured 6 times during the clinic visit, and mean blood pressure was calculated by averaging three supine, one standing, and two seated measurements, with 5 minutes rest in between measures. Height, weight, and waist and hip circumference were measured. Height and weight were used to calculate body mass index (BMI) as weight (kg) divided by height (m^2). Waist-to-hip ratio (WHR) was calculated from circumference measurements.

Statistical Analyses

All statistical analyses were completed in SPSS v24. Significance was set at an alpha level of 0.05. Participants were divided into quartiles based on FSH levels. Baseline characteristics were compared across quartiles using ANOVAs or chi-squared tests.

Linear regression was used to evaluate the relationship between FSH and lipid levels. Lipid variables were log transformed to improve normality and included TC, LDL-C, HDL-C, and TG. Model 1 adjusted *a priori* for age, date of examination, estradiol, SHBG and testosterone levels, age of menarche, last menstruation age, oral contraceptive use, and history of hysterectomy or oophorectomy. Model 2 included all covariates in model 1 as well as waist-to-hip ratio, body mass index, systolic and diastolic blood pressure, physical activity levels, and smoking status. The fully adjusted model included covariates in model 2 and use of lipid-lowering medications. Model 2 was also run in participants not taking lipid lowering medications. The median FSH value for each quartile was modeled as a continuous variable to assess linear trends. To evaluate if

relations of FSH and lipids varied by age and body mass index, we conducted analyses stratified by these factors and tested for effect modification.

We then used logistic regression to evaluate whether FSH levels were associated with dyslipidemia, as well as high TC (>6.20mmol/L), high LDL-C (≥4.1 mmol/L), low HDL-C (<1.0 mmol/L) and high TG (≥2.30 mmol/L)(128). Dyslipidemia was defined as meeting any of the criteria for abnormal lipid levels, as defined above, or the use of lipid-lowering medications. All abnormal lipid outcomes also included use of lipid lowering medications. Covariates were selected based on which variables led to the greatest change in the odds ratio for FSH. FSH was included as a continuous variable. Covariates meeting selection criteria included body mass index, waist-to-hip ratio, SHBG, estradiol, and age.

Results

When comparing participant characteristics based on FSH quartiles, there were significant, though modest, differences across groups (Table 6.1). BMI, WHR, estradiol, and SHBG were lower at higher FSH quartiles. Physical activity was higher at higher FSH quartiles. Despite these differences, many variables were similar across groups including age at menopause, past hormone therapy use, smoking status, and parity.

There was an increase in non-adjusted LDL-C (P=0.15) and TC (P<0.001) levels with increasing FSH quartiles (Table 6.2). We observed a positive linear relationship between FSH and TC (model 3, P for trend 0.001), and LDL-C (model 3, P for trend 0.007). Results from minimally and fully adjusted models for these lipids were virtually identical.

HDL-C levels were higher among women in quartiles 2-4 for FSH than quartile 1 in model 1 (P for trend = 0.03), with results somewhat attenuated in our fully adjusted model (P = 0.06). FSH was not consistently associated with triglyceride levels. We also evaluated model 2 in participants not taking lipid-lowering medications and found that this did not influence our results for any lipid variable (data not shown).

We assessed the FSH–lipids relation in fully adjusted models stratified by age (younger: 53-62 years; older: 64-73 years, Appendix I). Similar to the main analysis, there was no relationship between FSH and HDL-C (younger, P for trend 0.41, older; P for trend 0.09) or TG (younger, P for trend 0.54; older, P for trend 0.28). We did not observe evidence of significant effect modification by age (all P >0.05), but results varied slightly between older and younger women. The relationship between FSH and TC and LDL-C was only significant in older women, though there appeared to be a stronger association in younger women, as suggested by larger beta coefficients (Table 6.3). We found no evidence of effect modification based on body mass index (all P>0.05; data not shown).

Finally, we assessed the association of continuous FSH levels with risk of dyslipidemia and abnormal levels of each lipid (Table 6.4). In addition to presenting betas for a 1-unit increase in FSH, Table 6.4 presents OR for a 40 IU/L increase in FSH, equivalent to the difference in the medians of quartiles 1 and 4. Follicle stimulating hormone levels were not associated with prevalent dyslipidemia. However, FSH was significantly associated with higher risk of high TC and LDL-C. A 40-unit increase in FSH was associated with a 62% higher risk of high total cholesterol and a 55% higher risk of high LDL-C. Results suggested that higher FSH was associated with lower risk of

abnormal HDL-C and TG, but were not significant. We also found no evidence of effect modification based on body mass index or age (all P>0.05; data not shown).

Discussion

Menopause is associated with adverse changes in cardiovascular disease risk factors, including lipid levels (108). While these changes have largely been attributed to changing estrogens, increasing evidence suggests that FSH may play an independent role. The findings from this study suggest that FSH is positively related to total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) in postmenopausal women. We further show that FSH is not associated with dyslipidemia but has a significant positive relationship with elevated LDL-C and TC. The results also suggest that higher FSH is associated with better high-density lipoprotein cholesterol (HDL-C) and triglyceride (TG) levels, although these associations did not reach statistical significance. Relations persisted even after adjustment for hormonal factors, CVD risk factors, and lipid lowering medication use. Finally, our data suggests that these relationships may vary somewhat by age, though our power to detect significant differences was low due to the small samples sizes of individual strata.

Rodent studies demonstrate that FSH may have direct effects on LDL-C and TC production. In ovariectomized mice, when FSH was elevated independent of estradiol, there was an increase in both TC and LDL-C. This increase was associated with reduced LDL-receptor expression. FSH receptors are also present in human liver tissue, and when exposed to FSH, there is reduced expression of LDL-receptors (169). FSH has also been shown to stimulate lipid biosynthesis in chicken adipose tissue (32) and lipid droplet

formation in human adipose tissue (100), again demonstrating that FSH may have direct effects on lipid metabolism and specifically on TC and LDL-C.

Few human studies have evaluated the effect of FSH on lipids. An analysis comparing Chinese postmenopausal women with high vs. low levels of FSH found that higher FSH was associated with higher levels of LDL-C and TC (169). Further, a 30% reduction in FSH due to hormone therapy, equivalent to ~25IU/L change, led to a reduction in LDL-C of 0.14mmol/L and a reduction in TC of 0.19 mmol/L. While these results show the same directional relationships as our current analysis, the relation of FSH with lipid levels is more modest than in our study. When comparing FSH quartile 1 to quartile 3 (23.3 IU/L difference in median FSH) there was a 0.2 mmol/L difference in LDL-C and a 0.2 mmol/L difference in TC in our analysis. The difference in magnitude of effect may be due to differences in FSH levels between populations. For example, the 50^{th} percentile cutoff in our sample was 50.0 IU/L compared to 78.3 IU/L in their sample. Therefore, a 25 IU/L difference in FSH represents a greater relative change in FSH in our study. Further, women in our study were older, also potentially contributing to the difference in magnitude of study results.

A changing relationship between FSH and CVD risk factors with age is supported by the literature. For example, in longitudinal data from the Study of Women's Health Across the Lifespan (SWAN) study, a lower compared to a higher rise in FSH from perimenopause to approximately 8 years after menopause was associated with reduced CVD risk (45). Conversely, in older postmenopausal women, higher FSH has been associated with lower cardiovascular disease risk (11, 198) and lower risk of diabetes (12). Further, Wide et al., reported differences in FSH isoforms in postmenopausal

compared to premenopausal women, potentially leading to a higher FSH half-life in postmenopausal women and therefore influencing its biological effects (200). Our data shows a stronger relationship between FSH and TC and LDL-C in younger compared to older postmenopausal women, further indicating a possible change in the effects of FSH with increasing age.

While we show a positive association between FSH and abnormal levels of TC and LDL-C, we observed an inverse association of FSH levels with abnormal (i.e, low) HDL-C and (i.e. high) TG. This is in line with data in the literature that has suggested a positive effect of high levels of FSH on cardiovascular disease risk. In postmenopausal women in China, there was a positive association between FSH and HDL-C and a negative relationship with TG and LDL-C (198). The difference in the relationship between FSH and LDL-C in that study compared to our current analysis may be due to the wider FSH range in our study and that we controlled for more variables, including WHR, in our analyses. Among postmenopausal women in the KIHD study, high levels of FSH have also been associated with reduced incidence of type two diabetes (12) and with lower carotid artery intima media thickness, especially in older women (11). The positive association of FSH with TG and HDL-C may represent one of the mechanisms by which FSH impacts CVD risk; however, given the positive relationships that we found with TC and LDL-C, the overall effects of FSH on CVD risk, through its impact on lipid levels remains questionable.

Limitations

While this study provides some novel insight into the relationship between lipid levels and FSH in postmenopausal women, it has several limitations. As study

participants were postmenopausal women in Finland, results may not be generalizable to other populations with greater racial and ethnic diversity. Further, this is a cross-sectional analysis; therefore, we were unable to evaluate the temporality of the relationship between FSH and lipid levels.

Conclusions

Overall, this study indicates that FSH may be associated with lipid levels in postmenopausal women and that relations vary between lipids. Consequently, the results suggest that FSH may be associated with chronic conditions related to lipid levels, including CVD. Further study of these relations and the physiologic mechanisms underlying them is warranted as this may allow for a clearer understanding as to whether FSH may be an important physiological target for reducing CVD risk with menopause.

Tables

FSH Quartile (n)	Quartile 1 (n=147)	Quartile 2 (n=149)	Quartile 3 (n=145)	Quartile 4 (n=147)	
FSH range (IU/l)	1-39.3	39.4-50.0	50.1-61.8	61.9 to 136.8	
	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	P
Age (yrs)	64.5 (6.9)	65.1 (5.9)	64.4 (6.1)	62.8 (6.9)	0.02
BMI (kg/m^2)	31.1 (5.7)	29.0 (5.4)	28.7 (5.0)	26.8 (4.4)	<0.001
WHR	0.87 (0.07)	0.85 (0.06)	0.84 (0.06)	0.83 (0.06)	<0.001
Estradiol (pmol/ml)	55.8 (77.6)	35.1 (19.8)	32.5 (12.5)	34.1 (19.5)	<0.001
Testosterone (nmol/l)	1.5 (2.9)	1.1 (0.5)	1.1 (0.5)	1.1 (0.5)	0.06
SHBG (nmol/L)	45.6 (23.2)	49.5 (21.2)	55.0 (24.3)	58.4 (28.0)	<0.001
SBP (mmHg)	140.8 (20.4)	141.1 (17.3)	136.2 (16.4)	135.8 (16.4)	0.01
DBP (mmHg)	80.9 (9.7)	80.5 (8.7)	79.6 (8.3)	80.4 (8.5)	0.66
Physical Activity (MET-hr/day)	45.2 (5.9)	46.1 (7.1)	46.3 (6.1)	47.4 (7.5)	0.05
Parity	2.8 (1.4)	2.7 (1.6)	2.8 (1.7)	2.3 (1.3)	0.08
	n (%)	n (%)	n (%)	n (%)	
Age at menopause (yrs)	49.7 (4.4)	49.4 (4.3)	48.9 (4.8)	49.2 (4.3)	0.50
Current Smoker	12 (8%)	14 (9%)	16 (11%)	7 (5%)	0.25
Past Smoker	19 (13%)	15 (10%)	11 (8%)	19 (13%)	0.37
Past HT use	38 (26%)	45 (30%)	48 (33%)	49 (33%)	0.58

Table 6.1. Participant Characteristics.

FSH: follicle stimulating hormone, BMI: body mass index, SHBG: sex hormone binding globulin, SBP: systolic blood pressure, DBP: diastolic blood pressure: HDL-C: high-density lipoprotein cholesterol, LDL-C: low-density lipoprotein cholesterol, HT: hormone therapy

		Model 1[b]			Model 2[c]			Model 3[d]		
	Mean (SD)[a]	Beta	SE	p	Beta	SE	p	Beta	SE	p
Total Cholesterol										
FSH Q1	5.6 (0.7)		Ref			Ref			Ref	
FSH Q2	5.8 (0.9)	0.014	0.008	0.09	0.014	0.008	0.10	0.014	0.008	0.08
FSH Q3	5.8 (0.9)	0.016	0.008	0.05	0.015	0.008	0.04	0.018	0.008	0.04
FSH Q4	5.9 (1.0)	0.025	0.008	0.004	0.025	0.009	0.003	0.025	0.009	0.004
p for trend	0.01*			0.002			0.001			0.001
LDL-C										
FSH Q1	3.6 (0.7)		Ref			Ref			Ref	
FSH Q2	3.8 (1.0)	0.013	0.013	0.29	0.014	0.013	0.26	0.015	0.013	0.23
FSH Q3	3.8 (0.9)	0.022	0.013	0.09	0.025	0.013	0.05	0.026	0.013	0.05
FSH Q4	3.9 (1.0)	0.031	0.013	0.02	0.035	0.013	0.01	0.035	0.013	0.01
p for trend	0.15*			0.01			0.01			0.007
HDL-C										
FSH Q1	1.3 (0.3)		Ref			Ref			Ref	
FSH Q2	1.3 (0.3)	0.021	0.011	0.06	0.018	0.011	0.12	0.018	0.011	0.12
FSH Q3	1.4 (0.3)	0.021	0.012	0.07	0.019	0.012	0.11	0.019	0.012	0.11
FSH Q4	1.4 (0.6)	0.025	0.012	0.04	0.018	0.012	0.13	0.018	0.012	0.13
p for trend	<0.001*			0.02			0.06			0.06
Triglycerides										
FSH Q1	1.4 (0.7)		Ref			Ref			Ref	
FSH Q2	1.3 (0.8)	0.015	0.021	0.47	0.025	0.02	0.23	0.021	0.02	0.29
FSH Q3	1.2 (0.5)	0.01	0.022	0.66	0.021	0.021	0.49	0.014	0.021	0.50
FSH Q4	1.2 (0.6)	0.014	0.022	0.51	0.021	0.022	0.09	0.037	0.021	0.08
p for trend	0.01*			0.67			0.21			0.17

Table 6.2. Association of Follicle Stimulating Hormone and Lipid Levels.

[a]Unadjusted, untransformed mean total cholesterol (mmol/l), low-density lipoprotein cholesterol (LDL-C, mmol/L), high-density lipoprotein-cholesterol (HDL-C, mmol/L), and triglycerides (mmol/l).

[b]Covariates included in model 1: age (continuous), date of study entry (continuous), estradiol (quartiles), testosterone (quartiles), sex-hormone binding globulin (quartiles), starting menstruation age (above and below median), last menstruation age (above and below median), oral contraceptive use (yes, no: yes-above and below median), uterus eliminated (yes, no), ovaries eliminated (yes, no), hormone therapy use (yes, no: yes-above and below median).

[c]Covariates included in model 2: model 1 covariates, systolic blood pressure (continuous), diastolic blood pressure (continuous), smoking status (pack-years+past, current, never; current-above and below median for pack-years), physical activity (quartiles), BMI (continuous), waist-to-hip ratio (continuous).

[d]Covariates included in model 3: model 2 covariates, lipid lowering medication use (yes, no)

*indicates p-value from ANOVA

	Younger women (n=244, 53-62 years)				Older women (n=344, 64-73 years)			
		Model 3[b]				Model 3[b]		
	Mean (SD)[a]	Beta	SE	p	Mean (SD)[a]	Beta	SE	p
Total Cholesterol					**Total Cholesterol**			
FSH Q1	5.6 (0.7)	Ref			FSH Q1	5.5 (0.8)	Ref		
FSH Q2	5.8 (1.0)	0.023	0.013	0.11	FSH Q2	5.7 (1.0)	0.005	0.011	0.64
FSH Q3	5.8 (0.9)	0.026	0.013	0.06	FSH Q3	5.7 (0.8)	0.008	0.011	0.49
FSH Q4	5.9 (1.0)	0.021	0.013	0.10	FSH Q4	6.0 (1.0)	0.024	0.012	0.06
p for trend				0.22	p for trend				0.05
LDL-C					**LDL-C**			
FSH Q1	3.6 (0.7)	Ref			FSH Q1	3.6 (0.7)	Ref		
FSH Q2	3.8 (1.0)	0.022	0.022	0.33	FSH Q2	3.8 (1.0)	0.002	0.016	0.89
FSH Q3	3.8 (0.9)	0.048	0.021	0.03	FSH Q3	3.7 (0.8)	0.003	0.017	0.84
FSH Q4	3.9 (1.0)	0.041	0.021	0.05	FSH Q4	3.9 (1.0)	0.026	0.019	0.16
p for trend				0.14	p for trend				0.01
HDL-C					**HDL-C**			
FSH Q1	1.3 (0.3)	Ref			FSH Q1	1.2 (0.3)	Ref		
FSH Q2	1.3 (0.3)	0.027	0.018	0.13	FSH Q2	1.3 (0.3)	0.011	0.016	0.48
FSH Q3	1.4 (0.3)	0.014	0.017	0.42	FSH Q3	1.4 (0.3)	0.024	0.017	0.15
FSH Q4	1.4 (0.3)	0.008	0.017	0.57	FSH Q4	1.4 (0.4)	0.022	0.018	0.23
p for trend				0.42	p for trend				0.09
Triglycerides					**Triglycerides**			
FSH Q1	1.4 (0.7)	Ref			FSH Q1	1.4 (0.7)	Ref		
FSH Q2	1.4 (0.8)	-0.018	0.035	0.61	FSH Q2	1.4 (0.6)	0.032	0.026	0.23
FSH Q3	1.2 (0.6)	-0.001	0.033	0.98	FSH Q3	1.2 (0.6)	0.021	0.028	0.46
FSH Q4	1.2 (0.6)	0.044	0.032	0.17	FSH Q4	1.2 (0.6)	0.03	0.030	0.32
p for trend				0.54	p for trend				0.28

Table 6.3. Association of Follicle Stimulating Hormone and Lipid Levels by Age.

[a]Unadjusted, untransformed mean Total cholesterol (mmol/l), low-density lipoprotein cholesterol (LDL-C, mmol/L), high-density lipoprotein-cholesterol (HDL-C, mmol/L), and triglycerides (mmol/l).

[b]Covariates included in model 3: age (continuous), date of study entry (continuous), estradiol (quartiles), testosterone (quartiles), sex-hormone binding globulin (quartiles), starting menstruation age (above and below median), last menstruation age (above and below median), oral contraceptive use (yes, no: yes-above and below median), uterus eliminated (yes, no), ovaries eliminated (yes, no), hormone therapy use (yes, no: yes-above and below median), systolic blood pressure (continuous), diastolic blood pressure (continuous), smoking status (pack-years+past, current, never; current-above and below median for pack-years), physical activity (quartiles), BMI (continuous), waist-to-hip ratio (continuous), lipid lowering medication use (yes, no).

	n	beta per 1 IU higher	SE	OR per 40 IU higher	P
Dyslipidemia (all lipids)[a]					
No*	294	reference			
Yes	292	0.003	0.005	1.13	0.57
Total Cholesterol[b]					
≤ 6.2 mmol/L*	399	reference			
>6.2 mmol/L	189	0.012	0.005	1.62	0.02
LDL-C[b]					
<4.1 mmol/L*	374	reference			
≥ 4.1 mmol/L	214	0.011	0.005	1.55	0.03
HDL-C[b]					
≥1.0 mmol/L*	491	reference			
<1.0 mmol/L	97	-0.01	0.007	0.67	0.15
Triglycerides[b]					
<2.3 mmol/L*	520	reference			
≥ 2.3 mmol/L	68	-0.012	0.008	0.62	0.17

Table 6.4. Association of Follicle Stimulating Hormone and Abnormal Lipid Levels.

OR: odds ratio. Lipid classifications based on ATP II guidelines (128). *includes lipid-lowering medication

[b]Covariates included age (continuous), date of study entry, sex hormone binding globulin (continuous), estradiol (continuous), body mass index (continuous), waist to hip ratio (continuous).

CHAPTER 7

SUMMARY

The aim of this dissertation was to evaluate variables related to changing cardiovascular disease risk with menopause. This was accomplished through two primary studies. The first evaluated the influence of menopausal status and cardiorespiratory fitness on measures of endothelial health before and after an acute bout of exercise. The second evaluated the influence of follicle stimulating hormone (FSH) on lipid levels in postmenopausal women. Together, these studies filled several important knowledge gaps in the literature (Figure 7.1), improving our understanding of how cardiovascular disease risk changes with menopause.

The first study in this dissertation evaluated markers of endothelial health. This study found that endothelial function was reduced in postmenopausal women, potentially due to the influence of high FSH levels, and that fitness was not protective of endothelial function in perimenopausal or in postmenopausal women. These findings corroborate what others have shown in the literature: that endothelial function is reduced in postmenopause and that there may be a resistance to the benefits of fitness on endothelial function in postmenopausal women. It also shows, for the first time, that this resistance may be present in perimenopausal women. However, although FMD is a well-validated technique, our data suggests that it may underestimate vasodilatory capacity, therefore we also evaluated max-FMD. The max-FMD analyses suggest that fitness may be protective of endothelial function; however, this technique requires further validation.

This study also evaluated endothelial microparticle (EMP) levels. Through this analysis, we found that EMPs are reduced with acute exercise, despite differences in

menopausal status and fitness. This corroborates the reduction in EMPs with moderate intensity exercise that others have shown in the literature and may suggest that some of the benefits of long-term exercise training on EMPs may be due to the acute effects of exercise on EMPs. Together, the FMD and EMP data highlight that these independent variables provide unique and independent information about endothelial health in menopausal women. For example, the FMD data indicates that both fitness and menopausal status influence endothelial function. Conversely, the EMP data suggests that EMPs may not be influenced by these variables, but are highly responsive to acute exercise.

The final study in this dissertation evaluated the relationship between FSH and lipid levels in postmenopausal women. This study found that FSH had a positive linear relationship with low-density lipoprotein cholesterol (LDL-C) and total cholesterol (TC) and that these relationships may be stronger in younger compared to older postmenopausal women. We also found that higher FSH is associated with increased risk of having high TC and high LDL-C, suggesting a clinically relevant relationship. Overall, these results suggest that higher levels of FSH in the postmenopausal years may have adverse effects on TC and LDL-C.

Future Directions

There are several important areas of future inquiry based on the findings from this dissertation. Given the low levels of endothelial function we report in high-fit women, it will be important to evaluate potential mechanisms for this reduced function and whether other mechanisms may be contributing to these findings. For example, it is possible that high-fit women have more compliant blood vessels than low-fit women or that they are

relying on vasodilatory factors other than nitric oxide. It will also be important to validate the max-FMD technique. Given that it appears to better match the low cardiovascular disease risk profile of the participants in this study, this may be a promising new approach for evaluating endothelial function in perimenopausal and postmenopausal women. From the EMP data, it will be important to evaluate the functional implications of the reduction in EMPs that we report with acute exercise, as they may represent a potential mechanism for some of the benefits of long-term exercise training. Finally, it will be important to evaluate the effects of FSH on lipid levels longitudinally.

Conclusions

Overall, this dissertation showed that menopause may negatively influence endothelial function and lipid levels. It further suggested that the effects of cardiorespiratory fitness on endothelial function may not be present in perimenopausal and postmenopausal women. Finally, we showed that acute moderate intensity exercise reduces endothelial microparticles in midlife women, despite differences in menopausal status and cardiorespiratory fitness. Together, these data suggest that continued evaluation of how cardiovascular disease risk changes with menopause and the influence of fitness on these markers is warranted.

Figures:

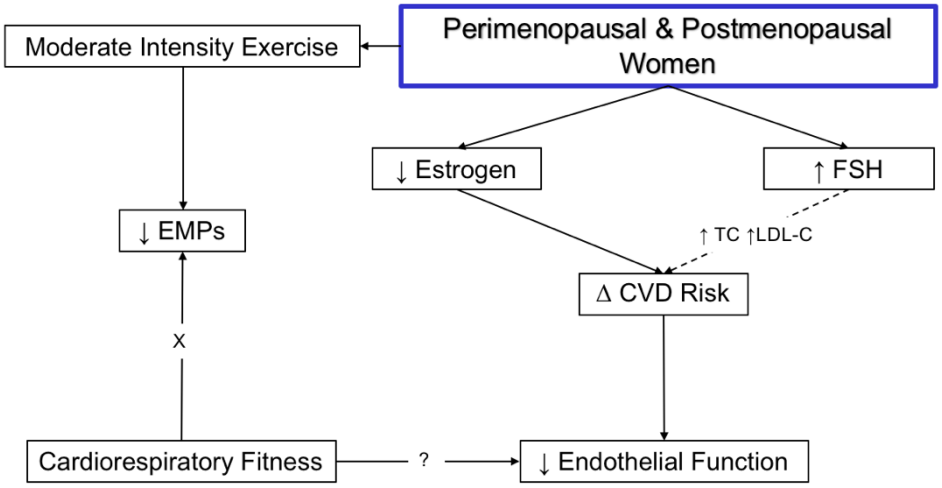


Figure 7.1. Summary of Findings

There is an increase in follicle stimulating hormone (FSH) and a reduction in estrogen with menopause that change a woman's cardiovascular disease (CVD) risk. The mechanisms behind the effects of estrogen on CVD risk are relatively well established, while the effects of FSH are not. This dissertation demonstrated that FSH may be exerting its effects on CVD risk through its effects on total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C). The change in CVD risk with menopause contributes to reduced endothelial function. While fitness is considered protective of endothelial function, this effect may be diminished in perimenopausal and postmenopausal women; however, what this relationship looks like still requires further research. Finally, we found that acute moderate intensity exercise reduces endothelial microparticles (EMPs) in perimenopausal and postmenopausal women and that that affect occurred despite differences in cardiorespiratory fitness and menopausal status across groups. x, indicates no relationship, ?, indicates an unclear relationship.

APPENDIX A

INFORMED CONSENT

Consent Form for Participation in a Research Study
University of Massachusetts
Amherst

Study Title: Vascular Function across the Menopausal Transition

Principal Investigators: Corinna Serviente, MS & Sarah Witkowski, PhD

Section One Overview of Study

WHAT IS THIS FORM?

This form is called a Consent Form. It will give you information about the study, so you can make an informed decision about participation in this research study. This consent form will give you the information you will need to understand why this study is being done and why you are being invited to participate. It will also describe what you will need to do to participate and any known risks, inconveniences or discomforts that you may have while participating. We encourage you to take some time to think this over and ask questions. If you decide to participate, you will be asked to sign this form and you will be given a copy for your records.

WHO IS ELIGIBLE TO PARTICIPATE?

- Participants must meet criteria for either of the following three categories and be
  65 years old or younger:
  - **Premenopause:** regular menstrual cycles
  - **Early perimenopause:** variable period length (more than 7 days different from normal)
  - **Late perimenopausal:** 60 days or more, but less than 1 year without a period
  - **Late postmenopause:** more than 5 years without a period
- Participants should:
  - Have normal blood pressure
  - Be non-diabetic (normal blood sugar levels)
  - Be participating in less than 150 min/week of moderate intensity physical activity or less than 75 min/week of vigorous intensity physical activity, accumulated in 10 minute bouts OR be participating in at least 300 min/week of moderate intensity physical activity or at least 150 min/week of vigorous intensity physical

activity, accumulated in 10 minute bouts and have been doing so for the past 2 years
    - Be nonsmokers
    - Have normal fat levels in the blood
    - Have a VO_2 max that is equal to or greater than the 80th percentile for their age, if classified as high-active
- Participants should not:
    - Have used hormone replacement therapy, menopausal symptom treatment, or birth control in the past 6 months
    - Have a history of heart or blood vessel diseases
    - Have had a previous heart attack or cardiovascular intervention (e.g.pacemaker implant)
    - Have had a hysterectomy prior to menopause or not have undergone natural menopause
    - Have long-term menstrual irregularities
    - Have used certain vitamin/supplements, fat lowering and/or anti-inflammatory medications in the past 4 week
    - Currently have and/or have a history of breast cancer (with radiation or chemotherapy treatment), vaginal bleeding, abnormal uterine/ovary structure, blood clots, acute liver or gallbladder diseases
    - Have high fat levels in the blood
    - Have a muscle, bone, or other condition that limits ability exercise
    - Have a reason that limits ability to take part in an exercise test
    - Have a reason to stop the exercise test early
    - Have a history of fainting with blood draws
    - Be pregnant
    - Are currently participating in a research study that conflicts with this study's protocols

3. WHAT IS THE PURPOSE OF THIS STUDY?

The purpose of this research study is to examine blood vessel function across the menopausal transition. We aim to determine if vessel function, and the inflammatory markers (MCP-1 and IL-8), are different at baseline and in response to a single exercise session and whether these responses vary in women who are more or less physically active

4. WHERE WILL THE STUDY TAKE PLACE AND HOW LONG WILL IT LAST?

The research for this study will be performed in the Department of Kinesiology at the University of Massachusetts in Amherst, MA. You will be asked to come to Amherst approximately 3 times for ~1-3 hours per visit. Your total participation will be about 6-8 weeks, depending on the ease of scheduling, the screening, and testing visits.

5. WHAT WILL I BE ASKED TO DO?

Your participation will involve the following procedures outlined below. Detailed procedures are described in section 2.

Visit Number	Tasks

Before Visit 1	• Pre-screening interview
	• For visit 1
	  ○ Refrain from eating or drinking (except water) for 12 hours before visit 1
	  ○ Early perimenopausal women arrive between days 2-5 of menstrual cycle for visits 1 and 3
	  ○ Wear loose fitting, nonmetallic clothing
	  ○ If desired, fill out questionnaires and read informed consent

Visit 1	• Fill out and review:
	  ○ Informed consent
	  ○ Health History
	  ○ Physical Activity Questionnaires
	  ○ Quality of life questionnaire
	• Measure height, weight, blood pressure
	• Complete blood draw
	• Receive low nitrate diet
	• Complete dual x-ray absorptiometry scan

Before Visit 2	• Fill out low nitrate diet log • Refrain from eating, drinking caffeine or alcohol, and smoking 3 hours before, and vigorous exercise 24 hours before visit 2
Visit 2	• Familiarization FMD* • VO_2 max test (exercise test) • Receive low nitrate diet log

Before Visit 3	• Fill out low nitrate diet log
	• For visit 3:
	  ○ Early perimenopausal women arrive between cycles days 2-5
	  ○ Refrain from eating 6 hours before, exercise, caffeine, smoking and alcohol 12 hours before, and any vitamins/supplements for 72 hours before the visit

Visit 3	• Baseline FMD and blood draw • Exercise session • Post-exercise FMD and blood draw

*FMD: flow mediated dilation

Possible Exclusion Time Points

Visit	Possible exclusion
Before Visit 1	Age; does not fit criteria for menopausal groups; history of cardiovascular disease, diabetes or other exclusion criteria asked about during pre-screening; regular exerciser; unable to exercise
Visit 1	Blood pressure; physical activity questionnaires suggest participant may be more active than inclusion criteria allows; answers on medical history that affect inclusion/exclusion criteria; pregnancy
Before Visit 2	High blood sugar levels or abnormal fat levels in the blood, VO_2 max less than the 80th percentile, if high active
Visit 2	Reason not to begin the exercise test; reason to stop the exercise test early

Section 2: Details of Study Visits

Day 1: If you agree to take part in this study, you will be asked to first come to the Molecular and Cardiovascular Physiology Laboratory at UMass Amherst. During your phone screening you will have been asked to arrive for this visit (today) having refrained from eating or drinking, except for water, for 12 hours. If you are classified as premenopausal or early perimenopausal, you will have been asked to arrive between menstrual cycles day 2-5. You will have been asked to wear loose-fitting non-metallic clothing. During this visit you will need to have the following exams, tests, and/or procedures. If you had some of these done recently, they may not need to be repeated.

- Forms that you filled out ahead of time will be reviewed. If you have not completed this paperwork yet, a medical history will be taken to be sure you don't have any conditions that could keep you out of the study. You will also be asked to fill out physical activity questionnaires to make a determination of whether you meet activity guidelines for this study. If you are classified as premenopausal or early perimenopausal and you are unsure or it is unclear if you are pregnant, you will be asked to complete a urine-based pregnancy test.

- Your heart rate, blood pressure, weight and height will be taken.
    - To evaluate your blood pressure, we will ask you to sit quietly for several minutes. A cuff will be placed around your upper arm. Either a lab member or a machine will inflate the cuff and then slowly release the pressure. If blood pressure is taken manually, we will listen for the blood pressure in the vessel with a stethoscope while the cuff deflates. We will repeat this procedure a few times to get an accurate reading. This reading will be used to determine if your blood pressure is at an acceptable level for this study.

- Blood will be collected for a fasting profile of the fats and sugar in your blood. This information will be used to determine if you are still eligible for the study. We will use a needle to withdraw blood from the vein in your arm. The amount of blood will be approximately 5 milliliters or 1 teaspoon.

- You will be given a quality of life questionnaire to complete and return on the next visit.

- You will be given instructions for following a low nitrate diet for the three days before your next visit. This will help get rid of any nitrates in your diet that could impact your blood vessel function study (flow mediated dilation). You will track this diet on a log sheet that will be given to you.

- You will be taken to University Health Services to complete a DEXA scan.
    - You will lie quietly on a machine in loose, non-metallic clothing, during which the arm of the machine will pass over your body emitting a very low level X-ray. The test takes approximately 15 minutes and allows us to measure muscle and fat mass and bone mineral density.

- You will be asked to avoid drinking caffeine, alcohol, or eating for 3 hours before your next visit and from exercising heavily for 24 hours before the next visit. This will allow us to obtain a more accurate reading of your exercise ability.

Day 2: On the second visit you will be asked to come to the Molecular and Cardiovascular Physiology Laboratory at UMass Amherst. You will return your diet log. During the visit you will have the following tests/procedures:

- We will familiarize you with our method for evaluation of blood vessel function in your arm, which will be used during the next visit. Before the test begins, you will lay quietly for approximately 10 minutes to relax. Your arm will be propped up with pillows and a small ball will be placed in your hand. We will use an ultrasound and Doppler machine to look at an artery in your arm. We will first image your artery at rest for 2 minutes and will then inflate a cuff placed around your forearm for 5 minutes. After 4 minutes, the cuff will be deflated and we will continue imaging for 3 more minutes. During the study, we will be taking blood pressures on your other arm.

- You will have a graded exercise treadmill test to make sure there are no problems with your blood pressure and heart rate and rhythm in response to exercise. This information will allow us to have you exercise at an appropriate intensity on your next visit. Based on previous screening criteria, your test will be supervised by a physician or by trained laboratory personnel.
    - For this test, we will place a blood pressure cuff around your upper arm to monitor your blood pressure and sensors on your chest to monitor your heart rate and rhythm during the test. The blood pressure cuff will remain on your arm for the rest of the test, but will only be inflated at the end of each stage of the test. Once we place all of the monitoring devices, we will take resting blood pressure and heart rate/rhythm measurements. We will explain a scale for how to rate your effort during the test and we will show you hand signals so that you can communicate with us throughout the test. Next, you will be asked to wear a snorkel-like mouthpiece and nose clip.

This allows us to look at how hard you are working. We will then have you chose a comfortable walking or jogging pace on the treadmill while we monitor you. After you are at a comfortable pace on the treadmill, the treadmill grade and/or speed will be increased every 2 minutes until you have reached your maximal capacity or until personnel see something that warrants them to stop the test. Once the testing is completed, we will have you walk for several minutes on the treadmill at a slow speed while we continue to monitor you. Then you will sit for several minutes until your heart rate and blood pressure start to return to pre-exercise levels.

- If, during the course of the test, laboratory personnel or a physician see anything that causes them to stop the test early, you will be advised to contact your physician.

- In the case that you remain qualified for the study, or you meet the inclusion criteria and do not meet the exclusion criteria, we will schedule you for the third visit. If possible, premenopausal and early perimenopausal women will be asked to schedule the visit between cycle days 2 and 5.

- We will ask you to follow a low nitrate diet for three days before the last visit and to log your food.

- We will ask you to refrain from eating 6 hours before, exercise, caffeine, smoking, and alcohol 12 hours before, and any vitamins/supplements 72 hours before the visit

Day 3: For the third testing visit, you will come to the Molecular and Cardiovascular Physiology Laboratory at UMass Amherst. We will check your blood pressure, draw blood to look for inflammatory markers (MCP-1 and IL-8), and perform measures of blood vessel function in your arm before and after exercise.

- When you arrive, we will have you rest quietly for about 10 minutes. Then we will check your blood pressure. Next, we will draw a sample of blood from the vein in your arm to analyze inflammatory factors found in the blood. This will be about 50 milliliters, or a little more than 3 tablespoons. Then, we will use the ultrasound and Doppler machine to look at blood vessel function in your arm (similar to the second visit). After we complete baseline measurements, we will inflate a cuff placed on your forearm for 5 minutes. The cuff will then be deflated and we will continue imaging for 4 more minutes. This will be repeated twice.

- Next, you will complete 30 minutes of exercise on a treadmill at what should feel like a moderate pace. We will ask you to wear a heart rate monitor during exercise so that we can adjust the treadmill to keep you at the desired heart rate. You will be given five minutes at the beginning and end of the test to warm up and cool down.

- 30 minutes after you cool down we will complete the same tests that were

done before the exercise. We will draw a sample of blood from your vein and use an ultrasound and Doppler machine to look at your blood vessel function two times.

WHAT ARE MY BENEFITS OF BEING IN THIS STUDY?

You may not directly benefit from this research; however, we hope that your participation in the study may help investigators to determine whether blood vessel function and inflammatory markers (MCP-1 and IL-8) vary across the menopausal transition. This information will help to determine if exercise causes short-term benefits on blood vessels and related inflammatory factors, and if exercise is a useful technique in all menopausal women.

You may benefit from the information we will gather from the screening, including blood pressure, fat levels in the blood, blood sugar, body composition, bone mineral density, and cardiovascular fitness. In the event that any of these factors are out of normal range, you will be advised to consult your personal physician. You will also be told of your results of the different risk factors that we measure and will be provided an explanation of these results.

Upon completion of the study, you will be entered into a raffle drawing. Prizes will include two $50 gift cards to a local business of choice, two gym memberships to the University of Massachusetts Amherst Body Shop, and two packages of three personal training sessions at the University of Massachusetts Amherst Body Shop. This drawing will be held once data collection has been completed on low-active participants and again when data collection has been completed on high-active participants.

Your participation in this research is completely voluntary and you may choose not to take part at all. You are free to ask questions at any time without penalty. If you decide to participate in this research, you may stop participating at any time. If you decide not to participate in this project or if you stop participating at any time, you will not be penalized or lose any benefits to which you otherwise qualify.

WHAT ARE MY RISKS OF BEING IN THIS STUDY?

The following risks are associated with your participation in this study:

1) **Blood draw:** There is risk of bruising and infection associated with blood drawing. These risks will be minimized by the use of sterile techniques and by having experienced personnel draw all blood samples. There may also be a chance of fainting during the blood draw. We will ask about any history of fainting during your screening visit. To minimize the risk of fainting or effects of fainting, blood draws will be performed with you in a reclined position.

2) **Physical Activity**: You may be at risk for injury from exercise. You may have sore muscles; you could sprain an ankle or fall and break a bone. When you start an exercise program, you could be at risk for heart problems. These risks may include high or low blood pressure, dizziness, skipped heartbeats, chest pain or heart attack when you

increase your physical activity. All exercise sessions will take place at the Molecular and Cardiovascular Physiology Laboratory at UMass Amherst, which is staffed by personnel trained in exercise testing and CPR. If necessary, a physician will be present during the graded exercise test. Trained personnel will be with you during each exercise session and will monitor your heart rate and ask how you are feeling.

3) **Vascular Ultrasound**: When we look at the blood vessel in your arm with the ultrasound, a blood pressure cuff positioned on your forearm will be inflated for 5 minutes. This might be uncomfortable and will feel tight. You will most likely feel some tingling like your hand is falling asleep while the blood pressure cuff is inflated. When the cuff is released, the tingling feeling will stop. If you have any discomfort during this procedure, let us know and we will do what we can to limit it.

4) **DEXA:** While radiation is involved in this procedure, the total amount of radiation you will be exposed to is relatively small (less than $1/20^{th}$ of the radiation received during a single chest x-ray).

5) **Timing:** Your involvement may lead to some inconveniences, such as time spent in travel and performing the screening and testing procedures.

6) **Fasting:** You will be asked to fast for 12 hours prior to your first visit and 6 hours prior to your third visit. Fasting for this time period may result in some discomfort. You will be allowed to drink water during this period. In addition, you will be given a snack and beverage on both of these visits. You will be asked to refrain from alcohol and caffeine for 3 hours before your second visit and 12 hours before your third visit. This can lead to temporary discomfort and irritability.

HOW WILL MY PERSONAL INFORMATION BE PROTECTED?

We will do our best to keep your personal information confidential. To help protect your confidentiality, all data are kept in a secure locked office with access available only to study personnel. Furthermore, all electronic files (e.g., database, spreadsheet, etc.) containing identifiable information will be password protected. Computers with data from this study will be protected by a password and only available to be accessed by authorized personnel involved with the study. Only the members of the research staff will have access to the passwords. Participants will be assigned IDs, which will not include any personally identifiable information. Medical information created by this research study (i.e. blood tests) will be given to the participant for their personal record. Blood samples will be labeled with a unique code, not your name or any other identifying information. For some analyses, samples cannot be analyzed immediately and will need to be stored until all samples from all participants are collected. These samples will only be identified by a unique code. The key to the codes will be kept in a locked and secure cabinet.

If we write a report or article about this research project, your identity will be protected to the maximum extent possible. Information will be presented in summary format and

you will not be identified in any publications or presentations. Your information may only be shared with representatives of the University of Massachusetts, or government authorities if you or someone else is in danger or if we are required to do so by law. Records of the research will be maintained for up to **10 years** following study completion.

WILL I RECEIVE ANY PAYMENT FOR TAKING PART IN THE STUDY?

You will not receive any monetary compensation for participating in this study.

WHAT IF I HAVE QUESTIONS?

Take as long as you like before you make a decision. We will be happy to answer any question you have about this study. If you have further questions about this project or if you have a research-related problem, you may contact the principal investigators, Sarah Witkowski at switkows@kin.umass.edu or 413-545-6102 or Corinna Serviente at cserviente@kin.umass.edu or 413-577-0392. If you have any questions concerning your rights as a research subject, you may contact the University of Massachusetts Amherst Human Research Protection Office (HRPO) at (413) 545-3428 or humansubjects@ora.umass.edu.

CAN I STOP BEING IN THE STUDY?

You do not have to be in this study if you do not want to. If you agree to be in the study, but later change your mind, you may drop out at any time. There are no penalties or consequences of any kind if you decide that you do not want to participate. You will be notified of all significant new findings during the course of the study that may affect your willingness to continue.

WHAT IF I AM INJURED?

The University of Massachusetts does not have a program for compensating subjects for injury or complications related to human subjects research, but the study personnel will assist you in getting treatment. Medical treatment will be available at the closest medical center at the expense of the participant.

Section 3
Subject Statement of Voluntary Consent

Release of blood samples is not a requirement of participation in this study. It is entirely your decision. Please read the statements below and check the appropriate box to reflect your decision about the use of your blood samples:

□YES, I agree to allow the investigators of this study to store my blood samples to use in future research

□NO, I do not allow the investigators of this study to store my blood samples for use in future research

I have read this form and decided that I will participate in the project described above. The general purposes and particulars of the study, as well as possible hazards and inconveniences have been explained to my satisfaction. I understand that I can withdraw at any time.

_____ _____ _____

Participant Signature: Print Name Date

By signing below I indicate that the participant has read and, to the best of my knowledge, understands the details contained in this document and has been given a copy.

_____ _____ _____

Signature of Person Obtaining Consent Print Name Date

APPENDIX B

HEALTH HISTORY FORM

Molecular and Cardiovascular Physiology Laboratory
University of Massachusetts
School of Public Health
Department of Kinesiology

Totman Building, 30 Eastman La, Amherst MA 01003
(413) 577-0392

Health History Questionnaire

GENERAL HEALTH

Height_____ Weight_____

How would you describe your overall physical health?
____ excellent ____ good ____ fair ____ poor

Have you undergone a physical examination in the last 5 years? □Yes □No

Are you on a special diet? □Yes □No
If yes, what type:_____

Have you gained or lost more than 10 lbs in the last 6 months? □ Yes □No

Have you had any illness in the last 2 weeks? □Yes □No
If yes, specify: _____

Do you have documented heart disease? □Yes □No
If yes, how long ago was it documented? _____ years

Has a doctor ever told you that you have an ulcer? □Yes □No

Has a doctor ever told you that you have any type of bleeding disorder?
□Yes □No

Have you ever experienced dizziness or fainting during a blood draw?
□Yes □No

Have you ever been advised by your doctor or any medical professional that you should not exercise or engage in any sort of strenuous physical activity?
☐ Yes ☐ No
If yes, please explain:_____

How many times a week do you exercise? _____

How many years have you been exercising? _____

What types of activity do you do?
_____

Do you have any limitations that you feel would hinder your ability to exercise? __
_____
_____

Have you ever had any of the following conditions/symptoms?
Leave blank for " no" . Indicate " yes" with a check mark and indicate year of onset.

Check for yes	Year of Onset	
	_____	High blood pressure
	_____	Heart attack /coronary problem
	_____	Heart murmur
	_____	Heart disease
	_____	Pain or tightness in the chest
	_____	Palpitations/rapid heart beat
	_____	Phlebitis
	_____	Stroke
	_____	Lung/respiratory problems
	_____	Diabetes
	_____	Varicose veins
	_____	High cholesterol
	_____	Anemia
	_____	Thyroid problems
	_____	Cancer
	_____	Liver disease
	_____	Gallbladder disease

MEDICATIONS

Are you currently taking any medications? □Yes □No

If yes, list all being taken. Include daily dosage and indicate for what condition. Include over-the-counter drugs (ibuprofen, aspirin, antihistamines, oral contraceptives.)

_____
_____
_____
_____

Do you take vitamins or herbal supplements? □Yes □No

If yes, please list :_____
_____
_____

Do you have any known drug allergies? □Yes □No

If yes, please list: :_____
_____
_____

FAMILY HISTORY

To your knowledge, have any immediate relatives (parent, grandparent, brother, sister) had a heart attack or been diagnosed with heart disease before the age of 60? □Yes □No

If so, indicate relative and age:

Have any immediate relatives had a high blood pressure before the age of 60? □Yes □No

If so, indicate relative and age:

MUSCULOSKELETAL/ORTHOPEDIC HISTORY
Have you ever had any of the following:
If yes, how long ago?

Complaint			Years Ago
Hernia or rupture?	Yes	No	
Present/recurrent back injury	Yes	No	
Osteoarthritis Arthritis	Yes	No	
Rheumatoid Arthritis	Yes	No	
Osteoporosis	Yes	No	
Spinal disc problem	Yes	No	
Joint dislocation	Yes	No	
Ligament strain	Yes	No	
Cartilage tear	Yes	No	
Tendon tear	Yes	No	
Intermittent leg cramps	Yes	No	
Swollen painful joints	Yes	No	
Polio	Yes	No	
Surgery	Yes	No	

If you have had surgery please explain:

SMOKING HISTORY
Have you always been a non-smoker? □Yes □No If yes, go to next section

Do you presently smoke?

Yes	No
Are you an ex-smoker?	
Yes	No

If yes, when did you stop? _____

MENOPAUSE:

How would you describe your menstrual cycle (please check box)

☐Variable (more than 7 days different from normal)

☐No period (60 days or more but less than 1 year)

☐No period (more than 5 years)

□Other (please describe) _____
Have you been treated for menopause-related symptoms? □Yes □No
If yes, please list type of treatment and dates:
_____

Have you ever taken oral contraceptives? □Yes □No
If yes, please specify the type of contraceptive used and the dates used _____
_____
_____

Are you currently or do you have a history of menopause related symptoms (e.g. hot flashes)? □Yes □No

If yes, please specify the intensity and frequency: _____
_____
_____

Do you have a history of irregular menstrual irregularities, vaginal bleeding, or abnormal vaginal or uterine anatomy? □Yes □No

OTHER
Please discuss any other significant medical concerns that you consider important for us to know:

APPENDIX C

QUALITY OF LIFE QUESTIONNAIRE

Utian Quality of Life Scale (UQOL)

Please rate the degree to which you agree with the following statements, as they apply to you *within the past month*. Be sure to *answer every question!* Please circle your answer using the following 5-point scale:

1 Not true of me	2	3 Moderately true of me	4	5 Very true of me

1.	I am able to control things in my life that are important to me.	1	2	3	4	5
2.	I feel challenged by my work.	1	2	3	4	5
3.	I believe my work benefits society.	1	2	3	4	5
4.	I am not content with my sexual life.	1	2	3	4	5
5.	I am content with my romantic life.	1	2	3	4	5
6.	I have gotten a lot of personal recognition in my community or at my job.	1	2	3	4	5
7.	I am unhappy with my appearance.	1	2	3	4	5
8.	My diet is not nutritionally sound.	1	2	3	4	5
9.	I feel in control of my eating behavior.	1	2	3	4	5
10.	Routinely, I engage in active exercise three or more times each week.	1	2	3	4	5
11.	My mood is generally depressed.	1	2	3	4	5
12.	I frequently experience anxiety.	1	2	3	4	5
13.	Most things that happen to me are out of my control.	1	2	3	4	5
14.	I am content with the frequency of my sexual interactions with a partner.	1	2	3	4	5
15.	I currently experience physical discomfort or pain during sexual activity.	1	2	3	4	5
16.	I believe I have no control over my physical health.	1	2	3	4	5
17.	I am proud of my occupational accomplishments.	1	2	3	4	5
18.	I consider my life stimulating.	1	2	3	4	5
19.	I continue to set new personal goals for myself.	1	2	3	4	5
20.	I expect that good things will happen in my life.	1	2	3	4	5
21.	I feel physically well.	1	2	3	4	5
22.	I feel physically fit.	1	2	3	4	5
23.	I continue to set new professional goals for myself.	1	2	3	4	5

APPENDIX D

INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE

4

1a. During the last 7 days, on how many days did you do **vigorous** physical activities like heavy lifting, digging, aerobics, or fast bicycling,?

Think about *only* those physical activities that you did for at least 10 minutes at a time.

_____ **days per week** ⇨

or

☐ **none**

1b. How much time in total did you usually spend on one of those days doing vigorous physical activities*?*

_____ **hours** _____ **minutes**

2a. Again, think *only* about those physical activities that you did for at least 10 minutes at a time. During the last 7 days, on how many days did you do **moderate** physical activities like carrying light loads, bicycling at a regular pace, or doubles tennis? Do not include walking.

_____ **days per week** ⇨

or

☐ **none**

2b. How much time in total did you usually spend on one of those days doing moderate physical activities*?*

_____ **hours** _____ **minutes**

3a. During the last 7 days, on how many days did you **walk** for at least 10 minutes at a time? This includes walking at work and at home, walking to travel from place to place, and any other walking that you did solely for recreation, sport, exercise or leisure.

_____ **days per week** ⇨

or

☐ **none**

3b. How much time in total did you usually spend walking on one of those days*?*

_____ **hours** _____ **minutes**

The last question is about the time you spent sitting on weekdays while at work, at home, while doing course work and during leisure time. This includes time spent sitting at a desk, visiting friends, reading traveling on a bus or sitting or lying down to watch television.

4. During the last 7 days, how much time in total did you usually spend *sitting* on a **week day?**

____ **hours** _____ **minutes**

This is the end of questionnaire, thank you for participating.

This is the final SHORT LAST 7 DAYS SELF-ADMINISTERED version of IPAQ from the 2000/01 Reliability and Validity Study. Completed May 2001.

APPENDIX E

STANFORD BRIEF ACTIVITY QUESTIONNAIRE

PhenX Toolkit

Data Collection Worksheet

PhenX Measure: Total Physical Activity-Screener (#150900)
PhenX Protocol: Total Physical Activity-Screener (#150901)

Date of Interview/Examination (MM/DD/YYYY): _____

Section I: On-The-Job Activity

Please check the box next to the **one** statement that **best** describes the kinds of physical activity you usually performed while on the job this last year. If you are not gainfully employed outside the home but perform work around the home **regularly**, indicate that activity in this section.

[ ] A.	If you have no job or regular work, check Box A and go on to Section II.
[ ] B.	I spent most of the day sitting or standing. When I was at work I did such things as writing, typing, talking on the telephone, assembling small parts or operating a machine that takes very little exertion or strength. If I drove a car or truck while at work, I did not lift or carry anything for more than a few minutes each day.
[ ] C.	I spent most of the day walking or using my hands and arms in work that required moderate exertion. When I was at work I did such things as delivering mail, patrolling on guard duty, mechanical work on automobiles or other large machines, house painting or operating a machine that requires some moderate activity. If I drove a truck or lift, my job required me to lift and carry things frequently.
[ ] D.	I spent most of the day lifting or carrying heavy objects or moving most of my body in some other way. When I was at work, I did such things as stacking cargo or inventory, handling parts or materials, or I did work like that of a carpenter who builds structures or a gardener who does most of the work without machines.
[ ] E.	I spent most of the day doing hard physical labor. When I was at work I did such things as digging or chopping with heavy tools, or carrying heavy loads (bricks, for example) to the place where they are to be used. If I drove a truck or operated equipment, my job also required me to do hard physical work most of the day with only short breaks.

Section II: Leisure-time Activity

Please check the box next to the **one** statement which **best** describes the way you spent your leisure-time during most of the last year.

[ ] F.	Most of my leisure time was spent without very much physical activity. I mostly did things like watching television, reading or playing cards. If I did anything else, it was likely to be light chores around the house or yard, or some easy-going game like bowling or catch. Only occasionally, no more than once or twice a month, did I do anything more vigorous, like jogging, playing tennis or active gardening.
[ ] G.	Weekdays, when I got home from work, I did few active things. But most weekend I was able to get outdoors for some light exercise- going for walks, playing a round of golf (without motorized carts), or doing some active chores around the house.
[ ] H.	Three times per week, on the average, I engaged in some moderate activity- such as brisk walking or slow jogging, swimming or riding a bike for 15-20 minutes or more. Or I spent 45 minutes to an hour or more doing moderately difficult chores- such as raking or washing windows, mowing the lawn or vacuuming, or playing games such a doubles tennis or basketball.
[ ] I.	During my leisure time over the past year, I engaged in a regular program of physical fitness involving some kind of heavy physical activity at least three times per week. Examples of heavy physical activity are: jogging, running or riding fast on a bicycle for 30 minutes or more; heavy gardening or other chores for an hour or more; active games or sports such as handball or tennis for an hour or more; or a regular program involving calisthenics and jogging or the equivalent for 30 minutes or more.
[ ] J.	Over the past year I engaged in a regular program of physical fitness along the lines described n the last paragraph (I), but I did it almost daily- five or more times per week.

Protocol Source: http://phenxtoolkitstage.rti.org/index.php?pageLink=browse.protocoldetails&id=150901

APPENDIX F

PHYSICAL ACTIVITY AND PERFORMANCE QUESTIONNAIRE

Molecular and Cardiovascular Physiology Laboratory
University of Massachusetts
DEPARTMENT OF KINESIOLOGY,
TOTMAN BUILDING, 30 EASTMAN LA, AMHERST MA 01003
(413) 545-6012 (413) 545-2906 FAX

Physical Activity and Performance Questionnaire

Physical Activity Information

How many times a week do you exercise?

_____

How many minutes do you spend exercising per session?

_____ minutes

Do you ever exercise twice a day? ☐ No ☐ Everyday ☐ Occasionally

_____

Mode(s) of exercise (check all that apply):

☐ Running ☐ Cycling ☐ Swimming ☐ Other

_____

What is the approximate pace (minutes/mile) for your exercise sessions?

_____

How many miles do you run/cycle/swim/etc. in an average week?

Running _____ Cycling _____ Swimming _____ Other

_____

Have you been exercising *continuously* since high school or college? ☐ Yes ☐ No

If no, how many years elapsed until you resumed exercising?

_____

How many years have you been exercising continuously in the last 20 years?

_____

Please outline a typical week's work out schedule giving distance covered and times registered:

DURATION	EXERCISE MODE (run/cycle/etc)	MILEAGE COVERED
SUNDAY		
_____	_____	_____
MONDAY		
_____	_____	_____
TUESDAY		
_____	_____	_____
WEDNESDAY		
_____	_____	_____
THURSDAY		
_____	_____	_____
FRIDAY		
_____	_____	_____
SATURDAY		
_____	_____	_____

Please use the space below to provide any additional information regarding your exercise/activity history that you feel will be helpful to us.

APPENDIX G

LOW NITRATE DIET

Vascular Function Across the Menopausal Transition Study

MOLECULAR AND CARDIOVASCULAR PHYSIOLOGY LAB
DEPARTMENT OF KINESIOLOGY,
UNIVERSITY OF MASSACHUSETTS, AMHERST

Dietary Instructions

Since diet influences your blood pressure, certain dietary restrictions (low nitrate diet) apply to this part of the study. We ask that for 3 days you minimize from your diet the foods and drinks that are listed on this page.

We will ask you to complete a 3-day diet record (included) for the 3 days prior to your testing. This will help ensure the accuracy of the results of your tests.

The diet restrictions include those items high in nitrates. Following is a list of foods we ask you to avoid for the 3-day period.

- All seafood (shellfish, salt water or fresh water fish)
- All cured, smoked, preserved, canned or processed meats, e.g., cold cuts, bacon, sausage, ham, hotdogs, pepperoni, corned beef, pastrami, salt pork
- Certain cheeses: cheddar, swiss, blue, american, mozzarella (cottage cheese, cream cheese are allowed)
- All canned meats and fish, including tuna
- Tofu, soybeans, legumes
- Vegetarian burgers made with lentils, soybeans or other legumes
- Nuts, peanut butter
- Pickles, olives, relish
- Raw or cooked garlic and onions
- Tomato-based foods
- bananas, strawberries, or melons
- Worcestershire sauce

APPENDIX H

RISK FACTOR STRATIFICATION

Guidelines for Risk Stratification

Provided by ACSM Guideline's for Exercise Testing and Prescription (137)

Low Risk	Asymptomatic men and women who have ≤1 cardiovascular risk factor
Moderate Risk	Asymptomatic men and women who have ≥2 cardiovascular risk factors
High Risk	Men and women who have ≥1 sign or symptom of cardiovascular disease or known cardiovascular, pulmonary, or metabolic disease

*the presence of a positive risk factor allows for the subtraction of one negative risk factor when classifying an individual; if the presence or absence of a risk factor is not available, that risk factor should be counted as a risk factor, except for prediabetes. If prediabetes status is unknown, it should be counted as a risk factor for those ≥ 45 years old, especially for those with a body mass index ≥ 25 kg/m^2 and those <45 years old with a body mass index index ≥ 25 kg/m^2 and additional risk factors for prediabetes.

Negative Risk Factors

- **Age:** Men ≥ 45 years old; Women ≥ 55 years old
- **Family History:** myocardial infarction, coronary revascularization, or sudden death before age 55 for first-degree male relatives (e.g. father) or before age 65 for first degree females relatives (e.g. mother)
- **Smoking:** current use of, or exposure to, cigarettes or use within the last six months
- **Sedentary lifestyle:** not participating in at least 30 minutes of moderate intensity physical activity (40%-<60% oxygen uptake reserve) on at least 3 days per week for at least 3 months
- **Obesity:** body mass index ≥ 30 kg/m^2 or waist girth >102 cm (40 in) for men and >88 cm (35 in) for women
- **Hypertension:** systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg, confirmed by measurements on at least 2 separate occasions, or antihypertensive medication
- **Dyslipidemia:** low density lipoprotein cholesterol ≥ 130 mg/dL or high density lipoprotein cholesterol <40 mg/dL or on lipid lowering medication or total serum cholesterol ≥ 200 mg/dL
- **Prediabetes:** fasting plasma glucose ≥ 100 mg/dL and ≤ 125 mg/dL or oral glucose tolerance test ≥ 140 mg/dL and ≤ 199 mg/dL confirmed by measurements on two separate occasion

Positive Risk Factor

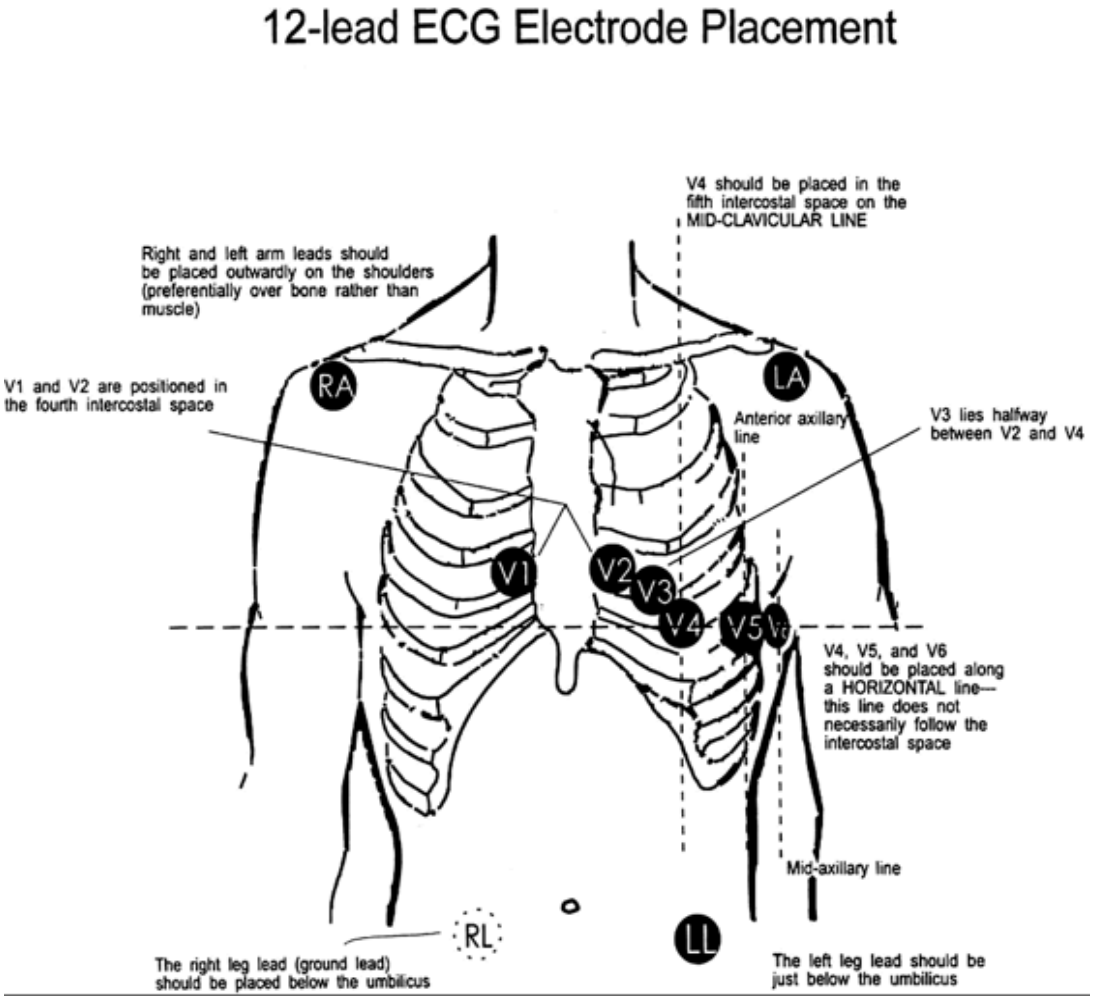
- **High density lipoprotein cholesterol:** $\geq$ 60 mg/dL

Symptoms

- Pain; discomfort (or other anginal equivalent) in the chest, neck, jaw, arms or other areas that may result from ischemia
- Shortness of breath at rest or with mild exertion
- Dizziness or syncope (fainting)
- Orthopnea (shortness of breath while lying down) or paroxysmal nocturnal dyspnea (shortness of breath during sleep)
- Ankle edema (swelling)
- Palpitations or tachycardia
- Intermittent claudication
- Known heart murmur
- Unusual fatigue or shortness of breath with usual activities

APPENDIX I

12-LEAD ECG PLACEMENT



(Fletcher et al. 2001)

APPENDIX J

EXERCISE TESTING CONTRAINDICATIONS & VO_{2MAX} DETERMINATION

ACSM Guidelines for Exercise Testing: Absolute/Relative Contraindications
Provided by ACSM Guideline's for Exercise Testing and Prescription (137)

Pre-test exclusion

- Recent change in resting ECG
- Unstable angina
- Cardiac dysrhythmia (uncontrolled, symptoms or hemodynamic compromise present)
- Severe symptomatic aortic stenosis
- Symptomatic, uncontrolled heart failure
- Acute pulmonary embolism or infarction
- Acute pericarditis or myocarditis
- Dissecting aneurysm (known or suspected)
- Acute systemic infection
- Left main coronary stenosis
- Moderate stenotic valvular heart disease
- Electrolyte abnormalities
- Arterial hypertension (SBP>200 mmHg and/or DBP >110 mmHg)
- Tachy- or bradydysrhthmia
- Hypertrophic cardiomyopathy or obstruction of outflow tract
- Musculoskeletal, neuromotor or rheumatoid disorders made worse with exercise
- high-degree AV block
- ventricular aneurysm
- uncontrolled metabolic disease
- chronic infectious diseases
- mental/physical impairment that hinders exercise

Test Termination

- SBP drop $\geq$10 mmHg with increased work load or SBP value below baseline measure (in same position) and with symptoms of ischemia
- Angina score $\geq$ 3 or increasing chest pain
- Worsening nervous system symptoms (i.e. ataxia, dizziness, near syncope)
- Poor perfusion (i.e. cyanosis or pallor)
- Technical problems with ECG or blood pressure
- Participant requests test termination
- Sustained ventricular tachycardia

- ST segment elevation ≥ 1 mm in leads without diagnostic Q (excluding V1 or AVR)
- ST segment or QRS changes (i.e. ST segment horizontal or downward depression >2 mm) or axis shift
- Arrhythmias such as multifocal PVCs, supraventricular tachycardia, heart block, or bradyrhythmias
- Fatigue, shortness of breath, wheezing, leg cramps, claudication
- BBB or intraventricular conduction delay that is indistinguishable from ventricular tachycardia
- SBP >250 mmHg and/or DBP >115 mmHg

Test Validation Criteria

- RER ≥ 1.1
- Plateau in oxygen consumption with increasing work
- Within 15 beats of age-predicted maximal heart rate (220-age)

APPENDIX K

LINEAR REGRESSION FIGURES

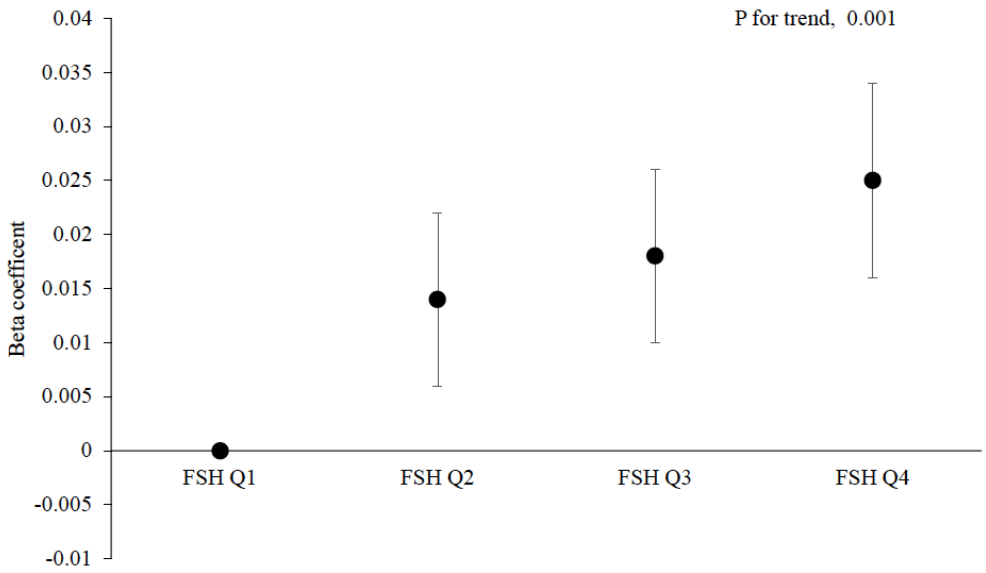


Figure 1. The Relationship Between Total Cholesterol and FSH.
There was a positive linear relationship between total cholesterol and follicle stimulating hormone (FSH). Data are presented as mean±SEM

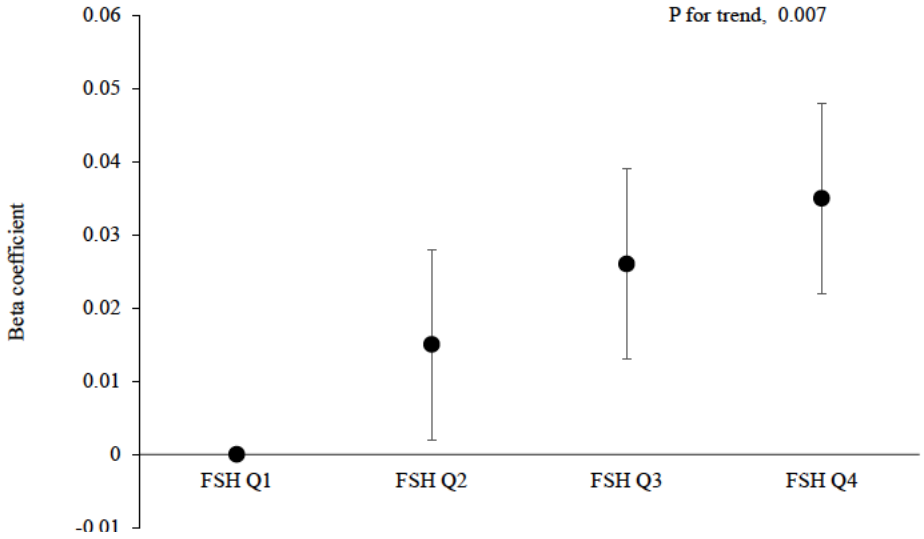


Figure 2. The Relationship Between LDL-C and FSH.
There was a positive linear relationship between low-density lipoprotein cholesterol (LDL-C) and follicle stimulating hormone (FSH). Data are presented as mean±SEM

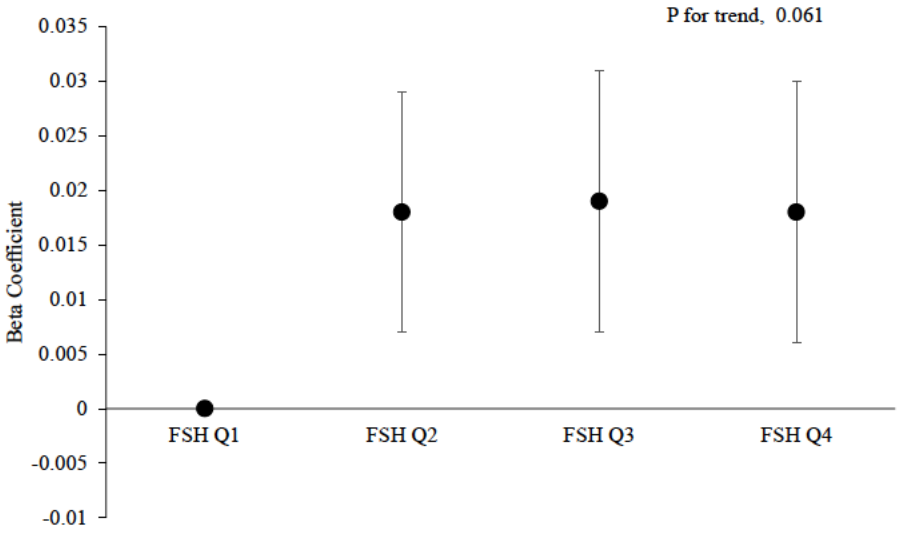


Figure 3. The Relationship Between HDL-C and FSH.
There not a linear relationship between high-density lipoprotein cholesterol (HDL-C) and follicle stimulating hormone (FSH). However, the relationship between HDL-C and FSH appears to vary by FSH level. Data are presented as mean±SEM

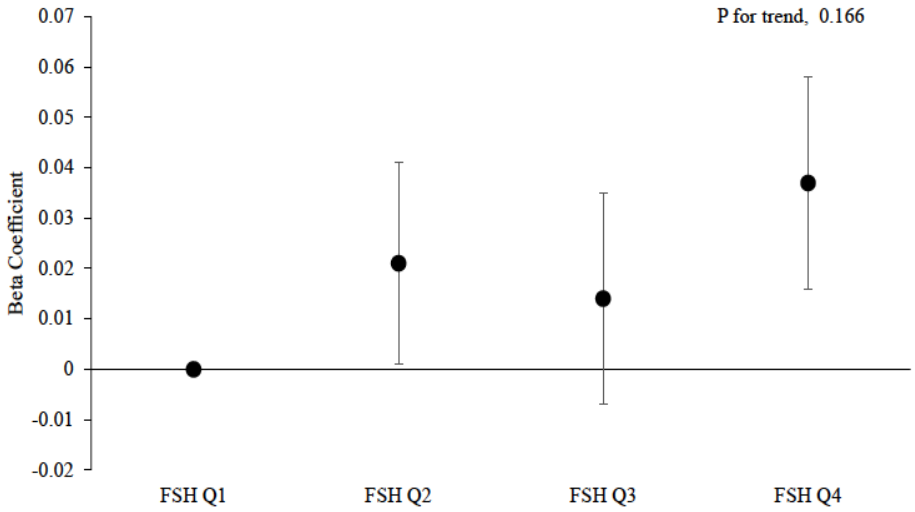


Figure 4. The Relationship Between Triglycerides and FSH.
There not a linear relationship between triglycerides and follicle stimulating hormone (FSH). However, the relationship between triglycerides and FSH appears to vary by FSH level. Data are presented as mean±SEM

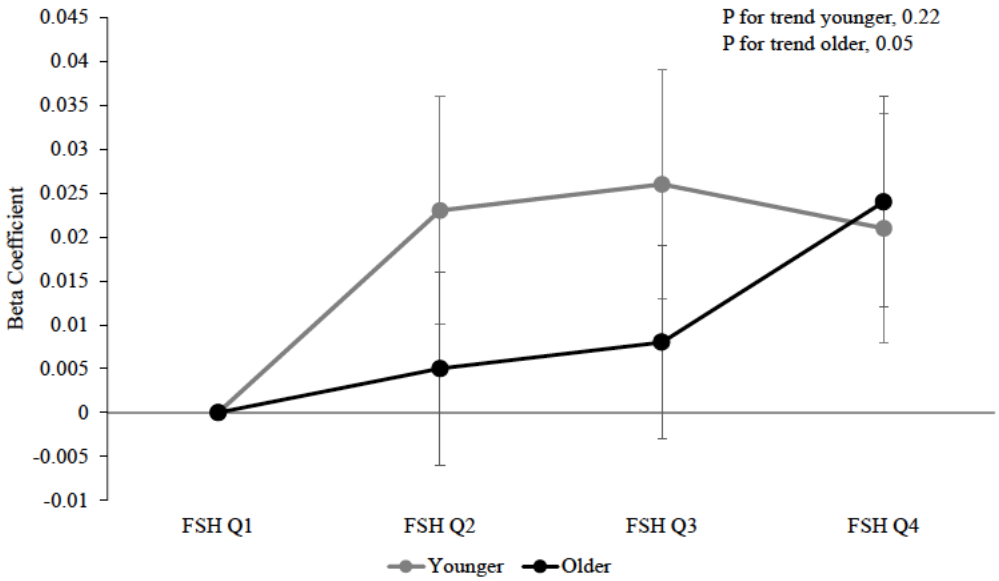


Figure 5. The Relationship Between Total Cholesterol and FSH by Age.
There was a linear relationship between FSH and total cholesterol in older, but not younger postmenopausal women. However, the beta coefficients were higher in younger postmenopausal women, suggesting that FSH has a greater effect on total cholesterol in younger women. Data are presented as mean±SEM

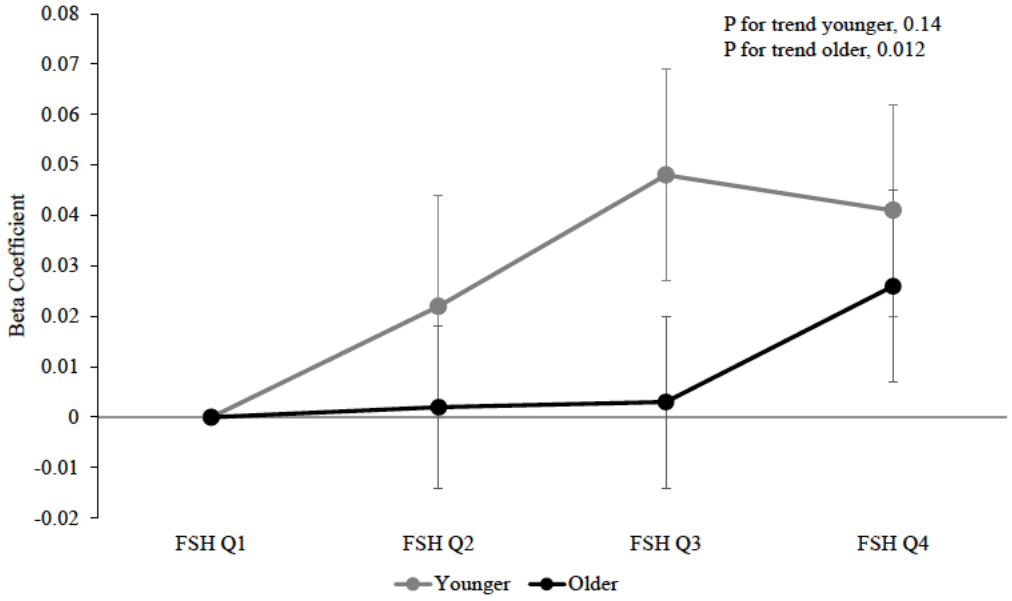


Figure 6. The Relationship Between LDL-C and FSH by Age.
There was a linear relationship between FSH and low-density lipoprotein cholesterol (LDL-C) in older, but not younger postmenopausal women. However, the beta coefficients were higher in younger postmenopausal women, suggesting that FSH has a greater effect on LDL-C in younger women. Data are presented as mean±SEM

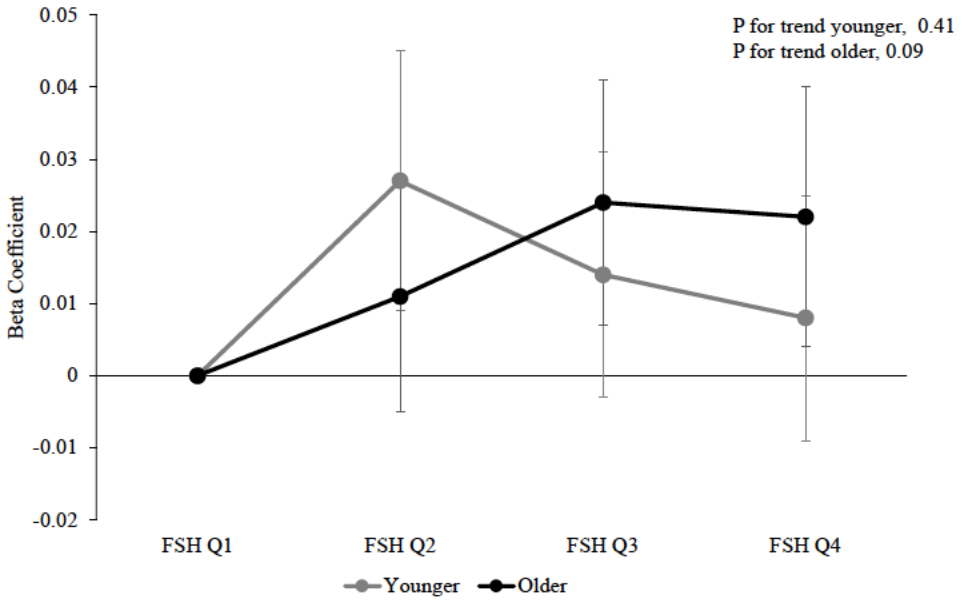


Figure 7. The Relationship Between HDL-C and FSH by Age.
There was not a linear relationship between high-density lipoprotein cholesterol (HDL-C) and follicle stimulating hormone (FSH) in younger or older postmenopausal women. Data are presented as mean±SEM

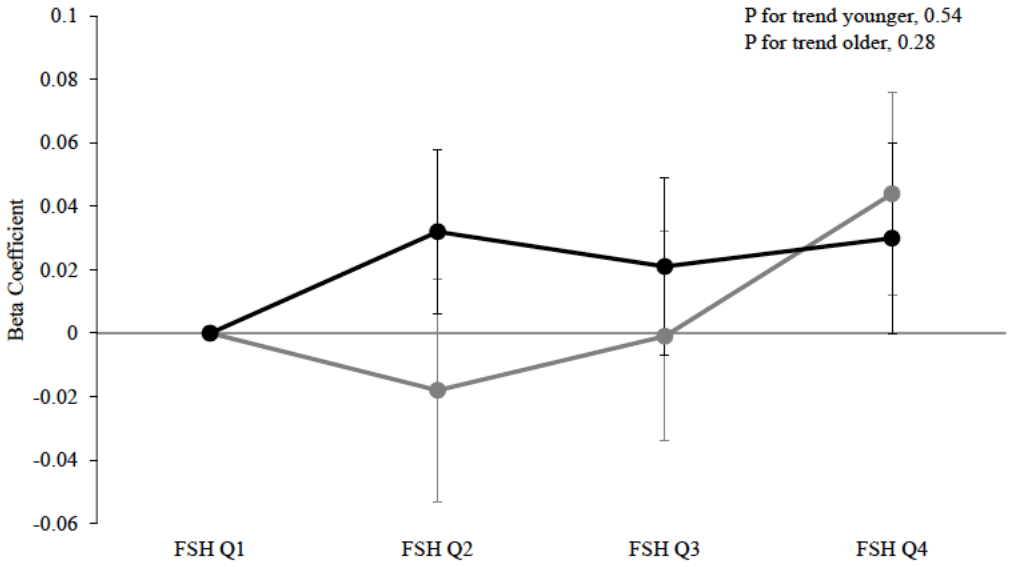


Figure 8. The Relationship Between Triglycerides and FSH by Age.
There was not a linear relationship between triglycerides and follicle stimulating hormone (FSH) in younger or older postmenopausal women. Data are presented as mean±SEM

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