HDL-C and Menopause in Women: The Contribution of Estradiol and Inflammation

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Abstract

Recent studies suggest a reversal in the protective association of HDL-C and cardiovascular disease (CVD) in women transitioning through menopause. Decreasing estrogen levels during the transition may explain this reversal, altering the structure and function of HDL particles and consequently increasing CVD risk. Inflammation may also contribute to this risk. We aimed to determine whether either estradiol concentration or C-reactive protein (CRP) concentration modifies the association between HDL-C and two measures of subclinical CVD: aortic calcification (AC) and coronary artery calcification (CAC).

Participants from the Study of Women's Health Across the Nation (SWAN) Heart ancillary study of either Black or White race, who had CAC/AC, estradiol, and CRP measures available were evaluated. AC and CAC presence were defined as Agatston score =>100 or =>10, respectively. Logistic regression was used to assess effect modification of estradiol and CRP levels on the relationship between HDL-C and AC/CAC presence, controlling for age, study site, race, hormone therapy use, waist circumference, triglycerides, and smoking status.

Of the 342 included women, 203 (59%) were pre/early perimenopausal and 139 (41%) were late peri-/postmenopausal. Average age of women was 51.2 (SD=2.8) years, and the sample was 38% Black. In unadjusted models, HDL-C was associated with a 3.7% lower odds of AC (95% CI: 0.943, 0.983) and 4.0% lower odds of CAC (95% CI: 0.940, 0.981). In adjusted models,

we found a significant interaction between HDL-C and estradiol with respect to AC but not CAC (p=0.0104 and 0.3172, respectively). For every one log-unit higher in estradiol concentration, the estimated OR between HDL-C and AC presence was 0.964 (95% CI: 0.938, 0.992). We found no statistically significant interaction between CRP and HDL-C with either outcome.

The protective cardiovascular association between AC and higher HDL-C levels was stronger at higher levels of estradiol, adjusting for confounders. Therefore, estradiol levels may impact the association of HDL-C and AC. However, we did not find evidence that inflammation impacts this association. These findings may have implications for public health in understanding factors which could contribute to CVD among midlife women.

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Preface

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1.0 Introduction

The term "cardiovascular disease" is an umbrella term that can refer to a number of conditions relating to the heart, brain, or vascular system[1]. However, it is most commonly used to refer to atherosclerotic heart disease, which includes heart attack, ischemic stroke, congestive heart failure, and hypertension[1].

1.1 The burden of Cardiovascular Disease

Cardiovascular disease (CVD) has been the leading cause of death worldwide for over two decades[2]. In the United States, CVD has been the leading cause of death since 1921[3]. In 2016, over 635,000 of 2.7 million deaths (23.5%) in the United States were reportedly due to heart disease[4]. However, age-adjusted death rates related to CVD have slightly decreased since 1960[5]. This decline is likely due to a decline in smoking rates, better nutrition, and the development of medication for high blood pressure and high cholesterol[6]. Despite this decline in age-adjusted deaths, CVD is still the number one cause of death nationwide. According to the American Heart Association, CVD is also the most expensive chronic disease, costing an estimated $555 billion in 2016[7]. This number is expected to reach $1.1 trillion by 2035, at which point the American Heart Association predicts that there will be 131.2 million Americans living with some form of CVD, an increase from 102.7 million in 2015[7]. From this information, it is evident that CVD is problematic in the United States, and it is important to fill gaps in research to decrease this burden. The American Heart Association (AHA) has defined the following seven key factors of ideal

cardiovascular health to address this burden: 1) total cholesterol of < 200 mg/dL, 2) glucose < 100 mg/dL, 3) systolic blood pressure of less than 120 and diastolic blood pressure of less than 80 mm Hg, 4) body mass index below 25 kg/m^2, 5) never smoking or quitting smoking more than 12 months previously, 6) at least 150 minutes of physical activity per week, and 7) between 4 or more of 5 ideal dietary habits: at least 4.5 cups of fruits and vegetables per day, two servings (3.5 ounces each) of fish per week, less than 1500 mg of sodium per day, no more than 450 kcal of sweets per week, and at least 3 servings of whole grains per day[8]. However, when these guidelines were created in 2010, less than 1% of adults in the US met all seven of these criteria[9]. The prevalence of ideal cardiovascular health was reportedly even lower among racial minorities in the US[9].

1.2 Sex-specific Differences in CVD

Men and women are approximately equally likely to die from CV[10]. Before midlife, women are less likely to die from CVD-related complications, but, as they approach midlife, this disparity between risk in men and women decreases[10]. On average, women develop CVD 7-10 years later than men[11]. An ACC/AHA systematic review from 2013 found that women typically have a lower 10-year risk estimate than men, with 77.5% of women included in this sample having a "low" 10-year risk of atherosclerotic CVD event (that is, a 10-year risk under 7.5%) compared to only 55.7% of men fitting this category[12]. However, women are more likely to encounter higher costs associated with CVD than men and have an overall greater burden in symptoms[13]. Additionally, the number of women who die from acute myocardial infarction each year has been higher than the number of men dying from the same cause since 1985[13].

In 2013, on average, nearly 45% of women had total cholesterol levels above the AHA recommended 200 mg/dL for ideal cardiovascular health, compared to 41% of men[14]. Women under age 45 tend to have lower blood pressure, on average, than males in the same age range. However, after age 65, women tend to have higher blood pressure than men[14]. Roughly 10 million adult women in the United States have been diagnosed with diabetes, compared to 9 million adult men[14]. These factors may all contribute to a portion of the disparity in the burden of CVD in women compared to men; however, men are less likely to have 5 or more metrics of ideal cardiovascular health than women (11% vs 25%, respectively)[14]. Furthermore, there is evidence that these traditional risk factors may contribute to the risk of developing CVD differently in men and women.

As of 1999, the American Heart Association has recognized differences in risk factors and recommendations with respect to CVD among women and men[15]. While factors like increased systolic blood pressure and increased BMI affect risk in both sexes roughly equally, certain factors, such as smoking and diabetes increase the risk in women more than in men[15]. Additionally, pregnancy and its complications can impact a woman's lifetime risk of CVD[15]. For example, preeclampsia, gestational diabetes, and preterm birth are all associated with a 1.5 to 2.5 times increased risk of CVD compared with women who did not suffer these complications[16 17]. Giving birth to one or more children was associated with a 1.5 times higher risk; giving birth to more than 5 children was associated with a 2.5 times higher risk[18].

Increasingly, evidence suggests that menopause may play a role in CVD risk. As noted above, women who are approaching midlife become approximately as likely as men to develop CVD[10]. This delay in CVD risk may, in part, be attributed to the cardio-protective effect of estrogen as a result of the decrease in estrogen concentration throughout the menopausal

transition[19]. Additionally, during the transition into menopause, women experience lipid changes, such as increases in total cholesterol, low-density lipoprotein cholesterol (LDL-C), and triglycerides, in addition to changes in body fat distribution. Some of these changes, such as increases in LDL-C, coincide with an increased risk of CVD, which may be related to the menopausal transition itself rather than the effects of aging[20].

1.3 Menopause and CVD Risk

The menopausal transition is a time in which a woman transitions out of a reproductive state, as marked by hormonal changes and changes in the menstrual cycle. This transition can be broken into several stages: pre-menopause (or the late reproductive stage), early perimenopause, late perimenopause, and postmenopause[21]. Early in the transition, women may start to notice changes in bleeding patterns, and hormonal changes[21]. These changes continue through each stage of the transition[21]. Bleeding becomes more varied, follicle stimulating hormone (FSH) concentration increases and estrogen concentration decreases[21]. Determination of reproductive stage is primarily based on bleeding patterns, hormonal changes, and physical symptoms, rather than age[21]. However, the timing of these changes often corresponds to the overall increase in CVD risk in women around this time.

The decrease in estrogen that is typically seen throughout the transition can be a risk factor for CVD. A study by Merz et al as part of the WISE (Women's Ischemia Syndrome Evaluation) study demonstrated that low estradiol (E2) levels before menopause are associated with an increased risk of coronary artery disease (CAD)[22]. Additionally, recent work from the Study of Women's Health Across the Nation (SWAN) demonstrated that (E2) and FSH concentrations were

associated with development of subclinical atherosclerosis, corresponding to an increased risk in CVD[23]. In this study, women with higher levels of E2 prior to the menopausal transition were less likely to show evidence of atherosclerosis, whereas women with higher levels after the transition were more likely to show signs of atherosclerosis. These findings suggest that hormonal changes could contribute to the increased risk of CVD. Notably, these studies refer to endogenous E2 only, as the role of exogenous estrogen and hormone replacement therapy has been highly controversial[24 25 26].

Previous research also suggests that the menopausal transition disrupts lipid metabolism, which is associated with an increased risk of CVD[27 28 29]. Transitioning through menopause is associated with increases low-density lipoprotein cholesterol (LDL-C), regardless of age[29]. However, studies investigating the association between menopause and changes in high-density lipoprotein cholesterol (HDL-C) have been inconsistent[30]. Understanding changes in HDL-C around the menopausal transition would improve the understanding of the increase in risk that occurs around midlife in women.

1.4 Types of Cholesterol

Cholesterol can be classified into two major types of molecules: low-density lipoprotein and high-density lipoprotein. Low density lipoprotein cholesterol (LDL-C) contributes to plaque buildup in the arteries and is consequently considered the "bad" cholesterol. LDL particles increase binding and adhesion[31]. This buildup increases the risk of CVD events such as heart attack and stroke by narrowing the arteries and decreasing blood flow with years of plaque accumulation. High density lipoprotein cholesterol (HDL-C) is typically considered the "good" cholesterol due

to its negative association with CVD[31]. HDL is thought to carry some circulating LDL molecules, along with triglycerides, back to the liver in a process known as reverse cholesterol transport[31]. The transport of these molecules prevents them from building up in the arteries, thereby reducing atherosclerosis[32]. High density lipoproteins are a heterogenous family of particles with different particle sizes and functions. Certain subspecies of HDL particles are more strongly associated with CVD than HDL-C, the crude measure of cholesterol carried by HDL[33 34]. These subspecies have different properties which may impact ion transport and consequently HDL functionality[34].

Previously, observational studies have shown a negative association of HDL-C on CVD risk[35 36]. Results from the Framingham Heart Study indicated that high HDL-C levels were associated with a significantly lower odds of death from coronary heart disease[35]. Similar results were found in a metanalysis of 68 studies on the association of HDL-C and CVD, which showed a significantly lower prevalence of CHD in both men and women in the high HDL-C concentrations, compared to the low concentrations[36].

There are two major metabolic pathways for HDL: direct uptake via the liver or via transfer to lipoproteins containing apo-B, by cholesteryl ester transfer protein[37]. The latter pathway typically involves a triglyceride exchange, which allows lipases (hepatic and endothelial) to modify the molecule, resulting in formation of a smaller HDL particle[37]. These pathways are complex and rely on several enzymes and proteins to maintain metabolism of HDL, which ultimately results in circulation of various types and sizes of HDL particles[37]. These particles may function differently, which may contribute to the complexities of understanding of the relationship between HDL and CVD[37].

There are multiple mechanisms that contribute to HDL's negative association with atherosclerosis. HDL reduces oxidation and inflammation in the body, which subsequently

decreases the accumulation of plaques[37]. Additionally, it downregulates the expression of adhesion molecules that contribute to plaque buildup[37]. Apolipoprotein A (apoA) attaches to HDL particles and aids in cholesterol transport[37]. ApoA likely contributes to the reduction of oxidation and may decrease rate of atherosclerosis[37].

1.5 Conflicting Evidence of HDL-C and CVD Risk

The National Heart, Lung, and Blood Institute (NHLBI) recommends a total blood cholesterol between 125 and 200 mg/dL for women over age 20 for optimal cardiovascular health. A healthy HDL-C concentration among this demographic is 50 mg/dL or higher, and a healthy LDL-C concentration is less than 100 mg/d.

Studies using statin therapies to lower LDL-C concentrations showed a decrease in CVD events in individuals using these treatments[38 39]. However, those who received the treatment were still at risk for CVD. Therefore, increasing HDL-C concentration was proposed as a mechanism to further decrease risk of CVD. An analysis of the Framingham Heart Study by Gordon et al. found that, among individuals with low levels of HDL-C, an increase of 1 mg/dL would decrease risk of CVD by 3% in women[40]. Consequently, in addition to decreasing LDL-C levels, clinical recommendations also focused on increasing HDL-C levels to at least 40 mg/dL in men and at least 50 mg/dL in women prevent CVD events[41]. However, recent evidence indicates that the association between HDL-C levels and CVD may be more complicated.

A recent analysis from the Framingham Heart Study offspring cohort study indicates that incident CVD may not be associated with HDL-C alone[42]. Researchers questioned whether low concentrations of HDL-C alone would predict incident CVD[42]. Holding LDL-C and triglyceride

concentrations constant, high levels of HDL-C (greater than or equal to 40 mg/dL for men and greater than or equal to 50 mg/dL for women) were associated with decreased risk of incident CVD events compared to low (less than 40 or 50 mg/dL) levels of HDL-C[42]. However, when HDL-C was greater than 40 or 50 but LDL-C and triglyceride concentrations were elevated (both greater than or equal to 100), risk of CVD was not significantly different from those with both low HDL-C and low (less than 100) triglycerides and LDL-C (OR: 0.9, 95% CI: 0.7-1.4)[42]. Based on this analysis, the authors concluded that concentration of HDL-C is not a robust predictor of CVD risk, compared to LDL-C and triglyceride concentration[42].

While several observational studies found a negative association between HDL-C concentration and CVD risk, clinical trials have suggested that the opposite may be true. The JUPITER trial found no significant association of HDL-C concentration and incident CVD[43]. Other clinical trials suggest that increasing HDL-C alone may only be a predictor of CVD in individuals who do not have other risk factors or who are otherwise healthy[43]. Furthermore, in some large-scale clinical trials, the effect of increased HDL-C was associated with increased incident CVD[44 45]. The Investigation of Lipid Level Management to Understand its Impact in Atherosclerotic Events (ILLUMINATE) trial randomized 15,000 participates to receive either atorvastatin (Lipitor) alone or atorvastatin plus torcetrapib, a drug that increases HDL-C concentration[44]. The researchers in this trial found that, despite a 73% increase in HDL-C levels and a 25% decrease in LDL-C levels, the treatment group had a 25% higher risk of CVD events like MI and angina and a 58% increased risk of death[44]. In a 2014 meta-analysis reviewing 39 clinical trials of HDL-C increasing medications, including niacin, fibrates, and CETP inhibitors, treatment groups did not show a significant difference in risk of CVD compared to placebo groups,

suggesting that simply raising HDL-C concentrations will not confer protection against events such as myocardial infarction and angina[45].

The inconsistent findings between results from observational and clinical studies may be attributed to factors relating to study populations and study design. In observational studies, individuals who receive treatment may have different risk factors that predispose them to CVD, or they may have more awareness of their risk. In a clinical trial, however, these factors would be distributed between the experimental and control groups, allowing researchers to determine a causal association between HDL-C levels and risk of CVD. Clinical trials offer a higher quality of evidence about the impact of HDL-C levels.

Regardless of the quality of evidence, these studies question the role of HDL in risk assessment and development of CVD. Risk estimators such as the Framingham Risk Score and Pooled Cohort Equation still rely on HDL-C concentrations to make predictions, despite the controversial evidence. However, the AHA no longer recommends medication for increasing HDL-C in its guidelines for primary prevention efforts for cardiovascular disease.

While the direction of the association between HDL-C and CVD risk has been questioned by recent research, recent literature has suggested that, in women transitioning through menopause, increasing HDL-C concentrations may be associated with an increased risk of CVD, further contradicting the notion that HDL is always cardioprotective.

1.5.1 HDL-C in menopause

Recent data from the MESA study and the SWAN Heart study suggest that, among midlife women, increased HDL-C levels are associated with an increased risk of atherosclerosis, which corresponds to increased risk of CVD[33 46 47]. In the context of one longitudinal SWAN study, an

increase in HDL-C before the final menstrual period (FMP) showed no association with subclinical CVD, whereas an increase in HDL-C after the transition was found to be associated with development of greater carotid intima-media thickness (cIMT) over a nine-year follow up period[46]. Further supporting this idea, a 2012 study by Woodard et al. analyzed risk of CVD by comparing measures of atherosclerosis in pre-/early perimenopausal women compared to late peri-/postmenopausal women[47]. The study found that pre-/early perimenopausal women were less likely to have atherosclerosis compared to late peri-/postmenopausal women[47]. Additionally, high HDL-C concentrations were associated with a lower risk of atherosclerosis in pre-/early perimenopausal women, but, in late peri-/postmenopausal women, this effect was reversed[47]. The late peri-/postmenopausal group showed an increased risk of atherosclerosis, controlling for age, site, race, blood pressure, glucose, BMI, smoking, and lipids[47]. The interaction between menopausal status and HDL-C was also evaluated and found to be significant, indicating that the protective effect of HDL-C with respect to CVD is weaker later in menopause[47].

In addition to the SWAN Heart sample, other studies have shown a similar effect among midlife women. Recent results from the Multi-Ethnic Study of Atherosclerosis (MESA) study indicate that HDL-C is positively associated with carotid plaque, which is indicative of an increased risk of CVD[33]. Additionally, this study suggests that this effect may result from a dysfunctionality of HDL molecules[33]. Emerging research has some theories surrounding this dysfunctionality, which may in turn lead to an increased risk of CVD.

1.6 Pathways which May Contribute to Increased Risk of CVD with Increasing HDL-C in Women During the Menopausal Transition

1.6.1 Estrogen

Women have a lower risk of CVD than men through approximately midlife, at which point the risk becomes approximately equal. It has been suggested that estrogen may confer a protective, anti-inflammatory effect, which explains women's decreased risk of CVD in early life with respect to men[48]. Therefore, the decline of estrogens throughout the menopausal transition may be a factor in this increasing risk. Previous work from SWAN Heart researchers has demonstrated an association between lower E2 and increased risk of atherosclerosis, supporting the notion that estradiol changes during the transition may be related to the risk of CVD[49].

Around menopause, research shows a flip/reversal in the protective effect of HDL-C with respect to CVD risk. This timeline coincides with the decrease in estradiol concentration that many women experience as they transition through menopause, the decrease in estradiol could be associated with the reversal in the protective effect of HDL. Low estradiol levels may impact HDL in several ways, explaining the reversal in the protective effect of HDL. Estradiol concentration may impact the composition or size of the HDL particles, both of which impact the functionality of HDL[50 51].

Estradiol concentration may impact the HDL proteome composition. In a 2014 study, Persson and colleagues evaluated lipid profiles as a function of estradiol concentration in women who were undergoing in vitro fertilization (IVF)[50]. Estradiol concentration was first suppressed and then induced as part of the IVF procedure[50]. Researchers found that, under high estradiol concentrations, apoB levels were decreased by 13%, and apoA levels were increased by 8%[50].

HDL-C levels remained stagnant, but the composition of HDL particles changed under high-estradiol concentrations[50]. As mentioned previously, apoA aids in HDL functionality by reducing oxidation and assisting in reverse cholesterol transport[37]. Based on the Persson et al. findings, high estradiol concentrations maintain apoA concentrations and, therefore, impact functionality of HDL. However, low estradiol concentrations could impact the structure of HDL, impacting its cholesterol-carrying ability and leading to an increased risk of atherosclerosis[37 50].

Estradiol may also impact the size and number of HDL molecules, impacting HDL's ability to transport cholesterol. This hypothesis is supported by a 2014 SWAN study investigating the role of endogenous estradiol on lipids, which noted that estradiol may influence lipid profiles[51]. The study reported that higher estradiol concentrations were associated with larger HDL particle sizes[51]. Based on previous research, HDL particle size is negatively associated with CVD risk[52]. Larger particles are associated with higher cholesterol efflux, indicating more efficient cholesterol transport[52]. Therefore, these results could also support the idea that decreasing estradiol concentrations seen around midlife increase CVD risk via changes in HDL particle concentrations. However, this evidence is based on cross-sectional studies.

In addition to affecting HDL, estradiol may also play an important role in decreasing inflammation. Since estradiol is an anti-inflammatory agent, its decrease may lead to an increase in inflammation[53 54 55].

1.6.2 Inflammation

Risk of cardiovascular disease and future coronary events such as sudden cardiac death, are positively associated with inflammation[56 57 58]. C-reactive protein (CRP) is part of the complement system of immune response, which promotes inflammation by promoting monocyte

binding in blood vessels, forming plaques in the vessels[59 60]. Results from the Women's Health Study show that CRP is a strong predictor of risk of incident CVD[61]. This risk may, in part, be attributed to structural changes that arise as a result of increased inflammation, resulting in dysfunctional HDL.

High-inflammatory states may induce conformational changes in HDL that render it dysfunctional. Chronic inflammatory conditions, such as psoriasis, are associated with an increase in serum amyloid A (SAA) and a decrease in apolipoprotein A-1 (apoA1)[62 63 64]. Some research indicates that apoA1 may be displaced by SAA under high inflammation and chronic inflammatory conditions[64 65]. During the acute phase immune response, serum amyloid A (SAA) may attach to HDL particles in place of apoA, which may consequently inhibit metabolism of HDL in the liver[65]. These SAA-rich particles may be denser and larger than apoA-rich particles[65]. This displacement of apoA may impact HDL by affecting its ability to transport cholesterol[65]. Consequently, different particles may not share the same functionality.

An increase in adhesion molecules produces an inflammatory state. Typically, the HDL molecules inhibit adhesion and decrease inflammation[32]. During acute infections such as influenza, HDL may lose its anti-inflammatory properties[32]. A possible mechanism for this pathway is that an attack on the immune system stimulates an inflammatory response, which then changes the properties of HDL, rendering it dysfunctional[32]. Because atherosclerosis is a chronic inflammatory state, understanding this pathway is crucial in understanding and predicting CVD.

Animal studies have shown a decline in cholesterol efflux under high-inflammatory states. In mice, acute inflammation induced by zymosan (an inducer of proinflammatory cytokines) reduced reverse cholesterol transport (RCT) by 20% compared to the control group[66]. The experimental mice showed an increase in SAA and increased SAA-enriched HDL compared to the

control group, which may decrease the ability of HDL to accept cholesterol for transport[66]. Similar RCT impairment has been shown in hamsters with simulated acute inflammation[67], as well as in other experiments with mice[68]. These studies, however, report only transient effects on RCT, indicating a need for more research investigating the effects of both acute and chronic inflammation on HDL structure and function.

Given that estradiol levels decrease during the menopausal transition, an increase in inflammation is expected, which may lead to dysfunctional HDL in midlife women. Consequently, understanding the relationship of inflammatory markers such as CRP with HDL and estradiol could provide insight into the paradox of the positive association of HDL-C levels and risk of CVD in midlife women.

1.7 Subclinical Measures of CVD: Arterial Calcification

Coronary artery calcification (CAC) and aortic calcification (AC) are measures of calcium buildup, a strong predictor of future CVD events[69]. The use of subclinical measures such as CAC and AC has been found to improve risk prediction in low- and intermediate-risk individuals compared to use of the Framingham Risk Score[70].

While several measures of sub-clinical CVD have been shown to predict risk of CVD, calcification scores (including CAC and AC) outperform measures such as carotid intima media thickness (CIMT)[71]. A recent study demonstrated that, while both CIMT and ankle-brachial index (ABI) were associated with incident cardiovascular events, CAC was a better predictor of risk in individuals at low or intermediate-risk, and that CAC was overall more predictive of CVD than these measures[71].

Both CAC and AC are measured via electron-beam computed tomography (EBCT). Three common methods exist to measure calcification: Agatston scoring, volume scoring, and mass scoring. An Agatston score is computed based on the density of observed calcification[72]. Calcified lesions of at least 1 square millimeter are weighted based on the density of the lesions (in Hounsfield units), and their areas are then summed to determine a total calcification score[72]. Volume scoring also uses density of lesions to calculate a calcium score, but this method simply sums the total voxels exceeding 130 Hounsfield units[72]. Finally, the mass score is a product of the volume and density of a lesion[72]. Clinical risk assessments rely on the Agatston score for risk assessment, due to its use in clinical studies[72]. Increasing Agatston scores are associated with increased mortality and increased risk of heart attack[72].

Calcification scoring via EBCT is non-invasive and can detect atherosclerosis before a major cardiac event[73]. Thus, this method is considered the gold standard in measurement of vascular calcification[73]. Additional benefits to using this method include its reproducibility, in addition to the ease of undergoing the scan[73]. However, this methodology has drawbacks. While the procedure is non-invasive, it does require exposure to radiation, which dissuades some practitioners from using it as a marker of subclinical CVD[74 75]. However, reports which estimate the total radiation exposure have been varied, and thus there is still uncertainty over the degree of this risk[73]. Additionally, because the Agatston score is a weighted measure (an area of 130-199 HU scores as 1, 200-299 HU scores as 2, 300-399 HU scores as 3, and 400 HU or higher scores as 4), a small difference in calcified lesion area may translate into a clinically significant difference in CAC or AC score, which then translates to an increased difference in CVD risk estimate[73].

Results from both the Healthy Women Study and the MESA estimate that calcification increases by around 6% annually, with older individuals progressing more rapidly[76 77]. There is

some evidence that suggests that postmenopausal women who use daily estrogen therapy have lower incidence rates of CAC compared to postmenopausal women who do not use HRT[78 79]. Additionally, two papers have examined the association between endogenous estradiol and calcification. A 2009 MESA paper determined that, while other endogenous hormones such as testosterone were associated with subclinical CVD, estradiol concentration was not significantly associated with CAC[80]. However, this population was strictly postmenopausal women[80]. A later SWAN Heart study found similar findings in a group of both pre and postmenopausal women, determining that estradiol was not significantly associated with either CAC or AC after adjustment for other CVD risk factors[81].

Previous research suggests that HDL-C levels are negatively associated with calcification. Orakzai et al reported that decreasing CAC scores with increasing HDL-C, although this study included only men[82]. MESA shared similar findings, reporting that a 1-mg/dL increase in HDL-C was associated with a 10% decreased odds of CAC[83]. MESA also found that HDL-C levels are associated with decreased risk of AC[84]. However, this association was only statistically significant in individuals between the ages of 65 and 74[84].

The negative association between HDL-C and calcification was also found among a population of healthy midlife women in a 1999 study. However, more recent literature has questioned this association in women. Studies from SWAN have shown an increased risk of subclinical CVD outcomes with increasing HDL-C concentrations in women as they transition through menopause. The Woodard et al. study was among the first papers to demonstrate that, in late peri-/postmenopausal women, every 1 mg/dL increase in HDL-C was associated with a 3% greater risk of AC and an 8 % greater risk of CAC[47]. Later studies have reported similar findings among midlife women, suggesting that increasing HDL-C concentrations may not have the

expected protective effect in midlife women[33 46]. While some other studies have reported similar effects with other outcome measures, such as cIMT, no other studies have reported a positive association between HDL-C and calcification measures. Furthermore, the mechanism for the positive relationship has not been demonstrated in previous literature. While there is speculation that estradiol or inflammation may play a role in HDL functionality and may affect risk of calcification, there is insufficient evidence to explain their roles in this effect. Therefore, this work aims to determine if estradiol or inflammation may modify the effect of HDL-C on calcification.

2.0 Summary and Objectives

Numerous studies have investigated the effect of increased levels of HDL-C on risk of CVD in midlife women[33 46 47 86 87]. While increased HDL-C concentration typically shows a protective effect in other populations (including premenopausal women and men), recent findings suggest that this association may be reversed during the menopausal transition[33 46 47]. In this demographic, increased HDL-C concentrations have shown a positive association with subclinical measures of CVD, including CAC, AC, IMT, and carotid plaque, which indicate an increased risk of CVD events [33 46 47 86 87]. This risk may not be reflected in traditional risk assessment and may consequently underestimate the risk of CVD among this demographic. Therefore, understanding the association between increased HDL-C and increased risk of atherosclerosis in this population is critical to reducing the annual incidence of CVD events such as heart attack, ischemic stroke, and heart failure which are often debilitating, expensive, or fatal.

2.1 Aims and Hypotheses

This thesis aims to build off prior findings that have shown a reversal in the association of HDL-C and CVD and understand how estradiol and inflammation concentrations may affect this reversal. Primarily, this analysis will assess whether estradiol levels contribute to the protective effect of HDL with respect to CVD, using CAC and AC as markers of early CVD. Additionally, this thesis will evaluate whether inflammation could explain the difference in cardioprotective effects of HDL at various concentrations of estradiol, or whether inflammation may modify the

effect of HDL on CAC and AC. Decreasing E2 levels commonly seen during the menopausal transition would explain the increased risk in CVD in women around this time. Lower E2 or high levels of inflammation may render HDL dysfunctional, leading to increased plaque and calcification via biological changes in HDL, which could contribute to increased atherosclerosis among postmenopausal women. The findings of this thesis may help to understand the factors that could contribute to the lessened protective effect of HDL-C among midlife women as seen in previous studies[33 46 47]. Additionally, these results may aid in the understanding and evaluation of risk assessment protocols for cardiovascular disease among midlife women. If estradiol or inflammatory markers are found to contribute to this diminished protective effect of HDL-C, then risk assessment measures like the FHS may consider further evaluating the contribution of HDL-C for CVD risk estimates in midlife women.

This thesis will evaluate effect modification of estradiol and inflammation on HDL-C with respect to calcification measures (CAC and AC) by assessing the interaction between these markers and HDL-C. We hypothesize that the interaction between HDL-C and estradiol or HDL-C and CRP will be significant, indicating that different levels of estradiol and CRP will have a different effect on the association of HDL-C and presence of CAC and AC. Low estradiol and/or high CRP levels may modify the effect of HDL-C on AC and CAC. **Figure 1** shows a conceptual model of this hypothesis.

The contribution of estradiol and inflammation on the association of HDL-C and calcification measures was analyzed using data from SWAN Heart, a cohort study of biological and psychological factors affecting women's risk of cardiovascular disease as they transition through menopause. The SWAN data repository contains a multitude of relevant biological variables which may contribute to calcification and subsequent risk of subclinical CVD. Results

from this analysis can contribute to the understanding of the relationships between hormone levels, HDL-C, and CVD risk in midlife women. Additionally, these findings may contribute to the evidence supporting clinical recommendations for CVD prevention in midlife women to decrease annual prevalence of CVD.

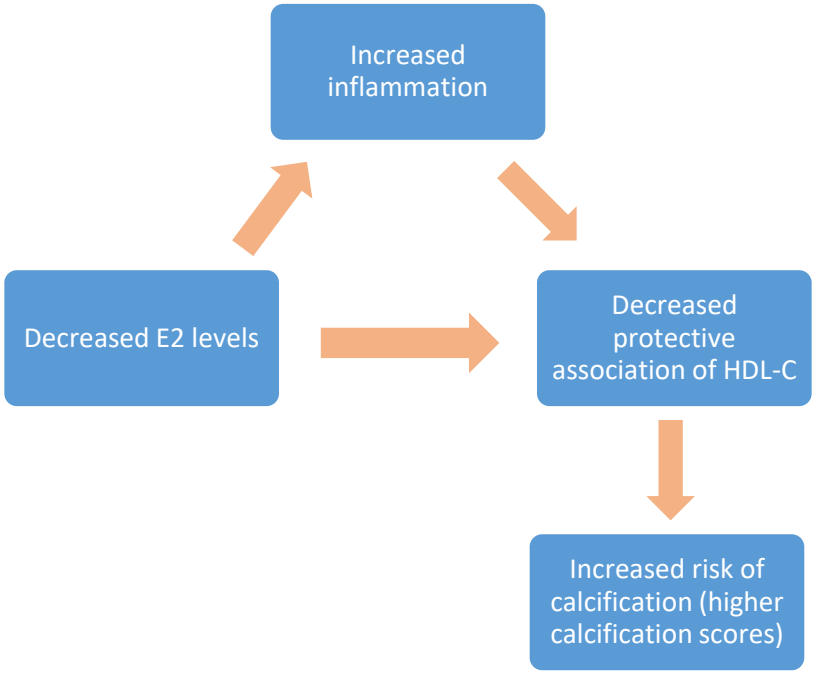

Figure 1: Conceptual model of proposed effect modification of estradiol and inflammation on the relationship between HDL-C and CAC/AC

3.0 Methods

3.1 Study Design

The Study of Women's Health Across the Nation is a longitudinal, multi-site study which focused on recruitment of a racially diverse population of women. The study began in 1994 and recruited 3302 mid-life women to understand biological factors as well as psychological factors that may impact health at midlife and throughout the menopausal transition. Women between the ages of 42 and 52 were recruited for this study, with each site targeting a specific racial/ethnic sample. Participants were recruited from seven research areas: Boston, MA, Chicago, IL, Pittsburgh, PA, Los Angeles, CA, Ypsilanti and Inkster, MI, Alameda and Contra Costa County, CA, and Hackensack, NJ. Participants were required to speak English, Cantonese, Japanese, or Spanish to be eligible to participate. The quality of evidence obtained from this study is higher due to the longitudinal nature. Thus, SWAN is one of the most prominent and respected longitudinal studies focusing on women's reproductive health.

Several ancillary studies have arisen from this original sample. SWAN Heart is an ancillary SWAN study which enrolled a total of 608 women from one Pittsburgh site and two separate Chicago sites. SWAN Heart baseline visits occurred between 2001 and 2003, at annual SWAN visits four through seven. Women from the SWAN cohort were ineligible for SWAN Heart if they were pregnant, had heart surgery (including CABG, PTCA, and bypass surgeries) were surgically menopausal, had a personal history of CVD, had diabetes and were receiving treatment, or had used hormone therapy in the past 3 months. This analysis used data from SWAN Heart baseline visit.

Of the 608 women enrolled in SWAN Heart, 11 were excluded from this analysis due to history of heart attack (n=4), stroke (n=2), or angina (n=5). Women who were surgically menopausal, either by hysterectomy or double oophorectomy, were also excluded from this analysis (n=16). Women who did not have CAC and AC scores were excluded (n=40). An additional 184 women were excluded for missing covariates of either estradiol concentration (n=39) or CRP concentration (n=136). It should be noted that all participants from one of the Chicago sites had no recorded CRP data. Thus, these participants were excluded from this analysis. Finally, women who were pre- or perimenopausal but had unknown menopausal status due to hormone therapy use in the past 3 months (n=24) were excluded, for a total sample size of 342 women (**Figure 2**).

Three sensitivity analyses were conducted using different sample sizes. The final models used in the main analysis were rerun using different sample sizes to compare to the main analysis with 342 women. Subjects who had estradiol measures but not CRP measures (n=136) were evaluated to assess effect modification of estradiol only on HDL-C for a sample size of 478. Subjects who had estradiol levels below the lower limit of detection of 7 pg/mL (n=9) were excluded from the analysis for a sample size of 333. Finally, a third sensitivity analysis was performed using the larger sample size (n=478) without subjects who had estradiol concentrations below the LLD (n=18), for a sample size of 460. The results of all sensitivity analyses are reported in the **Appendix**.

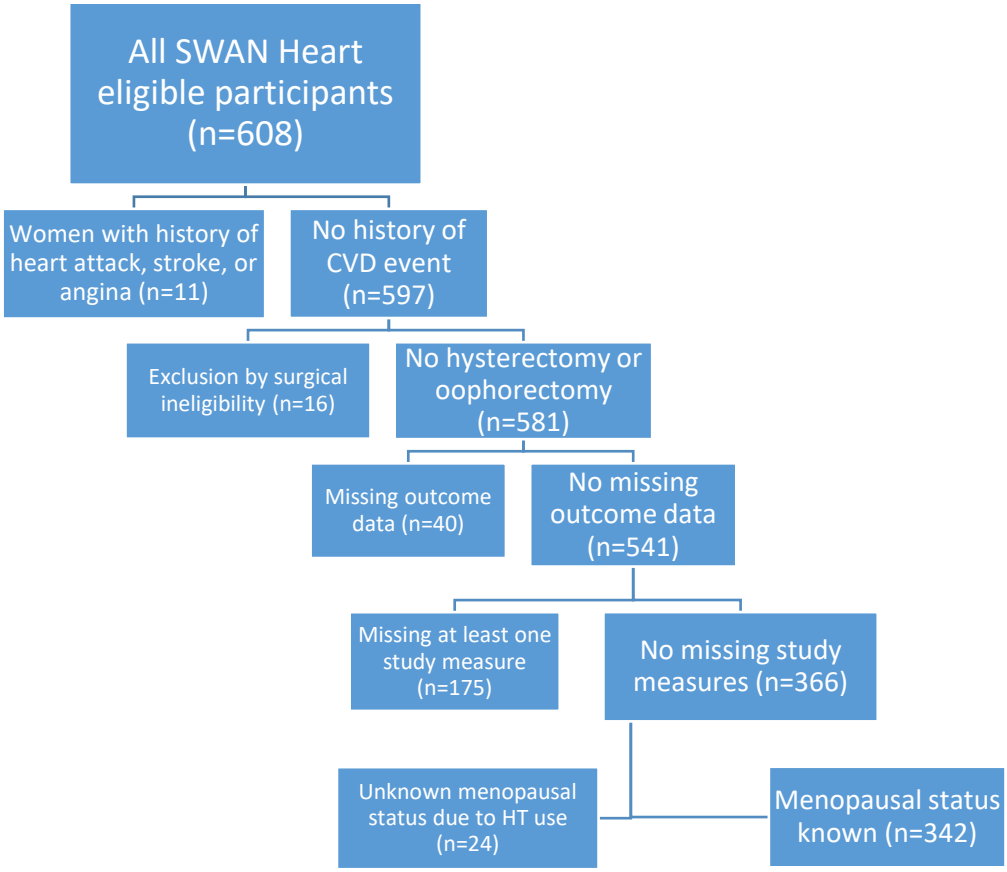

Figure 2: Sample selection criteria

Missing covariate criteria include missing CRP measurement, missing estradiol measurement, or missing HDL-C measurement.

3.2 Outcomes

Two subclinical measures of CVD were used in this analysis. Coronary artery calcification (CAC) and aortic calcification (AC) were measured via non-contrast CT scan at one visit for each woman.

An Agatston score was calculated for both outcomes. This score is based on Hounsfield units (HU), which measures density and area of calcification[88]. A density score of 1 indicates HU

of 130-199, a density score of 2 indicates 200-299, a density score of 3 indicates 300-399, and a density score of 4 indicates 400 or higher Hounsfield units[88]. The Agatston score is calculated by multiplying the unit weight by the total area of observed calcification, in millimeters[88]. Two passes were made; on the second pass, 30 to 40 contiguous 3-mm images were gathered for scoring. Calcium presence was defined as having 3 contiguous pixels of higher than 130 HU[47]. The CAC score was the sum of the Agatston scores from the left main, left anterior descending, left circumflex, and right coronary arteries[47]. AC score was the total calcium score from the aorta, based on the Agatston measures[47]. These scans were performed with an Imatron C-150 Ultrafast CT Scanner [47]. Results from scans were recorded and sent to Pittsburgh for scoring using a DICOM station. These scores report relatively high reproducibility ratings, with high intraclass correlation between readers (0.98 for AC and 0.99 for CAC)[88].

3.3 Study Variables

3.3.1 Anthropomorphic measurements and bloodwork

Waist circumference, body mass index, and systolic blood pressure were measured at SWAN Heart baseline. Blood pressure was measured using a sphygmomanometer and was recorded and averaged over two readings. BMI was calculated using height and weight measurements from each visit, which were taken without shoes and wearing light clothing. Waist circumference was recorded as the smallest circumference between the ribs and the top of the hip, or at the natural waist.

HDL-C, total cholesterol, LDL-C, triglycerides, glucose, CRP, and estradiol were measured at SWAN Heart baseline. Blood panels were performed after an overnight fasting of at least 10 hours, and on days 2-5 of a menstrual cycle that coincided with a scheduled annual visit. Researchers made two attempts to collect a specimen within this time frame, and, if unsuccessful, a random sample was collected within 90 days of the current visit. Cycle day of blood draw was recorded as an ordinal variable between 1 and 7. Samples which were not collected while a woman was menstruating were coded as missing (n=29). For this analysis, missing cycle day was recoded as unknown so that women with missing values were not excluded from the final analysis.

For blood panels, plasma was the preferred medium. If plasma was not obtained, most concentrations were measured using serum. All assays were performed at the Medical Research Lab in Lexington, KY.

Plasma for HDL-C measures was treated with heparin and manganese chloride, yielding a variation of roughly 4%. Total cholesterol was measured in plasma with a variation of roughly 0.8%. This measurement, in addition to triglycerides and HDL-C concentration, was then used to calculate LDL-C (LDL-C = Total cholesterol – 1/5 triglycerides – HDL-C). Triglycerides were measured primarily in plasma in an enzymatic assay with a variation of 1.2%. Serum glucose was measured using a Hitachi 747-200 analyzer, which has a variation of roughly 1.6%. CRP was measured as either serum or plasma concentration. However, these measurements yielded a 5% variation in results due to differences in media, so study leaders opted to exclude datapoints which used serum measurements. Estradiol was measured via E2-6 immunoassay, which can detect E2 levels as low as 7.0 pg/mL and has a variation of 3-12%. E2 was measured twice and were reported as the mean of these values. Women who had values below the lower limit of detection (n=9) were

assigned a random number between 1 and 7 to represent their estradiol concentrations so as not to exclude them from the dataset.

3.3.2 Self-reported covariates

Participants completed a questionnaire which reported factors about physical health, medical history, mental/psychosocial health, demographic characteristics, and prior medical history, including the use of hormonal contraception and hormone therapy.

Age in years was calculated at the time of the CT scan based on the birth date given at enrollment. Race was self-reported as either Black or White. Smoking status was reported as either currently smoking or not currently smoking at SWAN Heart baseline. Study site was dichotomized as either Pittsburgh or Chicago (excluding the Chicago site with no CRP data in the main analysis). Household income was dichotomized into less than $50,000 or greater than or equal to $50,000 annually. Alcohol consumption was reported as an average number of drinks per week, which was then dichotomized into less than 2 drinks per week or greater than or equal to 2 drinks per week. Education was also dichotomized into those having less than a bachelor's degree and those who obtained a bachelor's degree or higher.

Menopausal status was determined by questions about bleeding pattern, reproductive surgeries, and hormone usage and was based on responses to questions of regularity of bleeding patterns. Women who had not noted a change in bleeding patterns within 12 months were premenopausal[89]. Those who noted a change in regularity in the prior 3 months were considered early perimenopausal[89]. Those who had no bleeding for 3 months or more, but less than 12 months were considered late perimenopausal[89]. Finally, women who had no bleeding for 12 months or more were considered postmenopausal[89]. Women who were surgically menopausal or whose status

was unknown due to hormone usage were excluded from this analysis. Menopausal status in the included sample was dichotomized into pre-or early peri-menopausal and late peri- or post-menopausal. This variable manipulation was performed to maintain approximately even distribution among menopausal groups, as previously performed in SWAN studies[47 90].

3.4 Statistical Analyses

All statistical analyses were performed using SAS version 9.4. We used an alpha of 0.05 to indicate statistical significance, with the exception of stepwise model building, which used an alpha of 0.10.

3.4.1 Outcomes

Coronary artery calcification (CAC) has many 0 values, thus, CAC was dichotomized into high and low categories. While there are no clear clinical guidelines for CAC, some literature has suggested using CAC of => 10 or < 10 Agatston units, while others have suggested using the 75th percentile of the sample [91 92 93]. Both options were analyzed, and, because results were similar, we opted to pursue the analysis using only CAC => 10 Agatston units. Aortic calcification (AC) is also highly skewed with many 0 values and was dichotomized into high and low categories. The analysis was performed using both the 75th percentile and AC => 100 Agatston units with similar results. Thus, we opted to focus only on AC => 100 Agatston units. To assess whether those who had CAC presence are the same ones who had AC presence, cross-tabulation was performed to calculate frequencies.

3.4.2 Baseline characteristics

All continuous variables were assessed for normality. Those which were not normally distributed were log-transformed or categorized. Estradiol, triglycerides, glucose, and C-reactive protein were log-transformed after determining that they were non-normally distributed. Estradiol was also categorized into quartile groups: <16.25 pg/mL (Q1), 16.25 to less than 31.45 (Q2), 31.45 pg/mL to less than 79.40(Q3), and => 79.40 (Q4).

Age, race, smoking status, BMI, waist circumference, education, annual income, alcohol consumption, systolic blood pressure (SBP), total cholesterol, HDL-C, LDL-C, triglycerides, estradiol concentration, menopausal status, AC, and CAC were compared between both included and excluded participants, using chi-square tests for categorical variables and t-tests or Wilcoxon rank-sum tests for continuous variables. Means and standard deviations were compared for normally distributed variables, and variables were scanned for outliers. Medians and inter quartile ranges were compared for non-normally distributed/skewed variables.

3.4.3 Univariate regression

Univariate logistic regression was performed using CAC and AC as outcome variables. The associations between each of the two outcomes with study variables were used to determine which variables to include in multivariate regression. Any variable that was significantly associated (alpha <= 0.10) with an outcome was included in model building for that outcome. These covariates were also used in a univariate regression with HDL-C as the outcome to determine the association between HDL-C and covariates, irrespective of AC and CAC.

3.4.4 Multivariate regression

The relationship of HDL-C with each of CAC and AC was assessed using multivariate logistic regression. Models were built using forward and backward processes, based on the association between either CAC or AC and significantly associated variables. Beginning with variables which showed the strongest association (based on smallest p-value) with the outcome of interest, in addition to HDL-C and estradiol, covariates were added in a stepwise fashion until the model included all variables which were significantly associated with the outcome in the fully adjusted model. Variables which were not significant predictors (alpha <=0.10) in the fully adjusted model were eliminated in a backward fashion, starting with the least significant (largest p-value) predictors. Per SWAN protocol, age, race, study site, and hormone therapy usage were included as covariates in all final models, regardless of their level of significance. Additionally, any variable which was not statistically significant in the fully adjusted model but, upon elimination from the model, moved the point estimate of the main association by more than 10% was retained in the models. All outcomes were assessed independently, and variables were treated independently with outcomes for which they showed a significant association. AIC, BIC, and C-statistics were assessed to determine model fit.

The interaction term was defined as the cross-product between HDL-C and the natural log of estradiol. This term was assessed for significance in each of the unadjusted, the fully adjusted, and final models. Similarly, the interaction between HDL-C and CRP level, which was the cross-product of HDL-C and the natural log of CRP level, was assessed in the unadjusted, the fully adjusted, and the final models. In analyzing the interaction between HDL-C and E2, estradiol was analyzed as both a continuous and categorical variable. E2 was categorized into quartiles, and the odds of AC and CAC were compared based on quartile group. CRP was analyzed only as a

continuous variable. Because CRP and waist circumference were moderately correlated ($r=0.390$, $p<0.0001$), models that included waist circumference and CRP as a covariate were analyzed using the waist circumferences residuals from a linear regression model between waist circumference and CRP to reduce collinearity in the models.

After determining the final models, the models were rerun with a stratification analysis, using quartiles of estradiol. This output was plotted using forest plots to visualize the effect of HDL-C on AC and CAC at various estradiol levels.

4.0 Results

4.1 Baseline Characteristics

4.1.1 Demographic characteristics

A total of 342 women were included in the main analysis. The average age of women included in this analysis was 51.2 years (SD: 2.80). Those included in the analysis were 37% Black and 14% were current smokers. The sample contained slightly more pre- or early peri-menopausal women (203, or 59%) than late peri- or post-menopausal women (139, or 41%). Although women whose menopausal status was unknown due to hormone therapy use were excluded from this analysis, some women whose status was known reported hormone therapy use after menopause and were therefore not excluded from the analysis. In total, there were 28 women (8.2%) in this sample who reported HT use at SWAN Heart baseline. Additional baseline characteristics can be found in **Table 1**.

Table 1: Baseline characteristics of included versus excluded participants

Demographic characteristics	Excluded (n=275)	Included (n=333)	p
Age, years, mean (SD)	50.4 (2.92)	51.2 (2.80)	0.001
Black, n (%)	99 (37.22%)	127 (37.13%)	0.98
Current smoker, n (%)	52 (19.55%)	49 (14.33%)	0.09
Site = Chicago, n (%)	31 (29.25%)	158 (46.20%)	0.002
Education < college degree, n (%)	110 (44.72%)	160 (46.78%)	0.61
Annual income < $50k, n (%)	71 (26.79%)	110 (32.35%)	0.13
Alcohol consumption, n (%)			0.0052
> 2x weekly	87 (32.71%)	72 (21.05%)	
>1x per month, <= 1x weekly	85 (31.95%)	127 (37.13%)	
< = 1x per month	94 (35.34%)	143 (41.81%)	
HT use at SH baseline, n (%)	47 (17.67%)	28 (8.19%)	0.0004
Menopausal status			
Early peri- or pre-menopausal, n (%)	125 (50.00%)	203 (59.36%)	0.64
Late peri-or post-menopausal, n (%)	93 (37.20%)	139 (40.64%)	
Body measurements			
Waist circumference, cm, mean (SD)	88.1 (13.5)	89.7 (14.8)	0.16
BMI, kg/m2, mean (SD)	28.9 (5.90)	29.6 (6.57)	0.15
SBP, mm Hg, mean (SD)	120.7 (15.9)	118.7 (17.2)	0.15
HDL, mg/dL, mean (SD)	58.6 (15.3)	56.9 (13.7)	0.15
Total cholesterol, mg/dL, mean (SD)	202.7 (35.6)	200.3 (38.2)	0.44
LDL, mg/dL, mean (SD)	120.3 (31.6)	119.5 (33.4)	0.78
Triglycerides, mg/dL, median (Q1, Q3)	101 (78.0, 146.0)	99 (74, 137)	0.62
Glucose, mg/dL, median (Q1, Q3)	89 (83.3, 97.0)	88 (82, 96)	0.34
Estradiol, pg/mL, median (Q1, Q3)	28.8 (15.9, 66.1)	31.2 (16.2, 79.0)	0.24
Estradiol (quartiles)			
Q1 (E2 < 16.25 pg/mL)	60 (26.67%)	85 (24.85%)	0.56
Q2 (16.25pg/mL <= E2 <31.45pg/mL)	59 (26.22%)	86 (25.15%)	
Q3 (31.45 =< E2 <79.40 pg/mL)	61 (27.11%)	85 (24.85%)	
Q4 (E2 => 79.40 pg/mL)	45 (20.0%)	86 (25.2%)	
CRP, mg/dL, median (Q1, Q3)	1.40 (0.70, 4.50)	1.70 (0.60, 4.30)	0.35
Outcome characteristics			
AC, median (Q1, Q3)	9 (0, 58)	18.0 (0, 89)	0.07
CAC, median (Q1, Q3)	0.0 (0, 6.9)	0 (0, 8.58)	0.45
AC => 100, n (%)	43 (19.82%)	82 (23.98%)	0.25
CAC => 10, n (%)	41 (18.72%)	81 (23.68%)	0.16

4.1.2 Anthropomorphic measures

Mean waist circumference in the included sample was 89.7 cm (SD:14.8 cm). Average body mass index was 29.6 kg/m^2 (SD:6.57 kg/m^2). Systolic blood pressure was 118.7 mm Hg on

average. Total cholesterol, LDL-C, and HDL-C, on average, were 200.3, 119.5, and 56.9 mg/dL, respectively.

Glucose, estradiol, triglycerides, and CRP were all found to be skewed. Therefore, we reported the median as well as first and third quartiles for these variables. In those who were included in this analysis, median triglyceride concentration was 99 mg/dL, and median glucose concentration was 88 mg/dL. Median estradiol concentration was 31.5 pg/dL, and CRP was 1.70 mg/L.

4.1.3 Outcome measures

Both CAC and AC were found to be highly skewed variables with many 0 values. Median CAC score was 0, and median AC score was 18 among the 342 included women. A total of 82 women (24.0%) had an AC score greater than or equal to 100, and 81 (23.7%) women had a CAC score of greater than or equal to 10. Forty-six women (13%) had both high CAC and high AC. Thirty-five women (10%) had high CAC but not high AC; thirty-six women (11%) had high AC but not high CAC. The prevalence of AC and CAC can be visualized in **Figure 3**.

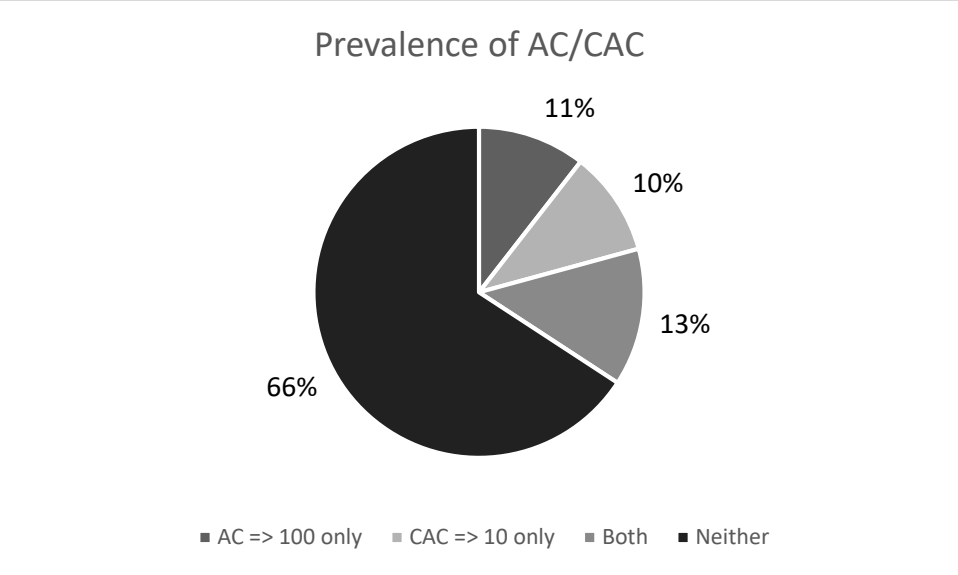

Figure 3: Prevalence of AC and CAC among study participants

4.1.4 Comparison of included and excluded groups

The women who were included in this analysis (n=342) were slightly older than those who were excluded (n=266), with an average age of 51.2 years and 50.4 years, respectively (p=0.0011). Additionally, those included in the analysis were less likely to report drinking alcohol two or more times per week (21% of the included group, compared to 33% of the excluded group). No other statistically significant differences were observed between included and excluded individuals. Anthropomorphic measurements and outcome characteristics showed no significant differences in included and excluded groups. These differences can be seen in **Table 1**.

4.2 Unadjusted Analysis

4.2.1 Univariate association of HDL-C with study variables (linear regression)

Age, race, study site, menopausal status, family history of CVD, education, and estradiol were not significantly associated with HDL-C concentration. Waist circumference, body mass index, systolic blood pressure, triglycerides, and CRP were all significantly negatively associated with HDL-C, along with low income and smoking. Alcohol consumption of at least two drinks per week was associated with a significantly higher HDL-C concentration. Results from this analysis can be found in **Table 2**.

Table 2: Univariate association of HDL-C with study variables

Variable	Beta estimate	SE	P-value
Age, per 1-year increase	0.10	0.27	0.71
Race=Black (ref: White)	-1.84	1.54	0.23
Study site=Pittsburgh (ref: Chicago)	-1.58	1.49	0.29
Menopausal status=Late peri-/post- (ref: pre-/early peri)	1.47	1.51	0.33
Family History of CVD=Yes (ref: no)	-0.780	1.58	0.62
Income < $50k/year (ref: =>$50k/year)	-4.52	1.57	0.004
Education < college (ref: => some college)	-0.898	1.49	0.55
Smoking=Yes (ref: non-smokers)	-4.58	2.11	0.031
Alcohol consumption < 2 drinks per wk (ref: => 2 drinks/ wk)	7.67	1.79	<.0001
Waist circumference, per 1-cm increase	-0.308	0.047	<.0001
BMI, per 1- kg/m^2 increase	-0.568	0.109	<.0001
SBP, per 1- mm Hg increase	-0.115	0.043	0.008
LDL-C, per 1- mg/dL increase	-0.039	0.022	0.08
Log (glucose) per 1-unit increase	-16.14	4.25	0.0002
Log (Trig), per 1-unit increase	-13.05	1.36	<.0001
Hormone therapy use=Yes(ref: no)	9.02	2.67	0.0008
Log (E2), per 1-unit increase	0.715	0.684	0.30
E2, per 1-quartile increase (ref: E2 => 79.40 pg/mL)			
Q1 (E2 < 16.25 pg/mL)	-1.60	2.096	0.45
Q2 (16.25pg/mL <= E2 <31.45pg/mL)	-4.69	2.090	0.026
Q3 (31.45 =< E2 <79.40 pg/mL)	-2.37	2.096	0.26
Log (CRP), per 1-unit increase	-2.06	0.57	0.0004

4.2.2 Univariate association of AC and CAC with study variables (logistic regression)

Logistic regression was performed using CAC and AC as outcome variables to determine the relationship between these outcomes and possible covariates. In general, variables were associated with both CAC and AC. However, some variables were significantly associated with one outcome but not the other. Therefore, each outcome was analyzed separately. Associations for both outcomes can be found in **Table 3**.

4.2.2.1 AC

Age, menopausal status, education, smoking, low alcohol consumption, BMI, waist circumference, systolic blood pressure, LDL-C, glucose, triglycerides, and CRP were all significantly associated with odds of high AC (AC =>100). Each 1-mg/dL increase in HDL-C was associated with a 3% reduction in odds of AC. Alcohol consumption was significantly associated with decreased odds of AC. Age, low education, being late peri-/postmenopausal, BMI, waist circumference, SBP, LDL-C, glucose, triglycerides, and CRP were all positively associated with odds of AC. Estradiol levels between 16.25 and 31.45 pg/mL were associated with a significantly higher odds of AC (OR=2.47, p=0.01), compared to high estradiol levels (greater than the 75th percentile of 79.4 pg/mL).

4.2.2.2 CAC

Age, alcohol consumption, BMI, waist circumference, systolic blood pressure, LDL-C, glucose, triglycerides, and CRP were all significantly associated with odds of high CAC (CAC =>10). Alcohol consumption, HDL-C, and log E2 showed a negative relationship with odds of high CAC. A 1-mg/dL increase in HDL-C concentration was associated with a 4% lower odds of

CAC => 10, while a 1-unit increase in log of estradiol was associated with a 25% lower odds of high CAC. Like AC, estradiol levels between 16.25 and 31.45 pg/mL were associated with a significantly higher odds of high CAC, compared to estradiol levels above the 75th percentile.

Table 3: Univariate association of CAC and AC with study variables

	CAC		AC	
Variable	**OR (95% CI)**	**P value**	**OR (95% CI)**	**P value**
Age, years	1.18 (1.09, 1.27)*	<.0001	1.10 (1.03, 1.19) *	0.009
Race=Black (ref: White)	1.49 (0.96, 2.32)	0.08	1.20 (0.77, 1.84)	0.42
Study site=Pittsburgh (ref: Chicago)	1.11 (0.67, 1.83)	0.69	0.73 (0.44, 1.20)	0.22
Menopausal status=Late peri-/post- (ref: pre-/early peri)	1.50 (0.91, 2.47)	0.12	1.77 (1.07, 2.91)*	0.026
Family History of CVD=Yes (ref: no)	0.91 (0.54, 1.55)	0.74	1.17 (0.68, 1.99)	0.57
Income < $50k/year (ref: =>$50k/year)	0.89 (0.52, 1.53)	0.68	1.01 (0.60, 1.71)	0.97
Education < college (ref: => some college)	1.15 (0.70, 1.89)	0.59	1.53 (0.93, 2.53)	0.09
Smoking=Yes (ref: non-smokers)	1.05 (0.52, 2.13)	0.89	3.51 (1.89, 6.58)*	<.0001
Alcohol consumption < 2 drinks per wk (ref: => 2 drinks/ wk)	2.99 (1.57, 5.69)*	0.0008	1.33 (0.79, 2.22) *	0.28
BMI, kg/m^2	1.22 (1.17, 1.27)*	<.0001	1.11 (1.07, 1.15) *	<.0001
Waist circumference, cm	1.10 (1.08, 1.12)	<.0001	1.06 (1.04, 1.08)	<.0001
SBP, mm Hg	1.04 (1.02, 1.05)*	<.0001	1.02 (1.01, 1.03) *	0.001
Total cholesterol, mg/dL	1.01 (1.00, 1.01)	0.0094	1.01 (1.00, 1.01)	0.002
HDL-C, mg/dL	0.96 (0.94, 0.98)*	0.0002	0.96 (0.94, 0.98) *	0.0002
LDL-C, mg/dL	1.01 (1.00, 1.01)*	0.0171	1.01 (1.00, 1.01) *	0.027
Glucose, mg/dL	1.03 (1.02, 1.05)*	0.0004	1.02 (1.01, 1.04)*	0.004
Log(Triglycerides)	2.78 (1.79, 4.33)*	<.0001	3.26 (2.09, 5.07) *	<.0001
Hormone therapy use=Yes(ref: no)	0.68 (0.25, 1.85)	0.45	0.85 (0.33, 2.18)	0.74
Log (CRP)	1.57 (1.27, 1.93)*	<.0001	1.57 (1.27, 1.93)*	<.0001
Estradiol (Quartile) (ref: E2 => 79.40 pg/mL)				
Q1 (E2 < 16.25 pg/mL)	1.96 (0.87, 4.43)	0.10	1.44 (0.69, 2.99)	0.33
Q2 (16.25pg/mL <= E2 <31.45pg/mL)	4.04 (1.87, 8.72)*	0.0004	2.47 (1.23, 4.96)*	0.01
Q3 (31.45 =< E2 <79.40 pg/mL)	1.96 (0.87, 4.46)	0.10	0.86 (0.39, 1.90)	0.71
Log (E2)	0.75 (0.61, 0.93)*	0.0076	0.88 (0.72, 1.08)	0.22

(*) indicates statistical significance at alpha < 0.05

4.3 Multivariate Analysis

4.3.1 AC

4.3.1.1 Interaction between HDL-C and E2

In the unadjusted model, the interaction between HDL-C and estradiol was statistically significant, controlling for cycle day (OR=0.969, p=0.0084). The interaction remained significant with the addition of all variables associated with AC. The fully adjusted model, which adjusted for cycle day, hormone use, age, race, site, waist circumference, smoking, triglycerides, SBP, glucose, LDL-C, menopausal status, education, and alcohol consumption, also showed a point estimate of 0.97 for the interaction term (p=0.029). The final model, which adjusted for HDL-C, estradiol, cycle day, hormone use, age, race, site, waist circumference, smoking status, and triglycerides also showed a significant interaction (OR=0.964, p=0.0103). The addition of CRP to the final model did not change the point estimate, despite the significant association of CRP with AC. Odds ratios and p-values for various models can be found in **Table 4**.

Table 4: Multivariate logistic regression: effect modification of estradiol on the association between HDL-C and calcification measures, per 1 mg/dL higher HDL-C in SWAN Heart participants

		Model 1		Model 2		Model 3		Model 4		Model 5		Model 6		Model 7		Model 8	
		OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P
AC => 100	HDL-C	0.963 (0.943, 0.983)	0.0004	0.963 (0.943, 0.984)	0.0005	1.069 (0.989, 1.156)	0.09	1.073 (0.993, 1.161)	0.08	1.18 (1.020, 1.225)	0.0170	1.140 (1.033, 1.257)	0.0089	1.119 (1.023, 1.225)	0.0144	1.139 (1.035, 1.253)	0.0078
	Log E2	------	------	0.834 (0.655, 1.064)	0.1440	4.583 (1.277, 16.448)	0.0196	5.414 (1.482, 19.770)	0.0106	5.599 (1.256, 24.963)	0.0239	7.242 (1.497, 35.022)	0.0138	5.660 (1.283, 24.97)	0.0221	6.909 (1.461, 32.681)	0.0148
	HDL* log E2	------	------	------	------	0.969 (0.947, 0.992)	0.0084	0.968 (0.946, 0.991)	0.0066	0.967 (0.942, 0.994)	0.0157	0.964 (0.937, 0.991)	0.0103	0.967 (0.942, 0.993)	0.0130	0.964 (0.938, 0.992)	0.0104
	Log CRP	------	------	------	------	------	------	------	------	------	------	1.625 (1.233, 2.143)	0.0006	------	------	1.616 (1.246, 2.096)	0.0003
CAC => 10	HDL-C	0.960 (0.940, 0.981)	0.0002	0.961 (0.941, 0.982)	0.0003	1.007 (0.931, 1.089)	0.86	1.012 (0.936, 1.094)	0.77	1.029 (0.937, 1.131)	0.55	1.036 (0.937, 1.145)	0.49	1.038 (0.944, 1.142)	0.44	1.038 (0.943, 1.141)	0.45
	Log E2	------	------	0.772 (0.602, 0.991)	0.0419	1.654 (0.466, 5.863)	0.44	1.903 (0.537, 6.737)	0.32	1.524 (0.332, 7.008)	0.59	1.649 (0.329, 8.269)	0.54	1.856 (0.378, 9.111)	0.44	1.836 (0.374, 9.012)	0.45
	HDL* log E2	------	------	------	------	0.986 (0.964, 1.009)	0.23	0.985 (0.963, 1.008)	0.20	0.988 (0.962, 1.016)	0.57	0.987 (0.959, 1.016)	0.38	0.986 (0.958, 1.014)	0.31	0.986 (0.958, 1.014)	0.32
	Log CRP	------	------	------	------	------	------	------	------	------	------	1.496 (1.139, 1.965)	0.0037	------	------	1.516 (1.173, 1.959)	0.0151

- Model 1: unadjusted models
- Model 2: main effects models, adjusted for cycle day.
- Model 3: main effects with interaction term, adjusted for cycle day.
- Model 4: main effects with interaction term, adjusted for cycle day, hormone use, age, race, site.
- Model 5: fully adjusted models, controlling for cycle day, hormone use, age, race, site, waist circumference, smoking, log triglycerides, SBP, log glucose, LDL-C, menopausal status, education, and alcohol consumption, without CRP
- Model 6: fully adjusted models, controlling for cycle day, hormone use, age, race, site, waist circumference, smoking, log triglycerides, SBP, log glucose, LDL-C, menopausal status, education, and alcohol consumption, with log CRP
- Model 7: reduced models (AC: controlling for cycle day, hormone use, age, race, site, waist circumference, smoking status, and log triglycerides; CAC: controlling for cycle day, hormone use, age, race, site, waist circumference, and SBP) without CRP.
- Model 8: reduced models (AC: controlling for cycle day, hormone use, age, race, site, waist circumference (residuals), smoking status, and log triglycerides; CAC: controlling for cycle day, hormone use, age, race, site, waist circumference residuals, and SBP) with log CRP

Because the interaction between HDL-C and estradiol was found to be significant with respect to AC, a stratification analysis was performed based on the final model. E2 was categorized into quartiles (Q1 < 16.25 pg/mL, Q2 16.25 to < 31.45 pg/mL, Q3 31.45 to < 79.40 pg/mL, and Q4 => 79.40 pg/mL) and odds ratios for AC were plotted based on E2 group (**Figure 4**). Women in the lowest quartile group showed a significantly higher odds of AC, while women in the highest quartile group showed a significantly lower odds of AC. The middle quartile groups did not show a significant association.

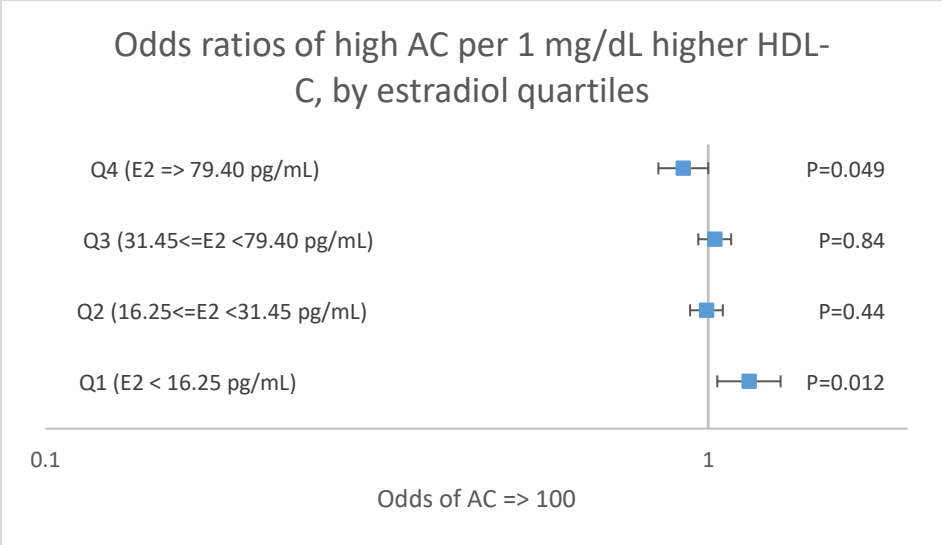

Figure 4: Odds ratio of high AC per 1 mg/dL higher HDL-C, by estradiol quartiles

Model is adjusted for cycle day, hormone use, age, race, site, waist circumference (residuals), smoking status, triglycerides, and CRP.

4.3.1.2 Interaction between HDL-C and CRP

The interaction between CRP and HDL-C did not show statistical significance with respect to AC in any model (**Table 5**). The unadjusted model (Model 1) showed a point estimate of 1.003 (p=0.74) for the interaction term. In the fully adjusted model (Model 4), this estimate changed only minimally but remained nonsignificant (p=0.68). The reduced model also showed a nonsignificant interaction (p=0.74).

Table 5: Multivariate logistic regression of effect modification of CRP level on association between HDL-C and calcification measures, per 1 mg/dL higher HDL-C in SWAN Heart participants

		Model 1		Model 2		Model 3		Model 4		Model 5	
		OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P
AC => 100	HDL-C	0.966 (0.941, 0.992)	0.0095	0.966 (0.941, 0.992)	0.0094	0.965 (0.940, 0.991)	0.0089	1.004 (0.972, 1.037)	0.82	1.006 (0.976, 1.038)	0.69
	Log CRP	1.276 (0.496, 3.280)	0.61	1.261 (0.492, 3.231)	0.63	1.163 (0.442, 3.062)	0.76	1.277 (0.452, 3.610)	0.64	1.345 (0.487, 3.717)	0.57
	HDL* log CRP	1.003 (0.986, 1.020)	0.74	1.003 (0.986, 1.020)	0.72	1.004 (0.987, 1.022)	0.65	1.004 (0.985, 1.023)	0.68	1.003 (0.985, 1.021)	0.74
	Log E2	------	------	0.889 (0.689, 1.147)	0.36	0.952 (0.729, 1.022)	0.72	0.949 (0.676, 1.332)	0.76	0.927 (0.674, 1.021)	0.64
CAC =>10	HDL-C	0.956 (0.929, 0.983)	0.0016	0.956 (0.930, 0.983)	0.0016	0.956 (0.930, 0.984)	0.0020	.982 (0.947, 1.018)	0.32	0.979 (0.949, 1.010)	0.19
	Log CRP	0.799 (0.305, 2.095)	0.65	0.789 (0.302, 2.065)	0.63	0.711 (0.266, 1.903)	0.50	0.739 (0.236, 2.314)	0.60	0.468 (0.152, 1.441)	0.19
	HDL* log CRP	1.012 (0.994, 1.030)	0.20	1.012 (0.994, 1.030)	0.19	1.013 (0.995, 1.032)	0.16	1.013 (0.992, 1.034)	0.22	1.013 (0.993, 1.034)	0.20
	Log E2	------	------	0.817 (0.629, 1.061)	0.13	0.870 (0.664, 1.141)	0.32	0.811 (0.578, 1.138)	0.22	0.825 (0.600, 1.135)	0.24

- Model 1: main effects models with interaction term (unadjusted)
- Model 2: main effects models with interaction term, adjusted E2 and cycle day.
- Model 3: main effects and interaction term, adjusted for E2, cycle day, age, race, site, and hormone use.
- Model 4: fully adjusted models, controlling for estradiol, cycle day, hormone use, age, race, site, waist circumference residuals, smoking, log triglycerides, SBP, log glucose, LDL-C, menopausal status, education, and alcohol consumption
- Model 5: reduced models (AC: adjusted for estradiol, cycle day, hormone use, age, race, site waist circumference residuals, smoking, and log triglycerides. CAC: adjusted for estradiol, cycle day, hormone use, age, race, site, waist circumference residuals, and SBP)

4.3.2 CAC

4.3.2.1 Interaction between HDL-C and E2

In the unadjusted model, the interaction between HDL-C and estradiol was not significant, controlling for cycle day (OR=0.986, p=0.23). The addition of variables to this model did not change the point estimate. The fully adjusted model, which adjusted for cycle day, hormone use, age, race, site, waist circumference, SBP, triglycerides, glucose, alcohol consumption, menopausal status, LDL-C, education, and smoking status, showed similar results (p=0.38). Backwards elimination of variables only slightly decreased the point estimate of the interaction term. The reduced model, which controlled for cycle day, hormone use, age, race, site, waist circumference, and SBP, also showed a nonsignificant interaction (p=0.31). The addition of CRP as a covariate to the final model did not change this result. While CRP was a significant predictor of CAC, the interaction of HDL-C and estradiol was not different in models which included CRP as a covariate. Odds ratios for various models can be found in **Table 4**. We found no statistically significant effect modification in these models.

Results from the stratification analysis (**Figure 5**) are consistent with these findings. Although not statistically significant, the direction of the association is similar to those seen in the AC stratification analysis.

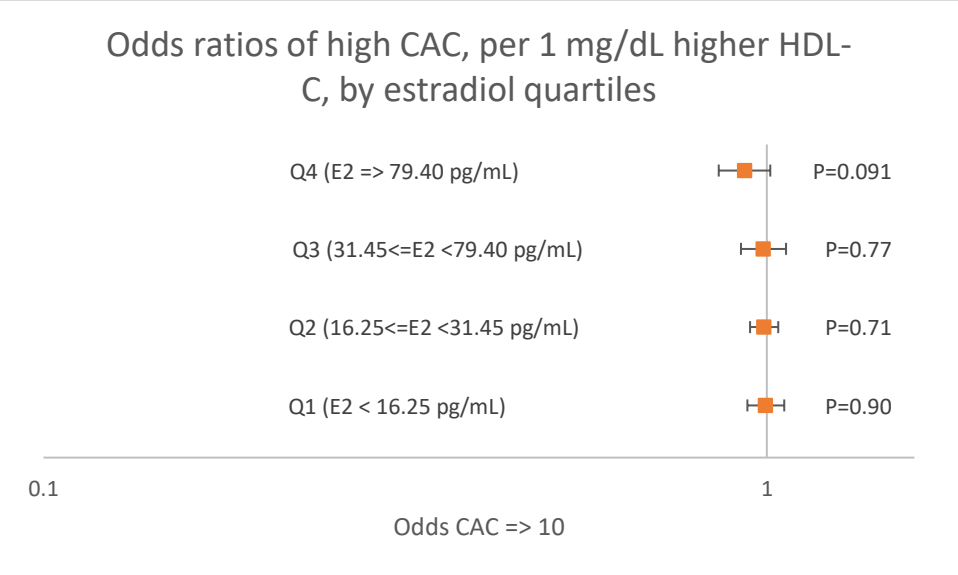

Figure 5: Odds ratios of high CAC per 1 mg/dL higher HDL-C, by estradiol quartiles

Model controlled for cycle day, hormone use, age, race, site, waist circumference residuals, SBP, and CRP.

4.3.2.2 Interaction between HDL-C and CRP

Like AC, the unadjusted model for CAC showed a non-significant interaction between HDL-C and CRP, with a point estimate of 1.012 (p=0.20). This estimate was maintained in the reduced model, which controlled for HDL-C, CRP, estradiol, cycle day, hormone use, age, race, site, waist circumference, and SBP and found no significant interaction (p=0.20). Odds ratios for these models can be found in **Table 5**.

4.3.3 Sensitivity analyses

Results from both sensitivity analyses can be found in **Appendix Table 1**. The inclusion of women who were excluded only due to missing CRP measures (n=136) yielded similar results to the analysis using the smaller sample size. There was a significant interaction between HDL-C and

estradiol with respect to AC (OR = 0.975, p = 0.0115), but not with respect to CAC (OR = 0.986, p = 0.21).

Nine women had estradiol levels below the lower limit of detection of 7 pg/mL. Leaving these observations out of the analysis, results were consistent with the results using a larger sample size of 342. With regards to AC, the interaction between HDL-C and estradiol level remained significant in the final model (OR = 0.97, p = 0.020). With regards to CAC, the interaction was still non-significant (OR = 0.99, p = 0.61).

Performing the analysis using subjects who had did not have CRP measures and had estradiol concentrations below the lower limit of detection yielded results consistent with the main analysis. Again, we observed a significant interaction with respect to AC (OR=0.974, p=0.01) but not with respect to CAC (OR= 0.986, p=0.28).

5.0 Discussion

5.1 Summary of Findings

5.1.1 Interaction of HDL-C and estradiol

There was a statistically significant difference in the association of HDL-C with risk of aortic calcification presence at different estradiol concentrations. This effect modification was observed across all models, controlling for other confounding factors such as age, race, study site, waist circumference, and systolic blood pressure. The addition of CRP as a covariate did not change the significance of this interaction, indicating that estradiol independently modifies the association of HDL-C with AC. At high concentrations of estradiol, HDL-C seems to exhibit a stronger negative association with respect to AC. Thus, it seems the association of HDL-C with AC presence varies by estradiol concentrations, which may, in part, be attributed to women transitioning through menopause.

This association was not seen with respect to CAC. There was not a statistically significant difference in the association of HDL-C on the odds of coronary artery calcification at different estradiol concentrations. Regardless of estradiol concentration, HDL-C was negatively associated with odds of CAC. Therefore, these findings do not suggest that estradiol modifies the association of HDL-C on CAC.

Results for both AC and CAC were similar using a different sample size, as done in the sensitivity analyses. With all sample sizes (n=478, n=460, and n=333), the interaction between HDL-C and estradiol remained significant with respect to AC but not CAC. Additionally, all estimates in

these analyses showed similar directionality, regardless of level of significance. The consistency of these results gives robust evidence of estradiol's apparent effect modification on HDL-C and AC which was reported in the main analysis.

5.1.2 Interaction of HDL-C and CRP

Although CRP/inflammation was significantly associated with atherosclerosis, it did not show a statistically significant impact on the association of HDL-C with either AC or CAC. Irrespective of CRP level, HDL-C was negatively associated with odds of CAC. The interaction between HDL-C and CRP was not statistically significant, thus there is insufficient evidence that the association of the effect of HDL-C on either CAC or AC changes based on CRP level.

5.2 Biological Mechanisms

Previous research suggests that estradiol and inflammation may cause conformational changes in HDL and may change the type of circulating particles, impacting their functionality[33 51 63 64]. Previous work has shown that HDL efflux capacity is strongly and positively associated with medium and large HDL particle sizes, whereas efflux capacity was strongly negatively associated with small HDL particle concentration[51]. Other SWAN work has shown that high estradiol concentration is associated with greater concentrations of large HDL particles[33], and other recent literature has suggested that mean HDL particle size is inversely associated with CVD risk[95]. Therefore, low estradiol may be associated with a more atherogenic particle profile compared to high estradiol levels due to changes in HDL, which would explain the interaction between HDL-C

and estradiol found in this analysis[33 95]. Our findings could potentially be explained by reduced cholesterol efflux as a result of low estradiol concentrations and changes in HDL particle size and concentrations[37].

Increasing inflammation is associated with the displacement of apoA, the main constituent of HDL, with SAA, which reduces HDL functionality by reducing reverse cholesterol transport[63 64]. Therefore, an increase in inflammation may lead to dysfunctional HDL and a reduction in cholesterol transport, promoting calcium buildup and atherosclerosis. These conclusions conflict with our results, however, in that the protective association of HDL-C did not change based on inflammation level. Future studies should assess these potential mechanistic pathways in a larger sample size.

Recent literature indicates that calcification may occur in some vessel beds before others[96]. While AC and CAC show a slight positive correlation (r = 0.35 between CAC and proximal aortic calcification and 0.31 between CAC and distal aortic calcification) [96], there are differences in the two that may explain different findings in this analysis. Allison et. al report that, among women over age 50, distal aortic calcification (DAC) is most prevalent compared to calcification in other beds[96]. Half (50%) of women between 50 and 60 showed DAC compared to 31% showing coronary artery calcification[96]. In women under 50, CAC was slightly more prevalent (27% vs 18% distal aortic), but distal aortic calcification was more prevalent than CAC in all age groups over age fifty[96]. Furthermore, this study reported that, in women, a 10-year increase in age was associated with a 6.1 times higher odds of calcification in the proximal aorta, a 5.5 times higher odds of calcification in the distal aorta, compared to a 2.2 times higher odds of calcification in the coronary artery[96]. Therefore, it is possible that calcification in the aorta develops earlier than in the coronary artery, or that atherosclerosis in the aorta occurs faster than in the coronary artery.

In addition, the Allison et. al results and results from the MESA suggest that AC is more strongly associated with CVD risk factors compared to CAC[96 97]. While results from univariate analysis with CAC and AC in this analysis (**Table 3**) only somewhat support the Allison et. al and MESA findings, differences in variables associated with AC versus those associated with CAC may partially explain the difference between their observed associations with HDL-C as a result of estradiol. Finally, prior research suggests inconclusive evidence of the association between calcification measures and cholesterol efflux[98 99]. It is possible that a decrease in cholesterol efflux impacts different measures of subclinical CVD differently, and AC may be more negatively impacted than CAC, which could explain the differing results in this analysis. At the present, there is no evidence to support this hypothesis, but future research may give insight into this conundrum.

5.3 Considerations with Other Literature

The findings of this analysis align with similar SWAN papers which suggest HDL-C is associated with a higher risk of subclinical CVD around the time of the menopausal transition[46 47]. In particular, these findings match the results of the Woodard et al. work and offer additional support of the reversal in the expected association of HDL-C. Woodard et al. tested the interaction of HDL-C and menopausal status with respect to CAC and AC and found a significant interaction between HDL-C and menopausal status with respect to AC, but not CAC[47]. Looking at left main CAC, there was a borderline significant interaction[47]. For AC, pre-/early perimenopausal women showed lower odds of calcification with high HDL-C, and late peri-/postmenopausal women showed higher odds with HDL-C[47]. Those findings are similar in directionality, but not statistically significant, with respect to CAC[47]. Researchers concluded that the protective effect of HDL-C is weaker in late peri-

/postmenopausal women with respect to AC[47]. Since estradiol concentrations are typically lower in late peri-/postmenopausal women, it is possible that the reversal in the protective effect of HDL-C is modified by estradiol level, as we have found in this analysis. Similarly, results from other SWAN researchers report increasing HDL-C levels as positively associated with greater cIMT production around the time of the menopausal transition[46]. While this study examined a different outcome, our results also highlight this unexpected positive association of HDL-C with subclinical CVD.

Though the findings of this analysis and the Woodard et al. work are similar, there are some key differences in methodology which may impact the strength of the observed association between HDL-C and calcification measures. We used different sample selection criteria and a smaller sample size than Woodard et al., due in part to the exclusion of women without CRP data in this analysis. This sample size was less than two-thirds the size of Woodard et al.'s work (342 vs. 540, respectively). Therefore, our smaller sample size would yield larger confidence intervals and requires a larger effect size to reach statistical significance. However, the comparison of characteristics among included and excluded women shows little difference between included and excluded groups, indicating that results should be similar despite the difference in sample selection. Additionally, the use of different calcification cut points (=> 75th percentile used by Woodard et al. compared to CAC => 10 or AC => 100 used in this work) could impact the strength of the findings. Defining CAC or AC presence as the 75th percentile would have labeled slightly more women as having CAC or AC presence (7 women and 6 women, respectively) in this analysis, which may have changed the strength of the observed interaction.

Several studies, such as the Persson et al. study[50] and the Malik et al. study[66] discussed in the introduction, give evidence of the impact of estradiol and inflammation using randomized clinical trials[50 66]. The Persson et al. study gives high quality evidence of the impact of estradiol on HDL-C

through a clinical trial, determining that high estradiol concentrations increase apoA levels and decrease apoB levels[50]. This study is a randomized trial and thus gives better evidence of a causal association between estradiol and structural changes in HDL, compared to an observational study. However, this study is limited by its small and very specific sample size of 31 women. All women were young, with an average age of 33.3 years, and all underwent in vitro fertilization, resulting in a very specific population. Research on the impact of CRP on HDL-C also gives high-quality evidence using clinical trials, determining that acute inflammation reduces reverse cholesterol transport and may lead to dysfunctional HDL[66 67]. However, past studies used animal models or humans with chronic health conditions to draw conclusions about the role of inflammation on HDL dysfunctionality. Thus, the results from these studies may not be generalizable to our target population of midlife women, and we cannot say with certainty that estradiol and inflammation are responsible for the reversal in the protective effect of HDL-C in this population. However, our research does provide insight and support for the theory by demonstrating a significant interaction between HDL-C and estradiol.

5.4 Strengths and Limitations

Although the overall included sample size was only a portion of the total SWAN Heart population, there were very few differences in those who were included compared to those who were excluded. For example, the age of those included vs excluded showed statistical significance, but the difference in average age was only 0.8 years between the two groups. Although this difference was statistically significant, the slightly older age of the included population is unlikely to be

clinically significant or to impact the results of the analysis. Thus, based on the differences between the groups, the results of this analysis are less prone to selection bias and are less likely artefactual.

One difference of note is the number of women who report alcohol consumption in the included vs excluded groups. Fewer women in the included group reported drinking two or more drinks per week. This variable was not included in the final model, and other baseline characteristics were similar between included and excluded women. Moderate alcohol consumption is negatively associated with CVD risk and positively associated with HDL-C[94]. However, alcohol consumption was not statistically significant in our models after adjusting for covariates such as age and race. While the fully adjusted model did adjust for alcohol consumption, its removal did not change the point estimate of the interaction term. In this population, controlling for alcohol consumption did not seem to impact this association. This conflicting finding could, in part, be attributed to differences in categorization of alcohol consumption in this study compared to others, or due to differences in age groups among these studies. Other studies measured alcohol consumption in drinks per day or grams of alcohol per day, rather than drinks per week[94]. Based on the conclusions of past research, having fewer regular drinkers in this analysis compared to the entire SWAN Heart cohort may bias the results toward the null. Despite these differences, we believe that these results are generalizable to a population of women with similar characteristics of those in this analysis. Additionally, results of the sensitivity analyses conducted as part of this work are consistent with results of the main analysis, indicating that the findings are robust and have high internal validity.

This study is limited by its cross-sectional nature. As a result, we cannot assess temporality and have less evidence of causation. While we observed that estradiol modifies the association of HDL-C with AC, there may be some residual confounding which influences this effect. Additionally, the use of CAC => 10 or AC => 100 as cut points may be somewhat arbitrary. These cutoffs were

chosen based on their use in prior studies, as there are no definitive clinical guidelines for calcification measures in low-risk populations, such as those in SWAN Heart. Finally, the use of odds ratios may overestimate the risk of CAC/AC in this analysis. Odds ratios are appropriate estimators for uncommon outcomes (< 10% of a population). In this analysis, approximately 24% of the included population had high AC or high CAC. Thus, our findings may overestimate the total risk of subclinical CVD. Future research could improve on these limitations by assessing effect modification of estradiol on the relationship between HDL-C and subclinical CVD with longitudinal data. Additionally, further research on novel metrics of HDL, such as HDL particle concentration or particle size, may be relevant to understand this association. Current research indicates that particle size may reflect HDL efflux capacity and thus reflects HDL functionality[49]. Efflux capacity is strongly and positively associated with large and medium HDL particle concentrations but inversely associated with small particle concentrations[49]. Considering these metrics rather than overall concentration of HDL-C may be a better estimator of the protective effect of HDL, but more research is needed to develop guidelines for the use of such metrics.

5.5 Clinical Significance

Estradiol level modified the association of HDL-C with odds of AC, such that, at high levels of estradiol, HDL-C showed a negative association with AC but a positive association at low levels of estradiol. This finding is both statistically and clinically significant. In women with low estradiol levels, recommendations to raise HDL-C may even have the opposite of the desired effect, as these findings suggest a positive association between HDL-C and AC risk at low estradiol levels, which is consistent with results from the statistical analysis. Future studies should assess this hypothesis in

different populations and using alternative study designs, such as longitudinal studies, as these improvements would give more evidence of this association.

While the risk of CAC was not positively associated with HDL-C at any estradiol level, the effect of the negative association seems stronger at high estradiol levels compared to low estradiol. While this association did not reach statistical significance, these results question the role of conventional HDL-C metrics for CVD risk estimation. Future research may see different results with a different sample size.

Although the interaction between CRP and HDL-C was not statistically significant with respect to CAC or AC, there may be a clinically significant impact of inflammation on HDL-C. However, in this analysis, CRP was positively associated with odds of CAC and AC, controlling for other CVD risk factors. Inflammation may be an important factor when evaluating CVD risk, but based on this analysis, it does not modify the association of HDL-C and AC or CAC. There may be other biological pathways which were not explored in this analysis which would explain the role of inflammation in CVD risk prediction. Additional research investigating these pathways may add improve understanding of the complex relationship between estradiol, inflammation, HDL-C, and subclinical CVD.

6.0 Conclusions and Public Health Significance

Despite current beliefs, HDL-C may not be cardioprotective in midlife women with respect to aortic calcification. Our results highlight the importance of considering estradiol concentrations in assessing CVD risk in women. The degree to which HDL-C protects against cardiovascular disease may vary based on estradiol concentration. With high levels of estradiol, the association between HDL-C and aortic calcification is in the expected direction. As HDL-C levels increase, risk of calcification, and, thus, risk of CVD, decreases. However, low estradiol levels show the opposite effect, with higher HDL-C levels showing an increase in AC risk. These results may explain the reversal in the protective effect of HDL that was seen in previous literature[33 46 47]. While this relationship was only observed with respect to AC, the implications may span to other subclinical measures of CVD as well. This association may not be observed across other measures of subclinical CVD, such as CAC, plaque, or intima media thickness, due to differences in the CVD risk factors associated with each, as well as differences in the pathogenesis of calcification across different vessels.

These findings support the reversal in the protective association of HDL-C with CVD risk which has been reported in previous literature in midlife women[33 46 47 86 87]. The results of this work may ultimately contribute to the understanding of mechanisms that compromise HDL-C functionality or otherwise increase risk of CVD among this population. This work could spawn additional research into the mechanisms for understanding the increase in risk of CVD around the menopausal transition. Finally, this work also underlines the limited information about the conventional HDL-C measure and calls for improvement in novel metrics for HDL which may better predict risk of CVD in certain populations, including midlife women.

Appendix Supplemental Table

Appendix Table 1 Results from Sensitivity Analyses

		Inclusion of those missing CRP[1]		Exclusion of those with E2 < 7pg/mL[2]		Inclusion of those missing CRP and exclusion of those with E2 <7 pg/mL[3]	
		n=478		n=333		n=460	
		OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P
AC => 100	HDL-C	1.093 (1.021, 1.171)	0.010	1.136 (1.022, 1.262)	0.018	1.09 (1.019, 1.183)	0.014
	Log E2	4.042 (1.308, 12.49)	0.015	6.501 (1.238, 34.13)	0.027	4.157 (1.254, 13.787)	0.020
	HDL* log E2	0.975 (0.956, 0.994)	0.012	0.965 (0.936, 0.994)	0.020	0.974 (0.953, 0.995)	0.014
CAC =>10	HDL-C	1.043 (0.966, 1.127)	0.28	1.013 (0.909, 1.130)	0.81	1.041 (0.955, 1.134)	0.36
	Log E2	1.859 (0.509, 6.792)	0.35	1.331 (0.233, 7.614)	0.75	1.764 (0.433, 7.182)	0.43
	HDL* log E2	0.986 (0.982, 1.008)	0.21	0.992 (0.961, 1.023)	0.61	0.986 (0.962, 1.011)	0.28

[1] Model based on final models, without CRP. AC: controlling for cycle day, hormone use, age, race, site, waist circumference, smoking status, and log triglycerides. CAC: controlling for cycle day, hormone use, age, race, site, waist circumference, and SBP.

[2] Model based on final models, with CRP. AC: controlling for cycle day, hormone use, age, race, site, waist circumference residuals, smoking status, log triglycerides, and log CRP. CAC: controlling for cycle day, hormone use, age, race, site, waist circumference residuals, SBP, and log CRP.

[3] Model based on final models, without CRP. AC: controlling for cycle day, hormone use, age, race, site, waist circumference, smoking status, and log triglycerides. CAC: controlling for cycle day, hormone use, age, race, site, waist circumference, and SBP.

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