High-Density Lipoprotein (HDL) Metrics in Midlife Women: Associations with Adiposity, Subclinical Atherosclerosis and Cardiovascular Health

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High-Density Lipoproteins (HDL) Metrics in Midlife Women: Associations with Adiposity, Subclinical Atherosclerosis and Cardiovascular Health

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Background: The clinical utility of high-density lipoprotein cholesterol (HDL-C) in evaluating the anti-atherogenic abilities of HDL is debated. With ovarian aging, HDL may become dysfunctional; this dysfunctionality may not be captured by HDL-C. Novel metrics of HDL function, lipid content, and subclasses may provide further information on role of HDL in cardiovascular risk.

Objectives: The objectives of this dissertation are to (1) determine whether higher abdominal and cardiovascular visceral adipose tissue (AT) volumes are associated with worse HDL metrics [lower HDL-cholesterol efflux capacity (HDL-CEC), HDL-phospholipids, and large HDL particles, smaller overall HDL size, and higher HDL-triglycerides and small HDL particles]; (2) identify whether clusters of HDL metrics during midlife are associated with future subclinical atherosclerosis; and (3) evaluate whether early midlife cardiovascular health (CVH) impacts future HDL metrics.

Methods: Participants from the Study of Women's Health Across the Nation (SWAN)-HDL ancillary study [n=558] were evaluated. For Aim 1, associations between visceral AT volumes with each future HDL metric were assessed by multivariable linear regression. For Aim 2, women were clustered by latent class analysis based on their baseline HDL metrics, and the relation between clusters with future subclinical atherosclerosis was evaluated by multivariable

linear/logistic regression. For Aim 3, the relationships between early midlife CVH and health behaviors with future HDL metrics were tested by multivariable mixed models.

Results: Higher visceral AT volumes were associated with a worse HDL metrics profile. Based on their HDL profile, women were classified into favorable (higher HDL-CEC, and phospholipid contents, and larger HDL subclasses) and unfavorable (lower HDL-CEC, and phospholipid contents, and smaller HDL subclasses) clusters; association between favorable HDL clusters at midlife and better future subclinical atherosclerosis was explained by body mass index. Better CVH, particularly lower BMI, early in midlife was associated with a better HDL metrics profile later in life.

Conclusions: A comprehensive HDL metric profile, particularly HDL subclasses and HDL phospholipids, may help decipher the increased risk of cardiovascular disease in midlife women. Obesity and fat distribution strongly impact these metrics. HDL metrics may be appropriate targets for novel therapeutic interventions to reduce cardiovascular disease burden in women.

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Preface

To start, I would like to sincerely express my appreciation and gratitude to my mentor, Dr. Samar El Khoudary. Over the past five years, she has provided me with the utmost support, enthusiasm, positivity and guidance. I thank her for believing in me, and pushing me to always give my best work. This dissertation would not have been possible without her efforts.

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To my friends in the program, I am thankful for having you by my side throughout this journey. Finally, my biggest thanks goes to my family; Mom, Dad, John, Jad, Jacky and Elsy, and my nieces and nephews. Thank you for your love, understanding and unwavering support. I wouldn't have been able to do it without you!

1.0 Introduction

Cardiovascular disease (CVD) is uncommon in women prior to midlife, but after menopause, the risk equalizes to that in men.[1] The mechanism of this increased risk of CVD is likely multifactorial, but the alterations in lipid metabolism that are prompted by the hormonal changes that accompany menopause have been suggested to play a critical role.[2]

High-density lipoprotein is presumed to carry anti-atherogenic qualities, and accordingly, older observational and interventional epidemiological studies focused on raising high-density lipoprotein cholesterol (HDL-C) levels as a mean to reduce CVD burden. However, the lack of evidence on successful CVD prevention with raising HDL-C has steered clinicians away from treating low HDL-C levels. In the 2019 American College of Cardiology /American Heart Association (ACC/AHA) guidelines on the primary prevention of CVD, there were no recommendations on raising HDL-C as a primary preventive method against CVD.[3] Similarly, the most recent ACC/AHA guidelines on secondary prevention in patients with pre-existing coronary or vascular disease did not include HDL-C-raising therapies as a target for future prevention of CVD.[4] Still, HDL-C is a component of the metabolic syndrome diagnosis,[2] is quantified in clinical practice when assessing dyslipidemia, and is a part of the pooled cohort equation for the assessment of CVD risk.[5]

Whether the athero-protective capabilities of the HDL are truly not sufficient to reduce CVD risk, or whether HDL-C, which exclusively measures the cholesterol content of the HDL particle, is not adequate to predict the cardio-protective abilities of the HDL is unknown.[6]

The HDL is highly heterogeneous, and particles vary in their cholesterol, lipid and protein composition, as well as shape, size and function.[7] It has been proposed that novel metrics of HDL,

which describe characteristics such as the function, lipid content, and subclasses, may provide further information on HDL quality and CVD risk prediction beyond the cholesterol content.[6]

In women specifically, a potential dysfunctionality in the HDL, which is not detected by HDL-C, has been suggested to occur during and after the menopause transition (MT).[8,9] We have recently shown that women around menopause experience a deterioration in HDL function as measured by the cholesterol efflux capacity per particle, and a shift in HDL particles towards a smaller size, with an accompanied increase in the triglyceride within the HDL.[10] This HDL metric profile has been linked to worse CVD outcomes. These results are in agreement with other studies that have shown that the characteristics of the HDL particle differ across the MT.[11,12]

It is unknown, however, how these HDL metrics in midlife women are associated with risk factors of CVD.[13] For instance, menopause is associated with accelerated accumulation of visceral fat, both in the abdomen and in various ectopic locations in the body.[13,14] This increase in visceral fat has been linked to alterations in the lipoproteins including HDL.[15] However, the exact link between visceral fat depots and different HDL metrics in women has not been elucidated. Moreover, since HDL is presumed to play a fundamental anti-atherogenic role, the impact of HDL metrics around menopause on future subclinical atherosclerosis, a well-known predictor of future cardiovascular events, would help identify how these metrics could be related to CVD outcomes. Finally, knowing whether better lifestyle factors in midlife women are linked to better HDL metrics later in life could aid in understanding whether modifying health behaviors can influence changes in characteristics of HDL and potentially, subsequent CVD.

It is crucial to identify the role of novel HDL metrics in the increased risk of CVD in midlife women to better guide the clinical management and prevention of CVD risk in this population. The overall objective of this work is to delineate the link between different HDL

metrics and CVD risk factors in midlife women. This would provide a better understanding of how the profile of HDL metrics during the menopausal period in women could impact future CVD.

2.0 Specific Aims

The Study of Women's Health Across the Nation (SWAN) is a longitudinal multi-ethnic, multi-site, community-based study that has characterized the hormonal, physiological and symptomatic changes that occur in women over the MT. The SWAN Heart and SWAN Cardiovascular Fat ancillary studies have collected measures of abdominal and ectopic visceral AT in a subset of SWAN women during midlife. Additionally, the SWAN HDL ancillary study evaluated a comprehensive profile of HDL metrics in 558 SWAN women over the MT. These studies provide an excellent opportunity to assess the relationship between HDL metrics and different CVD risk factors in midlife women. Our specific aims for this dissertation are:

Specific Aim 1: To characterize the associations of midlife abdominal visceral and cardiovascular adipose tissue (AT) volumes with HDL metrics, independent of potential confounders, to evaluate whether these associations differ by race, and to assess whether insulin resistance mediates the associations between visceral AT depots and HDL metrics.

Hypothesis 1: Higher volumes of abdominal and cardiovascular visceral AT around midlife will be associated with a worse HDL metric profile [lower HDL cholesterol efflux capacity (HDL-CEC), lower concentrations of HDL phospholipids (HDL-PL) and large HDL particles (HDL-P), higher concentrations of HDL-Triglycerides (HDL-Tg) and small HDL-P, and smaller overall HDL size], independent of potential confounders.

Hypothesis 2: The association of midlife abdominal and cardiovascular visceral AT volumes with a worse HDL metric profile will be more pronounced in White women compared to Black women.

Hypothesis 3: Insulin resistance, as measured by HOMA-IR, will partially mediate the observed associations between visceral AT and HDL metrics.

Specific Aim 2: To classify women into clusters based on their HDL metrics profiles and to investigate the independent associations between these clusters and future subclinical atherosclerosis, as measured by carotid intima media thickness (cIMT), plaque presence and inter-adventitial diameter (IAD), and to investigate whether those associations vary by race/ethnicity.

Hypothesis 1: Women will be classified into an unfavorable HDL cluster (lower HDL-CEC, HDL-PL, and large HDL-P concentrations, higher HDL-Tg and small HDL-P concentrations, and smaller overall HDL size) or a favorable HDL cluster (higher HDL-CEC, HDL-PL, and large HDL-P concentrations, lower HDL-Tg and small HDL-P concentrations, and larger overall HDL size).

Hypothesis 2: The favorable HDL cluster will be associated with less subclinical atherosclerosis (lower cIMT and IAD, and less odds of plaque presence) later in life compared to the unfavorable HDL cluster.

Hypothesis 3: The associations between the favorable HDL cluster and lower subclinical atherosclerosis will be strongest in Chinese women compared to Blacks and Whites.

Specific Aim 3: To evaluate the associations between baseline lifestyle factors, as measured by the American Heart Association (AHA) Life's Simple 7, and HDL metrics later in life in women, and to assess how each health behaviors component [body mass index (BMI), diet, physical activity, and smoking] associates with these HDL metrics. We will also assess the relation between baseline Life's Simple 7 score and health behaviors components with changes in HDL metrics.

Hypothesis 1: A higher baseline Life's Simple 7 score at baseline will be associated with a better future HDL metrics profile (higher HDL-CEC, HDL-PL, and large HDL-P concentrations, lower HDL-Tg and small HDL-P concentrations, and larger overall HDL size) independent of potential confounders.

Hypothesis 2: Ideal baseline BMI, physical activity, smoking and diet categories will independently be associated with better future HDL metrics profile compared to poor categories.

Hypothesis 3: Higher Life's Simple 7 score and ideal health behavior categories will be associated with favorable changes in HDL metrics over time.

3.0 Literature Review

3.1 Cardiovascular Disease in Women

Between 2015 and 2018, around 8.4% of adult US women aged ≥20 years were diagnosed with CVD, defined as coronary heart disease (CHD), stroke or heart failure.[1] However, at older ages of 60-79 years and ≥80 years, up to 18.2% and 31.3% of women were diagnosed with CVD, respectively. When hypertension was added to the diagnosis list of CVD, these estimates increased to 75.4% and 90.8% in those respective age groups. Moreover, 48% of all CVD deaths in 2019 occurred in women.[1] Despite the declines in CVD mortality in males and females over time, CHD still accounts for the majority of deaths in the US in both sexes.

In both men and women, prevalence of CVD increases with age. In early adulthood, however, prevalence of CVD is significantly lower in women compared to men, but equalizes between the 2 sexes in middle-aged and elderly adults.[1] It has been suggested that premenopausal women are protected against CVD by the effects of estrogen;[16] however, randomized clinical trials on hormone therapy (HT), such as the Heart and Estrogen/progestin Replacement Study (HERS) and the Women's Health Initiative (WHI), failed to show a cardio-protective effect of estrogen supplementation in postmenopausal women, with some studies even showing a detrimental effect.[17-19] A potential timing effect, termed the "timing hypothesis", has been suggested, implying that HT may be cardio-protective if started <10 years within the MT.[20,21]

Further, diagnosis of CVD in women lags behind the diagnosis in men by around 10 years.[22] However, studies have shown that by midlife, more than 80% of women have at least one risk factor for CVD, such as obesity, dyslipidemia, diabetes, hypertension, lack of physical

activity, or smoking.[23,24] A higher number of cardiovascular risk factors is associated with higher risk of CVD later in life.[25] The mechanism of this increased risk of CVD is likely multifactorial, but the estrogen deficiency that accompanies menopause may impact lipid metabolism, which could play a crucial role in the increased risk of CVD during that time.[2]

3.2 Cardiovascular Disease and High-Density Lipoprotein-Cholesterol

Dyslipidemia, which is characterized by elevated total cholesterol, low-density lipoprotein-cholesterol (LDL-C), triglycerides, and/or reduced high-density lipoprotein -cholesterol (HDL-C) levels, is associated with increased risk of CVD in women.[26,27] In particular, the role of elevated LDL-C in promoting atherosclerosis, and the impact of interventions that reduce LDL-C on alleviating CVD risk have been well-recognized. Both primary and secondary prevention efforts have been established to lower LDL-C as a mean to protect against CVD.[3,4,28] Nevertheless, despite the success of these interventions, residual CVD risk persists.

On the other hand, the role of high-density lipoproteins (HDLs) in reducing CVD, particularly in women, remains unclear.[29] Higher HDL-C levels are presumed to be cardio-protective,[2] where they are thought to reflect the major anti-atherogenic role of the HDL.[30]

Between 2015 and 2018, approximately 8.2% of US adult women were diagnosed with low HDL-C (defined as HDL-C≤40 mg/dL). However, large population-based studies have shown that prevalence of low HDL-C decreased with age, from 10.3% in women aged 20-39 years, to 7.8% in those aged 40-59 years, to 6.4% in those aged ≥ 60 years.[31]

Numerous epidemiological studies that have assessed the relation between HDL-C and CVD in the general population have shown an inverse relationship between HDL-C and CVD;

however, these results have not been consistent across all studies, with some studies showing no associations, while others showing an increased risk of CVD with higher HDL-C. **Table 3-1** summarizes the findings from population-based epidemiological studies that have assessed the relation between HDL-C and CVD in adult women.

Nevertheless, the design of observational studies renders them unable to determine the causal pathway between HDL-C and CVD risk. Unfortunately, studies of Mendelian randomization[32,33] have failed to show an association between HDL-C and CVD risk. For instance, Voight et el.[32] identified a genetic variant in the endothelial lipase *LIPG* gene that solely increases plasma HDL-C levels without affecting other lipoproteins; this gene, however, was not associated with reduced risk of myocardial infarction. Moreover, Haase et al.[33] reported no association between a SNP in the Lecithin-Cholesterol Acyltransferase (LCAT) gene, which is a marker of isolated reductions in HDL-C, with CVD outcomes. Other studies which investigated genetic variations in LCAT and apolipoprotein A-1 (ApoA-1)[34-36] which lead to massive reductions in HDL-C similarly did not show an accompanying increase in CVD risk.

Additionally, evidence from randomized clinical trials which tested the impact of raising HDL-C with anti-lipid treatments on CVD risk has been conflicting; i.e. one large clinical trial of 17,802 participants found no relation between raising HDL-C and future risk of CVD events,[37] whereas another trial in 9,770 subjects with history of CHD showed that higher HDL-C was associated with lower risk of CVD events in those on low dose but not on high-dose statins.[38]

The efficacy of newer therapies that raise HDL-C, such as cholesteryl ester transfer protein (CETP) inhibitors, in reducing CVD have also been investigated. These trials showed that CETP inhibitors failed to reduce risk of recurrent CVD events or mortality,[39] or progression of coronary atherosclerosis.[40] Moreover, some trials reported an increase in mortality and cardiovascular

Table 3-1: Population-based studies on the associations between HDL-C and CVD in women

Study	Design	Country	Cohort	Population	Outcome	Results
Castelli, 1986, JAMA[41]	Prospective (12 years)	USA	Framingham Study	n=985, mean age 62 years	CHD incidence	Inverse Association
Stensvold, 1992, Eur Heart J[42]	Prospective (6.8 years)	Norway	Norwegian Cohort	n=23425, ages 40-54 years	CHD mortality CVD mortality	Inverse Association
Zimetbaum, 1992, Arterioscler Thromb[43]	Prospective (10 years)	USA	The Bronx Aging Study	n=226, mean age 79 years	CVD mortality MI	No Association
Bass, 1993, Arch Intern Med[44]	Prospective (14 years)	USA	Lipid's Research Clinic's Follow-up Study	n=1405, mean age 58.2 years	Subsequent CVD mortality	Inverse Association
Corti, 1995, JAMA[45]	Prospective (4.4 years)	USA	EPESE study	n=2527, mean age 78.9 years	CHD mortality	Inverse Association
Simons, 1995, Atherosclerosis[46]	Prospective (5.2 years)	Australia	Dubbo Study of Australian Elderly	n=1569, age >60 years	CHD incidence	No Association
Frost, 1996, Circulation[47]	Prospective (4.5 years)	USA	SHEP study	n=2046, mean age 72 years	CHD events	No Association
Jousilahti, 1999, Circulation[48]	Prospective (10 years)	Finland	WHO MONICA project	n=7969, ages 25-64 years	CHD incidence CHD death	Inverse Association
Psaty, 1999, Arch Intern Med[49]	Prospective (4.8 years)	USA	Cardiovascular Health Study	n=2979, mean age 72 years	Coronary Events	No Association
Alencar, 2000, Arquivos Brasileiros de Cardiologia[50]	Cross-sectional	Brazil	Primary-care based cohort	n=364 women, mean age 75.6 years	Atherosclerotic complications*	No Association
Sharrett, 2001, Circulation[51]	Prospective (10 years)	USA	ARIC	n=6907, ages 45-64 years	CHD incidence	Inverse Association

Walldius, Lancet, 2001[52]	Prospective (5.4 years)	Sweden	AMORIS database	n=76831, mean age 49.7 years	CHD mortality	Inverse Association
von Mühlen, 2003, Am J Cardiol[53]	Prospective (6.3 years)	USA	The Rancho Bernardo Study	n=1305, mean age 69 years	CHD Mortality CVD mortality	No Association
Weverling-Rijnsburger, 2003, Arch Intern Med[54]	Prospective (2.6 years)	Netherlands	Leiden 85-Plus Study	n=373, >85 years	CVD Mortality	Inverse Association
Shai, 2004, Circulation[55]	Nested Case-Control (8 years)	USA	NHS	n=683, mean age 61 years	CHD incidence	Inverse Association
Mazza, 2005, Intern Med J[56]	Prospective (12 years)	Italy	CArdiovascular STudy in the ELderly	n=3257 women ≥65 years old	CHD mortality	Inverse Association
Ridker, 2005, JAMA[57]	Prospective (5 years)	USA	WHS	n=15632, mean age 54.4 years	CVD incidence	Inverse Association
Zyriax, 2005, Eur J Clin Nutr[58]	Case-Control	Germany	CORA study	n=200, mean age 64 years	CHD prevalence	No Association
Cooney, 2009, Atherosclerosis[26]	Prospective (8.1 years)	Europe	SCORE project	n=43,544 adults	CVD mortality	Inverse Association

EPESE: Established Populations for Epidemiologic Studies of the Elderly; SHEP: Systolic Hypertension in the Elderly Program; WHO MONICA: World Health Organization Monitoring of Trends and Determinants in Cardiovascular Disease; ARIC: Atherosclerosis Risk in Communities; AMORIS: Apolipoprotein-related MOrtality RISk study; NHS: Nurse's Health Study; WHS: Women's Health Study; CORA: Coronary Risk Factors for Atherosclerosis in Women; SCORE: Systematic COronary Risk Evaluation

* Atherosclerotic complications: CHD, cerebrovascular disease, or peripheral artery disease

morbidity with these drugs despite significant increases in HDL-C.[59] It is important to note though, that in some subjects who achieved very high levels of HDL-C, there was a regression in coronary atherosclerosis.[60] Thus, the results from randomized clinical trials have been conflicting, and a direct conclusion on the athero-protective indications of HDL-C cannot be established.

In midlife women specifically, the relation between HDL-C and reduced CVD risk seems to be even more controversial. Several studies have shown that an increase in HDL-C may be associated with higher cardiovascular events[61] and subclinical atherosclerosis[62] in women[63] but not in men. As such, and since CVD appears to increase significantly after the MT, there has been an interest in evaluating whether the changes in HDL-C over the MT could drive this increased CVD risk. In an attempt to better understand this phenomena, cross-sectional studies aimed to compare HDL-C by menopause status, whereas longitudinal studies assessed how HDL-C changes over the MT. One cross-sectional study[64] showed no difference in HDL-C by menopausal status (premenopausal, perimenopausal, naturally menopausal and surgically menopausal) stratifying by age. Longitudinally, in a study of middle-aged women [mean age 47 years], postmenopausal women experienced a decline in HDL-C levels after 2.5 years of follow-up compared to women who remained premenopausal, despite similar age.[65] In SWAN, midlife women who achieved menopause over the study follow-up period experienced an increase in HDL-C until one year before menopause, which then leveled off.[66] Similar findings were reported in the Melbourne Midlife Women's Health Project.[67] These results show that the change in HDL-C over the MT is not consistent with the expected decline that would put midlife women at increased CVD risk.

Moreover, several studies have tried to understand how the levels of HDL-C over the MT are linked to future CVD risk, as assessed by measures of subclinical atherosclerosis. Cross-sectionally,[68] SWAN showed that higher HDL-C was associated with protective effects against

aortic calcification (AC), carotid intima media thickness (cIMT) and carotid plaque in premenopausal/early perimenopausal women but not in late peri/postmenopausal women. These were consistent with findings reported by MESA, where higher HDL-C was associated with higher carotid plaque, particularly for women who were >10 years postmenopausal.[69] In a longitudinal analysis of 213 women [mean age at baseline 45.7 years], larger increases in HDL-C prior to the MT were associated with less progression of cIMT but increases in HDL-C after menopause were associated with greater cIMT progression.[8] These results suggest that HDL-C may not be a good indicator of cardio-protection in midlife women, and that HDL could potentially become dysfunctional in this population.

3.3 High-Density Lipoproteins: Structure, Lipid Content and Function

For decades, HDL-C has been utilized as a clinical and conventional marker of the athero-protective functions of the HDL. HDL-C, which is termed "the good cholesterol", is a measure of the cholesterol content within the HDL. However, the HDL is an exceptionally complex particle that varies in lipid and protein contents, subclass distribution and function, making the simple HDL-C measure insufficient to understand the full picture of the anti-atherogenic abilities of the HDL, and the impact of this particle on CVD risk.[7]

The individual HDL molecule, termed the HDL particle (HDL-P), contains varying concentrations of numerous lipids and proteins.[6] Different HDL particles vary in composition, size and shape. ApoA-I dominates the protein composition of the HDL and constitutes around 75% of the total protein content of the HDL. Additional proteins include ApoA-II, ApoA-IV, ApoA-V, ApoE, ApoJ, ApoM, LCAT, and cholesteryl ester transfer proteins (CETP), among others.[6] The

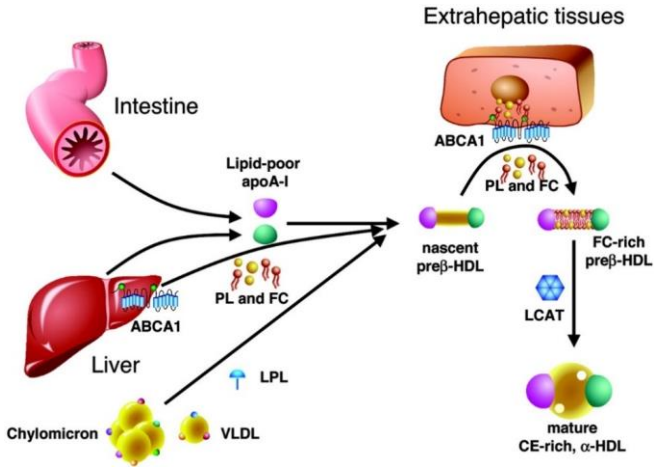
lipidome of the HDL includes mainly phospholipids (20-30%), cholesteryl esters (14-18%), free cholesterol (3-5%) and triglycerides (3-6%). Phospholipids and unesterified free cholesterol form the surface of the HDL, whereas the triglycerides and cholesteryl ester form the core of the HDL.[6,70] Studies have suggested a potential link between HDL size and content, where with decreasing HDL particle size, the abundance of cholesteryl esters and triglycerides increases on the surface[71] altering the content of the HDL particle.

The size and shape of HDL particles also varies, and ranges between the very small discoidal pre-β-1 HDL (which contains ApoA-I and phospholipids only), the very small discoidal α4 HDL (which contains ApoA-I, phospholipids and free cholesterol), the small spherical α3 and the medium spherical α2 HDLs (which contain ApoA-I, ApoA-II, phospholipids, free cholesterol, cholesteryl ester and triglycerides), and the large spherical α1 HDL(which contains ApoA-I, phospholipids, free cholesterol, cholesteryl ester and triglycerides).[72]

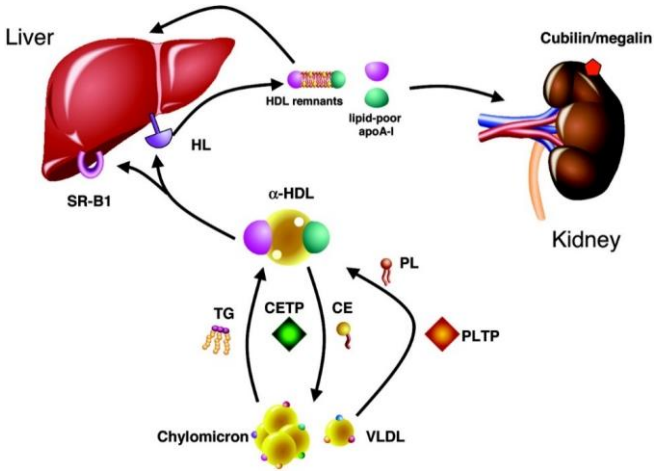
ApoA-I, the most abundant protein on the surface of the HDL, plays an important role in the ***cholesterol efflux process*** where the HDL acquires and transports excess cholesterol away from cells.[73] The ***reverse cholesterol transport*** (RCT) is the process where unesterified cholesterol is transported from non-hepatic tissue to the liver **[Figure 1(a)].** This occurs mainly via the ATP-binding cassette transporter 1 (ABCA1), which is the major pathway for efflux of cholesterol from macrophages. Pre-β1 HDL is the preferred acceptor of cholesterol that is removed from the macrophages through the ABCA1 pathway,[40] thereby participating in the maintenance of the net cholesterol balance in the arterial wall. Where preβ-1 HDL particles are most efficient in interacting with the ABCA1 to promote efflux of cholesterol into ApoA-I, and are responsible for around 60% of efflux via the ABCA1 pathway,[74] the ATP-binding cassette transporter G1 (ABCG1) promotes cholesterol efflux from macrophages to mature α-HDL particles.[75,76]

Cholesterol efflux capacity depends on the concentration and functionality of HDL particles.[74] Patients with coronary heart disease (CHD) appear to have elevated levels of the small pre-β1 HDL, but lower pre-β1 HDL functionality as measured by the ABCA1- specific cholesterol efflux capacity normalized to pre-β1 HDL levels.[77]

After cholesterol is moved into the HDL, the RCT is completed by the uptake of cholesterol by liver **[Figure 1(b)].** CETP mediates the transfer of cholesteryl esters from HDL particles to ApoB-containing lipoproteins such as the very low-density lipoprotein (VLDL), intermediate density lipoprotein (IDL) and low-density lipoprotein (LDL) particles, which are then taken by the liver, in exchange for triglycerides.[78] This results in the cholesteryl ester depletion and triglyceride enrichment of the HDL. Thus, CETP inhibition increases the concentration of the mature, cholesterol-rich α HDLs. Phospholipid transfer protein (PLTP) transfers surface phospholipids from triglyceride-rich lipoproteins to HDL.[79] Hepatic lipase and endothelial lipase also play a role in HDL metabolism, where they hydrolyze triglycerides and phospholipids within the HDL particle and generate smaller HDLs and HDL remnant particles.[70] Eventually, the cholesterol that has been moved by the HDL to the liver gets excreted in bile; else, it is carried, stored and utilized for other purposes such as steroidal hormone production.[2]



(a) Secretion, lipid acquisition, and maturation of HDL particles. Lipid-poor ApoA-1 is secreted from the liver and intestines, where it acquires phospholipids and unesterified cholesterol to form the small, discoidal pre-β1 HDL-P. This occurs via the ATP-binding cassette transporter 1 (ABCA1), the major pathway for efflux of cholesterol from macrophages. Pre-β1 HDL is the preferred acceptor of cholesterol that has been removed from the macrophages through the ABCA1 pathway,[40] thereby participating in the maintenance of the net cholesterol balance in the arterial wall. The discoid, cholesterol-nascent, HDL particle also obtains excess cholesterol from peripheral tissues and atherosclerotic plaques, or cholesterols released from the lipolysis of triglyceride -rich very-low density lipoproteins (VLDLs) and chylomicrons by lipoprotein lipase (LPL). This leads to the increase in the size of the pre-β1 HDL particle. Lecithin-cholesterol acyltransferase (LCAT) then esterifies the free cholesterol on the pre-β1 HDL to cholesteryl ester [80,81] to form the larger, mature, spherical HDLs (α1, α2, and α3 HDL particles).



(b) Intravascular modeling of HDL. Intravascular modeling of the HDL occurs via the scavenger receptor B1 (SR-B1) pathway mediates bidirectional lipid transport within the macrophage depending on the content of the cholesterol within the lipid-laden macrophage,[82] passive diffusion,[83] or by the cholesteryl ester transfer protein (CETP) enzyme.

Figure 3-1: The Reverse Cholesterol Transport (RCT) Pathway[79]

Higher concentrations of HDL-C have been deemed a reflection of higher cholesterol efflux capacity. Unfortunately, the concentration of HDL-C may not be able to fully capture the complexities of structure and function of the HDL.[7] In particular, the inability of high HDL-C to reduce CVD risk in prospective studies suggests the possibility that the continuous turnover and state of flux of the HDL may not be well detected by HDL-C alone. Over time, and due to the inconsistent and non-encouraging findings on the link between HDL-C and risk of CVD, clinical guidelines and practices have discarded HDL-C as a relevant target for primary and secondary prevention efforts.[3,4]

However, the question remains on whether the HDL is truly not related to future CVD risk, or whether HDL-C is only an insufficient measure of the underlying cardio-protective HDL abilities. This suggests the need to investigate more direct measures of the HDL particle to provide better and more comprehensive understanding of the anti-atherogenic potentials of the HDL. This could help identify those measure's role in identifying high-risk individuals or to potentially guide the development of novel therapies and preventive strategies against CVD.

3.3.1 Novel Metrics of HDL Assessment

As previously mentioned, the HDL-C quantifies the amount of cholesterol that is carried by the HDL. However, HDL-C may not be a sufficient surrogate marker of the anti-atherogenic properties of HDL due to the complexities in structure, composition and function of this particle.[6] On the contrary, direct quantitative HDL metrics of subclasses, lipid content and function may be superior to HDL-C in predicting risk of CVD.[84-86]

3.3.1.1 HDL Subclasses

Several methodologies have been developed to separate and quantify HDL subclasses based on the composition, density, charge, and/or the size of the HDL. **Table 3-2** provides a summary of these methods, the characteristics of the resulting HDL subclasses, and the advantages and disadvantages of each method.

Studies that assessed HDL particles have shown a strong correlation between HDL-C and large HDL-P,[87] suggesting that the HDL-C concentrations are a reflection of large, cholesterol-rich large HDL particles. However, over the years, there has been a growing interest in understanding the direct associations between HDL subclasses and CVD risk, and in determining whether these measures are superior to HDL-C in predicting disease. **Table 3-3** summarizes the studies that evaluated HDL subclasses, as quantified by different methodologies, in relation to CVD risk. The results of these studies show that, regardless of the method of HDL assessment,

Table 3-2: Summary of the methodologies that assess HDL subclasses[7,88]

Method	Basis of Method	Classification of HDL Subclasses	Advantages	Disadvantages
Analytical Centrifugation[94,95]	- Size and density based on ultracentrifugal flotation rate ($F_{1.2}$)	- HDL2 (1.063-1.125 g/mL) - HDL3 (1.125-1.210 g/mL)	- Gold Standard	- Separates HDL into either large (HDL2) or small HDL (HDL3) - High ionic strength and centrifugal force, shear force and high salt concentrations may disrupt particles.
Density Gradient Ultracentrifugation[89]	- Density	- HDL2b (1.063-1.087 g/mL) - HDL2a (1.088-1.110 g/mL) - HDL3a (1.110-1.129 g/mL) - HDL3b (1.129-1.154 g/mL) - HDL3c (1.154-1.170 g/mL)	- Single ultracentrifugal step; allows for quantitative recovery of highly-resolved HDL fractions - Avoids major contamination with plasma proteins - Facilitates HDL isolation in a non-denatured, non-oxidized states.	- High ionic strength and centrifugal force, shear force and high salt concentrations may disrupt particles.
Vertical Auto Profile (VAP) [89]	- Density	- HDL2-C - HDL3-C	- Fast, precise, and more practical for clinical use - Short single vertical spin centrifugation makes it less destructive than other centrifugation methods	- Limited comparison to other methods - Cannot isolate all HDL subpopulations
Gradient Gel Electrophoresis (GEE)[90]	- Density and particle diameter	- HDL2b (9.7-12.9 nm) - HDL2a (8.8-9.7 nm) - HDL3a (8.2-8.8 nm) - HDL3b (7.8-8.2 nm) - HDL3c (7.2-7.8 nm)	- Most utilized method for lipoprotein subclass characterization	- Labor-intensive and time-consuming - Requires a specialized laboratory

2-Dimensional Gel Electrophoresis[72]	- Size and Charge	- Pre-β-1 HDL (5.0-6.0 nm) - α4 HDL (7.0-7.5 nm) - α3 HDL (7.5-8.5 nm) - α2 HDL (9.0-9.4 nm) - α1 HDL (10.8-11.2 nm)	- Allows for accurate diagnosis of HDL metabolism disorders - Reproducible and standardized	- Requires a specialized laboratory
Nuclear Magnetic Resonance Spectroscopy (NMR)[91]	- Size	- Large HDL-P (9.4-14 nm) - Medium HDL-P (8.2-9.4 nm) - Small HDL-P (7.3-8.2 nm)	- Does not require physical separation of lipoprotein particles - Provides overall HDL size	- Does not provide information on chemical composition of lipoprotein - Small pre-β-1 HDL may not be detected
Ion Mobility[92]	- Charge	- HDL2b (10.5-14.5 nm) - HDL3+2a (7.65-10.5 nm)	- Utilizes gas-phase differential electrophoresis with short ultracentrifugation and in the absence of salt	- Cannot separate HDL3 from HDL 2a - Does not provide HDL size

there is a tendency for larger HDL particles to play a cardioprotective role, whereas smaller HDL particles tend to be associated with a higher risk of CVD; however, some inconsistencies exist, where some studies have shown that small HDL-P may be protective.[93]

3.3.1.2 HDL Lipid Content and Cholesterol Efflux Capacity

As mentioned earlier in this section, the HDL particle contains, along with cholesterol, different concentrations of lipids and proteins.[6] The lipidome of the HDL includes mainly cholesteryl esters, free cholesterol, triglycerides, and phospholipids. Phospholipids comprise the majority of the lipidome, contributing to 20-30% of the total HDL mass, whereas triglycerides contribute to 3-6%[6]. Proteins, such as CETP and PLTP, determine the content of the HDL by the dynamic process of interchanging triglycerides and phospholipids with other lipoproteins.[94]

Animal studies have suggested that the concentration of HDL-phospholipids (HDL-PL) is a major modulator of cholesterol efflux. In vitro studies have shown that depleting the HDL of phospholipids reduces free cholesterol efflux, whereas enriching lipoproteins with phospholipids improves the efflux capacity.[95] Studies in humans have shown a high positive correlation between HDL-PL and cholesterol efflux capacity.[96] HDL-PL levels may regulate the size and content of HDL in the circulation. On the other hand, in states of hypertriglyceridemia and in the presence of triglyceride-rich proteins, HDL becomes enriched with triglycerides and depleted of cholesterol due to activity of CETP. This indicates that the content of triglycerides in the HDL particle could be an indication of altered HDL metabolism. Moreover, triglyceride-rich HDLs produce smaller particles that are of higher catabolic rates, leading to a lower number of HDL particles.[97] The macrophage is the major cell involved in the process of HDL cholesterol efflux[70] since macrophages are the primary cells that accumulate cholesterol within the atherosclerotic plaque,

Table 3-3: Studies of the relationship between HDL subclasses and CVD

Study	Design	Population	Results	Direction of Findings
Analytical Ultracentrifugation				
Salonen, 1991, Circulation[98]	Prospective (1-5 years of follow-up)	**Kuopio Ischemic Heart Disease Risk Factor Study:** 1799 men, ages 42-60 years	↑ HDL2-C associated with ↓ AMI risk	HDL2-C protective
Williams, 2011, Atherosclerosis[99]	Prospective (up to 29 years)	**Gofman's Livermore Cohort:** 1905 men, baseline mean age 35.2 (8.6) years	- ↑ HDL2 and ↑ HDL3 mass associated with ↓ CHD risk - Associations stronger for HDL2 versus HDL3	HDL2 and HDL3 protective
Analytical Ultracentrifugation and Gradient Gel Electrophoresis				
Johansson,1991, Arterioscler Thromb[100]	Prospective (4-7 years)	60 men, mean age 46.5 (3.2) years with premature MI	- ↑ HDL2b (by GGE) associated with lower disease severity and coronary atherosclerosis progression - ↑ HDL3b correlated with ↑ progression of atherosclerosis in hypertriglyceridemic men - ↑ HDL2-C (by analytical ultracentrifugation) associated with lower disease severity in normo-triglyceridemic men	- HDL2b and HDL2-C protective - HDL3b detrimental
Wilson, 1993, Clin Chim Acta[101]	Case-Control	100 men with previous MI (mean age 54 years) and 100 health controls (mean age 52 years)	- Cases had ↓ proportion of HDL2b, and ↑ proportion of HDL3a, HDL3b, HDL3c - Cases had ↓ HDL2-C	- HDL2b and HDL2-C protective - HDL3a, HDL3b. HDL3c detrimental
2-Dimensional Gel Electrophoresis				
Asztalos ,2003, Arterioscler Thromb Vasc Biol[102]	Prospective (3 years)	**HATS Study:** 123 subjects with clinical CHD, majority men, <70 years	- Decrease in α1 HDL was associated with an increase in coronary stenosis	- α1 HDL protective
Asztalos, 2004, Arterioscler Thromb Vasc Biol[103]	Nested Case-Control (2 years)	**Framingham Offspring Study:** 169 men with incident CHD and 1277 healthy controls (matched in HDL-C levels); mean age 58 (10) years	- Cases had ↓ α1 HDL and pre- α HDL - Cases had ↑ pre-β1 and α3 HDL	- α1 HDL and pre- α HDL protective - Pre-β1 and α3 HDL detrimental

Asztalos, 2005, Arterioscler Thromb Vasc Biol[104]	Nested Case-Control	**VA-HIT Study/ Framingham Offspring Study:** - 398 men with history of CVD who developed incident CVD events, mean age 65 (7) years - 1097 men with history of CVD who did not develop new CVD events, mean age 64 (7) years - 431 men with HDL-C <40 mg/dL, no history of CVD (Framingham Offspring Study), mean age 58 (10) years	- Pre-β1 and α3 HDL particles were positively associated with new-onset CVD events risk - All other particles were negatively associated with this risk. - α1 HDL particles decreased the hazard ratio for a new CVD event.	- α1, α2, α3, α4 HDL protective - Pre-β1 and α3 HDL detrimental
Nuclear Magnetic Resonance Spectroscopy				
Freedman, 1998, Arterioscler Thromb Vasc Biol[105]	Cross-sectional	158 men with angiographic CAD, mean age 63 years	- Large HDL-P inversely related to disease severity - Small HDL-P directly associated with disease severity.	- Large HDL-P protective - Small HDL-P detrimental
Rosenson, 2002, Am J Cardiol[106]	Prospective (3 years)	**PLAC-I Cohort:** 241 middle-aged subjects with CAD, majority men	- Large HDL-P inversely with change in minimum lumen diameter (MLD) - Small HDL-P directly associated with MLD	- Large HDL-P protective - Small HDL-P detrimental
Kuller, 2002, Arterioscler Thromb Vasc Biol[107]	Case-Cohort (5 years)	**Cardiovascular Health Study:** 453 cases (with MI or angina), 750 healthy controls; 57% women, mean age 73 (5.5) years	- In women and men, ↑ large HDL-P associated with ↓ odds of MI and angina	- Large HDL-P protective
Otvos, 2006, Circulation[108]	Nested Case-Control	**VA-HIT:** 364 men with incident CHD and 697 health controls, mean age 64.2 (7.2) years	Baseline and treatment levels of total and small HDL-P levels were inversely associated with CHD incidence	- Total HDL-P and small HDL-P detrimental
van der Steeg, 2008, J Am Coll Cardiol[109]	Nested Case-Control	**EPIC-Norfolk:** 858 cases with incident CHD and 1491 controls; mean age 65 (8) years, 37% women	- HDL size inversely associated with odds of major coronary event - Adjusting for ApoB resulted in reversal of association	- HDL size detrimental
El Harchaoui, 2009, Ann Intern Med[110]	Nested Case-Control	**EPIC-Norfolk:** 822 cases with CHD, 1401 controls; 36% women, mean age 65 (8) years	- Total and large HDL-P higher in controls	- Total and large HDL-P protective

			- HDL size (by NMR and GEE) was smaller in cases, but association attenuated after adjusting for triglycerides and ApoB	
Mora, 2009, Circulation[111]	Prospective (11 years)	**Women's Health Study:** 27673 healthy middle-aged women	- Baseline: Subjects who did not develop CVD had ↑ large HDL-P, ↓ small HDL-P, larger overall HDL size. - Follow-up: ↑ large HDL-P and larger overall HDL size associated with ↓ risk of CVD events.	- Large HDL-P protective - Larger HDL size protective - Small HDL-P detrimental
Mackey,2012, J Am Coll Cardiol[112]	Prospective (6 years)	**MESA:** 5598 subjects, mean age 61.5 (10.3%), 53% women	-↑ Total HDL-P associated with ↓ carotid intima media thickness and CHD incidence - No difference by sex	- Total HDL-P protective
Mora, 2013, Circulation[113]	Prospective (2 years)	**JUPITER:** 10886 subjects, median age 66 (60-71) years, 64% women	- In the placebo group: inverse association between baseline and treatment total HDL-P with CVD risk - In the treatment group, baseline and treatment total HDL-P were positively associated with CVD. - Larger HDL size associated with increased risk of CVD incidence and death - No difference by sex	- Total HDL-P undetermined - Larger HDL size detrimental
Ion Mobility				
Musunuru, 2009, Arterioscler Thromb Vasc Biol[93]	Prospective (12.2 years)	**Malmo Diet and Cancer Study Cardiovascular Cohort:** 4594 subjects, 40% women	- Small and large HDL associated with ↓ risk of CHD	- Small HDL-P and large HDL-P protective

AMI: Acute Myocardial Infarction; CHD: Coronary Heart Disease; MI: Myocardial Infarction; HATS: HDL-Atherosclerosis Treatment Study; VA-HIT: Veterans Affairs High-Density Lipoprotein Cholesterol Intervention Trial Study; EPIC-Norfolk: the European Prospective Investigation into Cancer and Nutrition (EPIC)-Norfolk; CAD: Coronary artery disease; PLAC-I: Pravastatin Limitation of Atherosclerosis in the Coronary arteries; MESA: Multi-Ethnic Study of Atherosclerosis; JUPITER: Justification for the Use of Statins in Primary Prevention: An Intervention Trial Evaluating Rosuvastatin

forming foam cells. There are several pathways by which HDL can remove excess cholesterol from cholesterol-laden foam cells: ABCA1, ABCG1, and SR-B1 or by aqueous diffusion.[82] In general, the ABCA1 pathway is the dominant process of cholesterol efflux from macrophages, but the ABCA1 and ABCG1 pathways can also act together to promote cholesterol efflux from macrophages.[70,114]

The process of HDL-CEC quantification is not yet standardized but involves measuring the amount of cholesterol that moves from a donor cell to either Apo-B depleted plasma or serum.[115] Previous studies have shown that individuals with similar HDL-C levels may differ in their ability to promote cholesterol efflux.[86] The rate of cellular cholesterol efflux capacity depends on the cholesterol content of the cell, expression of cholesterol transporters, and the composition and concentration of extracellular cholesterol acceptors, i.e. the HDL and its subfractions.[116]

HDL-CEC is also stable over time in individuals.[117] In a study of 90 individuals, HDL-CEC measured at baseline was comparable to HDL-CEC measured 10-12 years later. This stability of HDL-CEC enhances the utility of this measure when used to assess the relation to CVD.

Few studies have assessed the link between HDL lipid content and function with CVD risk. In a cross-sectional analysis of 203 healthy subjects [mean age 51 (SD: 8) years, 54% men], Khera et al. reported an inverse association between macrophage HDL-CEC and cIMT independent of HDL-C and ApoA-I; however, no association was observed between HDL-C and cIMT.[118] In the same study, 442 subjects with angiographically-confirmed coronary artery disease (CAD) [mean age 57 (SD: 9) years, 69% men] were compared to 351 healthy controls who were free of angiographic CAD [mean age 62 (SD: 9) years, 48% men]. Cases had lower levels of HDL-C, ApoA-I and HDL-CEC compared to controls. Similar findings were reported in the EPIC-Norfolk cohort[119] and the Dallas Heart Study.[120]

On the other hand, Li et al.[121] recruited participants who either had signs of stable angiographic CAD or who were healthy, and participants from outpatient clinics who either had known CAD or were healthy controls. High HDL-CEC was inversely associated with presence of CAD in the outpatient cohort after adjusting for HDL-C, but not in those with angiographic disease. After participants with angiographic disease were followed for 3 years, those in the highest HDL-CEC tertile were at a higher risk of cardiovascular events.

In a study of 55 cases with high HDL-C and CAD versus 120 controls with high HDL-C but no CAD,[122] concentration of HDL-PL was significantly lower, whereas that of HDL-Tg was higher in cases versus controls. Total cholesterol efflux capacity and efflux capacity to HDL-C ratio were lower in cases compared to controls. There was no difference in HDL particles between the groups. Moreover, HDL-PL appeared to be a significant predictor of efflux capacity. In a nested case-control study within the China Kadoorie Biobank, HDL-Tg concentrations within small, medium and very large HDL (as measured by NMR) were associated with significantly higher odds of myocardial infarctions.[123]

It is important to note that comparing results from different studies on cholesterol efflux capacity is complicated since methods for cholesterol efflux capacity quantification can vary. For instance, the studies by Khera et al.[118] and the EPIC-Norfolk study[119] utilized the J774 mouse macrophages with ^{3}H-labeled cholesterol, whereas the Dallas Heart Study[120] used the J774 cells and BODYPI-labeled cholesterol, and Li et al.[121] utilized the RAW 264.7 mouse macrophage. However, in general, these studies show that cholesterol efflux capacity shows tendency for an athero-protective role, independent of HDL-C levels, which indicates that HDL-C may not reflect the athero-protective capacities of the particle.

3.4 HDL Dysfunction in Midlife Women

As discussed in Section 3.2, studies on the relationship between HDL-C and CVD risk in women have led researchers to postulate that a dysfunction in HDL occurs as women transition through menopause.

During menopause, alterations in hormone levels, specifically a decline in estradiol (E2), occurs. E2 generally increases the expression and activity of nitric oxide synthase (NOS) and could affect hepatic lipid metabolism, resulting in elevated nitric oxide levels. Nitric oxide is a potent vasodilator of the endothelial cells and plays a role in atherosclerosis prevention.[124] E2 could also inhibit the vasoconstrictor endothelin-1 and vascular smooth muscle cells.[9] It also possesses anti-oxidative and anti-inflammatory abilities, and in premenopausal women, it inhibits the production and activity of proinflammatory cytokines, growth factors and vasoconstrictors, and reduces the growth of vascular smooth muscle.[9,125,126]

Reductions in E2 levels after menopause may lead to increased lipid peroxidation and formation of reactive oxygen species (ROS), causing an increased susceptibility of lipoproteins to oxidation. The alterations in E2 after menopause could also lead to a change in the composition of the HDL resulting in a decline in the anti-oxidative and anti-inflammatory abilities of HDL;[8] it has been previously reported that the HDL particles in postmenopausal women have impaired abilities to limit the oxidation of LDL.[127]

Additionally, hepatic lipase (HL), which converts larger HDL particles into smaller particles for uptake by the liver, may be more active in postmenopausal women suggesting that the MT may impact the structure, content and function of HDL particles,[29,128,129] leading to dysfunctions in HDL.

In SWAN HDL, we have previously assessed how novel metrics of HDL vary over the MT. When linking these metrics to the final menstrual period, we found that despite increases in HDL-C over the MT, large HDL-P and HDL size declined and small HDL-P and HDL-Tg increased. Moreover, HDL-CEC per HDL-P declined. These results indicate that the HDL may undergo pro-atherogenic changes that are not reflected by the HDL-C. Moreover, we have shown that higher E2 concentrations are associated with larger HDL size, large HDL-P and HDL-CEC.[130] This supports a plausible link between the decline in E2 that is experienced over the MT and loss of the anti-atherogenic capabilities of the HDL.

Despite the SWAN HDL study being the only study that assessed a comprehensive HDL metric profile in women over the MT, other analyses have looked at individual metrics in relation to menopause. Several cross sectional studies have reported lower levels of large HDL-P,[131,132] smaller overall HDL size[131] and a lower HDL2/HDL3 ratio[131] (by gel electrophoresis) in postmenopausal compared to premenopausal women. Similar findings were reported by analytical centrifugation.[133,134] However, in one study, when HDL levels where standardized by age, the smaller HDL3-C appeared to be lower in postmenopausal women, whereas the larger HDL2-C was similar between the 2 groups.[134] In a small analysis from the SWAN study (19 pre/early-perimenopausal and 34 late peri/postmenopausal women), Woodard et al. reported that postmenopausal women had more total and small HDL-P and a smaller overall HDL size, as measured by NMR.[68]

Longitudinally, in an analysis of 105 women who progressed from perimenopause to postmenopause, HDL2-C was lower and HDL3-C was higher during the postmenopausal stage, despite similar HDL-C levels.[11] Badeau et al.[12] reported that in 29 women with high E2 levels who were presumed to be premenopausal [mean age 37 years], and 31 women with low estradiol who

were presumed to be postmenopausal [mean age 55 years], HDL-C did not differ between groups, but HDL-Tg was higher in women with low E2 versus high E2. Activities of LCAT, CETP, and PLTP, and macrophage cholesterol efflux capacity were similar between the 2 groups. In a prospective study of 3,312 midlife women in the UK who were followed for an average of 2.5 years, small HDL-P (by NMR) increased as women progressed from premenopause to postmenopause, without significant changes in overall HDL size, HDL-Tg, HDL-PL or HDL-C.[135]

In a small analysis of 46 women from SWAN,[136] it was shown that HDL-C, total and large HDL-P (by calibrated ion mobility), and macrophage and ABCA1-specific HDL-CEC were higher after menopause. Overall sizes of small and medium HDL-P were lower after menopause. Larger declines in E2 over the MT were correlated with larger increases in small and decreases in large HDL-P, whereas macrophage and ABCA1 cholesterol efflux capacity were correlated with E2 levels only before menopause. Collectively, these findings show that the MT is accompanied by changes to the HDL that could limit its anti-atherogenic abilities. Moreover, the clinical utility of HDL-C when assessing this dysfunction is limited.

3.5 Goals of this Proposal

In summary, since studies have shown that HDL-C may not be able to fully capture the risk of CVD, and due to the proposed dysfunction in HDL that accompanies the MT in midlife women, it has been suggested that the focus of studies should shift to evaluate other HDL metrics. This may allow for better clarification of the relation between HDL and CVD, and for identification of potential targets of therapy in this population.

However, there is a lack of data in the literature on how these HDL metrics are associated with risk factors that are known to worsen in midlife women. For instance, it has been established that the period around the MT is associated with accumulation of visceral fat, both in the abdomen and in various ectopic locations in the body. This increase in visceral fat has been linked to alterations in the lipoproteins including HDL, but the exact link between visceral fat depots and HDL metrics in women has not been elucidated. Moreover, since HDL is presumed to play a fundamental anti-atherogenic role, the impact of HDL metrics around menopause on future subclinical atherosclerosis, a well-known predictor of future CVD, would help identify how these metrics could be related to CVD. Finally, whether lifestyle factors in midlife women are linked to changes in HDL metrics later in life could aid in understanding whether modifying health behaviors can influence the changes in characteristics of HDL and, potentially, subsequent CVD.

In this proposal we attempt to better comprehend how the various HDL metrics relate to different risk factors of CVD in women around menopause and in later life, and how lifestyle factors could affect these metrics. The overall aim is to improve our understanding of the role of HDL metrics in cardiovascular risk in women.

3.6 Visceral Adipose Tissue and HDL in Midlife Women

The relationship between health and regional distribution of adipose tissue (AT), rather than the amount of total body fat, has recently become a focus of research interest. Typically, with weight gain, excess fat accumulates in the natural body fat storage compartment, which is the subcutaneous AT (SAT) depot.[137] Metabolic complications may arise when the subcutaneous AT depot is unable to store fat as required,[138] due to oversaturation of the AT or the limited ability to

expand.[139] This eventually results in the accumulation of free fatty acids within the abdominal visceral AT and in ectopic locations such as the liver, pancreas, skeletal muscle, and the heart.[140] Excess abdominal visceral AT and SAT depots differ in their composition, activity, and function.[141]

Around 40% of postmenopausal women are considered obese.[142] Studies have shown that obesity increases with surgical menopause,[143] suggesting that the change in the hormonal milieu around menopause prompts weight gain at midlife. Women prior to menopause accumulate more fat in the subcutaneous depots, a feature that may be related to cardio-protection against the metabolic consequences of obesity.[144] However, women at midlife experience redistribution of adipose tissue that is independent of weight gain; after menopause, fat accumulation shifts towards the visceral AT depots.[13,145] This is accompanied by a parallel increase in risk of metabolic disturbances.[144,146] Thus, understanding the relationship between visceral AT and metabolic disturbances during this critical period of life in women is important.

3.6.1 Abdominal Visceral AT and HDL Metrics in Women

Abdominal visceral AT accumulation has been linked to increased risk of CVD, particularly through alterations in glucose-insulin homeostasis, and through a dyslipidemic profile (high triglycerides and ApoB levels, increased small, dense LDL particles, and low HDL-C).[15,147]. In midlife and older women specifically, higher visceral AT area has been correlated with lower HDL-C.[148]

Abdominal visceral AT, but not SAT, is related to aortic plaque presence in obese subjects,[149] coronary artery disease incidence,[150] and higher cIMT in midlife women.[151] In older women (70-79 years old), excess visceral AT has been identified as a predictor of myocardial

infarction.[152] A recent study from SWAN showed that abdominal visceral AT accumulation peaks during late-perimenopause, and the increase in visceral AT closest to the MT, but not before or after menopause, is related to higher future cIMT.[153]

Since HDL-C may not be a sufficient measure of HDL, and since HDL is a family of heterogeneous particles that differ in size, function, and contents, it is important to evaluate how abdominal visceral AT accumulation impacts those metrics in women, and whether it could play a role in the dysfunctionality of HDL.

Only a few studies have investigated the relation between abdominal visceral AT and novel HDL metrics. Two cross sectional analysis in middle aged[149] and older[154] men and women showed that overall HDL size is inversely associated with abdominal visceral AT but not with SAT. These associations did not differ by gender, or between Whites, Blacks and Hispanics.[149]

In a longitudinal study[155] of 24 young women [mean age 29 (2) years at baseline, 71% obese] who were followed over a mean duration of 5.5 years, larger-sized HDL subclasses decreased (by gel electrophoresis), whereas intermediate-sized HDL subclasses increased, over the study duration. HDL-C did not change. Percent increase in total body fat, abdominal visceral AT and trunk fat were correlated with decreases in large HDL subclasses. However, the sample size was small, and the women were young, and thus the results cannot be generalized to midlife and older women. The analysis was done by ANOVA, and the results were not adjusted for potential confounders. In a small cross-sectional analysis of 52 obese premenopausal women [mean age 37.5 (5.5) years], higher abdominal visceral AT was negatively correlated with HDL-C, ApoA-I, and large HDL-2C (as measured by precipitation) but not with HDL3-C.[156] The women in this study were young and premenopausal, and only correlations were tested. In a subsample of 16 women from the previous analysis,[157] plasma levels of hepatic-triglyceride lipase (TGL) were

positively correlated with abdominal visceral AT after adjusting for total adiposity. TGL was inversely correlated with HDL2-C but not HDL3-C. TGL transfers triglycerides from triglyceride-rich lipoproteins (such as VLDL) to the HDL, eventually resulting in smaller HDL particles, suggesting that in women with high abdominal visceral AT, elevated triglyceride lipases could be responsible for the declines in HDL2-C. In 233 midlife and older women [45 to 73 years old, mean age 59 (6) years] who were either perimenopausal or postmenopausal, Nicklas et al.[148] reported that women in the lowest abdominal visceral AT quintile had significantly higher HDL-C and HDL2-C compared to higher quintiles, along with lower fasting glucose and insulin concentrations. These analyses were adjusted for age and race only, and thus, the results could be confounded by other variables that were not assessed in this study. Moreover, the study was cross-sectional, and causality cannot be implied.

Most importantly, none of these studies focused on midlife women, and none investigated whether abdominal visceral AT is related to HDL lipid contents and function, which would allow for a more thorough understanding of the impact of abdominal visceral AT on the HDL.

3.6.2 Cardiovascular AT and HDL metrics in women

As mentioned before, excess visceral AT can accumulate in ectopic locations, such as around the heart and vasculature. The major fat depots in the heart consist of epicardial fat and paracardial fat. Epicardial adipose tissue (EAT) is the fat located closest to the surface of the heart, between the myocardium and the visceral pericardium.[158] It surrounds the coronary arteries in the absence of fascia that separates the AT from the myocardium or the arteries.[159] Paracardial adipose tissue (PAT) is located outside of the visceral pericardium, on the surface of the external pericardium **[Figure 3-2]**.[160] Epicardial fat originates embryonically from the splanchnopleuric

mesoderm, similar to abdominal visceral AT, and is vascularized by the coronary arteries,[161] whereas paracardial fat originates from the primitive thoracic mesenchyme and is vascularized by noncoronary arteries.[159] Epicardial and paracardial fat, along with intramyocardial fat, may cover up to 80% of the heart surface and form 20% of its total weight.[162]

It is crucial to note that in the literature, pericardial fat and paracardial fat are sometimes used interchangeably, whereas in other studies, pericardial fat can be defined as the fat around the heart and coronary arteries,[163] or the fat within the pericardial fat.[164] For consistency within this review, we termed the adipose tissue that has been defined as fat within the pericardium as epicardial adipose tissue (EAT), that outside the pericardium as paracardial fat, and the sum of both as total cardiac adipose tissue.

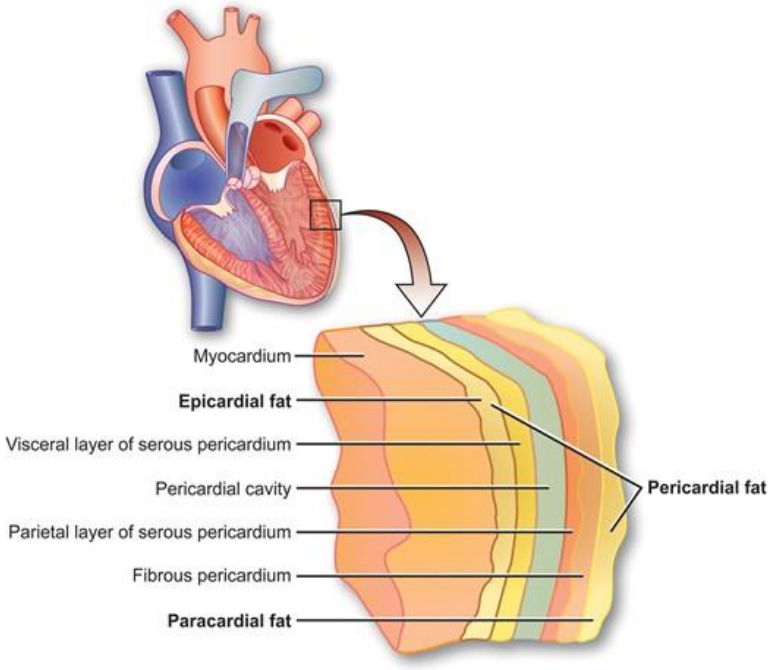


Figure 3-2: Illustration of cardiac fat depots[165]

In close proximity to the heart, within the thoracic cavity, lies the perivascular aortic adipose tissue (PVAT), also known as peri-aortic fat, which is the fat surrounding the aorta. The close proximity of these fat tissues to the heart makes them of extensive importance in understanding CVD. Adipocytokines and reactive oxygen species released from cardiovascular fat depots may lead to the formation of a pro-atherogenic environment by paracrine or vasocrine mechanisms, promoting coronary artery disease development.[15]

PVAT has been linked to higher incidence of CVD.[166] Total cardiac fat volume has been linked to the presence of coronary artery calcification, independent of abdominal visceral AT,[164,167] and to the presence of coronary plaques.[168] This suggests that cardiovascular fat depots may play a more localized role in the promotion of vascular calcification and atherosclerosis. Postmenopausal women have higher volumes of cardiac fat, particularly PAT, compared to premenopausal women,[14] and this has been linked to higher risk of CAC in women after menopause.[169]

Due to the close proximity of cardiovascular fat depots to the cardiac tissue, the release of local inflammatory markers such as adipokines and cytokines from these fat tissues may promote the progression of atherosclerosis.[170] Adipocytokines, such as interleukin-6 (IL-6), that are released by these ectopic adipose tissues may induce insulin resistance and alter the lipoprotein profile, including that of HDL.[139]

Several studies investigated the associations between cardiovascular fat depots and HDL-C. In a cross-sectional analysis, Grief et al.[168] reported that total cardiac AT volume (PAT+EAT) was negatively correlated with HDL-C and adiponectin, and positively with TNF-α and CRP. In another cross-sectional analysis in an Amish population,[171] PAT and total cardiac adipose tissue AT (EAT + PAT) were associated with lower HDL-C. When the analysis was stratified by gender,

the association between total cardiac AT and lower HDL-C was only significant in men. On the other hand, in an analysis from the Framingham Offspring Study, Rosito et al.[164] [n=1,155, 55% women, mean age 63 (9) years] reported that after adjusting for BMI or waist circumference, higher TAT and EAT were associated with lower HDL-C levels in women, but not in men. All associations were not independent of abdominal visceral AT in both genders.

In an analysis of 650 postmenopausal women from the KEEPS study [mean age 52.9 (2.6) years], Huang et al.[172] reported that after adjusting for age, race, education, smoking, alcohol, physical activity, site and BMI, total cardiac AT, EAT (fat inside the pericardial sac) and pericardial AT (PAT; fat outside the pericardial sac) were negatively associated with HDL-C; the association was stronger for EAT compared to PAT. When waist circumference was added to the models instead of BMI, total cardiac AT became positively associated with HDL-C. In 573 healthy, postmenopausal women [mean age 66.8 (5.5) years], those in highest quartile of EAT thickness (around the coronary arteries) had lower HDL-C compared to the lowest quartile. However, adjusting for age, weight, hip circumference, systolic blood pressure, triglycerides, smoking, and glucose levels attenuated these associations.[173]

In the Framingham Offspring Study[174] [n=1067, 56% women, mean age 59 (9) years], PVAT was correlated negatively with HDL-C after adjusting for age, and after stratifying by sex. In women, this persisted independent of smoking, alcohol use, menopause status, hormone therapy, BMI, and waist circumference, but this was attenuated when abdominal visceral AT volumes were added to the models. In men, the associations between HDL-C and PVAT persisted and was independent of abdominal visceral AT volumes.

One single study from MESA [n=5407, 53% women, mean age 62.5 years] investigated the relationship between cardiac fat depots and novel HDL subclasses as measured by NMR.[170]

Total cardiac fat volume was associated with lower HDL-C and with lower total, large and medium HDL-P, but with higher small HDL-P after adjusting for age, sex, and race. After additional adjustment for education, smoking, alcohol, BMI, metabolic risk factors, inflammation, and VLDL-P, the association between small HDL-P and cardiac fat volume persisted. Interaction by race showed that this association was only significant in Whites.

3.6.3 Pathophysiological Link between Visceral Adipose Tissue and HDL metabolism

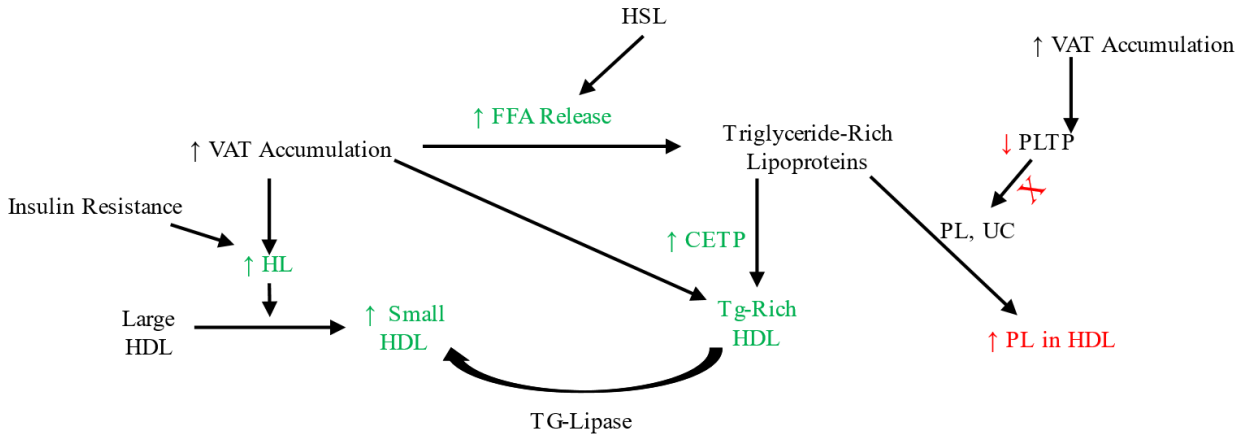
Abdominal and ectopic visceral AT are pro-inflammatory tissues that secrete cytokines and adipokines, and that have endocrine, paracrine and autocrine functions.[175,176] Visceral obesity is associated with a hyperlipolytic state, which results in excess lipolysis of portally-drained adipocytes.[177] Lipolysis is the biochemical pathway that primarily occurs in adipocytes, and results in the release of large amounts of free fatty acids (FFA). The released adipokines and FFAs are then delivered to the liver leading to higher hepatic glucose production and hyperinsulinemia, and the release of pro-inflammatory cytokines.[178,179] Studies have shown that surgical removal of visceral fat tissue lowers insulin resistance and diabetes.[180]

Moreover, increased lipolysis alters the lipid-lipoprotein profile, resulting in reduced triglyceride synthesis in hypertrophic lipid-storing adipocytes. It also increases fatty acid flux to lean tissues, which induces lipotoxicity and increased triglycerides storage in ectopic sites,[15] resulting in fat deposition in ectopic locations. This indicates that abdominal visceral AT and ectopic fat depots potentially share common physiological links.

Hyperinsulinemia is also associated with low levels of large HDL particles and smaller overall HDL particle size.[181] In a study of midlife women with either normal glucose tolerance (NGT) or impaired glucose tolerance (IGT), it has been found that IGT women had higher

abdominal VAT compared to NGT women. HDL-C and HDL2-C were also lower in IGT women.[182]

Figure 3-3 provides a model for the possible pathophysiological link between visceral AT and HDL.



* HL: Hepatic Lipase; FFA: Free Fatty Acid; HSL: Hormone-Sensitive Lipase; CETP: Cholesterol Ester Transfer Protein; PLTP: Phospholipid transfer protein; VAT: Visceral Adipose Tissue; PL: Phospholipids; UC: Unesterified Cholesterol

Figure 3-3: Model for Potential Physiological Link between Visceral Fat Accumulation and HDL Metabolism

A number of different hormones that are involved in the metabolism and anti-atherogenic functions of HDL may be impacted by visceral fat accumulation. Visceral AT has been related to an increase in hepatic lipase (HL).[183] HL hydrolyzes the large HDL particles to form smaller, dense HDL. HL activity is increased in states of obesity and insulin resistance.[184] Cholesterol ester transfer protein (CETP), which transfers triglycerides from triglycerides-rich lipoproteins to HDL particles in exchange for cholesteryl esters, is increased in states of obesity,[184] and in visceral adipose tissue.[185] This results in more triglyceride-rich HDL particles.[186] These triglyceride-rich HDL particles then become favorable substrates for hepatic triglyceride lipase (TGL), which result in the depletion of the lipid core of the HDL and formation of smaller HDL particles.[139] Furthermore, the activity of hormone-sensitive lipase (HSL), an enzyme that drives the release of free fatty acids (FFAs) from adipose tissue, is enhanced in states of obesity and insulin resistance.[187,188] This leads to increased production of triglyceride-rich lipoproteins such as VLDL, and hence, leading to increased transfer of triglycerides to the HDL core.[189] Phospholipid transfer protein (PLTP) is responsible for the majority of transfer of phospholipids between lipoproteins in plasma.[190] PLTP promotes the transfer of unesterified cholesterol and phospholipid from the surface of VLDL and chylomicrons undergoing lipoprotein-lipase induced hydrolysis, to HDL.[191,192] PLTP also plays a role in the conversion of HDL particles and results in the release of large HDL-P's and lipid-poor pre-β- HDL particles, which are acceptors of cholesterol.

In midlife women, and specifically after the MT, the accumulation of VAT increases; this can be reduced by exogenous estrogen supplementation.[193] Previous studies have shown that the activity of HL is impacted by the hormonal milieu in humans. For instance, the activity of HL during the luteal phase of the menstrual cycle, when estrogen levels are the highest, declines significantly,[194] whereas increases in testosterone levels may increase HL activity.[195] This suggests that the decline of estrogen after menopause, and the hyperandrogenism that could accompany visceral AT accumulation may enhance HL activity in women. Previous studies have also shown a positive correlation between CETP activity and visceral adipose tissue in postmenopausal women.[196]

However, not all visceral AT depots are the same. For instance, human and animal studies have shown that epicardial AT consists of smaller adipocytes, has high rates of free-fatty acid release, has a high protein content and low oxidative capacity and ability for utilization of glucose when compared to other AT depots.[159] It also has a unique transcriptome enriched in genes associated with inflammation and endothelial function.[197] PVAT is known to secrete adiponectin, which plays a role in vasodilation; in obesity states, however, adiponectin release is reduced, and tumor necrosis factor (TNF)-α is increased.[198] This indicates that different visceral AT tissues could impact HDL metabolism differently through various enzymes, insulin resistance, and inflammatory pathways.

3.6.4 Racial differences in the associations between visceral AT and HDL

Black women have higher rates of CVD-related morbidity and mortality compared to White women.[1] Paradoxically, at a similar weight, White women appear to have higher

accumulation of abdominal VAT compared to Black women.[199-201] Similarly, previous studies have shown that Black women tend to have less cardiac fat accumulation (EAT, PAT and PVAT) compared to White women, independent of total body adiposity.[202] This discrepancy between the increased risk of CVD in Blacks despite lower visceral fat is termed the racial-obesity paradox.

Blacks have lower prevalence of dyslipidemia compared to Whites, including higher HDL-C concentrations.[203] One previous study has shown that Blacks have higher HDL-C levels compared to Whites, despite similar levels of SAT and total body fat between both races;[204] however, Whites had significantly higher VAT levels compared to Blacks.

In women from the Dallas Heart Study, the median concentration of HDL-C was similar between White and Black participants; however, total HDL-P levels were slightly higher in Whites versus Blacks, whereas overall HDL size was lower in Whites.[205] Moreover, it has been reported that while the associations between HDL-C and CHD differs between Blacks and non-Blacks, the association between HDL-P and CHD was consistent across ethnicities,[205] indicating that HDL-P may be superior when assessing associations of HDL with CVD risk factors. In postmenopausal women, Whites had lower levels of HDL-C and large HDL-P compared to non-Whites [predominantly (84%) Blacks], with higher medium HDL-P and smaller HDL size.[206] Another analysis from the Dallas Heart Study showed that HDL-C was higher in Blacks compared to Whites, whereas the total HDL-P was similar in both races. However, HDL-CEC was lower in Blacks compared to non-blacks.[120] These results indicate that despite the higher HDL-C that we observe in Blacks compared to Whites, the cholesterol efflux capacity could be diminished; this may not be captured by the HDL-C alone. As such, it is important to assess whether the visceral AT depots contributes differently to lipoprotein metabolisms, and particularly HDL, among women of different racial backgrounds.

In summary, as women progress through menopause, they experience increased accumulation of visceral adiposity that is independent of weight gain. This coincides with the hormonal alterations of menopause, as well as with changes in HDL metabolism as has been previously reported. Whether visceral fat accumulation promotes the changes in HDL metabolism in midlife women has not been investigated. Understanding these associations can potentially help explain the underlying basis of altered HDL metabolism in midlife.

3.7 Subclinical Atherosclerosis and HDL in Midlife Women

Subclinical atherosclerosis is an antecedent and predictor of clinical CVD.[207-209] Carotid intima media thickness (cIMT), carotid plaque and inter-adventitial diameter (IAD) are well-established markers of subclinical atherosclerosis.[8]

cIMT is defined as the "space" between the intimal-luminal and medial-adventitial interfaces of the carotid artery.[210] Increased cIMT has been associated with increased risk of CVD incidence,[211] including coronary events such as nonfatal myocardial infarction and coronary death in participants with diagnosed coronary artery disease,[212] and in asymptomatic individuals.[213] In a meta-analysis of 119 randomized control trials (n=100,667 subjects, 42% women, mean age 62 years), it was reported that a 10μm/year reduction in cIMT was associated with a relative risk of 0.91 (95% CI: 0.87-0.94) for developing CVD (defined as MI, stroke, revascularization, or CVD-related death).[214] Presence of carotid plaque and other characteristics of plaque such as echogenicity, morphology and stenosis can also be predictive of future risk of stroke.[215] IAD, which is a measure of the adventitia-to-adventitia interfaces of the carotid artery, may reflect both atherosclerosis and arterial remodeling. IAD has also been linked to higher CVD risk.[216] cIMT,

carotid plaque and IAD are assessed non-invasively by ultrasonography, and have been shown to be reproducible measures of subclinical atherosclerosis.[210,217]

As women transition through menopause, subclinical atherosclerosis worsens. Multiple cross-sectional analyses from different cohorts have shown that cIMT[218-220] and odds of carotid plaque presence[218,221] are higher in postmenopausal compared to premenopausal women.[218] In SWAN, it was reported that after adjusting for age, common carotid artery IMT did not differ by menopause status, but IAD was higher in late-perimenopause and postmenopause women compared to premenopausal women. IAD was also higher with lower E2 levels.[222] The worsening of subclinical atherosclerosis over menopause has been confirmed by prospective studies which showed accelerated progression of cIMT with faster menopause transition,[223] larger increases in cIMT measures in women who achieved menopause compared to women who stayed premenopausal,[224] and an increase in cIMT and IAD during the late-perimenopausal period compared to the early peri/premenopause period.[225]

3.7.1 Subclinical Atherosclerosis in Relation to HDL-C

Atherosclerosis in the arteries occurs subsequent to an imbalance between deposition and removal of cholesterol following endothelial injury.[226] The cholesterol-laden macrophage is the primary cell involved in atherosclerotic lesions, and hence, macrophage-specific cholesterol efflux capacity is hypothesized to be the most involved in the atherosclerotic process.[227]

Several studies have aimed to investigate whether HDL-C, with its presumed anti-atherogenic properties, impacts measures of subclinical atherosclerosis. In healthy adults from the general population,[63] higher HDL-C was linked to less cIMT progression. When evaluated by sex,

lowest HDL-C quintiles were associated with faster cIMT progression in men; however, this was reversed in women, where higher HDL-C was associated with higher cIMT progression.

Studies focusing on midlife women have shown that the presumed cardio-protective effects of HDL-C on subclinical atherosclerosis may not extend to the time during the MT. Matthews et al.[228] reported that in previously healthy women [n=863, mean age at subclinical atherosclerosis assessment 59.6 (2.7) years] who had measures of subclinical atherosclerosis after menopause, smaller increases in HDL-C around menopause were independently associated with larger IAD, but not with cIMT or carotid plaque presence.

However, in one SWAN analysis,[68] HDL-C was associated with lower cIMT and carotid plaque presence in pre/early perimenopausal women, but not in the later peri/postmenopausal women. Moreover, SWAN has shown that larger increase in HDL-C and ApoA-I over the MT are associated with a larger increase in cIMT.[8] In particular, larger increases in HDL-C before the final menstrual period were associated with lower cIMT, but larger increases in HDL-C after the final menstrual period were associated with higher cIMT.[8] There were no observed associations between HDL-C and either carotid plaque or IAD in this study.

An analysis from Multi-Ethnic Study of Atherosclerosis (MESA) showed that in women [n=1380, mean age 61.8 (10.3) years], higher HDL-C was associated with higher carotid plaque, and this was particularly significant for women who were >10 years postmenopausal.[69] More recently, SWAN reported that higher HDL-C is associated lower cIMT in Chinese and Hispanic women, but not in Black and White women.[229]

These findings led to the speculation that higher HDL-C may not appropriately reflect the anti-atherogenic properties of HDL, or that the HDL may lose those protective abilities in midlife women.

3.7.2 Measures of Subclinical Atherosclerosis and Novel Metrics of HDL

To better understand the effect of the HDL molecule on subclinical atherosclerosis, several studies have looked at novel HDL metrics in relation to cIMT, carotid plaque and IAD. In MESA, higher total HDL-P was associated with lower cIMT independent of HDL-C; however the association between HDL-C and cIMT was not independent of total HDL-P and large density lipoprotein particles.[112] Higher CEC mass was associated with higher odds of carotid plaque progression over 10 years.[230] Khera et al.[118] reported no association between HDL-C and cIMT, but a significant inverse association between macrophage CEC and cIMT in a 203 healthy subjects, independent of HDL-C and ApoA-I. In another MESA study, Mora et al.[231] reported that in adults free of CVD [n=5,538, 53% women, mean age 61.4 (10.3) years], HDL-C, total and large HDL-P, and HDL size were all inversely associated with cIMT. In models that additionally adjusted for LDL and VLDL particles, the associations persisted for large, medium, and small HDL-P. It is important to note that in these models, there were potentially high correlations between certain particles, making these models less reliable. Longitudinally,[232] MESA reported that annual changes in mean cIMT or carotid plaque score were not associated with any HDL particles (by ion mobility). In an older Japanese cohort of 657 participants[233] [66% women, mean age 73 years], HDL3-C, as measured by ultracentrifugation, was negatively associated with maximal cIMT, but this was attenuated with multivariable adjustments. In a cohort of older adults from the Chicago Health Aging Study[234] [n=402, mean age 73 (5) years, 35% women], HDL-CEC, total HDL-P (by NMR) and HDL size were not associated with prevalence of lipid-rich necrotic core plaque in the carotid artery after adjusting for confounders.

In a cross-sectional analysis of 502 patients with features of the metabolic syndrome [mean age 61 (52-67) years, 51% women, 33% with carotid plaque presence], total HDL-P, but not HDL-

C or HDL-Tg, was inversely correlated with cIMT. HDL-Tg was higher in subjects with present carotid plaque. However, these results were not adjusted for potential confounders.[94]

In a study of women from the MESA study[69] [n=1,380 subjects, mean age 61.8 (10.3) years], HDL-C, total HDL-P, HDL2b and HDL3+2a, as assessed by ion mobility, were all independently and negatively associated with cIMT. Moreover, the association between total HDL-P and cIMT was independent of HDL-C, but not vice versa. In models that included HDL2b and HDL3+2a with HDL-C, HDL3+2a, but not HDL-2b, was associated with lower cIMT levels. HDL2b was significantly associated with lower cIMT further away from menopause. For carotid plaque, however, HDL-C was marginally associated with greater odds of carotid plaque presence, but this was observed in women with older age at menopause. This suggests that there is an adverse effect on large HDL-P close to menopause, but this may be restored later in life. Moreover, large HDL-P is more vulnerable to oxidative modifications compared to small HDL-P.[235] Interestingly, there has been no previous reports on the associations between HDL metrics and adventitial diameter.

In summary, studies have shown that there appears to be an association between certain HDL metrics and measures of subclinical atherosclerosis independent of HDL-C; however, the findings are not consistent across studies. In addition, only one cross-sectional study has focused on midlife women and included only measures of HDL subclasses. No previous study has evaluated a comprehensive profile of HDL metrics in midlife women, in relation to future subclinical atherosclerosis.

3.7.3 Hormonal Changes of Menopause and Vascular Remodeling

Transition through menopause is associated with an increase in body weight and body fat redistribution, increase in blood pressure, changes in the lipid profile, insulin resistance, and changes in adiponectin levels, which could influence subclinical atherosclerosis.[220] Weight gain and fat redistribution may induce inflammation, and the hyperinsulinemia that occurs with insulin resistance may stimulate the sympathetic nervous system and induce vascular smooth muscle growth, whereas elevated glucose may cause glycation of the proteins in arterial walls and promote atherosclerosis.[224] In insulin resistance states, the potent vasodilatory effects of insulin through endothelium-derived nitric oxide may be compromised.[224]

The changes in the hormonal milieu around menopause may alter the carotid structure of connective tissue and vascular wall.[236] For instance, the vasodilatory effects of estradiol and their effects on smooth muscle cells may be lost. Estrogen may also function by accelerating the re-endothelialization after vascular injury and leading to the inhibition of smooth muscle proliferation and the thickening of the intima media.[237] Estradiol is a known antioxidant, and thus the loss of this effect after menopause may lead to increased lipid peroxidation and the formation of reactive oxygen species,[9,125] leading to dysfunction of the HDL.

As previously discussed, in midlife women, states of inflammation, which can be induced by changes in sex hormones, visceral fat accumulation and alterations in lipid profile can impact the functional anti-atherogenic capabilities of HDL. Since it has been reported that the MT may result in changes in HDL subclasses distribution, size, contents, and function, and since it has been shown the associations between changes in HDL-C and future subclinical atherosclerosis measures vary by stage of metrics, it is critical to understand how early HDL metrics relate to future

measures of clinical atherosclerosis. This could guide future preventative and treatment measures to reduce CVD risk in midlife and older women.

3.8 Cardiovascular Health

In 2011, the Goals and Metrics Committee of the Strategic Planning Task Force of the American Heart Association (AHA) introduced a novel cardiovascular health (CVH) metric with a goal "to improve the cardiovascular health of all Americans by 20% while reducing deaths from cardiovascular diseases and stroke by 20%" by the year 2020.[238] This CVH metric, termed the Life's Simple 7 (LS7) score, encompasses a set of four health behaviors [diet quality, physical activity, smoking and BMI] and three health factors [total cholesterol, fasting blood glucose and blood pressure]. To assess the CVH using the LS7 score, each component is termed as either ideal, intermediate, or poor, and a score is calculated by adding all individual scores **[Table 3-4]**. A higher score indicates better cardiovascular health. To achieve an "ideal" cardiovascular health, all seven components must be classified as ideal.[239]

Since the development of the AHA's LS7, numerous studies have investigated its relationship to CVD[240-244] and have consistently shown that higher scores are inversely associated with prevalence of CVD events, CAC presence,[245,246] cIMT[247] and carotid plaque presence.[247] According to data from the National Health and Nutrition Examination Survey (NHANES), between 1988 and 2012, <2% of US adults met an ideal CV health for all seven categories of the LS7.[240] Prevalence of smoking decreased, whereas prevalence of consuming a healthy diet, having

a healthy BMI, and fasting blood glucose (FBG) declined[240] over time. No change occurred in prevalence in the other CVH metrics.

Not surprisingly, CVH metrics were also significantly associated with cost of healthcare in the US.[248] In middle-aged adults, it was reported that the annual health care expenditures in participants with optimal CVH (6-7 ideal categories) was significantly lower compared to those with poor CVH (0-2 ideal categories).

Table 3-4: Definitions of Poor, Intermediate and Ideal Cardiovascualr Health for each Life's Simple 7 Goals in Adults[238]

	Level of Cardiovascular Health for Each Metric		
	Poor	**Intermediate**	**Ideal**
Current Smoking	Yes	Former ≥ 12 months	Never or quit > 12 months
Body Mass Index	≥30 kg/m^2	25-29.9 kg/m^2	<25 kg/m^2
Physical Activity	None	1-149 min/week of moderate or 1-74 min/week of vigorous or 1-149 min/week moderate +2x vigorous	≥ 150 min/week of moderate or ≥75 min/week of vigorous or ≥150 min/week of moderate + 2x vigorous
Diet Pattern (No. of components of AHA diet score)[1]	<2 (0-39)	2-3 (40-79)	4-5 (80-100)
Total Cholesterol	≥240 mg/dL	200-239 mg/dL or treated to goal	<200 mg/dL
Blood Pressure	SBP ≥ 140 mmHg or DBP ≥ 90 mmHg	SBP 120-139 mmHg or DBP 80-89 mmHg or treated to goal	SBP <120 and DBP <80 mmHg
Fasting plasma glucose	≥126 mg/dL	100-125 mg/dL or treated to goal	<100 mg/dL

SBP: Systolic Blood Pressure; DBP: Diastolic Blood Pressure.

[1] Consistent with a Dietary Approaches to Stop Hypertension (DASH)–type eating pattern: ≥4.5 cups/d of fruits and vegetables, ≥2 servings/week of fish, and ≥3 servings/d of whole grains, ≤36 oz/week of sugar-sweetened beverages and 1500 mg/d of sodium. The consistency of one's diet with these dietary targets can be described using a continuous AHA diet score, scaled from 0 to 100.

3.8.1 Associations between Cardiovascular Health Metrics and HDL metrics

Despite the fact that no previous study has investigated the direct relation between the CVH and novel HDL metrics, per se, several studies investigated the association between the individual components of the LS7, particularly the health behaviors, and various HDL metrics.

3.8.1.1 Body Mass Index

The body mass index (BMI) is the most frequently used measure for assessment of obesity in clinical practice.[139] Even though BMI is not a behavior, per se, it has been identified as a health behavior in the original definition of the LS7[238] and previous analyses.[249,250] The relation between higher BMI and risk of CVD morbidity and mortality has been well established, and numerous studies have assessed the relation between BMI reductions and the amelioration of CVD risk.

In general, higher BMI is linked to lower HDL-C,[251] but some studies showed the inverse.[252] In the Insulin Resistance Atherosclerosis Study (IRAS) study,[253] HDL-C, large and total HDL-P and overall HDL size were inversely, whereas small HDL-P was positively, correlated with BMI adjusting for age, sex, and ethnicity. In a study of overweight/obese versus normal weight women, HDL-C was higher in the former group.[252] Higher BMI was associated with higher small and medium HDL-P, smaller overall HDL size, and less large HDL-P (by NMR). Other studies have similarly shown that healthy obese women have shown a shift in HDL subclasses to smaller size in obese versus lean women.[254,255]

The studies on the relation between BMI and cholesterol efflux capacity have been inconsistent. In a group of obese subjects with a known history of CHD, BMI was not correlated with ABCA1-specific HDL-CEC.[77] In a small study of 12 overweight/obese women and 9 normal

weight controls [23-51 years old], overweight/obese women exhibited lower total and free HDL-C, cholesteryl ester and HDL-PL, and higher HDL-Tg.[256] ABCA1-dependent HDL-CEC was positively correlated with BMI.[256]

The link between obesity and the remodeling of the HDL metrics could be through alterations to the CETP, hepatic lipase, and lipoprotein lipase enzymes.[139,255] In summary, there is evidence that obesity can impact HDL metrics in the general population; however, how this could impact the HDL metrics in midlife women specifically have not been assessed.

3.8.1.2 Smoking

HDL-C is, in general, lower in smokers compared to non-smokers; however, the studies that assessed the relationship between smoking and novel metrics of HDL are limited. Zaid et al.[252] evaluated the association between smoking status and HDL subclasses by NMR in the INTERLIPID study, and found that in Japanese women [n=403, mean age 49.0 (SD: 5.3) years], current smoking was inversely associated with total HDL-P, whereas the number of cigarettes smoked per day was inversely associated with large HDL-P. In a study of current smokers with low HDL-C [n=1,504 smokers, 58% women, mean age 45 (SD: 11.1) years], participants were randomized to a smoking cessation program, and 923 (61%) participants were tested after one year (58% women). In those who stopped smoking, HDL-C, total HDL-P, and large HDL-P increased significantly compared to those who continued smoking, and this was stronger in women. Other studies showed that smoking is associated with lower large HDL subclasses by gradient gel electrophoresis[257] or ultracentrifugation.[258]

Smoking has been shown to increase the activity of CETP,[259] and limit the activity and concentration of LCAT,[260] which could impair the maturation of the HDL particle, and lead to the

rapid clearance of nascent HDL from the circulation.[261] Smoking could also increase the activity of HL; Kong et al. reported that in type 2 diabetics, smoking was associated with increased HL activity and a reduction in HDL2 levels.[262] However, Zaratin et al.[263] found lower HL, PLTP, and CETP activity in smokers with an increase in phospholipid contents of the small HDL3. Smoking also results in the formations of reactive oxygen species, which can cause the oxidation of HDL affecting the efflux ability of HDL, oxidation of LDL, and vascular inflammation.[261,264,265]

3.8.1.3 Physical Activity

Physical activity, whether aerobic or resistance, has been linked to lower CVD risk in the general population. Increases in physical activity raise HDL-C levels, and reduce BMI, blood pressure, total cholesterol, and LDL-C.[266] The Women On the Move through Activity and Nutrition (WOMAN) study[267] evaluated the association between leisure physical activity, which was calculated from the Modifiable Activity Questionnaire, in relation to HDL subclasses as assessed by NMR. In 482 overweight, postmenopausal women [mean age 56.9 (SD: 2.9) years], this cross-sectional analysis reported that physical activity was associated with higher HDL-C and total HDL-P, after adjusting for age, BMI, hormone therapy (HT) use and fat intake; leisure physical activity was associated with larger HDL size in non-HT users, but not in HT users. The authors suggested that hormone therapy could mask the beneficial impact of physical activity on the lipoprotein profile in users. In 35 obese women who were randomized to either a control groups 12-weeks of aerobic and resistance training, HDL-C and percent of large or intermediate HDL-P (to total particles) as measured by gel electrophoresis did not differ with the intervention, whereas percent of small HDL-P decreased significantly in the exercise group,[266] independent of BMI changes. No changes were observed for HDL-CEC or HDL anti-inflammatory functions as

measured by changes in vascular cell adhesion molecule (VCAM). At baseline, higher BMI was correlated with lower HDL-CEC and large HDL-P, but changes in BMI were not related to changes in HDL metrics.[266]

Several mechanisms have been proposed to contribute to the changes in HDL metrics. Those include changes to CETP, HL and LPL. Exercise have been shown to reduce CETP concentrations and increase nitric oxide (NO) bioavailability that decreases the oxidative modifications of HDL, and to the upregulation of ABCA1 and ABCG1 pathways.[268,269] Studies have suggested that exercise may also increase LPL activity, which could by induced by the improvements of exercise in insulin resistances.[268] Other mechanisms may include the effects on PON activities, immune and endothelial functions.

Table 3-5 provides a summary of the potential mechanisms that could contribute to the effect of smoking, BMI, and physical activity on the HDL metabolism pathways.

Table 3-5: Summary of Potential Impact of smoking, BMI and physical activity on HDL metabolism

	Smoking	BMI	Physical Activity
CETP	↑	↑	↓
Hepatic Lipase	-	↑	-
PLTP	↓	-	-
LCAT	↓	-	-
Hepatic Triglyceride Lipase	-	↑	-
Lipoprotein Lipase	-	↑	↑
Oxidation	↑	-	↓
Impact on HDL Metrics	Lower total HDL-P Lower large HDL-P Lower HDL-CEC	Smaller HDL size Higher small HDL-P Higher HDL-Tg Less large HDL-P Less HDL-PL	Larger HDL size Higher total HDL-P Lower small HDL-P

CETP: Cholesteryl ester transfer protein; PLTP: Phospholipid Transfer Protein; LCAT: lecithin- cholesterol acetyl transferase; HDL-P: HDL particles; HDL-Tg: HDL triglycerides; HDL-PL: HDL phospholipids

3.8.1.4 Diet Pattern

Investigating the associations between HDL metrics and dietary patterns is complicated due to the complex nature of dietary habits since food and nutrients are not consumed as single components or in isolation. Diet, along with weight loss, is often associated with reductions in HLD-C.[270] In the Women's Health Initiative study, dietary interventions to reduce fat intake by 20% in postmenopausal women resulted in reductions in HDL-C, HDL2-C, HDL3-C, with no change in the HDL size by NMR.[271] Bogl et al.[272] investigated the association between habitual dietary pattern and lipoprotein subclasses in young adults. Adjusting for age, sex, sucrose and dietary fiber intake, a diet rich in meat was linked to higher HDL-C and small and medium HLD-P by NMR, whereas increased protein intake was correlated with larger overall HDL size. In a study of 6-months dietary modification by caloric restriction and weight loss in 90 overweight/obese women [mean age 46 years, range 22-67], ABCA1, ABCG1- and SR-BI-mediated HDL-CEC did not significantly change with diet changes and weight loss. In 115 non-diabetic, obese women [n=35-55 years old],[273] after 2 years of intensive lifestyle interventions with a Mediterranean diet and weight loss, overall HDL size did not significantly change.

High-carbohydrate diets can lead to unfavorable changes in HDL, particularly decreases in large HDL particles. Some studies have reported a reduction in small HDL particles with carbohydrate-rich diets, but those are likely induced by the weight loss achieved with the diet changes.[274] High-carbohydrate, low-fat diets lead to increases in plasma triglycerides, which lead

to reductions in LPL levels. Polyunsaturated fats increase LPL activity, and omega-3 fatty acids have been shown to shift the distribution of HDL subclasses into higher HDL2 and less HDL3.

These studies suggest that health behaviors could have an impact on the HDL metric profile. Assessing whether the Life's Simple 7 score, and its health behavior components, affects HDL metrics would provide an integrated perception on whether lifestyle factors could improve HDL quality. This is of particular importance since these lifestyle factors are usually interrelated, and even share similar mechanisms in their effect on HDL. For instance, dietary changes and physical activity could impact BMI, which, in turn, could impact insulin resistance, cholesterol and blood pressure. Moreover, the Life's Simple 7 provides an easy way to assess these lifestyle factors, and gives an overall view on whether behavioral risk factors are "ideal" or "poor"; this could be easily translated into interventions that can be used in clinical settings and epidemiological studies. In women, specifically, this would allow the understanding of whether interventions on lifestyle factors early in midlife could impact the dysfunctionality of HDL that is presumed to occur around the MT.

4.0 Manuscript 1: Associations of abdominal and cardiovascular adipose tissue depots with HDL metrics in midlife women: The SWAN Study

4.1 Abstract

Context: The menopause transition is accompanied by declines in the atheroprotective features of high-density lipoprotein(HDL), which are linked to deleterious cardiovascular (CV) outcomes.

Objective: To assess the relationship between abdominal and cardiovascular (CV) visceral adipose tissues(AT) with future HDL metrics in midlife women, and the role of insulin resistance on these associations.

Design: Temporal associations of abdominal and cardiovascular fat with later measures of HDL metrics.

Setting: Community-based cohort

Participants: 299 women, baseline mean age 51.1 (SD: 2.8) years, 67% White, 33% Black, from the Study of Women's Health Across the Nation (SWAN) HDL ancillary study.

Exposures: Volumes of abdominal visceral AT, epicardial AT(EAT), paracardial AT(PAT), or perivascular AT(PVAT).

Main Outcomes: HDL cholesterol efflux capacity(HDL-CEC), HDL phospholipid(HDL-PL), triglycerides(HDL-Tg), and cholesterol(HDL-C), apolipoprotein A-I(ApoA-I), HDL particles(HDL-P) and size.

Results: In multivariable models, higher abdominal visceral AT was associated with lower HDL-CEC, HDL-PL, HDL-C, and large HDL-P and smaller HDL size. Higher PAT was associated with lower HDL-PL, HDL-C and large HDL-P and smaller HDL size. Higher EAT was associated with higher small HDL-P. Higher PVAT volume was associated with lower HDL-CEC. HOMA-IR partially mediated the associations between abdominal AT depots with HDL-CEC, HDL-C, large HDL-P and HDL size; between PVAT with HDL-CEC, and PAT with HDL-C, large HDL-P and HDL size.

Conclusions: In midlife women, higher visceral AT volumes predict HDL metrics 2 years later in life, possibly linking them to future CVD. Managing insulin resistance may preclude the unfavorable impact of visceral fat on HDL metrics.

4.2 Introduction

The 2020 American Heart Association (AHA) Scientific Statement on "Menopause Transition and Cardiovascular Disease Risk: Implications for Timing of Early Prevention" highlighted the detrimental role of the menopause transition (MT) on CVD risk in women.[275] This statement emphasized the importance of understanding the deleterious changes in CVD risk factors that accompany this vulnerable period in women's lives.[275]

The MT has been linked to unfavorable changes in cardiometabolic risk factors related to lipids, metabolic syndrome, vascular health and adipose tissue (AT) distribution.[275] Alterations in the lipoprotein profile, as evidenced by increases in total cholesterol and low-density lipoprotein cholesterol (LDL-C), occur as women progress through menopause.[66] However, the impact of the

MT on high-density lipoproteins (HDL) is more complex and the findings from earlier studies are conflicting.[29]

The major anti-atherogenic function of HDL is mediated by the efflux of cholesterol from macrophages through a process called reverse cholesterol transport.[2,30] The well-known athero-protective abilities of HDL may be compromised during the MT, and levels of HDL-cholesterol (HDL-C) during and after the MT may not fully reflect those changes.[10] Recent studies suggest that more novel metrics, which evaluate the function, lipid contents and subclasses of the HDL, may provide a better understanding of the alterations in the cardio-protective functions of the HDL that occur as women progress through menopause. Findings from the Study of Women's Health Across the Nation (SWAN) HDL ancillary study and Multi-Ethnic Study of Atherosclerosis (MESA) suggest that despite increases in HDL-C over the MT, adverse changes in HDL function, content and subclass distribution may occur, indicating a potential dysfunctionality in HDL during midlife.[10,69] For instance, the SWAN HDL ancillary study reported that the triglyceride content of HDL increases over the MT, as HDL function declines per particle and that the subclass distribution shifts towards smaller particles.[10] These features have been linked to higher CVD risk profile in multiple,[111,118,122,123] yet not all [109,113] studies.

The MT is also accompanied by redistribution of AT depots,[145] as characterized by increased accumulation of visceral AT, independent of weight gain. The accumulation of abdominal visceral AT (VAT) in women peaks during the perimenopausal stage;[153] this has been linked to elevated future risk of subclinical atherosclerosis.[153] Beyond the abdomen, visceral AT accumulation extends to ectopic locations in the body such as the heart. Similar to abdominal VAT, postmenopausal women have higher volumes of cardiovascular AT, particularly paracardial fat

(PAT),[14] and this is associated with higher risk of coronary artery calcification in postmenopausal women.[169]

Visceral AT plays a role in the regulation of lipid metabolism including that of the HDL. In midlife and older women, higher abdominal visceral AT is linked to lower levels of HDL-C.[148] However, no studies have assessed how visceral AT associates with the function, composition and size of the HDL in women during midlife. Understanding the link between visceral AT and HDL metrics could help delineate the metabolic consequences of visceral AT accumulation and understand how visceral AT could affect future CVD risk in women, specifically during the vulnerable midlife period. Since visceral AT accumulation is often accompanied by a state of insulin resistance (IR) which can, in turn, contribute to HDL metabolism through several lipoprotein-related enzymes,[276,277] it is important to understand the role of IR on the associations between visceral AT depots and HDL metrics.

The SWAN Heart, Cardiovascular fat and HDL ancillary studies provide a unique opportunity to assess the temporal associations between different AT depots and a future comprehensive HDL metric profile in midlife women. In this study, we evaluate the relationship between visceral AT depots and HDL metrics measured on 2 years later average in White and Black midlife women and investigate the mediation effect of IR on those associations. We hypothesize that higher volumes of abdominal and CV AT will be associated with a worse HDL metric profile [lower HDL function, higher triglyceride, and lower phospholipid contents, and with smaller HDL subclasses and overall size]. Insulin resistance will mediate these associations, such that higher visceral AT will be associated with more insulin resistance and, thus, a worse HDL profile.

4.3 Materials and Methods

The Study of Women's Health Across the Nation (SWAN) is an ongoing, multi-site, multi-ethnic, community-based, longitudinal study that aims to characterize the physiological and psychological changes in women as they traverse menopause. The design of the SWAN study has been previously described.[278] Briefly, 3,302 women aged 42 to 52 years were recruited between 1996 and 1997 at seven different sites across the United States (Pittsburgh, PA, Chicago, IL, Boston, MA, Newark, NJ, Detroit, MI, Los Angeles, CA and Oakland, CA). Eligibility criteria for recruitment into SWAN were: (i) having an intact uterus and at least one ovary, (ii) not being pregnant or lactating at recruitment time, (iii) having had at least one menstrual period within the last 3 months prior to recruitment, (iv) not using hormone therapy, and (v) identifying as either White, Black, Hispanic, Chinese or Japanese.

The SWAN HDL study is an ancillary study to SWAN. This study aims to characterize the biological changes in HDL composition and function that accompany ovarian aging, and to identify how these changes influence the athero-protective capacities of HDL in women. For the SWAN HDL study, frozen serum samples from 558 SWAN women at different SWAN visits (1,461 samples) were used to quantify HDL function, lipid content and subclasses. Women were selected into SWAN HDL if they had participated in at least one visit before and at least 2 visits after the final menstrual period, with available blood samples at the selected visits.[10] The SWAN Heart ancillary study aimed to evaluate subclinical atherosclerosis in SWAN participants at the Chicago and Pittsburgh sites; the SWAN Cardiovascular Fat ancillary study measured cardiovascular fat in SWAN Heart study participants who had electron beam computed tomography scans available at the SWAN Heart baseline visit.[14]

For this study, we performed a prospective analysis where each of the exposures (visceral AT depots) was measured at one visit (coinciding with SWAN follow-up visits 4-7) and each of the outcome (HDL metrics) was measured at a later visit (coinciding with SWAN follow-up visits 5-7, 9 or 12) (Figure 1a). Women who had missing data on all AT measure [abdominal VAT, epicardial AT (EAT), PAT and perivascular aortic AT (PVAT)] were excluded. We included 299 women who had any AT measure (abdominal VAT, EAT, PAT or PVAT) followed by a later HDL metrics measure [HDL-cholesterol efflux capacity (HDL-CEC), HDL lipid contents, HDL subclasses, HDL size, HDL-C and apolipoprotein A-I (ApoA-I)]. For each women, one HDL metric measure was selected after the AT measure. The median duration between the assessment of AT and HDL metrics was 2.0 (Q1:1.8, Q3:2.2) years. Compared to excluded women (n=259), the included women had higher BMI. Age, physical activity score, anti-lipid medication use, and HOMA-IR were similar between groups. All participants provided written informed consent at every study SWAN visit, and study protocols were approved by the institutional review board at each study site.

Measures of Visceral Adipose Tissue Depots:

Abdominal Visceral Adipose Tissue:

The GE-Imatron C150 Electron-Beam Computed Tomography (EBCT) Scanner (GE Medical Systems, South San Francisco, CA, USA) was used to quantify abdominal adipose tissue volume within a single 6-mm thick cross-sectional image between the L4 and L5 vertebral space.[279] Scans were read by a single reader at the University of Pittsburgh. Adipose tissue was distinguished from other tissues of the abdomen by the threshold of -190 to -30 Hounsfield units (HU) by methods of image analysis software (AcuImage Software, South San Francisco, CA, USA). A line was drawn along the fascial plane at the interior of the abdominal muscles, and fat

within this line was considered visceral AT. Intra-observer reliability for abdominal VAT quantification was reported with an intra-class coefficient of 0.94.[279]

Cardiovascular Adipose Tissue:

Epicardial (EAT) and paracardial (PAT) adipose tissues were quantified on images that were previously acquired for the measurement of coronary artery calcification.[14] The images were obtained by the GE-Imatron C150 EBCT Scanner (GE Medical Systems, South San Francisco, CA, USA).[14] For EAT and PAT, 3-mm thick transverse images were read at the Los Angeles Biomedical Research Institute, Harbor-UCLA Medical Center, CA, USA. EAT and PAT areas were determined between 15 mm above and 30 mm below the superior extent of the left main coronary artery, which allowed for the evaluation of fat around the proximal coronary arteries. The chest wall was the landmark for the anterior border and the aorta and bronchus were the landmarks for the posterior border. Adipose tissue was distinguished from other tissues of the heart by the threshold of -190 to -30 HU by methods of volume analysis software (GE Healthcare, Waukesha, WI, USA). EAT and PAT were measured manually by tracing the border of the area of interest every 2-3 CT slices and using the software to automatically trace the slices in between. EAT volume was defined as the fat inside the pericardium and PAT was defined as the fat outside of the pericardium. PAT volume was calculated by subtracting the EAT volume from the total cardiac fat volume. The between- and within-reader Spearman correlation coefficients for EAT and PAT volumes were 0.97.[14]

To quantify perivascular aortic AT (PVAT) volume, 6-mm thick transverse images that were previously obtained for evaluation of aortic calcification were read at the University of Pittsburgh Ultrasound Research Lab (URL) with Slice-O-Matic version 4.3 software (Tomovision, Magog, Quebec, Canada).[14] PVAT was defined as the adipose tissue surrounding the descending

aorta. The pulmonary bifurcation was the landmark to determine the proximal border, the first lumbar vertebrae was the landmark for the distal border, the vertebral foramen was the landmark for the posterior border, and the line crossing through the left bronchus to the interior border of the crus of the diaphragm was the anterior border. PVAT was distinguished from other surrounding tissue by a -190 to -30 HU threshold. The borders were identified manually for every slice. Since the length of the evaluated part of the descending aorta varies across participants, the length of the descending aorta was estimated from table position number at first included CT slice and table position number at last included CT slice, and accounted for in multivariable analysis. The between- and within-reader Spearman correlation coefficients for PVAT volumes were 0.998 and 0.999, respectively.[280]

All readers of the cardiovascular AT depots at the Los Angeles Biomedical Research Institute and the University of Pittsburgh URL were blinded to the participants' characteristics.

Blood Data Collection:

Phlebotomy was performed after a minimum of a 10-hour overnight fast. This was scheduled 2-5 days after a spontaneous menstrual bleed when possible. When menstrual cycles were less predictable, phlebotomy was randomly performed within 90 days of the annual SWAN visit. Stored samples that have been frozen at -80°C and never been thawed were used for SWAN HDL assays to guarantee the validity of results.

Cholesterol efflux capacity and lipid content of HDL:

HDL-CEC, HDL-phospholipids (HDL-PL) and HDL-triglycerides (HDL-Tg) were measured at a CDC-certified lipid laboratory at the University of Pennsylvania. HDL-CEC was measured by the efflux of fluorescence-labeled cholesterol as has been described previously.[122] Briefly, J774 mouse macrophage cells were plated and labeled with 2 μCi/mL of ^{3}H cholesterol

overnight. The cells were then incubated in the presence of 0.3 mM 8-(4-chlorophenylthio)-cyclic AMP (cAMP), an upregulator of ATP-binding cassette transporter-1 (ABCA1), for 4 hours. Lipoproteins containing apolipoprotein B (ApoB) were removed from plasma by polyethylene glycol precipitation resulting in ApoB-depleted serum. The cells were then incubated with the equivalent of 1% ApoB- depleted serum or plasma for 2 hours at a temperature of 37°C. Cells incubated with media alone were used as baseline controls. Each medium was then collected and passed through a 0.22 μM filter to remove cell debris. Isopropanol extraction was then used to extract lipids from cells. ^{3}H cholesterol was quantitated in media and cells by scintillation counting. Percent efflux capacity was calculated by the following formula: [(cpm of ^{3}H cholesterol in the media - cpm of ^{3}H cholesterol in serum free media) / (cpm of ^{3}H cholesterol in the cells + cpm of ^{3}H cholesterol in the media)] × 100. The intra and inter-assay coefficients of variation were 3.7% and 10.1%, respectively.

For HDL-PL and HDL-Tg quantification, HDL was isolated from serum by phophotungstic acid precipitation (FujiFilm Wako Pure Chemical Corporation) and HDL-PL and HDL-Tg were measured by the Roche Cobas C311 clinical analyzer according to manufacturer's protocol (Wako: 433-36201 and Roche: 20767107322, respectively). The inter-assay coefficients of variation were 3.5% and 3.9% for HDL-PL and HDL-Tg, respectively.

HDL Subclasses:

HDL subclasses and size were measured at LabCorp (Morrisville, NC, USA) by the Nuclear Magnetic (NMR) Spectroscopy LipoProfile-3 algorithm,[281] by the Vantera Clinical Analyzer, which is an automated 400 MHz NMR spectroscopy platform. In brief, lipoprotein particle quantification by NMR utilizes composite signal envelopes at 0.8 ppm, which contain the signals emitted by terminal methyl group protons of the phospholipids, unesterified cholesterol,

cholesterol ester and triglycerides that are carried in each HDL lipoprotein particle. Signal amplitudes that contribute to the composite plasma signal are produced as a result of the deconvolution of the composite signal. Each lipoprotein subclass produces unique NMR signals that are specific in frequency and shape. The amplitude of the signal is proportional to the number of particles that are releasing the signal.

To obtain the amplitude of each subpopulation of subclasses, the line shape of the signal envelope was modeled as a sum of all lipoprotein signals. The areas of different subpopulations were multiplied by conversion factors to quantify the concentrations, which were then grouped into small (7.3-8.2 nm), medium (8.2-9.4 nm) or large (9.4-14.0 nm) HDL subclasses. The total HDL particle (HDL-P) concentration was obtained by summing the concentrations of all subclasses. The average size of HDL particles was calculated by adding the diameter of each subclass multiplied by its relative mass percentage from NMR signal amplitude. Due to the magnetic property of lipoproteins which produces signals of different shapes and frequencies for different lipoproteins, NMR spectroscopy does not require the separation of lipoprotein subclasses as is required by electrophoresis or ultracentrifugation.

The intra- and inter-assay coefficients of variation for HDL-P concentrations and size ranged from 0.6% to 3.7% (intra-assay) and 1.5% to 4.0% (inter-assay).

HDL-Cholesterol (HDL-C) and Apolipoprotein A-I (ApoA-I):

Lipid fractions were determined in EDTA-treated plasma. Fasting HDL-C[282,283] was separated with heparin-2M manganese chloride (Medical Research Laboratory, Lexington, KY for SWAN baseline visit until follow-up visit 7; University of Michigan Pathology (UM), Ann Arbor MI at SWAN follow-up visits 9 and 12). HDL-C was calibrated by converting the results at SWAN visits 9 and 12 to equivalent Medical Research Laboratory (MRL) values.

ApoA-I was quantified by the immunonephelometry using Behring reagents on the Behring Nephelometer II at MRL between SWAN baseline visit and follow-up visit 7, and by reagents from Beckman-Coulter (Brea, Ca) at the University of Pittsburgh Heinz lab (UP) at SWAN visits 9 and 12. ApoA-I results from UP were calibrated by converting to equivalent MRL values.

Study Covariates:

Race and economic hardship were self-reported at SWAN baseline visit. Economic hardship was defined as difficulty paying for basics (not hard or somewhat/very hard). Other study covariates were measured at the time of AT depots assessment. Age was calculated as the difference between the visit date at which AT was assessed and the date of birth. Body mass index (BMI in kg/m^2) was calculated as measured weight in kilograms divided by the square of height in meters. Physical activity score was measured by the Modified Kaiser Permanente Health Plan Activity Survey.[284] Menopause status was self-reported at every SWAN visit and categorized based on bleeding patterns over the past 12 months. Menopause status was classified as either pre/early-perimenopausal (no changes in menstrual bleeding patterns over the past 12 months, or at least one bleed within the last 3 months with some perceived changes in menstrual cycle intervals), late perimenopausal/postmenopausal (no bleed within the past 3 months but at least one cycle within the last 12 months, or lack of menstrual bleeding for 12 or more consecutive months, either naturally or surgically due to bilateral salpingo- oophorectomy), or unknown menopause status (due to hysterectomy or hormone therapy use). Triglyceride levels were analyzed in plasma at the MRL by an automated glycerol kinase enzymatic assay on a Hitachi 747-200 clinical analyzer. HOMA-IR was calculated as fasting glucose (mg/dL) x fasting insulin (mIU/mL)/405.

Statistical Analysis:

Descriptive statistics using mean (SD), median (Q1, Q3) or frequencies (%) were used to summarize participants' characteristics at the time of AT assessments. The distribution of continuous variables was assessed, and skewed variables [abdominal VAT, PAT, EAT, PVAT, HDL-Tg, large HDL-particles (HDL-P), medium HDL-P, triglycerides, and HOMA-IR] were log-transformed to reduce skewness. Correlations between different AT depots were tested by Spearman's Correlations.

Lagged analyses were used to assess the relation between different visceral AT volumes in relation to future HDL metrics, where one HDL metric measure was selected after each AT measure. Multivariable linear regression models were used to evaluate the associations between each visceral AT depot at one visit with each HDL metric at a later visit, separately. Variables that were univariately associated with both exposures and outcomes were considered as confounders, and the best parsimonious model was chosen by comparing models using the R-squares, without introducing multi-collinearity. Final models were adjusted for race, time between the visceral AT and HDL metric assessment, economic hardship, and the following measures at time of AT assessment: age, physical activity, menopause status, and log-transformed triglycerides. PVAT models were additionally adjusted for length of the descending aorta. Interaction between race and each HDL metrics was tested in final models to assess whether associations differed between White and Black women. For graphical representation of the significant associations between AT depots and HDL metrics, volumes of AT depots were categorized into tertiles, and means of HDL metrics within each tertile were estimated in the final models. Tertiles were used to ensure sufficient numbers of participants per group. Post hoc Bonferroni adjustment was applied to correct for multiple testing.

Whether insulin resistance mediated the associations between AT depots and HDL, causal mediation analysis was used to test the effect of log-transformed HOMA-IR on these associations. To ensure temporality between exposure, mediator and outcome, this was done in a subset of women (n=251) who had HOMA-IR evaluated at visits between the fat depot and HDL metrics measures, that is, after adipose tissue was evaluated but before HDL metrics were assessed (Figure 1b). The women included in this sub-analysis were similar to the excluded women (n=48) on all characteristics (data not shown). All analyses were conducted using SAS v9.4 (SAS Institute, Cary, NC).

4.4 Results

Characteristics of women included in this analysis:

The characteristics of women included in this analysis at time of AT assessment are presented in Table 1. Women, on average, were 51.1 (SD: 2.8) years old, 67% were White, and 57% were pre/early-peri menopausal. AT depots were measured 2.0 (Q1:1.8, Q3:2.2) years before HDL metrics measures.

Abdominal VAT was strongly correlated with EAT (r's: 0.67-0.74, all p-values <0.0001). All cardiovascular AT volumes were also correlated: EAT was correlated with PAT and PVAT; PAT was correlated with PVAT (r's: 0.62-0.68; all p-values <0.0001).

Univariate correlations between abdominal and cardiovascular visceral AT depots and HDL metrics:

The univariate correlations between AT depots and HDL metrics are presented in Table 2. All AT depots were inversely correlated with HDL-PL and HDL-C, and all except EAT were inversely correlated with ApoA-I. Abdominal VAT and PVAT were negatively correlated with HDL-CEC. Abdominal VAT and EAT were positively correlated with HDL-Tg. All AT depots were inversely correlated with large HDL-P and HDL size, and positively with small HDL-P. There were no correlations between the AT depots with total or medium HDL-P.

Associations between abdominal and cardiovascular visceral AT and HDL metrics:

Figures 2 and 3 present the significant multivariable associations between AT depots tertiles and HDL metrics. The multivariable adjusted associations between the AT depots and HDL function and content metrics are presented in Figure 2. Higher PVAT tertiles were associated with borderline lower HDL-CEC (p-trend=0.06), whereas higher abdominal VAT volumes were associated with lower HDL-PL (p-trend=0.02), and higher abdominal VAT and PAT tertiles were associated with lower HDL-C (p-trends=0.008 and 0.04, respectively).

The multivariable adjusted associations between the AT depots and HDL subclasses and size are presented in Figure 3. Abdominal VAT and PAT tertiles were associated with lower levels of large HDL-P (p-trends= 0.0002 and 0.008, respectively) and smaller overall size (p-trends=0.004 and 0.001, respectively). Higher tertiles of EAT were associated with more levels of small HDL-P (p-trend=0.02). All other associations between the different AT depots and HDL metrics were attenuated after adjusting for potential confounders.

The adjusted associations between the four continuous AT depots and each HDL metric are presented in Supplementary Tables 1 and 2 and are largely consistent with the findings from the analysis by tertiles[285].

After adjusting for multiple comparisons, interactions between HDL metrics and race in final models showed that the associations did not differ between White and Black women (data not shown).

Mediation by HOMA-IR on the association between visceral AT and HDL metrics:

Table 3 and Figure 4 present the significant results of the mediation analysis on the observed associations between AT depots and HDL metrics. For abdominal VAT, IR mediated 62.0% of the associations with HDL-CEC, 50.9% of the associations with HDL-C, 32.1% of the associations with large HDL-P, and 34.5% of associations with HDL size. For PAT, IR mediated 51.6% of the associations with HDL-C, 44.9% of associations with large HDL-P and 37.1% of associations with HDL size. IR mediated 34.7% of the associations between PVAT and HDL-CEC. The natural indirect effect (NIE) represents the effect of visceral AT on each HDL metric that is mediated by IR. The controlled direct effect (CDE) represents how much the HDL metric would change on average, if IR was fixed at sample mean and AT depot volumes are increased by 1-unit. The CDE shows that if IR was held constant, the decrease in each of the HDL metrics (HDL-CEC, HDL-C, large HDL-P and HDL size) with the increase in AT depots would have been less pronounced than observed in this analysis.

4.5 Discussion

In a sample of midlife women, we show that visceral fat accumulation in the abdomen and around the heart and vasculature may be linked to future dysfunction in HDL. Higher volumes of abdominal and cardiovascular AT depots were associated with lower HDL cholesterol efflux

capacity, lower HDL-PL, HDL-C, large HDL-P, and higher small HDL-P concentrations, and with smaller overall HDL size, independent of potential cardiovascular and metabolic confounders. This profile of HDL metrics has been linked to worse cardiovascular outcomes.[111,118,122] Furthermore, insulin resistance appears to mediate and drive some of the deleterious associations between AT depots and HDL metrics, particularly the associations between AT depots with HDL-CEC, large HDL-P and HDL size.

Only a limited number of studies had investigated the relation between abdominal VAT and novel HDL metrics. In a cross-sectional analysis of 1,200 obese participants from the Dallas Heart Study, Neeland et al.[149] reported that higher abdominal VAT mass was associated with smaller overall HDL size and lower HDL-C concentrations. These associations were similar for men and women. Consistent with our results, the associations did not differ by race. In another cross-sectional analysis of 382 subjects, Sam et al.[154] similarly reported an inverse association between abdominal VAT and overall HDL size. In women specifically, Woudberg et al.[155] followed 24 young women [mean age 29 (2) years] for an average of 5.5 years, and showed that an increase in abdominal VAT over time was associated with a decline in large HDL subclasses as measured by gel electrophoresis; no changes in HDL-C were observed. Nicklas et al.[148] reported that perimenopausal and postmenopausal women [mean age 59 (SD: 6) years old] in the lowest abdominal VAT quintile had significantly higher HDL-C and large HDL2-C subclasses as measured by ultracentrifugation. Results of these studies are in agreement with our findings; however, none of these studies focused on women during midlife, and none investigated whether abdominal VAT is related to composition and functional measures of HDL.

Only a single study from MESA had assessed the relation between cardiovascular AT depots and HDL subclasses as measured by NMR [n=5407, 53% women, mean age 62.5 years].[170]

Greater pericardial AT volume, defined as the fat around the proximal coronary arteries, was associated with lower HDL-C, total, large and medium HDL-P levels, but with higher small HDL-P. Interaction by race showed that this association was only significant in Whites; no interaction by gender was tested. In an analysis of 650 postmenopausal women from the KEEPS study [mean age 52.9 (2.6) years], Huang et al.[172] reported that EAT (fat inside the pericardial sac), pericardial fat (fat outside the pericardial sac) and total AT (EAT + pericardial AT) were independently and negatively associated with HDL-C.

The potential mechanism that links the different visceral AT depots to HDL metrics is complex. An array of enzymes that are influenced by the visceral AT accumulation has been proposed to be involved in the pathway linking adiposity to HDL metabolism. Hepatic lipase (HL) and cholesterol ester transfer protein (CETP) are elevated with higher visceral AT accumulation.[184,185] HL hydrolyzes large HDL particles to form smaller, dense HDL,[184] whereas CETP transfers triglycerides from triglycerides-rich lipoproteins to HDL particles in exchange for cholesteryl esters.[185] This leads to the formation of more triglyceride-rich HDL particles[186] which are become favorable substrates for hepatic triglyceride lipase (TGL), and eventually smaller HDL particles.[139] Another enzyme that could be involved in the mechanistic link between visceral AT and HDL is the lecithin-cholesterol acyltransferase (LCAT), which esterifies free cholesterol in HDL particles and increase the size of the HDL.[286] In one study that assessed the relation between obesity, as measured by BMI, with HDL metrics, it was reported that a BMI>30kg/m^2 in young women was associated with a shift in HDL subclasses towards smaller subclasses, and with less phospholipid contents within the HDL.[287] Obesity was also associated with increased CETP and LCAT activities. Higher LCAT activity in obesity may form HDLs rich in cholesteryl ester, which become substrates for increased CETP activity.[287] It is important to note, however, that this study

assessed general obesity by BMI. Weight gain in midlife has been found to be an age-related occurrence, whereas accumulation of visceral AT is menopause-related;[288,289] and independent of weight gain and obesity status, women transitioning through menopause accumulate more visceral AT in the abdomen and ectopically. This could predispose them to the alterations in HDL metabolism that were observed in this analysis. The hormonal alterations that occur around midlife may also play a role in this link. The activity of HL during the luteal phase of the menstrual cycle, or when estrogen levels are the highest, declines significantly,[194] whereas increases in testosterone levels may increase HL activity.[195] In SWAN HDL, we have previously shown that higher estradiol was associated with larger HDL size, large HDL-P and HDL-CEC.[130] This suggests that the decline of estrogen after menopause, and the hyperandrogenism that could accompany visceral AT accumulation may enhance HL activity in women leading to smaller HDL particles. Smaller HDL particles, elevated triglyceride content and diminished phospholipids in the particle, in turn, could lead to detrimental effects on the cholesterol efflux capacity and function of the HDL.[76,290,291] Moreover, insulin resistance, is associated with an increase in the CETP.[292] This enrichment of the HDL with triglycerides can also be induced by the reduction in lipoprotein lipase (LPL) activity that has been observed in states of insulin resistance,[293] leading to a lower cholesterol content in the HDL, as observed in our results. Further, elevated activity of hepatic TGL and HL with IR may eventually lead to the depletion of the lipid core of the HDL conversion of HDLs into smaller particles.[139,181,184] Another important marker that may play a role in the association between visceral fat accumulation and HDL metrics is adiponectin. Adiponectin is an adipokine that plays an anti-inflammatory and insulin-sensitizing role. Obesity and visceral fat accumulation lead to a state of hypoadiponectinemia.[294] Hypoadiponectinemia is associated with increased HL activity[295] and reduced HDL-CEC,[296] indicating that the impact of visceral fat on HDL metabolism could be

influenced by the role of adiponectin. Moreover, low adiponectin is associated with insulin resistance, further supporting the role of adiponectin in this relationship.

In this analysis, we show that insulin resistance mediates the detrimental effect of visceral adiposity on HDL metrics, and that controlling insulin resistance may reduce part of the impact of AT on HDL dysfunctionality. A HOMA-IR of 2.5 may clinically differentiate insulin sensitive from insulin resistant individuals,[297] which is close to the average of HOMA-IR in our sample. Thus, achieving a HOMA-IR of 2.5 in midlife women may limit the dysfunctionality in HDL that occurs with visceral AT accumulation. This suggests that interventions such as insulin-sensitizing agents may protect HDL against the changes in composition and function that occur with visceral fat accumulation. In-vitro studies have indeed shown that metformin prevents HDL modifications and subsequent impairments in cholesterol efflux capacity.[298,299] Future clinical trials should test our hypothesis.

Despite the similarities in the links between abdominal and CV visceral AT depots with higher risk of cardiometabolic and cardiovascular risk factors, previous studies have suggested visceral AT associations with CVD risk may vary based on location, adipocyte number, size, and other factors. Human and animal studies have shown that EAT consists of smaller adipocytes and has a unique transcriptome that is enriched in genes associated with elevated inflammation.[197] PAT, but not EAT, has been linked to lower estradiol concentrations[14] and to higher risk of CAC presence[169] in midlife women, suggesting that PAT is a stronger menopause-related CVD risk factor. We thus postulate that PAT may play a more significant role in the metabolic consequences of ectopic fat accumulation compared to EAT, as evidenced by the stronger associations with HDL metrics changes and the role of insulin resistance. On the other hand, EAT may play a more

localized role by factors such as inflammation on the heart compared to other cardiovascular fat depots.

The results of this study should be interpreted with caution regarding some potential limitations. We only assessed the cholesterol efflux capacity as a measure of HDL; however, visceral fat could affect other functions such as the anti-inflammatory and anti-oxidative functions of the HDL. Future studies should aim to assess other functions of HDL in relation to fat volumes. Even though the study was prospective, the intervals between adipose tissue and HDL metrics assessment were relatively short, and thus, reverse causality cannot be ruled out. The sample size per race group is low, and we may be underpowered to detect a significant difference by race. Moreover, HOMA-IR was used as a convenient and simple proxy for IR. HOMA-IR is a robust measure of IR and has good agreement, despite lower accuracy, compared to the gold-standard euglycemic-hyperinsulinemic clamp.[300] However, thresholds to determine insulin resistance have not been standardized, particularly across different genders, races and age groups. Compared to the excluded women, the subjects included in this analysis had a higher BMI. BMI is highly and positively correlated with visceral fat, and as such, we can assume that those who were excluded had lower visceral fat accumulation. Higher BMI has been previously linked to smaller HDL size and higher concentrations of large HDL-P. In our sample, overall HDL size and large HDL-P concentrations were comparable between included and excluded women. This indicates that the exclusion of women with lower BMI may have biased the results away from the null. Moreover, we do not have data on enzymes such as CETP and LCAT activities, which could play a significant role on the associations between visceral AT and HDL metabolism. Future studies should assess the role of such markers on these associations. On the other hand, the major strengths include the

design of the analysis where HDL metrics were measured after the visceral fat depots, and the use of CT scans to directly quantify visceral adipose volumes.

This is one of the first studies to investigate the associations between different visceral AT depots and a comprehensive HDL metric profile in midlife women. The pathways that link visceral AT and HDL metrics are not straightforward, especially in midlife women for whom the hormonal changes of menopause add to the complexity of these associations. In Figure 5, we present a conceptual model that summarizes the potential pathways between AT and HDL metrics. Our results elucidate a part of the relationship, but future studies should aim to further describe the roles of other factors such as estradiol, adiponectin and inflammation on those associations.

Our analysis shows that the abdominal and cardiovascular visceral AT depots in predict HDL subclass distribution, size, lipid content and potentially function 2 years later, and that insulin resistance could be of major influence on these associations. Clinically, examining the roles of modifiable risk factors that may impact visceral AT (such as diet and physical activity) in ameliorating the detrimental changes in HDL metrics could further elucidate the role of adiposity on HDL metabolism and future cardiovascular risk. One study have shown that a very low calorie diet for 16 weeks reduced abdominal VAT and cardiac fat in diabetics,[301] whereas resistance training lowered PAT in abdominally obese subjects.[302] Moreover, studies on pharmaceutical interventions such as metformin, which affect insulin resistance, have shown to reduce visceral[303] and cardiac fat.[304] Whether this could play a role in preventing the deleterious changes on HDL metabolism that accompany visceral AT accumulation should be investigated.

In summary, the current analysis shows that increased abdominal and cardiovascular AT in women during midlife may be linked to a subsequent worsening in HDL metrics, including the main anti-atherogenic function of HDL in effluxing of cholesterol from macrophages in the reverse

cholesterol transport process. Controlling insulin resistance during midlife period may prevent the detrimental effect of visceral AT accumulation on HDL.

4.6 Tables and Figures

Table 4-1: Characteristics of women included in this analysis

Participant Characteristics	**N=299**
Characteristics at Time of Fat Assessment	
Age (years), mean (SD)	51.1 (2.8)
Race, n(%)	
White	201 (67.2%)
Black	98 (32.8%)
Menopause Status, n(%)	
Pre/Early-peri menopause	169 (56.5%)
Late-peri/postmenopause	106 (35.5%)
Unknown	24 (8.0%)
Difficulty Paying for basics	
No	208 (69.6%)
Yes	91 (30.4%)
BMI, kg/m^2, median (Q1, Q3)	27.6 (24.4, 33.0)
Physical Activity Score, mean (SD)	7.97 (1.76)
HOMA-IR, median (Q1, Q3)	1.89 (1.42, 3.06)
Triglycerides (mg/dL), median (Q1, Q3)	104 (77, 143)
Abdominal Visceral AT Volume (cm^3), median (Q1, Q3)	66.3 (42.4, 95.0)
EAT Volume (cm^3), median (Q1, Q3)	37.0 (28.0, 51.7)
PAT Volume (cm^3), median (Q1, Q3)	8.5 (4.4, 14.1)
PVAT Volume (cm^3), median (Q1, Q3)	29.2 (23.7, 37.7)
HDL Measures at Subsequent Visit:	
HDL- CEC (%), mean (SD)	3.84 (0.64)
HDL-Tg (mg/dL), median (Q1, Q3)	17 (15, 21)
HDL-PL (mg/dL), mean (SD)	53.9 (10.0)
Total HDL-P (µmol/L), mean (SD)	35.4 (6.6)
Large HDL-P (µmol/L), median (Q1, Q3)	7.4 (4.9, 9.6)
Medium HDL-P (µmol/L), median (Q1, Q3)	9.6 (5.6, 14.4)
Small HDL-P (µmol/L), mean (SD)	17.2 (7.2)
HDL Size (nm), mean (SD)	9.37 (0.56)
HDL-C (mg/dL), mean (SD)	57.3 (14.0)
ApoA-I (mg/dL), mean (SD)	165.9 (27.7)

HDL-C: 1 mg/dL=0.0259 mmol/L; Triglycerides: 1 mg/dL=0.0113 mmol/L

Abbreviations: BMI: Body mass index; VAT: visceral adipose tissue; EAT: epicardial adipose tissue; PAT: paracardial adipose tissue; PVAT: perivascular aortic adipose tissue; HDL-CEC: HDL cholesterol efflux capacity;

HDL-Tg: HDL-triglycerides; HDL-PL: HDL-Phospholipids; HDL-P: HDL particles; HDL-C: HDL-cholesterol; ApoA-I: Apolipoprotein A-I.

Table 4-2: Spearman's correlations between visceral AT depots and HDL metrics

	HDL Function and Contents									
	HDL-CEC (%)		HDL-Tg		HDL-P		HDL-C		ApoA-I	
AT Depots	r	p-value	r	p-value	r	p-value	r	p-value	r	p-value
Abdominal VAT	-0.13	0.03	0.17	0.004	-0.26	<0.0001	-0.39	<0.0001	-0.23	<0.0001
EAT	-0.12	0.06	0.16	0.009	-0.14	0.03	-0.22	0.0006	-0.08	0.21
PAT	-0.12	0.06	0.10	0.13	-0.20	0.002	-0.30	<0.0001	-0.17	0.009
PVAT	-0.13	0.03	0.11	0.08	-0.18	0.002	-0.27	0.0001	-0.17	0.007
	HDL Subclasses and Size									
	Total HDL-P		Large HDL-P		Medium HDL-P		Small HDL-P		HDL Size	
AT Depots	r	p-value	r	p-value	r	p-value	r	p-value	r	p-value
Abdominal VAT	-0.06	0.30	-0.42	<0.0001	-0.10	0.10	0.20	0.0005	-0.38	<0.0001
EAT	0.02	0.80	-0.26	<0.0001	-0.11	0.07	0.22	0.0003	-0.24	0.0002
PAT	-0.03	0.68	-0.31	<0.0001	-0.03	0.60	0.15	0.01	-0.28	<0.0001
PVAT	-0.02	0.69	-0.30	<0.0001	-0.02	0.69	0.13	0.03	-0.25	<0.0001

Table 4-3: Mediation analysis for HOMA-IR on the associations between AT depots and HDL metrics*

	Natural Indirect Effect		Controlled Direct Effect		Total Effects		Percent Mediated[%]
	β (SE)	p-value	β (SE)	p-value	β (SE)	p-value	
	Abdominal VAT[#]						
HDL-CEC	-0.06 (0.03)	0.0	-0.04 (0.05)	0.45	-0.10 (0.05)	0.03	62.0%
HDL-C	-1.26 (0.55)	0.02	-1.13 (1.02)	0.27	-2.47 (0.89)	0.006	50.9%
Large HDL-P[#]	-0.04 (0.02)	0.02	-0.08 (0.03)	0.03	-0.12 (0.03)	<0.0001	32.1%
HDL Size	-0.04 (0.02)	0.03	-0.07 (0.04)	0.07	-0.13 (0.03)	0.0003	34.5%
	PAT[#]						
HDL-C	-0.90 (0.37)	0.02	-1.05 (0.94)	0.26	-1.73 (0.90)	0.05	51.6%
Large HDL-P[#]	-0.03 (0.01)	0.007	-0.05 (0.03)	0.12	-0.08 (0.03)	0.01	44.9%
HDL Size	-0.04 (0.01)	0.01	-0.06 (0.04)	0.07	-0.09 (0.03)	0.005	37.1%
	PVAT[#]						
HDL-CEC	-0.04 (0.02)	0.03	-0.07 (0.05)	0.12	-0.11 (0.04)	0.02	34.7%

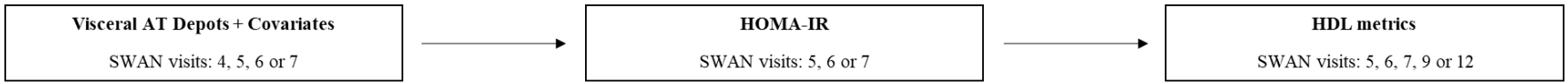
Models adjusted for race, time between adiposity and HDL metric assessment, economic hardship, and the following measures at time of AT assessment: age, physical activity, menopause status, and log-transformed triglycerides. PVAT models additionally adjusted for length of the descending aorta.

* We present data for significant mediation results, where the total effect is significant.

[#] Log-Transformed

[%] Calculated as: Natural Indirect Effect/Total Effects x 100%.

(a) Timeline for data collection of variables for analysis of associations between visceral AT depots and HDL metrics



(b) Timeline for data collection of variables for analysis of mediation of HOMA-IR on associations between visceral AT depots and HDL metrics.

Figure 4-1: Timeline of collection of variables in this analysis

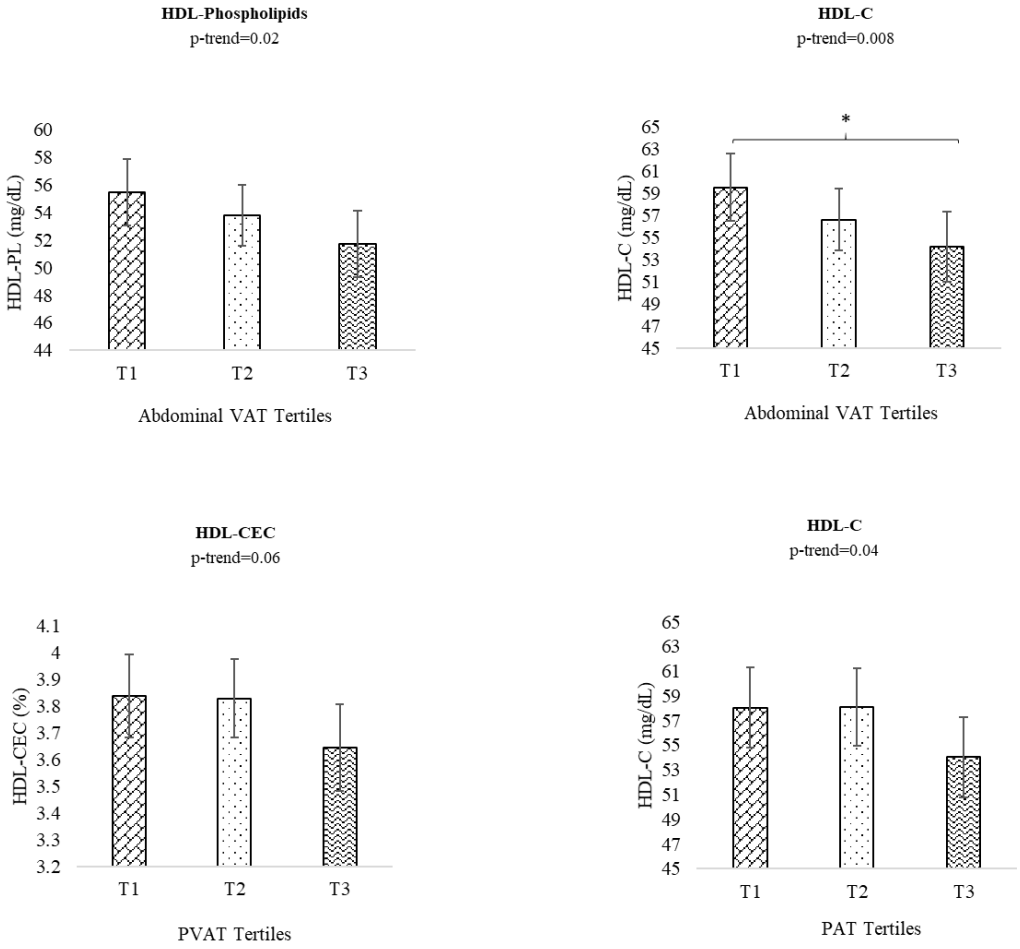


Figure 4-2: Associations between visceral AT tertiles and HDL function and content metrics

Models adjusted for race, time between adiposity and HDL metric assessment, economic hardship, and the following measures at time of AT assessment: age, physical activity, menopause status, and log-transformed triglycerides. PVAT models additionally adjusted for length of the descending aorta.
Bonferroni adjustments for multiple comparisons.
* Groups significantly different
Abdominal VAT volume tertiles: Tertile 1 (T1)≤ 50.6 cm^3; 50.6<Tertile 2 (T2)≤ 83.8 cm^3 ; Tertile 3 (T3)≥ 83.3 cm^3
PAT volume tertiles: Tertile 1 (T1)≤ 5.4 cm^3; 5.4<Tertile 2 (T2)≤ 11.5 cm^3 ; Tertile 3 (T3)≥ 11.5 cm^3
PVAT volume tertiles: Tertile 1 (T1)≤ 25.5 cm^3; 25.5<Tertile 2 (T2)≤ 34.6 cm^3 ; Tertile 3 (T3)≥ 34.6 cm^3

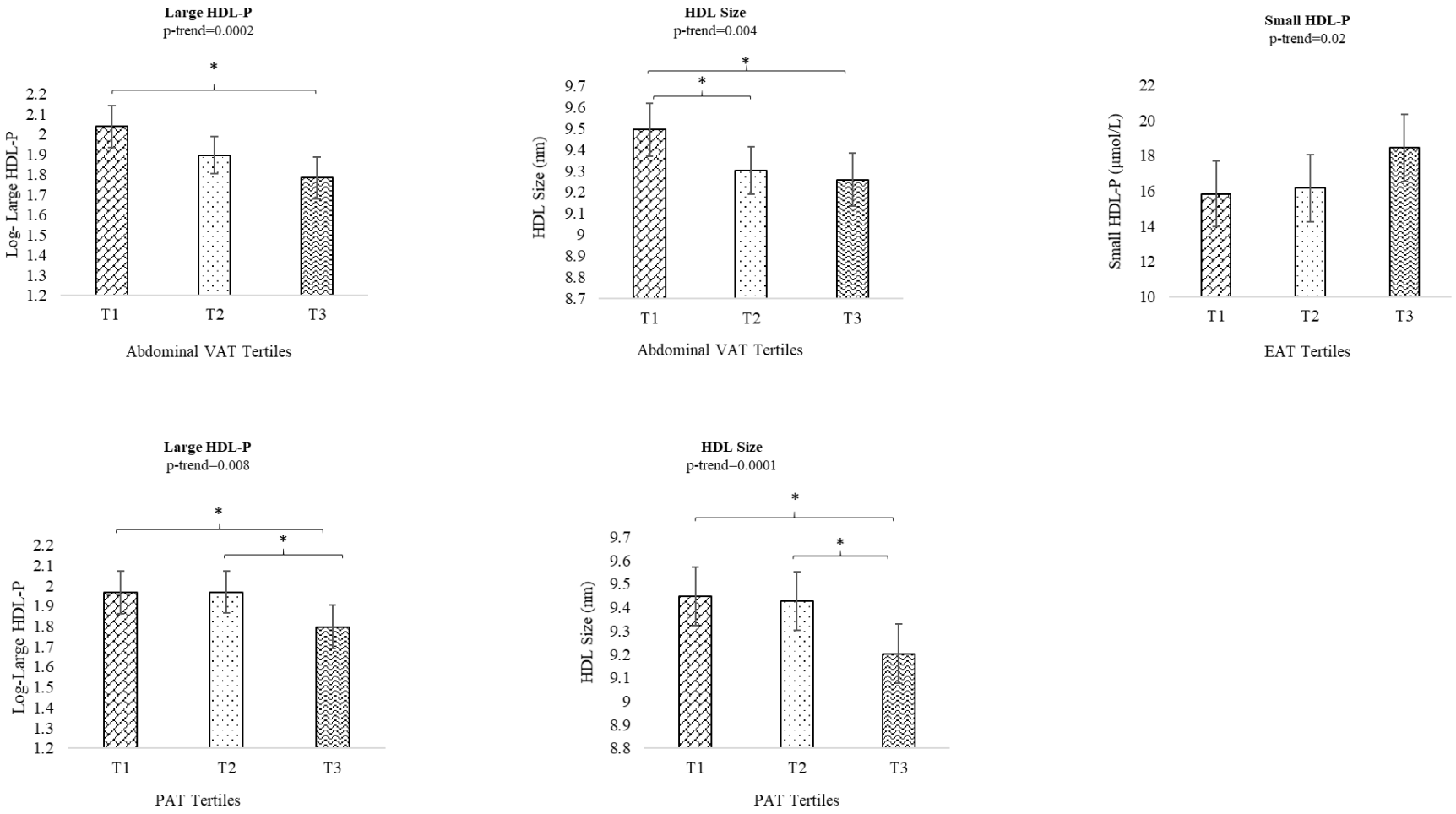


Figure 4-3: Associations between AT tertiles and HDL subclasses and size

Models adjusted for race, time between adiposity and HDL metric assessment, economic hardship, and the following measures at time of AT assessment: age, physical activity, menopause status, and log-transformed triglycerides.

Bonferroni adjustments for multiple comparisons.

* Groups significantly different

Abdominal VAT volume tertiles: Tertile 1 (T1)≤ 50.6 cm^3; 50.6<Tertile 2 (T2)≤ 83.8 cm^3 ; Tertile 3 (T3)≥ 83.3 cm^3

EAT volume tertiles: Tertile 1 (T1)≤ 31.7 cm^3; 31.7<Tertile 2 (T2)≤ 45.1 cm^3 ; Tertile 3 (T3)≥ 45.1 cm^3

PAT volume tertiles: Tertile 1 (T1)≤ 5.4 cm^3; 5.4<Tertile 2 (T2)≤ 11.5 cm^3 ; Tertile 3 (T3)≥ 11.5 cm^3

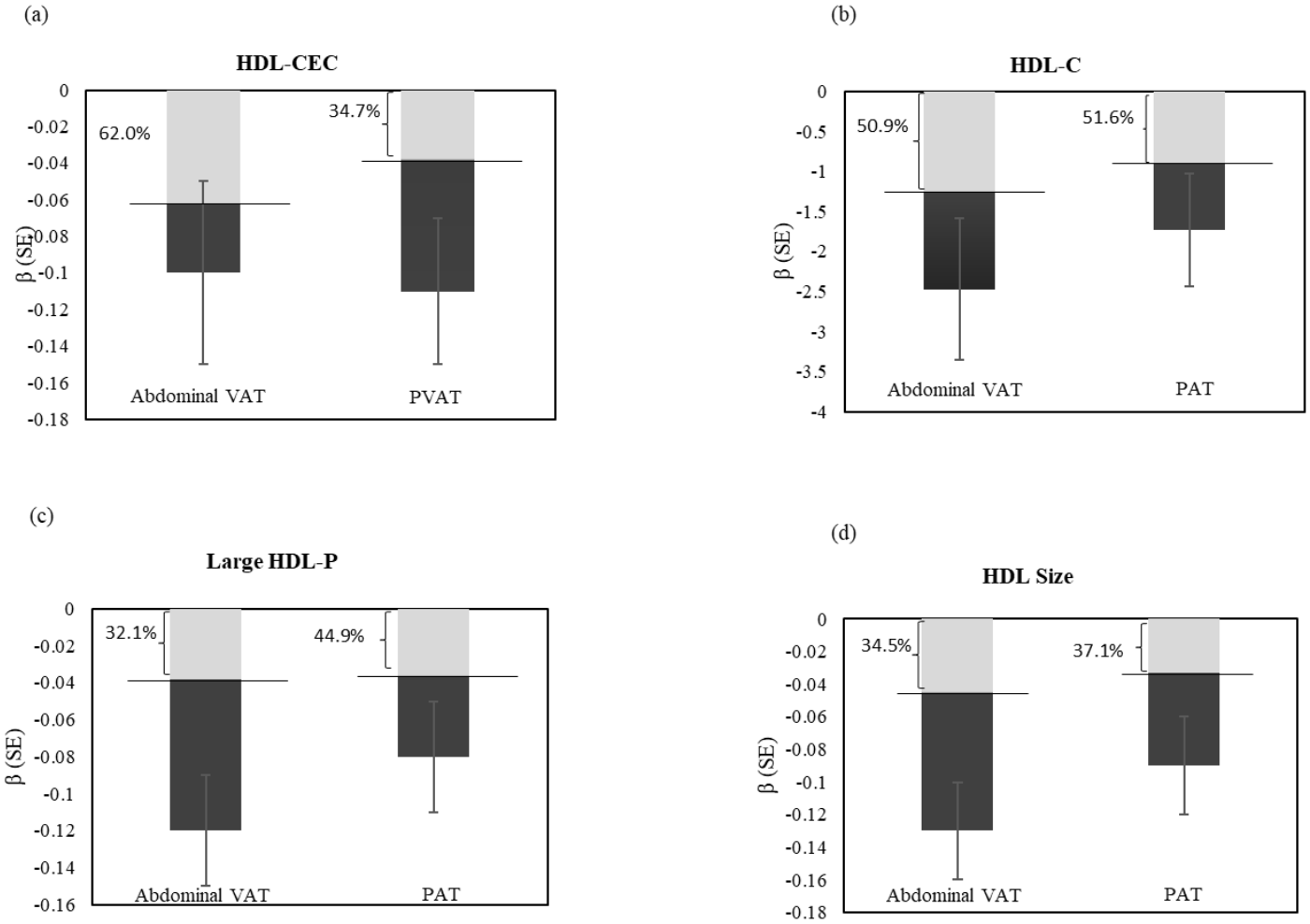


Figure 4-4: Mediation effect by HOMA-IR on the associations between AT depots and HDL metrics

Effect of HOMA-IR as a mediator on the associations between AT depots and (a) HDL-CEC; (b) HDL-C; (c) large HDL-P and (d) HDL size. Models adjusted for race, time between adiposity and HDL metric assessment, economic hardship, and the following measures at time of AT assessment: age, physical activity, menopause status, and log-transformed triglycerides. PVAT models additionally adjusted for length of the descending aorta.
The gray + black area represent the total effect of the association between the AT depot and the HDL metric. The gray area represents the percentage mediated by HOMA-IR (natural indirect effect/total effect x 100%).

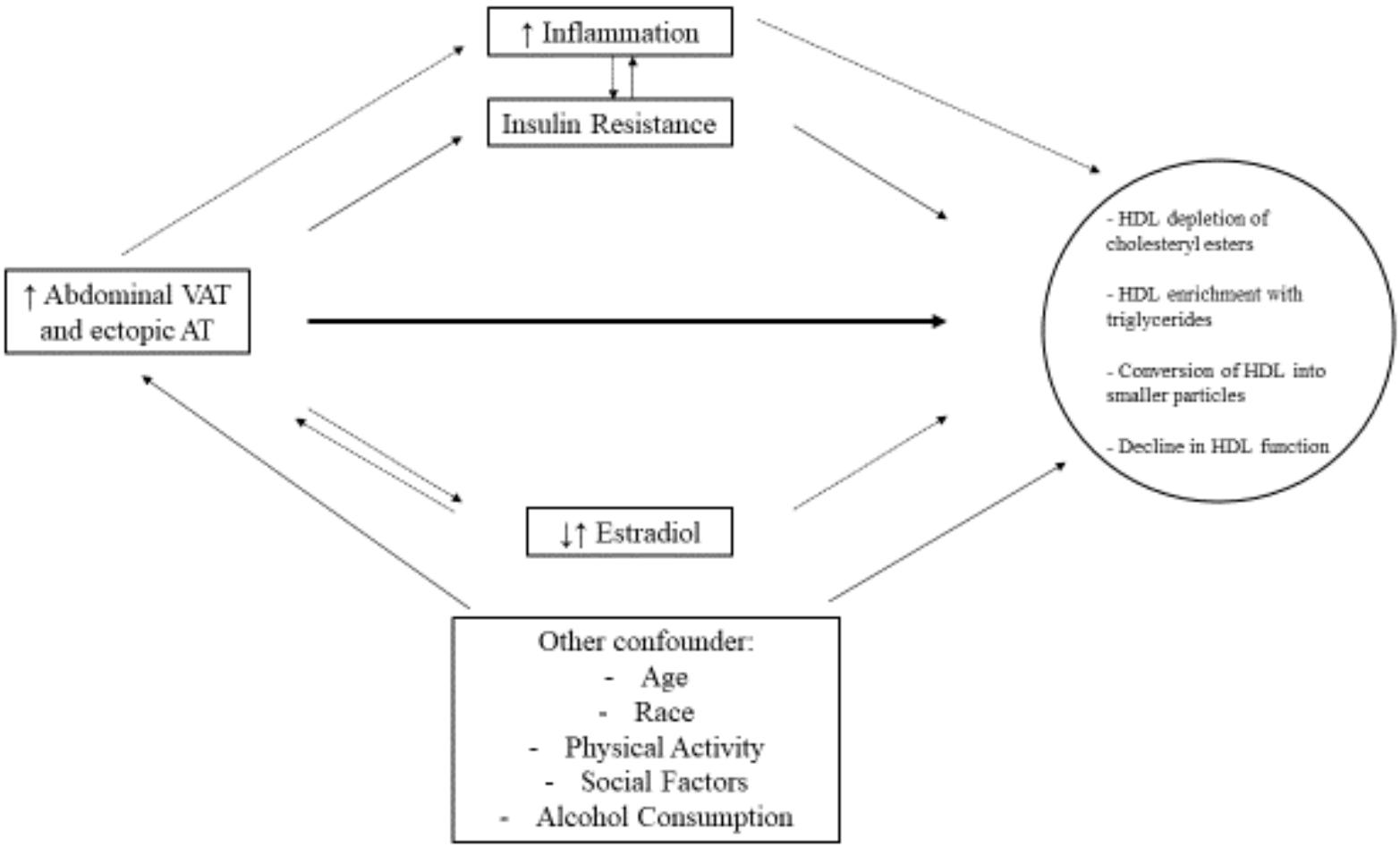


Figure 4-5: Conceptual model of the association between visceral AT and HDL metrics

Conceptual model for the potential pathways between visceral AT and HDL metabolism: Higher visceral AT can be linked to altered HDL metabolism through several pathways. Visceral AT accumulation could alter HDL composition, function and subclass distribution through its effect on enzymes involved in HDL metabolism (such as hepatic lipase (HL), cholesteryl ester transfer protein (CETP), triglyceride lipase (TGL), phospholipid-transfer protein (PLTP) and others). Other pathways could be through the induction of insulin resistance by visceral AT accumulation; insulin resistance could additionally alter the enzymes related to HDL metabolism. Visceral AT accumulation is also linked to a status of chronic inflammation status, which could directly impact HDL metabolism, and which could be indirectly interrelated to the state of insulin resistance. Moreover, previous studies have shown that alterations in HDL may induce a state of chronic inflammation. In women specifically, higher visceral AT accumulation is linked to elevated estradiol (E2) release through conversion of androgens to estradiol. However, lower E2 during menopause could lead to higher accumulation of visceral AT. E2 is also linked independently to changes in HDL metabolism.

4.7 Supplemental Material

Table 4-4: Supplementary Table 1: Multivariable adjusted associations between AT depots and HDL function and contents[a]

	HDL Function and Contents									
	HDL-CEC (%)		HDL-Tg[b]		HDL-PL		HDL-C		ApoA-I	
AT Depots	β (SE)	p-value	β (SE)	p-value	β (SE)	p-value	β (SE)	p-value	β (SE)	p-value
Abdominal VAT	-6.91 (-12.77, -0.66)	0.03	0.49 (-2.24, 3.30)	0.72	-68.77 (-88.34, -16.37)	0.02	-85.72 (-95.89. -50.43)	0.002	-79.48 (-98.67, 216.44)	0.26
EAT	-5.88 (-12.47, 1.20)	0.10	2.32 (-0.71, 5.44)	0.13	-29.74 (-76.56, 110.61)	0.53	-37.81 (-84.70, 152.73)	0.51	29.83 (-93.97, 2696)	0.87
PAT	-5.14 (-10.62, 0.68)	0.08	0.18 (-2.26, 2.68)	0.89	-46.15 (-78.00, 31.75)	0.17	-70.80 (-90.62, -9.10)	0.03	-63.08 (-96.98, 351.88)	0.43
PVAT	-7.76 (-14.16, -0.88)	0.03	0.18 (-2.79, 3.25)	0.83	-54.27 (-84.90, 38.51)	0.17	-70.80 (-93.17, 24.76)	0.10	-75.98 (-98.95, 448.32)	0.37

[a] Data presented per % change relative to mean AT volume. Models adjusted for race, time between adiposity and HDL metric assessment, economic hardship, and the following measures at time of AT assessment: age, physical activity, menopause status, and log-transformed triglycerides. PVAT models additionally adjusted for length of the descending aorta.

[b] Log-transformed

Table 4-5: Supplementary Table 2: Multivariable adjusted associations between AT depots and HDL subclasses and size[a]

	HDL Subclasses and Size									
	Total HDL-P		Large HDL-P[b]		Medium HDL-P[b]		Small HDL-P		HDL Size	
AT Depots	β (SE)	p-value	β (SE)	p-value	β (SE)	p-value	β (SE)	p-value	β (SE)	p-value
Abdominal VAT	-5.44 (-50.86, 81.95)	0.87	-8.82 (-12.57, -4.90)	<0.0001	0.15 (-7.99, 9.00)	0.97	143.50 (17.82, 403.39)	0.02	-9.35 (-13.79, -4.68)	0.0001
EAT	9.97 (-46.58, 126.39)	0.80	-3.31 (-7.64, 1.21)	0.15	-4.73 (-12.23, 3.40)	0.24	174.50 (21.81, 518.58)	0.02	-4.23 (-9.35, 1.17)	0.12
PAT	9.39 (-39.49, 97.76)	0.77	-5.04 (-8.50, -1.46)	0.006	1.69 (-4.97, 8.82)	0.63	75.58 (-10.24, 243.44)	0.10	-6.41 (-10.48, -2.15)	0.004
PVAT	-20.46 (-61.78, 65.55)	0.54	-4.58 (-9.07, 0.13)	0.06	8.77 (-0.97, 19.46)	0.08	14.90 (-49.42, 160.97)	0.74	-3.89 (-9.28, 1.81)	0.18

[a] Data presented per % change relative to mean AT volume. Models adjusted for race, time between adiposity and HDL metric assessment, economic hardship, and the following measures at time of AT assessment: age, physical activity, menopause status, and log-transformed triglycerides. PVAT models additionally adjusted for length of the descending aorta.

[b] Log-transformed

5.0 Manuscript 2: HDL Metrics during Midlife and Future Subclinical Atherosclerosis in Women: The SWAN HDL Study

5.1 Abstract

Background: The clinical utility of high-density lipoprotein cholesterol (HDL-C) in assessing the anti-atherogenic properties of HDL is limited. Novel metrics of HDL function, lipid contents and subclass distribution may better reflect these properties of HDL. This is of particular interest in midlife women when a potential loss in the anti-atherogenic properties of the HDL may occur. In this analysis, we aimed to investigate the relation between a comprehensive profile of HDL metrics in midlife women with future measures of subclinical atherosclerosis [carotid intima media thickness (cIMT), interadventitial diameter (IAD) and carotid plaque presence].

Methods: We included 461 women from the Study of Women's Health Across the Nation (SWAN) HDL ancillary study who had measures of HDL function by cholesterol efflux capacity (HDL-CEC), lipid contents [HDL-phospholipids (HDL-PL) and HDL-triglycerides (HDL-Tg)], and HDL particle (HDL-P) distribution and size, followed by a carotid ultrasound for subclinical atherosclerosis assessment [on average 12.9 (SD: 2.6) years later]. Using latent cluster analysis, women were grouped into clusters based on their HDL profiles. Multivariable associations between HDL clusters and future subclinical atherosclerosis were analyzed by linear or logistic regression.

Results: At time of HDL assessment, women were, on average, 50.4 (SD: 2.7) years old, 59% were White, 30% were Black, and 11% were Chinese. Women were classified as either

having a favorable HDL cluster (high HDL-CEC, HDL-PL, large and medium HDL-P, less HDL-Tg and small HDL-P and larger overall size) or an unfavorable HDL cluster (low HDL-CEC, HDL-PL, large and medium HDL-P, more HDL-Tg and small HDL-P and smaller overall size). The favorable HDL cluster was associated with lower cIMT, IAD and odds of plaque presence. This association was attenuated by BMI, except in Chinese women where the association with cIMT persisted after multivariable adjustment. Systolic blood pressure appeared to be a significant mediator for the associations between HDL cluster and cIMT and IAD, but not plaque.

Conclusion: A favorable HDL metrics cluster may be associated with better subclinical atherosclerosis profile in women after menopause. This could be mediated by blood pressure.

5.2 Introduction

Cardiovascular disease (CVD) is the major cause of death in women in the US.[305] During midlife, women experience an increase in numerous cardiovascular risk factors, such as visceral adipose tissue accumulation, dyslipidemia, and increase in subclinical atherosclerosis risk.[275] However, studies have been inconclusive in defining the changes in high-density lipoprotein cholesterol (HDL-C) that accompany the menopause transition. Some studies have shown that HDL-C, which measures the cholesterol content of the HDL, does not differ[64] or is lower[65] in postmenopausal versus premenopausal women. In contrast, longitudinal studies have shown an increase in HDL-C[10] in midlife. Characterizing the changes in high-density lipoproteins (HDLs) around menopause is crucial to understand the changes in the athero-protective functions of the HDL during that time period.

The HDLs are complex molecules that vary in function, size and contents of proteins and lipids. It has been suggested that HDL-C may not sufficiently reflect the complexity of the HDL molecule, and may not mirror the changes in HDL that occur during midlife. Novel metrics of the HDL that directly evaluate the function, lipid content and distribution of subclasses may provide better understanding of the HDL's athero-protective capacities, particularly in midlife women. The interest in this population arises from findings that have suggested a dysfunctionality in HDL and loss of the anti-atherogenic functions over the menopause transition, a phenomena that cannot be fully captured by HDL-C[10,29]. Higher HDL cholesterol efflux capacity (HDL-CEC), a measure of HDL's ability to efflux cholesterol from a cell to an acceptor, has been linked to lower risk of CVD.[122] Moreover, a higher phospholipid (HDL-PL) and lower triglycerides (HDL-Tg)

contents,[123] and a distribution of subclasses towards more large HDL particles (HDL-P) and less small HDL-P[111] have all been linked to lower CVD risk, despite some inconsistent findings.[109,113]

Subclinical atherosclerosis assessment can provide information on CVD prior to clinical manifestation.[207] Carotid intima media thickness (cIMT), inter-adventitial diameter (IAD) and presence of plaque are measures of subclinical atherosclerosis that can predict future CVD events.[208,209] Studies on the link between HDL-C and subclinical atherosclerosis in midlife women have shown that an increase in HDL-C during midlife is associated with higher cIMT after menopause.[8,68] However, studies on the relationship between novel HDL metrics and subclinical atherosclerosis in midlife women are limited. Cross-sectionally, in women from the Multi-Ethnic Study of Atherosclerosis (MESA), higher total HDL-P (by ion mobility) was associated with lower cIMT, whereas higher HDL-C was associated with higher risk of carotid plaque.[69] No studies have investigated direct measures of HDL function or lipid contents with future subclinical atherosclerosis in midlife women.

The SWAN HDL ancillary study provides a unique opportunity to investigate a comprehensive HDL metric profile during early midlife, prior to the dysfunction in HDL and before the worsening of subclinical atherosclerosis that occurs during the perimenopausal period.[224,225] Given the biological interrelations between different HDL metrics, we aimed to cluster women based on their comprehensive HDL profiles. We then aimed to investigate whether these cluster are associated with future subclinical atherosclerosis. We hypothesized that women will be classified as favorable (higher HDL-CEC, HDL-PL and large HDL-P, lower HDL-Tg and small HDL-P, and larger overall HDL size) or unfavorable (lower HDL-CEC, HDL-PL and large HDL-P, higher HDL-Tg and small HDL-P, and smaller overall HDL size) cluster; and that the unfavorable HDL metrics profile will be associated with greater carotid intima media thickness

(cIMT), larger inter-adventitial diameter (IAD) and higher odds of plaque presence postmenopausally. Since previous studies from SWAN have shown that the associations between HDL-C and subclinical atherosclerosis differ by race/ethnicity,[229] we also aimed to assess whether race/ethnicity modifies these associations. Moreover, since it is known that HDL may affects the vasculature and blood pressure,[306] we aimed to assess whether blood pressure mediates the associations between HDL metrics and subclinical atherosclerosis.

5.3 Subjects and Methods

The Study of Women's Health Across the Nation (SWAN) is an ongoing multi-site, multi-ethnic, community-based longitudinal study. The aim of SWAN is to characterize the physiological and psychological that occur as women traverse the menopause transition. In brief, 3,302 women aged 42-52 years were recruited between 1996-1997 at seven different sites across the United States: Boston, MA; Chicago, IL; Pittsburgh, PA; Detroit, MI; Newark, NJ; Los Angeles, CA; and Oakland, CA.[278] Women were eligible for recruitment to SWAN if they identified as White, Black, Hispanic, Chinese or Japanese; if at the time of recruitment they were not pregnant or lactating; had an intact uterus and at least one ovary, and were not using hormone therapy; and if they had at least one menstrual period within the last 3 months of recruitment.

The SWAN HDL study is an ancillary study to SWAN which aims to characterize the biological changes in composition and function of HDL that accompany ovarian aging. SWAN HDL also aims to understand how these changes in function and composition affect the athero-protective abilities of HDL in women. For this study, 558 participants (1,461 observations) from

the original SWAN were selected into SWAN HDL if they had frozen serum samples at least one visit before and two visits after the final menstrual period. These frozen serum samples were utilized to quantify HDL function, lipid composition and subclasses.

In this analysis, we included women from SWAN HDL who had an HDL metric measure at SWAN HDL baseline visit (coinciding with SWAN follow-up visits 1, 3-9, or 12) and one measure of subclinical atherosclerosis [carotid intima media thickness (cIMT), inter-adventitial diameter (IAD) and/or carotid plaque]; coinciding with SWAN follow-up visits 12/13 or 15. For women who had measures at both visits 12/13 and 15, priority was to select measures at subclinical atherosclerosis at visit 15. The average time between SWAN visits 12/13 and visit 15 was 5.4 (SD: 0.5) years. SWAN did not measure subclinical atherosclerosis at the Los Angeles site. Thus, Japanese women, who were only recruited at the Los Angeles site, were not included in this study. Moreover, Hispanics were only recruited at New Jersey, so due to the small number of women from the New Jersey site in SWAN HDL, and particularly the Hispanic women, all 5 women from this site were excluded. The final analysis included 461 women, who were either White, Black or Chinese. All participants provided written informed consent at every SWAN visit, and study protocols were approved by the institutional review board at each site.

Blood Data Collection:

Phlebotomy was performed after a minimum overnight fast of 10 hours. When menstrual cycles were predictable, this was done 2-5 days after a spontaneous menstrual bleed. When cycles were less predictable, this was randomly done within 90 days of the annual SWAN visit. To improve the validity of the results, blood analysis was done on stored samples that have been frozen at -80°C and never been thawed.

HDL Cholesterol Efflux Capacity and Lipid Contents:

Analysis of HDL cholesterol efflux capacity (HDL-CEC), HDL-phospholipids (HDL-PL) and HDL-triglycerides (HDL-PL) was performed at a CDC-certified laboratory at the University of Pennsylvania. HDL-CEC was measured by fluorescence-labeled cholesterol efflux as has been described before.[122] J774 mouse macrophage cells were plated overnight and labeled with 2 μCi/mL of ^{3}H cholesterol. Cells were then incubated for 4 hours with 0.3 mM 8-(4-chlorophenylthio)-cyclic AMP (cAMP), which upregulates the ATP-binding cassette transporter-1 (ABCA1). Then, lipoproteins containing apolipoprotein B (ApoB) were removed from plasma by polyethylene glycol precipitation to result in ApoB-depleted serum. Cells were then incubated at 37°C for 2 hours with the equivalent of 1% ApoB- depleted serum or plasma. Baseline controls were created by cells incubated with media alone. Then, each medium was collected and passed through a 0.22 μM filter to remove cell debris and lipids were extracted from cells using isopropanol. Scintillation counting was used to quantify ^{3}H cholesterol in media and cells and percent efflux capacity was calculated by the following formula: [(cpm of ^{3}H cholesterol in the media - cpm of ^{3}H cholesterol in serum free media) / (cpm of ^{3}H cholesterol in the cells + cpm of ^{3}H cholesterol in the media)] × 100. The intra and inter-assay coefficients of variation for HDL-CEC were 3.7% and 10.1%, respectively.

To measure HDL-Tg and HDL-PL, HDL was isolated from serum by phophotungstic acid precipitation (FujiFilm Wako Pure Chemical Corporation). HDL-Tg and HDL-PL were quantified by the Roche Cobas C311 clinical analyzer according to manufacturer's protocol (Roche: 20767107322 and Wako: 433-36201, respectively). The inter-assay coefficients of variation were 3.9% and 3.5% for HDL-Tg and HDL-PL, respectively.

HDL Subclasses:

HDL subclasses and overall size were measured at LabCorp (Morrisville, NC) by the Nuclear Magnetic (NMR) Spectroscopy LipoProfile-3 algorithm[281] using the Vantera Clinical Analyzer. This is an automated 400 MHz NMR spectroscopy platform. The NMR lipoprotein particle quantification utilizes composite signal envelopes at 0.8 ppm, which contain the signals emitted by terminal methyl group protons of phospholipids, triglycerides, unesterified cholesterol and cholesterol ester of each HDL lipoprotein particle. Signal amplitudes that contribute to the composite plasma signal are produced as a result of the deconvolution of the composite signal. Each lipoprotein subclass produces unique NMR signals that are specific in frequency and shape, and the amplitude of the signal is proportional to the number of particles that are releasing the signal. To obtain the amplitude of each subpopulation of subclasses, the line shape of the signal envelope was modeled as a sum of all lipoprotein signals. The areas of different subpopulations were multiplied by conversion factors to quantify the concentrations, which were then grouped into small (7.3-8.2 nm), medium (8.2-9.4 nm) or large (9.4-14.0 nm) HDL subclasses. The total HDL particle (HDL-P) concentration was obtained by adding the concentrations of all three subclasses and the overall HDL particle size was calculated by adding the diameter of each subclass multiplied by its relative mass percentage from NMR signal amplitude. The intra- and inter-assay coefficients of variation for HDL-P concentrations and size ranged from 0.6% to 3.7% (intra-assay) and 1.5% to 4.0% (inter-assay).

Subclinical Atherosclerosis Assessment:

Carotid intima media thickness (cIMT), inter-adventitial diameter (IAD) and carotid plaque were obtained by the Terason t3000 Ultrasound System (Teratech Corp, Burlington, MA) which is equipped with a variable frequency 5-12 MHz linear array transducer. Two digitized

images of the near and far walls of the left and right distal common carotid arteries (CCA), 1 cm proximal to the carotid bulb, were obtained during end-diastole.

cIMT: cIMT represents the thickness of the innermost two layers of the carotid artery walls. cIMT was measured by electronically tracing the lumen-intima interface and the media-adventitia interface over a 1-cm segment at each of the 4 locations described above using the artery measurement system (AMS) semi-automated edge detection software.[307] One measurement per pixel was generated over the area for a total of approximately 140 measures per segment, and the average of the measures was calculated for all 4 images, indicating the average cIMT.

IAD: IAD represents the diameter of the CCA, which includes the lumen and the intimal and medial layers of the CCA. IAD was measured directly as the distance from the adventitia-media interface on the near wall to the media-adventitia interface on the far wall of the CCA segments that were used for cIMT assessment.

Plaque Presence:

The presence and extent of carotid plaque were measured at 5 segments of the left and right carotid arteries: the distal CCA, the proximal CCA, the carotid bulb, the proximal internal carotid artery and the proximal external carotid artery.[308,309] Plaque was defined as an area with protrusion into the vessel lumen that is at least 50% thicker than the adjacent IMT, as described by the Mannheim and ASE consensus statement.[310] The degree of plaque within each segment was graded between 0 (no observable plaque) to 3 (plaque covering 50% or more of the vessel diameter). The plaque index was calculated as the sum of plaque grades across all carotid segments.[311] Plaque presence was defined as a plaque index ≥2.

All technicians were centrally trained by the University of Pittsburgh Ultrasound Research Laboratory (URL), and were monitored throughout the study period for reliability to ensure

accurate and consistent assessment of subclinical atherosclerosis measures across study sites. All carotid scans were read at the SWAN Ultrasound Reading Center at the URL. Two trained readers who had no knowledge or access to participants' data read the images. Reproducibility of cIMT and IAD measures was excellent with an intra-class correlation coefficient (ICC) between sonographers of ≥0.77 and >0.85 respectively and ICC between readers of >0.90 and 0.80, respectively. The ICC for plaque index ranged between 0.86 and 0.93. The scanning and reading protocols have been used in numerous previous studies.[311-313]

Study Covariates:

Race/ethnicity and education level were self-reported at SWAN baseline visit. All other covariates were assessed at each SWAN follow-up visit. Age was calculated as the difference between date of a SWAN visit and date of birth. Body mass index (BMI in kg/m^2) was calculated as measured weight (kg) divided by the square of measured height (m). Systolic and diastolic blood pressure (SBP and DBP) were assessed after a woman was seated for 5 minutes with flat feet on the ground using the appropriate size arm cuff. Blood pressure was measured twice and the average of the two measures was reported. Physical activity score was assessed by the modified Kaiser Permanente Health Plan Activity Survey.[314]

Smoking status (never or past/current) and menopause status were self-reported by the subjects at each SWAN visit. Menopause status was obtained at each visit based on the menstrual bleed frequency and patterns and hormone therapy use over the past 12 months. Menopause status was categorized as postmenopausal (no menstrual bleed within the last 12 months, either due to natural menopause or surgical bilateral salpingo-oophorectomy), late perimenopausal (no menstrual bleed within the last 3 months but at least one bleed in the last 12 months), early perimenopausal (at least one menstrual bleed in the last 3 months with some perceived changes in

cycle intervals and pattern), premenopausal (no perceived changes in menstrual cycles), or unknown (due to hormone therapy use or hysterectomy). Hormone therapy use was self-reported. Cardiovascular medication use [anti-lipids (statins or non-statins), anti-diabetics and/or anti-hypertensives] was self-reported.

Statistical Analysis:

Characteristics of the women who were included in this analysis were described at SWAN HDL baseline visit and at time of subclinical atherosclerosis assessment, using mean (SD), median (Q1, Q3) or frequencies (%), as appropriate. Results were compared using paired sample t-tests, Wilcoxon signed rank sum, or McNemar's tests.

Latent class analysis (PROC LCA) was used to cluster women based on medians of HDL metrics.[315] Measures of HDL function (HDL-CEC), lipid contents (HDL-PL and HDL-TG), subclasses distribution (large, medium and small HDL-P) and HDL size were included in the clustering analysis. We fit models with 2 to 5 clusters. The optimal number of clusters was chosen based on Bayesian information criterion (BIC), entropy, and biological interpretability. Accordingly, women were classified into two clusters: favorable and unfavorable.

Characteristics of women at time of subclinical atherosclerosis assessment were compared by clusters using Student's t-tests for normally distributed variables, Mann-Whitney U tests for skewed variables, or Chi-square for categorical variables. cIMT was log-transformed to reduce skewness.

Multivariable linear regression was used to evaluate the associations between clusters of HDL metrics and cIMT or IAD. Multivariable logistic regression was used to evaluate the associations between clusters of HDL metrics and odds of plaque presence (plaque score ≥2). Potential confounders were selected either a priori, or if they were associated with the tested

exposure and outcomes. Models were selected based on higher R^2 or lower Akaike Information Criterion (AIC), as appropriate, without introducing collinearity. Final models were adjusted for race/ethnicity, site, time between HDL measure and subclinical atherosclerosis assessment, age, BMI, smoking status and physical activity at time of outcome.

We tested whether SBP mediated the associations between HDL clusters and subclinical atherosclerosis in final models using causal mediation analysis. This analysis provides data on the "total effect" which represents the independent effect of exposure on outcome; the "controlled direct effect" which represents the difference in the outcome between the exposure classes if the mediator was fixed; and the "natural indirect effect" which represents the effect of exposure on outcome that is mediated by the examined mediator. For this analysis, we selected SBP at a visit between the time of HDL assessment and subclinical atherosclerosis measures. All analyses were conducted using SAS v9.4 (SAS Institute, Cary, NC).

5.4 Results

Characteristics of women included in this analysis:

The characteristics of women included in this analysis at SWAN HDL baseline visits and at time of subclinical atherosclerosis assessment are presented in **Table 1**. Women, on average, were 50.4 (SD: 2.7) years old at SWAN HDL baseline visit and 63.3 (SD: 3.7) years old at time of subclinical atherosclerosis assessment; 59% were White and 30% were Black. At SWAN HDL baseline visits, around 80% of women had not yet reached menopause, and almost all were postmenopausal at time of subclinical atherosclerosis assessment. Women appeared to gain weight

and report less physical activity with time. In this group of women, 35% had plaque. The time difference between HDL metrics assessment and subclinical atherosclerosis was 12.9 (SD: 2.6) years.

Clustering of HDL metrics and characteristics of women by clusters:

Based on BIC values, entropy and the interpretability of the results, women were clustered into 2 groups: favorable and unfavorable. The probabilities of group membership for each metric are presented in **Supplementary Table 1.** The comparison of HDL metrics by clusters are presented in **Supplementary Table 2.** HDL-CEC, HDL-PL, total, large, and medium HDL-Ps were significantly higher, whereas HDL-Tg and small HDL-P were significantly lower in the favorable compared to the unfavorable HDL cluster.

Compared to women in the unfavorable HDL cluster, those in the favorable HDL cluster had lower age, BMI, SBP and DBP, and higher physical activity score **(Table 2)**. Women in the favorable HDL cluster were more likely to be Chinese and never smokers, and were less likely to use CVD medications. cIMT, IAD and presence of plaque were significantly lower in the favorable group.

Associations between HDL clusters and measures of subclinical atherosclerosis:

In unadjusted models (Model 1), the favorable HDL cluster was associated with significantly lower cIMT, IAD and odds of plaque presence (all p-values<0.05), **Table 3**. After adjusting for age, site, race/ethnicity (Model 2), and time between measures, the associations between the HDL clusters and cIMT and IAD were weakened but remained statistically significant, whereas for plaque presence, the association was completely attenuated. Adding BMI, smoking status and physical activity score to the models (Model 3) explained the observed associations between HDL clusters and the subclinical atherosclerosis endpoints.

Interaction by race/ethnicity:

Interaction between race/ethnicity and HDL clusters was borderline significant for the associations with cIMT (p-interaction=0.05), **Table 4.** The association between HDL clusters and cIMT was not significant among White and Black women; however, the association of lower cIMT with the favorable HDL cluster was more pronounced and statistically significant among Chinese women. The difference between White and Black women and Chinese women was statistically significant. Interaction between race/ethnicity and HDL clusters for the associations with IAD and plaque were not significant (p-interaction=0.29 and 0.82), data not shown.

Mediation Analysis for the role of SBP on the relation between HDL clusters and subclinical atherosclerosis:

In the final models, SBP significantly mediated the associations between HDL clusters and cIMT and IAD **(Table 5)**. The total effect, which represents the estimate for the association between favorable HDL cluster compared to unfavorable HDL cluster on measures of subclinical atherosclerosis, was not significant (p-values>0.05). However, the natural indirect effect, which represents the effect of HDL clusters on measures of subclinical atherosclerosis that is mediated by systolic blood pressure, was significant for cIMT and IAD (p-values=0.05). SBP did not mediate the relationship between HDL clusters and plaque presence (p-value>0.05, data not presented).

5.5 Discussion

In this research work, metrics of HDL function, lipid content and subclasses were clustered to two main profiles, favorable and unfavorable, in a cohort of midlife women to evaluate their association with future subclinical atherosclerosis. As hypothesized, higher HDL cholesterol efflux capacity, higher phospholipid contents, larger HDL subclasses and larger overall HDL size were classified together in a "favorable" cluster, whereas lower cholesterol efflux capacity, lower phospholipid contents, and smaller subclasses and overall size were classified in the "unfavorable" cluster. The favorable HDL cluster was linked to a better postmenopausal subclinical atherosclerosis profile, particularly lower cIMT in Chinese women. In Black and White women, the observed association was explained by cardiometabolic factors, particularly BMI. We also found that the association between HDL metrics and subclinical atherosclerosis was mediated by systolic blood pressure, such that a favorable HDL profile was linked to lower SBP, leading to less subclinical atherosclerosis.

Studies that have investigated the correlations between HDL-CEC and different metrics of size, subclass distribution and contents have mainly shown that cholesterol efflux capacity is positively correlated with large and medium HDL-Ps, and with HDL size, but inversely with small HDL-Ps.[120,234] Moreover, studies in humans have shown a high positive correlation between HDL-PL and cholesterol efflux capacity.[96] In women, a strong positive correlation between HDL-CEC and large and medium HDL-Ps, but not with small HDL-Ps has been reported.[136] Thus, it would be intriguing to identify how a combination of HDL metrics that describe functional and structural properties may predict future subclinical atherosclerosis. We found that, as expected, higher HDL-CEC was clustered with higher phospholipid contents, higher large HDL-P levels and less small

HDL-P, and larger overall HDL size to form the more "favorable" cluster. Even though HDL-Tg were significantly lower, whereas medium HDL-Ps were significantly higher in the favorable cluster, the probability of distribution among the 2 groups for these 2 metrics was close to 50% per group, thus segregation was not very conclusive for these metrics.

In our analysis, we found that the association between the favorable HDL cluster with lower subclinical atherosclerosis was not independent of BMI. Consistent with our findings, in a cohort of older adults from the Chicago Health Aging Study,[234] it was reported that higher HDL-CEC, but not HDL size, was associated with lower odds of necrotic carotid plaque, but this was not independent of traditional cardiovascular risk factors. In contrast, in MESA, higher cholesterol mass efflux capacity was weakly associated with higher odds of carotid plaque progression over 10 years.[230] Moreover, in MESA, Khera et al.[118] reported a significant inverse association between macrophage CEC and cIMT, independent of HDL-C and ApoA-I in a cohort of healthy White subjects. In another MESA study, Mora et al.[231] found that large HDL-P and HDL size were all inversely associated with cIMT. In a cross-sectional analysis of subjects with features of the metabolic syndrome,[94] total HDL-P, but not HDL-Tg, was inversely correlated with cIMT; HDL-Tg was higher in subjects with present carotid plaque. However, none of these studies adjusted for measures of obesity, such as BMI. Longitudinally,[232] MESA reported that HDL particle subclasses (by ion mobility) are not associated with annual changes in mean cIMT or carotid plaque score. It is noteworthy that none of these studies focused on midlife women.

In women, an analysis from MESA[69] [n=1,380 subjects, mean age 61.8 (10.3) years] reported higher total HDL-P by ion mobility was associated with lower cIMT, whereas higher HDL-C was associated with higher risk or carotid plaque. Moreover, small HDL-P and large HDL-P were both associated with lower cIMT, but the association between large HDL-P and cIMT was

only observed with longer time since the final menstrual period. The comparison between the findings of these studies and our results can be challenging, since we used an approach to cluster together a more comprehensive profile of HDL metrics early in midlife.

The HDL reduces the atherosclerotic burden mainly through its role in the reverse cholesterol transport, where cholesterol is removed from macrophages and transported to the liver for excretion in bile or storage.[2,73] Macrophages are the major cells involved in the process of HDL cholesterol efflux,[70] since these are the primary cells that accumulate cholesterol within the atherosclerotic plaque to form the foam cells. The individual HDL molecule contains varying concentrations of numerous lipids and proteins,[6] and different HDL particles vary in composition, size and shape. Studies have suggested a correlation between the size, contents and function of the HDL. For instance, with smaller HDL particle size, the abundance of cholesteryl esters and triglycerides increases on the surface of the HDL[71] altering the content of the HDL particle. Animal studies have suggested that the concentration of HDL-PL is a major modulator of cholesterol efflux. In vitro studies have shown that depleting the HDL of phospholipids reduces free cholesterol efflux, whereas enriching lipoproteins with phospholipids improves the efflux capacity.[95] Studies in humans have shown a high positive correlation between HDL-PL and cholesterol efflux capacity.[96] On the other hand, triglyceride-rich HDLs produce smaller HDL particles that are of higher catabolic rates, leading to a lower number of HDL particles.[97] Collectively, these findings support our findings of clustering of high HDL-CEC and HDL-PL, lower HDL-Tg and larger particles together in a favorable cluster.

Even though we did not see an association between HDL clusters and measures of subclinical atherosclerosis after adjusting for BMI, we found that SBP mediated the associations between HDL clusters and cIMT and IAD, but not plaque. The lack of observed associations in

the main models could be attributed to the opposite direction of effects of the associations mediated by SBP, which could mask the effect of exposure on outcome. These results indicate that a favorable HDL cluster was associated with lower SBP, which in turn, is associated with lower cIMT and IAD. The natural indirect effect represents the amount of the association mediated by SBP. The difference in the results between cIMT, IAD and plaque may be due to the different aspects of the atherosclerotic process that each of these measures represent. cIMT and IAD reflect the vascular adaptation to cumulative risk factors, such as hypertension or turbulent flow.[316] High systolic blood pressure increases shear stress and stretching of the arterial wall, leading to adaptations in the intimal and medial sections of the artery.[317] This leads to arterial dilation and reductions in arterial elasticity, increasing susceptibility to tears in the intima layer of the artery, and predisposing the intima to atherosclerotic changes[318] and thickening of the arterial wall. The HDL preserve the activity of endothelial nitric oxide synthase (eNOS), which reduces blood pressure,[319] which could explain why SBP appears to mediate the relationship between HDL metrics and cIMT and IAD, but not plaque. Plaque, on the other hand, is a more direct measure of atherosclerosis and represents focal thickening of the wall, whereas cIMT and IAD may reflect earlier subclinical vascular changes. The development of atherosclerosis is a life-long process, where it starts with asymptomatic vascular changes early in life and results in symptomatic lesions with older age,[320] thus, longer follow-up into older age may identify a relationship between HDL metrics and plaque presence.

Based on prior SWAN findings, which reported that the association between higher HDL-C and lower cIMT is only observed in Chinese and Hispanic women, but not in White and Black women,[229] we tested whether the associations between HDL clusters differed by race. We found that in Chinese women, the association between HDL clusters and cIMT persisted after adjustment

for potential confounders. Similar to the findings from this analysis, the associations between HDL-C and IAD and plaque presence did not differ by race/ethnicity. Previous analyses in SWAN have shown that Chinese women have generally better cardiometabolic risk profile compared to other racial and ethnic groups[229] including lower BMI and less smoking prevalence.

Some limitations to this study need to be considered. First, we used latent cluster analysis to classify HDL metrics for women. This statistical method of clustering does not handle continuous measures, and we had to divide HDL metrics into medians. It is important to note that there are no clinically validated cut points for these novel HDL metrics, and previous studies have used sample-based cut-points to investigate these HDL metrics.[234,321] Moreover, the posterior probabilities of cluster membership (Supplementary Table 1) ranged between 0.52 and 0.99, indicating that some metrics (such as HDL-Tg and medium HDL-P) did not do as well as others when segregating into groups. Our analysis was limited to Black, White and a small number of Chinese women. Thus, the results may not be generalizable to other ethnic groups. We could also have volunteer bias in this analysis, and the women included in SWAN and SWAN HDL may be healthier than the general population. Whether similar clustering would be observed in the general population needs to be assessed. Finally, we included one point of HDL assessment, which was before menopause. As we have previously shown that changes in HDL metrics may not be linear over the menopause transition, longitudinal studies with repeated HDL metrics should be performed to better show how changes in HDL metrics over menopause relate to subclinical atherosclerosis. The strengths of this analysis are in the novelty of the design, where a comprehensive profile of HDL metrics was assessed, and the prospective design of the analysis.

In conclusion, an HDL profile of higher cholesterol efflux capacity, higher phospholipids, and larger subclasses and size may be associated with a better subclinical atherosclerosis profile

after menopause. However, this is dependent on cardiometabolic risk factors such as BMI. The role of SBP on these associations needs to be investigated further, as it may play a role of mediating these associations. In Chinese women, who have better CVD risk factor profile than other racial/ethnic groups, the association was independent of BMI, suggesting that along with a better overall cardiovascular risk, a better HDL profile may play a role in future subclinical atherosclerosis. Optimizing of these HDL metrics may be achieved via a healthier lifestyle, where we have shown that in midlife women, a better cardiovascular health and health behaviors is associated with an improved HDL metrics profile (data not published). Other methods to optimize HDL metrics could be through novel interventions such as cholesterol poor reconstituted HDL,[322] Liver X receptors (LXR) activator treatment,[323] and Resverlogix.[324] These interventions are still early, and more research is needed to assess their effect on HDL metrics.

5.6 Tables and Figures

Table 5-1: Characteristics of women at SWAN HDL Baseline and at time of subclinical atherosclerosis assessment (n=461 women)

	SWAN HDL Baseline	Time of Subclinical Atherosclerosis	p-value
Age, years, mean (SD)	50.4 (2.7)	63.3 (3.7)	<0.0001
Race, n (%)			
White	272 (59.0%)	272 (59.0%)	-
Black	137 (29.7%)	137 (29.7%)	-
Chinese	52 (11.3%)	52 (11.3%)	-
Education, n (%)			
≤High School	76 (16.6%)	76 (16.6%)	-
Some College/College Graduate	240 (52.3%)	240 (52.3%)	-
Post-Graduate Degree	143 (31.2%)	143 (31.2%)	-
BMI, kg/m^2, median (Q1, Q3)	27.1 (23.5, 32.4)	28.4 (24.3, 33.6)	<0.0001
Physical Activity Score, mean (SD)	7.95 (1.70)	7.67 (1.80)	0.0002
Smoking Status, n (%)			0.32
Never Smoker	296 (64.5%)	295 (64.3%)	
Past/Current Smoker	163 (35.5%)	164 (35.7%)	
Menopause Status, n (%)			0.50
Premenopausal	51 (11.1%)	-	
Early perimenopausal	281 (61.0%)	2 (0.4%)	
Late perimenopausal	34 (7.4%)	2 (0.4%)	
Post (Natural/BSO)	68 (14.8%)	453 (98.3%)	
Unknown (HT/Hysterectomy)	27 (5.9%)	4 (0.9%)	
Systolic Blood Pressure, mmHg, mean (SD)	115.8 (16.5)	123.2 (16.3)	<0.0001

Diastolic Blood Pressure, mmHg, mean (SD)	73.9 (10.6)	75.1 (9.6)	0.01
Cardiovascular Disease Medications*, n (%)			<0.0001
No	380 (82.4%)	212 (49.0%)	
Yes	81 (17.6%)	221 (51.0%)	
cIMT, mm, median (Q1, Q3)	-	0.798 (0.730, 0.896)	-
IAD, mm, mean (SD)	-	7.33 (0.71)	-
Plaque Presence (≥ 2), n (%)	-		-
No	-	296 (64.6%)	-
Yes	-	162 (35.4%)	-

BMI: Body Mass Index; cIMT: carotid intima media thickness; IAD: inter-adventitial diameter

Cardiovascular Disease Medication use was defined as use of either anti-diabetics, anti-hypertensives and/or lipid-lowering medications.

Table 5-2: Characteristics of women at time of subclinical atherosclerosis by HDL clusters

Characteristics	**Unfavorable (n=249)**	**Favorable (n=212)**	**p-value**
Age, years, mean (SD)	63.8 (3.8)	62.7 (3.6)	**0.0002**
Race, n (%)			**0.0002**
White	155 (62.3%)	117 (55.2%)	
Black	80 (32.1%)	57 (26.9%)	
Chinese	14 (5.6%)	38 (17.9%)	
Education, n (%)			0.92
≤High School	41 (16.6%)	35 (16.5%)	
Some College/College Graduate	131 (53.0%)	109 (51.4%)	
Post-Graduate Degree	75 (30.4%)	68 (32.1%)	
BMI, kg/m^2, median (Q1, Q3)	30.2 (26.7, 35.4)	26.2 (22.1, 30.5)	**<0.0001**
Physical Activity Score, mean (SD)	7.46 (1.78)	7.91 (1.81)	**0.009**
Smoking Status, n (%)			**0.002**
Never Smoker	143 (57.9%)	152 (71.7%)	
Past/Current Smoker	104 (42.1%)	60 (28.3%)	
Systolic Blood Pressure, mmHg, mean (SD)	125.9 (15.7)	120.0 (16.4)	**<0.0001**
Diastolic Blood Pressure, mmHg, mean (SD)	76.2 (9.3)	73.9 (9.9)	**0.01**
CVD Medications, n (%)			**<0.0001**
No	91 (37.5%)	121 (63.7%)	
Yes	152 (62.6%)	69 (36.3%)	
cIMT, mm, median (Q1, Q3)	0.814 (0.742, 0.917)	0.782 (0.711, 0.867)	**0.003**
IAD, mm, mean (SD)	7.42 (0.74)	7.23 (0.66)	**0.005**
Plaque Presence (≥ 2), n (%)			**0.02**
No	147 (59.8%)	149 (70.3%)	
Yes	99 (40.2%)	63 (29.7%)	

Table 5-3: Associations between HDL clusters and measures of subclinical atherosclerosis

	cIMT (mm)		IAD (mm)		Plaque Presence	
	Adjusted Means (95% CI)*	**p-value**	**Adjusted Mean (SE)**	**p-value**	**OR (95% CI)**	**p-value**
Model 1		0.001		0.005		0.02
Unfavorable	0.824 (0.808, 0.839)		7.42 (0.05)		1.00 (Ref)	
Favorable	0.787 (0.771, 0.803)		7.23 (0.05)		0.63 (0.43, 0.93)	
Model 2		0.03		0.04		0.06
Unfavorable	0.820 (0.799, 0.841)		7.42 (0.06)		1.00 (Ref)	
Favorable	0.796 (0.777, 0.815)		7.28 (0.06)		0.67 (0.45, 1.02)	
Model 3		0.34		0.59		0.36
Unfavorable	0.814 (0.794, 0.834)		7.40 (0.06)		1.00 (Ref)	
Favorable	0.803 (0.783, 0.823)		7.36 (0.06)		0.82 (0.53, 1.26)	

Model 1: Unadjusted; Model 2: Adjusted for study site, race/ethnicity, time between HDL measure and measure of subclinical atherosclerosis and age at time of outcome; Model 3: Model 2 + BMI, smoking and physical activity score at time of outcome

* Presented as adjusted mean (95% CI) since cIMT was log-transformed in original models.

Table 5-4: Interaction by ethnicity for the associations between HDL clusters and cIMT

	Estimates for favorable compared to unfavorable HDL cluster by ethnicity*		P-difference compared to Chinese	P-interaction between race/ethnicity and HDL cluster
	Adjusted Means (95% CI)*	**p-value**		
White		0.73	0.02	0.05
Unfavorable	0.794 (0.773, 0.815)			
Favorable	0.790 (0.768, 0.811)			
Black		0.71	0.02	
Unfavorable	0.860 (0.825, 0.895)			
Favorable	0.869 (0.832, 0.908)			
Chinese		0.005	-	
Unfavorable	0.820 (0.703, 0.955)			
Favorable	0.721 (0.630, 0.825)			

Model adjusted for site, time between HDL measure and measure of subclinical atherosclerosis, age at time of outcome, BMI, smoking and physical activity score at time of outcome

* Adjusted means obtained by analysis stratified by race/ethnicity

Table 5-5: Mediation analysis of systolic blood pressure on the association between HDL clusters with cIMT and IAD

	Total Effect		Controlled Direct Effect		Natural Indirect Effect		% Mediated*
	β (SE)	p-value	β (SE)	p-value	β (SE)	p-value	
Log-cIMT	-0.013 (0.01)	0.34	-0.008 (0.01)	0.57	-0.005 (0.003)	0.05	40.9%
IAD	-0.037 (0.07)	0.58	-0.006 (0.07)	0.92	-0.030 (0.02)	0.05	82.4%

Model adjusted for study site, time between HDL measure and measure of subclinical atherosclerosis, age at time of outcome, BMI, smoking and physical activity score at time of outcome

Total Effect represents the effect of HDL clusters on the various measures of subclinical atherosclerosis. Controlled direct effect represents how much each outcome would change on average, if systolic blood pressure was fixed, and for favorable versus unfavorable HDL clusters. Natural indirect effect represents the effect of HDL clusters on measures of subclinical atherosclerosis that is mediated by systolic blood pressure.

* Calculated as: Natural Indirect Effect/Total Effects x 100%.

5.7 Supplementary Tables

Table 5-6: Supplementary Table 1: Classification of women by median into favorable/unfavorable

	Unfavorable Cluster n=249 (54%)	Favorable Cluster n=212 (46%)
HDL-CEC		
Low Median	0.666	0.299
High Median	0.334	0.701
HDL-PL		
Low Median	0.760	0.161
High Median	0.240	0.839
HDL-Tg		
Low Median	0.477	0.570
High Median	0. 523	0.430
Large HDL-P		
Low Median	0.913	0.011
High Median	0.087	0.989
Medium HDL-P		
Low Median	0.571	0.411
High Median	0.430	0.589
Small HDL-P		
Low Median	0.344	0.689
High Median	0.656	0.312
HDL Size		
Low Median	0.888	0.124
High Median	0.112	0.876

Median Cut-offs: HDL-CEC<3.887%; HDL-Tg<17 mg/dL; HDL-PL <54 mg/dL; Large HDL-P <8 µmol/L; Medium HDL-P <10.9 µmol/L; Small HDL-P <15.5 µmol/L; HDL size <9.5 nm

Table 5-7: Supplementary Table 2: HDL metrics at Time of HDL Assessment by Clusters

	Unfavorable (n=249)	Favorable (n=212)	p-value
HDL-CEC, %, mg/dL, mean (SD)	3.69 (0.55)	4.27 (0.71)	<0.0001
HDL-Tg, mg/dL, median (Q1, Q3)	18 (15, 22)	16 (14, 21)	0.03
HDL-PL, mg/dL, mean (SD)	48.8 (7.3)	61.9 (9.9)	<0.0001
Total HDL-P, µmol/L, mean (SD)	33.7 (6.0)	36.9 (6.2)	<0.0001
Large HDL-P, µmol/L, mean (SD)	5.8 (1.8)	11.4 (2.4)	<0.0001
Medium HDL-P, µmol/L, median (Q1, Q3)	10 (6, 13.9)	12 (8.8, 17.1)	<0.0001
Small HDL-P, µmol/L , mean (SD)	17.4 (6.7)	12.4 (6.9)	<0.0001
HDL Size, nm, mean (SD)	9.16 (0.35)	9.94 (0.36)	<0.0001
HDL-C, mg/dL, mean (SD)	50.4 (8.8)	69.5 (12.2)	<0.0001

6.0 Manuscript 3: Early midlife cardiovascular health influences future HDL metrics in women: The SWAN HDL Study

6.1 Abstract

Background: Utility of high-density lipoprotein cholesterol (HDL-C) in assessing the anti-atherogenic properties of HDL may be limited in midlife women. Novel metrics of HDL function, lipid contents and subclasses may better reflect the atheroprotective capacities of HDL, supporting the need to evaluate how cardiovascular health affects these metrics in women. We assessed the relationship of early midlife Life Simple 7 (LS7) score and its health behavior components with future HDL function [cholesterol efflux capacity (HDL-CEC)], HDL-phospholipid (HDL-PL) , HDL-triglyceride (HDL-Tg), HDL particles (HDL-P) and size, and the relationship between LS7 score and changes in HDL metrics over time.

Methods: We analyzed 529 women [baseline age: 46.4 (2.6) years, 57% White] from the SWAN HDL study who had baseline LS7 followed by future repeated HDL metrics. Multivariable linear mixed models were used.

Results: Higher LS7 score was associated with favorable future HDL profile (higher HDL-PL, total HDL-P and large HDL-P, lower HDL-Tg and larger overall HDL size). Ideal body mass index (BMI) was associated with higher HDL-CEC, HDL-PL, and large HDL-P, lower HDL-Tg and small HDL-P and larger overall HDL size. Ideal physical activity was associated with higher HDL-PL, total, large and medium HDL-P. Ideal smoking was associated with less HDL-

Tgs. Diet was not related to HDL metrics. Higher LS7 score and ideal BMI were associated with slower progression of HDL size over time.

Conclusion: Novel HDL metrics may better reflect the clinical utility of HDL. Improving lifestyle at midlife, particularly maintaining ideal BMI, is associated with better future HDL phenotype.

6.2 Introduction

The midlife period in women is often accompanied by an increase in risk of cardiovascular disease (CVD) and in cardiometabolic risk factors.[275] Independent of aging, the menopause transition (MT) plays a critical role in accelerating CVD risk. During the MT, changes in lipoproteins, body fat composition, metabolic syndrome and vascular health contribute to the increased risk of CVD, the leading cause of death in midlife and older women.[275]

Previous studies that have assessed the alterations in the lipoprotein profile around the MT have consistently shown that this period is accompanied by increases in total cholesterol, large-density lipoproteins cholesterol (LDL-C), and apolipoprotein-B (ApoB).[66,289] However, changes in high-density lipoproteins (HDLs) are more complex. Earlier cross-sectional studies have shown that levels of HDL-cholesterol (HDL-C) do not differ by menopause status or are lower in postmenopausal compared to premenopausal women.[64,65] Nonetheless, more recent longitudinal studies have shown that HDL-C may increase after menopause,[10] and that higher HDL-C after menopause, but not before, is associated with higher risk of CVD.[8,68,69,325] This cluster of observations suggests that HDL may lose its athero-protective function during the MT. Additionally, lifestyle and pharmacological interventions that raise HDL-C have failed to show a beneficial effect on reducing risk of CVD.[326]

HDLs are complex particles that vary in composition, size and function. The major anti-atherogenic function of HDL is through its role in the reverse cholesterol transport process,[2,30] where cholesterol is removed from cells for clearance. Studies have suggested that novel metrics, which directly evaluate the function, lipid contents and subclasses distribution of the HDL, may provide a better understanding of the cardioprotective qualities of the HDL compared to HDL-C.

Despite some inconsistencies in certain studies,[109,113] findings have suggested that higher cholesterol efflux capacity and more phospholipid content within the HDL, as well as a larger subclass distribution are associated with a cardio-protective risk profile.[111,118,122,123]

In efforts to measure CVD burden in adults, the Goals and Metrics Committee of the Strategic Planning Task Force of the American Heart Association (AHA) introduced a comprehensive new metric in 2011 to monitor the cardiovascular health (CVH) of all Americans through 2020.[238] This CVH metric, termed the Life's Simple 7 (LS7), encompasses a set of seven components: 4 health behaviors [body mass index (BMI), physical activity, smoking status and diet quality] and 3 health factors [blood pressure, total cholesterol and fasting blood glucose].[238] A higher LS7 score is associated with reduced CVD-related morbidity and mortality.[327] However, only 3% of the US population in 2016 had ideal scores on ≥5 components of the LS7,[328] and CVD remains the most common cause of mortality in men and women.[328] This indicates that more action is needed to successfully achieve reductions in CVD morbidity and mortality.

Behavioral modifications and lifestyle changes are linked to increases in HDL-C in different populations.[329] However, the lack of consistent associations of HDL-C with CVD, particularly in midlife women, raises the question whether modifiable health behaviors affect other direct measures of HDL. Thus, in this analysis, we aimed to assess whether a better LS7 score and the modifiable health behavior components of the score (BMI, physical activity, diet and smoking) early in midlife are associated with a better future HDL metric profile, and whether this early-midlife LS7 score is associated with anti-atherogenic changes in HDL metrics over the MT. We hypothesized that a higher LS7 score and ideal BMI, physical activity, smoking and diet status at early midlife will be associated with higher HDL-CEC, higher phospholipid and less triglycerides contents, more large and less small HDL subclasses, and a larger overall HDL size in the future as

women traverse menopause, and that a better LS7 score and ideal health behavior components will be associated with favorable changes in HDL metrics over time.

6.3 Subjects and Methods

SWAN is an ongoing, multi-site, multi-ethnic, longitudinal study that aims to characterize the physiological and psychological changes as women traverse menopause. The design of the SWAN study has been previously described.[278] In brief, 3,302 women aged 42 to 52 years were recruited between 1996 and 1997 at seven different sites across the United States: Pittsburgh, PA; Detroit, MI; Chicago, IL; Los Angeles, CA; Oakland, CA; Boston, MA; and Newark, NJ. Women were eligible for recruitment to SWAN if they had an intact uterus and at least one ovary, were not pregnant or lactating at the time of recruitment, had at least one menstrual period within the last 3 months prior to recruitment, were not on hormone replacement therapy, and self-identified as either White, Black, Hispanic, Chinese, or Japanese.

The SWAN HDL study is an ancillary study to SWAN. This ancillary study aims to characterize the changes of HDL function and composition that accompany ovarian aging, and to understand how these changes influence the athero-protective associations of HDL in women as they progress through the MT. For SWAN HDL, frozen serum samples from 558 SWAN women (1,461 samples over the study period) were used to quantify the function, lipid content and subclasses of HDL. Women were selected into SWAN HDL if they had participated in at least one visit before and 2 visits after the final menstrual period, with available blood samples at the selected visits.[10]

For this analysis, 29 women who had missing data on the LS7 metrics at SWAN baseline visit were excluded. The final analysis included 529 women who had baseline LS7 score (SWAN visit 0), followed by at least one HDL metric at a later visit (coinciding with SWAN follow-up visits 1, 3-9 and/or 12). Out of the 529 women, 13 had HDL metrics measured once, 220 had HDL metrics measured twice, 258 had HDL metrics measured 3 times, 31 women had HDL metrics measured 4 times and 7 had HDL metrics measured 5 times over midlife. Written informed consent was provided by all participants prior to enrollment in SWAN, and the study protocols were approved by the institution review board at each study site.

Blood Data Collection:

Phlebotomy was performed after a minimum of 10-hour overnight fast. The blood draw was scheduled 2-5 days after a spontaneous menstrual bleed when possible or randomly within 90 days of the annual SWAN visit when the date of the menstrual cycle could not be determined. Stored samples that have been frozen at -80°C and never been thawed before were used for SWAN HDL assays to enhance the validity of results.

HDL cholesterol efflux capacity (HDL-CEC) and phospholipids (HDL-PL) and triglycerides (HDL-Tg) contents:

HDL-CEC, HDL-PL and HDL-Tg were measured at a CDC-certified lipid lab at the University of Pennsylvania. HDL-CEC was measured by the efflux of fluorescence-labeled cholesterol as has been previously described.[122] In summary, J774 mouse macrophage cells were plated and labeled with 2 μCi/mL of 3H cholesterol overnight. The cells were then incubated for 4 hours in the presence of 0.3 mM 8-(4-chlorophenylthio)-cyclic AMP (cAMP), an upregulator of ATP-binding cassette transporter-1 (ABCA1). To create apolipoprotein B (ApoB)-depleted plasma, lipoproteins containing ApoB were removed from plasma by polyethylene glycol

precipitation. Cells were then incubated for 2 hours with the equivalent of 1% ApoB- depleted serum or plasma at a 37°C. Cells incubated with media alone were used as the baseline controls. To remove the cell debris and quantify radioactivity by liquid scintillation counting, each medium was collected and passed through a 0.22 μM filter. Isopropanol extraction was then used to quantify radioactive cellular cholesterol that was incorporated into the cellular lipids. Percent efflux capacity was calculated by the following formula: [(cpm of 3H cholesterol in the media - cpm of 3H cholesterol in serum free media) / (cpm of 3H cholesterol in the cells + cpm of 3H cholesterol in the media)] × 100. Samples from participants were normalized to a pooled plasma control that was included on each plate. The intra and inter-assay coefficients of variation were 3.7% and 10.1% respectively.

For HDL-PL and HDL-Tg measurement, HDL was isolated from serum by phosphotungstic acid precipitation (FujiFilm Wako Pure Chemical Corporation). HDL-PL and HDL-Tg were then measured by the Roche Cobas C311 clinical analyzer according to manufacturer's protocol (Wako: 433-36201 and Roche: 20767107322, respectively). The inter-assay coefficients of variation for HDL-PL and HDL-Tg were 3.5% and 3.9%, respectively.

HDL Subclasses by Nuclear Magnetic Resonance (NMR) Spectroscopy:

The number of HDL subclasses and overall HDL size were measured by the Nuclear Magnetic (NMR) Spectroscopy LipoProfile-3 algorithm[281] using the Vantera Clinical Analyzer, an automated 400 MHz NMR spectroscopy platform. Lipoprotein particle quantification by NMR utilizes composite signal envelopes at 0.8 ppm, which contain the signals emitted by the terminal methyl group protons of the HDL contents (phospholipids, triglycerides, cholesteryl ester and unesterified cholesterol) that are carried in each HDL particle. Signal amplitudes that contribute to the composite plasma signal are produced as a result of the deconvolution of the composite

signal. Each lipoprotein subclass produces unique NMR signals, which are specific in frequency and shape. The amplitude of the signal is proportional to the number of particles that are releasing the signal. The line shape of the signal envelope is modeled as a sum of all lipoprotein signals to obtain the amplitude of each subpopulation of subclasses. To quantify the concentration of each subclass, areas of different subpopulations were multiplied by conversion factors and the subclasses were then grouped into small (7.3-8.2 nm), medium (8.2-9.4 nm) or large (9.4-14.0 nm) HDL subclasses. The total HDL particle concentration was calculated as the sum of the concentrations of individual subclasses. The average size of HDL particles was calculated by adding the diameter of each subclass multiplied by its relative mass percentage from NMR signal amplitude. Due to the magnetic property of lipoproteins, which produces signals of different shapes and frequencies for different lipoproteins, NMR spectroscopy does not require the separation of lipoprotein subclasses as is required by electrophoresis or ultracentrifugation. The intra- and inter-assay coefficients of variation for HDL-P concentrations and size ranged from 0.6% to 3.7% (intra-assay) and 1.5% to 4.0% (inter-assay).

HDL-C and Apolipoprotein A-I (ApoA-I):

Lipid fractions were determined in EDTA-treated plasma. From SWAN baseline visit until follow-up visit 7, fasting HDL-C[282,283] was separated with heparin-2M manganese chloride, whereas ApoA-I was measured using the immunonephelometry using Behring reagents on the Behring Nephelometer II at the Medical Research Laboratory (Lexington, KY). For SWAN follow-up visits 9 and 12, fasting HDL-C was separated with heparin-2M manganese chloride at the University of Michigan Pathology (Ann Arbor, MI), and ApoA-I was measured by using reagents from Beckman-Coulter (Brea, Ca) at the University of Pittsburgh Heinz lab. The results

of HDL-C and ApoA-I from University of Michigan Pathology and the University of Pittsburgh Heinz lab were calibrated by converting them to equivalent Medical Research Laboratory values.

American Heart Association's Life's Simple 7 (LS7):

The LS7 was evaluated at the SWAN baseline visit. Each component of the LS7 (BMI, physical activity, smoking, diet, total cholesterol, fasting blood glucose and blood pressure) was categorized into either ideal (score=2), intermediate (score=1) or poor (score=0), based on AHA guidelines[238] and as described in **Table 1**. A total LS7 score was calculated by summing the score of the each of the seven components. In SWAN study, the LS7 components were assessed as follows:[330]

Body Mass Index:

BMI was calculated as measured weight (kg)/height $(m)^2$.

Physical Activity:

The physical activity component was assessed by the Kaiser Physical Activity Survey (KPAS), which is an adaptation of the Baecke physical activity questionnaire.[314] Participants were asked about the frequency, duration and intensity of sports. Women were classified based on the minutes per week of activity for ≥9 months/year.

Smoking Status:

Smoking status was derived from self-reported questionnaires where women were asked about their current smoking status, if they had stopped smoking and the month and year when they last smoked.

Diet Quality:

The diet component was assessed by the food frequency questionnaire (FFQ). Five components contributed to the diet quality score: fruits and vegetables, fiber-rich whole grains, fish consumption, sugar sweetened beverages and daily sodium intake. In the context of a healthy dietary pattern that is consistent with a Dietary Approaches to Stop Hypertension (DASH)–type, consuming ≥4.5 cups/d of fruits and vegetables, ≥2 servings/week of fish, ≥3 servings/d of whole grains, ≤36 oz/week of sugar-sweetened beverages and ≤1500 mg/d of sodium was considered within target. Diet was classified into ideal, intermediate or poor based on the number of components met.

Blood Pressure:

Systolic and diastolic blood pressure were measured according to a standardized protocol, with readings taken on the right arm, while the subject was seated with feet flat on the floor for at least 5 minutes prior to measurement. An average of two sequential blood pressure was reported. Blood pressure treatment was determined from self-administered questionnaires.

Total Cholesterol:

Total cholesterol was quantified at Medical Research Laboratory at SWAN baseline visit. The Hitachi 747-200 clinical analyzer was used to measure total cholesterol by an automated cholesterol oxidase assay.[66] Lipid treatment was determined by self-administered questionnaires.

Fasting Blood Glucose:

Fasting blood glucose was quantified at Medical Research Laboratory at SWAN baseline visit. The Hitachi 747-200 clinical analyzer was used to measure fasting blood glucose by the hexokinase reaction.[66] Fasting blood glucose levels at SWAN baseline visit were calibrated to increase comparability with later SWAN glucose measures, which were measured at other laboratories. Anti-diabetic medication use was determined by self-administered questionnaires.

Study Covariates:

Study covariates were assessed at the SWAN baseline visit, coinciding with the time of LS7 assessment. Race, education status, economic hardship (defined as difficulty paying for basics), and alcohol use were self-reported. Race was categorized as White, Black or Other (Hispanics, Chinese or Japanese). Age was calculated as the difference between the visit date and the date of birth. Time between HDL measures and the LS7 measure was calculated as the difference between the dates of the two visits. Menopause status was self-reported based on bleeding patterns over the past 12 months, and was categorized as either premenopausal (no changes in menstrual bleeding patterns over the previous 12 months) or early perimenopausal (at least one bleed within the last 3 months with some perceived changes in menstrual cycle intervals). C-reactive protein (CRP) was measured at the Medical Research Laboratory on serum or plasma collected at SWAN baseline visit by immunonephelometry using Behring reagents on the Behring Nephelometer II. Medical Research Laboratory results were calibrated to the high-sensitivity (hs-CRP) ELISA assay. A random sample from SWAN women was selected and the distribution of CRP within different menopausal status groups, race/ethnicity groups and study visits were checked to confirm a representative sample of the SWAN cohort, and calibration equations were applied.

Statistical Analysis:

Descriptive statistics including mean (SD), median (Q1, Q3) or frequencies (%) were used to summarize participants' characteristics at the SWAN baseline visit, as appropriate. Distribution of continuous variables was assessed, and skewed variables (HDL-Tg and hs-CRP) were log-transformed to reduce skewness. Due to the small number of women in the intermediate smoking

and physical activity categories, poor and intermediate women for these components were combined in regression analysis.

Linear mixed models with random intercepts were used to evaluate the independent associations between baseline LS7 score and repeated measures of each HDL metric later in life. Potential confounders were added to the models and the parsimonious model with the best fit was chosen based on likelihood ratio tests. Final models were adjusted for time between LS7 score and HDL metrics measures, race, education status, and the following covariates at baseline: age, economic hardship, menopause status, alcohol use and log-transformed hs-CRP.

To investigate the associations between each health behavior component at baseline and HDL metrics later in life, linear mixed models with random intercepts were used to model baseline BMI, physical activity, smoking and diet quality categories, separately, in relation to repeated measures of HDL metrics in final models. In these analyses, HDL metrics were modeled as z-scores to increase the comparability of the metrics between the different components. Z-scores were calculated for each HDL metric from the mean and standard deviation.

To assess whether baseline LS7 score is associated with changes in future HDL metrics, random-intercept linear mixed effect models with repeated measures of each HDL metric were fitted as a function of the baseline LS7 score, time since first HDL measure and their interaction in final models. Time since first HDL measure was calculated as the time difference between each repeated HDL metric and the first available HDL metric. The beta estimate of the interaction represents the yearly change in each HDL metric per 1 standard deviation increase in LS7 score. To assess whether baseline health behavior components are associated with changes in future HDL metrics, random-intercept linear mixed effect models of repeated measure of each HDL metric were fitted as function of each health behavior component, time since first HDL measure and their

interaction. The beta estimate for the interaction represents the yearly change in the z-score of the HDL metric per group (ideal, intermediate or poor).

All analyses were run using SAS v9.4 (SAS Institute, Cary, NC).

6.4 Results

Characteristics of women included in this analysis at the SWAN baseline visit:

The baseline characteristics of women included in this analysis are presented in **Table 2**. Women, on average, were 46.4 (SD: 2.6) years old, mostly White (56.5%) or Black (26.7%), and were all premenopausal or early perimenopausal at time of LS7 assessment. The average LS7 score was 8.5 (SD: 2.0), which is ranked as intermediate, and ranged between 2 and 13. Time between the LS7 assessment and the first HDL metric measure was 3.9 (SD: 1.4) years.

Thirty-five percent of SWAN women had an ideal LS7 score (between 10-14), 46% had ideal BMI and 88% had an ideal smoking status (**Figure 1**). However, no woman achieved ideal diet quality and 79% had poor physical activity status.

Associations between baseline LS7 score with HDL metrics later in life:

Associations between baseline LS7 score and future metrics of function and contents, HDL-C and ApoA-I are presented in **Table 3**. In the univariate analysis (Model 1), a higher LS7 score was associated with higher HDL-CEC, HDL-PL, HDL-C and ApoA-I, and lower HDL-Tg. After adjusting for potential confounders (Model 2), the results for HDL-CEC were attenuated, but the other adjusted associations remained statistically significant.

Associations between baseline LS7 score and future HDL subclasses and size are presented in **Table 4**. In the univariate analysis (Model 1), a higher LS7 score was associated with higher concentrations of total HDL-P, large HDL-P, and medium HDL-P, lower small HDL-P, and larger overall HDL size. Adjusting for potential confounders (Model 2), the results for medium and small HDL-P were attenuated, but the other associations remained statistically significant.

Associations between baseline health behavior components and HDL metrics later in life:

The significant associations between baseline health behavior components and HDL function and contents are presented in **Figure 2**. In final models, an ideal BMI was associated with higher HDL-CEC (p-trend=0.0002), higher HDL-PL levels (p-trend=0.0006) and lower HDL-Tg (p-trend <0.0001). Ideal physical activity status was associated with higher HDL-PL (p-trend=0.01). Ideal smoking status was associated with lower HDL-Tg (p-trend=0.001). Diet was not associated with any of the HDL function and content metrics.

The significant associations between the baseline health behavior components and HDL subclasses and size are presented in **Figure 3**. Ideal BMI was associated with higher large HDL-P levels and larger HDL size (p-trends <0.0001 for both), and less small HDL-P levels (p-trend=0.02). Ideal physical activity was associated with higher total HDL-P (p-trend <0.0001), large HDL-P (p-trend=0.04) and medium HDL-P (p-trend=0.02). Diet and smoking were not associated with HDL subclasses and size.

Ideal BMI and physical activity were associated with higher HDL-C and ApoA-I concentrations **(Figure S1).** For clinically interpretable results, the adjusted means of HDL metrics for each health behavior component are presented in **Table S1**.

Association of baseline LS7 score and individual components of health behaviors with changes in HDL metrics since first HDL measure:

In final models, higher baseline LS7 score was associated with a slower increase in HDL size as time progressed (p-interaction=0.005; **Table S2**). Compared to women with ideal BMI, women in the poor BMI group had larger increases in HDL size (p-interaction=0.006**, Figure 4a**) over time. Medium HDL-P did not significantly change with time within each physical activity group, but the interaction with time was significant between women with ideal compared to poor/intermediate physical activity (p-interaction=0.02, **Figure 4b**), indicating that the latter group had a larger increase in medium HDL-P compared to the former.

6.5 Discussion

In this cohort of midlife women, better CVH is associated with a favorable profile of HDL function, lipid content and subclasses distribution. Healthier modifiable lifestyle risk factors during early midlife are linked to higher future cholesterol efflux capacity and phospholipid contents, lower triglyceride contents, and a shift of the subclasses distribution towards larger HDL particles. In particular, poorer BMI status appears be associated with lower anti-atherogenic cholesterol efflux capacity of the HDL, lower phospholipid contents and smaller size of HDL subclasses. A sedentary lifestyle with lack of sufficient physical activity is associated with lower phospholipid contents, whereas smoking is associated with higher triglyceride contents of the HDL. In light of the current uncertainty of the clinical utility of HDL-C in midlife women, the current findings

provide critical information on modifiable risk factors that can improve other metrics of HDL that could be more relevant to midlife women.

Despite the perception that HDL-C is associated with reduced CVD morbidity and mortality, most recent clinical guidelines have discarded HDL-C as a target for primary and secondary prevention.[3,4] Whether novel metrics of HDL could be potential targets to guide novel CVD therapies and preventive strategies is still unclear. This is of particular importance in midlife women when a loss in the athero-protective functions of the HDL, which is not captured by HDL-C, may occur. We have previously shown that the MT is accompanied by a decline in large HDL-P and overall HDL size, an increase in HDL-Tg and small HDL-P, and a decline in HDL function close to the final menstrual period.[10,136] This HDL profile has been linked to worse CVD risk in various cohorts,[111,118,122,123] including midlife women.[331] Moreover, in SWAN HDL, higher estradiol in midlife was linked to higher HDL-CEC, larger HDL subclass distribution and higher HDL-Tg; however, the positive association between higher estradiol and HDL-Tg was strongest after 2 years from the final menstrual period.[130] These results indicate a potential loss of the protective effects of estradiol on HDL as women transition into menopause. This has been corroborated in SWAN, where the presumably protective association between HDL-C on arterial calcification was only apparent with high estradiol levels, but tended to be the opposite at lower levels of estradiol.[325] It is important to understand how other interventions, including behavioral modifications, may impact other metrics of HDL found to be more relevant to midlife women.

Our results propose that lifestyle factors during midlife are linked to the functionality and composition of HDL. **Table S3** summarizes potential pathways linking BMI, physical activity, and smoking with HDL metrics. In particular, a healthy midlife BMI appears to be strongly associated with better cholesterol efflux capacity, higher phospholipids, and more large HDL-Ps.

These results are consistent with previous findings on the relation between weight and HDL metrics in different cohorts. In the INTERLIPID and Insulin Resistance Atherosclerosis Study cohorts, higher BMI was associated with more small and medium HDL-P, smaller overall HDL size, and less concentrations of large HDL-P.[252,253] However, in the Multi-Ethnic Study of Atherosclerosis (MESA) cohort, HDL cholesterol mass efflux did not differ by BMI classes.[230] The differences in findings may be due to the cross-sectional design of the analysis, the fact that groups were compared by unadjusted ANOVA, or the variation in BMI categorization compared to our study. Moreover, this study included both men and women, and did not assess whether this relation differed by sex.

An increase in activity of enzymes linked to HDL metabolism, such as cholesterol ester transfer proteins (CETP) and hepatic lipase (HL) may be observed in states of obesity.[332] CETP mediates the transfer of cholesteryl esters from HDL particles to triglyceride-rich proteins, which are then taken up by the liver, in exchange for triglycerides.[78] Triglyceride-rich HDLs produce smaller HDL particles.[97] Smaller HDL particles, elevated triglyceride content and diminished phospholipids in the particle, in turn, could lead to detrimental effects on the cholesterol efflux capacity and function of the HDL.[76,290]. This is of particular relevance to midlife women, since women gain significant weight as they transition through midlife.[288] It is important to note that BMI was identified as a health behavior in the original LS7 score,[238] but BMI is a biological variable that is consequence of genetic factors, energy intake, and energy expenditure, among others.

Furthermore, in our study, higher physical activity was associated with more phospholipid and less triglycerides content and higher concentrations of total, large and medium HDL-Ps. Physical activity, whether aerobic or resistance, has been linked to lower CVD risk in the general

population. In the Women On the Move through Activity and Nutrition (WOMAN) study, higher leisure physical activity was associated with higher total HDL-P and larger HDL size,[267] Exercising has been linked to reductions in CETP concentrations and increases in nitric oxide (NO) bioavailability,[268] and to increases in lipoprotein lipase activity and alterations in immune and endothelial function.

Our study showed that in women, smoking was associated with higher triglyceride contents in the HDL, but not associated with other HDL metrics. In healthy participants from the MESA study, Shea et al.[230] reported that HDL cholesterol mass efflux capacity did not differ by smoking status. However, in women from the INTERLIPID study,[252] smoking more cigarettes was associated with less total and large HDL-P by NMR, despite no differences in HDL-C. Smoking increases the activity of CETP[259] and HL,[262] and could inhibit the activity and concentration of lecithin–cholesterol acyltransferase (LCAT),[260] impairing HDL maturation, and potentially reducing levels of larger HDL particles.[261] The lack of associations between smoking and other metrics of HDL, particularly HDL subclasses, may be due to the small number of smokers in this analysis were, resulting in lower power to detect between-group differences for other HDL metrics. We did not find any significant associations between diet and HDL metrics in women. It is important to note that in our study population, no women achieved ideal diet, which could explain the lack of observed associations with HDL metrics between groups. This pattern has been reported previously in SWAN women.[330] Despite the fact that we did not observe an association between diet and HDL metrics, this finding of low frequency of healthy diet in midlife women calls attention to the need for more awareness about diet in US women. Moreover, investigating the associations between HDL metrics and dietary patterns is not straightforward due to the complex nature of dietary habits, since food and nutrients are not consumed as single components or in

isolation. Further, we used the food frequency questionnaire to categorize diet based on DASH patterns. Other approaches to diet assessment may result in different findings.

Additionally, we found that lower BMI and increased physical activity were associated with a smaller increase in HDL size and a larger increase in medium HDL-P, respectively. This is in an unexpected direction to we hypothesized. However, in the SWAN HDL ancillary study, we have reported that the changes in HDL metrics in midlife women are not linear over the MT,[10] thus a linear association may not reflect the actual changes in HDL metrics over time. Moreover, SWAN has previously reported that large HDL-P increases after menopause[136] and that greater levels of large HDL-P and a larger HDL size could be associated with less HDL-CEC close to menopause, suggesting a decline in the efficiency of the larger HDLs to promote efflux capacity,[10] and indicating a potential dysfunctionality in larger HDLs close to menopause. Similar findings were also observed in women from MESA study.[69]

The results of this analysis should be interpreted with care as some potential limitations exist. Except for BMI, the SWAN HDL study had a small number of participants in the intermediate CV health groups, thus we had to combine the poor and intermediate groups. Given the likely larger differences expected to exist between the poor and ideal groups, this may have biased the results towards the null. Moreover, smoking status, diet and physical activity were assessed by self-reported questionnaires. This may have introduced recall bias. We also included one measure of HDL function; however, lifestyle factors may affect other functions of the HDL such as the anti-oxidative and anti-inflammatory capacities, which were not measured in this study. The results may not be generalizable to all racial/ethnic groups, particularly Hispanics, due to the small number of Hispanics included in this analysis. The major strengths of this analysis are the inclusion of a comprehensive profile of HDL properties, which was measured repeatedly over the

MT. CVH was measured by the AHA's LS7 score, which has been frequently investigated in relation to future CVD. This measure is simple, easy, and could be readily computed in research and clinical settings. Moreover, this score provides a simultaneous evaluation of several lifestyle factors that are often interdependent.

In summary, achieving better CVH during early midlife may be tied to a better future HDL metric profile in women. This indicates that following a healthy lifestyle during early midlife may reverse or limit the dysfunction that occurs in the HDL as women transition through menopause. Even though women, in general, are advised to follow a healthy lifestyle including maintaining a healthy weight, being physically active, eating a healthy diet and avoiding smoking, only 20% of adult American women have an ideal score on more than 5 CVH metrics.[333] Future studies, and particularly interventional studies, should assess whether modifying lifestyle factors would impact HDL metrics and, potentially, reduce burden of future CVD. Since behavioral modifications and lifestyle changes are the initial targets for CVD prevention and risk reduction, assessing whether these lifestyle factors contribute to the prevention of the HDL dysfunction around menopause would be compelling.

6.6 Tables and Figures

Table 6-1: Definitions of Poor, Intermediate and Ideal Cardiovascular Health for each Life's Simple 7 Goal in Adults[237]

	Level of Cardiovascular Health for Each Metric		
	Poor	**Intermediate**	**Ideal**
Current Smoking	Yes	Former, quit ≤ 12 months	Never or quit > 12 months
Body Mass Index	≥30 kg/m^2	25-29.9 kg/m^2	<25 kg/m^2
Physical Activity	None	1-149 min/week of moderate or 1-74 min/week of vigorous or 1-149 min/week moderate +2x vigorous	≥ 150 min/week of moderate or ≥75 min/week of vigorous or ≥150 min/week of moderate + 2x vigorous
Diet Pattern (No. of components of AHA diet score)*	0-1 (0-39)	2-3 (40-79)	4-5 (80-100)
Total Cholesterol	≥240 mg/dL	200-239 mg/dL or treated to goal	<200 mg/dL
Blood Pressure	SBP ≥ 140 mmHg or DBP ≥ 90 mmHg	SBP 120-139 mmHg or DBP 80-89 mmHg or treated to goal	SBP <120 and DBP <80 mmHg
Fasting blood glucose	≥126 mg/dL	100-125 mg/dL or treated to goal	<100 mg/dL

SBP: Systolic Blood Pressure; DBP: Diastolic Blood Pressure.

Total cholesterol: 1mg/dL=0.026 mm/L; fasting blood glucose: 1 mg/dL =0.056 mm/L

* Consistent with a Dietary Approaches to Stop Hypertension (DASH)–type-eating pattern: ≥4.5 cups/d of fruits and vegetables, ≥2 servings/wk of fish, and ≥3 servings/d of whole grains, ≤36 oz/week of sugar-sweetened beverages and 1500 mg/d of sodium. The consistency of one's diet with these dietary targets can be described using a continuous AHA diet score, scaled from 0 to 100.

Table 6-2: Characteristics of women included in this analysis

Characteristic	N=529
At SWAN Baseline Visit:	
Age, years, mean (SD)	46.4 (2.6)
Race, n (%)	
White	299 (56.5%)
Black	141 (26.7%)
Other*	89 (16.8%)
Menopause Status, n (%)	
Premenopause	328 (62.4%)
Early Perimenopause	198 (37.6%)
Education Level, n (%)	
Post-College Degree	154 (29.2%)
Some College/College Degree	281 (53.3%)
≤ High School Degree	92 (17.5%)
Economic Hardship, n (%)	
No	364 (68.9%)
Yes	164 (31.1%)
Alcohol use, n (%)	
<1 drink/month	253 (47.8%)
≥ 1 drink/month	276 (52.2%)
Lipid medication users, n (%)	
No	527 (99.6%)
Yes	2 (0.4%)
Life's Simple 7 Score, mean (SD)	8.5 (2.0)
hs-CRP, mg/dL, median (Q1, Q3)	1.5 (0.5, 4.0)
HDL Metrics at first available visit:	
HDL-CEC (%), mean (SD)	3.96 (0.7)
HDL-Tg, mg/dL, median (Q1, Q3)	17 (14, 21)
HDL-PL, mg/dL, mean (SD)	54.8 (10.6)
Total HDL-P, µmol/L, mean (SD)	35.1 (6.3)
Large HDL-P, µmol/L, mean (SD)	8.4 (3.6)
Medium HDL-P, µmol/L, mean (SD)	11.3 (6.2)
Small HDL-P, µmol/L, mean (SD)	15.4 (7.1)
HDL size, nm, mean (SD)	9.5 (0.5)
HDL-C, mg/dL, mean (SD)	59.5 (14.3)
ApoA-I, mg/dL, mean (SD)	165.6 (27.1)

BMI: body mass index; hs-CRP: high-sensitivity C-reactive protein, HDL-CEC: HDL cholesterol efflux capacity; HDL-Tg: HDL triglycerides; HDL-PL: HDL-phospholipids; HDL-P: HDL particles; HDL-C: HDL cholesterol; ApoA-I: Apolipoprotein A-I

Hs-CRP: 1mg/dL=10 mg/L; HDL-C: 1mg/dL=0.026 mmol/L; ApoA-I: 1mg/dL=0.01 g/L

* Other Race category included 1 Hispanic, 47 Chinese and 41 Japanese women

Table 6-3: Unadjusted and multivariable-adjusted associations between baseline LS7 score and future HDL function, lipid contents and ApoA-I

	HDL Function, Contents and ApoA-I									
	HDL-CEC (%)		HDL-Tg*		HDL-PL (mg/dL)		HDL-C (mg/dL)		ApoA-I (mg/dL)	
	β (SE)	p-value	β (SE)	p-value	β (SE)	p-value	β (SE)	p-value	β (SE)	p-value
Model 1	0.11 (0.03)	<0.0001	-0.05 (0.01)	<0.0001	2.38 (0.42)	<0.0001	4.29 (0.58)	<0.0001	5.53 (1.10)	<0.0001
Model 2	0.02 (0.03)	0.60	-0.06 (0.01)	<0.0001	1.23 (0.49)	0.01	3.02 (0.67)	<0.0001	3.54 (1.28)	0.006

Data presented as increase or decrease in HDL metric per 1-SD increase in LS7 score.

Model 1: Unadjusted

Model 2: Adjusted for time between LS7 score and HDL metrics measures, race, education status and baseline age, economic hardship, menopause status, alcohol use and log-hs-CRP

* Log-transformed

Table 6-4: Unadjusted and multivariable-adjusted associations between baseline LS7 score and future HDL subclasses and overall size

	HDL Subclasses and Size									
	Total HDL-P (µmol/L)		Large HDL-P (µmol/L)		Medium HDL-P (µmol/L)		Small HDL-P (µmol/L)		HDL Size (nm)	
	β (SE)	p-value	β (SE)	p-value	β (SE)	p-value	β (SE)	p-value	β (SE)	p-value
Model 1	1.06 (0.24)	<0.0001	1.00 (0.15)	<0.0001	0.61 (0.22)	0.005	-0.57 (0.26)	0.03	0.12 (0.02)	<0.0001
Model 2	0.62 (0.27)	0.03	0.66 (0.17)	<0.0001	0.45 (0.26)	0.09	-0.51 (0.31)	0.10	0.10 (0.03)	<0.0001

Data presented as increase or decrease in HDL metric per 1-SD increase in LS7 score.

Model 1: Unadjusted

Model 2: Adjusted for time between LS7 score and HDL metrics measures, race, education status and baseline age, economic hardship, menopause status, alcohol use and log-hs-CRP

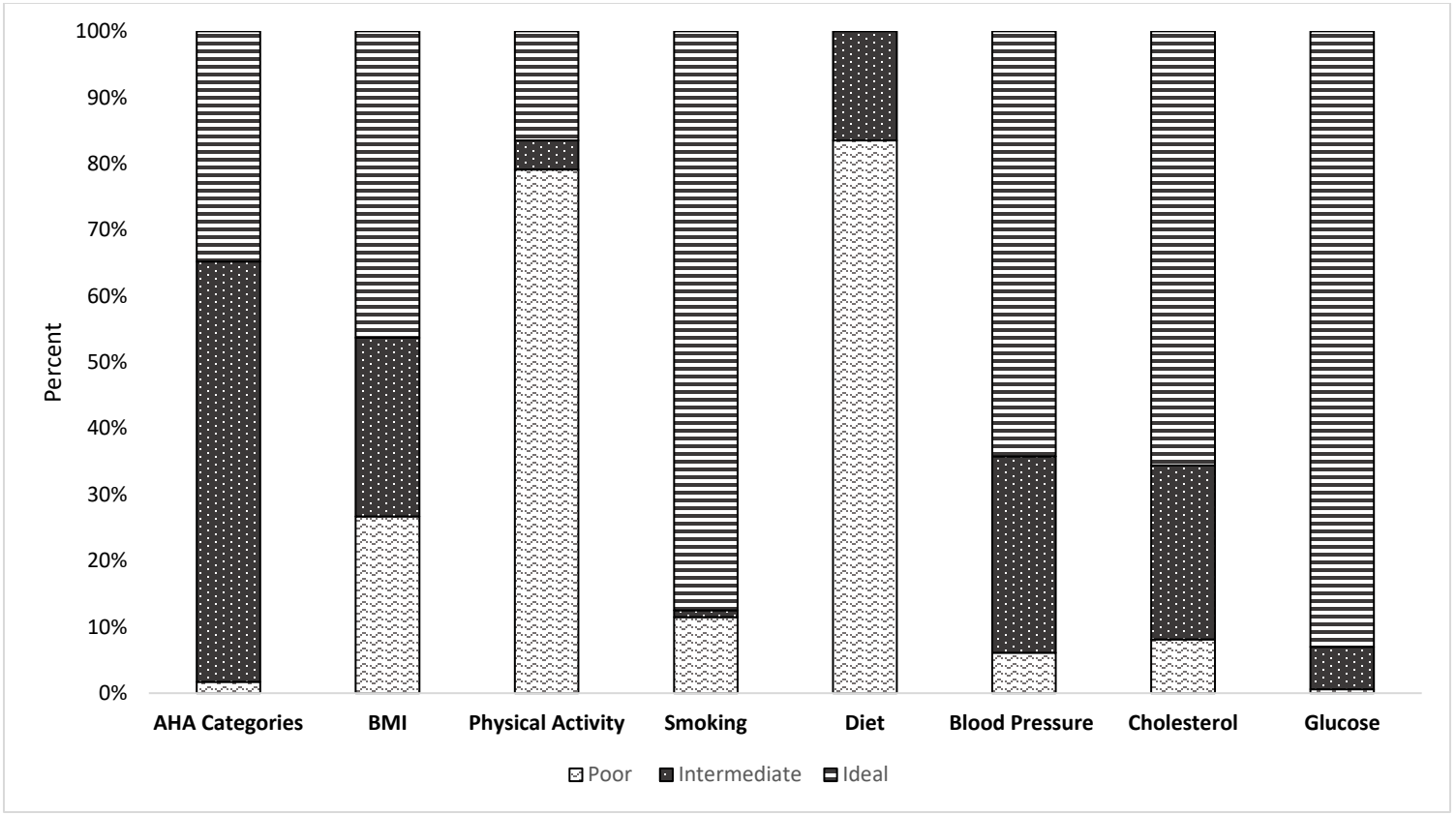


Figure 6-1: Distribution of LS7 components among study participants at SWAN baseline visit

* AHA LS7 Categories Classification: Poor: LS7 score 0-4; Intermediate: LS7 score 5-9; Ideal: LS7 score 10-14

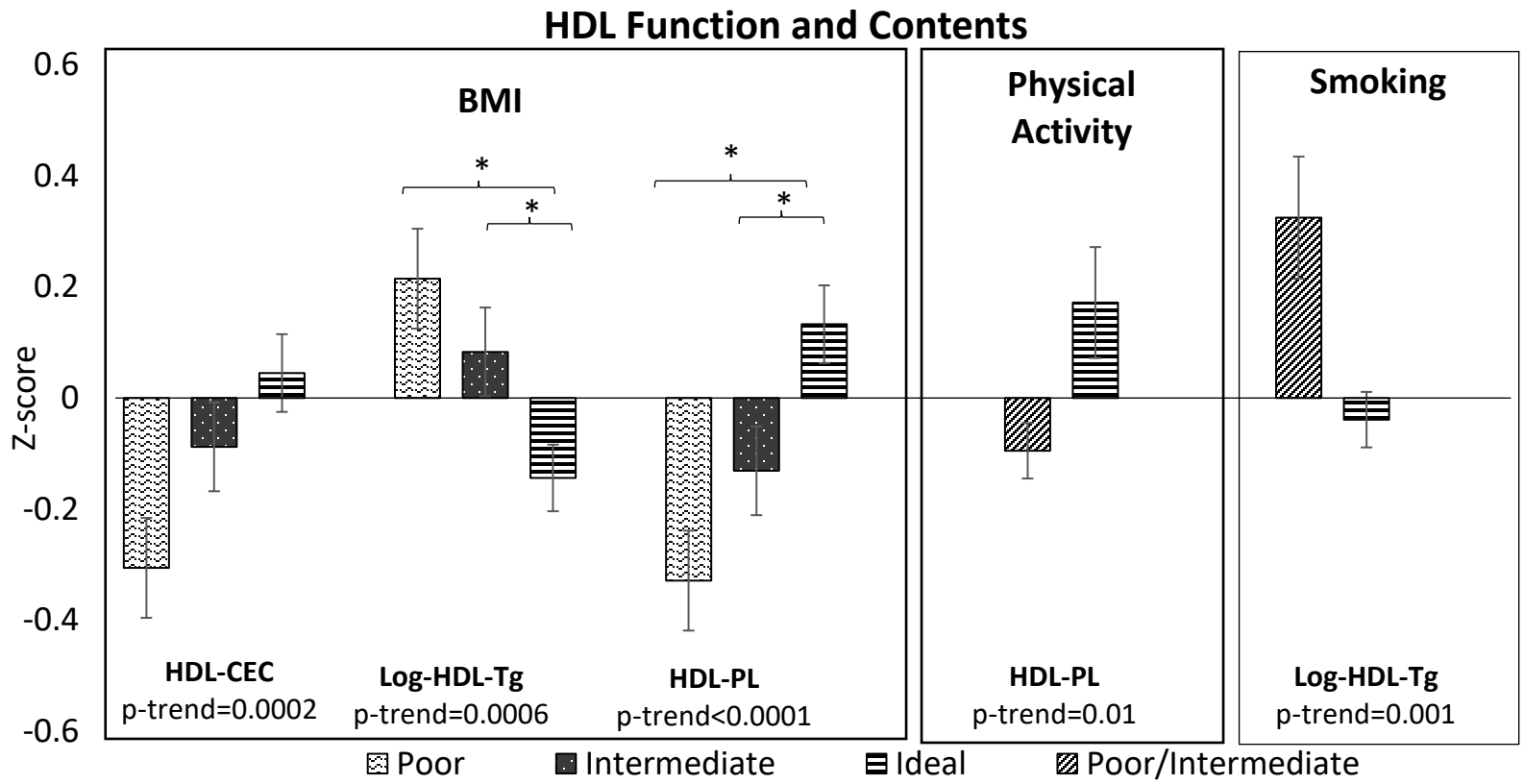


Figure 6-2: Associations between baseline LS7 components and HDL function and contents

Data presented as the z-score of each HDL metric per group.

Models adjusted for time between LS7 score and HDL metrics, race, and baseline age, education level, economic hardship, menopause status, alcohol use and log-hs-CRP. Bonferroni's adjustment used for multiple group comparisons.

* Significant difference between groups

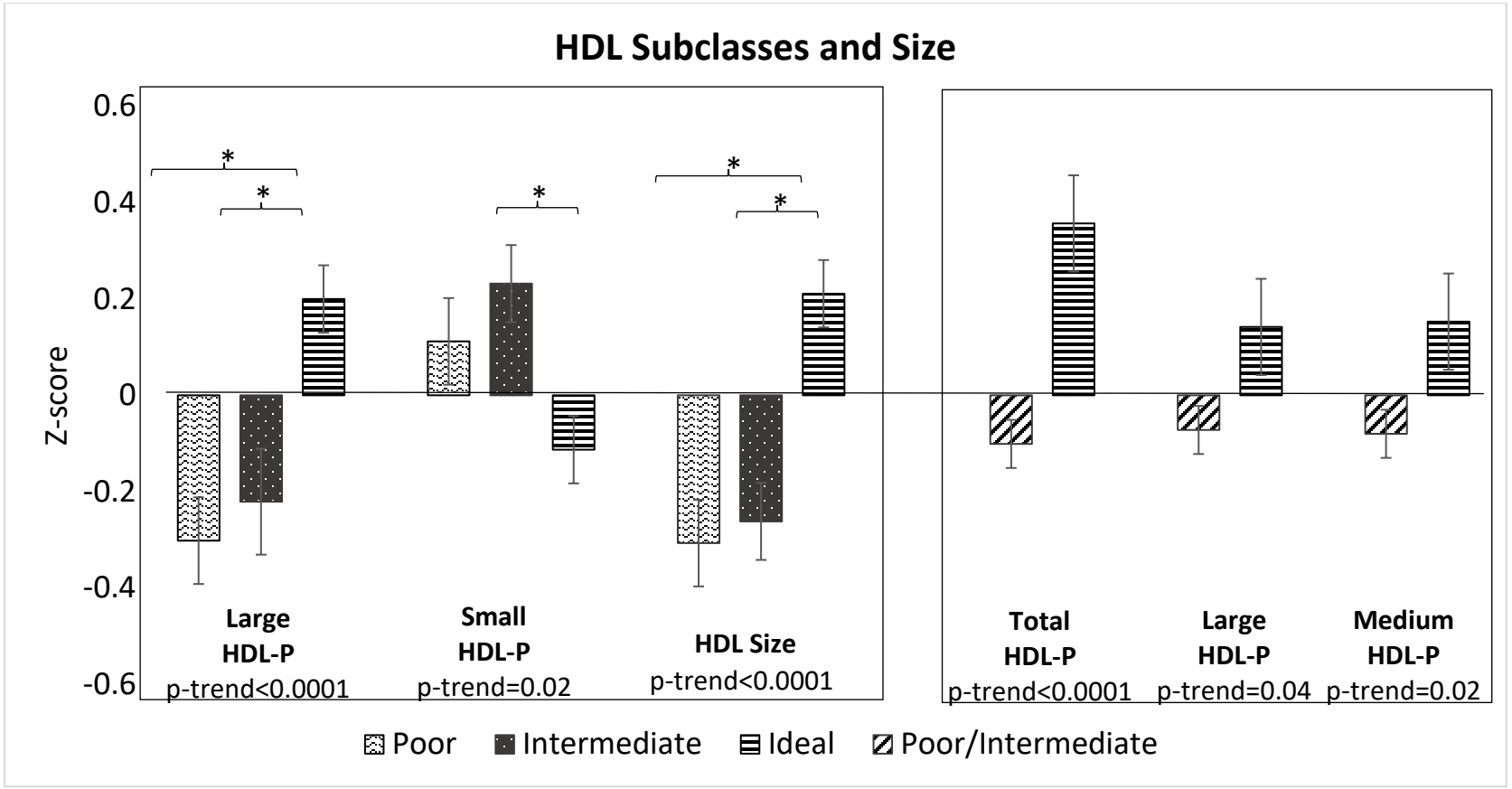


Figure 6-3: Associations between baseline LS7 component and HDL subclasses and size

Data presented as the z-score of each HDL metric per group.
Models adjusted for time between LS7 score and HDL metrics, race, and baseline age, education level, economic hardship, menopause status, alcohol use and log-hs-CRP.
* Significant difference between groups

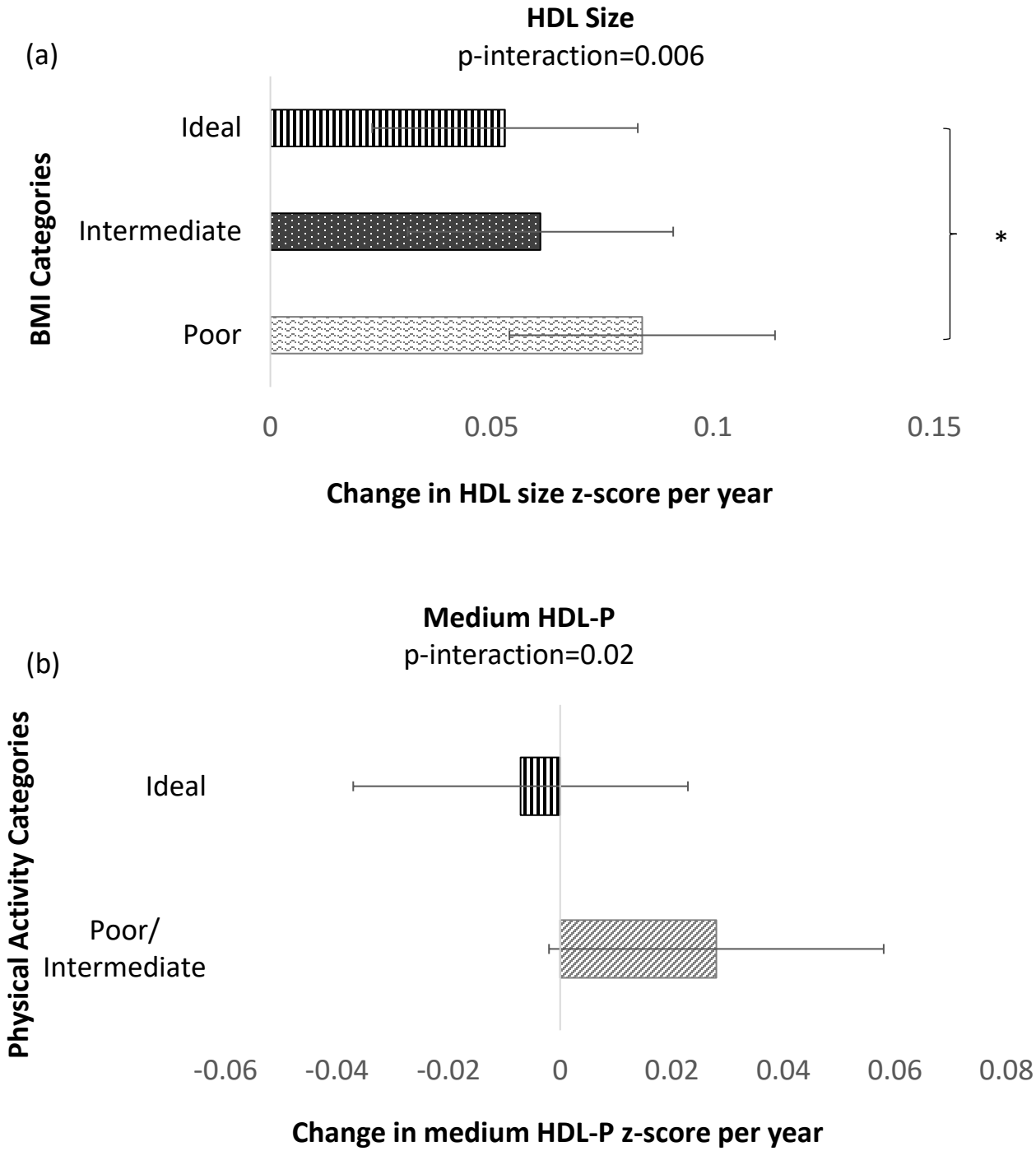


Figure 6-4: Associations between baseline LS7 components and change in HDL metrics

Data presented as the yearly change in z-score of each HDL metric per group.

Models adjusted for time between Life's Simple 7 score and HDL metrics, race, and baseline age, education level, economic hardship, menopause status, alcohol use and log-hs-CRP.

6.7 Supplemental Material

Table 6-5: Table S1: Adjusted means for the associations between LS7 components and future HDL metrics

	HDL-CEC (%)	HDL-Tg (log)	HDL-PL (mg/dL)	Total HDL-P (µmol/L)	Large HDL-P (µmol/L)	Medium HDL-P (µmol/L)	Small HDL-P(µmol/L)	HDL size (nm)	HDL-C (mg/dL)	ApoA-I (mg/dL)
	β (SE)	β (SE)	β (SE)	β (SE)	β (SE)	β (SE)	β (SE)	β (SE)	β (SE)	β (SE)
AHA BMI										
Poor	3.82 (0.06)*	2.94 (0.02)*	51.97 (0.97)*	34.77 (0.55)	7.56 (0.33)*	10.59 (0.52)	16.65 (0.61)	9.35 (0.05)*	55.42 (1.33)*	157.72 (2.52)*
Intermediate	3.97 (0.05)	2.90 (0.02)*	54.15 (0.88)*	35.95 (0.50)	7.86 (0.31)*	10.60 (0.47)	17.48 (0.56)*	9.37 (0.05)*	58.90 (1.21)*	164.37 (2.32)
Ideal	4.06 (0.05)	2.84 (0.02)	57.05 (0.73)	36.18 (0.42)	9.44 (0.25)	11.67 (0.39)	15.08 (0.47)	9.63 (0.04)	64.17 (1.01)	170.53 (1.92)
p-trend	0.0002	0.0006	<0.0001	0.053	<0.0001	0.07	0.02	<0.0001	<0.0001	<0.0001
AHA Diet										
Poor	3.98 (0.04)	2.88 (0.01)	54.81 (0.59)	35.69 (0.33)	8.54 (0.21)	11.09 (0.31)	16.05 (0.37)	9.49 (0.03)	60.36 (0.82)	165.0 (1.54)
Intermediate	3.95 (0.06)	2.88 (0.03)	55.56 (1.05)	35.94 (0.59)	8.53 (0.37)	11.22 (0.55)	16.25 (0.66)	9.51 (0.05)	61.11 (1.46)	167.4 (2.75)
p-trend	0.75	0.90	0.51	0.69	0.97	0.84	0.66	0.70	0.63	0.44
AHA Physical Activity										
Poor/ Intermediate	3.96 (0.03)	2.89 (0.01)	54.54 (0.56)	35.32 (0.31)	8.43 (0.20)	10.91 (0.30)	16.00 (0.36)	9.48 (0.03)	59.87 (0.78)	164.1 (1.46)
Ideal	4.01 (0.07)	2.85 (0.03)	57.48 (1.12)	38.21 (0.62)	9.23 (0.39)	12.33 (0.59)	16.65 (0.71)	9.56 (0.06)	64.30 (1.55)	173.88 (2.92)
p-trend	0.50	0.24	0.01	<0.0001	0.04	0.02	0.37	0.19	0.006	0.001
AHA Smoking										
Poor/ Intermediate	4.00 (0.08)	2.97 (0.03)	54.79 (1.25)	35.12 (0.70)	8.28 (0.43)	10.97 (0.66)	15.90 (0.79)	9.48 (0.06)	58.42 (1.73)	164.65 (3.26)
Ideal	3.97 (0.03)	2.87 (0.01)	55.00 (0.57)	35.83 (0.32)	8.58 (0.20)	11.14 (0.30)	16.12 (0.36)	9.49 (0.03)	60.83 (0.78)	165.65 (1.48)
p-trend	0.65	0.001	0.87	0.32	0.51	0.81	0.78	0.78	0.18	0.77

Models adjusted for time between Life's Simple 7 score and HDL metrics, race, and baseline age, education level, economic hardship, menopause status, alcohol use and log-hs-CRP. Bonferroni's adjustment used for multiple group comparisons when appropriate.

* Significantly different from the ideal groups.

Table 6-6: Table S2: Associations between baseline LS7 score with changes in HDL metrics

	HDL Function and Contents									
	HDL-CEC		HDL-Tg*		HDL-PL		HDL-C		ApoA-I	
	β (SE)	p-value	β (SE)	p-value	β (SE)	p-value	β (SE)	p-value	β (SE)	p-value
LS7 Score	0.002 (0.003)	0.40	-0.0002 (0.002)	0.91	0.03 (0.05)	0.53	-0.04 (0.06)	0.52	0.17 (0.15)	0.28
	HDL Subclasses and Size									
	Total HDL-P		Large HDL-P		Medium HDL-P		Small HDL-P		HDL Size	
	β (SE)	p-value	β (SE)	p-value	β (SE)	p-value	β (SE)	p-value	β (SE)	p-value
LS7 Score	-0.0001 (0.03)	0.99	-0.01 (0.02)	0.34	-0.05 (0.03)	0.15	0.06 (0.04)	0.07	-0.006 (0.002)	0.005

The beta estimate represents the yearly change in each HDL metric per 1 standard deviation increase in LS7 score.

Models adjusted for time between LS7 assessment and HDL metrics, race, baseline age, education status, economic hardship, alcohol use and log-hs-CRP

* Log-transform

Table 6-7: Table S3: Summary of Potential Impact of Smoking, BMI and Physical Activity on HDL Metabolism

	Ideal Smoking Status	Ideal BMI Status	Ideal Physical Activity
CETP	↓	↓	↓
Hepatic Lipase	↓	↓	-
PLTP	↓	-	-
LCAT	↑	-	-
Lipoprotein Lipase	-	-	↑
Oxidation	↓	-	↓

CETP: Cholesteryl ester transfer protein; PLTP: Phospholipid Transfer Protein; LCAT: lecithin- cholesterol acetyl transferase

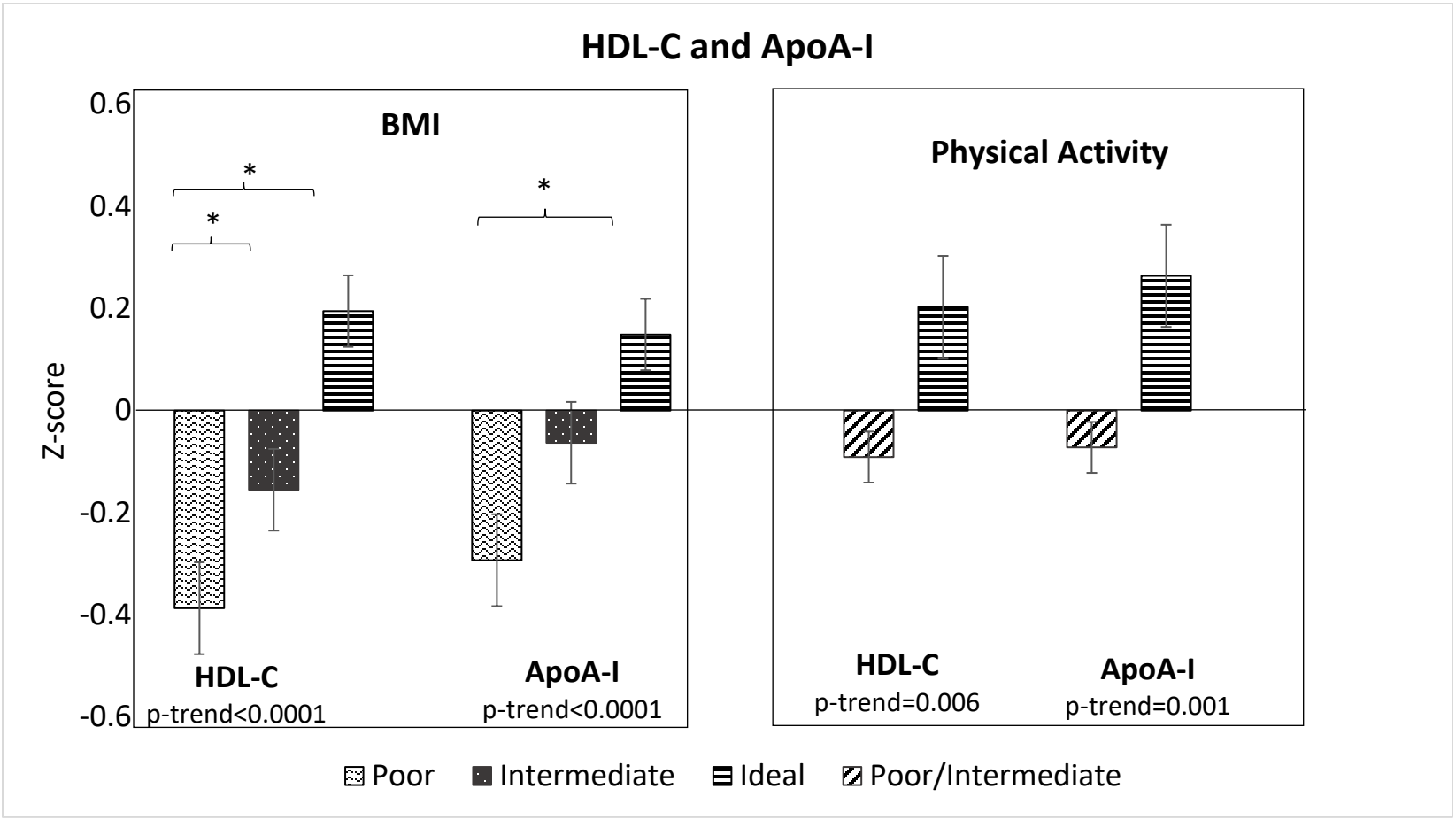


Figure 6-5: Figure S1: Associations between baseline BMI and Physical Activity with traditional HDL-C and ApoA-I

Models adjusted for time between Life's Simple 7 score and HDL metrics, race, and baseline age, education level, economic hardship, menopause status, alcohol use and log-hs-CRP.

* Significant difference between groups

7.0 Discussion

7.1 Summary of Findings

The midlife period in women is accompanied by a notable increase in CVD risk factors, including weight gain, visceral fat redistribution, increase in insulin resistance and dyslipidemia.[275] The changes in the presumably athero-protective HDL-C over midlife, however, are not well-characterized. It has been suggested that women experience a loss in the athero-protective capacity of the HDL during the menopause transition, a manifestation that is not detected by HDL-C. Studies have suggested that novel metrics of HDL may provide a more definite picture of the changes that occur in the HDL during midlife. The aims of this dissertation were to investigate the relationship between novel HDL metrics and different cardiovascular risk factors that are relevant to midlife women, and to identify how lifestyle factors and markers of cardiovascular health (CVH) predict these metrics. The *first* manuscript evaluated the associations between abdominal visceral AT volumes and cardiovascular AT volumes and different HDL metrics, assessed whether these associations differ by ethnicity, and identified the role of insulin resistance in these associations.[334] In the *second* manuscript, women were classified into clusters based on their comprehensive HDL metrics profile, and the associations between the clusters and future measures of subclinical atherosclerosis were tested. Effect modification by race/ethnicity, and mediation role of blood pressure were additionally tested. The *third* manuscript investigated the associations between

early midlife CVH with future HDL metrics, and assessed the relationships between separate health behaviors (BMI, physical activity, smoking and diet) and HDL metrics.

In the first manuscript, we showed that in women [mean age at time of AT assessment: 51.1 (SD: 2.8) years], higher abdominal visceral AT volume was associated with a shift in the contents of the HDL towards less phospholipids, and a shift in subclasses towards smaller size, independent of potential confounders. Cardiovascular fat depots associated differently with HDL metrics: 1) higher EAT volume was associated with more of the smaller HDL subclasses, 2) higher PAT volume was associated with less concentrations of the large HDL-P and HDL-C, and smaller overall HDL, whereas 3) higher PVAT volumes were associated with lower levels of cholesterol efflux capacity of the HDL. The associations did not vary between White and Black women. Moreover, we found that insulin resistance partially mediated the observed associations of the AT depots with HDL-CEC, large HDL-P, HDL size and HDL-C, such that higher AT volumes increase insulin resistance, which in turn, could be linked to lower levels of large HDL-P and HDL-C, and lower HDL size.

In the second manuscript, women were grouped into two clusters based on their HDL profile: a favorable HDL cluster (higher cholesterol efflux capacity, larger subclasses and a shift in contents towards more phospholipids and less triglycerides) and an unfavorable HDL cluster (lower cholesterol efflux capacity, smaller subclasses and a shift in contents towards less phospholipids and more triglycerides). The favorable HDL cluster formed 46% of the cohort, and the unfavorable HDL cluster formed 54% of the cohort. In unadjusted models, the favorable cluster was associated with lower future cIMT and IAD, and lower odds of carotid plaque presence [average age of women at time of outcome: 63.3 (SD: 3.7) years]. However, this was not independent of cardiometabolic factors like BMI. The association between the

favorable HDL cluster and lower cIMT was specific to Chinese women. Finally, we showed that SBP partially mediated some of the observed associations, such that a favorable HDL cluster would be associated with lower SBP, which in turn, could be linked to lower cIMT.

In the third manuscript, we showed that in early midlife [average age 46.4 (SD: 2.6) years], better CVH was independently associated with higher phospholipid and lower triglyceride contents, more total particles and a shift in particle size toward larger subclasses later in life. We also showed that an ideal BMI status was associated with higher phospholipids and less triglycerides contents, a shift in size toward larger particles and higher efflux capacity of the HDL. Ideal physical activity was associated with higher levels of phospholipids within the cell, more total, large and medium particles. Ideal smoking was associated with less triglycerides. Diet was not associated with any of the HDL metrics.

In summary, we reported that in midlife women, higher abdominal and cardiovascular fat accumulation are related to a worse HDL metrics profile. Moreover, a worse HDL profile may be linked to worse subclinical atherosclerosis in the future. We also reported that a healthier lifestyle, and particularly lower BMI and higher levels of physical activity early in midlife are associated with better HDL profile.

7.2 Interrelation between visceral adiposity, subclinical atherosclerosis, cardiovascular health and HDL metrics

Ovarian aging during midlife has been linked to an accumulation of several cardiovascular risk factors, including visceral fat build up, loss of lean mass, worsening of subclinical atherosclerosis, increases in metabolic syndrome risk, and blood pressure and potential loss of the athero-protective features of the HDL.[275]

The SWAN study is one of the few studies that have investigated, in detail, changes of cardiovascular risk factors over the menopause transition. SWAN has shown that independent of weight gain, women during midlife accumulate more visceral AT in the abdomen[153] and in ectopic areas, particularly paracardial fat.[14] Interestingly, the rate of accumulation of visceral fat speeds up during the perimenopausal period; this increase predicted higher future internal carotid IMT in women.[153] Along with our results from the analysis of the associations between different AT depots and HDL metrics, this could indicate that the rapid accumulation of visceral AT during the perimenopausal period may contribute to the decreased ability of HDL to efflux cholesterol from cells during that period. In addition, in our second manuscript, we report an association between favorable HDL metrics and less average cIMT and interadventitial diameter, which was explained by cardiometabolic risk factors such as BMI. Thus, controlling for weight gain early in midlife and limiting the accumulation of visceral fat could have a favorable impact on HDL metrics and, ultimately, subclinical atherosclerosis. We also report that insulin resistance mediates the association between visceral AT accumulation and HDL dysfunction, whereas systolic blood pressure mediates the association between HDL metrics and subclinical atherosclerosis. Thus, intervening on insulin resistance and blood pressure, whether by a healthier lifestyle, insulin

sensitizers or anti-hypertensives, in midlife women could alter the associations observed between HDL and these CVD risk factors, and future studies should aim to investigate these hypotheses. Finally, in our third manuscript, we found that better lifestyle (lower BMI, higher physical activity, non-smoking, following a healthy diet, and having low blood glucose, blood pressure and cholesterol) is associated with better HDL metrics profile later in life, which raises the question regarding the role that cardiovascular health plays in the interrelation between visceral fat, subclinical atherosclerosis and HDL metrics.

7.3 Public Health Significance

Over the past 40 years, there has been significant improvement in the diagnosis, prevention and treatment of CVD.[3] However, CVD remains the leading cause of death in the US population.[1] In midlife women specifically, CVD risk lags behind that of men by around 10 years.[1] Nevertheless, as women go through the MT, they experience a significant increase in CVD risk, such as an increase in visceral fat accumulation, blood pressure, dyslipidemia, insulin resistance, and worsening of vascular health,[275] leading to an increase in CVD risk to match that in men.

However, only until recently did CVD in women start to receive the required attention, and female-specific studies and guidelines began to target this population. Unfortunately, a recent survey by the AHA recognized that awareness of CVD as the most common cause of death in women has decreased from 65% in 2009 to 44% in 2019.[335] In addition, the latest guidelines that were published on the prevention of CVD in women[336] failed to include the midlife period as a significant factor for CVD development, since this was published in 2011, and missed on the recent

findings from research that was performed in this population. The AHA recently released a scientific statement, published in 2020,[275] which highlighted the importance of midlife as a period of increased CVD risk in women, and stressed the importance of targeting preventive measures to reduce the burden of CVD in this population.

HDL has been promoted as an anti-atherogenic marker, and research in the past decades has focused on raising HDL-C as a marker to reduce CVD. However, these efforts have failed to successfully impact CVD risk, and attention has shifted away from HDL-C,[3] In addition, women specifically seem to experience a decline in the ability of the HDL to efflux cholesterol from the cells, during midlife that is not captured by HDL-C.[10]

The life expectancy in the women in the US population is around 80 years.[337] With a median age of natural menopause of 50 (Q1: 48, Q3: 53) years,[338] women are expected to spend around 40% of their lifetime in the menopause. Subsequently, identifying novel risk factors specific to this high-risk period allows for targeted preventive measures and therapies.

In this dissertation, we identify novel markers of CVD that could be potential targets for disease prevention in midlife women. Metrics of HDL function, contents and subclasses, other than HDL-C, may allow for stratification of midlife women as high risk versus low risk. This could identify women who would benefit from targeted therapies and interventions. In addition, we found that lifestyle factors might impact these HDL metrics. There have been ongoing efforts to improve lifestyle factors in the adult population; however, over time, only rates of ideal smoking are improving, whereas those of ideal BMI and ideal status are decreasing.[1] This is especially important in midlife women, since around 40% of postmenopausal women are considered obese,[142] and weight increases continuously over the menopause transition.[339] Thus, identifying high-risk subjects based on HDL metrics may further push the need for weight loss or maintenance,

increased physical activity and smoking cessation, in those high-risk women who are approaching the menopause transition period.

7.4 Clinical Implications

Studies that aimed to identify preventive methods against CVD in midlife women focused mainly on estradiol and hormone replacement therapies. However, clinical trials have consistently shown that hormone replacement therapy is not beneficial in preventing CVD, and may in fact be associated with higher CVD risk, especially further away from menopause.[17,340]

Recent research has identified a "window of opportunity" in women, where preventions and interventions could be beneficial to reduce cardiovascular burden later in life. This is the time closest to the perimenopausal period, when hormonal, physiological and symptomatic changes related to menopause begin and last until after the final menstrual period.

This dissertation work identified that during the midlife period, accumulation of visceral fat may be associated with a worse HDL metrics profile, particularly a lower phospholipid content of the HDL and a shift toward smaller particles. Our work is the only work that identifies how different metrics tend to cluster together in women. As hypothesized, higher cholesterol efflux capacity, higher phospholipids, lower triglycerides and larger HDL particles were grouped together in a "favorable" cluster, and this was associated with a better future subclinical atherosclerosis, particularly in Chinese women. Moreover, we also found that worse lifestyle factors early in midlife are associated with worse HDL metrics.

Put together, these results suggest that in midlife women 1) preventing visceral AT accumulation may limit the adverse profile of HDL in women. Additionally, intervening on insulin resistance may also help limit the unfavorable effect of visceral AT on HDL metrics; 2) improving HDL metrics may be associated with a better subclinical atherosclerosis profile, specifically in Chinese women, and 3) improving lifestyle, specifically reducing BMI, and increasing physical activity could improve HDL metrics. Clinically, those HDL metrics can help identify women who are at higher risk, and who could benefit from early preventive therapies during perimenopause.

Previous research on novel metrics of the HDL have shown that higher efflux capacity, total HDL particles, larger HDL subclass distribution and an increase on phospholipid contents have been linked to CVD outcome, such as coronary heart disease,[112] composite CVD endpoints,[113] lower prevalence and incidence of CAD,[118,119,122] and cardiovascular mortality.[120] Out of all the HDL metrics, it appears that the large HDL-P and overall HDL size, and the phospholipid contents of the HDL seem to show the most consistent relationships with CVD risk factors in women, and these should be the strongest targets for prevention or intervention. Clinical trials that tested the effect of interventions that raise HDL-C, such as statins, niacin and CETP inhibitors, failed to show any success in reducing cardiovascular risk burden. Studies have shown that these interventions only modestly increase HDL-CEC,[341,342] and do not increase the number of HDL particles.[341-344]

Newer work has identified therapies that increase the efflux capacity and alter the composition of the HDL. These include infusions of cholesterol poor reconstituted HDL,[322] or Liver X receptors (LXR) activator treatments which upregulate ABCA1/ABCG1, the major proteins involved in the reverse cholesterol transport process, and improve glucose tolerance.[323] Other emerging therapies such as Resverlogix, which is an ApoA-I up-regulator, can increase

cholesterol efflux and pre-β HDL particles,[324] which are the precursors and main acceptors of cholesterol effluxed from macrophages. These interventions are still early. Future research should assess how those interventions affect large HDL-P and the phospholipid contents of the HDL. The benefit of these treatments on reducing CVD is still not confirmed, but they could be potential targets of treatment in high-risk midlife women.

7.5 Strengths and Limitations

The major strengths of this dissertation stem from the design of the SWAN and SWAN HDL. The longitudinal data collection of these studies allowed for the assessment of the exposures and outcomes in a prospective design. This reduces the chance of reverse causality. SWAN collected yearly data on its participants and is one of the few studies that was able to characterize the exact stage of the MT for each woman. Another important strength is the novelty of the measures, where SWAN HDL is the only study in women that has collected a comprehensive profile of HDL metrics, evaluating various characteristics such as function, composition and subclasses.

It is important to acknowledge the limitations of this dissertation. First, the HDL-CEC as a measure of function is an assessment of cholesterol efflux capacity of the HDL, which is a major anti-atherogenic function of the HDL. However, HDL has various other anti-oxidative and anti-inflammatory functions that were not tested in this study. These other functions could be impacted by AT accumulation and lifestyle factors, and could affect future subclinical atherosclerosis. Moreover, HDL subclasses can be classified using different methods other than NMR

(ultracentrifugation, precipitation, and ion mobility), and these classifications may not always overlap and show comparable results. This is especially important since NMR does not detect the very small HDL particles.

Additionally, SWAN collected AT measures only in Black and White women, thus the results of the analysis pertaining to VAT and HDL metrics may not be generalizable to other ethnic/racial groups. Similarly, our analysis on HDL metrics and subclinical atherosclerosis was performed in Black and White women, and a small number of Chinese women. Thus, the results of this analysis should be interpreted with care. Finally, we used measures of subclinical atherosclerosis as a surrogate marker of clinical CVD event. The prevalence of clinical CVD events in the SWAN HDL study is low, thus a proxy was used in this analysis. However, previous studies on HDL metrics and CVD outcomes have shown that, by NMR, higher large HDL-P levels are associated with lower odds of myocardial infarction and angina,[107] whereas higher levels of small HDL-P are associated with lower CHD incidence,[108] and larger overall HDL size is associated with lower risk of CVD events.[111] Moreover, subjects with coronary artery disease have lower HDL-CEC and HDL-PL concentrations, but higher HDL-Tg levels compared to healthy controls.[118,122]. As these studies show a similar direction to what was reported in the dissertation, this provides additional support to our results. In SWAN and SWAN HDL, the prevalence of hard CVD events is low, but future studies should assess the relationships between HDL metrics and CVD outcomes after a longer follow-up period.

7.6 Future Directions

The work in this dissertation has created a foundation for future research. This work has shown that the loss of the athero-protective abilities of the HDL in midlife women, as estimated by a decline in cholesterol efflux capacity, a change in the composition of the HDL particle, and a shift towards smaller HDLs, is related to several cardiovascular disease risk factors. Since our research is novel, it is important to replicate these findings in other cohorts of midlife women. It is of particular importance to replicate these findings across different racial/ethnic groups, since there appears to be effect modification by race/ethnicity on certain associations between HDL metrics and CVD risk factors.

Future research should also determine whether a comprehensive metric of HDL function, structure and contents improves risk prediction of cardiovascular disease in midlife women at the time of menopause transition. First of all, even though HDL-C has not been an effective target for preventive or treatment therapies in adults,[3,4] it is still a part of the pooled cohort equation for the assessment of CVD risk,[5] where lower HDL-C is considered to pose an elevated risk. However, since this may not apply to midlife women, future studies should assess whether a comprehensive metric of HDLs would more consistently improve disease risk prediction compared to HDL-C.

In addition, we identified that certain lifestyle factors, such as maintaining a healthy BMI and meeting the guidelines for physical activity may be linked to a better HDL profile. In midlife and postmenopausal women, a very small proportion of women meet the recommendations for ideal physical activity and diet status.[330] Future clinical trials should assess whether improving lifestyle and health behavior in midlife women alters HDL metrics. Moreover, whether intervening

on insulin resistance and blood pressure alters the associations between HDL metrics and CVD risk factors should be tested.

In the research setting, even though in our study we included several measures of HDL function, structure and composition, there are still other characteristics of the HDL that could help identify CVD risk factors. These include the proteome, the anti-oxidative and the anti-inflammatory functions of the HDL. Future research should extend our findings to include these metrics for an even better picture of the change sin HDL over menopause and their relation to future CVD risk.

Finally, as we have discussed previously, the alterations in E2 which occur with the menopause transition may be linked to the loss of athero-protective functions that occurs in HDL in midlife women. This happens mainly through the loss of nitric oxide synthase activity, increase in vascular smooth cell proliferation, and the loss of the anti-oxidative and anti-inflammatory functions of the E2 with the declines of the hormone level with menopause. Thus, the role of E2 in these associations should be identified, and whether estrogen supplementation early after the menopause transition may prevent a dysfunction in HDL, and improve CVD risk profile.

7.7 Conclusion

In this dissertation, we aimed to understand the clinical utility of HDL metrics other than HDL-C, by linking those metrics to CVD risk factors that are relevant to midlife women. We showed that visceral fat accumulation in women is associated with a worse HDL profile; and that a worse HDL profile may be linked to future subclinical atherosclerosis. We also identified that a

healthier lifestyle is associated with better HDL metrics. This novel work highlights the importance of emphasizing future research on these novel HDL metrics to identify whether they could be predictors of future CVD outcome and better therapeutic targets than HDL-C.

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