EVALUATING POTENTIAL MECHANISMS OF CARDIOVASCULAR DAMAGE IN WOMEN ACROSS THE LIFECOURSE

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ABSTRACT

The risk of cardiovascular disease (CVD) is low in premenopausal women, but after menopause it increases to match that in men. Most of this increased risk has been attributed to loss of the favorable effects of endogenous estrogen on CVD risk factors; however, the loss of estrogen is unable to explain all of the risk. Furthermore, in type 1 diabetes, CVD risk in premenopausal women already matches that in men, suggesting that premenopausal women with type 1 diabetes do not benefit from endogenous estrogen as premenopausal women without diabetes do. Traditional CVD risk factors and the higher prevalence of ovarian dysfunction are unable to explain this risk increase, and type 1 diabetes is associated with twice the relative increase in CVD risk in women compared with men.

In this thesis, we examined the potential role of two mechanisms of cardiovascular damage in women prior to and following menopause. We used cross-sectional data from the biracial Coronary Artery Risk Development in Young Adults (CARDIA) study to test a differential association by sex of pericardial adipose tissue (PAT), a proinflammatory ectopic fat surrounding the heart, with more deleterious cardiac structure and diastolic function in premenopausal women. PAT was associated with cardiac structure and diastolic function independent of sex and menopause. Second, we used pilot data from the Estrogen, Diabetes, and Endothelial Function (EDEN) ovarian suppression study to show lower vascular endothelial expression of estrogen receptors (ER) in women with type 1 diabetes compared with non-diabetic women, independent of serum estradiol.

Our findings contribute to the understanding of CVD risk in premenopausal women with and without type 1 diabetes, and to how CVD risk may change with surgical or natural menopause. We were able to inform future hypotheses about 1) a potentially inflammatory fat depot in a large, population-based study, and 2) cellular mechanisms contributing to complications of type 1 diabetes. These results provide a basis for future studies that examine temporal trends, mediation, and pathophysiology of the mechanisms presented.

The form and content of this abstract are approved. I recommend its publication.

Approved: Janet K. Snell-Bergeon

For Rabia Alauddin.

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CHAPTER I

INTRODUCTION

Background

Cardiovascular disease (CVD), including coronary artery disease, hypertension, heart failure, and stroke, is the leading cause of mortality in the United States.[1,2] CVD prevalence increases with age, and it continues to rise as incidence increases, mortality decreases, and life expectancy is prolonged.[2,3] Although the number of CVD-related deaths has decreased since 1979, CVD was still the cause of more than 800,000 deaths in 2015[4] and nearly 5 million hospital discharges in 2014.[4,5] In general, premenopausal women have a lower risk of CVD than similarly aged men, but menopause increases CVD risk in women.[6,7] Approximately 44 million men in the United States have a CVD diagnosis and the CVD mortality rate among men is driven by the number of deaths over the age of 45.[5] Approximately 48 million women in the United States have a CVD diagnosis and the CVD mortality rate among women is driven by the number of deaths over the age of 65.[5]

In adults with type 1 diabetes mellitus, women do not benefit from the same premenopausal protection against CVD that women without diabetes do.[8,9] Type 1 diabetes is associated with increased risk of atherosclerosis and coronary artery disease,[10,11] and premenopausal women with type 1 diabetes have up to a 40-fold increase in their risk of cardiovascular mortality compared with premenopausal women without diabetes.[12] Traditional cardiovascular risk factors, such as lipids, blood pressure, and adiposity, do not fully explain the differential risk by diabetes status in premenopausal women.[9,13] The sex hormone estrogen, which is thought to play a major role in premenopausal protection against CVD, also does not

differ between women with and without type 1 diabetes, although testosterone and sex hormone-binding globulin (SHBG) do.[14]

The work presented in this dissertation explores two potential mechanisms by which CVD may manifest in women. First, we examined whether accumulation of proinflammatory pericardial adipose tissue (PAT), the ectopic fat depot surrounding the heart, was associated with differences in cardiac structure and function by sex and by menopause status. The mechanisms underlying sex and menopausal differences in cardiac structure and function are unknown. Because PAT also differs by sex and by menopause status, lies on the surface of the heart, and is associated with inflammation, it may contribute to differences in cardiac structure and function. We used cross-sectional data from the Year 25 (Y25) visit of the Coronary Artery Risk Development in Young Adults (CARDIA) study to examine the relationship between PAT volume and cardiac structure and function first in a sample of men and premenopausal women, and second in a sample of premenopausal, naturally postmenopausal, and surgically postmenopausal women. The Y25 study visit was selected for these analyses due to the availability of measures of cardiac structure and function derived from the echocardiogram, and availability of PAT volume derived from cardiac CT scans.

Second, we examined whether endothelial cell expression of three proteins – estrogen receptors (ER) α and β, and nuclear factor (NF)-κB, a pro-inflammatory transcription factor – were different in premenopausal women with type 1 diabetes and without diabetes. Despite similar estrogen concentrations in women with and without type 1 diabetes,[14,15] women with type 1 diabetes are at increased risk of CVD and atherosclerosis. Impairment of ER expression is associated with CVD risk,[16] and NF-κB expression is associated with inflammation;[17] thus, we hypothesized that type 1 diabetes was associated with lower ER and higher NF-κB expression,

which could potentially explain the increased CVD risk. We used data from the pilot Estrogen, Diabetes, and Endothelial Function (EDEN) study, in which endothelial cells were harvested from a sample of 34 premenopausal women with and without type 1 diabetes.

Specific Aims

Aim 1: Evaluate whether PAT volume is associated with sex differences in LV cardiac structure and function.

Hypothesis 1: PAT volume is significantly associated with adverse cardiac structure and function outcomes independent of sex.

Aim 2: Evaluate whether PAT volume is associated with differences in LV cardiac structure and function by menopause status (premenopausal, naturally postmenopausal, or surgically postmenopausal).

Hypothesis 2: PAT volume is significantly associated with adverse cardiac structure and function outcomes regardless of menopause status.

Aim 3: Determine if there are differences in endothelial cell expression of ERα, ERβ, and NF-κB in premenopausal women by type 1 diabetes status.

Hypothesis 3: ER expression will be lower and NF-κB expression will be higher in type 1 diabetes, indicative of the higher inflammatory burden.

Significance

The analyses presented in this dissertation test hypothesized mechanisms by which sex and menopausal differences exist in CVD risk. Cross-sectional (Aims 1 and 2) or pilot (Aim 3) data were used to characterize associations that can be tested robustly in future longitudinal studies for which the data are currently unavailable. Analyses for the first two aims used a large, comprehensive dataset collected on a biracial, population-based cohort of men and women in

different menopause groups (i.e., premenopausal, naturally postmenopausal, and surgically postmenopausal), and provided evidence for adverse associations between PAT and cardiac structure and function.

Results of the third aim may help clarify a potential cellular mechanism by which women with type 1 diabetes are at increased risk of CVD. Little is known about why women with type 1 diabetes lose the premenopausal protection against CVD that non-diabetic women have, and results of this pilot analysis may suggest differences by diabetes status in how estrogen receptors (ERα and ERβ) or inflammatory proteins (NF-κB) are expressed prior to menopause. The procedures and results described for Aim 3 can be examined further in a larger study population.

CHAPTER II

LITERATURE REVIEW

Background

CVD is a high-volume and costly public health concern, accounting for nearly 25% of all adult deaths[18] and nearly 17% of all money spent on health care.[19,20] The American Heart Association estimates that as the United States population ages, the prevalence of CVD will increase from 41% to 45% of the total population by 2035.[21] The associated economic burden is projected to increase substantially to $1.1 trillion over the next 20 years as CVD risk factors worsen in the aging population.[21]

Heart failure (HF) is a clinical syndrome defined by the heart's inability to pump blood efficiently. It accounts for 9% of all CVD deaths in the United States,[4] and is characterized by impairment of the heart's structure and function preventing the ability to fill or eject blood to full capacity.[22] HF may be diagnosed via non-invasive echocardiography, which uses ultrasound to capture measures of cardiac structure, cardiac function, and blood velocity during systole (i.e., cardiac contraction) and diastole (i.e., cardiac relaxation). More than 6.5 million adults in the United States have a HF diagnosis.[4] The aging of the United States population has resulted in increasing HF incidence and overall prevalence, with current projections estimating that HF prevalence will increase by 46% in the next 15 years.[23] Despite the growing number HF cases, survival after diagnosis has improved[24] and mortality has decreased, in large part due to development and implementation of treatments including angiotensin-converting enzyme (ACE) inhibitors, beta blockers, and the use of surgical interventions.[4] Nevertheless, more elderly Americans are still hospitalized for HF than for any other condition and more than 40% of HF

patients are readmitted to the hospital within 90 days,[25] contributing to substantial medical and economic burdens.

Measures of cardiac structure and function

Pathophysiologic changes to the heart's structure and function, generally measured via echocardiography, predict HF.[26,27] Measures of structure refer to anatomical changes and abnormalities, whereas measures of cardiac function refer to blood flow efficiency. While these measures may apply to any chamber of the heart, HF frequently results from structural damage to the left ventricle (LV), and so all measures discussed in this project will focus on the LV.

HF may be described in the context of systolic and diastolic dysfunction, both of which are defined by characteristic changes in cardiac structure and function. Eccentric remodeling, where the LV cavity dilates or expands, characterizes systolic dysfunction.[28] In systolic dysfunction, the damaged heart muscle weakens and is unable to maintain its output. LV stroke volume increases to fill the ventricle with blood, but both the ejection fraction (i.e., the proportion of total blood pumped from the heart) and the cardiac output (i.e., the amount of blood pumped from the LV per minute) decrease.[29]

Concentric remodeling, where the LV muscle wall thickens, characterizes diastolic dysfunction by preventing the ventricle from being able to relax completely during diastole.[28] The ratio of LV mass to LV end-diastolic volume increases as the muscle mass increases.[30] Diastolic dysfunction is further defined by blood flow across the mitral valve from the left atrium (LA): if the ratio of transmitral blood flow velocity in early diastole (E wave) to transmitral blood flow velocity in late diastole (A wave) is greater than 1 (i.e., $E < A$), the LV muscle may not fill properly due to hypertrophy and stiffness. Similarly, a ratio of the E wave to the blood

flow velocity across the mitral annulus (e' velocity) that is greater than 15 (i.e., a low e') indicates increased LV filling pressure.

The two major types of left-sided HF have different presentations: HF with reduced (≤ 40%) ejection fraction (HFrEF), which is commonly diagnosed in men and the primary target of existing medical treatment, and HF with preserved (≥50%) ejection fraction (HFpEF), which is more prevalent in older women but does not have any adequate medical interventions.[31,32] Reduced systolic function is associated with HFrEF, while the presence of diastolic dysfunction characterizes HFpEF.[33]

Natural history of CVD progression through HF

We can examine the natural history of CVD progression using the American College of Cardiology (ACC) and AHA's stages of HF risk through advanced HF (**Figure II- 1**). Stages A and B represent HF risk, while Stages C and D represent HF. During Stage A, individuals are at risk of HF due to the presence of non-structural comorbidities, including a family history of HF or cardiomyopathy, diabetes, metabolic syndrome, hypertension, coronary artery disease, and history of drug and alcohol abuse.[34] Stage B is defined by the presence of structural heart disease but the absence of HF symptoms: individuals in this stage have signs of LV dysfunction, such as reduced ejection fraction or LV hypertrophy, and may have experienced an acute CVD event such as MI.[34] Individuals in Stage C have structural heart disease and also begin showing the classic symptoms of HF, specifically dyspnea and fatigue.[34] Finally, Stage D indicates advanced HF, or refractory HF, where severe symptoms affect quality of life despite medical therapy and intervention to manage the illness, and where patients may require specialized care.[34]

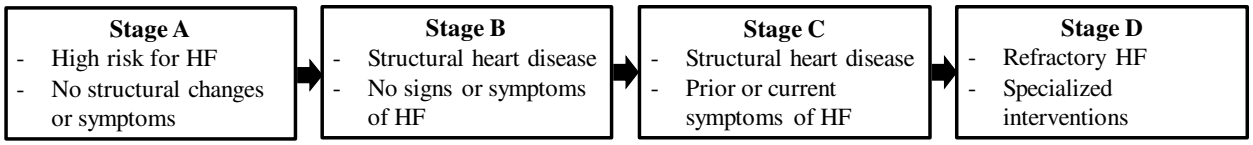

Figure II- 1. ACC/AHA Stages of HF[31]

CVD, including atherosclerosis, myocarditis, and cardiomyopathy, may lead to HF;[35] similarly, the presence of inflammatory cytokines may be associated with HF severity as they can drive fibrosis of cardiac muscle.[36] These cytokines, including interleukin (IL)-4, transforming growth factor (TGF)-β, and IL-1β, are produced during immune responses to inflammation and are important for wound repair, but may be detrimental to the cardiovascular system.[35] Tumor necrosis factor α (TNFα), IL-6, and C-reactive protein (CRP) are inflammatory cytokines that show a direct relationship with HF severity: higher levels of both of these cytokines are associated with reduced functional status in HF patients as measured by the New York Heart Association's classifications.[37–39] TNFα is further associated with dilated cardiomyopathy and hypertrophy in both animal models and humans.[40,41]

Sex differences in CVD

The sex differences apparent in overall CVD extend to the specific case of HF. Men with HF tend to be younger than women with HF,[42] and HFrEF is more common among men while HFpEF is more common among women, with women making up twice as many HFpEF cases as men (63% of all HFpEF cases).[33] The underlying cause of HF in men is generally ischemic heart disease, generally coupled with systolic dysfunction.[33,43] The underlying cause of HF in women is generally diastolic dysfunction with a history of hypertension.[6,33,44] HF is associated with poor prognosis and survival regardless of sex, with a 5-year mortality rate of 50 to 60%.[45] Survival analysis has shown that HFpEF is associated with greater short-term (i.e., within 3 years) survival than HFrEF,[46–49] although patients with HFrEF have seen improvements in survival attributed to the development of medical interventions.[24,50] Indeed, women have a greater relative

survival following HF onset than men (median 3.2 compared with 1.7 years);[48,51] however, they have poorer quality of life outcomes (e.g., Minnesota living with HF score) following HF hospitalization than men.[49,52] As a result of differences in survival, overall HF prevalence is similar between men and women and increases with age.[4,24,42]

Differences in specific risk factor prevalence may account for the sex differences in HF incidence and type of HF. In women, hypertension is the greatest risk factor for developing HF and is more prevalent in women than in men after age 65.[43,53] In men, MI is the greatest risk factor for HF,[43] and HFpEF in men is often associated with coronary artery disease rather than hypertension.[54] Sex modifies the effect of hypertension on HF risk. A review of 35 studies found that women with hypertension are 3 times more likely to develop HF compared with normotensive women, while men with hypertension have only twice the risk of HF compared with normotensive men.[43] In addition to hypertension, obesity is more prevalent in women than men,[4] and body mass index (BMI) is associated with HF to a greater extent in women than in men. Obese women demonstrated greater increases in LV mass and relative wall thickness compared with non-obese women than observed in men.[55] Similarly, the presence of diabetes is associated with a higher risk of HF in women compared with men.[56–58]

Sex differences also exist in cardiac structure and function. Compared with healthy men, healthy women have lower stroke volume and LV mass.[59,60] With aging, LV mass in women increases (i.e., worsens) slightly as the LV wall thickens, but it remains relatively stable in men.[61,62] Women generally have higher heart rates and better diastolic function compared with men, but premenopausal women also see greater changes to their cardiac structure than men due to the increasing prevalence of hypertension in women with aging.[63]

Menopausal changes in CVD risk

Menopause refers to the termination of menstruation as ovarian function decreases and ultimately ceases.[64] Women transition from premenopausal (i.e., having a menstrual cycle) to perimenopausal (i.e., having irregular menstrual cycles) to postmenopausal when they have not had a menstrual period for at least 12 months (natural menopause) or have had a hysterectomy and removal of both ovaries (surgical menopause). Postmenopausal women have a higher incidence of CVD compared with premenopausal women independent of age,[65] and thus the menopause transition is a unique period to study CVD in women as their cardiovascular risk increases dramatically. After menopause, ovarian production of estrogen ceases and women lose their premenopausal protection against CVD.[66] Estrogen is associated with lower LDL and higher HDL cholesterol levels,[67] and menopause is associated with a shift towards more atherogenic levels of total cholesterol, HDL cholesterol, and LDL cholesterol.[68,69] Coronary artery disease rates are 3 times higher in postmenopausal compared with premenopausal women, and CVD-associated morbidities develop at a greater rate after menopause.[6,65] Mouse models have shown that estrogen deficiency results in impaired vascular function.[70] Furthermore, both early menopause[71–73] and bilateral oophorectomy (i.e., surgical removal of both ovaries)[74–76] are associated with increased overall cardiovascular risk in women, suggesting an important role of ovarian estrogen in premenopausal CVD risk protection.

Menopause is associated with changes in HF risk factors as the body ceases production of ovarian estrogen and adapts to changes in sex hormone levels in the body, potentially resulting in the increased risk of incident HF. Postmenopausal women undergo a dramatic difference in their lipid profile compared with premenopausal women, as menopause is associated with higher total and LDL cholesterol and lower HDL cholesterol.[77,78] The loss of estrogen is directly associated

with worse lipid levels. Postmenopausal rates of hypertension are also higher than they are in premenopausal women. Approximately 40% of postmenopausal women have hypertension,[79] potentially as a result of worsening risk factors predisposing women to hypertension, such as weight gain during menopause, and poor control of hypertension in women.[80] Furthermore, postmenopausal women see greater increases in their blood pressure over time compared with premenopausal women.[81]

Menopause has also been associated with worse measures of cardiac structure and function, as postmenopausal women have poorer diastolic function compared with premenopausal women.[82] A small study of 14 early and late postmenopausal women found that greater time spent in menopause was associated with reduced cardiac contractility as measured by LV end-systolic volume.[83] Menopause is associated with greater LV wall thickness[84] and reduced ejection fraction.[85] Unfortunately, many of the studies examining cardiac structure and function by menopause status have small sample sizes or cross-sectional designs that do not control for other factors that may influence cardiac structure and function, making inference about the effect of menopause difficult.[60] However, animal models have shown that ovariectomy (i.e., surgically induced menopause) is associated with increased LV mass[86,87] and diastolic dysfunction[88,89] independent of the effect of age.[89]

Other risk factors for HF that change during menopause include coronary artery disease incidence, insulin resistance, and weight gain. Coronary artery disease is characterized by the development of atherosclerosis in the coronary arteries, and although it is more common in men at risk of HF, it still occurs at greater rates after menopause in women.[90] Postmenopausal women have increased incidence of plaque formation in the carotid artery[91] and a more atherogenic lipid profile[90] compared with premenopausal women. Similarly, women are at increased risk of insulin

resistance after menopause,[92] ultimately amplifying their risk of diabetes and subsequent HF. Women frequently report increases in body weight as they go through menopause.[93–95] However, menopause is independently associated with changes in overall fat distribution, with a shift in preferential deposition from subcutaneous, or peripheral, adipose tissue to visceral adipose tissue.[93]

Type 1 diabetes mellitus and CVD

Type 1 diabetes mellitus is a condition characterized by autoimmune destruction of the insulin-producing β cells of the pancreas. Incidence of type 1 diabetes is increasing globally,[96] and improvements to diabetes and complications management have resulted in increased life expectancy.[97] Adults with type 1 diabetes nonetheless have increased risk of CVD, specifically of coronary artery disease,[98] and as they live to older ages it is imperative to understand how cardiovascular complications in type 1 diabetes progress.

CVD events are more common in type 1 diabetes, and they may occur a decade or more earlier than they do in adults without diabetes.[99] The natural history of CVD in type 1 diabetes including risk factors, subclinical disease presentation, and clinical disease presentation is presented in **Figure II- 2.** Type 1 diabetes mellitus is most commonly diagnosed early in life, with peak incidence occurring in adolescents between the ages of 10 and 14 years.[96] As such, individuals diagnosed with type 1 diabetes have prolonged exposure to dysglycemia and increased risk of developing insulin resistance.[100] Hyperglycemia is associated with oxidative stress,[101] and insulin resistance is partially linked to the increasing prevalence of overweight and obesity in type 1 diabetes.[102,103]

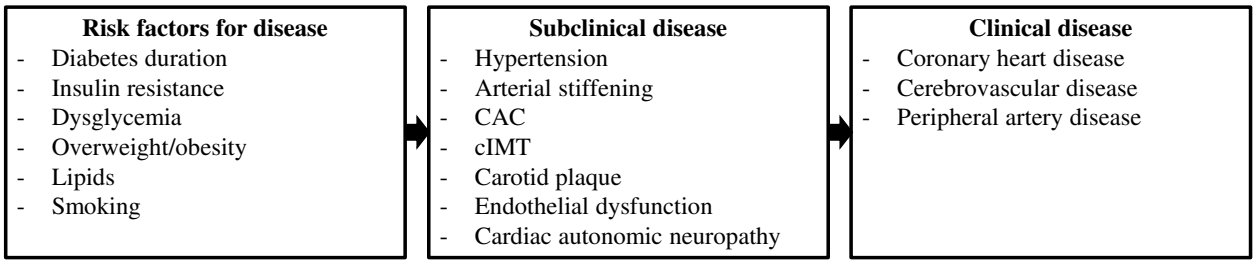

Figure II- 2. Natural history of CVD in type 1 diabetes.[10,104]

Type 1 diabetes is associated with a persistent state of systemic inflammation,[105,106] which likely exacerbates CVD risk factors and contributes to development of CVD. Adults with type 1 diabetes are more likely to have hypertension[107] and have more coronary artery calcium (CAC)[108] than adults without diabetes. Carotid-femoral pulse wave velocity is higher and brachial distensibility is lower in type 1 diabetes compared with non-diabetic controls,[109–111] indicating greater arterial stiffness. Children with type 1 diabetes have higher carotid intima-media thickness (cIMT) compared with children without diabetes,[111,112] and endothelial function measured by brachial artery flow-mediated dilation may be impaired in children with type 1 diabetes as early as between ages 6 and 13 years.[113,114]

Ultimately, subclinical CVD progresses into clinical disease. In type 1 diabetes, the most common CVD outcomes are coronary heart disease (i.e., coronary artery disease), cerebrovascular disease, and peripheral artery disease.[10,98]

Type 1 diabetes and sex differences in CVD

Among individuals with type 1 diabetes, CVD is the leading cause of mortality and men are diagnosed with CVD at a higher rate than women; however, type 1 diabetes confers a greater increase in CVD risk in women than in men.[12,115,116] Compared with non-diabetic women, women with type 1 diabetes have nearly 9 times the risk of CVD; prior to the age of 40, this increase in risk is between 30- and 40-fold.[12] Men with type 1 diabetes have 4.5 times the risk of CVD, and prior to the age of 40, this increase in risk is between 8- and 17-fold.[12] A study of

more than 7,000 adults with type 1 diabetes and 38,000 adults without diabetes in the United Kingdom found similar differences by sex in overall risk of CVD, where type 1 diabetes was associated with a HR of CVD of 3.6 in men and 7.6 in women.[99] The larger increase in risk may be associated with the greater insulin resistance that women with type 1 diabetes experience compared with men, as insulin resistance is associated with increased CVD risk and levels of CAC.[117,118] Women with type 1 diabetes may also have more atherogenic lipids[119] or adverse fat distribution patterns[9] compared with women without diabetes, resulting in the larger difference in risk than seen among men with and without type 1 diabetes.

Women face a unique set of risk factors that influence their CVD risk: the menstrual cycle, sex hormones, pregnancy, and menopause.[4,6,120] While estrogen is thought to protect women against CVD, an irregular menstrual cycle can contribute to an increased risk of heart disease due to hypoestrogenemia.[121] During pregnancy, women who experience complications such as preeclampsia[122,123] and gestational diabetes[124] are at higher risk of future CVD. Women are uniquely subject to peripartum cardiomyopathy during and within 5 months following delivery,[125] and pregnancy-related cardiac remodeling can persist for up to 6 months postpartum.[126,127] Preeclampsia contributes to the risk of future chronic hypertension and cardiac remodeling,[122,128–130] and more severe preeclampsia is associated with a greater degree of diastolic dysfunction[131] and with significantly higher risk of future HF and CVD.[132]

Women with type 1 diabetes do not appear to have the same premenopausal protection against CVD that women without diabetes have despite having similar estrogen and progesterone concentrations.[14] **Table II- 1** shows sex hormone concentrations stratified by phase of the menstrual cycle in 311 women in the Coronary Artery Calcification in Type 1 Diabetes (CACTI)

study.[14] In this study, women with type 1 diabetes had higher testosterone and SHBG concentrations, but similar estrogen concentrations.

Table II- 1. Sex hormone levels by type 1 diabetes status, least square geometric means, adjusted for BMI and age[14]

	Early follicular		Late follicular		Luteal		Anovulatory	
	T1DM (n=27)	**Control (n=26)**	**T1DM (n=61)**	**Control (n=54)**	**T1DM (n=58)**	**Control (n=68)**	**T1DM (n=14)**	**Control (n=3)**
Testosterone (ng/dL)	38.9	30.1	47.7*	39.2	43.0*	35.8	44.0	35.6
SHBG (nmol/L)	151.3	112.3	137.9*	108.4	147.9**	89.6	108.0	111.9
Free androgen index	0.89	1.06	1.20	1.24	0.97**	1.39	1.50	1.08
Estradiol (pg/ml)	43.9	43.2	126.0	143.0	120.9	105.5	51.3	64.3
Free estradiol index	0.11	0.14	0.34*	0.48	0.30*	0.43	0.18	0.21
LH (mIU/ml)	5.9	4.6	9.6	9.9	4.2	3.9	3.0	9.1
FSH (mIU/ml)	9.6	8.1	8.2	8.1	4.3	4.4	4.5**	14.3
Progesterone (ng/ml)	0.55	0.59	0.81	0.73	10.0	8.7	0.89	0.73

Abbreviations: T1DM, type 1 diabetes mellitus; SHBG, sex hormone-binding globulin; LH, luteinizing hormone; FSH, follicle-stimulating hormone
* $p < 0.0125$, ** $p < 0.001$
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Despite the similar estrogen concentrations regardless of diabetes status, women with type 1 diabetes nevertheless have a higher prevalence of ovarian dysfunction than women without diabetes. Amenorrhea, irregular menstrual cycles, and polycystic ovary syndrome (PCOS) are all more prevalent in type 1 diabetes.[133–135] In addition to the higher prevalence of ovarian dysfunction, type 1 diabetes is also associated with pregnancy complications, such as preeclampsia,[136] and adverse birth outcomes.[137,138]

Type 1 diabetes and menopause

Among women with type 1 diabetes, menopause is associated with greater progression of CAC volume over time. Not only do premenopausal women with type 1 diabetes have increased baseline risk of CVD as measured by CAC, but among women followed longitudinally in the CACTI study, CAC volume increased to a greater extent across menopause than in women

without diabetes. This increase in CAC volume persisted after adjustment for traditional cardiovascular risk factors, including lipids, blood pressure, adiposity, and ovarian dysfunction,[13] and so the mechanism by which menopause affects women with type 1 diabetes to a greater degree is unknown.

Potential mechanisms for CVD: sex hormones and inflammation

The sex and menopausal differences observed in HF and in the risk factors leading to HF may partially be explained by differing levels of sex hormones in males, premenopausal females, and postmenopausal females. Testosterone and estrogen levels affect male and female adaptations to cardiac stress. Endogenous testosterone is believed to be protective against CVD risk in men, but testosterone is also associated with apoptosis in cardiac myocytes, resulting in eccentric remodeling. [139] Indeed, myocyte apoptosis occurs at 3 times the rate in men compared with women[140] and at more than 200 times the rate in HF versus normal hearts.[141]

Estrogen is believed to provide protection against cardiac risk in premenopausal women due to the changes in CVD incidence seen after menopause. The change in sex hormone levels with menopause is associated with increased risk of CVD.[142,143] In contrast to testosterone, estrogen – an inclusive term that refers to the three major hormones of estradiol, estrone, and estriol – is protective against myocyte apoptosis,[35] and so premenopausal women undergo less cardiac remodeling and do not see the same decrease in myocyte number that men do.[60,144] Estrogen interacts with numerous biological pathways, and is associated with downregulation of proteins that promote cardiac stiffness,[144] inflammatory cytokines,[145–147] and cardiac fibrosis.[148] It also activates synthesis of nitric oxide (NO) in vascular endothelial cells, resulting in greater levels of NO.[149] NO is associated with cardiac contractility and reduced inflammation,[150] and

reduced bioavailability of NO is associated with greater vasoconstriction and increased risk of CVD, inflammation, and smooth muscle proliferation.[150,151]

Expression of estrogen receptors (ERs) is reduced with menopause as production of ovarian estrogen ceases.[152] Reductions in ER activity may be associated with atherosclerosis[152] and cardiac inflammation,[153] whereas normal ER expression is associated with reduced cardiac hypertrophy and fibrosis.[154] Low estrogen concentrations in postmenopausal women may ultimately lead to diastolic dysfunction through activation of the renin-angiotensin-aldosterone system (RAAS), a system that normally regulates blood pressure but can lead to hypertension through overactivation.[155] Animal models confirm that the loss of ovarian estrogen production through ovariectomy has resulted in cardiac hypertrophy and fibrosis,[87,156] emphasizing the important role of estrogen in cardiovascular health.

Estrogen is associated with a more favorable lipid profile, as premenopausal women have better lipids compared with either men or postmenopausal women. Premenopausal women have lower total and LDL cholesterol and increased HDL cholesterol compared with both men and postmenopausal women. LDL cholesterol can interact with free radicals, resulting in its oxidation.[157] Oxidized LDL is proatherogenic and associated with increased risk of CVD: it is an important factor in the formation of foam cells and their accumulation into fatty streaks in the arteries.[158,159] Estrogen prevents LDL oxidation and thereby may prevent the lipid accumulation that leads to coronary artery disease.[6] Furthermore, estrogen triggers expression of the LDL receptor, allowing for increased uptake and removal of LDL cholesterol.[160] Estrogen is associated with downregulation of inflammatory cytokines such as TNFα and IL-1β[161] that may explain the lower CVD and subsequent mortality risk in premenopausal compared with postmenopausal women.

Inflammation may contribute to CVD in postmenopausal women, as they no longer benefit from the anti-inflammatory effects of estrogen. Increasing age is associated with increased levels of inflammation, and inflammatory markers are correlated with diastolic dysfunction and subsequent HFpEF.[162] Epidemiologic studies of menopause have shown that estrogen deficiency is associated with increased levels of TNFα, IL-1, and IL-6,[163] and animal models provided further evidence that ovariectomy was associated with marked increases in inflammatory cytokine levels.[164]

Aims 1 and 2: Pericardial adipose tissue in CVD

Prevalence of overweight and obesity in the United States continues to increase,[4] and so it is imperative to understand the effect on cardiovascular and overall health. Adipose tissue stores fat in the form of triglycerides and fatty acids, and it is an endocrine organ that may secrete hormones or other proteins.[165] Subcutaneous adipose tissue (SAT) is the major fat depot in the body, accounting for about 80% of total body fat.[166] It accumulates under the skin and deposits largely in the gluteal-femoral region or peripherally, whereas visceral adipose tissue (VAT) deposits near the internal organs and contributes to central obesity.[167] VAT is associated with higher concentrations of IL-6, TNFα, CRP, and plasminogen activator inhibitor 1 (PAI-1), all of which are associated with atherosclerosis and increased systemic inflammation. [168] VAT is further associated with increased insulin resistance, and increased VAT levels are a predictor of metabolic disease.[169]

Men have more VAT, whereas premenopausal women have more SAT, particularly in their lower body (i.e., the gluteal-femoral region).[170,171] Among men and women matched on BMI, women have a greater percentage of body fat compared with fat-free mass than men.[170,172] Estrogen may play a role in fat deposition, as SAT expresses estrogen receptors that may

promote accumulation of fat in SAT rather than VAT depots.[173] This potential role of estrogen in the modulation of fat accumulation may protect against metabolic syndrome,[172] as animal models have shown that deletion of estrogen receptors in female mice causes insulin resistance, increased overall adiposity, and cholesterol levels.[174,175]

With menopause, women's overall VAT levels and their risk for cardiovascular and metabolic disease increases.[176] VAT levels as measured by whole-body CT scans were more than 2.5 times greater in postmenopausal than premenopausal women.[172] Hormone therapy with estrogen and progesterone, however, may decrease preferential VAT accumulation in postmenopausal women.[177] Ovariectomized animal models have suggested an association between menopause and increased VAT compared with intact animals, and estrogen administration was associated with reductions in VAT mass.[178]

Pericardial adipose tissue (PAT) is a specific ectopic fat depot that surrounds the heart and is correlated with overall VAT burden (r=0.81).[179] It includes fat on the inside of the pericardial sac that is in direct contact with the cardiac muscle (i.e., epicardial adipose tissue) as well as fat that is immediately external to the pericardium (i.e., paracardial adipose tissue).[180] Epicardial adipose tissue is located inside the pericardium and shares a blood supply with the heart.[180,181] Because of differences in the embryological origin of epicardial and paracardial adipose tissue, as well as potential differences in function of the two fat depots, epicardial adipose tissue is believed to be a better measure of CVD risk.[181,182] However, available imaging techniques may not be sensitive enough to isolate a measure of epicardial adipose tissue, and many studies have instead chosen to measure total PAT instead of differentiating between epicardial adipose tissue and paracardial adipose tissue. PAT is easier to measure using various imaging tools, and is significantly correlated with epicardial adipose tissue (0.92).[183]

PAT is of particular concern in the context of HF due to its location and its association with inflammation. As a VAT depot, PAT is correlated with inflammatory markers and markers of oxidative stress, including CRP, IL-6, and MCP-1,[184] and it is associated with subclinical atherosclerosis and coronary artery disease,[185,186] insulin resistance,[187,188] and diabetes mellitus.[187,189] PAT is associated with general risk factors for HF, changes in cardiac structure and function, and CVD events after adjusting for other comorbidities.

Like VAT, men have more PAT compared with women, and postmenopausal women have more PAT compared with premenopausal women. A cross-sectional analysis of PAT volume in the Study of Women's Health Across the Nation (SWAN) found that decreased levels of estrogen were associated with higher levels of PAT: postmenopausal women had 21% more PAT volume than premenopausal women.[190] A second analysis in SWAN showed an inverse association between PAT volume and estrogen concentration, with greater declines in estrogen levels associated with greater increases in PAT volume.[190] Greater PAT volume is further associated with increases in LV mass,[191] reduced cardiac output, and reduced stroke volume.[192] Similarly, it is associated with worse diastolic dysfunction;[191,192] excess PAT may result from obesity and is also associated with cardiomyopathy.[193] Weight loss preferentially reduces VAT, and weight loss is associated with a reduction in PAT and improvements in cardiac remodeling.[194] An examination of cardiac MRI-derived PAT volume found that PAT is associated with reduced cardiac output and stroke volume.

Aim 3: Estrogen receptors in type 1 diabetes

Ovarian dysfunction, systemic inflammation, and PCOS are all more prevalent in women with type 1 diabetes and are further associated with future development of CVD.[195,196] Estrogen deficiency is also associated with CVD, but women with type 1 diabetes have similar estrogen

concentrations as women without diabetes. Expression of estrogen receptors (ERs), which mediate the genomic and non-genomic actions of estrogens on cells, may differ by diabetes status and potentially be a mechanism by which ovarian dysfunction and CVD risk are increased in type 1 diabetes.

The two primary ERs are ERα and ERβ, which bind with estrogen and act as nuclear transcription factors to regulate gene expression in cells.[197] Genetic polymorphisms to ERα and ERβ have been implicated in development of CVD.[198,199] Furthermore, in ERα- and ERβ-knockout animal models, estrogen was unable to downregulate the production of inflammatory cytokines, and thus was unable to exert its anti-inflammatory properties.[200] The anti-inflammatory and potentially cardioprotective effects of estrogen, then, may not be realized if there is an impairment or deficiency in ER expression.

NF-κB is a nuclear transcription factor that is associated with chronic inflammatory and autoimmune disease, including type 1 diabetes.[201] A preliminary analysis of 27 adults with type 1 diabetes and 20 adults without diabetes showed that endothelial cell expression of NF-κB normalized to human umbilical vein endothelial cell expression (HUVEC) was significantly higher in type 1 diabetes after adjusting for age and sex.[202] NF-κB and ER expression may interact, and ER expression may repress NF-κB expression to downregulate the inflammatory response.[203]

CHAPTER III

APPROACH

Aims 1 and 2: The CARDIA study

Study design

The Coronary Artery Risk Development in Young Adults (CARDIA) study is a prospective cohort study that enrolled 5,115 study participants between 1983 and 1985 to examine predictors of CVD prior to middle age.[204] The CARDIA study had two primary aims: to characterize the distribution of cardiovascular risk factors, and to identify behavioral risk factors associated with baseline and longitudinal changes in cardiovascular risk factors. Participants were between the ages of 18 and 30 years at baseline and were recruited from one of four study sites: Birmingham, AL; Chicago, IL; Minneapolis, MN; or Oakland, CA. The biracial cohort was sampled to have approximately equal numbers of black and white participants and men and women. Potential study participants were excluded at baseline if they had a chronic illness or disability that would prevent completion of the study or if they were pregnant.

Participants completed study visits at baseline and at 2, 5, 7, 10, 15, 20, 25, and 30 years of follow-up, and there was 71% retention of all surviving participants at the Year 30 (Y30) study exam. In September 2017, CARDIA was funded for a limited Y35 study visit to take place between June 2020 and July 2021.[205] A summary of the number of study participants at the completed visits (Y0-Y30) is presented in **Table III- 1.**

Table III- 1. Number of CARDIA participants completing visits at each year.

Year 0	Year 2	Year 5	Year 7	Year 10	Year 15	Year 20	Year 25	Year 30
5,115	4,624	4,352	4,086	3,950	3,672	3,547	3,499	3,358

Data collection

Components of visits at each study year, including study protocols, forms, and questionnaires, are publicly available on the CARDIA study website.[206] In brief, study participants completed a series of questionnaires regarding their medical history and provided information about cardiovascular morbidity. The endpoints of interest were CVD events, including MI, HF, stroke, coronary artery disease, and hypertension; procedures, including coronary revascularization; and other comorbidities, including diabetes mellitus and asthma. In the case of a participant death, study staff attempted to determine the cause and location.[207] A subcommittee of physicians from CARDIA centers reviewed all endpoints to adjudicate event occurrence, confirm morbidity occurrence, and/or verify cause of death.[207]

All study visits included a clinical exam, during which participants' resting blood pressure and anthropometric measures, including height, weight, and waist circumference, were measured. Participants provided a fasting blood sample, and plasma lipids and lipoproteins were assayed at each study visit. As of the Y7 study visit, serum insulin and glucose have also been assayed at each study visit. Furthermore, trained interviewers administered questionnaires to collect data on medical history; illicit drug, tobacco, and alcohol use; sociodemographic information; and physical activity at all study visits. As of Y7, diet questionnaires were also administered, and as of Y15, a specific reproductive history questionnaire was administered to all women in the CARDIA study.

Study population

The analyses presented in Aims 1 and 2 of this dissertation use cross-sectional data collected at the Y25 study visit. At this study visit, participants were between the ages of 43 and 55 and completed both CT scans and echocardiograms. While CT scans and echocardiograms

had been done at previous study visits, they were not completed at the same study visit and so the association between the CT scan-derived adipose tissue measures and the echocardiogram-derived measures of cardiac structure and function could not be examined.

At Y25, 3,499 participants completed all or part of the study visit, and 3,173 of the returning participants completed echocardiograms and had PAT and VAT volumes from cardiac and abdominal CT scans, respectively.

Measures of adiposity

We evaluated two measures of adiposity: PAT volume and abdominal VAT volume. PAT volume was the primary measure of interest, and was measured from gated cardiac electron beam computed tomography (EBCT) scans.

Outcomes: cardiac structure and function

The following echocardiogram measures of cardiac structure and function were chosen *a priori* to examine as outcomes: LV ejection fraction, a clinical measure of systolic function; LV mass, a measure of cardiac structure; E/A ratio, a measure of transmitral velocity; and average E/e' ratio, a measure of LV filling pressure. The E/A and E/e' ratios define diastolic function.

Statistical analysis

Linear regression was used to examine the relationship between PAT volume and the outcomes of interest. First, the statistical interaction between sex and PAT volume was tested to identify a differential association between PAT volume and the outcome between men and premenopausal women. If the PAT volume by sex interaction term was found significant at $p<0.05$, sex-stratified β estimates were examined. If the PAT volume by sex interaction was not found to be significant at $p<0.05$, the interaction term was removed and the sex-adjusted association of PAT volume with the outcome of interest was examined.

The second set of models further adjusted for potential confounders as described in the following aim-specific sections. The third and final set of models further adjusted for abdominal VAT volume to test the independent association between PAT volume and the outcomes of interest.

Aim 1: PAT volume and cardiac structure and function in men vs. women.

Study population

Aim 1 included men and premenopausal women who attended the Y25 study visit. Participants were excluded from the study sample if they were missing any of the variables of interest, and women were excluded from the study sample if they reported any of the following on a reproductive history questionnaire:

1) Menopause, either by surgery or naturally
2) No menstrual cycle for ≥ 12 months
3) Hysterectomy

Ultimately, the study sample for Aim 1 included 1,170 men and 620 premenopausal women who had complete data on each of the 4 measures of cardiac structure and function and on the measures of adiposity of interest.

Risk adjustment

We generated the following list of candidate variables for potential inclusion in our fully adjusted model: age, race, education, alcohol intake, systolic blood pressure, diastolic blood pressure, use of statins, use of antihypertensive medications, smoking, total physical activity intensity score, and diabetes mellitus. The final adjusted model included variables that were associated with a 10% or greater change to the association between PAT and the outcome, and additional variables believed to be important to include (e.g., age). The final adjusted model

included age, race, education, alcohol intake, blood pressure, use of antihypertensive medications, and diabetes mellitus.

Expected outcomes and power

We hypothesized that there would be a significant relationship between PAT volume and the measures of cardiac structure and function independent of sex, confounders, and VAT volume. The minimum detectable difference between men and premenopausal women for each of the outcomes of interest at 80% power is presented in **Table III- 2**.

Table III- 2. Minimum detectable difference determined by power calculation assuming a sample of 1,790 and alpha=0.05

Outcome measure	Standard deviation	Minimum detectable difference
LV ejection fraction, %	7.34	0.93
LV mass index, $g/m^{2.7}$	11.37	1.46
E/A ratio	0.36	0.05
E/e' ratio	2.17	0.29

Aim 2: PAT volume and cardiac structure and function in premenopausal, surgically postmenopausal, and naturally postmenopausal women.

Study population

Aim 2 included premenopausal women and both naturally and surgically postmenopausal women who attended the Y25 study visit. Women were categorized as premenopausal based on the same criteria as those used in Aim 1: they had to report at least 1 menstrual cycle in the previous 12 months, they reported not having undergone menopause, and they reported not having had a hysterectomy. Women were categorized as naturally postmenopausal if they reported having undergone menopause, not having a menstrual cycle in at least 12 months and that their menstrual cycles stopped naturally. Women were categorized as surgically postmenopausal if they reported having a hysterectomy, either with ovarian preservation or with

bilateral oophorectomy (BLO). The study sample for Aim 2 included 620 premenopausal women, 487 naturally postmenopausal women, 230 women with hysterectomy with ovarian preservation, and 104 women with hysterectomy with BLO. The participants included in these analyses had complete data on the 4 measures of cardiac structure and function and the measures of adiposity of interest.

Risk adjustment

We generated the following list of candidate variables for potential inclusion in our model: age, race, education, alcohol use, systolic blood pressure, diastolic blood pressure, use of statins, use of antihypertensive medications, smoking status, total physical activity intensity score, diabetes mellitus, use of hormones, history of oral contraceptive use, and parity. We used the same approach to risk adjustment described in Aim 1: we tested each candidate variable as a potential confounder. The final model included variables believed to be important to account for, as well as variables associated with at least a 10% change in the association between PAT volume and the outcomes of interest. The final model included age, race, education, alcohol use, blood pressure, use of antihypertensive medication, physical activity intensity score, and diabetes mellitus.

Expected outcomes and power

We hypothesized that there would be a larger association between PAT volume and the outcomes of interest for the surgical and natural menopause groups compared with the premenopausal group due to an adverse cardiovascular risk profile[75] and extended exposure to the absence of ovarian estrogen in the case of natural menopause and hysterectomy with BLO. The power to detect differences between mean values of each menopause group is presented in **Table III- 3**. Given the menopause group-specific mean levels of each outcome measure, we

would have at least 99% power to detect differences across groups for all outcomes except LV ejection fraction given our sample size of 1,551 women.

Table III- 3. Power to detect differences between menopause groups.

Outcome measure	Premenopausal	Naturally postmenopausal	Hysterectomy, ovarian preservation	Hysterectomy, BLO	SD	Power
LV ejection fraction, %	62.5	62.8	62.2	63.8	6.98	0.79
LV mass index, $g/m^{2.7}$	38.5	38.7	42.6	43.5	11.93	0.99
E/A ratio	1.35	1.24	1.26	1.2	0.37	0.99
E/e' ratio	7.71	8.25	8.39	8.71	2.38	0.99

Aim 3: Endothelial cell protein expression by type 1 diabetes status in the EDEN study

Study design

The Estrogen, Diabetes, and Endothelial Function (EDEN) study was a pilot substudy of the Women, Insulin, and Sex Hormones (WISH) study. The WISH study recruited a study population of premenopausal women with and without diabetes between the ages of 18 and 45 years. Women were eligible to participate in the WISH study if they were not pregnant or using any hormonal contraceptives, had normal thyroid function, and had regular menstrual cycles. Participants completed a total of 4 study visits during the following phases of their menstrual cycle: early follicular (days 2-5), late follicular (days 6-9), early luteal (days 6-9 after ovulation), and luteal (days 11-13 after ovulation).

The EDEN study recruited 20 women with type 1 diabetes and 20 women without diabetes to examine differences by diabetes status in endothelial function and protein expression in vascular endothelial cells. Measures of endothelial function and protein expression were examined before and after an ovarian suppression intervention with randomization to concurrent estradiol add-back or placebo.

Data collection

EDEN participants completed a screening visit during which their hemoglobin A1c and thyroid-stimulating hormone concentration were measured to ensure eligibility for the study. They then completed a fasted baseline study visit which took place during days 2-5 (i.e., the early follicular phase) of their menstrual cycle when estrogen and progesterone concentrations are naturally at their lowest.[208] During this visit, a trained research assistant collected measures of anthropometry, brachial distensibility, and cIMT. Endothelial cells were harvested via an intravenous catheter placed in an antecubital vein.[209,210] Endothelial function was measured first following an intravenous saline infusion and second after an intravenous vitamin C infusion to determine the contribution of oxidative stress to endothelial function. During this visit, participants self-reported demographic, medical history, reproductive history, current medication, depression, menstrual discomfort,[211] physical activity, tobacco use, and alcohol use via questionnaires. Participants with type 1 diabetes further self-reported their insulin use.

All participants then underwent hormone suppression with the drug Cetrotide, a gonadotropin-releasing hormone antagonist, for the 1-week period following their baseline visit. They were concurrently randomized to receive either a transdermal estradiol patch or a placebo patch. After 1 week, participants returned for a follow-up visit during which they repeated all procedures.

Study population

Of the 40 women enrolled in the EDEN study, endothelial cells were successfully harvested from 34 women. These 18 women with type 1 diabetes and 16 women without diabetes were included in the analyses presented in this dissertation.

Outcomes: Endothelial cell protein expression

The protocol used to process and analyze endothelial cells is described in detail in Chapter VI. Briefly, the endothelial cells harvested during the baseline and follow-up EDEN study visits were isolated via centrifugation, fixed to slides, and stored at 70°C until further analysis.

Expression of total and nuclear ERα, ERβ, and NF-κB was measured using quantitative immunofluorescence, and control human umbilical vein endothelial cells (HUVEC) were included in each batch of EDEN slides examined. The stored endothelial cells and HUVEC control cells were incubated with a primary and fluorescent secondary antibody chosen to identify the endothelial protein of interest, and then with von Willebrand factor to identify endothelial cells. Cells were finally stained with DAPI (4′, 6′-diamidino-2-phenylindole hydrochloride) to confirm integrity of the nucleus.

Slides were then systematically scanned and analyzed using a fluorescence microscope and NIS-Elements BR Software. This software quantified the intensity of the fluorescent secondary antibody staining. Endothelial cell expression in study participants was normalized to HUVEC expression to control for variability with staining across batches.

Statistical analysis

Linear regression was used to examine baseline endothelial cell expression of ERα, ERβ, and NF-κB by diabetes status after adjustment for age and estradiol concentration. The change in protein expression between the baseline and follow-up visits was calculated, and linear regression was used to identify differences in the change in protein expression by diabetes status after adjustment for age, estradiol concentration, and randomization to either the estradiol or placebo patch.

Expected outcomes and power

We hypothesize that women with type 1 diabetes will have lower ER expression and higher NF-κB expression. The minimum detectable difference between women with and without type 1 diabetes for each of the endothelial proteins at 80% power is presented in **Table III- 4**. These power calculations used the sample size of 34 women, standard deviations of the sample, and assumed an alpha of 0.05. Given the small sample size of this pilot study, the results will primarily inform hypotheses and design of future studies.

Table III- 4. Minimum detectable difference determined by power calculation assuming a sample of 34 and alpha=0.05.

Endothelial protein	Standard deviation	Minimum detectable difference
ERα/HUVEC, total	0.15	0.149
ERα/HUVEC, nuclear	0.17	0.168
ERβ/HUVEC, total	0.10	0.099
ERβ/HUVEC, nuclear	0.10	0.099
NF-κB/HUVEC, total	0.11	0.109
NF-κB/HUVEC, nuclear	0.12	0.119

CHAPTER IV

PERICARDIAL ADIPOSE TISSUE VOLUME IS ASSOCIATED WITH SEX DIFFERENCES IN CARDIAC STRUCTURE AND FUNCTION

Abstract

Background. Sex differences in the risk of cardiovascular disease (CVD) exist, with premenopausal women having lower CVD risk than men. Pericardial adipose tissue (PAT), a depot of cardiac ectopic fat, may be a mechanism by which underlying cardiac structural and functional differences related to sex exist.

Methods. We conducted a cross-sectional analysis of 1,170 men and 620 premenopausal women (mean age 50 ± 4 and 48 ± 3 years, respectively) in the Coronary Artery Risk Development in Young Adults (CARDIA) study. A statistical interaction term was tested in linear regression models to evaluate if PAT volume was differentially associated with 4 measures of cardiac structure and function between men and premenopausal women. These 4 outcomes were left ventricular (LV) ejection fraction, LV mass indexed to height$^{2.7}$, E/A ratio, and E/e' ratio. We then tested for an association between PAT volume and these outcomes after further adjustment for abdominal visceral adipose tissue (VAT) volume.

Results. The cross-sectional linear relationship between PAT volume and LV ejection fraction (p=0.20) and average E/e' ratio (p=0.051) was not differential by sex. There were sex differences in the linear relationships between PAT volume and both LV mass index and E/A ratio: women had greater differences in LV mass index (3.2 ± 0.4 g/m$^{2.7}$ in women vs. 2.4 ± 0.3 g/m$^{2.7}$ in men, p<0.0001 for both) and in E/A ratio (-0.10 ± 0.04 in women vs. -0.06 ± 0.01 in men, p<0.0001 for both) than men per 1-standard deviation (SD) increment in PAT volume. A 1-SD increment change in PAT volume was associated with higher E/e' ratio independent of sex

(0.27 ± 0.05, p<0.0001). PAT was not associated with LV ejection fraction (p=0.33), but remained significantly associated with LV mass index, E/e' ratio, and E/A ratio in premenopausal women only after adjustment for VAT volume.

Conclusions. PAT is associated with adverse cardiac structure and function in men and premenopausal women and may be mechanism for cardiovascular damage. Although men have more PAT than premenopausal women, PAT was associated with worse LV mass index and E/A ratio in premenopausal women than men. Future studies must examine longitudinal relationships to elucidate this mechanism.

Introduction

Heart failure is a leading cause of cardiovascular disease (CVD) and subsequent mortality among both men and women in the United States,[2,212] but sex differences exist in clinical manifestation and cardiovascular outcomes. Premenopausal women have significantly lower CVD risk compared with men of similar ages, and indeed, men are diagnosed with CVD and heart failure earlier than women.[7] Measures of cardiac structure and function predict heart failure in middle-aged and young adults prior to manifestation of the disease.[59] These measures also differ between men and women, presumably leading to some of the sex differences in heart failure.[59] Men are likely to have eccentric cardiac remodeling resulting from ischemic heart disease,[213] which is more commonly associated with a diagnosis of heart failure with reduced ejection fraction (HFrEF).[33,35] Conversely, women are more likely to have concentric cardiac remodeling resulting from prolonged exposure to hypertension, more commonly associated with a diagnosis of heart failure with preserved ejection fraction (HFpEF).[33,44]

Proinflammatory fat depots may be CVD risk factors associated with sex-specific changes to cardiac structure and function. Men generally carry higher levels of visceral adipose

tissue (VAT) than women,[171] which is associated with systemic inflammation due to its secretion of proinflammatory cytokines.[168] Pericardial adipose tissue (PAT) is the ectopic adipose tissue depot that surrounds the heart, and may contribute to localized inflammation.[180,214] PAT volume, as measured by cardiac computed tomography (CT) scan, has been shown to be correlated with overall VAT volume.[179] However, due to its location and sex-specific differences in adipose tissue distribution, it may be an independent mechanism for cardiac structure and function. In this study, we examined whether a linear relationship between PAT volume and measures of cardiac structure and function differs between men and premenopausal women in the population-based Coronary Artery Risk Development in Young Adults (CARDIA) study. Additionally, the relation between PAT volume with cardiac structure and function independent of abdominal VAT volume was examined. We hypothesized an association between PAT volume and cardiac structure and function in men and women independent of abdominal VAT volume.

Methods

Study population

The biracial CARDIA study began enrollment in 1985-1986 with 5,115 black and white men and women between the ages of 18 and 30 and has followed participants for more than 30 years. Study participants were recruited from 4 sites across the United States (Birmingham, AL; Chicago, IL; Minneapolis, MN; and Oakland, CA).[204] All study participants provided written informed consent at each visit, and all protocols were reviewed approved by the site institutional review boards.

Analyses for the current study include cross-sectional data collected at the CARDIA Year 25 (Y25) study visits due to the availability of both echocardiograms and computed tomography (CT) scans. At Y25, 72% of the surviving members of the initial cohort completed study

visits,[215] and 1,170 men and 620 premenopausal women were included in these analyses based on availability of PAT volume, VAT volume, and measures of cardiac structure and function as described in the following sections. Premenopausal status was ascertained based on self-reported reproductive history. Women were considered premenopausal if they reported having a menstrual cycle at any time during the 12 months prior to the Y25 study visit, had not had a hysterectomy, and had not undergone menopause.

Study data collection

All CARDIA study sites followed standardized study protocols for all exam components.[204] Clinical examination components included measurement of pulse rate, blood pressure after at least 5 minutes at rest, height, weight, and waist circumference. Blood pressure was measured 3 times and the second and third measurements were averaged and used in all analyses. Height was measured using either a wall-mounted ruler or a stadiometer, and weight was measured using balance-beam scale. Height and weight were recorded without shoes and with the participant in light clothes; body mass index (BMI) was calculated as the participant's weight in kilograms over their height in meters, squared (kg/m^2). Waist circumference was measured horizontally at a level laterally midway between the iliac crest and the lowest lateral portion of the rib cage, and anteriorly midway between the xiphoid process of the sternum and the umbilicus.

Hypertension was defined according to the most recent guidelines as systolic blood pressure ≥ 130 mmHg, diastolic blood pressure ≥ 80 mmHg, or use of antihypertensive medications.[216,217] Diabetes mellitus status was ascertained based on fasting glucose concentration ≥ 126 mg/dL, oral glucose tolerance ≥ 200 mg/dL, hemoglobin A1c ≥ 6.5%, or use of diabetic medications. Total physical activity intensity score was estimated based on self-

reported moderate and heavy physical activity on the CARDIA Physical Activity History questionnaire.[218]

Study participants completed questionnaires to provide data on their family and medical history, current and past use of medications, and smoking status. Interviewers also administered questionnaires to obtain or confirm sociodemographics, highest level of education achieved, and socioeconomic status.

PAT and VAT volume

PAT volume was measured from cardiac CT scans performed at the CARDIA Y25 study visit. Three CARDIA investigators trained to analyze CT scans used a validated protocol[219] to identify and measure PAT volume described previously.[189] Briefly, analysts manually segmented CT scan slices from 15 mm above and 30 mm below the left main coronary artery and identified PAT as tissue with density between -190 and -30 Hounsfield units. PAT volume in cubic centimeters (cm^3) was quantified by summing all pixels containing tissue within the density range.

VAT volume was measured from non-contrast abdominal CT scans performed at the CARDIA Y25 study visit. A 10mm slice was taken at the L4-5 vertebrae and segmented. The same tissue density range was applied to identify adipose tissue, and VAT volume in cubic centimeters (cm^3) was similarly quantified by summing all pixels within this range.

Cardiac structure and function

At the Y25 CARDIA visit, study participants underwent echocardiographic assessment according a standardized protocol[220] and equipment at all study sites (Toshiba Medical Systems, Otawara, Japan). M-mode, 2-dimensional (2D), and Doppler images were obtained at 3 consecutive cardiac cycles. Images were examined by one of four experienced analysts at the

CARDIA Echocardiography Reading Center to obtain measures of cardiac structure and function according to American Society of Echocardiography (ASE) guidelines.[221,222]

We examined 4 clinically relevant measures of cardiac structure and function as outcomes. Two outcomes measured systolic structure and function: left ventricular (LV) ejection fraction and LV mass. LV ejection fraction was calculated as the ratio of stroke volume to end-diastolic volume from 2D echocardiography.[221] LV mass was derived from M-mode echocardiography as a function of ventricular septal and posterior wall thicknesses and internal dimension at end-diastole,[221] and LV mass was indexed to height exponentiated to a power of 2.7 due to sex differences in height and BMI.[223] The remaining outcomes were obtained during Doppler echocardiography and measured diastolic function: E/A ratio, a measure of transmitral velocity; and E/e' ratio, a measure of LV filling pressure.

To estimate the prevalence of LV hypertrophy, we characterized LV mass index as normal or mildly, moderately, or severely abnormal based on ASE sex-specific reference values.[224] To estimate the prevalence of diastolic dysfunction, we characterized normal diastolic function vs. Grades I through III of diastolic dysfunction based on both the E/A ratio and early diastolic filling velocity (e' wave).[225]

Statistical methods

We compared demographic information, clinical data, measures of adiposity, non-outcome echocardiogram data, and the cardiac structure and function outcomes by sex. Independent sample t-tests were used to test differences by sex in all continuous variables, and chi-square tests were used to test differences by sex in all categorical variables.

Model 1 examined the relationship of PAT volume with each of the outcomes of interest after accounting for the sex. To evaluate if there was a difference in the association of PAT

volume with each of the 4 outcomes between men and premenopausal women, a statistical interaction term was tested in separate linear regression models. If interaction was detected at $p<0.05$, we interpreted the sex-specific β estimates representing the difference in each outcome associated with a 1 standard deviation (SD) increment change in PAT volume. If no significant interaction was found, the interaction term was removed and sex was included in the model as a confounder.

Model 2 examined the relationship of PAT volume and the outcomes after adjustment for age (continuous), race (black vs. white), education level attained (continuous), blood pressure (continuous), use of statins (yes vs. no), use of antihypertensive medications (yes vs. no), total physical activity intensity (continuous), and diabetes mellitus (yes vs. no). Based on prior analyses that have shown significant race and sex differences in LV cardiac structure and function,[59] we also tested the interaction between race and sex in all models and included it in Model 2 if significant.

Model 3 tested whether there was an independent association of PAT volume with the outcomes after further adjustment for continuous abdominal VAT volume.

All analyses were conducted using SAS Version 9.4 (SAS Institute, Cary, NC). Statistical tests were considered significant at a two-sided $p<0.05$.

Results

Participant characteristics by sex are presented in **Table IV- 1.** On average, men were older and had higher prevalence of diabetes mellitus and hypertension, as well as higher use of both statins and antihypertensive medications compared with premenopausal women. While premenopausal women had higher BMI and more total abdominal adipose tissue volume than

men, this was likely driven by higher subcutaneous adipose tissue volumes. Men had larger waist circumferences, as well as more VAT and PAT volumes, compared with premenopausal women.

Table IV- 1. Participant characteristics at the Y25 CARDIA study visit.

	Men (n=1,170)	Premenopausal women (n=620)	p-value
Demographics			
Age, years	50 ± 4	48 ± 3	<0.0001
Race, n (% black)	516 (44%)	309 (50%)	0.02
Clinical information			
Diabetes mellitus, n (%)	131 (11%)	34 (5%)	0.0003
Hypertension, n (%)	575 (32%)	241 (13%)	<0.0001
Average systolic BP, mmHg	122 ± 14	116 ± 16	<0.0001
Average diastolic BP, mmHg	76 ± 10	72 ± 12	<0.0001
Current use of statins, n (%)	199 (17%)	53 (9%)	<0.0001
Current use of antihypertensive medications, n (%)	292 (25%)	118 (19%)	0.03
Measures of adiposity			
Waist circumference, cm	97.4 ± 12.7	88.4 ± 15.3	<0.0001
BMI, kg/m^2	28.9 ± 5.2	29.7 ± 7.6	0.01
Total abdominal adipose tissue volume, cm^3	427.6 ± 185.2	475.0 ± 222.9	<0.0001
Subcutaneous adipose tissue volume, cm^3	262.5 ± 130.7	356.5 ± 175.5	<0.0001
VAT volume, cm^3	147.8 ± 74.9	102.0 ± 59.0	<0.0001
PAT volume, cm^3	64.4 ± 35.1	41.2 ± 19.8	<0.0001
Echocardiography measures			
Stroke volume, mL	97.0 ± 22.4	82.3 ± 20.0	<0.0001
Cardiac output, L/min	6.1 ± 1.7	5.5 ± 1.5	<0.0001
Fractional shortening, %	39.1 ± 7.1	40.4 ± 6.3	<0.0001
Pulmonary vein systolic/diastolic velocity ratio	1.22 ± 0.29	1.27 ± 0.31	0.001
Peak circumferential strain, %	-14.9 ± 2.8	-15.7 ± .9	<0.0001
Peak radial strain, %	37.8 ± 12.4	35.9 ± 11.4	0.002
Peak longitudinal strain, %	-14.6 ± 2.3	-15.8 ± 2.4	<0.0001
Diastolic function grades			<0.0001
Normal, n (%)	680 (58%)	447 (72%)	
Grade 1 diastolic dysfunction, n (%)	62 (5%)	15 (2%)	
Grade 2 diastolic dysfunction, n (%)	368 (31%)	141 (23%)	
Grade 3 diastolic dysfunction, n (%)	60 (5%)	17 (3%)	
Global hypertrophy, %	21 (2%)	9 (1%)	0.59

Table IV- 1, cont'd.

	Men (n=1,170)	Premenopausal women (n=620)	p-value
LV mass index categories			0.03
Normal, n (%)	945 (81%)	481 (78%)	
Mildly abnormal, n (%)	120 (10%)	55 (9%)	
Moderately abnormal, n (%)	56 (5%)	45 (7%)	
Severely abnormal, n (%)	49 (4%)	39 (6%)	
Outcomes			
LV ejection fraction, %	60.3 ± 7.6	62.5 ± 6.6	<0.0001
LV mass index, $g/m^{2.7}$	41.3 ± 11.2	38.5 ± 11.4	<0.0001
E/A ratio	1.32 ± 0.35	1.35 ± 0.38	0.12
E/e' ratio	7.5 ± 2.1	7.7 ± 2.2	0.03
E/e' ratio, septal	8.7 ± 2.6	8.8 ± 2.6	0.41
E/e' ratio, lateral	6.7 ± 2.2	7.0 ± 2.3	0.008

Abbreviations: BP, blood pressure; BMI, body mass index; VAT, visceral adipose tissue; PAT, pericardial adipose tissue; LV, left ventricular

Measures of cardiac structure and function derived from the echocardiogram were within the range of normal for both sexes;[226] nevertheless, there were differences by sex in nearly all of them. Men had higher stroke volumes and cardiac output, and lower fractional shortening and the ratio of pulmonary vein systolic to diastolic velocity than premenopausal women. Men also had higher peak circumferential, radial, and longitudinal strain. A higher proportion of premenopausal women compared with men had normal diastolic function (72% vs. 58%), and while prevalence of global hypertrophy was low in both men and premenopausal women, a higher proportion of men were categorized as having normal (81% vs. 78%) LV mass index.

Of the outcomes of interest, men had significantly lower LV ejection fraction, higher LV mass index, and lower E/e' ratio. Men and premenopausal women had similar E/A ratios.

The results of the models examining outcomes for which there was a significant PAT volume by sex interaction are presented in **Table IV- 2**. Interaction between sex and race was not detected for any of these measures, but a significant PAT volume by sex interaction was detected for LV mass index (p-value for interaction=0.0001) and E/A ratio (p-value for

interaction<0.0001). In Model 1, a 1-SD increment in PAT volume was significantly associated with 3.2 ± 0.4 g/m$^{2.7}$ higher LV mass index in women and 2.4 ± 0.3 g/m$^{2.7}$ higher LV mass index in men (p<0.0001 for both). The differential association of PAT volume with LV mass index remained (interaction p-value = 0.0009) in Model 2 after adjustment for age, race, education, blood pressure, statin use, antihypertensive medication use, diabetes mellitus, and physical activity intensity. In Model 3, a 1-SD increment in PAT volume was associated with 2.4 ± 0.5 g/m$^{2.7}$ greater LV mass index in women and 1.9 ± 0.4 g/m$^{2.7}$ greater LV mass index in men (interaction p-value=0.003) after further adjustment for VAT volume.

Table IV- 2. Sex-stratified β estimates for the relationships between PAT volume and LV mass index and E/A ratio.

	PAT volume by sex		Interaction p-value	VAT volume	
	β ± SE	p-value		β ± SE	p-value
LV mass index					
Model 1			0.0001	--	--
Premenopausal women	3.2 ± 0.4	<0.0001			
Men	2.4 ± 0.3	<0.0001			
Model 2			0.0009	--	--
Premenopausal women	2.9 ± 0.4	<0.0001			
Men	2.6 ± 0.3	<0.0001			
Model 3			0.003	1.1 ± 0.4	0.004
Premenopausal women	2.4 ± 0.5	<0.0001			
Men	1.9 ± 0.4	<0.0001			
E/A ratio					
Model 1			<0.0001	--	--
Premenopausal women	-0.10 ± 0.01	<0.0001			
Men	-0.06 ± 0.01	<0.0001			
Model 2			0.002	--	--
Premenopausal women	-0.07 ± 0.01	<0.0001			
Men	-0.03 ± 0.01	0.003			
Model 3			0.007	-0.04 ± 0.01	0.0003
Premenopausal women	-0.04 ± 0.02	0.005			
Men	-0.006 ± 0.01	0.67			

Model 1: Sex, PAT volume, sex*PAT volume interaction
Model 2: Model 1 + age, race, education level, blood pressure, statin use, antihypertensive medication use, diabetes mellitus, physical activity intensity
Model 3: Model 2 + VAT volume
β estimates for PAT volume and VAT volume are presented as the change in the outcome per 1-SD increment.

The magnitude of association of PAT volume with E/A ratio was larger in women than in men (p-value for interaction<0.0001). A 1-SD increment in PAT volume was associated with 0.10 ± 0.01 lower E/A ratio in premenopausal women and 0.06 ± 0.01 lower E/A ratio in men. This relationship remained significant but differential for both sexes after adjustment for age, race, education level, blood pressure, statin use, antihypertensive medication use, diabetes mellitus, and physical activity in Model 2 (p-value for interaction=0.002). Inclusion of VAT volume in Model 3 abolished the relationship between PAT volume and E/A ratio in men (p=0.69), but a 1-SD increment in PAT volume was still inversely associated with E/A ratio in premenopausal women (-0.04 ± 0.02 per SD increment, p=0.005, interaction p-value=0.007).

Significant interactions between PAT volume and sex and between race and sex were not detected for either LV ejection fraction or E/e' ratio. Results of the models examining these measures are presented in **Table IV- 3**. Sex-adjusted PAT volume was not associated with LV ejection fraction in Model 1 (p=0.33), and thus the relation between PAT volume and LV ejection fraction was not examined in subsequent adjusted models.

Table IV- 3. β estimates for the associations of sex, PAT volume, and VAT volume with LV ejection fraction and E/e' ratio.

	Sex, men vs. women		PAT volume		VAT volume	
	β ± SE	p-value	β ± SE	p-value	β ± SE	p-value
LV ejection fraction						
Model 1*	-2.4 ± 0.4	<0.0001	0.18 ± 0.18	0.33	--	--
E/e' ratio						
Model 1	-0.42 ± 0.11	0.0002	0.27 ± 0.05	<0.0001	--	--
Model 2	-0.69 ± 0.11	<0.0001	0.24 ± 0.06	<0.0001	--	--
Model 3	-0.69 ± 0.11	<0.0001	0.21 ± 0.07	0.005	0.05 ± 0.07	0.48

Model 1: PAT volume, sex
Model 2: Model 1 + age, race, education level, blood pressure, statin use, antihypertensive medication use, diabetes mellitus, physical activity intensity
Model 3: Model 2 + VAT volume
β estimates for PAT volume and VAT volume are presented as the change in the outcome per 1-SD increment.
* Models 2 and 3 are not presented for LV ejection fraction due to no association with PAT volume.

PAT volume was associated with higher E/e' ratio (0.27 ± 0.05 per SD increment, p<0.0001) independent of sex in Model 1. Adjustment for additional confounders in Model 2 attenuated the estimate slightly, but the association remained significant (p<0.0001). PAT volume was still significantly and independently associated with E/e' ratio after further adjustment for abdominal VAT volume in Model 3 (p=0.004).

Discussion

In this cross-sectional analysis, PAT volume was consistently associated with LV mass index, E/A ratio, and E/e' ratio in both men and premenopausal women, independent of age, race, education level, blood pressure, use of statins and antihypertensive medications, diabetes mellitus, physical activity, and abdominal VAT volume. The linear relationships between PAT volume and greater cardiac remodeling and dysfunction was larger in magnitude in premenopausal women compared to men in the context of LV hypertrophy (LV mass index) and diastolic function (E/A ratio), but not LV filling pressure (E/e' ratio). Examination of PAT may help predict future cardiovascular damage and subsequent heart failure.

VAT is associated with a persistent inflammatory state[227] and numerous cardiovascular risk factors, including insulin resistance and diabetes mellitus.[169,228] PAT, which is correlated with overall VAT burden, is comprised of both epicardial and paracardial adipose tissues surrounding the heart.[180] Epicardial adipose tissue shares its circulation with the heart itself, whereas paracardial adipose tissue is external to the pericardium.[180] PAT is associated with insulin resistance,[187,188] metabolic syndrome,[229] and diabetes mellitus,[189] and there may be a differential association of PAT with these risk factors by sex.[230] Indeed, prior analyses in the CARDIA study have identified an association of PAT volume with diabetes mellitus,[189] as well as suggested that a higher ratio of PAT volume to subcutaneous adipose tissue volume is

associated with insulin resistance,[188] but the mechanisms for these associations are not fully understood. PAT is also associated with subclinical atherosclerosis and coronary artery disease,[231] and may affect CVD risk through compression of the myocardium[232] or through localized secretion of inflammatory proteins.[184]

Our study adds to the existing body of literature by characterizing the association of PAT with cardiac structure and function in a sample of only premenopausal women and men. Our findings are consistent with results of previous cross-sectional studies that have shown associations between PAT and measures of cardiac structure and function.[233] In the Jackson Heart Study, PAT and VAT volume were associated with both LV mass and E/A ratio, but neither was associated with LV ejection fraction in a sample of 1,402 African American adults.[191] In the Framingham Heart Study, PAT was significantly correlated with LV mass in both men and women, but the correlation coefficient was stronger in women ($\rho = 0.20$ in women and 0.19 in men) after age adjustment.[232] Prior studies have also suggested an independent inverse association between PAT and diastolic function.[234–237]

In our study, we identified sex differences in all cardiac structure and function outcomes except E/A ratio. The sex differences in LV ejection fraction and mass index are consistent with what has been reported in the literature previously: women tend to have higher LV ejection fraction than men.[238] Women also have lower LV mass compared with men, even after indexing to body size.[59,60]

We did not find significant sex differences in E/A ratio, and while average E/e' ratio was numerically lower in men than in premenopausal women, the statistically significant difference in E/e' ratio may not be clinically significant. Studies examining diastolic function have largely focused on older adults than those included in our sample. A study of adults with HFpEF found

significantly lower E/A ratio in women (1.1 ± 0.7 vs. 1.2 ± 0.8, 0.01); however, these adults were in their early 70s and therefore notably older than our study population.[239] The lack of a sex difference in E/A ratio observed in our study is likely due to our inclusion of only premenopausal women, and the relatively young age in our cohort (49 ± 4 years). Diastolic dysfunction is more prevalent in postmenopausal women compared to premenopausal women.[240] Furthermore, we did not detect a significant interaction between race and sex, although a prior analysis of Y25 participants in CARDIA showed significant race and sex differences in E/A ratio.[59] Nevertheless, even in this relatively young population that had a high prevalence of normal diastolic function, PAT volume was associated with worse E/A and E/e' ratios regardless of sex, a relationship that agrees with those found in previous studies.[191,192,234] However, it should be noted that while we examined E/A ratio as a continuous outcome representing diastolic function, we were unable to differentiate between study participants who had normal vs. pseudonormal E/A ratio values, although we were able to approximate the overall prevalence of Grade II diastolic function in the study population.

Our study has several limitations related to the cross-sectional nature of the data precluding conclusions about causal relationships. We are unable to elucidate a clear temporal mechanism explaining the association between PAT and cardiac structure and function. Furthermore, because the reproductive history questionnaire administered does not adequately capture perimenopausal women who may begin to transition through menopause but who still had a menstrual cycle within a year of their study visit,[241] we likely included perimenopausal women in our premenopausal group.

Nevertheless, our findings may inform future longitudinal collection and analysis of these measures to test PAT volume as a potential mediator of the relationship between sex and cardiac

structure and function. Our findings are strengthened by use of a large, population-based biracial cohort, allowing for greater generalizability. These results provide a groundwork for examining the relationship between PAT burden and CVD risk in further detail.

Conclusion

In conclusion, higher PAT volume was associated with LV structural and functional remodeling, and the association was greater in magnitude for women when examining LV mass index and E/A ratio. The association between PAT volume and these measures of structure and function was independent of VAT volume. As the prevalence of obesity increases in both men and women, it is vital that we understand the role of body fat distribution and adipose tissue function so that we can promote therapeutic interventions that preferentially target ectopic fat depots.

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CHAPTER V

PERICARDIAL ADIPOSE TISSUE VOLUME IS ASSOCIATED WITH ADVERSE LEFT VENTRICULAR MASS AND DIASTOLIC FUNCTION IN WOMEN INDEPENDENT OF MENOPAUSE

Abstract

Background. Risk of cardiovascular disease (CVD) in women increases with the loss of ovarian estrogen, which is also associated with adverse weight distribution. Surgical menopause (i.e., hysterectomy with and without bilateral oophorectomy [BLO]) is associated with increased CVD risk. We examined the relation between pericardial adipose tissue (PAT) with measures of cardiac structure and function between premenopausal and postmenopausal women (both natural and surgically induced) independent of visceral adipose tissue (VAT).

Methods. We conducted a cross-sectional analysis of 620 premenopausal women, 487 naturally postmenopausal women, 230 women with hysterectomy with ovarian preservation, and 104 women with BLO in the Coronary Artery Risk Development in Young Adults (CARDIA) study. Study participants were between the ages of 43 and 55 years. Statistical interaction was evaluated in linear regression to determine if associations between PAT volume and 4 measures of cardiac structure and function (left ventricular (LV) ejection fraction, LV mass index, E/A ratio, E/e' ratio) differed by menopause status. The association of PAT volume and these outcomes was evaluated after adjustment for abdominal VAT volume.

Results. There was a differential association between PAT volume and E/A ratio by menopause status (p=0.02), but this interaction was fully attenuated after risk adjustment (p=0.07). PAT volume was not associated with E/A ratio after adjustment for abdominal VAT volume (p=0.15). The associations of PAT volume with LV mass index and E/e' ratio did not

differ by menopause status. PAT volume was significantly associated with higher LV mass index (4.07 ± 0.30 g/$m^{2.7}$ per 1-standard deviation (SD) increment) and higher E/e' ratio (0.43 ± 0.06). PAT volume was not associated with LV ejection fraction, but the association of PAT volume with LV mass index and E/e' ratio remained significant ($p<0.0001$ and $p=0.0003$, respectively) after adjustment for abdominal VAT volume.

Conclusions. PAT volume was associated with adverse LV mass index and E/e' ratio independent of menopause status. These associations were significant even after adjusting for confounding and abdominal VAT volume, suggesting the importance of cardiac adipose tissue in cardiac structure and function.

Introduction

Menopause is associated with an increase in cardiovascular disease (CVD) risk in women. Women who go through menopause prematurely, such as via hysterectomy with or without bilateral oophorectomy (BLO), may be at greater risk for CVD than women who undergo natural menopause, independent of other CVD risk factors.[242,243] Menopausal changes in fat distribution from preferential subcutaneous to ectopic fat deposition are thought to be related to declines in estrogen concentrations.[172,174,244] Increased fat deposition into visceral and ectopic depots is associated with increased CVD risk and contributes to an overall systemic inflammatory state through the release of proinflammatory cytokines.[168,172] These cytokines include tumor necrosis factor α, interleukin (IL)-1, and IL-6,[161,163] which in turn are associated with diastolic dysfunction and subsequent heart failure in women.[37,245] Postmenopausal women indeed have more adverse measures of cardiac structure and function compared with premenopausal, particularly when examining diastolic function and left ventricular (LV) wall thickness.[76,82–84]

The greater abdominal visceral adipose tissue (VAT) after menopause is associated with inflammation, insulin resistance, and atherosclerotic heart disease, [169] further contributing to the menopause-associated increase in CVD risk in women. Pericardial adipose tissue (PAT), the cardiac ectopic fat depot, may contribute to localized inflammation of the coronary arteries and the myocardium in CVD due to its proximity to the heart.[214,246]

PAT is significantly correlated with VAT,[191] and both PAT and VAT increase with menopause. We examined the relation between PAT volume and measures of cardiac structure and function among 1,441 postmenopausal women in the Coronary Artery Risk Development in Young Adult (CARDIA) study classified by hysterectomy status (premenopausal, natural menopausal, hysterectomy with ovarian preservation, or hysterectomy BLO). We further evaluated if the relationship was independent of abdominal VAT volume.

Methods

Study population

The CARDIA study is a longitudinal cohort study that enrolled 5,115 black and white men and women aged 18 to 30 years in 1985-1986. [204] Study participants were recruited from 4 urban sites across the United States (Birmingham, AL; Chicago, IL; Minneapolis, MN; and Oakland, CA) and have been followed for over 30 years to examine the development of cardiovascular disease over time. All study participants provided written informed consent at each visit, and all protocols were reviewed approved by the site institutional review boards.[204]

This study used cross-sectional data collected from women who attended the CARDIA Year 25 (Y25) study visit where both echocardiograms and computed tomography (CT) scans were completed. Study participants were between the ages of 43 and 55 at this study visit. Of the baseline cohort, 72% of survivors completed a Y25 exam,[215] and women who were pregnant or

who reported cessation of their menstrual cycle for any reason other than surgery or menopause (e.g., chemotherapy) were excluded. 1,441 women with available PAT and VAT volumes and measures of cardiac structure and function were included in these analyses.

Premenopausal, naturally postmenopausal, and surgically postmenopausal status was ascertained based on self-reported reproductive history.[241] Women were classified as surgically postmenopausal if they reported a history of hysterectomy with preservation of one, partial, or no ovaries (n=230) or with BLO (n=104). Women were classified as naturally postmenopausal (n=487) if they reported having undergone menopause naturally and had not had a menstrual cycle within 12 months of their visit date not due to pregnancy or birth control. Women were classified as premenopausal (n=620) if they reported a having a menstrual cycle within 12 months of their study visit, had not had a hysterectomy, and had not undergone menopause. Time since cessation of the menstrual cycle was calculated for all postmenopausal women based on self-reported age of menopause or age at hysterectomy.

Data collection

Standardized protocols were used at each CARDIA study site for all exam components to ensure consistent and high quality data collection.[204] Study participants underwent a clinical examination in which pulse rate, resting blood pressure, and measures of anthropometry were collected. Blood pressure was measured 3 times after 5 minutes at rest, and the second and third measures were averaged for analysis. Height and weight were measured using either a wall-mounted ruler or stadiometer and balance-beam scale, respectively. Body mass index (BMI) was calculated as the study participant's body weight (kg) divided by height squared (m^2), and all heights and weights were measured with the participant wearing light clothing and not wearing shoes. Waist circumference was measured horizontally at a point laterally midway between the

iliac crest and the lowest portion of the rib cage, and anteriorly midway between the xiphoid process and the umbilicus.

Hypertension was defined as having a systolic blood pressure ≥ 130 mmHg, diastolic blood pressure ≥ 80 mmHg, or use of antihypertensive medications.[216,217] Diabetes mellitus was defined as fasting glucose concentration ≥ 126 mg/dL, oral glucose tolerance ≥ 200 mg/dL, hemoglobin A1c ≥ 6.5%, or use of diabetic medications. Physical activity intensity was estimated based on responses to the CARDIA Physical Activity History questionnaire.[218]

Participants self-reported family and medical history, smoking status, and medication use, including use of statins, antihypertensive medications, oral birth control, and other hormones. Smoking status, sociodemographic data, and highest level of education achieved were collected via interviewer-administered questionnaires.

PAT and VAT volume

Three independent reviewers analyzed cardiac CT scans performed at the CARDIA Y25 study[189] and used a validated protocol to measure PAT volume.[219] Analysts manually segmented CT scan slices starting at 15mm above and ending at 30mm below the left main coronary artery. PAT was identified as tissue with density in the range of -190 and -30 Hounsfield units, and total PAT volume in cubic centimeters (cm^3) was calculated as the sum of all pixels containing tissue within the density range.

Non-contrast abdominal CT scans performed at the Y25 study visit were analyzed to measure VAT volume. A 10mm slice taken at the L4-L5 vertebrae was manually segmented, and VAT was similarly identified as any tissue within the range of -190 and -30 Hounsfield units. VAT volume in cubic centimeters (cm^3) was calculated as the sum of all pixels within this range.

Cardiac structure and function

Study participants underwent M-mode, 2-dimensional (2D), and Doppler echocardiographic assessment according to standardized protocols and equipment (Toshiba Medical Systems, Otawara, Japan).[220] Images were obtained from 3 consecutive cardiac cycles and transmitted to the CARDIA Echocardiography Reading Center where one of four experienced analysts obtained the measures of cardiac structure and function according to American Society of Echocardiography (ASE) guidelines.[221,222]

We examined 2 measures of systolic structure and function: LV ejection fraction, which was derived from 2-dimensional echocardiography and calculated as the ratio of stroke volume to end-diastolic volume, and LV mass, which was derived from M-mode echocardiography as a function of septal and posterior wall thicknesses and internal dimension at end-diastole.[221] LV mass was estimated as a function of ventricular septal thickness at end-diastole, LV internal dimension at end-diastole, and posterior wall thickness at end-diastole.[247] LV mass was indexed to height raised to a power of 2.7.[223]

We also examined 2 outcomes measuring diastolic function obtained from Doppler echocardiography: E/A ratio, a measure of transmitral velocity, and average E/e' ratio, a measure of LV filling pressure. Average E/e' ratio was derived using the average of septal and lateral e' wave values.

LV mass index was characterized as normal, mildly abnormal, moderately abnormal, or severely abnormal based on ASE reference values to estimate LV hypertrophy prevalence in the study cohort.[224] Prevalence of diastolic dysfunction was estimated using definitions of normal diastolic function and Grades I through III of diastolic dysfunction based on the E/A ratio and early diastolic filling velocity (e' wave).[225]

Statistical methods

Demographic information, clinical data, measures of adiposity, echocardiogram-derived measures, and the cardiac structure and function outcomes were compared by menopause status. Analysis of variance was used to test differences across menopause status in mean values of all continuous variables, and chi-square tests were used to test differences by menopause status in all categorical variables.

Multiple linear regression was used to test the relationship of PAT volume with cardiac structure and function. Model 1 evaluated the relationship of PAT volume with each of the outcomes of interested after accounting for menopause status. First, a statistical interaction term was tested in linear regression models to test whether there was a differential association of PAT volume with each of the 4 outcomes of interest by menopause status. If a significant interaction was detected at $p<0.05$, we interpreted β estimates stratified by menopause status. If a significant interaction between menopause status and PAT volume was not found, the interaction term was removed from all subsequent models and menopause status was included in the model as a confounder.

Model 2 evaluated the association of PAT volume with each outcome of interest after adjustment for age (continuous), race (black vs. white), education level attained (continuous), blood pressure (continuous), use of antihypertensive medications (yes vs. no), physical activity intensity (continuous), and diabetes mellitus (yes vs. no).

Model 3 tested whether there was an independent relationship between PAT volume and cardiac structure and function independent of continuous abdominal VAT volume, after adjusting for all variables included in Model 2.

All β estimates are reported as the change in outcome associated with a 1-standard deviation (SD) increment in PAT volume. Analyses were conducted using SAS Version 9.4 (SAS Institute, Cary, NC). Statistical tests were deemed significant at a two-sided $p<0.05$.

Results

Participant characteristics

Y25 characteristics by menopause group are presented in **Table V- 1**. Compared with the natural and surgical menopause groups, premenopausal women were younger and had lower prevalence of diabetes mellitus and hypertension, and they had lower use of both statins and antihypertensive medications. Age at menstrual cycle cessation was highest in the natural menopause group and lowest in the hysterectomy with ovarian preservation group, while history of oral contraceptive use was comparable across all groups. Women who had a hysterectomy with BLO had the highest prevalence of hormone use other than birth control.

Table V- 1. Participant characteristics by menopause status.

	Premenopausal (n=620)	Natural menopause (n=487)	Hysterectomy, ovarian preservation (n=230)	Hysterectomy, BLO (n=104)	p-value
Demographics					
Age, years	48 ± 3	53 ± 2	50 ± 3	51 ± 3	<0.0001
Race, n (% black)	309 (50%)	187 (38%)	172 (75%)	66 (63%)	<0.0001
Clinical information					
Diabetes, n (%)	34 (5%)	43 (9%)	32 (14%)	19 (18%)	0.0001
Hypertension, n (%)	241 (39%)	210 (43%)	144 (63%)	63 (61%)	<0.0001
Average systolic BP, mmHg	116 ± 16	118 ± 18	122 ± 18	120 ± 16	0.0001
Average diastolic BP, mmHg	72 ± 12	74 ± 12	78 ± 10	76 ± 12	<0.0001
Current use of statins, n (%)	53 (9%)	71 (15%)	44 (19%)	20 (19%)	0.0004
Current use of antihypertensive medications, n (%)	118 (19%)	118 (24%)	99 (43%)	48 (46%)	<0.0001
Female-specific measures					
Age at which periods stopped, years	--	49 ± 4	40 ± 7	42 ± 6	<0.0001
History of oral contraceptive use, n (%)	509 (82%)	386 (79%)	179 (78%)	81 (78%)	0.73
Current use of other hormones, n (%)	22 (4%)	35 (7%)	27 (12%)	31 (30%)	<0.0001
Measures of adiposity					
Waist circumference, cm	88.4 ± 15.3	88.4 ± 14.7	94.1 ± 14.7	93.8 ± 17.8	<0.0001
BMI, kg/m^2	29.7 ± 7.6	29.0 ± 7.1	32.5 ± 7.3	32.9 ± 7.1	<0.0001
Total abdominal adipose tissue volume, cm^3	475.0 ± 222.9	480.8 ± 208.9	561.2 ± 214.3	575.5 ± 210.9	<0.0001
Subcutaneous adipose tissue volume, cm^3	356.5 ± 175.5	350.6 ± 165.6	428.5 ± 178.4	426.9 ± 163.5	<0.0001
VAT volume, cm^3	102.0 ± 59.0	111.3 ± 57.2	115.9 ± 58.1	129.9 ± 68.8	<0.0001
PAT volume, cm^3	41.2 ± 19.7	50.2 ± 24.9	48.1 ± 23.2	56.6 ± 31.0	<0.0001
Echocardiography measures					
Stroke volume, mL	82.3 ± 20.0	82.9 ± 19.8	84.2 ± 20.8	87.2 ± 21.2	0.11
Cardiac output, L/min	5.5 ± 1.5	5.4 ± 1.5	5.7 ± 1.8	5.8 ± 1.5	0.02
Fractional shortening, %	40.4 ± 6.3	41.0 ± 6.6	40.8 ± 7.2	41.0 ± 7.3	0.51
Pulmonary vein systolic/diastolic velocity ratio	1.27 ± 0.31	1.32 ± 0.31	1.27 ± 0.32	1.30 ± 0.32	0.06

Table V- 1, cont'd.

	Premenopausal (n=620)	Natural menopause (n=487)	Hysterectomy, ovarian preservation (n=230)	Hysterectomy, BLO (n=104)	p-value
Peak circumferential strain, %	-15.7 ± 2.9	-15.8 ± 2.8	-15.3 ± 3.0	-15.5 ± 3.1	0.17
Peak radial strain, %	35.9 ± 11.9	37.9 ± 11.9	37.5 ± 12.2	38.9 ± 11.7	0.01
Peak longitudinal strain, %	-15.8 ± 2.4	-15.5 ± 2.3	-14.9 ± 2.6	-14.7 ± 2.6	<0.0001
Diastolic function categories					<0.0001
Normal, n (%)	447 (72%)	265 (54%)	128 (56%)	45 (43%)	
Grade 1 diastolic dysfunction, n (%)	15 (2%)	35 (7%)	18 (8%)	8 (8%)	
Grade 2 diastolic dysfunction, n (%)	141 (23%)	176 (36%)	79 (34%)	48 (46%)	
Grade 3 diastolic dysfunction, n (%)	17 (3%)	11 (2%)	5 (2%)	3 (3%)	
LV mass index categories					<0.0001
Normal, n (%)	481 (78%)	375 (77%)	140 (61%)	65 (63%)	
Mildly abnormal, n (%)	55 (9%)	47 (10%)	47 (20%)	18 (17%)	
Moderately abnormal, n (%)	45 (7%)	37 (8%)	21 (9%)	12 (12%)	
Severely abnormal, n (%)	39 (6%)	28 (6%)	22 (10%)	9 (9%)	
Outcomes					
LV ejection fraction, %	62.5 ± 6.6	62.8 ± 7.1	62.2 ± 7.7	63.8 ± 6.9	0.29
LV mass index, $g/m^{2.7}$	38.5 ± 11.4	38.7 ± 11.5	42.6 ± 13.3	43.5 ± 12.0	<0.0001
E/A ratio	1.35 ± 0.38	1.24 ± 0.35	1.26 ± 0.37	1.20 ± 0.32	<0.0001
E/e' ratio	7.7 ± 2.2	8.2 ± 2.5	8.4 ± 2.4	8.7 ± 2.4	<0.0001
E/e' ratio, septal	8.8 ± 2.6	9.3 ± 3.1	9.4 ± 2.8	10.3 ± 3.2	<0.0001
E/e' ratio, lateral	7.0 ± 2.3	7.6 ± 2.5	7.8 ± 2.7	7.9 ± 2.5	<0.0001

Abbreviations: BP, blood pressure; BMI, body mass index; VAT, visceral adipose tissue; PAT, pericardial adipose tissue; LV, left ventricular

Significant differences by menopause status were detected for all measures of adiposity examine. Women who underwent surgical menopause had the highest adiposity across all measures. Postmenopausal women with hysterectomy with BLO had the highest average BMI, and total abdominal adipose tissue, VAT, and PAT volumes of all groups. Women with hysterectomy with ovarian preservation had the largest waist circumference and highest subcutaneous adipose tissue volume of all groups.

Postmenopausal women with hysterectomy with BLO had the highest cardiac output, peak radial strain, and peak longitudinal strain, while no significant differences by menopause status were detected in stroke volume, fractional shortening, ratio of pulmonary vein systolic to diastolic velocity, or peak circumferential strain. The prevalence of normal diastolic function was highest in premenopausal (72%) and lowest in postmenopausal women with hysterectomy with BLO (43%). Women who underwent surgical menopause had a higher prevalence of abnormal LV mass index compared with both premenopausal and naturally postmenopausal women.

Of the outcomes of interest, women who underwent hysterectomy with BLO had the highest LV mass index and E/e' ratio, and the lowest E/A ratio. Conversely, premenopausal women had the lowest LV mass index and E/e' ratio, and the highest E/A ratio. Only LV ejection fraction was not significantly different by menopause status.

Regression models

A significant PAT volume by menopause status interaction was only detected for the E/A ratio outcome (p-value for interaction=0.02), and results examining E/A ratio as an outcome are presented in **Table V- 2**. In Model 1, PAT volume was significantly associated with lower E/A ratio in women who were premenopausal, naturally postmenopausal, and who had hysterectomy with ovarian preservation, and these associations were comparable in magnitude. A 1-SD

increment in PAT volume was associated with 0.10 ± 0.01 lower E/A ratio in premenopausal women ($p<0.0001$), 0.09 ± 0.02 lower E/A ratio in naturally postmenopausal women ($p<0.0001$), and 0.08 ± 0.02 lower E/A ratio in women with hysterectomy with ovarian preservation ($p=0.001$). PAT volume was not associated with E/A ratio in women with hysterectomy with BLO ($p=0.44$).

Table V- 2. β estimates for the association between PAT volume and E/A ratio stratified by menopause status.

	Menopause status		PAT volume			VAT volume	
	β ± SE	p-value	β ± SE	p-value	Interaction p-value	β ± SE	p-value
Model 1		0.0002					
Premenopausal (ref.)	--	--	-0.10 ± 0.01	<0.0001			
Natural menopause	-0.13 ± 0.05	0.006	-0.09 ± 0.02	<0.0001	0.02	--	--
Hysterectomy, ovarian preservation	-0.14 ± 0.06	0.02	-0.08 ± 0.02	0.001			
Hysterectomy, BLO	-0.31 ± 0.08	<0.0001	-0.03 ± 0.03	0.44			
Model 2		0.81					
Premenopausal (ref.)	--	--					
Natural menopause	-0.01 ± 0.02	0.61	-0.05 ± 0.01	<0.0001	0.07	--	--
Hysterectomy, ovarian preservation	0.01 ± 0.03	0.61					
Hysterectomy, BLO	-0.01 ± 0.04	0.72					
Model 3		0.73					
Premenopausal (ref.)	--	--					
Natural menopause	-0.02 ± 0.02	0.43	-0.02 ± 0.01	0.15	0.13	-0.05 ± 0.01	0.0002
Hysterectomy, ovarian preservation	0.01 ± 0.03	0.70					
Hysterectomy, BLO	-0.02 ± 0.04	0.61					

Model 1: menopause status, PAT volume, menopause*PAT volume interaction
Model 2: menopause status, PAT volume, age, race, education, blood pressure, use of antihypertensive medications, physical activity, diabetes mellitus
Model 3: Model 2 + VAT volume
*β estimates for PAT and VAT volume are presented per standard deviation.

In Model 2, the differential relationship between PAT volume and E/A ratio by menopause status was fully attenuated. A 1-SD increment in PAT volume was nonetheless still significantly associated with 0.05 ± 0.01 lower E/A ratio ($p<0.0001$) independent of menopause status, age, race, education, blood pressure, use of antihypertensive medications, physical activity, and diabetes mellitus. In Model 3, the association between PAT volume and E/A ratio was abolished after adjusting for abdominal VAT volume.

Significant interactions between PAT volume and menopause status on LV ejection fraction, LV mass index, and E/e' were not detected. Results of the models examining these measures are presented in **Table V- 3**. PAT volume was not associated with LV ejection fraction in Model 1 (0.29), and so the association between PAT volume and LV ejection fraction was not examined in subsequent models (i.e., Model 2 and 3).

Table V- 3. β estimates for the associations of menopause status, PAT volume, and VAT volume with LV ejection fraction, LV mass index, and E/e' ratio.

	Menopause		PAT volume		VAT volume	
	β ± SE	**p-value**	**β ± SE**	**p-value**	**β ± SE**	**p-value**
LV ejection fraction						
Model 1*		0.36				
Premenopausal (ref.)	--	--				
Natural menopause	0.15 ± 0.43	0.73	0.20 ± 0.19	0.29	--	--
Hysterectomy, ovarian preservation	-0.37 ± 0.54	0.49				
Hysterectomy, BLO	1.09 ± 0.75	0.15				
LV mass index						
Model 1		<0.0001				
Premenopausal (ref.)	--	--				
Natural menopause	-1.41 ± 0.68	0.04	4.07 ± 0.30	<0.0001	--	--
Hysterectomy, ovarian preservation	2.90 ± 0.86	0.0008				
Hysterectomy, BLO	2.36 ± 1.19	0.049				
Model 2		0.11				
Premenopausal (ref.)	--	--				
Natural menopause	-1.48 ± 0.72	0.04	3.92 ± 0.30	<0.0001	--	--
Hysterectomy, ovarian preservation	0.24 ± 0.82	0.77				
Hysterectomy, BLO	0.30 ± 1.12	0.79				
Model 3		0.19				
Premenopausal (ref.)	--	--				
Natural menopause	-1.18 ± 0.72	0.10	2.93 ± 0.39	<0.0001	1.53 ± 0.39	0.0001
Hysterectomy, ovarian preservation	0.36 ± 0.82	0.66				
Hysterectomy, BLO	0.46 ± 1.12	0.68				

Table V- 3, cont'd.

	Menopause		PAT volume		VAT volume	
	β ± SE	**p-value**	**β ± SE**	**p-value**	**β ± SE**	**p-value**
E/e' ratio						
Model 1		<0.0001				
Premenopausal (ref.)	--	--				
Natural menopause	0.37 ± 0.14	0.01	0.43 ± 0.06	<0.0001	--	--
Hysterectomy, ovarian preservation	0.55 ± 0.18	0.002				
Hysterectomy, BLO	0.72 ± 0.25	0.004				
Model 2		0.73				
Premenopausal (ref.)	--	--				
Natural menopause	0.11 ± 0.16	0.55	0.33 ± 0.07	<0.0001	--	--
Hysterectomy, ovarian preservation	0.04 ± 0.18	0.88				
Hysterectomy, BLO	0.26 ± 0.24	0.12				
Model 3		0.49				
Premenopausal (ref.)	--	--				
Natural menopause	0.12 ± 0.16	0.45	0.31 ± 0.09	0.0003	0.03 ± 0.09	0.71
Hysterectomy, ovarian preservation	0.05 ± 0.18	0.79				
Hysterectomy, BLO	0.26 ± 0.24	0.28				

Model 1: menopause status, PAT volume
Model 2: Model 1 + age, race, education, blood pressure, use of antihypertensive medications, diabetes mellitus
Model 3: Model 2 + VAT volume
β estimates for PAT and VAT volume are presented as the change in the outcome per 1-SD increment.
* Models 2 and 3 are not presented for LV ejection fraction due to no association with PAT volume.

In Model 1 examining LV mass index, PAT volume was associated with higher LV mass index (4.07 ± 0.30 g/$m^{2.7}$ per SD increment, p<0.0001) independent of menopause status. In Model 2, the association between PAT volume and LV mass index persisted independent of age, race, education, blood pressure, use of antihypertensive medications, physical activity, and diabetes mellitus (p<0.0001), and in Model 3, PAT was independently associated with LV mass index after further adjustment for abdominal VAT volume (p<0.0001).

Finally, PAT volume was associated with higher average E/e' ratio in all models. In Model 1, a 1-SD increment in PAT volume was associated with 0.43 ± 0.06 higher E/e' ratio independent of menopause status. This association remained significant after adjustment for confounders in Model 2 (p<0.0001) and for abdominal VAT volume in Model 3 (p=0.0003).

Discussion

PAT volume, an inflammatory fat depot that surrounds the heart, was significantly associated with measures of cardiac structure and function in a cross-sectional comparison of premenopausal, naturally postmenopausal, and surgically postmenopausal women. PAT volume was associated with greater LV mass index and average E/e' ratio in all women regardless of menopause status. These analyses provide a robust basis for future research that can examine PAT as a mediator of heart failure risk in women.

We were able to use comprehensive CT and echocardiography data from a long-running study to show a clear and logical association between PAT volume and both LV mass index and a measure diastolic function. Our results are bolstered by our ability to test this relationship in a large sample of 1,441 women who had reported their reproductive history consistently over at least 10 years. They were further strengthened by the comparison of premenopausal, and

postmenopausal women classified according to natural vs. surgical menopause, as each of these groups has different levels of CVD risk.

Our study expands upon the existing literature to suggest a potential mechanism by which CVD risk increases after menopause in women. Menopause is a unique time window during which CVD risk in women increases, regardless of surgical vs. natural menopause. A previous comparison of 508 naturally and 317 surgically postmenopausal women (hysterectomy with and without and BLO) in the CARDIA study showed that whereas surgical menopause is associated with adverse cardiac structure and function, it was explained by an adverse baseline risk profile prior to surgery.[75] Findings of the current study further suggest a similarity between surgical and natural menopause, because most menopausal differences in the outcome were fully attenuated with the inclusion of confounders across all models. Furthermore, the only outcome that showed a significant menopause by PAT volume interaction was E/A ratio, and this interaction was fully attenuated after risk adjustment.

Much of the increased CVD risk in postmenopausal women has been attributed to the loss of vascular protection offered by endogenous estrogen. In addition to its potential role in fat distribution, estrogen prevents apoptosis of myocytes, prevents cardiac remodeling,[35] and downregulates inflammatory cytokine production.[146] Experimental studies have examined the effect of estrogen deficiency on health outcomes in animal models, showing an increased risk of coronary artery disease,[70] adverse cardiac remodeling,[87] and diastolic dysfunction independent of age.[88,89] PAT accumulation is inversely associated with estrogen concentration[190] and may be associated with inflammation in response to the loss of ovarian estrogen; however, it is also associated with increased adiposity and obesity,[248] which may explain PAT accumulation in women who underwent surgical menopause in our study. Women who undergo natural

menopause have decreasing production of ovarian estrogen prior to the final menstrual period,[190,249] and women who undergo surgical menopause with BLO have an abrupt drop in their estrogen production, but women who undergo hysterectomy with ovarian preservation continue to produce ovarian estrogen. In this study, women who underwent surgical menopause had notably higher adiposity compared with both premenopausal and naturally postmenopausal women regardless of ovarian preservation, which may explain their higher PAT volumes.

Diastolic dysfunction coupled with hypertension and advancing age are the primary causes of heart failure in women,[44] specifically of heart failure with preserved ejection fraction. We found significant differences by menopause status in blood pressure, with women undergoing surgical menopause having the highest average blood pressure compared with both premenopausal and naturally postmenopausal groups. Differences across groups were observed despite a higher prevalence of antihypertensive medication use among surgically postmenopausal women. This may be related to the increased time in surgical vs. natural menopause: postmenopausal women with hysterectomy with ovarian preservation reported their final menstrual period at 40 years, BLO women at 42 years, and naturally postmenopausal women at 49 years. Although women in these groups experienced menopause at different ages on average, there was a difference of only 5 years between the average ages of the oldest vs. the youngest groups (53 vs. 48 years).

This study has several limitations that must be considered when interpreting the results. The data used in these analyses were cross-sectional, preventing any inference about causal relationships. We cannot elucidate a clear mechanism relating PAT volume with cardiac structure and function without longitudinal and more controlled data. Furthermore, although self-reported data reliably capture natural and surgical menopause, [250,251] they do not identify

perimenopausal women who are transitioning through menopause.[241] These women were likely included in the premenopausal category. Finally, we are unable to draw conclusions about the pathophysiological function of PAT, and can only hypothesize about a potential role in inflammation and CVD risk.

In conclusion, we found that PAT was associated with greater LV mass index and reduced E/e' ratio, independent of menopause and VAT. Although abdominal VAT is correlated with PAT, it does not attenuate the association between PAT and cardiac structure and function. Future studies should examine longitudinal changes to PAT volume and inflammatory function of PAT to better understand the mechanism underlying the associations seen in this study.

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CHAPTER VI

ENDOTHELIAL ESTROGEN RECEPTORS α AND β ARE REDUCED IN PREMENOPAUSAL WOMEN WITH TYPE 1 DIABETES

Abstract

The mechanism for the loss of premenopausal cardiovascular protection in women with type 1 diabetes women is unclear. In this study, 18 premenopausal women with type 1 diabetes (age 33 ± 9 years) and 16 premenopausal women without diabetes (age 29 ± 5 years) underwent 1 week of gonadotropin-releasing hormone antagonist therapy ($GnRH_{ant}$) and were randomized to add-back of transdermal estradiol (E2) or placebo (PL) to understand the role of ovarian hormones in type 1 diabetes. Endothelial cell expression of estrogen receptors (ER) α and β and nuclear factor (NF) κB standardized to expression in human umbilical vein endothelial cell (HUVEC) compared by diabetes status before and after the GnRHant intervention.

Total ERα/HUVEC (0.90 ± 0.03 vs. 1.00 ± 0.03, p=0.04) and ERβ/HUVEC (1.01 ± 0.02 vs. 1.09 ± 0.02, p=0.03) expression were significantly reduced in type 1 diabetes independent of age and estradiol concentration, despite similar estradiol concentration at baseline. After 1 week of hormone suppression, total and nuclear ERβ/HUVEC were increased in type 1 diabetes but decreased in women without diabetes in the $GnRH_{ant}$+PL group only (p=0.01 and 0.003, respectively).

Reduced ER expression may be mechanisms for the loss of premenopausal protection against cardiovascular disease in women with type 1 diabetes.

Introduction

Women with type 1 diabetes mellitus have a higher risk of cardiovascular disease (CVD) than women without diabetes. Premenopausal women with type 1 diabetes mellitus have

increased coronary artery calcium (CAC) compared with women without diabetes independent of cardiovascular risk factors, including lipids and blood pressure.[9,13] They further have up to 40 times the risk of CVD-related mortality compared with premenopausal women without diabetes due to the low risk of CVD in premenopausal women without diabetes.[12] It is unclear why type 1 diabetes is associated with a loss of the protection against CVD that premenopausal women without diabetes experience. Women with type 1 diabetes report a higher prevalence of ovarian dysfunction than women without diabetes, suggesting a potential role for hormonal irregularities in their manifestation of CVD. However, the role of estrogen in the increased CVD risk in women with type 1 diabetes mellitus is unclear. Estrogen promotes a more favorable lipid profile,[157,252] increases insulin sensitivity, reduces oxidative stress and inflammation, and enhances endothelial function,[253] while whereas with type 1 diabetes have impaired endothelial function, oxidative stress,[254] systemic inflammation,[255] and insulin resistance.[256] A better understanding of the actions of estrogen in type 1 diabetes may elucidate a mechanism by which women with type 1 diabetes are at increased cardiovascular risk.

Estrogen is associated with cardiovascular protection in women given its potent effects on endothelial function, and its antioxidant and anti-inflammatory effects.[257,258] It has direct and rapid effects on the vasculature through its activation of endothelial nitric oxide synthase (eNOS), which subsequently promotes vasodilation,[259] and it also has genomic effects where it activates gene transcription in the nucleus. The effects of estrogen are mediated through estrogen receptor (ER) α and ERβ, nuclear transcription factors that are expressed in the vascular endothelium.[260] ER expression may also suppress expression of nuclear factor (NF)-κB, another nuclear transcription factor expressed in the endothelium. NF-κB is associated with

inflammation, and its expression is upregulated in type 1 diabetes and other autoimmune disorders.[17,105]

We conducted a pilot study of premenopausal women with and without type 1 diabetes who underwent an ovarian suppression intervention using gonadotropin-releasing hormone antagonist ($GnRH_{ant}$) to understand the relationship between ovarian hormones and vascular endothelial cell proteins. Participants were randomized to either $GnRH_{ant}$ with placebo (+PL) or $GnRH_{ant}$ with transdermal estradiol (+E2). We examined vascular endothelial cell protein expression of ERα, ERβ, and NF-κB harvested from peripheral veins of premenopausal women with and without type 1 diabetes before and after 1 week of $GnRH_{ant}$. We hypothesized that 1) ER expression would be lower and NF-κB expression would be higher in women with type 1 diabetes prior to ovarian hormone suppression independent of estradiol levels, 2) that $GnRH_{ant}$+PL would decrease ER expression and increase NF-κB expression regardless of type 1 diabetes status, and 3) that $GnRH_{ant}$+E2 would increase ER expression and increase NF-κB expression regardless of type 1 diabetes status.

Research design and methods

Study population

Women with and without type 1 diabetes were recruited to participate in the pilot Estrogen, Diabetes, and Endothelial Function (EDEN) study. The study cohort consisted of 20 women with type 1 diabetes and 20 women without diabetes between the ages of 18 and 45 who did not have a history of CVD. Inclusion criteria included normal thyroid function and no use of any hormonal contraceptives for ≥ 6 months, and women were excluded if they were pregnant, breastfeeding, did not experience a menstrual cycle in the previous 6 months, or had a diagnosis of polycystic ovary syndrome. Additional inclusion criteria for women with type 1 diabetes were

that they had ≥ 5 years duration of type 1 diabetes, started using insulin within 1 year of diagnosis, and were currently treated with insulin. Women without type 1 diabetes were also excluded if they had a hemoglobin A1c value ≥ 5.7% (39 mmol/mol) to exclude anyone with prediabetes or with type 2 diabetes mellitus. All study participants provided written informed consent, and all study procedures were approved by the Colorado Multiple Institutional Review Board.

Study visits

Study participants completed two visits. The baseline study visit took during the early follicular phase of the menstrual cycle, and participants returned for follow-up after the $GnRH_{ant}$ intervention. Both study visits included a clinical exam where anthropometric measures and a fasting blood sample were collected. Carotid intima-media thickness (cIMT) was measured at the baseline visit by ultrasound (CardioHealth Station, Panasonic, Yokohama, Japan).

Intervention

Beginning the day of the baseline visit, all participants underwent ovarian sex hormone suppression with $GnRH_{ant}$ therapy (cetrorelix acetate, 0.25 mg/day) delivered daily as subcutaneous injections for a 1-week period. Participants were randomized to one of two concurrent intervention groups: transdermal estradiol patch (0.075 mg/day) or placebo patch. Of the 18 women with type 1 diabetes mellitus, 8 were randomized to receive the estradiol patch. Of the 16 women without diabetes mellitus, 9 were randomized to receive the estradiol patch. Investigators and participants were blinded to randomization status.

Endothelial cells

Of the 40 women originally enrolled in the EDEN study, 18 women with type 1 diabetes and 16 women without diabetes completed the endothelial cell harvest and were included in our

analyses. Endothelial cells were harvested from an antecubital vein using 2 J-wires advanced through an intravenous catheter and placed into a dissociation buffer, as previously described.[209,210] Cells were then isolated via a washing and centrifugation protocol, and fixed with 3.7% formaldehyde on poly-L-lysine coated slides (Sigma Chemical, St. Louis, MO). Slides were stored at -80°C until they were analyzed for protein expression.

Quantitative immunofluorescence was used to assess total and nuclear protein expression. Cells were first rehydrated with a phosphate buffer solution and made permeable with 0.1% Triton X-100 (Fischer Scientific, Fair Lawn, NJ) for subsequent staining. Non-specific binding sites were blocked with 5% donkey serum (Jackson Immunoresearch, West Grove, PA), and cells were incubated with monoclonal antibodies for the endothelial protein of interest: ERα (Cell Signaling Technology, Danvers, MA), ERβ (Santa Cruz Biotechnology, Dallas, TX), NF-κB (Novus Biologicals, Littleton, CO). The cells were secondly incubated with conjugated secondary antibodies (Alexa Fluor, Thermo Fisher Scientific, Waltham, MA).

All cells were stained with 4′, 6′-diamidino-2-phenylindole hydrochloride (DAPI; Vector Laboratories, Burlingame, CA). Slides were systematically examined by a single reviewer using a fluorescence microscope (Eclipse 80i, Nikon, Melville, NY) who was blinded to the diabetes status of the study participant and to whether they received the estradiol or placebo patch. Endothelial cells were identified by positive staining of von Willebrand factor, and only endothelial cells with confirmed nuclear integrity were included in analysis. Cell images were captured by a Photometrics CoolSnap ES2 digital camera (Roper Scientific, Inc., Tucson AZ) and analyzed using NIS-Elements BR Software (Nikon Instruments, Inc., Melville, NY). Total and nuclear protein expression were quantified as the pixel intensity of secondary antibody staining for each cell. Mean expression of ERα, ERβ, and NF-κB are reported as the ratio of

endothelial cell protein expression to control human umbilical vein endothelial cells (HUVEC), thereby accounting for potential differences in intensity across staining sessions. All analyses used these standardized ratios.

Laboratory assays

The Colorado Clinical and Translational Sciences Institute (CCTSI) Clinical and Translational Research Centers Core Laboratory conducted all laboratory assays. Fasting plasma lipids, glucose, insulin (Beckman Coulter, Brea, CA), and adiponectin (Millipore, Billerica, MA), were measured at the baseline visit. Plasma total antioxidant capacity (TAC, Cell Biolabs, San Diego, CA) was assayed at both visits to quantify the antioxidant ability to reduce Cu(II) to Cu(I). Plasma glutathione peroxidase activity (Randox Laboratories, UK) was also assayed at both visits. Serum samples were assayed at both visits to measure the following sex hormones: estradiol, sex hormone-binding globulin (SHBG), testosterone, luteinizing hormone (LH), follicle-stimulating hormone (FSH), and progesterone (Beckman Coulter, Brea, CA). Free estradiol index (FEI) was calculated for all women at both visits as the ratio of estradiol to SHBG.

Statistical methods

We compared the following participant characteristics at the baseline visit by diabetes status: age, blood pressure, measures of adiposity, cIMT, lipid concentrations, and hormone concentrations. All variables were tested for normality using the Shapiro-Wilk test. Independent t-tests were used to compare mean values of normally distributed characteristics by diabetes status. Wilcoxon exact tests were used to compare non-normally distributed characteristics by diabetes status.

Linear regression was used to test cross-sectional associations of baseline ERα/HUVEC, ERβ/HUVEC, and NF-κB/HUVEC expression with type 1 diabetes mellitus status after controlling for age and baseline estradiol concentration.

Change in sex hormone concentrations between the baseline and follow-up study visits was examined by diabetes status and by treatment group. Paired t-tests were used to identify differences in normally distributed sex hormone concentrations between the baseline and follow-up visit. Wilcoxon signed rank tests were used to identify differences in non-normally distributed sex hormone concentrations between the baseline and follow-up visit.

Linear regression was then used to evaluate changes in endothelial protein expression at the follow-up visit by diabetes status and $GnRH_{ant}$ group. These models were adjusted for age and change in estradiol concentration, and stratum-specific estimates were obtained using a statistical interaction term between diabetes status and $GnRH_{ant}$ treatment group.

Finally, the independent relationship between glycemic control and endothelial protein expression was evaluated by adding hemoglobin A1c to the baseline and change models.

Results

Cross-sectional analysis: baseline visit

Participant characteristics at the baseline visit by type 1 diabetes status are presented in **Table VI-** 1**.** Women with type 1 diabetes mellitus were slightly older than women without diabetes mellitus. Average blood pressure and BMI did not differ by diabetes status, but women with type 1 diabetes mellitus had significantly higher waist (p=0.02) and hip (p=0.01) circumferences compared with women without diabetes mellitus. Women with type 1 diabetes mellitus further had significantly higher maximum cIMT compared with women without

diabetes (p=0.005), but there were no significant differences in lipid concentrations or in triglycerides, adiponectin, or creatinine (p>0.20) for all).

Table VI- 1. Participant characteristics at baseline visit by diabetes status.

	Type 1 diabetes (n=18)	No diabetes (n=16)	p-value
Demographic information			
Age, years	33 ± 9	29 ± 5	0.18
Age of diabetes diagnosis, years	15 ± 8	--	--
Duration of diabetes, years	17 ± 7	--	--
Clinical information			
Systolic blood pressure, mmHg	114 ± 8	112 ± 8	0.57
Diastolic blood pressure, mmHg	66 ± 6	66 ± 8	0.95
BMI, kg/m^2	26.0 ± 3.1	24.0 ± 4.6	0.13
Waist circumference, cm	80.1 ± 8.7	73.0 ± 8.8	0.02
Hip circumference, cm	106.0 ± 11.1	94.9 ± 13.3	0.01
Maximum cIMT, mm	0.886 ± 0.426	0.545 ± 0.101	0.005
Laboratory results			
HbA1c, % (mmol/mol)	7.7 ± 1.3 (61 ± 14.2)	5.2 ± 0.2 (33 ± 2.2)	<0.0001
Fasting glucose, mg/dL	146 ± 44	80 ± 9	<0.0001
Insulin, µIU/mL	17.5 (12, 34)	6 (4, 9)	0.0002
TAC, µM CRE	857 ± 93	965 ± 81	0.001
Glutathione peroxidase, U/L	7207 ± 1655	7265 ± 2019	0.93
Oxidized LDL cholesterol, U/L	47 ± 12	56 ± 17	0.08
HDL cholesterol, mg/dL	59 ± 15	55 ± 14	0.39
Total cholesterol, mg/dL	154 ± 24	150 ± 16	0.49
Triglycerides, mg/dL	49 (43, 55)	52 (43, 68)	0.70
Adiponectin, µg/mL	12.9 (11.7, 17.2)	13.1 (9.5, 15.1)	0.25
Creatinine, mg/dL	0.75 (0.70, 0.80)	0.75 (0.70, 0.80)	0.81
Hormone concentrations			
Estradiol, pg/mL	49 (43, 56)	51 (37, 63)	0.97
Progesterone, ng/mL	0.3 (0.1, 0.6)	0.3 (0.2, 0.6)	0.54
Testosterone, ng/dL	38 ± 12	36 ± 13	0.64
SHBG, nmol/L	73 ± 44	57 ± 20	0.17
FSH, mIU/mL	4.7 (4.1, 7.4)	5.6 (5.0, 6.7)	0.32
LH, mIU/mL	3.7 (2.0, 5.0)	4.5 (2.5, 5.5)	0.46
FEI	0.85 (0.61, 1.25)	0.80 (0.63, 1.27)	0.98

Median (interquartile range) are presented for the following non-normally distributed variables: insulin, triglycerides, adiponectin, creatinine, estradiol, progesterone, FSH, LH, and FEI. Mean ± SD are presented for all other variables.

Abbreviations: BMI, body mass index; cIMT, carotid intima-media thickness; HDL, high-density lipoprotein; LDL, low-density lipoprotein; TAC, total antioxidant capacity; CRE, copper reducing equivalents; SHBG, sex hormone binding globulin; FSH, follicle-stimulating hormone; LH, luteinizing hormone; FEI, free estradiol index

Results of the first model examining baseline protein expression by diabetes status after adjusting for age and baseline estradiol concentration are presented in **Figure 1**. Women with type 1 diabetes had significantly lower total ERα/HUVEC expression (0.90 ± 0.03 vs. 1.00 ± 0.03, p=0.04) and ERβ/HUVEC expression (1.01 ± 0.02 vs. 1.09 ± 0.02, p=0.03) compared with women without diabetes. Although the differences in nuclear ERα and ERβ did not reach statistical significance, they were in the same direction of lower expression in women with versus without type 1 diabetes (0.92 ± 0.04 vs. 1.00 ± 0.04, p=0.13 and 1.00 ± 0.02 vs. 1.04 ± 0.03, p=0.17, respectively). On the contrary, there were no differences in either total or nuclear NF-κB/HUVEC by diabetes status.

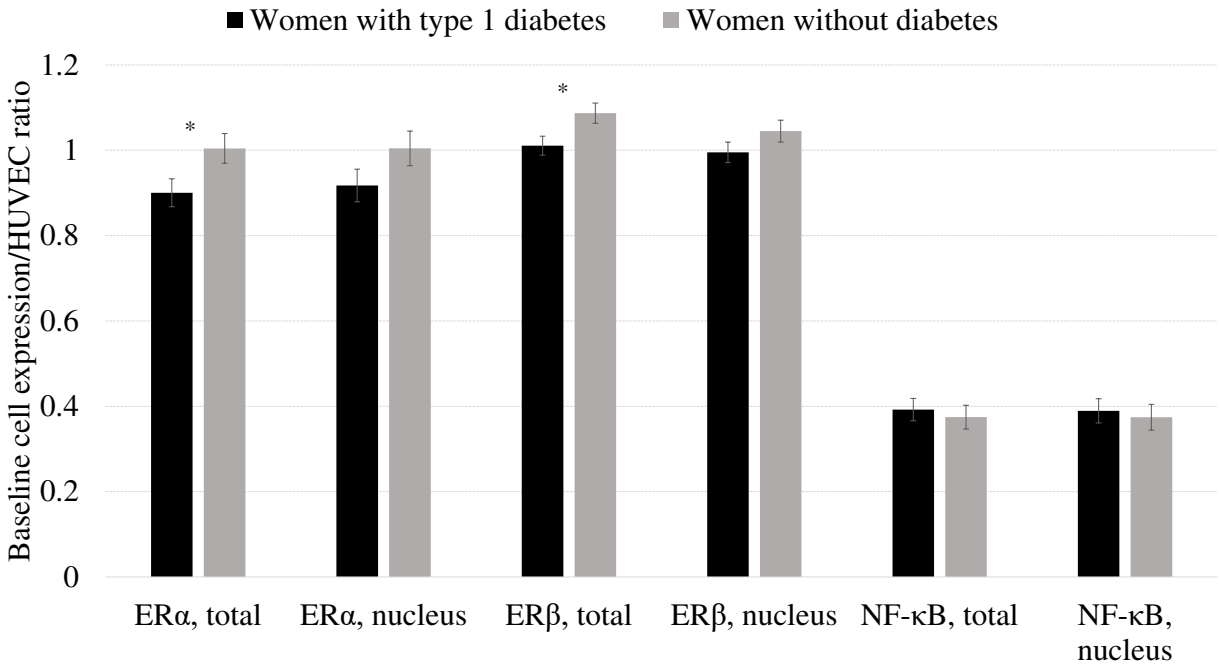

Figure VI- 1. Mean endothelial total and nuclear expression/HUVEC ratios of ERα, ERβ, and NF-κB. * *p < 0.05*

$GnRH_{ant}$ intervention: follow-up visit

Sex hormone concentrations at the baseline visit (i.e., before the $GnRH_{ant}$ intervention) and at the follow-up visit (i.e., after the $GnRH_{ant}$ intervention) are presented in **Table VI- 2**. Among women with type 1 diabetes mellitus, testosterone concentration decreased significantly in the $GnRH_{ant}$+PL group, and estradiol concentration and FEI increased significantly in the $GnRH_{ant}$+E2 group. Among women without diabetes, LH concentration decreased significantly in the $GnRH_{ant}$+PL group. In the GnRHant+E2 group, progesterone concentration decreased significantly while estradiol concentration and FEI increased significantly.

Table VI- 2. Differences in sex hormone concentrations before and after the intervention.

	Type 1 diabetes				No diabetes			
	$GnRH_{ant}$+PL (n=10)		$GnRH_{ant}$+E2 (n=8)		$GnRH_{ant}$+PL (n=9)		$GnRH_{ant}$+E2 (n=7)	
	Baseline	**Follow-up**	**Baseline**	**Follow-up**	**Baseline**	**Follow-up**	**Baseline**	**Follow-up**
Estradiol, pg/mL	49 (46, 68)	50 (36, 56)	46 (37, 54)*	173 (133, 196)	58 (34, 88)	44 (27, 79)	48 (39, 55)*	129 (75, 206)
Progesterone, ng/mL	0.4 (0.1, 0.6)	0.3 (0.1, 0.9)	0.3 (0.2, 0.3)	0.2 (0.1, 0.3)	0.2 (0.1, 0.3)	0.2 (0.1, 0.3)	0.4 (0.3, 1.1)*	0.2 (0.1, 0.3)
Testosterone, ng/dL	38 ± 13*	31 ± 9	38 ± 11	40 ± 16	37 ± 10	30 ± 12	35 ± 15	30 ± 15
SHBG, nmol/L	79 ± 44	80 ± 40	66 ± 46	66 ± 45	57 ± 20	51 ± 13	56 ± 21	55 ± 22
FSH, mIU/mL	5.0 (3.8, 6.3)	6.2 (4.9, 7.5)	4.7 (4.4, 7.9)	3.9 (2.6, 6.1)	5.4 (4.9, 6.2)	5.7 (4.9, 7.9)	5.8 (5.1, 6.9)	4.2 (2.6, 5.5)
LH, mIU/mL	3.8 (2.3, 5.8)	3.3 (2.2, 5.0)	2.8 (2.0, 4.8)	2.7 (1.1, 6.3)	5.2 (2.6, 5.5)*	1.8 (1.5, 3.4)	4.3 (2.3, 5.2)	1.9 (1.6, 2.9)
FEI	0.78 (0.51, 1.26)	0.63 (0.33, 1.32)	0.87 (0.69, 1.21)*	3.19 (2.45, 4.24)	1.10 (0.58, 1.52)	1.22 (0.53, 1.74)	0.76 (0.68, 0.91)*	2.43 (1.81, 3.17)

Mean ± SD are presented for testosterone and SHBG. Median (IQR) are presented for all remaining hormones.
Abbreviations: SHBG, sex hormone-binding globulin; FSH, follicle-stimulating hormone; LH, luteinizing hormone; FEI, free estradiol index
* $p<0.05$

The 1-week change in endothelial protein expression by diabetes status and $GnRH_{ant}$ intervention group after adjusting for age and change in estradiol concentration is presented in **Figure VI- 2.** In the $GnRH_{ant}$+PL group, there was a significant difference in total (p=0.01) and nuclear (p=0.003) ERβ/HUVEC expression by diabetes status. Women with type 1 diabetes and in the $GnRH_{ant}$+PL group had an increase in total and nuclear ERβ/HUVEC expression (0.09 ± 0.04 pg/mL and 0.10 ± 0.04 pg/mL, respectively). Women without diabetes in the $GnRH_{ant}$+PL group had a decrease in total and nuclear ERβ/HUVEC expression (-0.08 ± 0.05 pg/mL and -0.09 ± 0.05 pg/mL, respectively). There were no differences in endothelial protein expression by diabetes status in the $GnRH_{ant}$+E2 group.

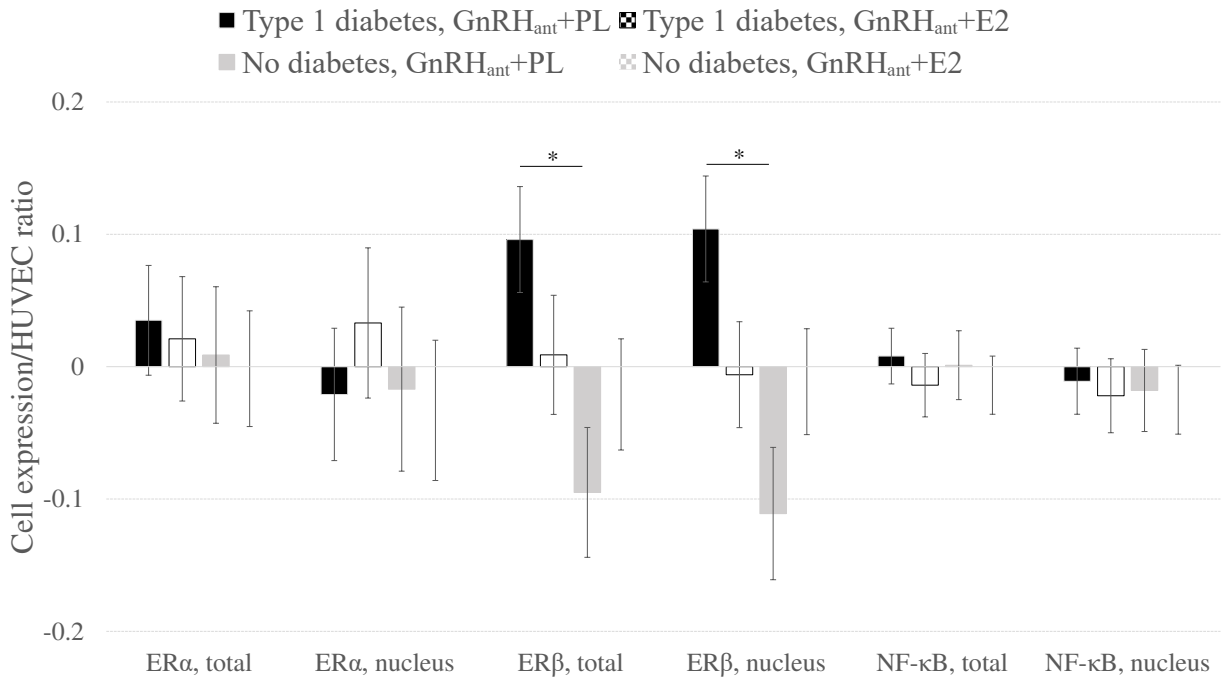

Figure VI- 2. Least squares mean change from baseline to follow-up in cell expression/HUVEC ratio of total and nuclear ERα, ERβ, and NF-κB by diabetes status and treatment group, adjusted for age and change in estradiol concentration

Association between endothelial protein expression and glycemic control

Results of the linear regression models testing the independent relationship of hemoglobin A1c with endothelial protein expression are presented in **Table VI- 3**. At the

baseline visit, hemoglobin A1c was significantly associated with ERβ, but not ERα or NF-κB, expression. After adjusting for age, diabetes status, and baseline estradiol concentration, a 1% increment of hemoglobin A1c was associated with lower total (-0.05 ± 0.02, p=0.03) and nuclear ERβ/HUVEC expression (-0.05 ± 0.02, p=0.03 for both).

Table VI- 3. β estimates for the change in endothelial protein expression associated with a 1% increment of hemoglobin A1c before and after ovarian hormone suppression, adjusted for age, diabetes status, and estradiol concentration.

	Association with baseline expression		Association with change in expression			
			$GnRH_{ant}$+PL		$GnRH_{ant}$+E2	
	β ± SE	**p-value**	**β ± SE**	**p-value**	**β ± SE**	**p-value**
ERα, total	-0.06 ± 0.03	0.08	0.003 ± 0.04	0.94	-0.02 ± 0.03	0.59
ERα, nuclear	-0.01 ± 0.04	0.79	-0.05 ± 0.06	0.46	-0.04 ± 0.02	0.13
ERβ, total	-0.05 ± 0.02	0.03	0.03 ± 0.04	0.52	0.01 ± 0.05	0.85
ERβ, nuclear	-0.05 ± 0.02	0.03	0.01 ± 0.04	0.79	-0.01 ± 0.05	0.86
NF-κB, total	0.02 ± 0.03	0.58	-0.02 ± 0.02	0.48	-0.04 ± 0.02	0.03
NF-κB, nuclear	0.02 ± 0.03	0.56	-0.02 ± 0.03	0.57	-0.03 ± .02	0.07

In the $GnRH_{ant}$+PL group, hemoglobin A1c was not associated with change in expression of any of the proteins at the follow-up visit. In the $GnRH_{ant}$+E2 group, however, a 1% increment of hemoglobin A1c was significantly associated with a decrease in total NF-κB/HUVEC expression of 0.04 ± 0.02 (p=0.03).

Discussion

In a pilot study of 34 premenopausal women with and without type 1 diabetes, we observed significantly lower endothelial protein expression of ERα and ERβ in women with type 1 diabetes compared to women without during the early follicular phase of the menstrual cycle, and an inverse association between glycemic control and ERβ expression.

We have previously shown that premenopausal women with type 1 diabetes are at increased risk of CVD independent of traditional cardiovascular risk factors, including lipids,

blood pressure, and ovarian dysfunction.[13] Prior to menopause, women with type 1 diabetes still have significantly higher CVD risk compared with women without diabetes despite a less atherogenic lipid profile and no adverse risk factors, and this difference only increases in magnitude over time.[13] The mechanism for the increased risk is unclear.

Estrogen affects gene expression through ERα and ERβ, but ERs may also play a role in the non-genomic effects of estrogen. Although both ERs are expressed in the vascular endothelium, ERα is of particular importance as it mediates activation of eNOS in addition to its function as a nuclear transcription factor.[260,261] ERα/HUVEC expression is correlated with expression of eNOS and phosphorylated eNOS expression (ρ=0.54 and 0.59, respectively).[262] Animal models have shown that ERα deficiency impairs production of nitric oxide (NO), which results in vasoconstriction.[263,264] While ERα and ERβ expression is associated with reduced cardiac damage,[154] reductions in ER activity (e.g., via methylation) may be associated with atherosclerosis and inflammation.[152] Indeed, previous studies have shown an inverse relationship between ERα and ERβ expression and atherosclerosis.[265,266] The lower ER expression in women with type 1 diabetes that we found in this study may be associated with the increased risk of subclinical atherosclerosis that has been observed in larger studies.

Estrogen is further implicated in hepatic glucose homeostasis; it improves hepatic insulin sensitivity by activating ER signals and suppressing glucose production in the liver.[267] Reduced estrogen levels are associated with reduced systemic insulin sensitivity due to increased hepatic insulin resistance.[256] Furthermore, animal models suggest that ERα may be associated with insulin sensitivity[268] and ERβ may be associated with impaired insulin function and insulin resistance.[269,270] In contrast, our study identified a significant inverse association between glycemic control and baseline ERβ/HUVEC expression during the early follicular phase of the

menstrual cycle. The observed relationship may be due to natural variation across the menstrual cycle in insulin sensitivity, as women with type 1 diabetes are most insulin sensitive during the early follicular phase.[271] Women with type 1 diabetes are nonetheless more insulin resistant than women without diabetes, [117] and the association of ERβ with insulin resistance may explain the differential response to $GnRH_{ant}$+PL by diabetes status.

Alternatively, as ERβ/HUVEC expression increased in the women with type 1 diabetes in response to $GnRH_{ant}$ therapy independent of estradiol concentration, this may reflect a compensatory response to the systemic inflammation in type 1 diabetes. ERβ expression may be upregulated in relation to inducible NO synthase (iNOS), which is associated with inflammatory cytokines and β cell destruction in type 1 diabetes.[272] A rat model of type 2 diabetes found more than twice the ERβ protein expression in the aortic smooth muscle cells of rats with vs. rats without diabetes, and concluded that ERβ overexpression in diabetes was associated with an impaired response to increased estradiol.[273] Although this study examined expression in vascular smooth muscle rather than endothelial cells, it suggests a potential mechanism for the increased vascular dysfunction in diabetes in combination with increased cytokine concentrations and iNOS.[274,275]

Although previous studies have found an association between type 1 diabetes and NF-κB activation,[276,277] we did not find any differences by diabetes status in endothelial NF-κB in our sample. NF-κB is a nuclear transcription factor that has been implicated in beta cell apoptosis and development of type 1 diabetes;[278] however, it is possible that these differences are more apparent in immune rather than endothelial cells. Furthermore, the women included in our study were young and free of CVD, and it is possible that endothelial NF-κB may be expressed more strongly in older women who may be experiencing more advanced subclinical CVD. The women

with type 1 diabetes in our study did have higher adiposity and cIMT, as well as reduced antioxidant capacity but not antioxidant enzyme activity (i.e., glutathione peroxidase), so differences in endothelial NF-κB expression may manifest later in the CVD process.

We must consider several limitations when interpreting the results of this pilot study. The small sample size limited the detectable difference by diabetes status in sex hormone concentrations and endothelial protein expression. Protein expression was measured in venous endothelial cells rather than arterial endothelial cells, and we did not have data on transcription activity (i.e., mRNA), which would provide specific information about the function of the endothelial proteins. Finally, these results are not generalizable to the larger population of women who use hormonal contraceptives.

Despite the limitations of this study, our findings provide the basis for future examination of endothelial protein expression in a larger sample of premenopausal women across a wider age range, and they are bolstered by several study design decisions. All study participants completed their baseline study visit during the early follicular phase of their menstrual cycle. At this phase, estrogen and progesterone concentrations are at their lowest, allowing us to naturally control for intra-subject variability in sex hormone concentrations across the cycle. We also measured both sex hormones and endothelial proteins at both the baseline and follow-up visit, allowing us to examine the 1-week change and consider how endothelial proteins were being expressed in response to changes in hormone levels.

Conclusion

In conclusion, the reduced expression of venous endothelial ERα and ERβ in type 1 diabetes present a potential mechanism by which CVD risk is increased in women with type 1 diabetes. Despite similar estradiol concentrations as women without diabetes, women with type 1

diabetes may have impaired ER function, even in the absence of inflammation as measured by NF-κB. Future studies may examine changes to endothelial protein expression, insulin sensitivity, and mRNA expression in a larger sample of women across the full menstrual cycle.

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CHAPTER VII

DISCUSSION

Summary of findings and public health implications

Aims 1 and 2

In Aim 1, we examined the relationship of PAT volume with measures of cardiac structure and function in a biracial population-based cohort of 1,790 men and premenopausal women from the CARDIA study. We found significant associations of PAT volume with cardiac structure as measured by LV mass index, and diastolic function as measured by both the E/A and average E/e' ratios. The association of PAT volume with LV mass index and E/A ratio differed by sex, and the magnitude of the association was greater in premenopausal women than in men. While men had more PAT volume than premenopausal women (64.4 ± 35.1 cm^3 vs 41.2 ± 19.8 cm^3), the same increment of PAT was associated with higher LV mass and lower E/A ratio (i.e., more adverse cardiac structure and diastolic function) in women than in men. These results align with existing literature indicating that PAT has more adverse associations with diastolic function,[232] fasting glucose concentration, and lipids[230] in women than in men.

We similarly saw an association of PAT volume with LV mass index and diastolic function in Aim 2, where we examined the relationship of PAT volume with measures of cardiac structure and function in a cohort of 1,441 premenopausal women, naturally postmenopausal women, and surgically postmenopausal women (i.e., hysterectomy with or without removal of both ovaries). In this aim, we did not find a differential association by menopause status of PAT volume with any of the 4 measures of cardiac structure and function examined, but PAT volume was associated with higher LV mass and E/e' ratio independent of menopause status, abdominal VAT volume, and confounding by age, race, education, blood pressure, use of antihypertensive

medications, and diabetes mellitus. Women who underwent surgical menopause with BLO had the highest PAT volumes of all menopause status groups (56.6 ± 31.0 cm^3 vs. 41.2 ± 19.7 cm^3 in premenopausal women, 50.2 ± 24.9 cm^3 in naturally postmenopausal women, and 48.1 ± 23.2 cm^3 in women with hysterectomy and ovarian preservation), possibly due to both increased overall adiposity[230] and the association of estrogen deficiency with ectopic fat deposition.[190,279]

Taken together, the findings of Aims 1 and 2 show differences in how PAT may affect diastolic function between sexes and across menopause. PAT volume was directly associated with E/A ratio in men and premenopausal women. However, when we examined premenopausal and postmenopausal women, the relationship of PAT volume with E/A ratio was fully explained by abdominal VAT volume. There may be a role of overall adiposity in women that is not seen in men. Indeed, women in all menopause status groups had higher adiposity than men in the CARDIA study, although men did have the highest PAT and abdominal VAT volumes. However, it should be noted that E/A ratio was examined as a continuous outcome in these analyses, and the prevalence of Grade II diastolic dysfunction (46% in women with hysterectomy and BLO, 31% in men, 23% in premenopausal women) indicates that these results are likely affected by the inclusion of pseudonormal E/A ratios.

Aims 1 and 2 also showed a consistent relationship between PAT accumulation and LV mass index, indicating that PAT may be associated with cardiac hypertrophy. LV hypertrophy is associated with future risk of HFpEF,[280] and indeed, the prevalence of abnormal LV mass index was highest in women who underwent surgical menopause and lowest in men. The overall association of PAT with LV mass index independent of menopause status in Aim 2 was higher than that seen in either premenopausal women or men in Aim 1, and was independent of abdominal VAT in both aims.

Aim 3

In the EDEN study, we found that premenopausal women with type 1 diabetes had significantly lower expression of ERα and ERβ compared with non-diabetic controls, independent of serum estradiol concentration. We did not observe a relationship between type 1 diabetes and NF-κB, although NF-κB has previously been shown to be higher in type 1 diabetes.[202,277] In healthy populations, circulating estrogen (specifically, estradiol) is associated with protection against atherosclerosis and inflammation. Premenopausal women are generally protected against CVD, but this cardiovascular protection is not observed in women with type 1 diabetes, who have a higher prevalence of traditional CVD risk factors, subclinical CVD, and clinical CVD compared with non-diabetic women.

Women with type 1 diabetes have higher prevalence of amenorrhea, menstrual irregularity, and PCOS than women without diabetes;[134] experience delayed onset of menarche;[134,281] and may experience premature ovarian aging.[133] Ovarian dysfunction is associated with increased CVD risk, and the high prevalence in type 1 diabetes suggests there may be a role for hormonal dysregulation in CVD risk. Current evidence regarding estradiol concentrations in type 1 diabetes conflicts and so is poorly understood,[14,15,282] but in our study we did not observe a difference in estradiol concentration by type 1 diabetes status during the early follicular phase, similar to what was observed previously in the CACTI study.[14] Despite similar estradiol levels, ER expression was significantly reduced in type 1 diabetes. ERs are activated in response to circulating estradiol concentration, and these results suggest that this response may be impaired in type 1 diabetes.

Less clear is the increased expression of ERβ in response to ovarian hormone suppression in type 1 diabetes, but not in the non-diabetic controls. ERβ is implicated in increased insulin

resistance and in type 2 diabetes risk, and so we hypothesized that the reduced ERβ expression prior to the hormone suppression intervention was also related to lower insulin resistance during the early follicular phase. The increased ERβ expression after hormone expression may be compensatory and possibly associated with increased insulin resistance following hormone suppression, but we did not measure insulin resistance in this study.

Furthermore, given the results of previous studies,[105,201,283] the association of NF-κB with inflammation and subsequent atherosclerotic disease,[284] and the observed the reduced ER expression, we expected but did not observe increased NF-κB expression. This may be due to inclusion of only CVD-free, young premenopausal women in the study. Increased NF-κB in type 1 diabetes may occur later in the CVD process.

Future research directions

Few studies have examined the role of PAT in the context of both sex and menopause, and the CARDIA cohort provided an ideal population to study this due to its large sample size, longitudinal follow-up to help characterize menopause status, high retention, and measurement of multiple facets of cardiovascular risk. We have so far been able to show cross-sectional observed associations of PAT with LV mass index and diastolic function, but whether PAT is a mediator of sex and menopausal differences in these outcomes is unclear. Future simultaneous collection of PAT volume, LV mass, and diastolic function at multiple time points will allow for robust analysis of PAT as a potential mediator of sex and menopausal differences in cardiac structure and function. Although still observational, temporality can be established and the indirect association of sex or menopause with outcomes of interest through PAT can be isolated.

Additionally, the pathophysiology and function of PAT in this population cannot be ascertained, and thus, we can only speculate on the mechanisms for the relationship between

PAT volume and cardiac structure and function. More research to examine normal and abnormal function of PAT in the context of sex differences and systemic inflammation (e.g., obesity, autoimmunity) will help to understand the role PAT may play in CVD.

Nevertheless, the method of estimating PAT volume and comparing it with non-invasive echocardiogram measures of structure and function can be applied to other studies where both imaging modalities are available, which has been done in both the Framingham Heart Study[184,232,285] and the Jackson Heart Study.[191] These relationships can be examined in younger populations than those presented here or in the context of autoimmune disease, such as type 1 diabetes.

The third aim was a pilot study and included a small study sample, but the results will lay the groundwork for larger studies examining ER and ovarian function in type 1 diabetes. Future studies should recruit women across a wider age distribution and also examine the association between type 1 diabetes and ER expression in the context of hormonal contraceptive use. To better understand the physiological response to endogenous estrogen in type 1 diabetes, it will also be vital to examine other endothelial proteins, such as eNOS and iNOS, which are associated with the beneficial effects of estrogen. Examination of mRNA will also provide insight into the genomic effects of the ERs. Future studies must consider differences in endothelial protein expression across the menstrual cycle. Sex hormone concentrations change across the menstrual cycle, and this pilot study only examined women in the early follicular phase – when estrogen and progesterone concentrations are naturally at their lowest. Endothelial protein expression should be considered in conjunction with changes to insulin sensitivity across the cycle, which may be estimated in the absence of clamp studies.

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