

**PERIVASCULAR ADIPOSE TISSUE OF THE DESCENDING THORACIC  
AORTA, VASCULAR CALCIFICATION, AND  
SYSTEMIC LUPUS ERYTHEMATOSUS**

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# **PERIVASCULAR ADIPOSE TISSUE OF THE DESCENDING THORACIC AORTA, VASCULAR CALCIFICATION, AND SYSTEMIC LUPUS ERYTHEMATOSUS**

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University of Pittsburgh, 2011

**Aims:** Women with systemic lupus erythematosus (SLE) have an increased risk of cardiovascular disease (CVD). Traditional CVD and disease related risk factors have been implicated, but do not fully account for this increased risk. Visceral adipose is most strongly associated with vascular dysfunction and metabolic disorder than other adipose regions. Perivascular adipose tissue (PVAT) is a visceral adipose depot in close proximity to blood vessels, which may have more direct influence over vascular function and progression of CVD. We hypothesized that SLE patients have increased PVAT surrounding the descending thoracic aorta than healthy control subjects. For both SLE and control subjects, we examined the relation between PVAT and vascular calcification of the aorta (AC) and coronary arteries (CAC).

**Methods:** Using EBT, we quantified the PVAT of the descending thoracic aortic PVAT in SLE (n=135) and age/race matched controls (n=152). Participants were non-diabetic and free of CVD. CAC and AC were quantified using Agatston scores and the PVAT was quantified using standard attenuation values for adipose tissue (-190 to -30 HU) on commercially available software. The total PVAT volume (tPVAT) was calculated by summing the areas and multiplying by the length of the participant's aorta.

**Results:** The SLE women were more likely to be hypertensive ( $p < 0.0001$ ) and had a larger waist to hip ratio ( $p = 0.001$ ) than controls reflecting a different adipose distribution. The SLE group had a greater tPVAT than the control group ( $p = 0.0071$ ) despite no differences in BMI

( $p=0.26$ ), or metabolic syndrome (MS,  $p=0.17$ ). Total PVAT remained associated with SLE after adjusting for CVD risk factors including BMI ( $1.025[1.0-1.1]$ ,  $p=0.022$ ), but was attenuated to non-significance with inflammatory factors ( $p=0.34$ ). In a logistic regression analysis, tPVAT was associated with AC ( $1.073[1.043- 1.103]$ ,  $p<0.0001$ ), which remained significant after adjustment for CVD and inflammatory factors including BMI ( $p<0.0007$ ) and MS ( $p<0.0004$ ). The tPVAT association with CAC ( $1.051[1.03-1.07]$ ,  $p<0.0001$ ) was non-significant with BMI ( $p>0.075$ ) and attenuated with MS ( $p<0.047$ ) when adjusted for CVD or inflammatory factors.

**Conclusions:** Total PVAT is greater in clinically CVD-free SLE versus control participants and is associated with AC independent of overall adiposity. This work contributes to the field of public health elucidating the mechanisms of CVD progression through PVAT in SLE patients and ultimately in the general population.

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## PREFACE

The pursuit of this degree was an unexpected opportunity in my academic career. In following this path, the countless blessings far outweighed the required additional effort. ‘So do not throw away your confidence; it will be richly rewarded. You need to persevere so that when you have done the will of God, you will receive what He has promised,’ Hebrews 10:35-36.

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I have been so very blessed to have all of you in my life. I covet your prayers, your love, and your guidance. I have not stopped giving thanks for you, remembering you in my prayers. Ephesians 1:16

## **1.0 INTRODUCTION**

### **1.1 CARDIOVASCULAR DISEASE AND WOMEN'S HEALTH**

Cardiovascular disease (CVD) and in particular coronary heart disease (CHD) and stroke are the leading causes of death of women in America and of all major developed countries including emerging economies. Each year, more women than men have strokes and the presence of hypertension in women is also higher than in men after 65 years of age.[1] Updated guidelines from the American Heart Association on women's health report that despite the increased awareness of CVD in women, reducing major risk factors, and improved treatment options, more deaths occur due to CVD than cancer, chronic lower respiratory disease, Alzheimer disease, and accidents combined.[1]

Along with unique risk factors associated with age and gender, there may also be differences in effective treatment and prevention between the genders as well. Lifestyle approaches such as weight loss, exercise and a healthy diet are perhaps the most cost effective measure to controlling adverse CVD outcomes. The presence of comorbidities and lack of consistent lifestyle modification can make lowering the risk of developing CVD or future CV events more difficult.[1] Other options for controlling the progression of CVD include preventive care measures through medication to manage hypertension and blood pressure. Current estimates from the World Health Organization suggest that if the currently available

guidelines were properly adhered to then adverse CVD outcomes could be reduced by nearly 80%.[2]

## 1.2 ATHEROSCLEROSIS

Lipid accumulation is a well established mechanism in the development of atheromas within the vascular wall, which burdens the vasculature resulting in CVD. However, studies have shown that atherosclerosis is not merely an imbalance of circulating and accumulating lipids within the vascular wall, which create cores susceptible to rupture and thrombosis. The pathophysiology of atherosclerotic lesion formation is intricate and is now regarded as an inflammatory disease.[3] With this new classification, the role of inflammation and immune response become important constituents in the initiation and progression of CVD. Initially the inflammation was thought to be more local within the vicinity of the athero-lesion and the idea of systemic inflammation was not considered.[4] In addition to the myriad of cellular components and the circulating lipid, a cascade of events and pro-atherogenic response of inflammatory factors including vascular cell adhesion molecule -1 (VCAM-1), monocyte chemoattractant protein (MCP-1), interferon- $\gamma$  (IFN-  $\gamma$ ), oxidized low density lipoprotein (ox-LDL), heat shock proteins ( HSPs), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and other soluble factors such as homocysteine, complement, c-reactive protein (CRP), CD40 and CD40-ligand (CD40L) also contribute to the progression and inflammatory cycle of atherosclerosis.

### 1.2.1 Athero-lesion Development and Immunity

Clinical manifestations of atherosclerosis do not occur until the atheroma becomes complex. An overabundance of smooth muscle cells (SMCs) contribute to extracellular matrix (ECM) thickening of the vascular wall creating a stenosis within the lumen, which either impedes blood flow or creates unstable plaque prone to rupture. In pro-atherogenic conditions, such as hypertension, hyperglycemia, hypercholesterolemia, and smoking, endothelial cells (ECs) express molecular adhesion molecules such as VCAM-1 and Pselectin attracting monocytes and other leukocytes. [5, 6]

Fatty streaks are precursors to plaques filled with lipid-laden macrophage foam-cells. The monocyte and macrophage are inflammatory cells of the innate immune system with a significant role and presence throughout the atheroma development and plaque formation. Chemoattractant factors such as MCP-1 are upregulated on endothelial cells (ECs) due to low shear stress, injury, or dysfunction and direct progression of leukocytes into the fatty streak. The monocytes release matrix metalloproteinase-9 (MMP-9) degrading the collagen type IV constituent of the ECM to enter the intimal layer. Once in the intimal layer, the monocytes mature into macrophages through upregulation of monocyte colony stimulating factor (M-CSF). [7] The pattern-recognition scavenger receptors on macrophages involved in innate immunity are also upregulated by M-CSF. Monocytes also express toll like receptors (TLR1, -2, and -4) which are also pattern recognition receptors and are upregulated during inflammation. [8]

The macrophage engulfs lipoproteins and other foreign factors within the developing lesion becoming a foam cell. Macrophages secrete many inflammatory propagating factors including TNF- $\alpha$  and interleukin-1 $\beta$  (IL-1  $\beta$ ) which upregulate adhesion molecules (e.g. VCAM-1), chemokines (e.g. MCP-1), growth factors (e.g. M-CSF), and proteases (e.g. MMP9)

instigating the inflammatory cycle again, but from within the athero-lesion. Other innate immune cells such as mast cells, natural killer cells, and neutrophils are not nearly as abundant as the monocyte/macrophage in the progression of the athero-lesion. Interestingly, high circulating neutrophil counts predict MI better than total white blood cell, lymphocyte or monocyte circulating count. [9] The complement cascade is also a part of the innate immune system. Complement protein C3 plays a critical role in complement activation and is required for both the classical and alternative pathways. C3 cleavage products become ligands for leukocyte complement receptors thus targeting the foreign body for clearance.[10] Complement protein C3 has been found in atheromas and is produced by adipose tissue.

Dendritic cells (DCs) are the messengers between innate and adaptive immunity and are found in atherosclerotic plaques with increasing numbers associated with lesion complexity.[11] oxidized LDL and TNF $\alpha$  attract and adhere dendritic cells to the endothelium and initiate their migration into the intima.[12] T helper 1 cells (CD4 $^{+}$ ) are the most common T-cells present in human and experimental models of atherosclerosis and are produced when DCs and pre-T helper cells form an immunological synapse. [13] [14] These same CD4 $^{+}$  cells express CD40 ligand (CD40L) which activates inflammatory cells including ECs, VSMCs, macrophages and platelets, which all contribute to atherosclerotic lesion development. [15] [16]

Adipocytes and the cellular constituents of adipose tissue release many of the aforementioned factors involved in both innate and adaptive immunity including complement components, cytokines and chemokines including TNF $\alpha$ , IL-6 and MCP factors and other circulating inflammatory markers. [17]

### 1.3 OBESITY AND ADIPOSE

Obesity is a CVD risk factor and is linked to mechanisms involved in coronary CVD including atherosclerosis, hypercholesterolemia, hypercoagulability, platelet dysfunction, insulin resistance, type 2 diabetes and metabolic syndrome.[18] Adipose tissue was once thought to be inert and more of a structural support, but is now considered the largest endocrine organ of the body involving a chronic inflammatory process inducing both innate and adaptive immune responses.[4, 19]

Adipose tissue is located either subcutaneously or viscerally and in many different depots. Adipose tissue can also be found ectopically within organs and muscle tissue. Studies have shown that differences in adipose distribution and in particular the intra-abdominal or visceral adipose volume is associated more strongly with CVD than subcutaneous adipose tissue volume.[20, 21] Adipose tissue also has its own anti- and pro-atherogenic cytokines which can initiate many of the mechanisms linking obesity to CVD including systemic inflammation.[22]

Recently, smaller visceral adipose depots such as epicardial and pericardial have garnered increased attention due to their anatomical proximity to the myocardium and coronary arteries and their dual cardioprotective and pathological pro-inflammatory roles.[23-26] The epicardial adipose surrounding the coronary arteries could also be considered a perivascular adipose depot and this concept has initiated studies involving the perivascular adipose tissue surrounding other vasculature including the aorta.[27] The perivascular adipose tissue surrounding the descending thoracic aorta is readily discernable through CT scans.[28, 29] An association between coronary artery and aortic calcification has been confirmed within CVD-free participants of the Framingham Heart Study Offspring cohort.[29] Further quantification of the perivascular adipose volume and determining the association with CVD risks and other subclinical CVD

measures will provide valuable information into the development and role of this small visceral adipose depot in the progression of CVD.

### **1.3.1 Cellular Components of Adipose**

Adipose is composed of a heterogeneous mixture of cell types, which play a role in both adipocyte cell function and overall adipose tissue metabolism and include adipocytes, preadipocytes, fibroblasts, and leukocytes which include lymphocytes (e.g. T cells and B cells), monocytes and macrophages.[30] In both mouse and human adipose tissue, different inflammatory cell types including macrophages and T cells, have distinct subpopulations dependent on a lean or obese state. [30-37] Resident macrophages (5-10% of adipose tissue) help to maintain a healthy adipose state. However, the proliferation of hypertrophic adipocytes and adipose dysfunction is marked by an increase in macrophage infiltration (60% of adipose tissue with weight gain) contributing to and sustaining an inflammatory state.[32, 38, 39] Most of these same cell types can be found in atherosclerotic lesions in an inflammatory capacity.

The adipocyte is sensitive to infections and cytokine-mediated inflammatory signals and can activate inflammatory response and induce expression and secretion of TNF- $\alpha$ , plasminogen activator inhibitor-1 (PAI-1), IL-1, IL-6, IL-8, IL-10, and IL-15, leukemia inhibitory factor, hepatocyte growth factor, serum amyloid A-3 (SAA3), macrophage migration inhibitory factor, haptoglobin, complement factors B, D, and C3, prostaglandin E2, and potential adipokine inflammatory modulators such as leptin, adiponectin, and resistin. [40] The relation of adipose to these inflammatory biomarkers shows a link that adipose is more similar to an immune organ, e.g. the liver.[4] Adipose tissue is not the only contributor to systemic inflammation, but may



also control the upregulation of secreted inflammatory proteins through the liver and other inflammatory producing tissue. These pro-inflammatory factors are modifiable, increasing with weight gain and decreasing with weight loss.[41, 42]

#### **1.4 INFLAMMATORY FACTORS OF ATHEROSCLEROSIS AND ADIPOSE**

Inflammatory markers may be considered cardiovascular risk factors due to the number of studies linking obesity, atherosclerosis, and inflammation. One of the most studied circulating inflammatory markers is C-reactive protein (CRP), which is related to obesity and CVD risk and is associated with insulin resistance, diabetes, metabolic syndrome, hypertension, smoking, and dyslipidemia [43] This acute phase reactant is produced in the liver, released during an inflammatory state, and predicts CVD in both healthy and unhealthy populations.[44] CRP is linearly correlated with BMI and in particular adipose distribution as measured by waist to hip ratio. Numerous epidemiological studies have defined significant associations between high sensitivity CRP (hsCRP) and underlying atherosclerosis, risk of recurring CV events among those with established disease, and incidence of first CV events among those at risk for atherosclerosis.[45]

Plasminogen Activator Inhibitor-1(PAI-1) is another circulating inflammatory marker which regulates the coagulation cascade and is enhanced in inflammatory and obese states. PAI-1 is derived from platelets and endothelium, but can also be produced by adipocytes.[46, 47] Increased PAI-1 decreases the rate of fibrinolysis , which creates a hypercoagulable state when combined with increased clotting factors and platelet activation and is associated with increased CVD risk [48]

TNF  $\alpha$  is produced by macrophages in an inflammatory state including atherogenesis and is also produced by adipose tissue. [17] An increase in circulating TNF  $\alpha$  is associated with an increase in adipose mass. Interleukin-6 (IL-6) stimulates acute-phase proteins including CRP and SAA3. This action instigates the innate immune system to protect tissue and causes inflammation. [49] IL-6 is secreted by Tcells, macrophages and adipose tissue and circulating levels are higher with increased adipose tissue.

Another inflammatory marker primarily secreted from visceral adipose depots with higher levels in obese populations is angiotensinogen.[50, 51] This circulating inflammatory marker contributes to angiogenesis, hypertension, and atheromatous alterations and contributes to vascular inflammation through upregulation of EC expression of VCAM-1, intracellular adhesion molecule-1 (ICAM-1), Eselectin and MCP-1.[51, 52] VCAM-1, ICAM-1 and Eselectin recruit leukocytes to athero-lesion sites and ICAM-1 and Eselectin are markers of subclinical atherosclerosis.[53] In addition, EC expression of ICAM and VCAM are increased with increased fat mass.[17] Vascular endothelial growth factor (VEGF) functions in the development of hypertension and angiogenesis and is particularly elevated in visceral adipose tissue. The volume of adipose tissue is able to expand through vascularization of the adipose depot through expression of VEGF, with increased obesity resulting in increased adipose expression of VEGF.[54]

Along with CRP, fibrinogen and SAA3 are also acute phase reactants that are increased in inflammatory states. Elevated levels of fibrinogen are associated with traditional CVD risk factors and are independent risk factors for CVD.[55] Fibrinogen binds and contributes to platelet aggregation, promotes fibrin formation, and increases plasma viscosity.[55] In addition, fibrinogen levels increase with increased fat mass.[17] Serum amyloid A3 is produced by many

tissues including adipocytes. Circulating levels are elevated in obese and diabetic patients and SAA3 also acts as a chemoattractant inducing remodeling through matrix metalloproteinases, stimulating Tcells, and binding HDL.[56] An additional circulating risk factor associated with increased risk of coronary events and strokes is the amino acid, homocysteine, which is a byproduct of meat consumption and high circulating levels are also associated with low levels of vitamins B6, -12, folate, and renal disease.[57, 58] Studies in the general population have determined homocysteine a risk factor of atherogenesis independent of other coronary risk factors, but may not be a cause of CVD.[58, 59]

#### **1.4.1 Adipokines**

In addition, adipocytes produce cytokines termed adipokines, two of which include leptin and adiponectin. These two adipokines in particular may also participate or lend to development of CVD. Adiponectin is anti-atherogenic, anti-inflammatory, and anti-diabetic adipokine, which inversely correlates with CVD and obesity and may have a protective role against cardiac hypertrophy, hypertension, hypertrophic cardiomyopathy, and ischemic heart disease.[17] Adipocyte secretion of adiponectin is suppressed in circulation of obese patients and in diabetic states affecting the liver, insulin sensitivity, and whole body metabolism. [60] *In vivo* murine models have shown inhibition of adiponectin during systemic inflammation.[61] Adiponectin levels are sensitive serum marker for future CV events even after controlling for traditional CV risk factors.[62].

Leptin is responsible for controlling energy expenditure and increases sympathetic activity to CV-relevant organs including the kidneys and adrenal gland potentially predisposing an individual to obesity related hypertension.[17] Circulating leptin levels correspond to obesity

and high levels of circulating CRP.[63] Leptin affects the Th1 immune response[64] and is associated with the development of atherosclerosis independent of insulin.[65]

In general, circulating pro-inflammatory markers increase and anti-inflammatory markers decrease with an increase in fat mass and in particular the visceral adipose depot

## **1.5 SYSTEMIC LUPUS ERYTHEMATOSUS AND CARDIOVASCULAR DISEASE**

Systemic Lupus Erythematosus (SLE or lupus) is an autoimmune disease associated with chronic systemic inflammation. SLE is more common in women than in men usually exacerbated during the early child-bearing years. All tissue types are susceptible to an autoimmune complication and symptoms of SLE vary from person to person. The disease is controlled through a myriad of medications including corticosteroids, immune suppressants, anti-malarials, and non-steroidal anti-inflammatory drugs (NSAIDs). Currently, there is no cure for SLE. There exists a bimodal mortality pattern in the SLE population with an early peak due to active disease or SLE-complications and a later peak attributed to atherosclerosis and CVD.[66] New treatment methods and earlier diagnoses has significantly improved survival rates within the first 5 years of diagnosis to over 90% in the 1990s[67]; however, the later peak due to exacerbated and premature CVD is proving to be difficult for the SLE population. In particular, young women with SLE aged 35-44 are over 50 times more likely to experience an MI when compared to women without SLE from the Framingham Offspring Study.[68, 69] Women with SLE may also have more vulnerable plaques. When compared to those without SLE, there is no difference in rates of coronary stenosis or total occlusion for those undergoing coronary revascularization. Conversely, cardiovascular outcomes at one year following the coronary

revascularization procedure, SLE women have a higher risk of MI and need of a repeat percutaneous coronary intervention even after adjusting for CV covariates. [70, 71]

The risk of hospitalization due to congestive heart failure (CHF) is also increased in SLE women aged 18-44years (>2.5 times) along with a 3.5 fold increased risk of mortality than the general population with CHF.[71, 72] Hypertension is also more common in women with SLE than the general population and is a univariate predictor of mortality and vascular events in SLE.[73-75] Traditional CVD risk factors within the general population include age, gender, smoking, HTN, hypercholesterolemia, diabetes, body composition in particular visceral adipose, insulin resistance, circulating hsCRP, and physical inactivity. For SLE, traditional risk factors do not account for the substantial increase in risk associated with MI and stroke at such an early age. Many of the traditional risk factors may be present, but SLE patients are most likely at increased risk due to some SLE-specific manifestation. The most obvious additional risk factor is the total inflammatory burden due to the chronic inflammation associated with SLE and also with atherosclerosis.[73-75]

Many circulating inflammatory markers associated with excess adipose tissue and atherosclerosis are also elevated in SLE patients when compared to the general population. CRP has been an associated risk factor for increased subclinical carotid plaque and IMT [76] along with increased coronary artery calcification (CAC) in SLE patients.[77-79] Soluble ICAM-1 has also been associated with an increase in CAC in sle patients. [77] Complement activation by immune complexes containing autoantibodies increases tissue inflammation/damage in SLE.[80] High levels of circulating complement protein C3 have been associated with greater CVD risk in both the general and SLE populations.[81] High C3 levels are also predictive of CAC [82] and

associated with plaque progression and aortic stiffness [79, 81] along with an increased waist circumference, postprandial lipemia, metabolic syndrome [83]

In addition to these elevated inflammatory components, SLE patients may be more likely to have dyslipidemia, [78, 84] and those with dyslipidemia have increased risk of vascular events.[68, 73] Other pro-CVD conditions within the SLE population include a pro-inflammatory form of HDL (iHDL) losing the benefit of having a higher HDL level [80], premature menopause thus losing the cardioprotective benefits [85], having hyperhomocysteinemia, which is associated with thrombosis, stroke, and increased CAC in SLE patients [86, 87], having hypertension and suffering from high BP (>130mmHg) [78], and an increased risk of developing diabetes and insulin resistance.[88, 89] SLE patients are also more likely to have metabolic syndrome than healthy counterparts.[90] Metabolic syndrome as defined by the American Heart Association includes a combination of factors including abdominal obesity, dyslipidemia, hypertension, insulin resistance, and when taken together has a much greater risk of CVD than individually.[4] The metabolically active visceral adipose of abdominal obesity and its inflammatory secretions including CRP, IL-6, TNF $\alpha$ , VEGF, PAI-1, angiotensinogen is the defining characteristic of metabolic syndrome.[40, 91]

Not all adipose depots confer the same CVD risk as visceral adipose. Evaluation of body fat distribution and not necessarily overall adiposity is essential when evaluating a CVD risk panel. Excess fat within the abdominal region is associated with pro-inflammatory and prothrombotic states along with metabolic syndrome thus increasing the risk of CVD.[92-94]

Abdominal fat is a surrogate measure of visceral adipose [95] which drains directly into the portal vein to be received by the liver thus increasing the upregulation of inflammatory cytokines produced by the liver. Simple weight measures and BMI calculations do not capture

adipose distribution or distinguish between fat and fat-free mass. Body composition is a univariate risk factor for atherosclerotic events and an SLE-forced sedentary lifestyle may play a role in excess adipose in the SLE population.[75] SLE patients develop sarcopenic obesity with a reduction in muscle mass and over fat more frequently than rheumatoid arthritis patients and the general population.[96] This loss of muscle mass is likely due to physical inactivity and may be associated with high dose steroid use. Steroids inhibit protein synthesis and stimulate protein degradation in skeletal muscle. Body composition changes including a decline in fat free mass and bone mineral density and concomitant increase in fat mass occur occurs with age and menopause, which also occurs prematurely in the SLE population. In addition, the systemic inflammatory nature of lupus may prove to be an additional risk factor for altered body composition.[97] Furthermore, high levels of circulating inflammatory markers such as TNF  $\alpha$  have been associated with loss of lean mass in RA patients, which may prove similar in SLE patients. [98]

Some additional CVD risk factors specific to SLE patients include the development of renal disease and lupus nephritis. Serum creatinine levels are independent predictors of IMT progression.[81, 87] Other issues with SLE patients that may complicate the CVD burden and inflammatory processes include complement deficiency, antiphospholipid antibodies, mannose binding protein gene complications, microalbuminuria, neuropsychiatric disease, and vasculitis.[99] Generally, SLE patients have a greater number of traditional CVD risk factors, but the same Framingham risk scores suggesting that the traditional risk factors have more influence within the SLE population and there may also be differences among SLE patients.[99] As mentioned, the traditional risk factors cannot account for the exacerbated CV risk in SLE population. There is still almost an 8 fold increase in occurrence of MI or stroke in SLE after

controlling for all traditional risk factors [72] indicating the influence of some SLE-related risk remaining. With this in mind, it may be just as important to try and treat the known traditional risk factors as well through lifestyle changes or medication.[80] Evaluation of subclinical CVD is an important method of determining risk and establish a baseline for CVD intervention protocols.

## **1.6 SUBCLINICAL CARDIOVASCULAR DISEASE MEASURES**

Subclinical CVD measures are non-invasive risk assessments providing a baseline from which to provide the best possible health intervention for reducing CVD risk. Atherosclerosis is a systemic disease with a strong correlation between atheroma found in the carotid and the presence of atheroma in the coronary. Numerous subclinical disease measures exist and include markers of arterial structure (IMT, plaque presence, calcification) and markers of arterial function (EC dysfunction, pulse wave velocity). Carotid ultrasounds can provide intima media thickness, adventitial diameter, and lumen diameter measures along with assessing plaque presence. Greater IMT thickness in the general population is associated with increased risk of stroke and MI in both men and women without a history of CVD.[100, 101] SLE patients have a significantly higher prevalence of carotid plaque when compared to controls. Older age at diagnosis, longer duration of SLE, and higher homocysteine concentration are independently related to atherosclerosis progression within the SLE population.[102]

Electron beam computed tomography (EBCT) is used to measure coronary calcification and aortic calcification. EBCT is a sensitive and specific marker in the general population and produces rapid images without the use of contrast media giving an accurate visualization of the



vasculature. Vascular calcification involves biomineralization through bone metabolism and occurs in the intima and media.[103] Intimal calcification is due to atherosclerosis where medial calcification is independent of atherosclerosis and called Monckeberg sclerosis. Atherosclerosis and calcification are more common at bifurcations and those with diabetes mellitus have both intimal and medial calcification. Hypertension is a known risk factor for atherosclerosis and distal aortic calcification, which increases vascular rigidity and the hemodynamics of blood flow.[103]

SLE patients have diffuse calcification over several vascular beds when compared to age and gender matched controls and the extent of calcification is also greater in SLE vs control.[104] CAC has been a better predictor of future CV events than thoracic aorta calcification. CAC is a marker of subclinical atherosclerosis strongly associated with CHD and mortality [105] CAC is more frequent in SLE than controls and the mean artery calcification score is significantly higher in patients when compared to healthy controls independent of traditional risk factors.[77]

Use of subclinical CVD measures and lupus – specific disease control methods through lifestyle changes and medications will relieve the overall cumulative burden of inflammation. The SLE-specific CVD risk factors still remain to be determined in order to develop improved treatment options. Smaller visceral adipose depots are receiving increased attention for their localized inflammatory role in cardiovascular disease. Mouse models have shown extensive complement protein deposition throughout the perivascular adipose tissue (PVAT) surrounding the descending thoracic aorta and bound to the ECM constituents of the vascular wall including collagen and elastin. We hypothesized that the PVAT surrounding the descending thoracic aorta

of lupus patients would be greater when compared to healthy controls and this difference would be associated with the increased coronary and aortic calcification of the SLE population.

## **2.0 METHODS**

### **2.1 INCLUSION / EXCLUSION CRITERIA**

A retrospective case-control study was performed on SLE participants and their respective 1:1 matched age and race controls. Women who fulfilled the 1987 revised American College of Rheumatology criteria of SLE[73] but with no prior history of cardiovascular event were non-selectively recruited from the Pittsburgh Lupus Registry to participate in the SLE study, which was designed to compare the prevalence and risk factors of coronary artery and aortic calcification in SLE women and healthy controls. These women were participating in the “Heart Effects on Atherosclerosis and Risk of Thrombosis in SLE” (HEARTS) study.

SLE women had at least 2 years of disease duration. This lupus registry includes women diagnosed with SLE who have been seen either at the UPMC inpatient and outpatient facilities or by practicing rheumatologists in the Pittsburgh metropolitan area. In this study, the healthy women (controls) were matched to the SLE women by age (+/- 5 years) and race. The recruitment methods for healthy controls were based on the following: 1) Voters registration list or Motor Vehicle License list depending on where the case (SLE woman) was found in these lists; 2) direct sample neighborhood control if the case was not in the previous lists. (SLE, n=161; Control, n=161)

Subjects with diabetes mellitus, as defined by history of diabetes or fasting glucose  $\geq 7$  mmol/L ( $\geq 126$  mg/dL) or hypoglycemic therapy, (SLE, n=8; Control, n=3) were excluded.

## 2.2 DATA COLLECTION/MEASURES

Information on patient demographics and potential risk factors was collected at the time of Electron Beam Computed Tomography (EBT) scan. The University of Pittsburgh Institutional Review Board approved these studies; all patients gave written informed consent. The study visit included anthropomorphic measurements (height, weight, and waist and hip circumferences), two consecutive blood pressure readings (with patients seated), and a blood draw after a required fast, which was uniform between groups. Blood samples were used to measure total cholesterol, triglycerides, and high-density lipoprotein (HDL) cholesterol at the Lipid Laboratory in the University of Pittsburgh, Graduate School of Public Health, which has been certified by the Centers for Disease Control and Prevention. Low-density lipoprotein (LDL) cholesterol was calculated from measured total cholesterol, HDL and triglycerides (Friedewald equation).[106] Metabolic syndrome was defined by the National Cholesterol Education Program Adults Treatment Panel III guidelines.[107] The homeostatic model assessment of insulin resistance (HOMA-IR) was calculated by  $[\text{insulin (mU/liter)} \times \text{glucose (mmoles/Liter)}] \div 22.5$ .[108, 109] Hypertension was defined by physician diagnosis, measured mean blood pressure  $\geq 140/90$  mm Hg or antihypertensive medication use. Other information was collected on family history of ASCVD (first-degree relative having myocardial infarction or stroke before age 60), cigarette smoking (current, past, or never), and menopausal status. Information was recorded on corticosteroid treatment (current use, ever used, and daily dosage) and current use of

hydroxychloroquine, immunosuppressants for SLE or other disease modifying anti-rheumatic drugs, non-steroidal anti-inflammatory drugs, antihypertensives, hormonal therapy, and lipid-lowering medications.

Fibrinogen was measured using a modified clot-rate assay. An enzyme-linked immunosorbent assay was used for determination of high sensitivity CRP (hsCRP). sICAM-1 was measured using commercial assays (Parameter Human sICAM-1 Immunoassay; R&D Systems, Minneapolis, MN) at the Laboratory for Clinical Biochemistry Research (University of Vermont, Burlington, VT).

### **2.3 EBT SCANS**

The EBT scans were performed using an Imatron C-150 scanner (Imatron, San Francisco, CA) using standard imaging procedures in all participants. The same scanner and methodology was used in the SLE and healthy controls. For CAC, 3-mm slices were scanned at the same point in diastole (at 80% of the patient's RR interval in electrocardiogram) during a single breath hold, starting at the aortic root to the apex of the heart. Following completion of the coronary scan, aortic scans (6mm transaxial images, 300 ms exposure time) were obtained from the aortic arch to the iliac bifurcation. All scan data was saved to optical disc and scoring of calcification (Agatston and volumetric) was performed using a DICOM work station and software by AcuImage. The individual region of interest scores were then summed for a total Agatston calcification score.[110] The analysis was based on the total Agatston calcification scores as previously described.[111] The participants' EBT scans for PVAT quantification that were not

included were due to missing files on the optical disk, bad optical disks, and perhaps being miscatalogued (n=24: n<sub>SLE</sub>=18, n<sub>Control</sub>=6).

## 2.4 PERIVASCULAR ADIPOSE TISSUE VOLUME

The 6mm transaxial EBCT images were used to quantify the PVAT surrounding the descending thoracic aorta using the commercially available software (Slice-O-Matic, Tomovision, Montreal, Canada). The aortic PVAT was distinguished from the remaining tissue using physiological landmarks. The posterior border is the anterior portion of the vertebral foramen. The anterior border and left/right lateral borders change as the images progress distally from the carina and includes the left bronchus, esophagus, and crus of diaphragm. The first proximal image analyzed includes the carina also known as the pulmonary bifurcation and each 6mm image is quantified through vertebral body, T12. Analysis stops at the pedicles of L1.(Figure 1) Adipose tissue areas were calculated by the range of attenuation values for



**Figure 1: Sample EBCT scan with yellow boundary and red adipose.**

adipose (−190 to −30 HU) tissue. A volume was calculated by summing the areas and multiplying by the length of the participant’s aorta. A reproducibility analysis was conducted for the quantification of PVAT volume and excellent intra- and inter-reader reproducibility for the measurement of thoracic PVAT (intra-class correlation coefficient 0.999 and 0.998, respectively) was demonstrated in a random subset of participants.

## **2.5 OUTCOMES**

Total aortic PVAT (tPVAT) volume along the length of the thoracic aorta was evaluated with traditional CVD risk factors and inflammatory factors associated with SLE. The PVAT volume was also evaluated against valid subclinical CVD measures including aortic calcification (AC) and coronary artery calcification (CAC). The contribution of tPVAT to vascular calcification including AC and CAC along with adjustments for CVD risk factors and circulating inflammatory markers was also evaluated.

Predictor variables included traditional CVD risk factors: smoking history, average systolic blood pressure (SBP), postmenopausal status, hypertension, log transformed HOMA-IR, cholesterol ratio (total cholesterol/high density lipoprotein cholesterol), and homocysteine serum levels. Inflammatory risk factors included log transformed C-reactive protein (logCRP) and log transformed plasminogen activating inhibitor (logPAI) along with serum levels of fibrinogen, intercellular adhesion molecule (sICAM) and eSelectin. Additional adjustments were made for BMI and metabolic syndrome.

## 2.6 STATISTICAL ANALYSIS

Descriptive statistics were calculated for demographic variables, cardio-metabolic factors, subclinical CVD measures including CAC and AC, and PVAT volume total. Differences between SLE and control outcomes were assessed with either t-tests or Mann-Whitney U tests for continuous covariates and chi square or Fisher's exact test for categorical variables. Spearman correlations were used to assess associations between cardio-metabolic factors, outcome (SLE vs control), tPVAT volume, and log transformed CAC and AC.

A multivariate linear regression was used to determine the CVD risk factors and circulating inflammatory risk factors that influenced tPVAT volume and this analysis was stratified by disease status. The models were developed with adjustment for predetermined CVD risk factors (history of smoking, postmenopausal status, hypertension, log HOMAIR, cholesterol ratio (total cholesterol/hdl) homocysteine and average systolic blood pressure) and inflammatory factors (log PAI, log CRP, fibrinogen, sICAM, and eselectin).

Logistic regression was used to determine the association between tPVAT, CAC, and AC with SLE. The association of tPVAT with SLE was evaluated with the control group being referent. The models were adjusted for predetermined CVD risk factors and inflammatory factors with the control group being referent. In a separate set of models, logistic regression was used to determine the contribution of tPVAT along with CVD risk factors and circulating inflammatory markers to any vascular calcification, AC or CAC separately. All analyses were performed using SAS (version 9.2, SAS Institute, Cary, North Carolina). The level of statistical significance was set at a 2-sided p-value of 0.05.



### **2.6.1 Reproducibility Analysis**

Eleven (n=11) HEARTS participants were chosen at random to determine the reproducibility of the EBCT scan reading protocol quantifying the PVAT of the descending thoracic aorta. Two readers (MG and KJS) were given the protocol and the EBCT scans of the 11 participants. Two separate readings were completed approximately 3 to 4 weeks apart to determine intra- and inter-reader variability. Spearman correlations, linear regression, descriptive difference statistics, intra-class correlations, and Bland-Altman plots were used to determine consistency, variability, and systematic bias.

### **3.0 RESULTS**

#### **3.1 DEMOGRAPHICS**

The characteristics of the study groups are summarized in Table 1. The SLE and control groups were comparable among many covariates including age, BMI, ethnicity, smoking status, metabolic syndrome prevalence, systolic and diastolic blood pressures, insulin and HOMA-IR.

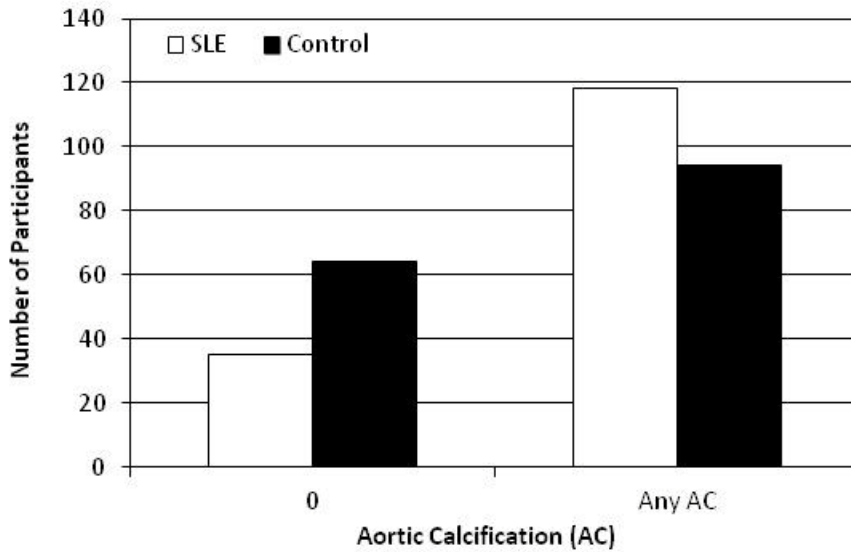
Women with SLE were slightly more likely to be postmenopausal than healthy controls ( $p=0.058$ ) and more likely to be hypertensive ( $p<0.0001$ ). The SLE group had lower pulse pressures ( $p=0.0049$ ) partially explained due to blood pressure (BP) lowering medications to control hypertension. In addition, the SLE group had lower serum glucose levels ( $p<0.0001$ ), total cholesterol ( $p=0.0027$ ) and LDLc ( $p=0.0009$ ) when compared to their healthy controls. Lower glucose levels for SLE women versus controls persisted when subjects with diabetes were excluded from the analysis. Likewise, significant group differences in lipid profiles persisted after controlling for lipid lowering medications. Despite the exclusion of diabetics from the analysis the differences in glucose could not be explained. More of the SLE group was on lipid lowering medications ( $p=0.044$ ), but that did not fully explain the differences in lipid panel. As expected, several inflammatory factors including CRP, PAI, sICAM, cd40, eSelectin, albumin, and homocysteine were significantly greater in the SLE study group. There were no control group measurements for complement C3 and C4 serum levels for group comparisons.

**Table 1: Demographic, clinical, and laboratory characteristics of SLE participants and controls.**

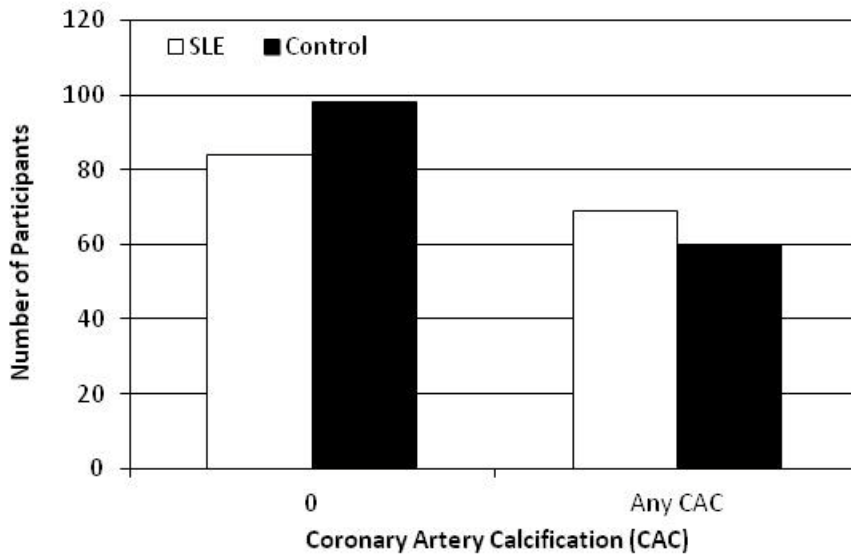
<i>Covariates</i>	<b>SLE (n=153)</b>	<b>Control (n=158)</b>	<b>p-value</b>
Age (yr)	49.5 (9.7)	51.1 (9.7)	0.14
Caucasian, n (%)	134 (87.6%)	143 (90.5%)	0.41
Current Smoker, n (%)	16 (10.5%)	20 (12.7%)	0.54
Post menopausal Status, n(%)	90 (58.5%)	76 (48.1%)	0.058
BMI (kg/m <sup>2</sup> )	27.0(22.6 - 31.9)	26.9 (23.8 - 32.1)	0.26
Metabolic Syndrome	39(25%)	29(31%)	0.17
Waist Circumference (cm)	83(75-94.5)	81.5 (73 -93)	0.46
Waist to Hip Ratio	0.833(0.79-0.88)	0.797(0.77-0.84)	0.001
SBP (mm Hg)	119(106-132)	120 (110-135)	0.087
DBP (mm Hg)	76.6(10.3)	76.2 (9.84)	0.71
pulse pressure	41(35-50)	45.8(37.5-59)	0.0049
Hypertensive	82(54%)	48(31%)	<0.0001
Glucose (mmol/L)	89 (82-94)	93 (88-101)	<0.0001
Insulin (pmol/L)	12.1 (9.1-17.6)	11.4 (9.2-14.8)	0.29
HOMA-IR (mmol/L x $\mu$ U/mL)#	7.01(4.9-10)	6.66(5.4-9.4)	0.88
<b><i>LIPID PANEL</i></b>			
Triglycerides (mmol/L)	111 (76-153)	103 (74-140)	0.14
Total Cholesterol (mmol/L)	185 (162-215)	203 (175-222)	0.0027
HDLc (mmol/L)	54.2 (43.1-62)	56 (46.4-63.8)	0.12
LDLc (mmol/L)	107 (87-107)	121 (101-141)	0.0009
Cholesterol Ratio (tot cho/hdl)	1.23 (1.1-1.4)	1.27 (1.1-1.5)	0.85
<b><i>INFLAMMATORY FACTORS</i></b>			
CRP (mg/L)	2.34 (0.93-5.5)	1.61 (0.61-3.6)	0.032
Fibrinogen (mg/dL)	337.4 (86.1)	344.7 (63.0)	0.4
PAI (ng/ml)	15.7 (9.6-28)	11.8 (6.0-25.4)	0.016
sICAM (ng/ml)	265 (233-319)	245 (212-276)	<0.0001
cd40 ligand	5543 (3759-7822)	6205 (4804-8136)	0.012
eSelectin (ng/ml)	47.1 (31.2-62.8)	36.6 (26.4-53.2)	0.0009
Albumin (g/dL)	4.6 (4.2-4.9)	4.3 (4-4.6)	<0.0001
Homocysteine ( $\mu$ mol/L)	9.7 (8-11.8)	8.9 (7.6-10.3)	0.0088
C3	96 (82-118)	N/A	
C4	19 (15-25)	N/A	
<b><i>MEDICATIONS</i></b>			
NSAID	59 (38.6%)	21 (13.3%)	<0.0001
Aspirin	20 (13.1%)	13 (8.23%)	0.17
Hormone Replacement Therapy	20 (13.1%)	19 (12.0%)	0.78
Lipid Lowering Medication,n (%)	13 (8.5%)	5 (3.2%)	0.044
Corticosteroids	63 (41.2%)	0	<0.0001
years on prednisone (n=59)	12(7-18)	N/A	
Immunosuppressant	27 (17.7%)	0	<0.0001
Plaquenil	71 (46.4%)	0	<0.0001
Family History MI <60yo	62(41%)	48 (31%)	0.071
Family History Stroke <60yo	35 (23%)	18 (12.0%)	0.0074

There was a significant difference between the SLE (median (25%-75%): 3.293 (1.39-5.46)) and control (1.946(0.00-4.28)) group in mean log aorta calcification score ( $p=0.0013$ ) and a mild association with mean log coronary calcification score (SLE: (0.000(0.00-2.74)) and

A.



B.



**Figure 2: Presence of vascular calcification between SLE and Control groups, (chi square p-value). A) Aortic Calcification, Chi Square, ( $p=0.0008$ ), B) Coronary Artery Calcification, ( $p=0.20$ ).**

control: (0.000(0.00-1.45)),  $p=0.086$ ) exists. When evaluating the presence of calcification using the chi square test, there is a preference for the SLE group to have AC ( $p=0.0008$ ), but there is not a strong preference with CAC ( $p=0.20$ ). (Figure 2) The SLE group was more likely to have a family history of stroke prior to 60 years old ( $p=0.0074$ ) and slightly more likely to have a family history of MI prior to 60 years old ( $p=0.071$ ).

### 3.2 TOTAL PERIVASCULAR ADIPOSE VOLUME

The SLE (median (25-75%): 32.2 (25-42)  $\text{cm}^3$ ) group had greater total PVAT (tPVAT,  $p=0.0071$ ) than controls (28.6 (22-37)  $\text{cm}^3$ ) with more SLE participants having higher tPVAT volumes. (Figure 3) There were no differences between the SLE group and controls in the participants' overall height or the length of the aorta. (not shown) Overall tPVAT was significantly correlated with all selected CVD risk factors and inflammatory factors regardless of SLE status. (Table 2) This was true within SLE and control groups except for homocysteine, sICAM, and eSelectin in the SLE group. (Table 2)

#### *Logistic Regression*

In a univariate logistic regression model, tPVAT alone was associated with SLE (1.019 [1.00-1.034],  $p=0.020$ ). The tPVAT odds ratio (OR) associated with SLE remained significant when adjusting for CVD risk factors (1.022[1.0-1.04],  $p=0.035$ ) and became non-significant when adjusting for inflammatory factors ( $p=0.34$ ).

The model was expanded to include obesity-related factors BMI, metabolic syndrome (MS) or metabolic syndrome components (MSc: triglyceride, HDL, and glucose levels, waist to

hip ratio, and average systolic blood pressure) along with appropriate CVD risk factors (hypertension, homocysteine serum levels, average systolic blood pressure, history of smoking, postmenopausal status, log HOMA-IR, and the cholesterol ratio) and inflammatory factors

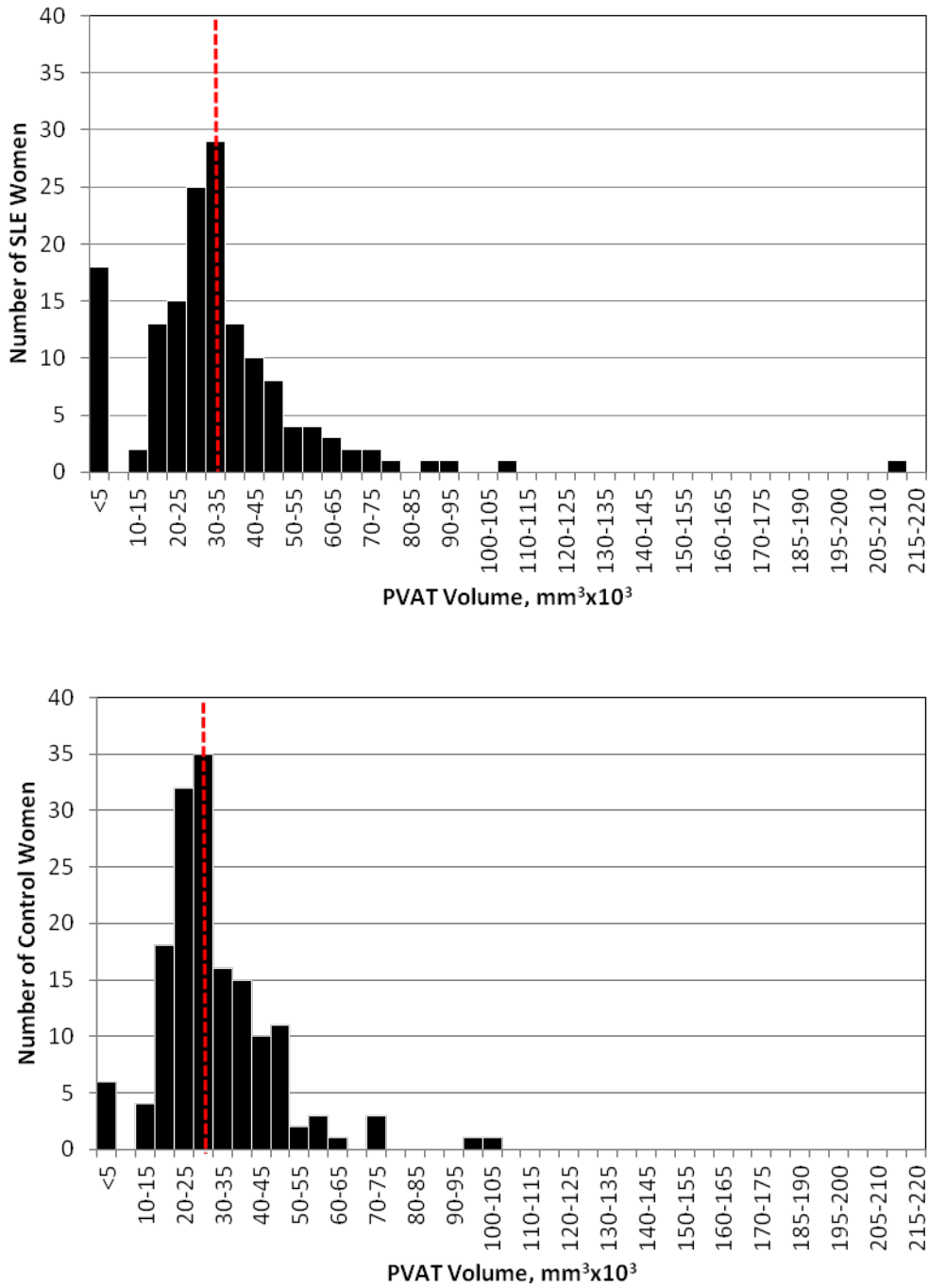


Figure 3: Total PVAT distribution for A) SLE; B) controls. (p=0.0071). Dash indicates median values.

(fibrinogen, sICAM, log CRP, log PAI and eSelectin) dependent on the obesity-related factor used thus ensuring not to have adjustment overlap. When evaluating BMI the CVD risk factors

Since the MS diagnosis included: history of smoking, postmenopausal status, hypertension, homocysteine, HOMAIR (log), cholesterol ratio, average SBP, and waist to hip ratio. includes some of the CVD risk factors, when evaluating the MS influence the CVD risk factors included: history of smoking, postmenopausal status, hypertension, and homocysteine. Lastly, when evaluating the components of an MS (MSc) diagnosis, the CVD risk factors included: history of smoking, postmenopausal status, hypertension, homocysteine, and log HOMAIR.

**Table 2: Spearman correlations between Total PVAT with clinical and laboratory outcomes, rho(p-value).**

<b>Covariate</b>	<b>SLE tPVAT</b>	<b>Control tPVAT</b>	<b>Overall tPVAT</b>
<b>HOMA-IR</b>	0.384(<0.0001)	0.422(<0.0001)	0.401(<0.0001)
<b>SBP</b>	0.300(0.0004)	0.427(<0.0001)	0.342(<0.0001)
<b>cholesterol ratio</b>	0.178(0.039)	0.216(0.0075)	0.191(0.0012)
<b>Homocysteine</b>	0.164(0.057)	0.170(0.036)	0.192(<0.009)
<b>PAI</b>	0.285(0.0008)	0.360(<0.0001)	0.348(<0.0001)
<b>Fibrinogen</b>	0.191(0.026)	0.221(0.0072)	0.197(<0.0009)
<b>sICAM</b>	0.130(0.13)	0.354(<0.0001)	0.269(<0.0001)
<b>eSelectin</b>	0.154(0.076)	0.276(0.0006)	0.230(<0.0001)
<b>C3</b>	0.215(0.013)	N/A	0.215(0.013)
<b>CRP</b>	0.382(<0.0001)	0.373(<0.0001)	0.404(<0.0001)
<b>BMI</b>	0.518(<0.0001)	0.572(<0.0001)	0.531(<0.0001)
<b>Metabolic Syndrome Score</b>	0.425(<0.0001)	0.547(<0.0001)	0.507(<0.0001)
<b>waist to hip ratio</b>	0.327(0.0001)	0.228(0.0048)	0.303(<0.0001)

The tPVAT remained significantly associated with SLE outcome when evaluated with BMI alone (tPVAT, p=0.0038) and MS components alone (tPVAT, p=0.0079). Both the BMI (0.993[0.91-0.995],p=0.030) and the glucose (0.933[0.91-0.96],p<0.0001) of the MSc were protective against SLE. The tPVAT association with SLE evaluating MS alone was attenuated

to non-significance ( $p=0.059$ ) while MS was also not a significant obesity-related factor ( $p=0.59$ ). Measures of adiposity such as BMI do not describe adipose distribution accurately. The waist to hip ratio is another anthropomorphic measure, which takes into consideration the distribution of adipose. When tPVAT was evaluated with the waist to hip ratio alone, the tPVAT was slightly attenuated but still significant ( $1.016[1.0-1.03], p=0.044$ ); however, the waist to hip ratio itself was not significantly associated with SLE ( $p=0.18$ ).

The tPVAT ( $p=0.022$ ) remained significantly associated with SLE when evaluated with the BMI ( $p=0.31$ ) and CVD risk factors; however, tPVAT ( $p=0.13$ ) did not remain significant when evaluated with BMI ( $p=0.0022$ ) and inflammatory risk factors. The tPVAT ( $p=0.17$ ) did not remain significantly associated with SLE when evaluated with MS ( $p=0.50$ ) and CVD risk factors or with inflammatory factors (tPVAT ( $p=0.33$ ) and MS ( $p=0.28$ )). The tPVAT ( $p=0.065$ ) was slightly attenuated to non-significance with MS components and both the CVD and inflammatory risk factors. (Table 3) The tPVAT ( $p=0.16$ ) loses the significant association with SLE when evaluated with waist to hip ratio ( $p=0.24$ ) and the CVD risk factors. This loss of significance trend continues of tPVAT ( $p=0.42$ ) with waist to hip ratio ( $p=0.18$ ) and inflammatory risk factors.

#### *MV Linear Regression*

Multivariate linear regression was used to determine the significant CVD and inflammatory risk factors affecting the tPVAT with significance held at  $p<0.25$  level. The waist to hip ratio was included with the obesity-related factor BMI to include an adipose distribution factor, which is taken into consideration with MS. The CVD risk factors that affect log tPVAT volume for SLE patients include BMI ( $0.0302\pm 0.0054, p<0.0001$ ), postmenopausal status ( $0.208\pm 0.066, p=0.0019$ ), and waist to hip ratio ( $0.6173\pm 0.34, p=0.073$ ) were significant, while



the CVD risk factors for controls included BMI (0.0196±0.0046,p<.0001), history of smoking (0.103 ±0.051,p=0.046), postmenopausal status (0.184±0.055,p=0.0010), logHOMAIR (0.115±0.065, p=0.077), and average SBP ( 0.00315± 0.0016, p=0.056). Evaluating the inflammatory factors alone within the SLE population log PAI-1 (0.0582±0.044,p=0.19) and log CRP (0.115±0.031, p= 0.0003) are significant, while for controls, log PAI-1 (0.108±0.033, p= 0.0012), log CRP(0.0874±0.025, p=0.0007), and sICAM (0.000611±0.00040, p= 0.13) are significant. The same sequence of analyses with MS instead of BMI show that CVD risk factors for SLE include, MS (0.311±0.057, p=0.0001) and postmenopausal status (0.222±0.070,p=0.0018) significantly affect log tPVAT while for the controls, MS (0.385±0.078,p<0.0001), history of smoking (0.145±0.053, p=0.0075), postmenopausal status (0.158±0.057, p=0.0063) and hypertension (0.0812±0.067, p=0.23) significantly affect log tPVAT. Metabolic syndrome with inflammatory factors for SLE include MS (0.284±0.087, p=0.0014), log CRP (0.119±0.030, p=0.0001), and sICAM (-0.000777±0.00044, p=0.087) while for controls include MS(0.290±0.082, p=0.0006), log CRP (0.0626±0.025, p=0.015) and log PAI-1 (0.0899±0.032, p=0.0054).

**Table 3: Logistic regression of total PVAT association with outcome status (SLE). Cardiovascular risk factors: CVD, Inflammatory risk factors: Inflamm, Metabolic Syndrome: MS, Components of Metabolic Syndrome: MSc**

	<b>Risk of SLE OR(95% Confidence Interval)</b>	<b>p-value</b>
<b>Model 1:</b>		
<b>tPVAT + CVD</b>	1.022 (1.00-1.04)	<b>0.034</b>
<b>tPVAT + Inflamm</b>	1.007(0.99-1.02)	0.34
<b>Model 2:</b>		
<b>tPVAT + CVD + BMI</b>	1.025 (1.00-1.05)	<b>0.022</b>
<b>tPVAT +CVD+MS</b>	1.013(0.99-1.03)	0.17
<b>tPVAT + CVD + MSc</b>	1.016 (0.99-1.03)	0.065
<b>Model 3:</b>		
<b>tPVAT + Inflamm + BMI</b>	1.014(0.99-1.03)	0.13
<b>tPVAT +Inflamm+MS</b>	1.01 (0.99-1.02)	0.33
<b>tPVAT + Inflamm + MSc</b>	1.016 (0.99-1.03)	0.065

Considering all factors with BMI, the significant covariates for the SLE group included BMI ( $0.0261 \pm 0.0061$ ,  $p < 0.0001$ ), postmenopausal status ( $0.197 \pm 0.067$ ,  $p = 0.0037$ ), waist to hip ratio ( $0.545 \pm 0.35$ ,  $p = 0.12$ ), and log CRP ( $0.0443 \pm 0.031$ ,  $p = 0.15$ ) while the control group included postmenopausal status ( $0.202 \pm 0.054$ ,  $p = 0.0003$ ), log CRP ( $0.0579 \pm 0.023$ ,  $p = 0.014$ ), BMI ( $0.0172 \pm 0.0049$ ,  $p = 0.014$ ), history of smoking ( $0.0969 \pm 0.051$ ,  $p = 0.057$ ), log HOMAIR ( $0.0791 \pm 0.065$ ,  $p = 0.23$ ), and average SBP ( $0.00320 \pm 0.0017$ ,  $p = 0.054$ ). Considering all factors with MS, the significant covariates for the SLE group include MS ( $0.298 \pm 0.085$ ,  $p = 0.0006$ ), postmenopausal status ( $0.201 \pm 0.069$ ,  $p = 0.0041$ ), log CRP ( $0.107 \pm 0.029$ ,  $p = 0.0004$ ), and sICAM ( $-0.000874 \pm 0.00043$ ,  $p = 0.044$ ) while those significant for the control group include MS ( $0.220 \pm 0.086$ ,  $p = 0.012$ ), history of smoking ( $0.114 \pm 0.054$ ,  $p = 0.037$ ), postmenopausal status ( $0.169 \pm 0.056$ ,  $p = 0.0032$ ), hypertensive status ( $0.0848 \pm 0.067$ ,  $p = 0.20$ ), log CRP ( $0.0696 \pm 0.024$ ,  $p = 0.0047$ ) and log PAI-1 ( $0.0634 \pm 0.031$ ,  $p = 0.042$ ). No interactions between the significant CVD or inflammatory risk factors and group were detected. (Table 4)

**Table 4: Multivariate Linear Regression covariate results per obesity-related factors and outcome. Forward selection,  $p < 0.25$ .**

BMI		MS	
SLE tPVAT	Control tPVAT	SLE tPVAT	Control tPVAT
BMI $0.0261 \pm 0.0061$ , $p < 0.0001$	BMI $0.0172 \pm 0.0049$ , $p = 0.014$	MS $0.298 \pm 0.085$ , $p = 0.0006$	MS $0.220 \pm 0.086$ , $p = 0.012$
Postmenopausal Status $0.197 \pm 0.067$ , $p = 0.0037$	Postmenopausal Status $0.202 \pm 0.054$ , $p = 0.0003$	Postmenopausal Status $0.201 \pm 0.069$ , $p = 0.0041$	Postmenopausal Status $0.169 \pm 0.056$ , $p = 0.0032$
log CRP $0.0443 \pm 0.031$ , $p = 0.15$	log CRP $0.0579 \pm 0.023$ , $p = 0.014$	logCRP $0.107 \pm 0.029$ , $p = 0.0004$	logCRP $0.0696 \pm 0.024$ , $p = 0.0047$
waist to hip ratio $0.545 \pm 0.35$ , $p = 0.12$	History of smoking $0.0969 \pm 0.051$ , $p = 0.057$	sICAM $-0.00087 \pm 0.0004$ , $p = 0.044$	History of Smoking $0.114 \pm 0.054$ , $p = 0.037$
	logHOMAIR $0.0791 \pm 0.065$ , $p = 0.23$		logPAI-1 $0.0634 \pm 0.031$ , $p = 0.042$
	avg SBP $0.00320 \pm 0.0017$ , $p = 0.054$		Hypertension $0.0848 \pm 0.067$ , $p = 0.20$

### 3.3 PVAT AND VASCULAR CALCIFICATION

Total PVAT is highly correlated with both logCAC(0.441,<0.0001) and logAC (0.420,<0.0001). The correlation of tPVAT is slightly greater in SLE (logCAC: 0.469,<0.0001; logAC: 0.418, <0.0001) than in controls (logCAC: 0.406,<0.0001; logAC: 0.384, <0.0001).

*Logistic Regression: SLE / Control on AC and CAC*

In logistic regression analysis, AC alone was associated with SLE by a factor of 2.3 (95%CI:1.4-3.8, p=0.001). When adjusting for the CVD risk factors and BMI the OR increases to 3.36((1.85- 6.12),p<0.0001) and is slightly attenuated with MS and associated CVD risk factors 2.12 ((1.26-3.59),p=0.0049). When adjusting for BMI and inflammatory factors the AC OR is attenuated (2.52 (1.42-4.47),p=0.0015) and even more so with MS and the inflammatory factors (1.83 (1.07-3.13)p=0.028). CAC did not show any statistical preference between SLE and the control group.

*Logistic Regression: tPVAT on AC and CAC*

In a logistic regression analysis, tPVAT alone was associated with any AC by a factor of 1.073 [1.04-1.1] (p<0.0001) (Table 5A) and with any CAC by a factor of 1.051 [1.03-1.07] (p<0.0001) (Table 5B). The model was expanded to include either tPVAT and BMI, MS, or MS components (triglyceride, waist to hip ratio, HDL, glucose, and average SBP) and stratified by outcome.

For the AC, adjusting for CVD and inflammatory risk factors slightly attenuated the effect of tPVAT on AC, but remained significant regardless of BMI, MS, or MS components. For the CAC, the significant effect of tPVAT on CAC was dependent whether BMI, MS, or MS components were used when adjusting for CVD and inflammatory risk factors. When adjusting

for BMI or MS components along with either CVD or inflammatory components, tPVAT did not remain a significant effect on CAC. However, when adjusting for MS alone with either CVD or inflammatory risk factors, tPVAT did remain a significant effect on CAC. (Table 5B)

Interestingly, when stratifying the logistic regression by outcome (SLE vs Control) BMI attenuates the influence of tPVAT on both AC and CAC to non-significance within the SLE group, but tPVAT remains a significant effect for both AC and CAC in controls. (Table 6A-B) The tPVAT remains significant for both SLE and control when controlling for MS with both AC and CAC.

**Table 5: Logistic regression evaluating A. tPVAT with any AC B. tPVAT with any CAC. Cardiovascular risk factors: CVD, Inflammatory risk factors: Inflammation, Metabolic Syndrome: MS, Components of Metabolic Syndrome: MSc**

A.

	<b>Risk of any AC OR(95% Confidence Interval)</b>	<b>p-value</b>
<b>Model 1: tPVAT</b>	1.073(1.043- 1.103)	<b>&lt;0.0001</b>
<b>Model 2: tPVAT+CVD+BMI tPVAT+CVD+MS tPVAT+CVD+MSc</b>	1.053(1.02-1.09) 1.063(1.03-1.1) 1.049(1.02-1.08)	<b>0.0006</b> <b>&lt;0.0001</b> <b>0.0037</b>
<b>Model 3: tPVAT + Inflammation+BMI tPVAT + Inflammation + MS tPVAT + Inflammation + MSc</b>	1.065(1.030-1.1) 1.061(1.03-1.1) 1.060(1.02-1.09)	<b>&lt;0.0001</b> <b>0.0003</b> <b>0.0004</b>

B.

	<b>Risk of any CAC OR(95% Confidence Interval)</b>	<b>p-value</b>
<b>Model 1: tPVAT</b>	1.051(1.03-1.07)	<b>&lt;0.0001</b>
<b>Model 2: tPVAT +CVD + BMI tPVAT + CVD + MS tPVAT + CVD + MSc</b>	1.007 (0.99-1.02) 1.022(1.0-1.04) 1.016(0.99-1.03)	0.41 <b>0.046</b> 0.059
<b>Model 3: tPVAT + Inflammation + BMI tPVAT +inflammation+MS tPVAT + Inflammation + MSc</b>	1.017 (0.99-1.04) 1.022(1.00-1.04) 1.020(0.99-1.04)	0.076 <b>0.040</b> 0.068

When adjusting for MS components (MSc) for CAC, the tPVAT of the control group remains significant while the tPVAT influence for the SLE group is attenuated to non-significance. In addition, when controlling for the MS diagnosis for both AC and CAC in both SLE and control, the tPVAT influence on the calcification remains significant and the OR for AC is increased.(Table 6A) Also of note, the OR for the MS influence on CAC is 3.13[1.3-7.6] (p=0.012) for SLE and 4.73[1.6-14](p=0.0046) for control while there is no significant influence of MS on AC for either SLE or controls.(not shown)

**Table 6: Logistic regression evaluating tPVAT with any AC or any CAC adjusting for BMI, MS, or MS components.**

A.

	<b>Risk of any AC OR(95% Confidence Interval)</b>	<b>p-value</b>
<b>SLE: tPVAT + BMI</b>	1.049(0.99-1.10)	0.065
<b>Control: tPVAT + BMI</b>	1.044(1.01-1.083)	<b>0.025</b>
<b>SLE:tPVAT + MS</b>	1.077(1.03-1.13)	<b>0.0036</b>
<b>Control: tPVAT + MS</b>	1.050(1.01-1.09)	<b>0.012</b>
<b>SLE: tPVAT + MSc</b>	1.063(1.01-1.12)	<b>0.025</b>
<b>Control: tPVAT + MSc</b>	1.048(1.009-1.089)	<b>0.016</b>

B.

	<b>Risk of any CAC OR(95% Confidence Interval)</b>	<b>p-value</b>
<b>SLE: tPVAT + BMI</b>	1.013(0.993-1.033)	0.21
<b>Control: tPVAT + BMI</b>	1.038(1.01-1.073)	<b>0.024</b>
<b>SLE:tPVAT + MS</b>	1.030(1.00-1.06)	<b>0.029</b>
<b>Control: tPVAT + MS</b>	1.040(1.01-1.08)	<b>0.020</b>
<b>SLE: tPVAT + MSc</b>	1.020(0.99-1.046)	0.13
<b>Control: tPVAT + MSc</b>	1.037(1.002-1.073)	<b>0.036</b>

### *Complement, Calcification and SLE*

Only the SLE participants had measures of complement proteins C3 and C4 quantified. Spearman correlations showed that C3 (0.215,  $p=0.013$ ) was correlated to tPVAT and that both C3 (0.249,  $p=0.0023$ ) and C4 (0.221,  $p=0.0099$ ) were correlated with log CAC, but not with log AC. A univariate linear regression confirmed the association of log C3 ( $0.282\pm 0.14$ ,  $p=0.043$ ) with log tPVAT and the non-significance of log C4 ( $p=0.37$ ).

## 4.0 DISCUSSION

This is the first study to compare the unique tPVAT volume surrounding the descending thoracic aorta within clinically cardiovascular disease free SLE participants and their age- and race-matched, healthy controls. Women with SLE have increased tPVAT when compared to their healthy controls. The tPVAT is also significantly correlated with cardio-metabolic risk factors in both the SLE and control participants. Total PVAT is independently associated with AC regardless of adjustments for CVD risk and inflammatory factors or obesity-related factors, while CAC retains significance to tPVAT when CVD risk and inflammatory factors are adjusted with MS. The premature AC in SLE participants regardless of obesity-related factors and CVD or inflammatory factors reflects the increased pre-clinical CVD burden for those with SLE.

Most traditional CVD risk factors and a few SLE-related factors [76, 77, 79] have been implicated in the accelerated progression of CVD in the SLE population. Obesity-related factors such as waist to hip ratio are surrogate measures for adipose distribution, which has been found to be significantly different between asymptomatic CVD SLE and control groups [99] despite limited [112] to no significant differences in BMI.[99] In this study there were no significant differences between BMI, waist circumference, and metabolic syndrome between the SLE and control participants. However, the distribution of adipose was significantly different when evaluating the waist to hip ratio (SLE: 0.833 (0.79-0.88), Control: 0.797(0.77-0.84),  $p=0.001$ ), which suggests the importance of evaluating separate and distinct adipose regions. [113]

Smaller, visceral adipose depots have been gaining more attention for their localized inflammatory role in CVD. Epicardial adipose is a small, visceral adipose depot found between the myocardium and the visceral layer of the pericardium, which can transform from a cardio-protective state into a pro-inflammatory, pro-thrombotic pathological state.[24] Strong evidence exists in linking excessive epicardial adipose with CVD development and progression. The descending thoracic aorta tPVAT is a distinct, small visceral adipose reservoir. In the multivariate linear regression analysis involving all factors, there were similarities and differences in CVD risk factors and inflammatory factors associated with the descending thoracic aorta tPVAT depending on the obesity-related factor used (BMI or MS) and on the outcome (SLE or control). The obesity-related factor, postmenopausal status, and log CRP were always significant regardless of outcome; however, the controls consistently had more significant factors including lifestyle or medication modifiable factors such as history of smoking and hypertension. In addition, the association of tPVAT to SLE remains significant after adjusting for CVD risk factor and BMI. This significance is lost when adjusting for inflammatory components or with the obesity-related factor MS, which is associated with elevated CRP and inflammation.[114] The loss of significance of tPVAT to SLE when evaluating inflammatory factors or obesity-related factors closely associated with inflammation underscores the influence of the inflammatory component(s) in SLE. The increased CV risk of SLE patients along with their chronic inflammatory disease state and the fewer number of CVD and inflammatory risk factors associated with tPVAT suggests an amplified inflammatory response and perhaps an increased dysfunction of the adipose of an SLE patient.

Adipocyte size and adipose cellular composition including inflammatory infiltrates such as mast cells and leukocytes (neutrophils, dendritic cells, lymphocytes and monocytes),



macrophages, fibroblasts, adipocytes and preadipocytes are responsible for the normal metabolic activity of adipose and the dysfunction of adipose including production of many of the circulating inflammatory and atherogenic factors.[17, 115] These same inflammatory markers link obesity, atherosclerosis, and inflammation to SLE resulting in an exacerbated chronic inflammatory state. As noted, not all adipose depots confer the same CVD risk. In particular, excessive visceral adipose tends to upregulate circulating inflammatory components and the distribution of adipose becomes more important than overall adiposity. SLE patients tend to develop a loss of muscle mass and fat free mass with a concomitant increase in fat mass. This altered macro body composition also extends to the tPVAT of the descending thoracic aorta, which despite no differences in BMI, waist circumference, hip circumference or metabolic syndrome, was greater in the SLE participants when compared to the controls. Increased adipose volumes of smaller visceral adipose depots may be the precursors to chronic inflammatory state leading to atherosclerosis, obesity and exacerbating the inflammatory response of a disease like SLE.

Women with SLE have a higher risk of CVD related events [68] and have a higher risk of subclinical CVD as measured through CAC [77], AC [104], pulse wave velocity [79, 116] when compared to healthy controls. Given the systemic nature of CVD, descending thoracic aortic calcification has been found to be a strong predictor of CAC independent of CVD risk factors and is thus another important subclinical CVD measure of vascular calcification.[117] Although the volume of aortic tPVAT is approximately two orders of magnitude less than total visceral adipose measures [28], there is a significant cardio-metabolic influence and association between this visceral adipose and AC and CAC. In a recent study of the Framingham Heart Study Offspring cohort including both men and women, the descending thoracic aorta PVAT volume

was significantly associated with thoracic AC (1.31[1.01–1.71], $p=0.04$ ), but only when adjusting for visceral adipose which was then attenuated when adjusting for any CVD risk factors [29]. This same study also reported higher OR associated with aortic PVAT and CAC in a clinically CVD free population; however, the population was 10 years older when compared to the population within this study.[29] Within this population, AC is associated with SLE and tPVAT independent of cardio-metabolic factors in this group of asymptomatic participants, while the CAC association with tPVAT is contingent on which obesity-related factor is adjusted within the model. This suggests that excessive tPVAT and AC are precursors to CAC and exacerbated CVD progression. By extension, this early calcification of the aorta may contribute to the increase in vascular stiffness [79] and the increased number of hypertensive SLE participants ( $p<0.0001$ ), despite no difference in waist circumference, BMI, or metabolic syndrome. Systemic hypertension is associated with increased frequency of CV events in SLE patients.[118] The visceral adipose, including aortic tPVAT, of the SLE group may follow a more similar pattern of dysfunction generally associated with that of chronic low grade inflammatory state of overweight or obese individuals.

Surprisingly, not all of the measured circulating inflammatory markers including sICAM and eSelectin, were significantly correlated with tPVAT within the SLE group, both of which are associated with atherosclerosis in the general population.[53, 119] ESelectin has been found to be higher with the presence of plaque in SLE patients [120] and general visceral adiposity [121] while sICAM has been found in atherosclerotic lesions, is a predictor of coronary heart disease, [53] and positively correlated with SLE disease activity index (SLEDAI, [122]). SICAM has also been found in human epicardial tissue in obese participants and those with coronary artery disease. [123] A mild association of sICAM ( $p=0.087$ ) to tPVAT was detected through the

multivariate linear regression analyses of MS, inflammatory factors, and SLE, which suggests that perhaps the other inflammatory markers such as CRP are more important in a clinically CVD free population and that inflammatory markers such as sICAM and eSelectin may require more progressive CVD development or more significant SLE disease activity. Additional metabolic characterization of aortic PVAT is necessary to determine if this visceral adipose depot indeed secretes increased amounts of pro-inflammatory products or perhaps upregulates the expression factors necessary to sustain inflammation.

Additional investigation into other visceral adipose depots including quantification of the general intra-abdominal and distinct pericardial, epicardial and aortic arch adipose depots relating to the tPVAT needs to be assessed in the SLE population. Evaluating these various adipose reservoirs along with the subclinical CVD measures will allow us to determine the adipose with the most developed cardio-metabolic influence and adipocyte dysfunction and elucidate if there are any early cardio-protective roles of aortic PVAT in both SLE patients and the broader population.

In addition, evaluation of the metabolic profile of serum adipokine levels, including leptin, resistin, visfatin, and adiponectin, with tPVAT is required. In the general US population, elevated circulating leptin levels are associated with MI and stroke independent of CVD risk factors [124], while adiponectin may modulate SLE activity and levels may be increased with CVD progression.[120] Perhaps changes in adipokine serum levels can be seen within a finer nuance of increasing tPVAT volume rather than larger depot of general visceral adipose volume. The control group within this study also lacked the analysis of circulating complement protein levels. Aortic stiffness of the SLE women is associated with SLE specific risk factors such as elevated complement C3.[79] A strong positive correlation exists between C3 levels and tPVAT

in SLE patients along with CAC, but no effect on AC. Quantifying the complement proteins of the control group will elucidate the role of elevated circulating complement proteins in subclinical CVD. In the SLE population, differentiating the apparent cause of a CV event whether related to atherosclerosis or active lupus can be difficult.[75] Detecting early adipose dysfunction through quantification of aortic tPVAT may aid in delaying or preventing later arterial structural changes leading to better outcomes. Determining if tPVAT volume is predictive of CV events may elevate the overall status of this visceral depot from risk factor to a subclinical CVD measure.

Several limitations within this study exist and included the cross-sectional study design. No prognostic evaluations of PVAT can be made at this time; however, subsequent data collection was done on this same population with CV events, which will allow us a longitudinal evaluation of tPVAT. The majority of this population is Caucasian and any extrapolation of the findings to other ethnicities must be done with caution. There may be other CVD risk factors not included in the multivariate analyses that need to be considered when evaluating adipose tissue, such as adipokines.

Cardiovascular disease is the leading cause of death of women in America and in developing countries and emerging economies.[1] Young women with SLE are over 50 times more likely to experience a myocardial infarction when compared to healthy controls.[68] Traditional CVD risk factors alone do not account for this exaggerated risk and it is suspected that there are SLE-related factors possibly linked with the inflammatory nature of the disease which exacerbates the pro-inflammatory conditions of atherosclerosis and CVD development. This existing pro-inflammatory state may also incorporate the chronic, low-grade inflammatory response of the adipose tissue. From an epidemiological and public health standpoint, both the

SLE population and the general population will benefit from knowledge gained in characterizing the role of small visceral adipose depots in the progression of CVD and its contribution to the inflammatory state.

Descending thoracic aortic PVAT is a viable, metabolically active visceral adipose depot with optimal location for influencing the surrounding arterial structures. Descending thoracic PVAT volume is greater in asymptomatic SLE population when compared to controls despite other anthropomorphic measures being the same. The PVAT volume is associated with both AC independently and CAC through BMI.

## APPENDIX

### REPRODUCIBILITY ANALYSIS

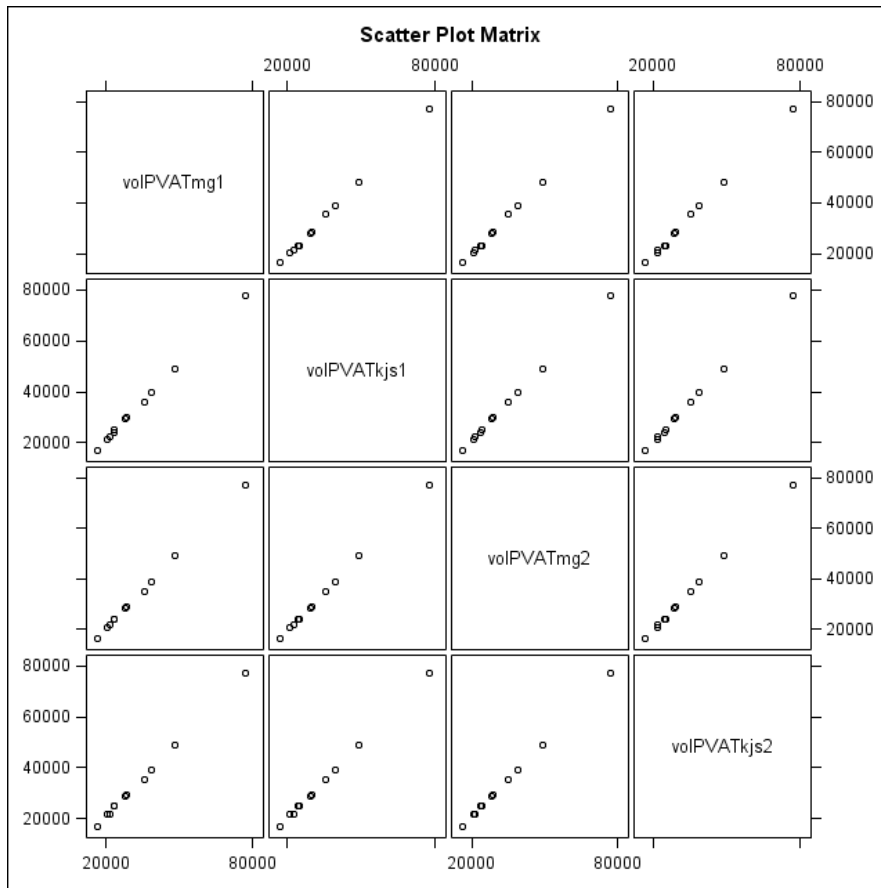
A reproducibility analysis was performed to determine the inter- and intra-reader variability in determining the PVAT volume using EBCT scans. Two readers were chosen (KJS and MG) and two time points approximately 3 to 4 weeks apart were chosen. Eleven participants were randomly selected to use in the analysis.

**Table A1: Descriptive statistics of PVAT volume at time point 1. Std: Standard deviation, IQR: Interquartile Range.**

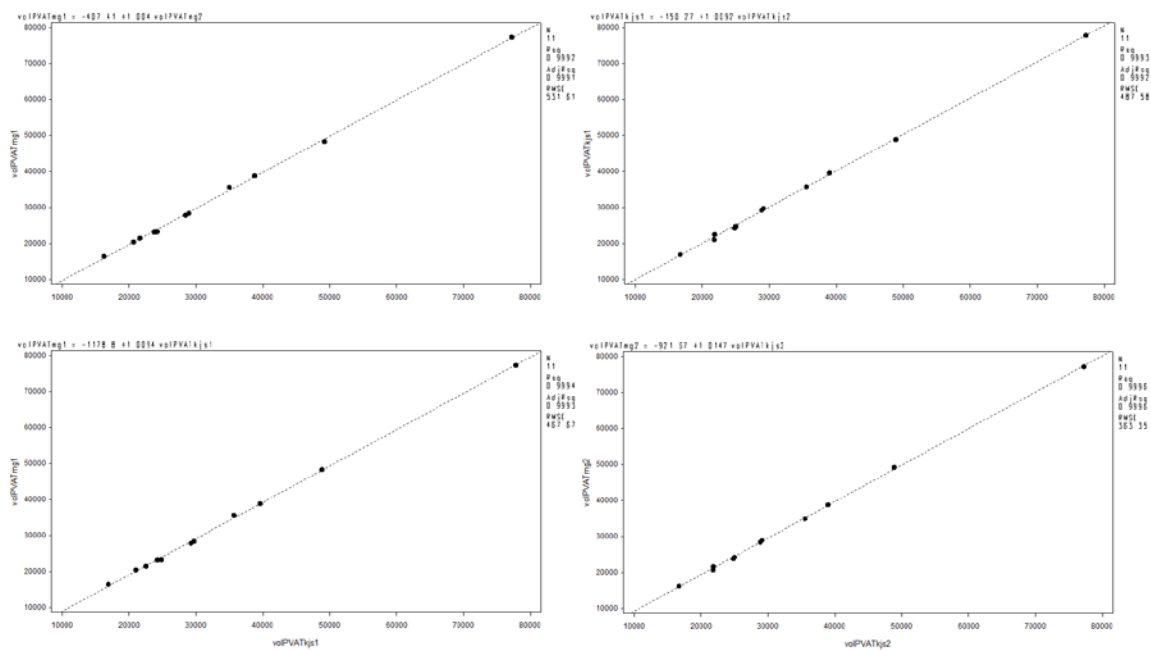
<b>PVAT (mm<sup>3</sup>)</b>	<b>Mean</b>	<b>Median</b>	<b>Std</b>	<b>IQR</b>
<b>MG Time 1</b>	32821.73	27844.86	17445.84	21511.98 38828.16
<b>KJSTime 1</b>	33682.28	29243.40	17276.96	22510.20 39599.34

**Table A2: Descriptive statistics of PVAT volume at time point 2.**

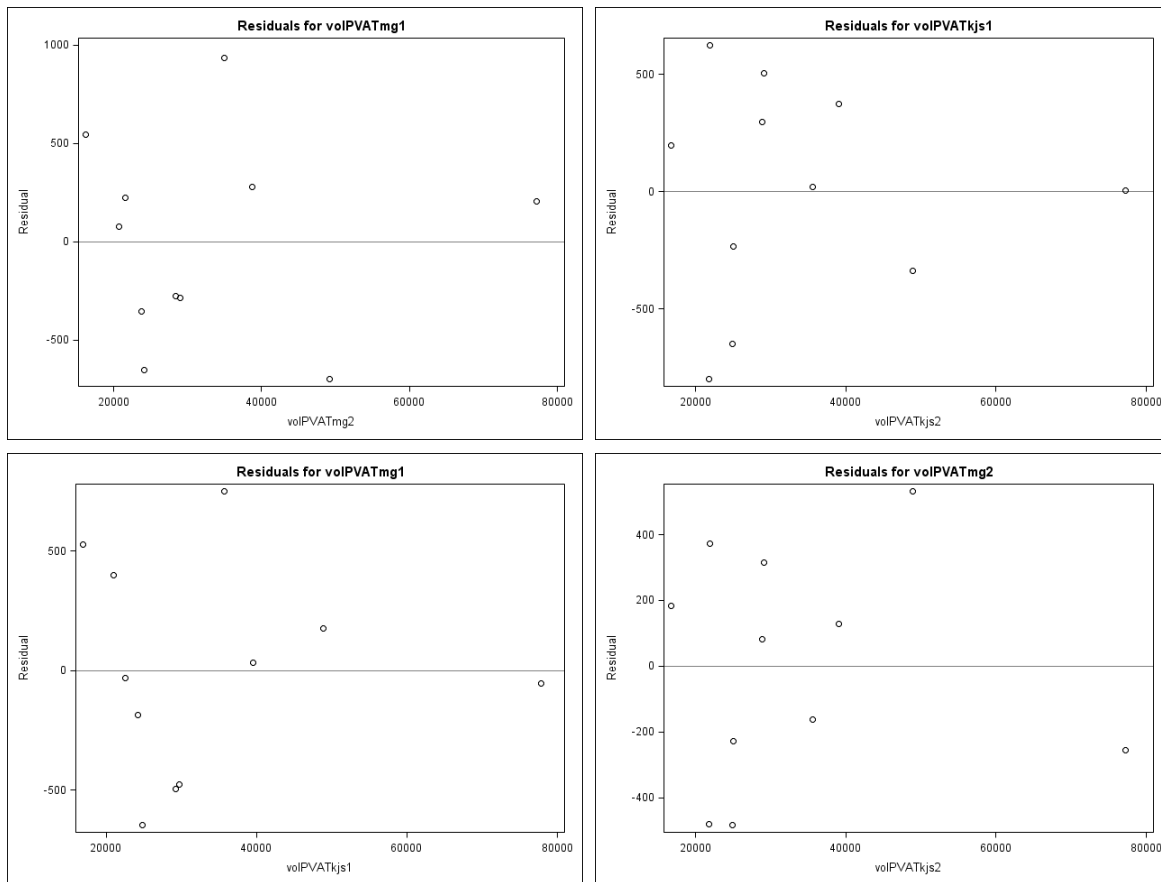
<b>PVAT (mm<sup>3</sup>)</b>	<b>Mean</b>	<b>Median</b>	<b>Std</b>	<b>IQR</b>
<b>MG Time 2</b>	33097.11	28416.12	17369.26	21610.20 38799.42
<b>KJSTime 2</b>	33525.31	28832.76	17113.93	21836.82 39019.32



**Figure A1: Spearman correlation matrix of PVAT volume between both readers and both time points.**



**Figure A2: Linear regression of PVAT volume between both readers and both time points.**



**Figure A3: Residuals of PVAT volume between both readers and both time points.**



**Table A3: Descriptive difference of PVAT volume between both readers and both time points. Std: Standard deviation, IQR: Interquartile Range.**

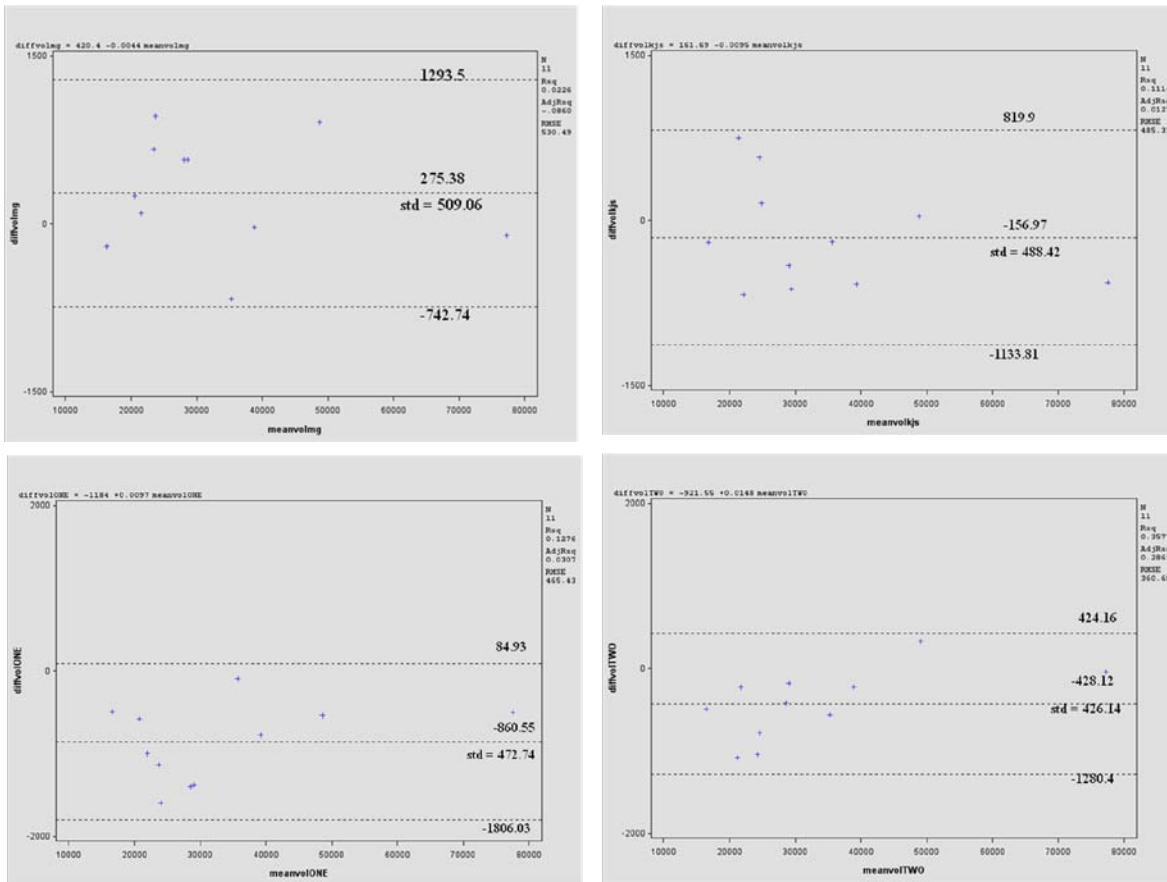
<b>Difference PVAT (mm<sup>3</sup>)</b>	<b>Mean</b>	<b>Median</b>	<b>Std</b>	<b>IQR</b>	<b>p-value <math>\mu = 0</math> Paired ttest</b>
<b>MG Time (2-1)</b>	275.38	246.96	509.06	-105.6 - 666.6	0.103
<b>KJSTime (2-1)</b>	-156.97	-199.5	488.42	-580.02 - 154.68	0.312
<b>MG1-KJS1</b>	-860.55	-771.18	472.74	-1375.74 - -498.0	0.0001
<b>MG2-KJS2</b>	-428.12	-416.64	426.94	-783.24 - -178.14	0.0077

**Table A4: Intraclass correlation of PVAT volume between both readers and both time points, DEYO Method.**

<b>ICC</b>	<b>MG</b>	<b>KJS</b>	<b>MG1 vs KJS1</b>	<b>MG2 vs KJS2</b>
<b>DEYO's</b>	0.999	0.999	0.998	0.999

**Table A5: Intraclass correlation of PVAT volume between both readers and both time points, SAS REML, TYPE I Method. ICC: Intraclass Correlation.**

<b>volPVAT</b>	<b>Between Subject Variance</b>	<b>Between Readings</b>	<b>Error Variance</b>	<b>ICC</b>
MG2-MG1*	302894580	26137.2	129573.1	0.999
KJS2-KJS1 <sup>+</sup>	295570734	1476.5	119277.4	0.999
MG1-KJS1*	301313560	360112.3	111743.1	0.998
MG2-KJS2*	297197722	83391.5	91138.7	0.999



**Figure A4: Bland-Altman plots of PVAT volume between both readers and both time points.**

PVAT volume data was consistent between participants with a good range of values. Spearman correlations were high, 1.000. Excellent intraclass correlations were calculated by hand (DEYO's Method) and through SAS (REML, TYPE I Method) ranging from 99.8-99.9% of the variability due to participants and not to the readers or the different time points. There was some systemic bias with KJS readings consistently higher than MG readings; however the differences seen within the Bland-Altman show that the limits of agreement were at least one order of magnitude less than the PVAT volumes.

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