ELEVATED CIRCULATING ALDOSTERONE AND PLATELET ACTIVITY IN OVERWEIGHT/OBESE YOUNG ADULTS: ROLES IN VASCULAR REMODELING AND CARDIOMETABOLIC HEALTH

by

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ELEVATED CIRCULATING ALDOSTERONE AND PLATELET ACTIVITY IN OVERWEIGHT/OBESE YOUNG ADULTS: ROLES IN VASCULAR REMODELING AND CARDIOMETABOLIC RISK

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Globally, cardiovascular disease (CVD) is the leading cause of morbidity and mortality. Overweight/obese individuals are at increased risk for CVD because the increased metabolic requirements and inflammation caused by excess weight drive adverse cardiovascular changes. Elevated circulating aldosterone and platelet activity are hypothesized to be important factors linking obesity to declining cardiometabolic health, but little longitudinal data is available in young adults with no clinically apparent obesity-related comorbidities. We sought to evaluate the roles of elevated serum aldosterone and plasma β-thromboglobulin, a marker of platelet activity, in vascular remodeling and cardiometabolic risk in overweight/obese young adults. These questions were investigated in a sample from the Slow Adverse Vascular Effects of excess weight trial, a randomized trial that evaluated the effects of a one year lifestyle intervention targeting weight loss, increased physical activity, and dietary sodium reduction on vascular health.

We found that lower circulating platelet activity at the end of the two year study was associated with smaller common carotid artery IMT and greater weight loss during the study. In addition, non-Hispanic white individuals carrying the T allele of rs168753 in the gene encoding PAR-1, the main thrombin receptor, had greater carotid bulb IMT than non-carriers at baseline but not at the end of the study. In another analysis, higher arterial stiffness over the course the

study was found to predict higher circulating platelet activity at the end of the study. However, this association was partly explained by the effect of obesity. Finally, in our study of serum aldosterone and obesity-related factors, we found that reductions in aldosterone were associated with reductions in insulin resistance, C-reactive protein, leptin, heart rate, tonic cardiac sympathovagal balance, and increases in adiponectin, independent of changes in dietary sodium and weight. In addition, weight loss and reduced intermuscular fat were associated with reduced aldosterone in individuals who had metabolic syndrome at baseline.

The public health relevance of these findings is that elevated aldosterone and platelet activity are important modifiable cardiometabolic risk factors in overweight/obese otherwise healthy young adults. These factors may be useful targets for therapies to reduce the burden of CVD is this population.

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PREFACE

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Key Terms:

Obesity

Platelet activity

Aldosterone

Cardiometabolic health

Carotid intima-media thickness

Arterial stiffness

1.0 DISSERTATION OVERVIEW AND OBJECTIVES

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality in the US and throughout the developed world (1). Worldwide, over 7.1 million people die from coronary heart disease (CHD) each year (1). Large epidemiologic cohort studies such as the Framingham Heart Study have shown that excess weight increases the risk of stroke, incident CVD, cardiovascular mortality, and all-cause mortality (2-5). It is also clear that obesity brings about early adverse vascular changes in adolescents (6, 7) and young adults (8). Obesity is a growing public health problem in much of the world. In the USA, almost two-thirds of the adult population is overweight (BMI≥25) and approximately one-third is obese (BMI≥30) according to a recent report from the National Health and Nutrition Examination Survey (9). In addition to CVD and stroke, obesity predisposes individuals to other serious chronic diseases, such as hypertension, type 2 diabetes, respiratory complications, and some cancers (10, 11). It has been hypothesized that elevated renin-angiotensin-aldosterone-system (RAAS) activity (12) and elevated platelet activity (13-15) are two important mechanisms linking obesity to the decline in vascular health that eventually can lead to clinical CVD events.

The objective of this dissertation was to evaluate the roles played by RAAS activity, as measured by circulating aldosterone, and platelet activity, as measured by plasma β-thromboglobulin (β-TG), in vascular remodeling and elevated cardiometabolic risk in overweight and obese young adults. These research questions were investigated in a sample of

normotensive overweight and obese young adults who participated in the Slow Adverse Vascular Effects of excess weight (SAVE) trial, a randomized clinical trial that evaluated the effects of a one year lifestyle intervention targeting weight loss, increased physical activity, and dietary sodium reduction on vascular health. In the study, circulating platelet activity was measured as plasma β-thromboglobulin (β-TG) at 24-month follow-up visits. Serum aldosterone was measured at all study visits (see Appendix I for table listing all data collected in the SAVE trial). Higher levels of β-TG are indicative of excess platelet activity and higher serum aldosterone levels are indicative of excess RAAS activity.

Research questions were addressed in a series of three studies that addressed these aims:

1. Are previously studied single nucleotide polymorphisms (SNPs) in platelet membrane receptor genes associated with segment-specific carotid intima-media thickness (CIMT) and circulating platelet activity? Do positive lifestyle changes counteract any excess risk brought about by these genetic variants? Is lower circulating platelet activity one year post-intervention associated with greater weight loss during the study or with smaller concurrently measured CIMT?

2. Independent of changes in urinary sodium excretion, are reductions in circulating aldosterone associated with weight loss and reduced abdominal visceral and subcutaneous adiposity, intermuscular adiposity, C-reactive protein, leptin, insulin resistance, cardiac sympathovagal balance and increased adiponectin and ghrelin over the course of the two year study? Independent of weight loss, are decreases in serum aldosterone and urinary sodium associated with decreases in blood pressure?

3. Is there a positive association between cumulative arterial stiffness exposure during the two year study and circulating platelet activity at the final study visit? Are associations between arterial stiffness and platelet activation independent of cumulative exposure to other cardiovascular and metabolic risk factors?

2.0 INTRODUCTION

2.1 OBESITY AND CARDIOVASCULAR DISEASE

Obesity leads to poor vascular health and increases the risk of cardiovascular disease (CVD) (16-18). The metabolic requirements of excess weight necessitate increases in total blood volume and cardiac output, and these hemodynamic changes elevate arterial wall stress, smooth muscle cell proliferation, vessel wall thickness and diameter, and eventually arterial stiffness (16, 19). Adverse hemodynamic factors work together with other features of obesity, such as chronic inflammation and endothelial dysfunction, to impair vascular structure and function in obese individuals (20). Weight loss reverses many adverse vascular changes (8, 21-23) and lowers CVD risk (23-25), but the mechanisms by which this occurs are not completely understood. In addition, weight loss is difficult to achieve and maintain through lifestyle modification. Thus, in addition to recommending weight loss, targeted treatment of cardiovascular and metabolic risk factors that are elevated in young overweight/obese individuals can be helpful to reduce long-term CVD risk.

2.2 SUBCLINICAL CARDIOVASCULAR DISEASE

In recent decades, large population-based and clinical studies have used non-invasive techniques to examine early functional and structural vascular changes. CIMT and pulse wave velocity (PWV) are two such measures. Because established cardiovascular risk factors are not sufficient to prospectively identify all individuals who will suffer from CVD, measures of functional and structural arterial characteristics can be useful to improve these predictions (26). Non-invasive measures of functional and structural arterial characteristics also allow us to study the pathophysiological mechanisms leading to CVD and the effects of various interventions on vascular health in much smaller samples than are required for studies of clinical events (26).

2.2.1 Carotid Intima-Media Thickness

Carotid intima-media thickness (CIMT), measured using B-mode ultrasound, is an established measure of subclinical CVD. CIMT is predictive of incident cardiac and cerebrovascular events (27) and has also been associated with numerous CVD risk factors. Traditional risk factors, such as male sex (28), increased age (28), excess weight (28), elevated blood pressure (29-34), high blood cholesterol (35-37), diabetes and insulin resistance (38-40) and cigarette smoking (41) have been associated with higher CIMT in numerous observational studies both in patients at elevated CVD risk and in the general population. Of these factors, hypertension appears to have the greatest effect on carotid IMT, probably as a result of its stimulation of medial hypertrophy (35-37).

CIMT has been found to be associated with other measures of subclinical and clinical CVD. For example, CIMT has been associated with angiographically assessed coronary artery

disease (42), electron beam computed tomographically (EBCT) assessed coronary artery calcification (43) and echocardiographic left ventricular hypertrophy (44-46). It has been linked to endothelial dysfunction, as measured by flow-mediated dilation of the brachial artery (47). The relations of CIMT with cardiovascular risk factors, other measures of cardiac and vascular health, and clinical vascular events suggest that increased CIMT may provide a comprehensive picture of the remodeling, both atherosclerotic and non-atherosclerotic, that occurs over time in the artery wall as a result of chronic exposure to risk factors (48). Interestingly, there is also evidence that the effects of both traditional (49-51) and genetic (52) cardiovascular risk factors differ depending on which segment of the carotid artery is being evaluated. The distinctive anatomy and hemodynamics in each segment of the carotid artery support different pathological mechanisms (49-51), such that risk factors may contribute differently to intimal thickening in the common carotid artery (CCA), internal carotid artery (ICA), and carotid bulb, and in turn, CIMT may present different predictive ability for vascular events depending on which segment is measured (53).

2.2.2 Pulse Wave Velocity

Arterial stiffness, often measured non-invasively as PWV, is an established measure of vascular health. Carotid-femoral pulse wave velocity (cfPWV), a measure of aortic stiffness, and brachial-ankle pulse wave velocity (baPWV), a mixed measure of central and peripheral arterial stiffness, are both predictive of incident vascular events and cardiovascular and all-cause mortality in the general population (54, 55), though cfPWV has been by far the more frequently reported predictor. Mechanisms behind arterial stiffening include elastin degeneration and altered collagen and fibronectin within the vascular wall, vascular smooth muscle cell (VSMC)

hypertrophy, damage from proinflammatory cytokines, and calcium deposition within the medial layer of the vascular wall (56). Recent studies have also highlighted associations between increased aortic stiffness and elevated renin-angiotensin-aldosterone system (RAAS) activity (56-59) or sympathovagal balance (60). CfPWV is associated with numerous CVD risk factors in apparently healthy adults. It has been found to be higher in males (61-63) and African Americans (64) in some studies. Greater aortic stiffness is associated with higher systolic (64-66), diastolic (62), and mean arterial blood pressure (61, 63), as well as pulse pressure (PP) (62), prevalent hypertension (67), and incident hypertension (68). Elevated cfPWV is also associated with higher fasting glucose (62), higher homeostasis model assessment of insulin resistance (HOMA-IR) (63, 64), cigarette smoking (64, 66), increased age (61-65), higher C-reactive protein (CRP) (59, 61, 65), lower adiponectin (63, 65), greater waist circumference (63), greater abdominal visceral fat (66), higher hemoglobin A1C (66), and higher heart rate (61, 63, 66). Individuals with elevated aortic stiffness have also been found to have poor vascular health according to other metrics such as increased CIMT (61, 69) and increased aortic (61) and coronary calcification (70).

Brachial-ankle pulse wave velocity (baPWV), a mixed measure of both central (aortic) and peripheral arterial stiffness, has also been found to be higher in males (71, 72) and African Americans (71). In apparently healthy adults, baPWV has been associated with higher systolic blood pressure (SBP) (71-74), prevalent hypertension (75), higher body weight (76), higher BMI (72), higher age (73, 75), cigarette smoking (71, 72), higher CRP (59, 77), higher heart rate (59, 78), higher triglycerides (71), and lower HDL cholesterol (71). BaPWV has also been found in one study to predict cardiovascular and all-cause mortality in a general population of older adults (55). Both peripheral and central arterial stiffness have been shown to be greater in obese

individuals at all ages (16). Such stiffening likely occurs as a result of hemodynamic forces, namely increased shear stress, blood volume, and blood pressure, as well as the nervous and hormonal alterations that accompany obesity (60).

2.3 RENIN-ANGIOTENSIN-ALDOSTERONE SYSTEM

The RAAS plays an important role in blood pressure regulation, water and electrolyte balance, and tissue growth. The RAAS functions both as an endocrine system in circulation and as a paracrine/autocrine system within tissues such as the heart, brain, kidney and vasculature. In the endocrine system, renin is released by the kidneys and converts angiotensinogen into angiotensin I, which is then converted to angiotensin II by angiotensin converting enzyme (ACE) (12). Angiotensinogen is produced primarily by the liver, but several studies over the last two decades have shown that angiotensinogen and other RAAS components are also secreted by adipose tissue (79). Under pathophysiological conditions such as hypertension or obesity, overactivation of the RAAS at the tissue level may contribute to the development and progression of cardiovascular and renal diseases (12, 80).

2.3.1 Aldosterone and obesity

Although obesity is well established as a cause of hypertension, the mechanisms behind this causal relationship remain poorly understood. Increased renal tubular sodium and water reabsorption in obesity is thought to be one of the most important mechanisms driving obesity-related hypertension (81). Mechanisms such as sympathetic nervous system activation by

hyperinsulinemia and hyperleptinemia, excess RAAS activation, and physical compression of the kidney, have been proposed to explain the altered sodium and fluid handling in obesity (82). Aldosterone, the most potent mineralocorticoid secreted by the adrenal cortex, plays an important role in the regulation of blood pressure by influencing salt-water balance (12). Its primary role is to induce sodium and fluid retention, resulting in increased intravascular volume (83). Aldosterone stimulates cytoplasmic mineralocorticoid receptors in distal renal tubular cells, which upregulates the activity and number of epithelial sodium channels, thus promoting transepithelial sodium transport (83). Both experimental and clinical studies suggest that aldosterone participates in the pathogenesis of hypertension (84-86). Primary aldosteronism, the classic clinical example of aldosterone excess, is a well-established cause of secondary hypertension and has an estimated prevalence of 5-10% in the general hypertensive population and 20% in severe or resistant hypertension (83). In addition to aldosterone's classical effect on sodium and water retention, animal and human studies suggest that aldosterone may play a pro-inflammatory and pro-fibrotic role in the heart and vasculature (12, 87-90).

Several clinical studies have found that aldosterone levels are elevated in overweight and obese individuals, especially in those with excess visceral fat (91-96). Several investigations have found a significant decrease in aldosterone with weight loss when subjects either maintained a moderate to high dietary sodium intake (93, 96-98) or severely restricted their calorie intake irrespective of dietary sodium intake (96, 99). Other factors that have been found to correlate with higher aldosterone levels include increased blood pressure (85), female sex (100), increased inflammation (101), increased triglycerides (102), and decreased HDL cholesterol (102). In addition, greater circulating aldosterone is associated with several metabolic abnormalities of obesity including insulin resistance (91, 95, 96, 102) and the metabolic

syndrome (103-107). Interestingly, adipose tissue contains all components of the RAAS, and increased adipose-tissue and circulating RAAS activity are thought to play key roles in the metabolic abnormalities and hypertension that often accompany obesity (79, 80, 98, 108-111). A few studies have also found that several different substances released by adipocytes are able to stimulate adrenal aldosterone secretion (112-115). The relationship between aldosterone and adiposity also appears to go in the reverse direction, with several studies finding that exposure to aldosterone and/or activation of the mineralocorticoid receptor in adipose tissue can affect the tissue's development and adipokine expression or secretion (116-118).

2.3.2 Role of Aldosterone in Cardiac and Vascular Decline

Studies in hypertensives have shown BP-independent associations between increased circulating aldosterone and reduced systemic arterial compliance (119), and studies in both hypertensives (58) and normotensives (59) have found BP-independent associations between elevated aldosterone and heart-femoral pulse wave velocity (hfPWV), a measure of aortic stiffness. In addition, individuals with primary aldosteronism have greater arterial stiffness than either normotensives or hypertensives with normal aldosterone matched for BP and duration of hypertension (120). One study in hypertensive individuals found that aldosterone-to-renin ratio (ARR), which reflects an excess secretion of aldosterone relative to renin secretion, is positively associated with several measures of arterial stiffness, including cfPWV (121). Another study found a positive correlation between ARR and carotid-femoral PWV in normotensive individuals (122). However, not all studies have found such a relationship (58, 123). Significant BP-independent reductions in conduit and resistance artery stiffness have been reported with the aldosterone antagonists spironolactone and eplerenone (124, 125). Elevated aldosterone levels

also contribute to adverse cardiac remodeling, particularly left ventricular hypertrophy and fibrosis, which can lead to heart failure and acute vascular events (126). Together this evidence suggests that aldosterone, through mineralocorticoid receptors present throughout the cardiovascular system, participates in cardiac and vascular damage, leading to hypertension and CVD. Because aldosterone is closely associated with obesity and cardiometabolic risk, overweight and obese individuals may be at an increased risk for the decline in cardiometabolic health caused by inappropriate aldosterone levels.

2.4 PLATELET ACTIVITY

2.4.1 Mechanisms and Measurement of Platelet Activation

Elevated platelet activity is another mechanism that may link obesity to cardiovascular decline. Platelets play a key role in the normal hemostasis response to injury, but they are also responsible for the formation of pathologic thrombi that cause such events as acute coronary syndrome (ACS), unstable angina, myocardial infarction (MI), ischemic stroke/transient ischemic attack and symptomatic peripheral artery disease (PAD) (127). It is well established that platelets also act as mediators of inflammation, contribute to atherogenesis, and have immunomodulatory activity (127). The initial step in primary hemostasis occurs when platelets adhere to the extracellular matrix. Platelets roll and spread on the collagen matrix to form an activated monolayer. At sites of vascular injury, platelet adhesion is mediated by the interactions between (1) the glycoprotein (GP) Ib/V/IX receptor complex on the platelet surface and von Willebrand factor (vWF) and (2) GPVI/GPIa and collagen (127). Under high shear, as found in

small arteries and arterioles, the interaction between vWF and GPIb/V/IX is necessary for the initial adhesion of platelets to the subendothelium. vWF interacts with GPIb/V/IX only when platelets become immobilized on exposed collagen at sites of injury. Platelet activation and recruitment to sites of vascular injury is stimulated by platelet secretion products and local prothrombotic factors (127). Many different pathways lead to platelet activation, including those stimulated by collagen, adenosine diphosphate (ADP), thromboxane A2, epinephrine, serotonin and thrombin. Activation results in platelet shape change, expression of pro-inflammatory molecules such as P-selectin and soluble CD40 ligand (sCD40L), procoagulant activity, and conversion of GPIIb/IIIa into an active form (127, 128). These changes enable platelet aggregation and provide the potential for pathologic thrombosis. Thrombin-mediated generation of fibrin from fibrinogen also contributes to the hemostasis, providing a link between platelets and the coagulation cascade (127, 128).

An important link between atherosclerosis progression and platelet function is the role played by platelets in the migration of vascular smooth muscle cells (VSMCs) from the media to the intima. Accumulation of platelets at sites of vascular injury can cause excessive proliferation of VSMCs. This proliferation is a contributing factor to a number of vascular disease states, including atherosclerosis and hypertension (129, 130). Platelets contain an assortment of growth factors (GFs), that play a crucial role in wound repair, angiogenesis, and defense against infectious agents (131). However, when platelets become hyperactive, they promote atherosclerosis and/or cause an acute thrombotic event. Antiplatelet drugs, such as aspirin, reduce the levels of platelet-secreted proteins (132) and reduce the risk of CVD events (133, 134). However, the percentage of people who do not respond adequately to antiplatelet therapies is high (130, 131). In addition, the increased bleeding risk associated with these therapies makes

non-pharmacologic options to reduce platelet activation a better option for primary CVD prevention in healthy individuals (134).

Many different methods are used to quantify platelet activation. Because platelet activation comprises a change in platelet shape, platelet aggregation, and the release of granule contents from within the platelet, activation can be quantified by diverse factors such as a change in shape and the corresponding tendency to aggregate or by measuring the blood or urine levels of platelet metabolic products (128). Methods to measure in vitro platelet activation are most frequently used. One such in vitro method involves the use of a platelet aggregometer to measure aggregation response to agonists such as ADP, collagen, or thrombin (128). The extent of aggregation is quantified by the amount of light that passes through platelet rich plasma in comparison with platelet-free plasma (128). Platelet adhesion response to a collagen or endothelial cell surface is another measure of in vitro activation (128). It is important to keep in mind that measures of in vitro platelet activation do not accurately quantify the true amount of activation occurring in vivo (135, 136). The basal level of activation taking place in vivo, which has a wide range in healthy adults, likely plays an influential role in atherosclerosis progression (128). Measures of in vivo platelet activation include flow cytometry detected expression of surface proteins expressed upon activation such as P-selectin (128) and measures of soluble markers in plasma or urine such as beta thromboglobulin (β-TG), platelet factor 4, soluble P-selectin, or thromboxane (128). Results from plasma β-TG measurement are comparable to those obtained by flow cytometry (137), but β-TG measurement is less costly and does not require immediate measurement using fresh blood samples. Thus plasma β-TG measurement is more logistically realistic for large epidemiology studies.

2.4.2 Platelet Activity and Cardiovascular Risk Factors

Numerous cardiovascular risk factors have been found to correlate with elevated platelet activity (15). Several studies have shown a positive relationship between platelet activation and BMI and/or a decrease in platelet activation with weight loss (13, 138-142). Larger volume platelets contain more dense granules, are more metabolically and enzymatically active, and have higher thrombotic potential than smaller platelets, which may explain the increased platelet activity in obese individuals (13). Obese individuals also have greater platelet activity as measured by urinary 11-dehyhdro-TxB_2 (138), plasma sCD40L (139), or agonist induced platelet aggregation (140). One small study (n=65), however, found no significant differences between obese and non-obese individuals in surface markers of activation detected with flow cytometry (14). High blood pressure and the presence of hypertension have been associated with increased platelet activation (143, 144). Increased waist-hip ratio, BMI, and CRP have been associated with multiple measures of platelet activation in obese women (138). Measures of platelet activation are higher in cigarette smokers and individuals with hypercholesterolemia (145), metabolic syndrome (146), or Type 2 diabetes (138, 146). Female gender is associated with increased in vitro platelet reactivity, but it remains unclear if in vivo activation varies by sex in healthy individuals (147-149). Leptin and insulin, which are elevated in individuals with metabolic syndrome and/or obesity, have been shown to participate in platelet aggregation and activation (13). Normal insulin levels inhibit platelet aggregation and activation, but insulin resistance has been linked to platelet hyperactivity (13, 150). Leptin has a strong positive effect on platelet activation (13). Decreased vascular endothelial production of prostacyclin and nitric oxide in insulin resistant individuals promote the activation of platelets, as does the osmotic effect of hyperglycemia (151). Together, these relationships help to explain the increased risk of

thrombosis in obesity. Fortunately, it appears that weight loss and other positive lifestyle changes may be able to reduce platelet activation (13, 138).

2.4.3 Platelet Activity and Subclinical Cardiovascular Disease

Platelet hyperactivity occurs simultaneously with vascular remodeling as measured by carotid intima-media thickening and arterial stiffening. Greater platelet activation has been associated with increased CIMT in several cross-sectional studies (152-159). Three of these studies specifically found plasma β-TG to be associated with CIMT (153-155). However, two of these three studies were small and none consisted of a majority of individuals free of prevalent CVD. In addition, none investigated associations between platelet activation and segment-specific CIMT. Some tudies have also shown a prospective association between greater platelet activation or aggregation and greater CIMT progression (160, 161) or a reduction in CIMT progression with antiplatelet drug treatment (162-164). In contrast, one study found no relationship between platelet activation, as measured by mean platelet volume (MPV), and CIMT (165). This study included only patients who had an indication for coronary angiography, thus the characteristics of this particularly high risk sample may explain the different results of this study.

Arterial stiffness has been shown to correlate with platelet activation in a small number of studies. Yamasaki et al. found that platelet activation, measured by flow cytometry detected surface expression of P-selectin, was positively correlated with both brachial-ankle PWV and heart-brachial PWV (166). Dotsenko et al. found that increased platelet activation, as measured by circulating platelet-monocyte complexes (PMC), was associated with aortic PWV (167). Both of these PWV studies had small sample sizes (N<60), so larger studies are needed to

reliably determine if platelet activation and arterial stiffness are related. One large study of apparently healthy Chinese adults found that MPV was positively associated with baPWV independent of other CVD risk factors (168). Importantly however, this study did not measure any inflammatory markers, and inflammation is an important confounder in the relationship between platelet activation and vascular health. Similarly to the studies of PWV, greater platelet activation has been shown to be positively correlated with carotid stiffness index β, a measure of local artery stiffness in one study (152). Although none of these cross-sectional studies of platelet activation and subclinical CVD measures could determine causality, it is possible that the accumulation of activated platelets at sites of vascular injury plays a causal role in vascular remodeling and atherosclerosis. Conversely, it is also possible that atherosclerotic plaque formation and changes in shear stress and endothelial structure influence platelet activation. The present research project investigates not only cross-sectional associations between subclinical CVD measures and platelet hyperactivity, but also determines whether platelet activity or increases in platelet activity that occur after the conclusion of a lifestyle intervention are associated with changes in or cumulative exposure to subclinical atherosclerosis and arteriosclerosis. These are key questions to address in the targeting of healthy overweight and obese adults for CVD prevention.

2.4.4 Genetic Variants in Genes Encoding Platelet Membrane Receptors

In addition to the CVD risk factors discussed above, genetic factors also influence in vivo platelet activation. A number of common functional variants in platelet membrane receptors have been associated with platelet function, and several have been linked to an increased risk of cardiovascular events. Many molecules are important in platelet activation and aggregation:

coagulation factors, inflammatory factors, endothelium-derived molecules, platelet receptors and others. Importantly, platelet receptors are the key mediators between platelets and other cells or molecules in both acute thrombosis and chronic atherosclerosis (169). Allelic variants in genes encoding platelet membrane receptors have been found to be associated with altered platelet activation or aggregation (170-175) and/or an increased risk of clinical CVD events (169, 176-189). Examples of such variants include single nucleotide polymorphisms (SNPs) present in the genes encoding glycoprotein (GP) IIIa (rs5918), $P2Y_{12}$ (rs2046934), protease-activated receptor 1 (PAR-1) (rs168753), GP VI (rs1613662), and GP Ib (rs2243093, rs6065). Each of these six SNPs has been found to be associated with the risk of cardiovascular events and/or with excess platelet activation, but the associations for each SNP have been inconsistent (169-191), likely due to the small sample size of many of the studies and the heterogeneity of outcomes. Each of the membrane receptors containing these genetic variants plays a critical role in platelet function. GP IIb/IIIa is a receptor for fibrinogen and von Willebrand factor (vWF) (169). $P2Y_{12}$ is an adenosine diphosphate (ADP) receptor that is essential for complete aggregation response to ADP (192). PAR-1 is the most potent thrombin activated receptor (193). GP Ib is part of the GP Ib-IX-V complex, which attaches platelets to vWF, a crucial step in plaque progression and thrombus development (169). GP VI is a collagen receptor that augments the transduction of signals initiated by GP Ib-IX-V (194). SNP Pl^{A1}/Pl^{A2} (rs5918) leads to a leucine to proline substitution at position 33 in GP IIIa. Results from studies evaluating the association of the Pl^{A1}/Pl^{A2} SNP with clinical CVD endpoints have been both positive and negative (169). SNP 744T/C (rs2046934) defines the H1/H2 haplotype in P2Y12, the latter of which has been associated with increased reactivity to ADP as well as with peripheral arterial disease (PAD) and coronary artery disease (CAD) (192). The intervening sequence-14 A/T dimorphism (rs168753)

has been linked to platelet PAR-1 density, activation, and aggregation (170, 171). The A allele has been linked to platelet hyperactivity in patients with CAD (170). SNP T13254C (rs1613662) is responsible for changing amino acid 219 in GP VI from serine to proline. Genotype 13254CC has been associated with risk of MI (194). GPIb is part of the GP Ib-IX-V complex, which attaches platelets to vWF, a crucial step in plaque progression and thrombus development (169). The "Kozak"-sequence SNP (rs2243093) is correlated with platelet surface density of GP Ibα (169, 195). The C allele has been associated with acute coronary syndrome (ACS) in some populations (169, 196). The Thr145Met SNP (rs6065) in GP Ibα is in linkage disequilibrium with a variable number of tandem nucleotide repeats (VNTR) polymorphism. The haplotypes composed of Met145 and VNTR A (four repeats) or VNTR B (three repeats) have been associated with MI and stroke (169, 180). According to HapMap data, each of the six SNPs chosen for our study has a minor allele frequency (MAF) of >5% in both Yoruba Africans and Caucasian Americans with northern and western European ancestry . Though a substantial amount of research has been performed on platelet receptor gene SNPs and clinical CVD outcomes, further study is needed to determine if these SNPs are associated with intermediate endpoints such as platelet hyperactivity and subclinical CVD in healthy adults.

2.5 ANALYSIS OF LONGITUDINAL STUDIES WITH MISSING DATA

2.5.1 Linear Mixed Effects Models

The primary goal of longitudinal analysis is to assess within-individual changes in characteristics of interest over time and to determine which factors influence heterogeneity among these within-individual changes. Mixed models are widely used in health studies for longitudinal analysis. Linear mixed effects models can be used to model continuous outcome variables and, as the name implies, these models assume that some of the regression parameters vary randomly between subjects (random effects) and some are common to all subjects (fixed effects). The introduction of random effects induces within-subject correlation among outcomes. Such correlation must be accounted for in order to avoid obtaining biased standard errors for both within- and between-subject factors (198). Another appealing aspect of linear mixed effect models, in addition to their capacity to differentiate within-subject and between-subject sources of variation, is their ability to accommodate imbalanced data. Linear mixed effects models, unlike univariate or multivariate repeated-measures analysis of variance (ANOVA), require neither the same number of observations nor the same timing of measurement occasions on all subjects. Thus, mixed models are particularly convenient for handling unbalanced longitudinal data.

In the simplest case of a linear mixed effects model, only the intercept is treated as random, thereby assuming that each subject has a latent underlying level of response that persists throughout the study duration:

$$Y_{it} = X'_{it}\beta + b_i + e_{it}$$

In this model, b_i is the random intercept for subject i and e_{it} is the measurement or sampling error for subject i and time t. It is typically assumed that $b_i \sim N(0,\sigma^2_b)$ and $e_i \sim N(0, \sigma^2 I_{ni})$ where I_{ni} is the n_i-dimensional identity matrix, though additional within-subject serial correlation beyond that accounted for by random effects can be investigated. In addition, b_i and e_{ij} are assumed to be independent of one another. In this model, the conditional mean of Y_{ij} given the subject-specific effect, is:

$$E(Y_{it}|b_i) = X'_{it}\beta + b_i$$

and the marginal mean of Y_{ij} in the population (averaged over the subject-specific effects) is:

$$E(Y_{it}) = X'_{it}\beta$$

Linear mixed effect models can also include random coefficients. For example, in longitudinal studies time is often treated as a random effect. In general, a linear mixed effect model is any model that satisfies the four properties below:

$$Y_i = X_i\beta + Z_i b_i + e_i$$

$$b_i \sim N(0, D)$$

$$e_i \sim N(0, \Sigma_i)$$

$$b_1,\ldots, b_N, e_1,\ldots,e_N \text{ independent}$$

where Y_i is the n_i-dimensional response vector for subject i, $1 \le i \le N$, N is the number of subjects, X_i and Z_i are (n_i x p) and (n_i x q) dimensional matrixes of known covariates, β is a p-dimensional vector containing the fixed effects, b_i is a q-dimensional vector containing the random effects, and e_i is an n_i-dimensional vector of residual components. D is a general (q x q) covariance matrix with (i, j) element $d_{ij} = d_{ji}$ and Σ_i is a (n_i x n_i) covariance matrix which depends on i only through its dimension n_i (199).

Though the main goal of a longitudinal study is to investigate within-individual changes in responses over time, longitudinal studies provide both longitudinal and cross-sectional information. These two sources of information can sometimes be at odds. Care must be taken in model specification in order to avoid the confounding of longitudinal effects with cross-sectional effects when the two differ. This can be accomplished by including separate parameters for the cross-sectional (between-subject) and longitudinal (within-subject) effects of time-varying variables in the model, as shown below:

$$Y_{ij} = Z'_i\beta_0 + X'_{i1}\beta^{(C)} + (X'_{ij} - X'_{i1})\beta^{(L)} + e_{ij}$$

where X'_{ij} is the row vector of q time-varying covariates for the j^{th} response on the i^{th} subject and Z'_i is the row vector of p – q time-stationary covariates. This model allows the simultaneous estimation of both cross-sectional effects, $\beta^{(C)}$, and longitudinal effects, $\beta^{(L)}$. When investigating the associations between time-varying covariates and an outcome of interest during an intervention, such as in this dissertation, it is mainly $\beta^{(L)}$ that is of interest (198).

2.5.2 Missing Data

Although mixed models for longitudinal data have many advantages, they are not guaranteed to produce unbiased parameter estimates in studies with missing data. Missing data are ubiquitous in longitudinal biomedical research, in which missing data usually occur in the form of dropouts. Since the form of the non-response process can never be fully known, assumptions must be made in any analysis of available data (200). According to widely used terminology first conceived by Rubin (201), missing data are missing completely at random (MCAR) if missingness is independent of both unobserved and observed outcome and covariate data, and missing at random (MAR) if, conditional on the observed outcome and covariate data, missingness is

independent of the unobserved data. Missing data that is neither MCAR nor MAR is termed missing not at random (MNAR). In the context of likelihood inference, which is used in linear mixed effects modeling, when the parameters describing the measurement process are independent of the parameters describing the missingness process, MCAR and MAR processes are ignorable whereas an MNAR missingness process is non-ignorable. Thus, as long as the observed outcome and covariate data included in a linear mixed effects model are sufficient to bring about a MAR mechanism for the missing data, the parameter estimates of the model will be unbiased. This is not the case for frequentist methods such as repeated-measures ANOVA, which require the missing data to be MCAR (200). In the past, simple methods for dealing with missing data, such as last observation carried forward (LOCF), single imputation, and complete case analysis have been popular. However, given the commercial software available today, there is little reason to use these simple, typically biased methods (200).

To examine the non-response process, one must first assume that the outcome vector, $\boldsymbol{Y}_i = (Y_{i1}, \ldots, Y_{in})$, contains a sequence of responses designed to be measured at occasions $j = 1, \ldots, n$ for all subjects $i = 1, \ldots, N$. Next, one can define a dropout indicator D_i for the occasion at which dropout occurs and assert that $D_i = n + 1$ for a complete sequence. $\boldsymbol{Y}_i$ can be split into observed ($\boldsymbol{Y}_{oi}$) and missing ($\boldsymbol{Y}_{mi}$) components. Generally the aim is to examine the full data density $f(\boldsymbol{y}_i, d_i|\boldsymbol{\theta},\boldsymbol{\psi})$, in which the parameter vectors $\boldsymbol{\theta}$ and $\boldsymbol{\psi}$ describe respectively the measurement and missingness processes. To examine the full data, one method that can be used is pattern-mixture modeling, which is based on the factorization

$$f(\boldsymbol{y}_i, d_i|\boldsymbol{\theta},\boldsymbol{\psi}) = f(\boldsymbol{y}_i|d_i,\boldsymbol{\theta})f(d_i|\boldsymbol{\psi})$$

Pattern-mixture models can easily be seen to be a mixture of subpopulations each characterized by a distinct non-response pattern. This method assume that covariates included in

the analysis are fully observed, often not the case for time-varying covariates in longitudinal studies (200). Using multiple imputation, however, both missing covariate and outcome data can be imputed consistent with an a priori hypothesis for the missing data process, often called an identifying restriction.

An important problem with pattern-mixture models is that they are always under-identified. There are two main strategies used for pattern-mixture modeling, and they handle the problem of under-identification quite differently (202). Little, Thijs, and others have advocated the use of identifying restrictions, in which data that are unavailable for a particular pattern are borrowed from a pattern or patterns in which such data are available (202, 203). Alternatively, model simplification can be used to identify parameters. With this technique, parameters are made to vary across patterns in a controlled parametric way by including pattern as a covariate in the pattern-mixture model. Though the second strategy is computationally simple, it requires the untestable assumption that it is appropriate to extrapolate time trends beyond the point of dropout. The first strategy, on the other hand, can accommodate a greater variety of hypotheses about the missing data mechanism through the use of multiple imputation (202).

Multiple imputation is a valuable tool for longitudinal biomedical research studies, especially in the area of sensitivity analysis. In multiple imputation, the imputation model can be easily changed to reflect hypothesized departures from the MAR assumption and the analytical model subsequently refitted to the imputed data (204). With a general (non-monotone) pattern of missingness, such as occurs in many clinical trials and observational epidemiologic studies, Bayesian methods based on Markov Chain Monte Carlo (MCMC) can be used to multiply impute missing covariate and outcome data. This method assumes that the missing data, given the observed data, follows a multivariate normal distribution. The method is based on the

construction of a Markov chain long enough for the distributions of the imputed variables to stabilize to a stationary distribution (204).

3.0 ASSOCIATIONS BETWEEN ALLELIC VARIANTS IN GENES ENCODING PLATELET MEMBRANE RECEPTORS, CAROTID INTIMA-MEDIA THICKNESS, AND CIRCULATING PLATELET ACTIVITY IN OVERWEIGHT AND OBESE YOUNG ADULTS

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3.1 ABSTRACT

Objectives Obesity is associated with increased platelet activation and elevated cardiovascular disease risk, but the role of platelet activation in early atherosclerosis and whether genetic variants influence activation in obese individuals remains unknown. We aimed to determine whether (1) allelic variants in genes encoding platelet membrane receptors are associated with

segment-specific carotid intima-media thickness (IMT) and circulating platelet activity, as measured by plasma beta-thromboglobulin (β-TG) (2) positive lifestyle changes counteract any excess risk associated with these genetic variants, and (3) lower circulating platelet activity one year after a behavioral weight loss intervention is associated with smaller carotid IMT and greater weight loss.

Methods This analysis included participants in the Slow Adverse Vascular Effects of excess weight (SAVE) trial, a study evaluating the effects of positive lifestyle changes on vascular health in normotensive overweight and obese young adults. For the genetic study, 266 non-Hispanic white participants were examined. For the study of platelet activity, 92 individuals were assessed.

Results At baseline, T allele carriers of rs168753 in the gene encoding PAR-1, the main thrombin receptor, had higher carotid bulb IMT after adjustment for cardiovascular risk factors ($p=0.02$). This association was no longer significant one year after the lifestyle intervention. Lower plasma β-TG one year after the intervention was associated with smaller common carotid artery IMT and greater weight loss ($p<0.05$ for both).

Conclusions Reduced circulating platelet activity may be one pathway by which weight loss reduces cardiovascular risk in young adults with excess weight. Weight loss may counteract any potential atherosclerotic risk associated with carrying the T allele of rs168753.

3.2 INTRODUCTION

Platelets play a key role in the processes of atherosclerosis and thrombosis that cause acute cardiovascular events. Platelets release inflammatory molecules and growth factors, thereby

stimulating the migration and proliferation of vascular smooth muscle cells and monocytes in early atherosclerosis (127, 169). In populations at moderate to high cardiovascular disease (CVD) risk, increased platelet activation has been associated with increased carotid intima-media thickness (IMT) (152-156, 159), a marker of subclinical atherosclerosis and an established predictor of cardiovascular events(27). In addition, platelet activation is increased in individuals with hypercholesterolemia, type II diabetes, and hypertension (135, 205), and several studies have detected excess platelet activation in obese individuals and a reduction in platelet activation with weight loss (13, 138, 159), which suggests that reduced platelet activation may be one of the numerous mechanisms by which weight loss slows the progression of atherosclerosis in obese individuals.

The ability of platelets to mediate atherosclerotic processes is dependent on their membrane receptors, which enable platelets to attach to leukocytes, endothelial cells, and molecules such as thrombin and fibrinogen. Platelets become activated when their membrane receptors contact specific agonists such as collagen, adenosine diphosphate (ADP), or thrombin (169). This activation enables platelets to adhere to extracellular matrix components and subsequently aggregate (127). Several allelic variants in genes encoding platelet membrane receptors have been repeatedly associated with altered platelet activation or aggregation (170-175) and/or an increased risk of clinical CVD events (169, 176-189). Examples of such variants include single nucleotide polymorphisms (SNPs) present in the genes encoding glycoprotein (GP) IIIa (rs5918), $P2Y_{12}$ (rs2046934), protease-activated receptor 1 (PAR-1) (rs168753), GP VI (rs1613662), and GP Ib (rs2243093, rs6065), each of which plays a critical role in platelet function (169, 192-194). Each of these SNPs has been associated with platelet activation and examined as a predictor of cardiovascular disease, but the associations for each SNP have been

inconsistent (169-191, 206), likely due to the small sample size of many of the studies and the heterogeneity of the outcomes studied. Because of the importance of platelet activation in atherosclerotic progression, further study is warranted to determine if these variants are associated with subclinical atherosclerosis in healthy adults. In addition, there is some evidence that both traditional (49, 50) and genetic (52) risk factors may have heterogeneous effects on the different segments of the carotid artery. The distinctive anatomy and hemodynamics in each segment of the carotid artery support different pathological mechanisms (49, 50), such that proatherosclerotic genetic variants may contribute differently to intimal thickening in the common carotid artery (CCA), internal carotid artery (ICA), and carotid bulb.

The Slow Adverse Vascular Effects of Excess Weight (SAVE) trial is a randomized clinical trial evaluating the effects of positive lifestyle changes on vascular health in normotensive overweight and obese young adults. Because obesity has been associated with both increased platelet activation and subclinical atherosclerosis, this trial provided a valuable opportunity to test whether (1) previously studied allelic variants in genes encoding platelet membrane receptors are associated with segment-specific carotid IMT and circulating platelet activity, as measured by plasma beta-thromboglobulin (β-TG) (2) positive lifestyle changes counteract any excess risk associated with these genetic variants, and (3) lower circulating platelet activity one year post-intervention is associated with smaller carotid IMT and greater preceding weight loss.

3.3 METHODS

3.3.1 Study Population

The Slow Adverse Vascular Effects of excess weight study (SAVE) is a randomized-controlled trial (NCT00366990) evaluating the effects of weight loss, increased physical activity, and reduced dietary sodium intake on vascular health. Participants were recruited from June 2007 through May 2009 using mass mailing.

Eligible participants were men and women 20-45 years of age who were overweight or obese (body mass index (BMI) 25-39.9 kg/m^2) and physically inactive (<8 months of consistent physical activity (PA) during the past 12 months). Exclusions included 1) diabetes, 2) hypertension or average screening blood pressure ≥140/90 mmHg, 3) cholesterol lowering, anti-psychotic, or vasoactive medication use and 4) current pregnancy or lactation. For the present genetic study, those participants who were of non-Hispanic white race and had genotype data for at least one SNP under investigation were included (n=266). Participants of any race who provided a blood sample for the measurement of β-TG at the final study visit were included in the analysis of platelet activity (n=92). All subjects signed informed consent, and the study was approved by the institutional review board of the University of Pittsburgh (Pittsburgh, PA).

3.3.2 Intervention

Three hundred and forty-nine participants received a 1-year lifestyle intervention consisting of diet and physical activity (PA). Participants were randomized to either 1) diet and PA alone (Control Na/lifestyle) or to 2) diet and PA plus reduced sodium intake (Low Na/lifestyle). The

lifestyle intervention was delivered in group sessions that occurred weekly for months 1-4, biweekly for months 5-8, and monthly for months 9-12. The goal of the intervention was a 10% reduction in body weight over 6 months and continued maintenance of weight loss thereafter. The additional goal of the sodium reduction intervention (Low Na) was to gradually reduce daily sodium intake to approximately 1 mg Na^+/1 kcal/day, an average reduction of about 50% from the participant's usual diet (207).

3.3.3 Clinic Visits

Participants were to complete clinic visits at screening, baseline, and 6, 12, and 24 months following randomization. Self-reported demographic information, self- and interviewer-administered questionnaires, anthropometric measurements, fasting blood draw, 24-hour urine collection, and non-invasive tests of vascular structure and function were collected at these visits.

3.3.4 Demographic and Physical Measures

Age, race, and smoking status were self-reported. Race was coded as White or Caucasian, Black or African American, Asian, American Indian or Alaska Native, Native Hawaiian or other Pacific Islander, or other. Ethnicity was coded as Hispanic or Non-Hispanic. Smoking status was assessed as current or past vs. never. Weight was measured in kilograms using a balance scale. Height was measured in centimeters using a stadiometer. BMI was calculated as weight in kilograms divided by height in meters squared. Waist circumference was measured against the participant's skin at the narrowest part of the torso between the ribs and the iliac crest. Blood Pressure (BP) was measured with a mercury sphygmomanometer after participants sat quietly for

5 minutes with feet flat on the floor. Final BP was the average of the last 2 of 3 readings taken 30 seconds apart.

3.3.5 Blood Assays

Blood analytes were measured at the Heinz Laboratory at the University of Pittsburgh's Graduate School of Public Health. Total cholesterol and high density lipoprotein cholesterol (HDL-C) were determined using the enzymatic method of Allain et al.(208). HDL-C was determined after selective precipitation by heparin/manganese chloride and removal by centrifugation of very low density lipoprotein and low density lipoprotein cholesterol (LDL-C)(209). LDL-C was calculated indirectly using the Friedewald equation. Triglycerides were assessed enzymatically using the procedure of Bucolo et al.(210). C-reactive protein (CRP) was measured using an enzyme-linked immunoassay (Alpha Diagnostic International, Inc., San Antonio, TX). Serum glucose was determined enzymatically with a procedure similar to that described by Bondar and Mead (211). Insulin, leptin, and adiponectin were measured using radioimmunoassays developed by Linco Research, Inc. (St. Charles, MO). The intra- and inter-assay CV% for insulin were 4.8% and 10.5% respectively. The CV% for the other assays were all <3%.

3.3.6 Urine Collection

Valid 24-hour urine collections had volume between 500 mL and 4000 mL, duration ≥22 hours and ≤26 hours, and total creatinine within the expected range(212). From April 13, 2007 to March 6, 2009, analytes were measured using an Ortho Vitros 950. Direct potentiometry was

used to measure sodium and colorimetry to assess creatinine levels. Afterward, results were determined using a Beckman Coulter DxC 800 instrument employing an indirect ion selective method for sodium and an alkaline picric kinetic method for creatinine.

3.3.7 Platelet Activity

Circulating platelet activity was measured as plasma β-TG, a platelet-specific alpha granule protein released upon activation (213). Participants were eligible to provide a blood sample for the measurement of plasma β-TG if they had not taken aspirin in the preceding 14 days or any NSAID, antiplatelet, or anticoagulant medication in the preceding 10 days. At the 24 month visit, blood for the measurement of β-TG was drawn into a 4.5 mL vacutainer tube (Becton-Dickinson, Franklin Lakes, NJ) containing an anticoagulant/antiplatelet mixture of citric acid, theophylline, adenosine, and dipyridamole (Thermo Fischer Scientific, Pittsburgh, PA). The tube was chilled on ice for 15-60 minutes then centrifuged at 2000G for 30 minutes at 4°C, after which platelet-poor plasma was obtained from the upper portion of the supernatant and kept frozen at -70°C until assayed. Plasma β-TG was determined using an enzyme linked immunosorbent assay (Asserachrom, Diagnostica Stago, Parsippany, NJ). The intra- and inter-assay CV% were 3.8% and 13.2% respectively.

3.3.8 Genotyping of Polymorphisms

Genomic DNA was extracted from peripheral blood leukocytes from a blood sample provided at the screening visit using a commercially available kit (Qiagen Inc., Hilden, Germany). Genotyping of each variant was performed using the TaqMan method. Assays were purchased

from Applied Biosystems (ABI) (Foster City, CA) and the TaqMan Universal PCR protocol was used to perform the amplification reaction. The plates were placed in the 7900HT Fast Real Time PCR System (ABI), and the fluorescence intensity in each well of the plate was read using the program SDS 2.2.1 (ABI). The call rate for each SNP was ≥96%. Based on the analysis of ≥25 pairs of blind duplicates for each variant, there was 100% concordance in genotyping. All SNPs were in accordance with Hardy-Weinberg equilibrium ($p>0.05$).

3.3.9 Carotid Ultrasound

Carotid ultrasound measures and readings were performed at the Ultrasound Research Laboratory of the Department of Epidemiology, University of Pittsburgh, by sonographers using an Acuson Sonoline Antares high resolution duplex scanner (Siemens, Malvern, PA). At baseline and 24 month visits, digitized images were obtained from 8 locations (4 locations each from the left and right carotid arteries): the near and far walls of the distal common carotid artery (1 cm proximal to the carotid bulb), the far walls of the carotid bulb (the point in which the near and far walls of the common carotid are no longer parallel, extending to the flow divider), and the internal carotid artery (from the flow divider to 1 cm distal to this point). At 6 and 12 month visits, digitized images were obtained from 4 locations, the near and far walls of the distal common carotid artery. IMT measures were obtained by electronically tracing the lumen-intima interface and the media-adventitia interface across a 1-cm segment for each segment; one measurement was generated for each pixel over the area, for a total of approximately 140 measurements for each segment. The reading software used was the AMS system developed by Dr. Thomas Gustavsson (214) which has an edge detection algorithm that allowed much of the reading to be done automatically. For this study the following IMT measures were used: 1) the

mean of the average readings at ICA, 2) the mean of the average readings at the carotid bulb, and 3) the mean of the average readings at the CCA. Reproducibility of IMT was excellent with intraclass correlation coefficients of ≥0.87 between sonographers and ≥0.92 between readers.

3.3.10 Statistical Methods

Descriptive statistics were calculated to summarize study variables at baseline and 6, 12, and 24 months follow-up and were presented as median/inter-quartile range (IQR) or mean (SD) for continuous variables and frequency and percentages for categorical variables. Whether the changes in body size, cardiometabolic risk factors, and IMT were statistically significantly different from zero at each follow-up visit was determined by testing the coefficient for time, as a nominal variable, in linear mixed models with unstructured covariance. Non-normally distributed variables were transformed as necessary before mixed modeling. Intervention arm (Low Na/lifestyle versus Control Na/lifestyle) was included as a covariate in every mixed model for consistency with trial design. Interactions between intervention arm and time since baseline were included only if statistically significant at $p<0.10$.

Next, mean (SD) for the mean ICA IMT, mean CCA IMT, and mean carotid bulb IMT were presented by genotype for each of the six SNPs of interest. A dominant genetic model was employed because of the small number of minor allele homozygotes for all variants. Segment specific carotid IMT measures were analyzed because 1) the different segments of the carotid artery show different relationships with both genetic(52) and traditional cardiovascular risk factors (49, 50) and 2) only CCA IMT was measured at all study visits.

Covariates of interest were the following cardiovascular and/or metaboolic risk factors, all of which are known or hypothesized to have a role in subclinical atherosclerosis: age, sex,

BMI, waist circumference, blood pressure, LDL-C, HDL-C, triglycerides, insulin, C-reactive protein (CRP), leptin, adiponectin, smoking status (ever vs. never), and 24-hour urinary sodium excretion. Pearson correlations or t tests were used to examine the associations between each baseline segment-specific carotid IMT and baseline covariates of interest. Analysis of covariance (ANCOVA) was used to evaluate the relationships between each SNP and IMT; covariates associated with any segment-specific IMT measure at $p<0.10$ were added in order of increasing p-value from correlation analysis and kept if statistically significant in the multivariable model at $p<0.10$. Linear mixed effects models were used to evaluate whether changes in segment-specific IMT over the course of the two year study varied by genotype for any SNP. This was accomplished by testing the interaction between time since baseline and genotype for each SNP. The mixed models included random intercepts and slopes as well as baseline age, sex, intervention arm, and concurrent values of the covariates included in the baseline multivariable models if statistically significant at $p<0.10$. Differences in segment-specific IMT by genotype at 24 months were also determined from the mixed models. The Benjamini-Hochberg method to control the false discovery rate (FDR) was used for each carotid segment individually to correct p values for multiple hypothesis testing.

After log transforming β-TG to normalize its distribution, the association between each SNP and plasma β-TG in non-Hispanic white individuals (n=70) was evaluated with the use of a two sample t test and with ANCOVA after adjustment for age, sex, race, and BMI. Associations between plasma β-TG and IMT or CVD risk factors in individuals of any race were evaluated using Pearson correlations or t tests. Multivariable linear regression models were used to evaluate the relationships between concurrent measures of IMT and β-TG after adjustment for age, sex, race, BMI, and SBP. Similar models were used to evaluate the relationships between

measures of change in body size during the two year study and plasma β-TG at the final study visit. P values ≤0.05 were considered statistically significant. Statistical analyses were performed using SAS (Statistical Analysis Software release 9.2, Cary, NC).

3.4 RESULTS

3.4.1 Genetic Study

Two hundred and sixty-six participants of non-Hispanic white race had both valid genotype and baseline IMT data and thus were included in the genetic study. The mean age of the sample was 37.9 (SD 6.3) years, 74.8% of the sample was female, and 39.5% were either past or current smokers. With the exception of race, there were no significant differences between these subjects and other SAVE trial participants. Key clinical characteristics and IMT measures over the course of the two year study are presented in Table 3.1.

All baseline segment-specific IMT measures were positively associated with male sex, greater age, BMI, waist circumference, and SBP. Baseline ICA IMT was additionally positively associated with LDL-C. Baseline carotid bulb IMT was additionally positively associated with LDL-C and triglycerides and negatively associated with HDL-C. Baseline CCA IMT was additionally negatively associated with HDL-C and positively associated with triglycerides ($P<0.05$ for all). Because BMI and waist circumference were highly correlated ($r=0.72$, $p<0.0001$) and correlations with IMT measures were stronger for BMI than waist circumference, only BMI was retained in multivariable regression models. In multivariable linear regression models, with adjustment for sex and baseline age, BMI, SBP, LDL-C, and HDL-C, C allele

carriers of rs5918 in the gene encoding GPIIIa had a higher mean CCA IMT, C allele carriers of rs2243093 in the gene encoding GPIbα had a higher mean bulb IMT, and T allele carriers of rs168753 in the gene encoding PAR-1 had a higher mean carotid bulb IMT at baseline (Table 3.2). However, only the association with rs168753 remained statistically significant after FDR-adjustment for multiple testing. There were no significant associations found between any SNP and ICA IMT.

Weight loss was statistically significant at all follow-up time points. In addition, the CVD risk profile was significantly improved from baseline at all follow-up visits, with the largest improvements seen at either 6 or 12 months (Table 3.1). CCA IMT was not significantly changed at six months but was increased at 12 and 24 months. Carotid bulb IMT also increased between baseline and 24 months (Table 3.1). From linear mixed models adjusting for intervention arm, the mean rates of progression of segment-specific IMT were 0.007 mm/yr (SE 0.001) for CCA IMT, -0.006 mm/yr (SE 0.004) for ICA IMT, and 0.01 mm/yr (SE 0.004) for carotid bulb IMT. Sodium excretion was the only measure that differed over time by intervention arm; the reduction in urinary sodium was greater in those randomized to the Low Na/lifestyle intervention at 6 and 24 months ($p<0.05$) but did not differ between groups at 12 months ($p=0.74$).

In longitudinal analyses, mixed modeling showed that changes in segment-specific IMT over the course of the intervention and post-intervention periods did not differ statistically significantly by genotype for any of the investigated allelic variants. Importantly, the significant baseline difference in mean carotid bulb IMT between T allele carriers and non-carriers at rs168753 was no longer statistically significant at the 24 month time point (mean carotid bulb

IMT = 0.71 (95% CI 0.68, 0.72) in T allele carriers and 0.70 (95% CI 0.68, 0.75) in non-carriers, p=0.34) in a linear mixed model adjusting for age, sex, and concurrent BMI, SBP, and LDL-C.

3.4.2 Study of Circulating Platelet Activity

Plasma β-TG was measured in 92 individuals at the 24 month time point. The demographic and clinical characteristics of this sample are shown in Table 3.3. With the exception of SBP, which was slightly lower among individuals in whom platelet activity was measured, characteristics at the 24 month visit did not differ between trial participants with and without platelet activity data. Median plasma β-TG was within the normal range and did not differ by age, sex, or race. None of the investigated SNPs was associated with plasma β-TG in non-Hispanic white individuals either in univariable or multivariable models. Higher plasma β-TG was correlated with higher concurrent BMI and serum leptin (Table 3.4), though the association between β-TG and leptin lost significance after adjustment for BMI (p=0.49). Plasma β-TG was also significantly correlated with concurrently measured CCA IMT but not with ICA IMT or carotid bulb IMT (Figure 1). However, the lack of statistically significant correlation between ICA IMT and plasma β-TG appeared to be due to the influence of one subject with unusually large ICA (1.64 mm) and bulb IMT (1.53 mm) measurements. When this individual was removed from the analysis, the correlation between plasma β-TG and ICA IMT became statistically significant (r=0.21, p=0.046), though the correlation between plasma β-TG and carotid bulb IMT did not (r=0.07, p=0.50). In multiple linear regression models adjusting for age, sex, race (black/non-black), BMI, and SBP, plasma β-TG was not significantly associated with any segment-specific IMT measure (p>0.20 for all). When the individual with unusually high ICA and bulb IMT was excluded, these associations remained nonsignificant. With regard to the effect of the lifestyle

intervention, lower β-TG at the 24 month follow-up visit was correlated with greater reductions in BMI (r=0.22, p=0.03) and weight (r=0.22, p=0.03) but not waist circumference (r=0.12, p=0.25) during the study. In multiple linear regression models, BMI reduction over the course of the study was a significant predictor of lower circulating platelet activity at the end of the study (Table 3.5).

3.5 DISCUSSION

An importantfinding in this study was that, among non-Hispanic white overweight/obese young adults, T allele carriers of SNP rs168753 in the gene encoding PAR-1, the main platelet thrombin receptor, had a higher mean carotid bulb IMT than non-carriers before but not one year after a lifestyle intervention. In addition, higher plasma β-TG, a measure of circulating platelet activity, was not associated with any of the investigated allelic variants in genes encoding platelet membrane receptors but was correlated with greater CCA IMT. In addition, lower plasma β-TG measured one year after the conclusion of the intervention was correlated with lower concurrent BMI as well as with greater reductions in body size. To our knowledge, this is the first study to show 1) an association between carotid IMT and an allelic variant in the gene encoding PAR-1 and 2) an association between weight loss and lower circulating platelet activity a considerable time after the weight loss intervention occurred.

Our findings suggest that the T allele of variant rs168753 in the gene encoding PAR-1 may be associated with increased carotid bulb IMT in healthy overweight and obese adults. The A allele in rs168753 has been linked to increased platelet PAR-1 density, increased sensitivity to a PAR-1 activating peptide, and higher P-selectin expression in one study of healthy white males

(171). However, in a small study of platelets from healthy Swedish adults of both sexes, no significant association between rs168753 genotype and PAR-1 receptor density or platelet reactivity was found (190). Furthermore, a recent case-control study in a European population found that this allelic variant was not associated with symptomatic carotid stenosis (206). Thus, it remains unclear if this SNP plays any role in platelet activation or atherosclerotic progression, and if so which allele may increase risk. Nevertheless, it is known that platelet PAR-1 activation plays an important role in inflammatory response and neointimal proliferation at sites of vascular injury (215). In addition, PAR-1 is expressed in other vascular cells such as leukocytes, smooth muscle cells, and endothelial cells, and in these cells it also mediates responses that contribute to atherosclerotic progression (127, 216). Given that in this study, rs168753 was associated with only carotid bulb IMT, which is the carotid segment most prone to atherosclerotic plaque development (217), it could be that this SNP plays a role in early intimal plaque formation but not medial hypertrophy, the primary response to elevated shear and tensile stress that drives CCA intima-media thickening in healthy adults (218). Interestingly, we found no associations between rs168753 and segment-specific CIMT at the end of the two year study or progression over the course of the study, thus it could be that positive lifestyle modifications eliminated the influence of this SNP on carotid bulb IMT. There is substantial evidence from gene-lifestyle interaction studies suggesting that lifestyle factors determine an individual's propensity to develop obesity and obesity-related conditions and that genetic susceptibility may be partly or even completely controlled by lifestyle modifications (219). Thus, it is possible that positive lifestyle changes during the intervention were sufficient to remove the atherosclerotic risk associated with the T allele of rs168753. However, it cannot be disregarded that the absence of this genetic association post-intervention could result from measurement error or selection bias

during follow-up. Importantly, it is also possible that rs168753 is in linkage disequilibrium with other SNPs that influence carotid IMT. Though no studies have reported an association between variants in the gene encoding PAR-1 and carotid IMT, three SNPs in this gene: an intronic SNP, a variant in the promoter region, and a SNP in the upstream regulatory region of PAR-1, have all been linked to an increased risk of CHD (220, 221). These three SNPs were not chosen for evaluation in this study because, to our knowledge, they had not been associated with altered platelet function in any previous studies. It could be that one or more of these SNPs is linked to rs168753, however we could not verify this post-hoc as rs168753 was not genotyped in the HapMap project. Finally, it is important to note that genome-wide association studies of segment-specific carotid IMT have not identified SNPs near or within genes encoding platelet membrane receptors, though SNPs in other genes relevant to platelet biology were identified (222, 223).

In this study, we found no association between circulating platelet activity and any of the studied allelic variants. One potential reason for the lack of association between rs168753 and plasma β-TG could be that, because the production of thrombin, the main PAR-1 ligand, is constrained to cell surfaces, it is short lived in circulation and thus its role in the response to vascular injury can only be measured locally (193). In contrast, plasma β-TG is a measure of circulating (systemic) platelet activity. We did detect a correlation between β-TG and CCA IMT, though this association lost significance in a multivariable model. This suggests that circulating platelet activity may influence the generalized response of the vascular wall to obesity and other risk factors, as represented by CCA IMT, but not local plaque development, as represented by carotid bulb IMT. Elevated platelet activation can accelerate atherosclerosis through the actions of numerous factors secreted upon activation (15). These factors include CD40 ligand and P-

selectin, both of which have been found to be associated with an increased rate of CCA IMT progression in individuals with type 2 diabetes mellitus (T2DM) (160). Our findings are in agreement with at least six cross-sectional studies reporting associations between elevated platelet activation and increased carotid IMT (152-156, 159), one of which specifically examined obese individuals (159) and three of which reported significant associations between plasma β-TG and CCA IMT (153-155). None of these studies, however, evaluated segment-specific IMT and all consisted of a study sample at substantially greater CVD risk than the young adults in the current study. The finding that associations between platelet activity and carotid IMT remained significant after adjustment for other cardiovascular risk factors in previous studies (152, 155, 156, 159) but not in this study may indicate that platelet activity plays a greater role in adverse vascular remodeling in individuals with more advanced atherosclerosis.

The correlation found between lower circulating platelet activity and reduced body size is in agreement with past studies showing positive associations between platelet activation and measures of obesity (13, 138, 139, 142) as well as decreases in platelet activation with weight loss (13, 138, 139, 224). Unlike the current study, however, no previous studies measured platelet activity a substantial time period after the weight loss occurred. Thus, this study additionally discovered that, after weight loss and partial weight regain, circulating platelet activity is lower in individuals who maintained greater weight loss. There are several reasons for the increased platelet activation that is present in obese individuals. Platelet size, typically measured as mean platelet volume, is elevated in individuals with excess weight (142) and is positively correlated with the metabolic and enzymatic activity as well as thrombotic potential of platelets (225, 226). In addition, platelet count has been found to be increased in overweight and obese individuals in some (14, 227) but not all studies (228), and platelet count correlates

positively with plasma β-TG (229). Thus, increased platelet size and count in obesity may at least partly explain the associations found between lower β-TG and lower BMI or more successful weight reduction. In addition, insulin resistance and elevated circulating leptin are potent promoters of platelet activation and aggregation (15, 140, 230), and the correlation between β-TG and serum leptin in this study suggests that leptin may play a role in platelet activation in overweight/obese otherwise healthy young adults, though in the present study this correlation was explained by BMI. The absence of statistically significant correlations between plasma β-TG and many established cardiometabolic risk factors in this study was surprising. In particular, in a recent study by Csongradi et al., significant correlations were detected between several measures of circulating platelet activity and blood pressure, insulin resistance, lipids, and CRP in middle-aged obese adults, the majority of whom had hypertension, T2DM, or dyslipidemia (159). In another study of non-diabetic women, both normal weight and overweight/obese, soluble P-selectin was associated with BMI, insulin resistance, and blood pressure in univariable but not multivariable models (231). These findings suggest that platelet activity may be more closely associated with insulin resistance, inflammation, and lipid levels in obese individuals with more advanced atherosclerosis, but that these factors may not be independently associated with platelet activity in overweight/obese adults with no other atherosclerotic comorbidities. Furthermore, it could be that other obesity-related factors, such as oxidative stress or endothelial dysfunction (15, 232), are more closely associated with platelet activity in young overweight/obese individuals at low CVD risk.

There were several important limitations to this study. First, the sample sizes were small for both the genetic study and the study of platelet activity. Therefore, we were unable to evaluate additive or recessive genetic associations, and the power available to detect associations

with plasma β-TG was low. Second, because plasma β-TG was measured at the 24 month study visit only, relationships between concurrent changes in IMT, weight, or other CVD risk factors and changes in circulating platelet activity could not be determined. However, the prospective association between weight loss and plasma β-TG does strongly suggest that platelet activity was reduced by weight loss. Third, we evaluated only one measure of platelet activity, plasma β-TG. There are several widely used measures of in vivo platelet activity, including platelet specific proteins released into circulation upon activation that can be detected in plasma or urine and platelet surface proteins expressed upon activation that can be detected with flow cytometry. Plasma β-TG is a more sensitive marker of circulating platelet activity than flow cytometric measures, but requires very careful sample collection to avoid ex vivo artifacts (137, 233). We attempted to minimize ex vivo activation by avoiding any trauma during blood draws, by drawing the blood samples used for β-TG measurement as the last of three samples, and by keeping the samples on ice prior to centrifugation. Finally, a notable strength of this study was that all participants were normotensive and not on antihypertensive, lipid lowering, or vasoactive medications, which enabled us to evaluate associations of interest independent of any potentially confounding treatment effects.

In conclusion, the T allele of rs168753, a common allelic variant in PAR-1, appears to be associated with increased carotid bulb IMT in non-Hispanic white overweight/obese adults prior to but not after modest weight loss. In addition, greater BMI is associated with greater circulating platelet activity in young adults with excess weight. Weight loss clearly improves cardiovascular risk profiles, and the detected effect of weight loss on platelet activity suggests that a reduction in circulating platelet activity may be one pathway by which weight loss reduces CVD risk. Future studies should examine whether elevated platelet activity is a causative factor in early

atherosclerosis and adverse vascular remodeling and whether other genetic variants influence early atherosclerosis and/or platelet activation in overweight and obese individuals.

3.6 TABLES AND FIGURES

Table 3.1 Clinical Characteristics and Carotid Intima-Media Thickness in Non-Hispanic White Study Participants

Characteristic	Baseline (N=266)	6 Months (N=221)	12 Months (N=199)	24 Months (N=182)
BMI (kg/m^2)	32.9 (4.0)	30.3 (4.2)*	30.3 (4.6)*	31.3 (4.6)*
Weight (kg)	92.8 (15.4)	85.6 (15.0)*	85.6 (15.6)*	88.8 (16.3)*
Waist Circumference (cm)	101.0 (11.8)	95.6 (11.6)*	95.5 (12.6)*	98.1 (13.0)*
SBP (mmHg)	113.1 (10.1)	109.6 (9.0)*	109.6 (9.2)*	111.6 (9.6)*
DBP (mmHg)	72.9 (8.6)	70.8 (8.0)*	72.1 (8.1)	74.0 (8.9)
LDL-C (mg/dL)	124.5 (33.2)	121.9 (30.9)	125.0 (30.8)	127.1 (32.4)
HDL-C (mg/dL)	51.6 (12.6)	52.2 (11.8)	54.9 (12.6)*	54.2 (12.8)*
Triglycerides (mg/dL)	121 (82, 175)	100 (73, 143.5)*	97 (72, 142)*	103 (77, 153)*
Insulin (μU/mL)	12.2 (9.3, 17.2)	11.1 (8.9, 15.4)*	11.7 (9.3, 15.2)	11.7 (9.4, 15.5)
CRP (mg/L)	2.6 (1.4, 5.6)	2.2 (0.99, 4.3)*	2.0 (0.94, 4.0)*	2.2 (0.90, 4.6)*
Leptin (ng/mL)	24.8 (12.3)	17.2 (11.3)*	19.6 (12.9)*	21.5 (12.4)*
Adiponectin (μg/mL)	12.2 (6.1)	12.4 (5.6)	12.4 (5.6)	11.1 (5.6)*
Sodium Excretion	187.2 (70.9)	158.2 (68.8)*	158.2 (59.8)*	154.1 (64.6)*
Mean ICA IMT (mm)	0.57 (0.15)	------	------	0.55 (0.11)
Mean Carotid Bulb IMT (mm)	0.68 (0.16)	------	------	0.70 (0.15)*
Mean CCA IMT (mm)	0.59 (0.07)	0.60 (0.07)	0.60 (0.08)*	0.61 (0.07)*

Data from trial participants of non-Hispanic white race are included in this table. Mean (SD) or median (IQR) are shown. *P<0.05 versus baseline in a linear mixed model with time since baseline as a nominal variable and with adjustment for intervention arm. Insulin, triglycerides, and CRP were log transformed for linear mixed modeling. BMI=body mass index; SBP=systolic blood pressure;

DBP=diastolic blood pressure; LDL-C=low density lipoprotein cholesterol; HDL-C=high density lipoprotein cholesterol; CRP=C-reactive protein; ICA=internal carotid artery; IMT=intima-media thickness; CCA=common carotid artery. [#]Baseline N=219, 6 Months N=171, 12 Months N=150, 24 Months N=134.

Table 3.2 Associations between Allelic Variants and Carotid Intima-Media Thickness

SNP	Gene	Genotype (N)	Baseline Mean* ICA IMT (mm)	Nominal P value[#]	Baseline Mean* Bulb IMT (mm)	Nominal P value[#]	Baseline Mean* CCA IMT (mm)	Nominal P value[#]
rs168753	PAR-1	AA (190) AT/TT (75)	0.56 (0.13) 0.58 (0.19)	0.27	0.67 (0.14) 0.72 (0.19)	0.004[$]	0.59 (0.08) 0.59 (0.07)	0.89
rs2046934	P2Y12	AA (182) AG/GG (72)	0.57 (0.16) 0.56 (0.12)	0.87	0.69 (0.16) 0.67 (0.13)	0.75	0.59 (0.07) 0.59 (0.07)	0.88
rs2243093	GPIbα	TT (195) TC/CC (61)	0.57 (0.16) 0.55 (0.13)	0.39	0.68 (0.16) 0.71 (0.15)	0.045	0.59 (0.08) 0.58 (0.07)	0.96
rs5918	GPIIb-IIIa	TT (180) TC/CC (77)	0.56 (0.13) 0.59 (0.19)	0.35	0.68 (0.15) 0.69 (0.16)	0.73	0.58 (0.07) 0.60 (0.08)	0.03
rs6065	GPIbα	CC (223) CT/TT (36)	0.56 (0.15) 0.58 (0.15)	0.94	0.68 (0.15) 0.70 (0.20)	0.84	0.59 (0.08) 0.59 (0.07)	0.40
rs1613662	GPVI	AA (189) AG/GG (74)	0.57 (0.15) 0.56 (0.14)	0.62	0.69 (0.17) 0.66 (0.12)	0.18	0.59 (0.07) 0.59 (0.07)	0.56

*Values shown are unadjusted Mean (SD). [#]Comparisons between non-carriers and carriers of the minor allele were adjusted for baseline age, sex, BMI, SBP, LDL-C, and HDL-C. [$]$P<0.05$ after correction for multiple hypothesis testing using FDR-adjustment (Benjamini-Hochberg method) for each carotid artery segment individually.

Table 3.3 Characteristics of Subjects in the Platelet Activity Sub-Study at the 24 Month Visit

Characteristic	Total (n=92)
Age (years)	40.2 (5.9)
Women (n, %)	60 (65.2)
Black Race (n, %)	13 (14.1)
Ever Smoker (n, %)	28 (30.4)
BMI (kg/m^2)	31.1 (4.2)
Waist Circumference (cm)	98.1 (12.6)
SBP (mmHg)	110.9 (8.7)
DBP (mmHg)	73.1 (8.5)
LDL-C (mg/dL)	123.0 (33.7)
HDL-C (mg/dL)	54.0 (14.2)
Triglycerides (mg/dL)	98.0 (78.5,
Insulin (μU/mL)	12.1 (9.5, 16.2)
CRP (mg/L)	2.3 (0.83, 4.5)
Leptin (ng/mL)	20.7 (13.3)
Adiponectin (μg/mL)	9.6 (5.5)
β-thromboglobulin (IU/mL)	25.8 (18.6, 35.9)
Mean CCA IMT (mm)	0.62 (0.08)
Mean ICA IMT (mm)	0.52 (0.47, 0.59)
Mean Carotid Bulb IMT (mm)	0.69 (0.62, 0.79)

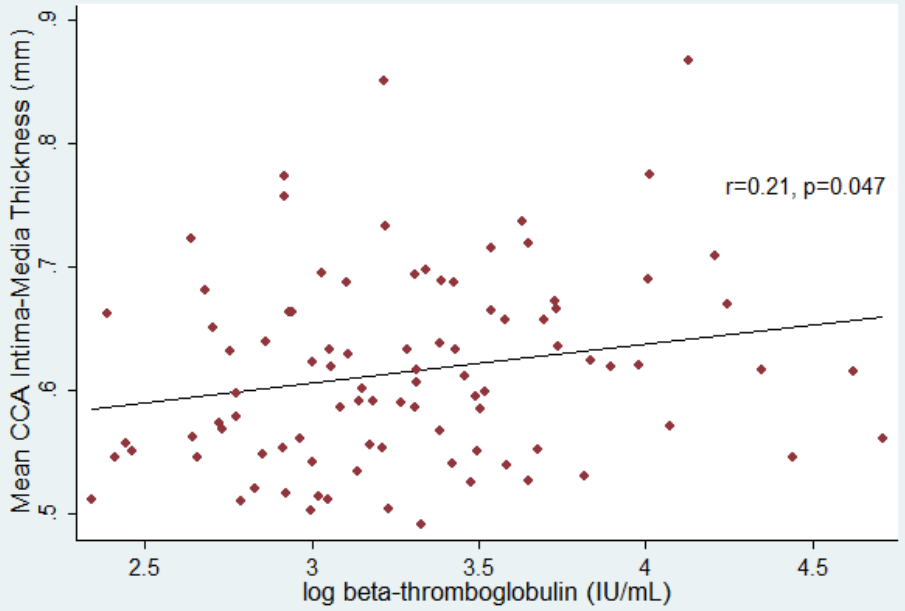
Mean (SD) or Median (IQR) values from 24 month visit. BMI=body mass index; SBP=systolic blood pressure; DBP=diastolic blood pressure; LDL-C=low density lipoprotein cholesterol; HDL-C=high density lipoprotein cholesterol; CRP=C-reactive protein; ICA=internal carotid artery; IMT=intima-media thickness; CCA=common carotid artery.

Table 3.4. Pearson Correlations between Circulating Platelet Activity and Cardiovascular Risk Factors at 24 Months

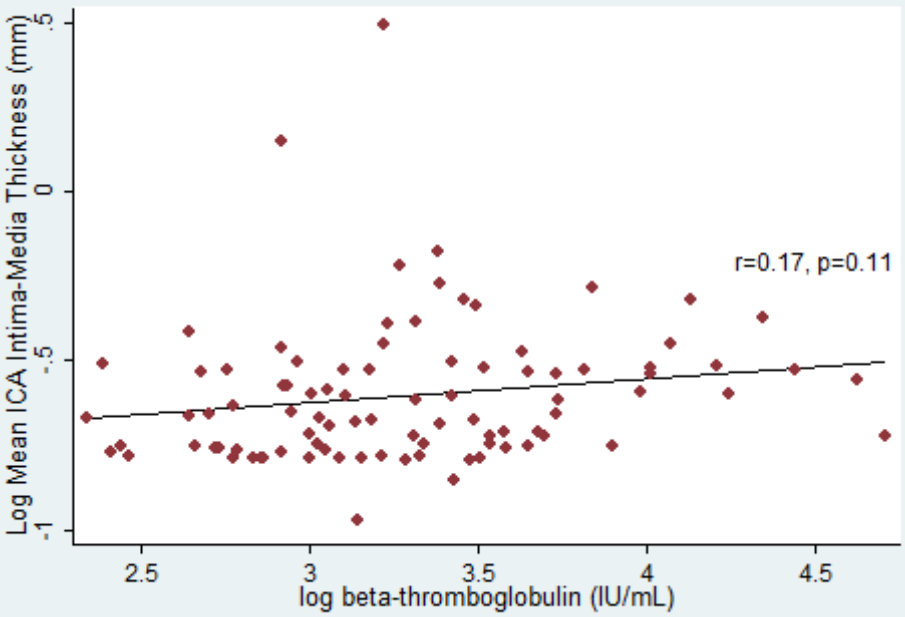
Variable	r	P value
Age	0.13	0.23
Female	0.09	0.42
Black Race	0.02	0.87
Ever Smoker	0.02	0.82
BMI	0.25	0.02
Waist Circumference	0.06	0.60
SBP	0.12	0.27
DBP	-0.04	0.68
LDL-C	0.09	0.40
HDL-C	0.03	0.79
Triglycerides	0.02	0.82
Insulin	0.02	0.85
CRP	0.12	0.27
Leptin	0.21	0.049
Adiponectin	-0.06	0.60

All variables were measured concurrently with plasma β-TG at the 24 month study visit. Plasma β-TG, triglycerides, insulin, CRP, ICA IMT, and Carotid Bulb IMT were log transformed. BMI=body mass index; SBP=systolic blood pressure; DBP=diastolic blood pressure; LDL-C=low density lipoprotein cholesterol; HDL-C=high density lipoprotein cholesterol; CRP=C-reactive protein. N=92.

(a)


Mean CCA Intima-Media Thickness (mm)
.5
.6
.7
.8
.9
r=0.21, p=0.047
2.5
3
3.5
4
4.5
log beta-thromboglobulin (IU/mL)

(b)


Log Mean ICA Intima-Media Thickness (mm)
-1
-.5
0
.5
r=0.17, p=0.11
2.5
3
3.5
4
4.5
log beta-thromboglobulin (IU/mL)

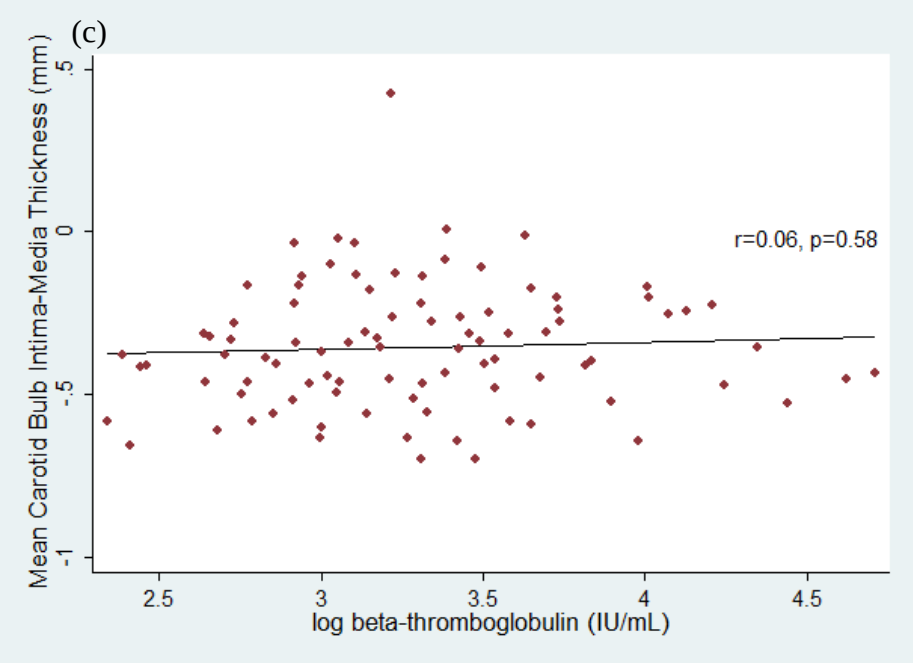


Figure 3.1 Correlations between Plasma β-thromboglobulin and (a) Common Carotid Artery (b) Internal Carotid Artery, and (c) Carotid Bulb Intima-Media Thickness

Table 3.5 Associations between Weight Loss during the Lifestyle Intervention and Plasma β-thromboglobulin One Year Post-Invention

Variable	Parameter Estimate (SE)	P value
Age (years)	0.01 (0.009)	0.15
Sex (Male vs. Female)	-0.09 (0.11)	0.42
Race (Black vs. Non-Black)	-0.02 (0.15)	0.91
Baseline BMI (kg/m^2)	0.03 (0.01)	0.07
Change in BMI (kg/m^2)	0.05 (0.02)	0.03

Plasmaβ-thromboglobulin at the 24 month study visit is the dependent variable in a multiple linear regression model including all listed independent variables. Plasma β-thromboglobulin was log transformed. Change in BMI indicates change between baseline and 24 months (time of plasma β-thromboglobulin measurement). BMI=body mass index. SE=standard error. N=92.

4.0 CHANGES IN SERUM ALDOSTERONE ARE ASSOCIATED WITH CHANGES IN OBESITY-RELATED FACTORS IN NORMOTENSIVE OVERWEIGHT AND OBESE YOUNG ADULTS

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4.1 ABSTRACT

Objectives Elevated aldosterone promotes cardiometabolic decline. This is particularly important in obesity because adipocytes secrete factors that increase aldosterone production. Weight loss is thought to lower aldosterone levels, but little longitudinal data is available. We

aimed to determine if, independent of changes in sodium intake, reductions in serum aldosterone are associated with reductions in weight, abdominal visceral and subcutaneous adiposity, thigh intermuscular adiposity, inflammation, leptin, insulin resistance, cardiac sympathovagal balance, blood pressure, and increases in adiponectin and ghrelin in normotensive overweight/obese young adults undergoing lifestyle modification.

Methods Participants were overweight/obese adults aged 20-45 years (20% male, 15% black) from a clinical trial evaluating the relationships between weight loss, dietary sodium, and vascular health. Subjects were randomly assigned to a regular or reduced sodium diet, and all received a one-year nutrition and physical activity intervention. For this study, individuals providing valid baseline 24hr urine collections were included (n=285). Linear mixed models were used to evaluate associations between changes in aldosterone and changes in obesity-related factors.

Results Weight loss was significant at 6 months (~7%), 12 months (~6%), and 24 months (~4%) ($p<0.0001$for all). Decreases in aldosterone were associated with decreases in C-reactive protein, leptin, insulin, homeostasis assessment of insulin resistance, heart rate, tonic cardiac sympathovagal balance, and increases in adiponectin ($p<0.05$ for all) in models that adjusted for baseline age, sex, race, intervention arm, time since baseline, and baseline and concurrent changes in sodium and potassium excretion. Decreases in aldosterone were associated with weight loss and reductions in intermuscular fat (IMAT) in the subgroup (n=98) with metabolic syndrome (MetS) at baseline (MetS x percent weight loss $p=0.04$, MetS x change in IMAT $p=0.04$). Though no associations were detected between changes in aldosterone and blood pressure, an association was found between reduced mean arterial pressure and reduced sodium excretion in those with MetS (MetS x sodium excretion reduction $p=0.07$).

Conclusions Changes in aldosterone are associated with changes in many obesity-related factors in overweight/obese normotensive young adults. In persons with MetS, weight loss and sodium restriction are particularly useful to reduce aldosterone and blood pressure respectively. Given the adverse effects of excess aldosterone on cardiometabolic health, future studies should investigate the benefits of aldosterone antagonists in individuals with MetS.

4.2 INTRODUCTION

Aldosterone plays important roles in blood pressure regulation and sodium and water balance, but inappropriately elevated levels have been shown to contribute to left ventricular hypertrophy (126, 234), cardiac fibrosis (235), and aortic stiffness (58, 59, 235). Aldosterone secretion by the adrenal cortex is normally regulated by extracellular potassium and angiotensin II (Ang II) in response to intravascular volume depletion (236). However, several studies have found that aldosterone levels are elevated in overweight and obese individuals, especially in those with excess visceral fat (91-96). Aldosterone has also been found to decrease with modest weight loss in studies in which individuals either maintained a moderate to high dietary sodium intake (57, 93, 96-98, 237) or severely restricted their calorie intake under any level of sodium intake (96, 99). In addition, elevated circulating aldosterone is associated with obesity-related abnormalities such as insulin resistance (91, 95, 96, 102) and the metabolic syndrome (103-106). This may be partially explained by the presence of numerous renin-angiotensin-aldosterone system (RAAS) components, including angiotensinogen, angiotensin converting enzyme, Ang II, and Ang II receptors, in adipose tissue, though there is contrasting evidence as to whether adipocytes produce aldosterone (236, 238, 239). Laboratory studies have demonstrated that adipocyte

mineralocorticoid receptor (MR) stimulation with aldosterone promotes inflammatory adipokine expression and lipid accumulation whereas such effects are removed by MR knockout or blockade (117, 240). Conversely, human adipocytes produce mineralocorticoid-stimulating factors that increase adrenal aldosterone secretion independently of AngII or potassium and that also sensitize adrenocortical cells to AngII (112, 241). Other studies have suggested that oxidized free fatty acids (114) or novel adipokines may stimulate aldosterone production in obese individuals (113). In addition, the imparied renal sympathovagal balance present in obese individuals stimulates renin release by the kidneys, which elevates RAAS activity (236). In addition, both renal and cardiac sympathovagal balance are worsened by inappropriately elevated aldosterone levels (242). Finally, increased formation of Ang II by large insulin-resistant adipocytes in overweight/obese individuals inhibits the recruitment and differentiation of preadipocytes, which leads to ectopic fat storage and decreased insulin sensitivity (243, 244). Together, this evidence suggests a vicious cycle exists in obese individuals wherein excess adiposity promotes aldosterone production and aldosterone and other RAAS components in turn act as drivers of adipose inflammation, insulin resistance, and cardiovascular decline.

Though several studies have reported decreases in aldosterone with weight loss (57, 93, 96-98, 237), to our knowledge no study of healthy normotensive young adults has examined the longitudinal associations between changes in aldosterone and changes in obesity-related factors while accounting for discretionary changes in dietary sodium intake, an important determinant of circulating aldosterone levels. Because aldosterone plays an important role in the development of hypertension, metabolic dysfunction, and cardiovascular disease, particularly in overweight and obese individuals (236, 245), it is important to understand its associations with obesity-related factors prior to the development of these clinical conditions. We hypothesized that, independent

of changes in 24-hour urinary sodium excretion, reductions in serum aldosterone would be associated with weight loss and reductions in abdominal visceral and subcutaneous adiposity, thigh intermuscular adiposity, inflammation, leptin, insulin resistance, and sympathovagal balance, and increases in adiponectin and ghrelin in normotensive overweight and obese young adults. We also hypothesized that, independent of weight loss, decreases in both serum aldosterone and sodium excretion would be associated with decreases in blood pressure over the course of the study. Subjects were assessed at baseline, 6, and 12 months during a one year behavioral weight loss intervention and again 12 months after the conclusion of the intervention.

4.3 METHODS

To study the associations between changes in serum aldosterone and obesity-related factors, we measured these factors at baseline and 6, 12, and 24 month follow-up visits in overweight and obese adults participating in the Slow Adverse Vascular Effects of excess weight study (SAVE), a randomized-controlled trial (NCT00366990) evaluating the effects of weight loss, increased physical activity, and reduced dietary sodium intake on vascular health.

4.3.1 Study Population

Participants were recruited from June 2007 through May 2009 using mass mailing. The study was approved by the University of Pittsburgh IRB and all participants provided written informed consent to participate in the study.

Eligible participants were men and women 20-45 years of age who were overweight or obese (body mass index (BMI) 25-39.9 kg/m^2) and physically inactive (<8 months of physical activity (PA) during the past 12 months). Exclusions included 1) diabetes, 2) hypertension or average screening blood pressure ≥140/90 mmHg, 3) cholesterol lowering, anti-psychotic, or vasoactive medication use and 4) current pregnancy or lactation.

4.3.2 Intervention

Three hundred and forty-nine eligible participants received a 1-year lifestyle intervention promoting diet and physical activity (PA). Participants were randomized to either 1) diet and PA alone (Control Na/lifestyle) or to 2) diet and PA plus reduced sodium intake (Low Na/lifestyle). The lifestyle intervention was delivered in group sessions that occurred weekly for months 1-4, biweekly for months 5-8, and monthly for months 9-12. The goal of the intervention was a 10% reduction in body weight over 6 months and continued maintenance of weight loss thereafter. The additional goal of the sodium reduction intervention (Low Na) was to gradually reduce daily sodium intake to approximately 1 mg Na^+/1 kcal/day, an average reduction of about 50% from the participant's usual diet (207).

4.3.3 Clinic Visits

Participants were to complete clinic visits at screening, baseline, and 6, 12, and 24 months following randomization. Self-reported demographic information, self- and interviewer-administered questionnaires, anthropometric measurements, fasting blood draw, and 24-hour urine collection were collected at these visits.

4.3.4 Demographic and Physical Measures

Age, race, and smoking status were self-reported. Race was re-coded as black vs. non-black. Smoking status was assessed as current vs. past or never. Weight was measured in kilograms using a balance scale. Height was measured in centimeters using a stadiometer. BMI was calculated as weight in kilograms divided by height in meters squared. Waist circumference was measured against the participant's skin at the narrowest part of the torso between the ribs and the iliac crest. Blood Pressure (BP) was measured with a mercury sphygmomanometer after participants sat quietly for 5 minutes with feet flat on the floor. Final BP was the average of the last 2 of 3 readings taken 30 seconds apart.

4.3.5 Blood Assays

Blood analytes were measured at the Heinz Laboratory at the University of Pittsburgh's Graduate School of Public Health. Serum glucose was determined enzymatically with a procedure similar to that described by Bondar and Mead (211). Total cholesterol, high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), triglycerides and glucose were determined using standard laboratory procedures (246). Insulin, leptin, adiponectin and total ghrelin were measured using radioimmunoassay (Linco Research, Inc., St. Charles, MO). Insulin resistance was estimated using the homeostasis model assessment of insulin resistance index (HOMA-IR) derived from fasting insulin and glucose values (247). HOMA-IR (mmol/L x μU/ml) = fasting glucose (mmol/L) x fasting insulin (μU/ml)/22.5. C-reactive protein (CRP) was measured using an enzyme-linked immunoassay (Alpha Diagnostic International, Inc., San Antonio, TX). Aldosterone was measured using an enzyme-linked immunoassay

developed by Diagnostic Systems Laboratories (Webster, TX). The intra- and inter-assay CV% for insulin were 4.8% and 10.5% respectively. The CV% for the other assays were all <3%.

4.3.6 Urine Collection

Valid 24-hour urine collections had volume between 500 mL and 4000 mL, duration ≥22 hours and ≤26 hours, and total creatinine within the expected range (212). From April 13, 2007 to March 6, 2009, analytes were measured using an Ortho Vitros 950. Direct potentiometry was used to measure sodium and potassium and colorimetry to assess creatinine levels. Afterward, results were determined using a Beckman Coulter DxC 800 instrument employing an indirect ion selective method for sodium and potassium and an alkaline picric kinetic method for creatinine.

4.3.7 Regional Measures of Adiposity

At the baseline and 12 month visits only, single-slice computed tomography (CT) scans of the abdomen and thigh were acquired using a C-150 Ultrafast CT Scanner (GE Imatron, San Francisco, CA). Slice thickness was set at 6 mm. Abdominal scans were transverse images between L4 and L5 obtained during suspended respiration; left thigh images were transverse images 15 cm above the patellar apex.

CT images were interpreted by two independent readers using Slice-O-Matic software. A pixel range of -30 to -190 Hounsfield units was used to define fat in the scan circumference and a pixel range of 0-100 was used to define muscle. Areas were calculated by multiplying the number of pixels of a given tissue type by the pixel area. Density values were determined by averaging the CT number (pixel density) values of the regions outlined on the images. For the

abdominal scan, region of interest lines were drawn along fascial planes. Fat above the more external fascial plane was considered superficial fat, fat between the external and internal fascial planes was considered deep fat, and fat below the internal fascial plane (i.e., plane at the interior of abdominal musculature) was considered visceral fat area. Subcutaneous fat was calculated as the difference between the whole image (total) and visceral fat area. For the thigh scan, a single region of interest line was drawn along the deep fascial plane surrounding the thigh muscle. Fat above this line was considered subcutaneous fat, and fat below this line was considered intermuscular fat (248). For these analyses, abdominal visceral and subcutaneous fat were examined and, as a measure of ectopic fat, thigh intermuscular fat was examined.

4.3.8 Heart Rate Variability

At the baseline and 6 month visits only, heart rate variability (HRV) was measured using an ANSAR monitor (ANX-3.0, ANSAR Group Inc, Philadelphia, PA), which provides continuous and noninvasive measurements of electrocardiogram signals (for HRV assessment) and bioimpedance plethysmography signals (for respiratory rate variability assessment; RRV) respectively. Testing began with attachment of electrocardiogram electrodes in a modified Lead-II configuration to the participant's chest, along with a blood pressure cuff to the left arm. Participants were asked to sit with their feet flat on the floor and refrain from sudden movements or talking. Resting measures at a normal breathing rate were taken for 5 minutes followed by deep breathing (6 breaths per minute) for 1 minute. Participants returned to their resting rate for 1 minute, performed Valsalva challenge for 1.5 minutes and returned to resting again for 2 minutes. To finish, participants remained in a standing position for 5 minutes. A spectral analysis of the HRV and RRV was generated using ANSAR software. The low-frequency area (LFa) was

centered on the HRV spectrum from 0.04 - 0.10 Hz, which is reflective of sympathetic cardiac activity. From the spectral analysis of the RRV, the frequency of the peak mode was defined as the fundamental respiratory frequency (FRF). A 0.12 Hz wide window from the HRV spectrum was centered at the FRF and was used to generate the respiratory frequency area (RFa), which is reflective of parasympathetic cardiac activity (249-252). During low FRF, the RFa shifts into the low-frequency bandwidth. The area under the spectral curve centered on the FRF is computed as RFa. The remaining area under the spectral curve in the low-frequency bandwidth is computed as LFa.

The measures of HRV used for this analysis were LFa/RFa during the initial 5 minute resting period (corresponding to tonic sympathovagal balance) and LFa/RFa during the 5 minute standing period (corresponding to sympathovagal balance during orthostatic challenge). Between technologist ICCs ranged from 0.73 to 0.93.

4.3.9 Statistical Methods

Descriptive statistics were calculated to summarize study variables overall and by intervention arm at baseline and 6, 12, and 24 months follow-up and were presented as median/inter-quartile range (IQR) or mean (SD) for continuous variables and frequency and percentages for categorical variables. Only trial participants with a valid baseline 24-hour urine collection were included in this analysis. Whether the changes in body size, serum aldosterone, and other variables of interest were statistically significantly different from zero in the total sample at each follow-up visit was determined by testing the coefficient for time, as a nominal variable, in linear mixed models with unstructured error covariance. Non-normally distributed variables were transformed as necessary prior to modeling. Intervention arm was included as a covariate in

every model for consistency with trial design. An interaction between intervention arm and time since baseline was used to test whether changes over time in study variables differed by intervention arm.

The main analysis began with linear mixed models for aldosterone at all study visits. Variables included in the models were age, sex, race (black/non-black), baseline and within-subject changes in sodium and potassium excretion, and baseline and within-subject changes in independent variables of interest. In all models, baseline and change from baseline factors were included as separate effects in order to evaluate the longitudinal associations between changes in aldosterone and changes in the independent variables of interest without confounding by the cross-sectional between-subject associations among the same factors. The independent variables of interest were the following obesity-related factors: BMI, weight, waist circumference, abdominal visceral and subcutaneous adipose tissue areas, thigh intermuscular adipose tissue area, insulin, HOMA-IR, adiponectin, leptin, ghrelin, CRP, resting supine heart rate, and sitting and standing HRV (cardiac sympathovagal balance). A quadratic time since baseline effect as well as random intercepts and linear and quadratic time since baseline effects were individually tested and included if found to be statistically significant at $p<0.10$ using likelihood ratio tests. To determine whether longitudinal associations between aldosterone and obesity-related factors varied over time or varied between subgroups defined by demographic factors or metabolic dysfunction at baseline, first order interactions between changes in the obesity-related factors of interest and time since baseline, race, sex, age, or the presence of metabolic syndrome (MetS) at baseline were evaluated. Next, to evaluate whether the longitudinal associations between aldosterone and obesity-related factors were independent of weight loss, all previous models

were reexamined after additional adjustment for baseline and within-subject changes in body size.

Next, to assess whether changes in urinary sodium excretion and/or serum aldosterone were associated with concurrent blood pressure changes independently of weight loss, mean arterial pressure (MAP), systolic blood pressure (SBP) and diastolic blood pressure (DBP) over the course of the study were individually modeled using linear mixed models that included baseline age, sex, race, weight, baseline and within-subject changes in serum aldosterone, sodium and potassium excretion, and percent weight loss. To determine if baseline demographic or metabolic factors influenced BP sensitivity to sodium, first order interactions between changes in sodium excretion and baseline serum aldosterone, race, sex, age, and baseline MetS status were evaluated.

Several sensitivity analyses were performed. First, criteria other than the presence vs. absence of MetS at baseline were evaluated to determine whether other baseline factors characterizing metabolically "at-risk" obese individuals (253, 254) better distinguished between subgroups of individuals showing stronger vs. weaker associations between changes in the obesity-related factors of interest. First, a dichotomous indicator of baseline insulin resistance (baseline HOMA-IR greater than vs. less than 65^{th} percentile) was used in place of MetS. Next, baseline abdominal visceral adipose tissue (VAT) (greater than vs. less than sex-specific 65^{th} percentile) was used in place of MetS. The 65^{th} percentiles were chosen so that these metabolically "at-risk" subgroups would be the same size as the subgroup with MetS at baseline. Finally, each of the five components of metabolic syndrome (255) was investigated individually to determine whether the presense of any particular component of MetS characterized a subgroup of individuals who showed stronger longitudinal associations among the variables of interest.

The five criteria used to define MetS were: (i) SBP/DBP ≥130/85 mm Hg (ii) fasting triglycerides ≥150 mg/dL, (iii) high-density lipoprotein <40 mg/dL in men and <50 mg/dL in women, (iv) fasting glucose ≥100 mg/dL and (v) waist circumference ≥102 cm for men and ≥88 cm for women (256). For the evaluation of individual MetS criteria, the Bonferroni method was used to correct for multiple comparisons, such that P values ≤0.01 were considered statistically significant.

Finally, because the quantity of missing data was considerable in this study, several sensitivity analyses were performed to evaluate potential effects of the missing data. First, to evaluate whether the associations of interest were maintained when all available urinary excretion data was used rather than data from only valid 24-hr urine collections, sodium/creatinine and potassium/creatinine excretion ratios were examined in place of 24-hr sodium and potassium excretion respectively. Second, to evaluate the associations of interest under the hypothesis that participants with incomplete data were on average less successful in achieving weight loss than participants with complete data, pattern-mixture modeling and multiple imputation were used. Multiple imputation was performed for each missing data pattern (dropout after baseline (n=73), dropout after 6 months (n=32), and dropout after 12 months (n=38)), and the assumption was made that the conditional multivariate distribution of the missing outcome and covariate data for each pattern, given the observed data, followed the corresponding distribution in the subgroup of participants with complete follow-up data who had achieved less than the mean percent weight loss at follow-up visits with unavailable data for that pattern. Intermittently missing data (n=48) was treated as missing at random and was not imputed. P values ≤0.05 were considered statistically significant. Statistical analyses were performed using SAS (Statistical Analysis Software release 9.3, Cary, NC).

4.4 RESULTS

The study population consisted of 285 participants in the SAVE clinical trial who provided valid baseline 24-hr urine collections and serum aldosterone data. These subjects were, on average, slightly older, less insulin resistant, and more likely to be female than the trial participants not included in this study (n=64) ($p<0.05$ for all). The sample had a mean age of 38.4 years (SD 5.8) at baseline and consisted of 20% males and 15% African-Americans. Eight percent of the study population identified themselves as current smokers at baseline. Mean values of key clinical characteristics over the course of the intervention are shown in Table 1. Average weight loss was 7.1% at 6 months, 6.4% at 12 months, and 3.5% at 24 months. The only measures that differed at least marginally by intervention arm were changes in 24-hour urinary sodium and serum aldosterone. Mean sodium excretion was decreased from baseline by 48.1 mmol/24hr (SD 79.7) at 6 months, 35.0 mmol/24hr (SD 80.5) at 12 months, and 42.0 mmol/24hr (SD 75.8) at 24 months in the Low Na/lifestyle arm, but decreased by only 9.1 mmol/24hr (SD 77.1) at 6 months, 20.5 mmol/24hr (SD 84.8) at 12 months, and 7.6 mmol/24hr (SD 78.2) at 24 months in the Control Na/lifestyle arm ($p<0.001$, $p=0.27$, and $p=0.01$ respectively for between-arm comparisons). Serum aldosterone was marginally higher in the Low Na/lifestyle arm compared to the Control Na/lifestyle arm at 6 months only ($p=0.06$).

At each follow-up visit, there were differences between participants who did vs. did not have missing data. Subjects missing 6 month data on at least one measure examined in the present study had slightly higher baseline BMI and serum leptin than those with complete 6 month data ($p<0.05$ for both). Subjects missing data at 12 months were more likely to be of black race and had higher baseline SBP than those with complete 12 month data ($p<0.05$ for both). Study subjects missing data at 24 months were more frequently of black race, had higher

baseline BMI and leptin and lower baseline adiponectin, and had achieved lesser reductions in all measures of body size at 12 months than those with complete 24 month data ($p<0.05$ for all). At all visits, valid urinary electrolyte excretion data was the most frequently missing (Table 4.1).

In linear mixed models for log aldosterone that included baseline age, sex, race (black/non-black), intervention arm, time since baseline, and baseline and within-subject changes in sodium and potassium excretion, no measures of change in body size or changes in abdominal adipose tissue areas were associated with changes in aldosterone over the course of the two year study. However, changes in circulating levels of adipokines and changes in measures of insulin resistance, inflammation, and tonic cardiac sympathovagal balance were strongly associated with changes in aldosterone (Table 4.2). When tonic sympathetic (LFa) and parasympathetic (RFa) components were evaluated individually, it was found that parasympathetic activity was increased ($p=0.02$) but sympathetic activity was unchanged at 6 months, though neither component individually showed a statistically significant longitudinal association with aldosterone. There was a trend towards an association between decreased intermuscular adipose tissue area and decreased serum aldosterone (Table 4.2). As expected, there were at least marginally significant associations between increases in serum aldosterone and both decreases in sodium excretion and increases in potassium excretion over time in all models ($p<0.10$ for all). When interactions between within-subject changes and baseline demographic factors or MetS status were examined in the mixed models for log aldosterone, weight loss was associated with a reduction in serum aldosterone only in the subgroup of subjects (n=98, 34%) who had MetS at baseline (Figure 4.1). The significance of the interaction between BMI reduction and MetS was similar ($p=0.046$) to that between percent weight loss and MetS, but the interactions between MetS and changes in waist circumference or abdominal adipose tissue depots were not

significant ($p>0.10$ for all). The interaction between baseline MetS and change in thigh intermuscular adipose tissue area was statistically significant (Figure 4.2), suggesting that reductions in this ectopic fat depot were more strongly associated with reductions in serum aldosterone in subjects with MetS. No other first order interactions were found to be significant. The associations between changes in aldosterone and changes in each evaluated obesity-related factor were unaltered by additional adjustment for baseline and within-subject changes in weight, BMI, or waist circumference (results not shown).

Neither changes in sodium excretion nor changes in serum aldosterone were associated with changes in BP during the two year study (Table 4.3). However, several marginally significant interactions were detected between changes in sodium excretion and baseline factors in the models for MAP and DBP. First, reductions in sodium excretion were found to be associated with decreases in DBP in subjects with MetS but not in subjects without MetS at baseline (Figure 4.3). This marginal interaction was similar for MAP ($p=0.07$) but did not approach statistical significance for SBP ($p=0.40$). Second, a marginal interaction between race and change in sodium excretion was detected in the model for DBP, such that decreases in sodium excretion were associated with decreases in DBP in black study participants but not in non-black participants (Figure 4.4). This interaction was not statistically significant in the models for MAP ($p=0.13$) or SBP ($p=0.33$).

When other baseline factors characterizing metabolically "at-risk" obese individuals were investigated in place of MetS status, having baseline insulin resistance (baseline HOMA-IR greater than vs. less than the 65^{th} percentile (3.66 mmol/L x μU/mL)) was associated with having greater log aldosterone at baseline ($\beta(se)=0.14(0.05)$, $p=0.007$), but interactions between baseline insulin resistance and changes in body size were not significant in linear mixed models for log

aldosterone that included baseline age, sex, race, intervention arm, time since baseline, and baseline and within-subject changes in weight and sodium and potassium excretion. In similar models, baseline abdominal VAT (greater than vs. less than the sex-specific 65th percentile (179.0 cm^2 in males, 115.4 cm^2 in females)) was associated with greater log aldosterone at baseline (β(se)=0.12(0.06), p=0.03), but interactions between baseline abdominal VAT and changes in body size were not statistically significant in mixed models for log aldosterone. In the models for blood pressure, it was found that reductions in sodium excretion showed at least marginally stronger associations with decreases in blood pressure in the more insulin resistant subjects (p=0.03, p=0.08, and p=0.04 for baseline HOMA-IR*change in sodium excretion interactions in models for MAP, SBP, and DBP respectively). For example, independently of baseline demographic factors, baseline weight, weight loss, and baseline and within-subject changes in potassium excretion and serum aldosterone, a 50 mmol/24hr reduction in sodium excretion was associated with a change in MAP of -1.04 mm Hg (95% CI -1.83, -0.25) among more insulin resistant individuals (baseline HOMA-IR > 3.66 mmol/L x μU/mL), whereas in less insulin resistant individuals, no associations between changes in sodium excretion and BP were detected. Interactions between baseline abdominal VAT (greater than vs. less than 179.0 cm^2 in males, 115.4 cm^2 in females) and changes in sodium excretion were not statistically significant in the mixed models for blood pressure. Finally, when each of the five components of the metabolic syndrome was investigated individually in the models for log aldosterone, no single component showed an interaction with weight loss or changes in sodium or potassium excretion that was statistically significant at $p \leq 0.01$. Similarly, in the models for blood pressure, no statistically significant interactions were detected between individual components of the metabolic syndrome and changes in sodium excretion (data not shown).

The final sensitivity analyses examined potential effects of the missing data. When sodium/creatinine and potassium/creatinine excretion ratios were used in place of 24-hr sodium and potassium excretion, an additional 55 subjects could be included in the analyses. However, the associations of interest were very similar to those from the original models (data not shown). Next, pattern-mixture modeling and multiple imputation were used under the assumption that, for each dropout pattern (subgroups of subjects missing all data after either baseline, 6 months, or 12 months), the distribution of the missing follow-up data followed that of subjects with complete follow-up data who had achieved less than the mean percent weight loss at visits with unavailable data for that pattern. Under this assumption, marginal associations (averaged over all patterns) differed little from those in the original fully-adjusted mixed models for log aldosterone and BP, though most associations were slightly weaker under this particular assumption of less successful weight loss among non-completers (Tables 4.4 and 4.5).

4.5 DISCUSSION

The main findings of this study were that, independent of the stimulatory effect of dietary sodium reduction on systemic RAAS activity, decreases in serum aldosterone were associated with reductions in fasting insulin, HOMA-IR, CRP, leptin, heart rate, and tonic cardiac sympathovagal balance as well as increases in adiponectin in normotensive overweight/obese young adults during a one year lifestyle intervention and one year post-intervention period. Another notable finding of this study was that, though changes in weight and serum aldosterone were unassociated in the total study sample, such an association was evident in individuals who

had MetS at baseline. In addition, reductions in intermuscular thigh fat were marginally associated with reductions in serum aldosterone in the total sample and significantly associated with reductions in serum aldosterone in individuals who had MetS at baseline. Finally, as was expected from previous studies (257, 258), associations between decreases in sodium excretion and decreases in blood pressure were more evident in individuals who had greater baseline metabolic dysfunction or who were of black race. These findings are important because this is the first longitudinal study in a large sample of overweight/obese otherwise healthy young adults to report associations between changes in circulating aldosterone and changes in a wide variety of obesity-related factors.

Previous small or moderate sized studies have reported decreases in serum aldosterone or aldosterone excretion with modest weight loss in obese postmenopausal women (98), young overweight/obese adults (96, 97), middle-aged overweight/obese adults (57, 259), and obese adults who submitted to very low calorie diets (99, 237). Surgical weight loss also reduced circulating aldosterone in severely obese hypertensive adults (260). Some studies have reported significant associations between reductions in circulating RAAS components and reductions in central adiposity (96, 98, 261) or insulin resistance (96) during periods of weight loss, though not all studies agree (97, 262). Reasons for the discrepant findings between studies include different levels of sodium intake and heterogeneous characteristics of the samples, such as varying levels of obesity, metabolic dysfunction, and blood pressure. Unlike the present study however, no past studies consisted of only normotensives, evaluated the effect of weight loss on aldosterone independent of changes in discretionary sodium intake, followed participants after weight loss, or examined as large a variety of obesity-related factors.

The importance of these findings lies in the recent insight that aldosterone is an important cardiovascular and metabolic risk factor, promoting not only hypertension, but also inflammation and remodeling of the heart, vasculature, kidneys, and adipose tissue (108, 111). Higher levels of circulating aldosterone predict incident hypertension (84, 263) and metabolic syndrome (104) in the general population. Furthermore, this study and several others have found that higher circulating aldosterone correlates with insulin resistance (91, 96, 105), an association that may be independent of anthropometric measures of body size (91). Thus, aldosterone appears to influence cardiometabolic health independently of BMI and other traditional risk factors.

A likely explanation for the stronger associations of circulating aldosterone with markers of inflammation, insulin resistance, adipokines, and cardiac autonomic activity than with body size or abdominal adipose tissue area is that it is the quality rather than the quantity of adipose tissue that determines cardiometabolic dysfunction (236, 264). 'Dysfunctional' adipose tissue is characterized by hypertrophied adipocytes, increased macrophage infiltration, hypoxia, and marked changes in adipokine and free fatty acid secretion (264, 265). Elevated production of leptin, angiotensinogen, and reactive oxygen species, and reduced production of adiponectin accompany the accumulation of dysfunctional fat (264). In addition, as excess energy-intake overwhelms the body's fat storage capacity, ectopic fat is stored in tissues such as skeletal muscle and the liver (264). These changes promote insulin resistance, chronic systemic inflammation, RAAS activation, sympathoactivation, and oxidative stress (264). In obese individuals with metabolic dysfunction, both visceral and subcutaneous adipose tissue depots are characterized by increased proinflammatory macrophage content and adipocyte hypertrophy (266, 267). However, these morphological changes in adipose tissue are not necessarily accompanied by significant changes in the amount of total body fat or visceral or subcutaneous

fat mass (266). Nonetheless, unfavorable adipose morphology is linked to intrahepatic and intramuscular fat storage, the latter of which has been found to be associated with metabolic syndrome (248), insulin resistance (268), and diabetes (269) even in normal weight individuals. In addition, intramuscular lipid content has been shown in animal studies to correlate more closely with insulin sensitivity, CRP and adiponectin levels than abdominal visceral fat (270). To our knowledge, this is the first study to examine associations between serum aldosterone and intermuscular fat. However, the findings from separate investigations showing that intermuscular fat (248, 268, 269) and serum aldosterone (104) predict metabolic abnormalities suggest both factors participate, perhaps in an interrelated way, in metabolic dysfunction in apparently healthy individuals.

Although it is impossible in this observational study to determine which obesity-related factors had the greatest impact on serum aldosterone or, in reverse, which factors were most influenced by serum aldosterone, in reality it appears that the investigated factors are both causes and consequences of cardiometabolic decline (271). Adipocytes produce angiotensinogen and Ang II and contribute to the elevated circulating levels of these hormones in obese individuals (272, 273). Though adipocytes are not believed to produce aldosterone (238), one recent study suggests otherwise (239). Additionally, it has recently been discovered that several adipocyte-derived factors increase adrenal aldosterone production independent of Ang II and serum potassium levels (112-114). These effects appear to be independent of well-known adipocyte-derived factors such as leptin, IL-6, TNF-α, adiponectin, and adipose Ang II (112). On the other hand, increased aldosterone secretion by adrenocortical cells results in greater binding and activation of adipocyte MRs, which in turn impacts adipose differentiation, expansion, and inflammation (271). Finally, there is evidence that both circulating and adipose RAAS are

influenced by autonomic activity. Sympathetic nerve stimulation increases the release of both renin and Ang II as well as the stimulatory effect of Ang II on adrenal aldosterone secretion (274, 275). In the reverse direction, elevated circulating aldosterone induces cardiac and renal sympathetic activation through angiotensin type-1 receptor induced mitogen-activated protein kinase signaling in the brain (242). Clearly there seems to be a cycle linking inappropriately high aldosterone levels to adipose dysfunction, and this cycle promotes cardiometabolic decline over time in individuals with excess weight. In animal studies, prolonged obesity causes visceral fat accumulation, insulin resistance and hepatic steatosis, and eventually results in impaired insulin secretion (276). This slow progression may explain why reductions in weight and intermuscular fat correlated more strongly with reductions in serum aldosterone in study subjects who had MetS at baseline; these individuals were likely further along on the spectrum of metabolic dysfunction and thus could reap larger benefits from weight loss. Nevertheless, physical activity and weight reduction are lifestyle changes known to improve cardiometabolic health and are central in reducing adipose tissue dysfunction even in individuals who have had persistent obesity (265).

The beneficial effects of weight loss on blood pressure were clear in this study, but it was somewhat surprising that changes in aldosterone and sodium were not statistically significantly associated with changes in blood pressure in the total study sample. It could be that chronically elevated aldosterone causes rises in blood pressure only over longer time periods, such as the 3 to 4 years over which persons were followed in studies that found aldosterone to predict incident hypertension (84, 263). In addition, the strong effect of weight loss on blood pressure may have overwhelmed the effects of concurrent changes in dietary sodium and serum aldosterone. However, the associations between reductions in sodium excretion and reductions in blood

pressure in individuals of black race or who had MetS or elevated HOMA-IR at baseline agree with past studies (257, 258) and suggest that these subgroups in the overweight/obese young adult population may particularly benefit from sodium reduction along with weight loss to achieve reductions in blood pressure.

Clinically, besides the well established adverse effects of RAAS hyperactivity on blood pressure, the effects of RAAS hyperactivity on adipose and metabolic dysfunction are beginning to be appreciated. These benefits are highlighted by the reduced incidence of type 2 diabetes in individuals treated with angiotensin-converting enzyme (ACE) inhibitors or angiotensin receptor blockers (ARBs) (277). In addition, in essential hypertensives, ACE inhibitors and ARBs lead to improvements in insulin sensitivity (244, 278), increases in adiponectin levels (244), and reductions in sympathetic activity (244, 278) without affecting body weight. In studies in animal models of obesity, MR blockade decreased obesity-related insulin resistance, adipose ROS production, induction of inflammatory cytokines, numbers of hypertrophic adipocytes and infiltrated macrophages in adipose tissue, and increased adipose expression of adiponectin (117, 118); aldosterone had the opposite effects (118). Whether MR blockers have these effects in obese humans remains to be determined.

There were several important limitations to this study. First, its observational design did not allow us to determine which obesity-related factors most influenced serum aldosterone or, in reverse, which factors were most affected by serum aldosterone levels. In reality, however, all of the factors investigated in this study are both causes and consequences of a worsening cardiometabolic profile; thus it is likely that most if not all of the associations detected in this study are in fact bidirectional (271). A second limitation of this study was the small number of males and African Americans, which provided insufficient power to stratify results by sex or

race. However, interactions with these factors were tested and found to be statistically not significant. Another limitation was the lack of data on other RAAS components, such as plasma renin activity and serum Ang II, the latter of which is known to also drive inflammation and insulin resistance (279). In addition, a measure of 24-hour aldosterone excretion might have reflected chronic circulating aldosterone exposure more accurately than a serum measurement. Another limitation of this study was the substantial loss to follow-up. Sensitivity analyses, however, suggested that the present findings were likely not biased by this missing data. One strength of this study was that all participants were normotensive and not using antihypertensive or vasoactive medications, which ruled out treatment related confounding. In addition, the large number and variety of obesity-related factors investigated provide novel insights into the complex role of aldosterone in cardiometabolic health.

In conclusion, in normotensive, overweight/obese young adults followed over the course of a one year lifestyle intervention and one year follow-up period, reductions in fasting insulin, HOMA-IR, CRP, leptin, heart rate, tonic cardiac sympathovagal balance, and increases in adiponectin are associated with decreases in serum aldosterone. Additionally, weight loss and reductions in intermuscular fat are associated with reduced serum aldosterone in individuals with MetS. Importantly, given these findings, along with the recognition that MR antagonists improve overall adipose tissue function in animal models of obesity (117, 118) and have proven benefit for patients with heart failure or hypertension (280), future trials should test the efficacy of these drugs for reducing cardiometabolic risk in normotensive overweight/obese patients. Of course, lifestyle changes must continue to be recommended to all individuals with excess weight, as these can produce improvements in cardiometabolic health even before substantial weight loss occurs (281).

4.6 TABLES AND FIGURES

Table 4.1 Clinical Characteristics across the One Year Intervention and at One Year Post-Intervention

Characteristic	Baseline (N=285)	6 Months (N=233)	12 Months (N=210)	24 Months (N=189)
Aldosterone (pg/mL)	108 (79, 156)	117 (84.3, 156)	104 (84, 140)	107 (83.1, 157.5)
Weight (kg)	91.8 (13.3)	84.6 (13.3)*	85.1 (14.2)*	88.1 (14.6)*
BMI (kg/m^2)	32.9 (3.7)	30.3 (4.1)*	30.4 (4.4)*	31.4 (4.5)*
Waist Circumference (cm)	100.1 (10.3)	94.8 (10.7)*	95.0 (11.8)*	97.5 (12.3)*
SBP (mmHg)	113.2 (10.1)	109.5 (9.0)*	109.7 (9.6)*	112.2 (9.9)
DBP (mmHg)	72.7 (8.5)	70.6 (8.2)*	71.7 (7.8)	73.8 (9.1)*
Glucose (mg/dL)	97.3 (7.9)	97.8 (8.4)	98.2 (8.3)	97.6 (9.5)
Insulin (μU/mL)	12.5 (9.4, 16.7)	11.4 (8.7, 15.4)*	11.8 (9.3, 15.3)	11.9 (9.5, 15.4)
HOMA-IR (mmol/L x μU/mL)	3.0 (2.2, 4.2)	2.7 (2.1, 3.8)*	2.9 (2.2, 4.0)	2.9 (2.2, 3.8)
LDL-C (mg/dL)	123.2 (32.8)	121.4 (29.1)	123.3 (30.8)	125.5 (32.6)
HDL-C (mg/dL)	52.5 (12.7)	52.8 (12.1)	55.5 (13.7)*	54.5 (13.3)*
Triglycerides (mg/dL)	115.5 (79, 169.5)	94 (67, 135)*	88 (71, 136)*	99 (75, 146)*
CRP (mg/L)	2.6 (1.4, 5.6)	2.2 (1.0, 4.6)*	2.1 (0.94, 4.2)*	2.3 (0.91, 5.0)*
Leptin (ng/mL)	26.2 (13.1)	18.6 (11.5)*	21.0 (13.5)*	22.7 (13.4)*
Adiponectin (μg/mL)	11.9 (6.1)	12.1 (5.6)*	12.1 (5.7)	10.6 (5.7)*
Ghrelin (pg/mL)	673.5 (547, 874.5)	774 (614, 1042)*	804.5 (629, 1121.5)*	875 (711, 1113)*

Table 4.1 continued

Sodium Excretion (mmol/24hr) [#]	185.8 (69.1)	154.5 (65.2)*	156.9 (58.9)*	157.6 (63.5)*
Potassium Excretion (mmol /24hr) [#]	60.7 (22.1)	62.0 (21.0)	63.9 (23.1)	61.6 (21.3)
Heart Rate (beats/min)	64.3 (9.2)	62.7 (8.4)*	64.0 (8.9)	63.6 (8.8)
Sitting LFa/RFa^	1.6 (0.87, 2.9)	1.1 (0.64, 2.6)*	-------	-------
Standing LFa/RFa^^	4.9 (2.3, 12.3)	4.4 (2.3, 10.0)	-------	-------
Abdominal visceral fat area (cm^2)[$]	117.8 (56.0)	-------	99.1 (53.5)*	-------
Abdominal subcutaneous fat area (cm^2)[$]	425.0 (122.4)	-------	361.0 (132.2)*	-------
Thigh intermuscular fat area (cm^2)[$]	13.0 (4.8)	-------	7.7 (3.7)*	-------

Mean (SD) or median (IQR) are shown. *P<0.05 versus baseline in a linear mixed model with time since baseline as a nominal variable and with adjustment for intervention arm. Aldosterone, insulin, HOMA-IR, triglycerides, CRP, ghrelin, sitting LFa/RFa, and standing LFa/RFa were log transformed. SBP=systolic blood pressure, DBP=diastolic blood pressure, LDL-C=low density lipoprotein cholesterol, HDL-C=high density lipoprotein cholesterol, HOMA-IR= homeostasis model assessment of insulin resistance, CRP=C-reactive protein, LFa=low frequency area, RFa=respiratory frequency area. [#]Baseline N=285, 6 Months N=184, 12 Months N=158, 24 Months N=136.^Baseline N=277, 6 Months N=227. ^^Baseline N=273, 6 Months N=221. [$]Baseline N=272, 12 Months N=200.

Table 4.2 Associations between Changes in Serum Aldosterone and Changes in Obesity-Related Factors Over the Course of the Study

Independent Variable (Change from Baseline)	Parameter Estimate ($x10^2$)	Standard Error ($x10^2$)	P value*
Weight (%)	2.9	27.0	0.92
BMI (kg/m^2)	-0.007	0.82	0.99
Waist Circumference (cm)	0.12	0.29	0.69
Insulin (μU/mL)	1.4	0.39	0.0005
HOMA-IR (mmol/L x μU/mL)	3.1	1.3	0.02
CRP (mg/L)	2.3	0.61	0.0002
Leptin (ng/mL)	0.62	0.21	0.003
Adiponectin (μg/mL)	-2.0	0.54	0.0002
Ghrelin (pg/mL)	0.0043	0.0053	0.42
Heart Rate (beats/min)	0.91	0.28	0.001
Sitting LFa/RFa	10.8	3.0	0.0004
Standing LFa/RFa	4.4	2.7	0.11
Abdominal visceral fat area (cm^2)	0.12	0.11	0.28
Abdominal subcutaneous fat area (cm^2)	0.042	0.040	0.29
Thigh intermuscular fat area (cm^2)	2.2	1.2	0.053

*P values are from linear mixed models for log aldosterone (pg/mL) that included baseline age, sex, race (black/non-black), intervention arm, time since baseline, and baseline and within-subject changes in 24-hr urinary sodium excretion, potassium, and the specified independent variable. Aldosterone, sitting LFa/RFa, and standing LFa/RFa were log transformed. HOMA-IR= homeostasis model assessment of insulin resistance. CRP=C-reactive protein. LFa=low frequency area. RFa=respiratory frequency area. To calculate percentage change in aldosterone for a one unit change in the independent variable, use the formula $100*(\exp(\beta)-1)$.

Table 4.3 Associations between Changes in Blood Pressure and Changes in Weight, Serum Aldosterone, and Urinary Electrolytes Over the Course of the Study

Independent Variable	Parameter Estimate	Standard Error	P value*
Mean Arterial Pressure			
Weight Loss (%)	22.91	4.18	<0.0001
Sodium Excretion (mmol/24hr)	0.0062	0.0043	0.15
Potassium Excretion (mmol/24hr)	-0.0083	0.015	0.59
Aldosterone (pg/mL)	-0.47	0.62	0.45
Diastolic Blood Pressure			
Weight Loss (%)	19.94	4.49	<0.0001
Sodium Excretion (mmol/24hr)	0.0075	0.0047	0.11
Potassium Excretion (mmol/24hr)	-0.0059	0.016	0.72
Aldosterone (pg/mL)	-0.35	0.67	0.60
Systolic Blood Pressure			
Weight Loss (%)	28.07	4.73	<0.0001
Sodium Excretion (mmol/24hr)	0.0031	0.0049	0.52
Potassium Excretion (mmol/24hr)	-0.013	0.017	0.45
Aldosterone (pg/mL)	-0.85	0.70	0.23

*P values are from linear mixed models for each respective blood pressure measure and included baseline age, sex, race (black/non-black), intervention arm, time since baseline, weight, percent weight loss, and baseline and within-subject changes in 24-hr urinary sodium, potassium, and serum aldosterone. Aldosterone was log transformed.

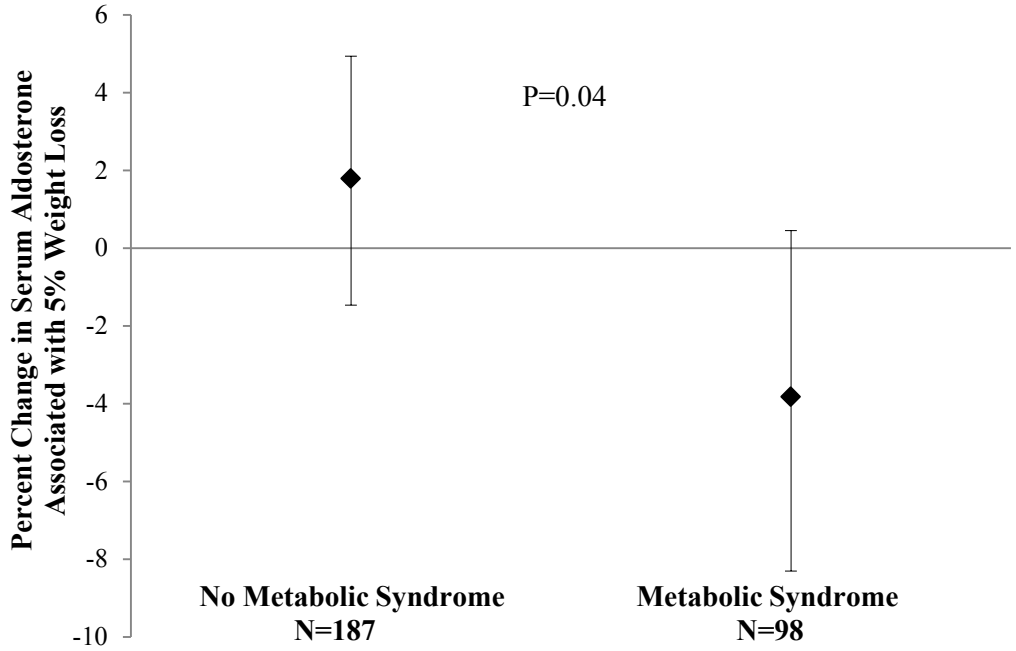

Figure 4.1 Change in Serum Aldosterone Associated with 5% Weight Loss: Differences by Baseline Metabolic Syndrome Status

Parameter estimates and 95% confidence intervals shown are from a linear mixed effects model for log aldosterone that included baseline age, sex, race (black/non-black), weight, intervention arm, time since baseline, baseline and within-subject changes in 24-hr urinary sodium and potassium excretion, percent weight reduction, baseline MetS status, and the interaction between percent weight reduction and baseline MetS status. The p value shown is for the interaction between percent weight reduction and baseline MetS status in this model.

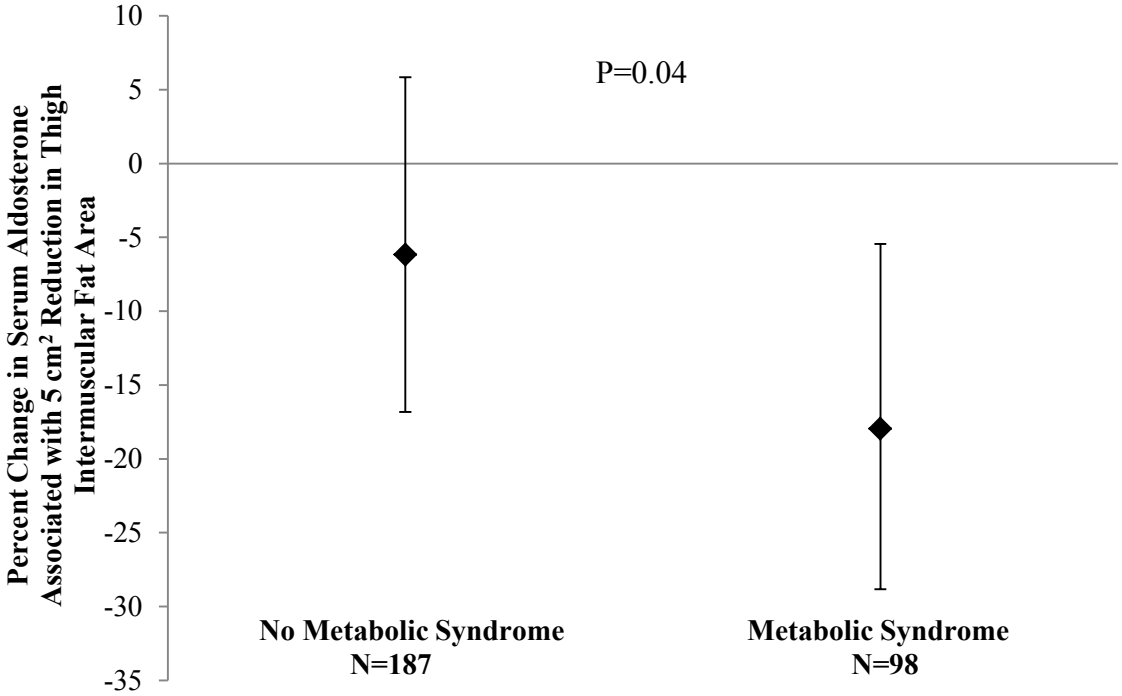

Figure 4.2 Change in Serum Aldosterone Associated with a 5 cm^2 Reduction in Thigh Intermuscular Fat Area: Differences by Baseline Metabolic Syndrome Status

Parameter estimates and 95% confidence intervals shown are from a linear mixed effects model for log aldosterone that included baseline age, sex, race (black/non-black), weight, intervention arm, time since baseline, baseline and within-subject changes in 24-hr urinary sodium and potassium excretion, change in thigh intermuscular fat area, baseline MetS status, and the interaction between change in thigh intermuscular fat area and baseline MetS status. The p value shown is for the interaction between change in thigh intermuscular fat area and baseline MetS status in this model.

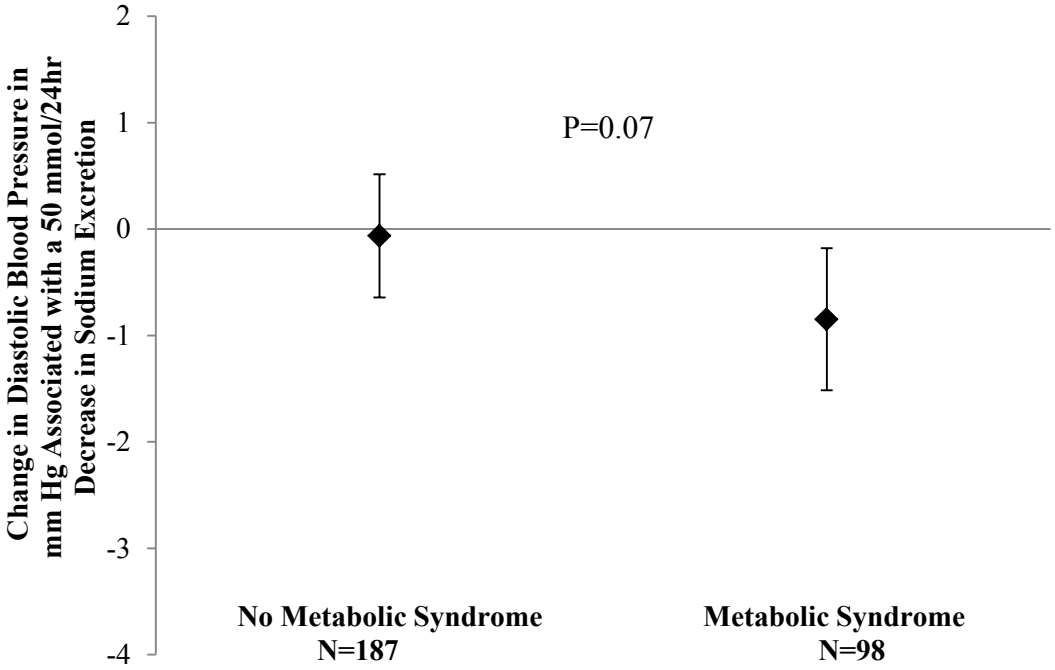

Figure 4.3 Change in Diastolic Blood Pressure Associated with a 50 mmol/24hr Decrease in Sodium Excretion: Differences by Baseline Metabolic Syndrome Status

Parameter estimates and 95% confidence intervals shown are from a linear mixed effects model for diastolic blood pressure that included baseline age, sex, race (black/non-black), weight, intervention arm, time since baseline, baseline and within-subject changes in urinary sodium and potassium excretion and serum aldosterone, percent weight reduction, baseline MetS status, and the interaction between change in sodium excretion and baseline MetS status. The p value shown is for the interaction between change in sodium excretion and baseline MetS status in this model. A decrease in sodium excretion of 50 mmol/day was approximately the median decrease from baseline to 24 months in the Low Na/lifestyle intervention arm.

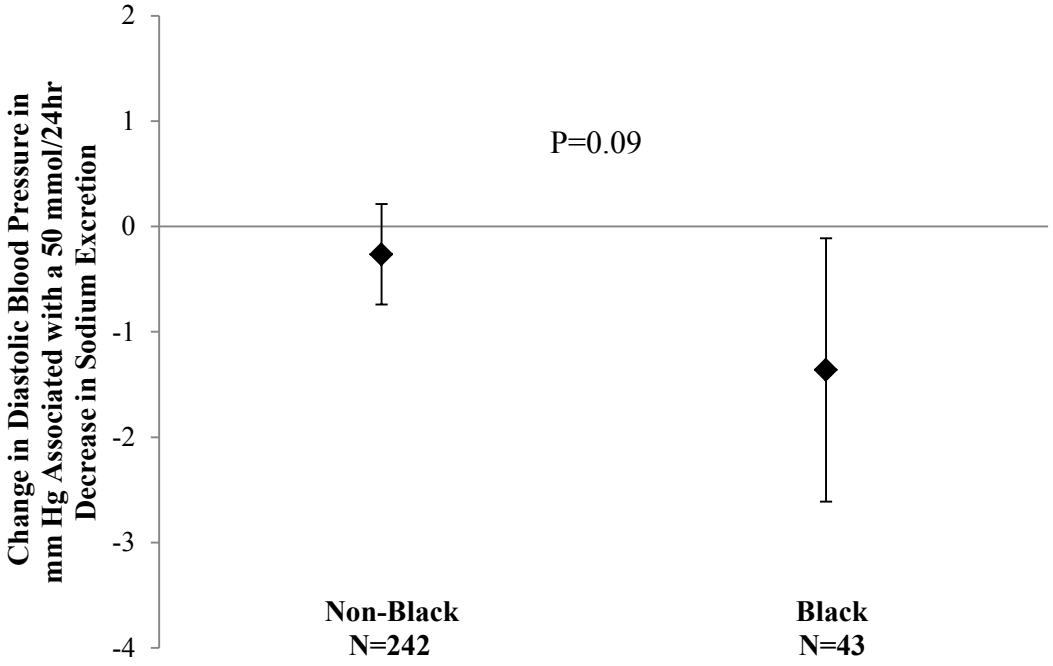

Figure 4.4 Change in Diastolic Blood Pressure Associated with a 50 mmol/24hr Decrease in Sodium Excretion: Differences by Race

Parameter estimates and 95% confidence intervals shown are from a linear mixed effects model for diastolic blood pressure that included baseline age, sex, race (black/non-black), weight, intervention arm, time since baseline, baseline and within-subject changes in urinary sodium and potassium excretion and serum aldosterone, percent weight reduction, and the interaction between change in sodium excretion and race. The p value shown is for the interaction between change in sodium excretion and race in this model. A decrease in urinary sodium excretion of 50 mmol/24hr was approximately the median decrease from baseline to 24 months in the Low Na/lifestyle intervention arm.

Table 4.4 Marginal Associations between Changes in Serum Aldosterone and Changes in Obesity-Related Factors: Results from Pattern-Mixture Models with Multiply Imputed Data

Independent Variable	Parameter Estimate ($x10^2$)	Standard Error ($x10^2$)	P value*
BMI (kg/m^2)	-0.28	1.09	0.80
Insulin (μU/mL)	16.25	6.90	0.02
CRP (mg/L)	4.47	2.66	0.09
Leptin (ng/mL)	0.49	0.26	0.06
Adiponectin (μg/mL)	-2.16	0.69	0.002
Ghrelin (pg/mL)	1.58	6.18	0.80
Heart Rate (beats/min)	0.63	0.31	0.04
Sitting LFa/RFa	10.28	3.55	0.005
Standing LFa/RFa	5.57	2.78	0.047
Abdominal visceral fat area (cm^2)	0.10	0.11	0.35
Abdominal subcutaneous fat area (cm^2)	-0.0016	0.045	0.97
Thigh intermuscular fat area (cm^2)	1.52	1.21	0.21

*P values are for marginal parameter estimates from linear mixed effects pattern-mixture models for log aldosterone that included baseline age, sex, race (black/non-black), intervention arm, time since baseline, baseline and within-subject changes in 24-hr urinary sodium, potassium, and the specified independent variable. Missingness pattern and its interactions with all time-varying covariates were also included in the models; however the estimates shown are marginalized over all patterns. Aldosterone, insulin, CRP, ghrelin, sitting LFa/RFa, and standing LFa/RFa were log transformed to meet the multivariate normality assumption needed for MCMC multiple imputation. HOMA-IR= homeostasis model assessment of insulin resistance. CRP=C-reactive protein. To calculate percentage change in aldosterone for a one unit change in the independent variable, use the formula $100*(\exp(\beta)-1)$.

Table 4.5 Marginal Associations between Changes in Blood Pressure and Changes in Weight, Serum Aldosterone, and Urinary Electrolytes: Results from Pattern-Mixture Models with Multiply Imputed Data

Independent Variable	Parameter Estimate	Standard Error	P value*
Mean Arterial Pressure			
Weight Loss (%)	24.54	4.41	<0.0001
Sodium Excretion (mmol/24hr)	0.003	0.003	0.36
Potassium Excretion (mmol/24hr)	-0.005	0.010	0.61
Aldosterone (pg/mL)	-0.43	0.62	0.49
Diastolic Blood Pressure			
Weight Loss (%)	20.99	4.68	<0.0001
Sodium Excretion (mmol/24hr)	0.004	0.003	0.30
Potassium Excretion (mmol/24hr)	-0.003	0.011	0.78
Aldosterone (pg/mL)	-0.36	0.66	0.59
Systolic Blood Pressure			
Weight Loss (%)	31.17	5.09	<0.0001
Sodium Excretion (mmol/24hr)	0.001	0.004	0.72
Potassium Excretion (mmol/24hr)	-0.009	0.011	0.41
Aldosterone (pg/mL)	-0.61	0.71	0.39

*P values are from linear mixed models for each respective blood pressure measure that included baseline age, sex, race (black/non-black), intervention arm, time since baseline, and baseline and within-subject changes in 24-hr urinary sodium, potassium, and serum aldosterone. Missingness pattern and its interactions with all time-varying covariates were also included in the models; however the estimates shown are marginalized over all patterns. Aldosterone was log transformed.

5.0 ELEVATED AORTIC STIFFNESS PREDICTS HIGHER PLATELET ACTIVATION IN NORMOTENSIVE OVERWEIGHT AND OBESE YOUNG ADULTS

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5.1 ABSTRACT

Objectives Obese individuals have elevated platelet activation and arterial stiffness, but the strength and temporality of the association between these two measures in overweight/obese individuals are unknown. We aimed to determine the effects of cumulative exposure to greater arterial stiffness, measured four times over the course of a two year study, on circulating platelet activity, measured at the end of the study, in overweight and obese young adults.

Methods This analysis included 92 participants (mean age 40 yrs, 60 women) in the Slow Adverse Vascular Effects of excess weight (SAVE) trial, a study evaluating the effects of positive lifestyle changes on vascular health in normotensive overweight and obese young adults. Carotid-femoral (cf), brachial-ankle (ba), and femoral-ankle (fa) pulse wave velocity (PWV) served as measures of arterial stiffness. Platelet activity was measured as plasma beta-thromboglobulin (β-TG).

Results Higher plasma β-TG was correlated with greater cumulative exposure to elevated cfPWV (p=0.02) and baPWV (p=0.04). After adjustment for serum leptin, greater exposure to elevated baPWV remained significant (p=0.03) and greater exposure to elevated cfPWV was marginally significant (p=0.054) in predicting greater plasma β-TG.

Conclusions Greater arterial stiffness, particularly central arterial stiffness, predicts greater platelet activation in overweight and obese individuals. This relationship might partly explain the association between arterial stiffness and incident atherothrombotic events.

5.2 INTRODUCTION

Platelets play an important role not only in thrombotic vascular events but also in the initiation and progression of atherosclerosis (127). Platelets release inflammatory molecules and growth factors that contribute to endothelial activation as well as the migration and proliferation of vascular smooth muscle cells, all of which are key processes in atherosclerosis (127). In the reverse direction, platelet activation is triggered by endothelial cell erosion and ruptured atherosclerotic plaques (127) as well as by elevated shear stress (282). Arterial stiffness is an established marker of vascular health, and stiffer central arteries promote greater wall shear and

tensile stresses, speed up the fatigue of arterial wall components, and promote the vulnerability of atherosclerotic plaques throughout the vasculature (283). Thus, it stands to reason that greater arterial stiffness may also promote a prothrombotic phenotype, including greater platelet activation, throughout the arterial tree. By measuring pulse wave velocity (PWV), arterial stiffness can be estimated in any region of the arterial tree (283). A previous small cross-sectional study of apparently healthy men observed an association between increased platelet activation and increased carotid-femoral pulse wave velocity (cfPWV) (166, 167), a measure of aortic stiffness and independent predictor of cardiovascular events (27, 54). Other studies of apparently healthy adults have found associations between several markers of in vivo platelet activation and brachial-ankle pulse wave velocity (baPWV), a measure of mixed central (aortic) and peripheral arterial stiffness (166, 168). Such associations may be explained by either the influence of activated platelets on the vasculature or the effect of vascular damage and dysfunction on circulating platelets.

Overweight and obese individuals have increased arterial stiffness (284) as well as greater platelet reactivity as measured by agonist induced platelet aggregation (140) and greater circulating platelet activity as measured by urinary 11-dehyhdro-TxB_2 (138), plasma sCD40L (139), soluble P-selectin levels (231), or mean platelet volume (MPV) (142). Several obesity related factors have been associated with increased circulating platelet activity, including elevated waist-hip ratio, body mass index (BMI), and circulating leptin, insulin, and C-reactive protein (CRP) in both adults (138) and children (232). In addition, increased oxidative stress and endothelial dysfunction promote the activation of platelets in obese individuals (15, 151, 232).

Though obesity is linked to both increased platelet activation and increased arterial stiffness, no studies have examined the temporality of this relationship. The aim of this study

was to determine the associations between cumulative exposure to greater arterial stiffness, measured three times during the course of a one year lifestyle intervention and again one year post-intervention, and circulating platelet activity, as measured by plasma β-thromboglobulin (β-TG) (137, 213) at the final study visit, in overweight and obese young adults. We hypothesized that, of cfPWV, baPWV, and femoral-ankle (fa) PWV, the association with platelet activity would be strongest for cfPWV, due to the influence of aortic stiffness on wall stress, blood flow patterns, and atherosclerotic progression throughout the arterial tree (283). We also hypothesized that the association between cumulative cfPWV exposure and platelet activity would be independent of the effects of cumulative exposure to other cardiovascular and metabolic risk factors during the two year study.

5.3 METHODS

5.3.1 Study Population

The Slow Adverse Vascular Effects of excess weight study (SAVE) is a randomized-controlled trial (NCT00366990) evaluating the effects of weight loss, increased physical activity, and reduced dietary sodium intake on vascular health. Participants were recruited from June 2007 through May 2009 using mass mailing.

Eligible participants were men and women 20-45 years of age who were overweight or obese (body mass index (BMI) 25-39.9 kg/m^2) and physically inactive (<8 months of consistent physical activity (PA) during the past 12 months). Exclusions included 1) diabetes, 2)

hypertension or average screening blood pressure ≥140/90 mmHg, 3) cholesterol lowering, anti-psychotic, or vasoactive medication use and 4) current pregnancy or lactation.

Participants who provided a blood sample for the measurement of β-TG at the final study visit were included in this analysis (n=92). All subjects signed informed consent, and the study was approved by the institutional review board of the University of Pittsburgh (Pittsburgh, PA).

5.3.2 Intervention

Three hundred and forty-nine participants received a 1-year lifestyle intervention consisting of diet and physical activity (PA). Participants were randomized to either 1) diet and PA alone (Control Na/lifestyle) or to 2) diet and PA plus reduced sodium intake (Low Na/lifestyle). The lifestyle intervention was delivered in group sessions that occurred weekly for months 1-4, biweekly for months 5-8, and monthly for months 9-12. The goal of the intervention was a 10% reduction in body weight over 6 months and continued maintenance of weight loss thereafter. The additional goal of the sodium reduction intervention (Low Na) was to gradually reduce daily sodium intake to approximately 1 mg Na^+/1 kcal/day, an average reduction of about 50% from the participant's usual diet (207).

5.3.3 Clinic Visits

Participants were to complete clinic visits at screening, baseline, and 6, 12, and 24 months following randomization. Self-reported demographic information, self- and interviewer-administered questionnaires, anthropometric measurements, fasting blood draw, 24-hour urine collection, and non-invasive tests of vascular structure and function were collected at these visits.

5.3.4 Demographic and Physical Measures

Age, race, and smoking status were self-reported. For this analysis, race was recoded as black vs. non-black. Ethnicity was coded as Hispanic or Non-Hispanic. Smoking status was assessed as ever vs. never. Weight was measured in kilograms using a balance scale. Height was measured in centimeters using a stadiometer. BMI was calculated as weight in kilograms divided by height in meters squared. Waist circumference was measured against the participant's skin at the narrowest part of the torso between the ribs and the iliac crest. Blood Pressure (BP) was measured with a mercury sphygmomanometer after participants sat quietly for 5 minutes with feet flat on the floor. Final BP was the average of the last 2 of 3 readings taken 30 seconds apart.

5.3.5 Blood Assays

Blood analytes were measured at the Heinz Laboratory at the University of Pittsburgh's Graduate School of Public Health. Total cholesterol and high density lipoprotein cholesterol (HDL-C) were determined using the enzymatic method of Allain et al.(208). HDL-C was determined after selective precipitation by heparin/manganese chloride and removal by centrifugation of very low density lipoprotein and low density lipoprotein cholesterol (LDL-C)(209). LDL-C was calculated indirectly using the Friedewald equation. Triglycerides were assessed enzymatically using the procedure of Bucolo et al.(210). C-reactive protein (CRP) was measured using an enzyme-linked immunoassay (Alpha Diagnostic International, Inc., San Antonio, TX). Serum glucose was determined enzymatically with a procedure similar to that described by Bondar and Mead (211). Insulin, leptin, and adiponectin were measured using radioimmunoassays developed by Linco Research, Inc. (St. Charles, MO). Aldosterone was

measured using an enzyme-linked immunoassay developed by Diagnostic Systems Laboratories (Webster, TX). The intra- and inter-assay CV% for insulin were 4.8% and 10.5% respectively. The CV% for the other assays were all <3%.

5.3.6 Urine Collection

Valid 24-hour urine collections had volume between 500 mL and 4000 mL, duration ≥22 hours and ≤26 hours, and total creatinine within the expected range (212). From April 13, 2007 to March 6, 2009, analytes were measured using an Ortho Vitros 950. Direct potentiometry was used to measure sodium and colorimetry to assess creatinine levels. Afterward, results were determined using a Beckman Coulter DxC 800 instrument employing an indirect ion selective method for sodium and an alkaline picric kinetic method for creatinine.

5.3.7 Platelet Activity

Circulating platelet activity was measured as plasma β-TG, a platelet-specific alpha granule protein released upon activation (213). Participants were eligible to provide a blood sample for the measurement of plasma β-TG if they had not taken aspirin in the preceding 14 days or any NSAID, antiplatelet, or anticoagulant medication in the preceding 10 days. At the 24 month visit, blood for the measurement of β-TG was drawn into a 4.5 mL vacutainer tube (Becton-Dickinson, Franklin Lakes, NJ) containing an anticoagulant/antiplatelet mixture of citric acid, theophylline, adenosine, and dipyridamole (Thermo Fischer Scientific, Pittsburgh, PA). The tube was chilled on ice for 15-60 minutes then centrifuged at 2000G for 30 minutes at 4°C, after which platelet-poor plasma was obtained from the upper portion of the supernatant and kept frozen at -70°C

until assayed. Plasma β-TG was determined using an enzyme linked immunosorbent assay (Asserachrom, Diagnostica Stago, Parsippany, NJ). The intra- and inter-assay CV% were 3.8% and 13.2% respectively.

5.3.8 Pulse Wave Velocity

Pulse wave velocity measures were generated using the VP2000 system (Omron Health Care Co., Kyoto, Japan), a noninvasive automated waveform analyzer. Aortic stiffness was assessed by cfPWV and peripheral arterial stiffness by faPWV; baPWV served as a mixture of central and peripheral arterial stiffness. Following ten minutes of rest in a supine position, the participant had occlusion and monitoring cuffs placed around both arms and ankles, ECG electrodes on both wrists and a phonocardiogram on the left edge of the sternum. Occlusion cuffs at the brachial and tibial arteries were connected to pressure sensors that measured blood pressure and pressure waveforms at these peripheral sites as previously described (285). Handheld tonometers were used to simultaneously obtain femoral and carotid pulse waveforms. PWV (in cm/sec) was calculated as the path length between arterial sites of interest divided by the time delay between the foot of the respective waveforms. For cfPWV path length, the distance between the carotid and femoral sites was measured (in cm) over the surface of the body with a tape measure. The path lengths for baPWV and faPWV were calculated using height-based formulas (285). For baPWV and faPWV, results for the right and left legs were averaged. For all PWV measures, data were collected twice for each participant, and the values were averaged. Participants with valid PWV measures (defined as between 300 m/s and 2500 m/s) were included in analyses. Intraclass correlation coefficients (ICC) for within technologist replicate measures were 0.76

(cfPWV), 0.97 (baPWV), and 0.96 (faPWV), and for between technologists replicates were 0.60 (cfPWV), 0.87 (baPWV), and 0.87(faPWV).

5.3.9 Statistical Methods

Descriptive statistics were calculated to summarize study variables at baseline and 6, 12, and 24 months follow-up and were presented as median/inter-quartile range (IQR) or mean (SD) for continuous variables and frequency and percentages for categorical variables. Whether the changes in body size, cardiovascular and metabolic risk factors, and PWV measures were statistically significantly different from zero at each follow-up visit was determined by testing the coefficient for time, as a nominal variable, in a linear mixed model with unstructured covariance. Non-normally distributed variables were transformed as necessary prior to modeling. Intervention arm was included as a covariate in every model for consistency with trial design. An interaction between intervention arm and time since baseline was used to test whether changes over time in study variables differed by intervention arm.

Similar descriptive statistics were presented for the area under the curve (AUC) of the four serial measurements of each factor of interest, and this served as a measure of cumulative risk factor burden over the two year study period. To calculate the AUC for each risk factor for every study participant, linear mixed models (growth curves) with the serial risk factor measurements as the dependent variable were used. For these models, the intercept, linear time since baseline, and quadratic time since baseline effects had both fixed and random components. Higher-order random and fixed effects were not kept in the model if not statistically significant at $p<0.10$. Intervention arm was also included as a covariate in each model for consistency with trial design. Time since baseline was centered at its mean value to minimize collinearity. The

AUC was then calculated by integrating the individual's estimated growth curve over his/her total follow-up period (286). Finally, this value was divided by the individual's total follow-up time, since there was subtantial variability in the timing of the 24 months visit due to participants being permitted to attend this visit outside of the prespecified visit window in order to maximize attendance.

Next, associations between β-TG and either risk factor levels at 24 months or cumulative risk factor exposures were tested by examining Pearson correlation coefficients. Risk factors of interest were cardiometabolic and vascular factors known to be associated with CVD risk and included BMI, waist circumference, mean arterial pressure (MAP), pulse pressure (PP), LDL-C, HDL-C, triglycerides, insulin, HOMA-IR, CRP, leptin, adiponectin, aldosterone, 24-hr urinary sodium excretion, heart rate, and each PWV measure (cfPWV, baPWV, and faPWV). The available sample size provided 80% power to detect correlations of r=0.29. For those AUC PWV measures that showed statistically significant correlations with β-TG, multiple linear regression models for β-TG were examined. After centering covariates to reduce collinearity, stepwise selection of covariates (other than PWV) was used with entry and removal p-values of 0.15 and 0.10 respectively. Covariates considered for inclusion included all measures of cumulative risk factor exposure that had shown statistically significant correlations with β-TG at $p<0.10$. In all of these analyses, non-normally distributed variables were transformed as necessary. Values of $p<0.05$ were considered statistically significant. Statistical analyses were performed using the statistical package SAS (Statistical Analysis Software release 9.3, Cary, NC, USA).

5.4 RESULTS

Plasma β-TG was measured in 92 individuals at the 24 month visit of the parent trial. The sample had an average age of 40.2 yrs (SD 5.9) and consisted of 60 women, 13 African-Americans, and 8 current and 20 past smokers. Clinical characteristics of this sample over the course of the two year study are shown in Table 5.1.With the exception of MAP, which was slightly lower among individuals in whom platelet activity was measured, characteristics were similar between trial participants with and without platelet activity data. Median plasma β-TG was 25.8 IU/mL (IQR 18.6, 35.9), which was within the normal range and did not differ by race, sex, age, or smoking status ($p>0.20$ for all).

In cross-sectional analyses, higher plasma β-TG was statistically significantly correlated with higher BMI ($r=0.25$, $p=0.02$), leptin ($r=0.21$, $p=0.049$), and baPWV ($r=0.22$, $p=0.04$), and marginally significantly correlated with higher cfPWV ($r=0.19$, $p=0.07$) and faPWV ($r=0.20$, $p=0.06$). Similarly, higher plasma β-TG was correlated with higher BMI and leptin when these factors were assessed as AUC from baseline to the 24 month study visit (Table 5.2). In addition, higher plasma β-TG was statistically significantly correlated with greater cfPWV and baPWV when the latter were assessed as AUC (Figure 5.1).

In multiple linear regression models derived from the stepwise selection of AUC exposures showing correlations with plasma β-TG at $p<0.10$, greater cumulative exposure to elevated baPWV and leptin were significant predictors and greater cumulative exposure to elevated cfPWV was a marginally significant predictor of greater plasma β-TG at the end of the two year study period (Table 5.3). Adding BMI AUC to these models resulted in no predictor being statistically significant in either model for plasma β-TG, but in these models cfPWV AUC

(p=0.15) and baPWV AUC (p=0.10) did come the closest to achieving statistical significance of all included predictors.

5.5 DISCUSSION

The main finding of this study was that, in overweight and obese but otherwise healthy young adults, greater circulating platelet activity, as measured by plasma β-TG, was predicted by greater exposure to stiffer arteries during the preceding two years. However, greater exposure to excess weight and serum leptin over the same time period, when examined together, removed the statistical significance of this association. Our findings suggest that elevated arterial stiffness, particularly central arterial stiffness, might be one of the mechanisms by which platelet activation is increased in overweight and obese individuals. Because of the key roles that platelets play in thrombotic events and the initiation and progression of atherosclerosis (127), these findings might also partly explain the association between arterial stiffness and incident cardiovascular events (54).

Arterial stiffness is an established marker of vascular health, and stiffer central arteries promote greater wall shear and tensile stresses, speed up the fatigue of arterial wall components, and promote endothelial damage and atherosclerotic plaque vulnerability throughout the arterial tree (283). The present findings agree with those from at least three cross-sectional studies of individuals at low to moderate CVD risk, in which associations were found between several markers of in vivo platelet activation and both cfPWV(167) and baPWV(166, 168). Though the present observational study cannot establish that arterial stiffening causes increased platelet activation, there are several potential mechanisms that may explain this prospective association.

First, platelet activation is triggered by endothelial damage (127), and several studies have found evidence of endothelial dysfunction, as measured by flow-mediated dilation (FMD), in individuals with elevated arterial stiffness, as measured by aortic PWV (287, 288), cfPWV (287, 288) or baPWV (289). Circulating levels of endothelial microparticles, which are released upon endothelial cell activation and closely relate to reduced endothelial structural and functional integrity, are positively associated with baPWV in type 2 diabetics and healthy adults (290). Second, shear stress plays an important role in platelet activation and is influenced by arterial stiffness. Pathologically high shear stress activates platelets and is an important driver of thrombus formation in occluded vessels (282). However, even in individuals with minimal atherosclerosis, as central arteries stiffen blood flow velocity increases, thereby increasing shear stress and creating a steep systolic pressure waveform that enhances the pulsatility of shear stresses in peripheral vessels (283, 291). Such oscillatory shear stress can induce a prothrombotic, prooxidant, and proinflammatory state in vascular endothelial cells, particularly in less compliant vessels (292-294). In addition, flow reversal may occur during diastole in peripheral vessels as the central arteries stiffen, which may trigger pathological changes in the endothelium (295). Finally, cyclic strain of the vascular wall induces endothelial cell expression of adhesion molecules and vascular smooth muscle cell migration and proliferation (296). Altogether, these changes promote platelet activation and atherosclerosis (127). It is therefore evident that the close relationship between arterial stiffness, shear stress, and endothelial damage might explain the association between greater arterial stiffness and greater circulating platelet activity.

Importantly, however, in this study the associations between plasma β-TG and both cfPWV and baPWV appeared to be explained by excess weight and serum leptin, when

considered together. Several studies have shown positive associations between platelet activation and excess body weight (13, 138, 139, 142) or reduced platelet activation with weight loss (13, 138, 139, 224). One possible mechanism for these relationships is that obese individuals have larger platelets than normal weight individuals (142). These larger platelets have greater metabolic and enzymatic activity as well as thrombotic potential (225, 226). In addition, platelets exhibit membrane receptors for both insulin and leptin, and insulin resistance and elevated circulating leptin promote platelet activation and aggregation (15, 140, 230). The correlation in this study between plasma β-TG and serum leptin, measured either concurrently or as a cumulative exposure, suggests that leptin may play a role in platelet activation in overweight and obese adults.

The lack of correlation between plasma β-TG and either MAP or PP in this study was somewhat surprising in light of the statistically significant correlations detected between β-TG and measures of arterial stiffness. Cross-sectional studies in obese and normal weight adults have found significant correlations between markers of in vivo platelet activation and both SBP and DBP (159, 231). One cross-sectional study of hypertensives also reported a correlation between pulse pressure and mean platelet volume (297). Furthermore, platelet activity is higher in hypertensives than normotensives (143, 298). It may be that the inclusion of only normotensives prevented the present study from detecting associations between plasma β-TG and blood pressure. Additionally, PP was measured at the brachial artery. Central PP is more closely associated with aortic stiffness and increases more with age and other cardiovascular risk factors (291, 299). Furthermore, central PP has a marginally better predictive ability for incident vascular events and is more closely associated with end-organ damage than brachial PP (299,

300). Thus, had central PP been measured, it may have shown a correlation with platelet activity similar to that of cfPWV.

There were several important limitations to this study. First, the small sample size limited the power to detect associations between plasma β-TG and cumulative exposures in multivariable models. Second, despite the prospective design of this study, reverse causation could exist due to the tracking of plasma β-TG levels over time. It is possible that activated platelets may influence arterial stiffness, perhaps through their release of vascular smooth muscle cell growth factors and extracellular matrix modulators (301). Third, it would have been informative to evaluate several measures of platelet activity. The measurement of other platelet specific proteins in plasma or urine or the detection of platelet surface proteins using flow cytometry might have improved the accuracy of platelet activity assessment. However, plasma β-TG is a more sensitive marker of circulating platelet activity than flow cytometric measures, though it requires very careful sample collection to avoid ex vivo artifacts (137, 233). We attempted to minimize ex vivo activation by avoiding trauma during blood draws, drawing blood samples used for β-TG measurement as the last of three samples, and keeping the samples on ice prior to centrifugation. A notable strength of this study is that all participants were normotensive and not on antihypertensive, lipid lowering, or vasoactive medications, which enabled us to evaluate associations of interest independent of potentially confounding treatment effects.

In conclusion, in overweight and obese but otherwise healthy young adults, greater exposure to arterial stiffness over a two year period is a predictor of greater circulating platelet activity, as measured by plasma β-TG. Greater exposure to excess weight and serum leptin over time are also associated with greater platelet activity. These findings suggest that elevated arterial stiffness, particularly central arterial stiffness, might be one of the mechanisms by which

platelet activation is increased in overweight and obese individuals. Future studies of lifestyle modification and other arterial "de-stiffening" strategies in overweight and obese adults should examine whether sustained reductions in arterial stiffness can reduce the risk of thrombotic events.

5.6 TABLES AND FIGURES

Table 5.1 Clinical Characteristics over the Course of the Study

Characteristic	Baseline (N=92)	6 Months (N=90)	12 Months (N=90)	24 Months (N=92)	Cumulative Risk (AUC) (N=92)
BMI (kg/m^2)	32.4 (3.6)	29.7 (4.0)*	29.9 (4.2)*	31.1 (4.2)*	30.3 (3.7)
Waist Circumference (cm)	101.6 (11.0)	95.0 (11.7)*	96.0 (12.2)*	98.1 (12.6)*	96.4 (10.6)
Mean Arterial Pressure (mmHg)	86.4 (7.6)	83.3 (7.2)*	84.1 (7.6)*	85.7 (7.8)	84.6 (5.4)
Pulse Pressure (mm Hg)	40.0 (7.6)	39.3 (7.6)	37.0 (7.1)*	37.8 (7.5)*	38.6 (4.8)
Glucose (mg/dL)	98.8 (9.2)	98.2 (9.2)	98.9 (8.8)	98.1 (10.4)	98.5 (6.1)
Insulin (μU/mL)†	12.5 (10.1, 16.2)	11.3 (9.1, 14.9)*	12.0 (9.3, 16.0)	12.1 (9.6, 16.2)	11.4 (9.7, 14.3)
HOMA-IR (mmol/L x μU/mL)†	3.0 (2.5, 4.2)	2.8 (2.2, 3.6)*	2.9 (2.2, 4.0)	3.1 (2.2, 4.0)	2.8 (2.4, 3.5)
LDL-C (mg/dL)	124.9 (34.3)	120.6 (33.4)	124.5 (33.5)	123.0 (33.7)	123.0 (29.2)
HDL-C (mg/dL)	51.3 (14.6)	52.5 (13.8)	54.3 (13.8)*	54.0 (14.2)*	53.4 (13.1)
Triglycerides (mg/dL)†	110.5 (74, 189)	89 (64, 138)*	99.5 (73, 135)*	98 (77, 146)*	94.9 (73.0, 137.9)
CRP (mg/L)†	2.5 (1.3, 5.2)	1.9 (0.85, 3.8)*	2.0 (0.80, 3.7)*	2.5 (0.83, 4.5)*	1.8 (0.76, 2.7)
Leptin (ng/mL)	22.7 (13.0)	15.0 (10.6)*	19.1 (14.2)*	20.7 (13.3)*	17.7 (10.1)
Adiponectin (μg/mL)	11.2 (5.9)	11.7 (5.9)	11.2 (5.7)	9.6 (5.5)*	11.0 (5.2)
Aldosterone (pg/mL)†	97.1 (78.5, 126.5)	107 (79.3, 147)	102 (83.9, 140)	108.5 (88, 160)*	108.0 (97.4, 131.8)

Table 5.1 continued

Sodium Excretion (mmol/24hr)[#]	187.3 (64.3)	156.2 (66.3)*	154.0 (61.2)*	154.0 (61.6)*	157.4 (27.3)
Heart Rate (beats/min)	62.7 (8.2)	60.7 (7.5)*	62.6 (8.8)	61.9 (8.3)	62.0 (5.2)
Carotid-femoral PWV (cm/s)[†]	817 (700, 934.5)	767.5 (683, 907.5)*	785.5 (680.3, 907.5)*	787.3 (690.3, 903)	774.6 (717.0, 875.0)
Brachial-ankle PWV (cm/s)	1218.7 (128.4)	1196.5 (140.8)	1201.2 (130.9)	1205.3 (130.5)	1207.9 (108.2)
Femoral-ankle PWV (cm/s)	946.1 (102.9)	945.0 (112.9)	939.8 (105.5)	941.0 (100.6)	945.3 (78.0)

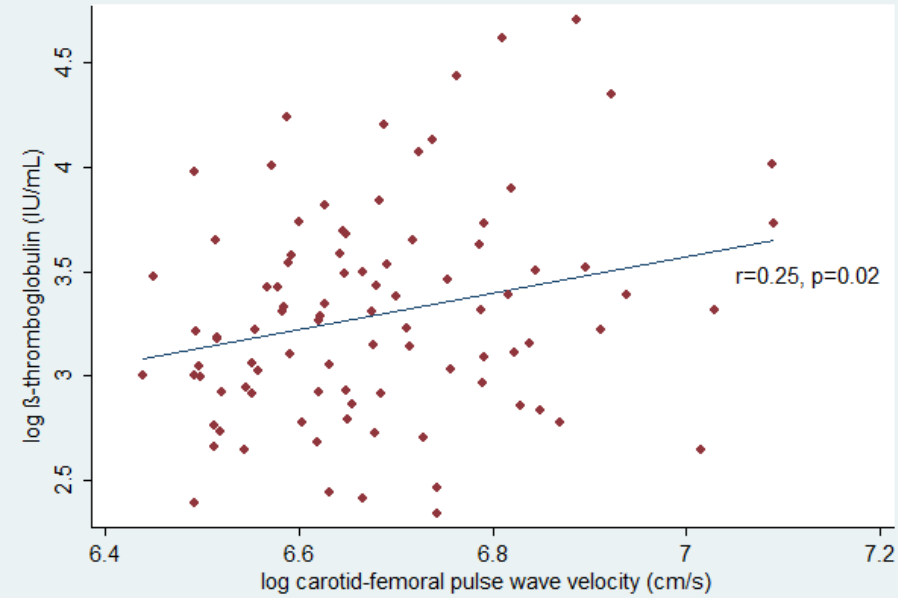
Mean (SD) or median (interquartile range (IQR)) are shown. *P<0.05 versus baseline in a linear mixed model with time since baseline as a nominal variable and with adjustment for intervention arm. LDL-C=low density lipoprotein cholesterol, HDL-C=high density lipoprotein cholesterol, HOMA-IR= homeostasis model assessment of insulin resistance, CRP=C-reactive protein, PWV=Pulse Wave Velocity, AUC=area under the curve divided by total follow-up time in years. [†]Log transformed for mixed modeling and AUC calculation; median and IQR AUC values shown were back exponentiated. [#]Baseline N=71, 6 Months N=68, 12 Months N=68, 24 Months N=60.

Table 5.2 Pearson Correlations between β-thromboglobulin and Cumulative Exposure to Cardiometabolic and Vascular Risk Factors Over the Preceding Two Years

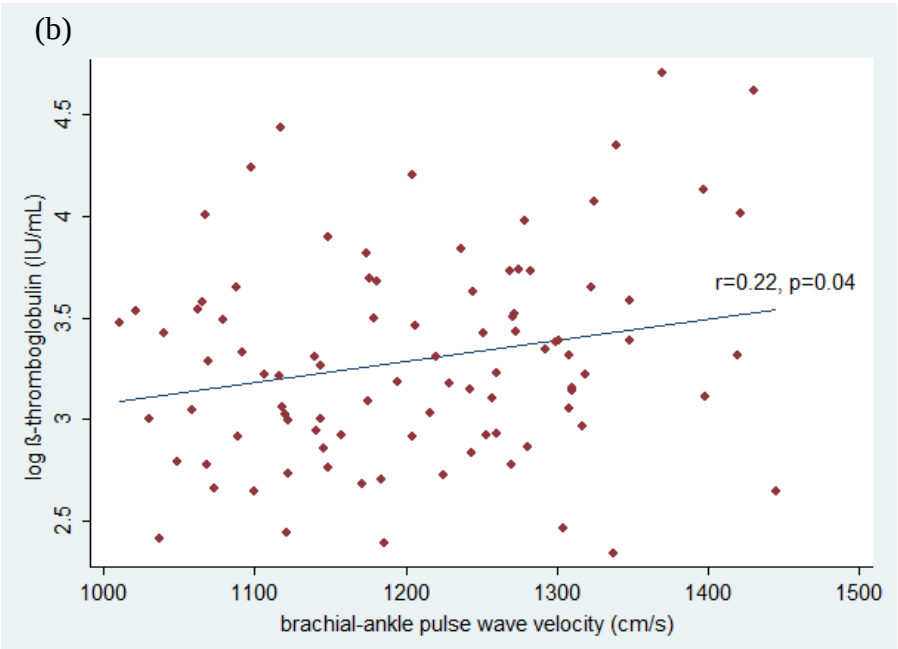
Variable	r	P value
BMI	0.28	0.007
Waist Circumference	0.07	0.48
Mean Arterial Pressure	0.05	0.63
Pulse Pressure	0.14	0.17
LDL-C	0.07	0.52
HDL-C	0.06	0.58
Triglycerides	0.09	0.39
Insulin	0.14	0.17
HOMA-IR	0.15	0.16
CRP	0.19	0.08
Leptin	0.25	0.01
Adiponectin	-0.07	0.48
Heart Rate	-0.04	0.71
24-hr Sodium Excretion	0.02	0.83

Plasma β-TG, triglycerides, insulin, HOMA-IR, and CRP were log transformed. BMI=body mass index; SBP=systolic blood pressure; DBP=diastolic blood pressure; LDL-C=low density lipoprotein cholesterol; HDL-C=high density lipoprotein cholesterol; CRP=C-reactive protein. N=92.

(a)


log ß-thromboglobulin (IU/mL)
2.5
3
3.5
4
4.5
6.4
6.6
6.8
7
7.2
log carotid-femoral pulse wave velocity (cm/s)
r=0.25, p=0.02

(b)


log ß-thromboglobulin (IU/mL)
2.5
3
3.5
4
4.5
1000
1100
1200
1300
1400
1500
brachial-ankle pulse wave velocity (cm/s)
r=0.22, p=0.04

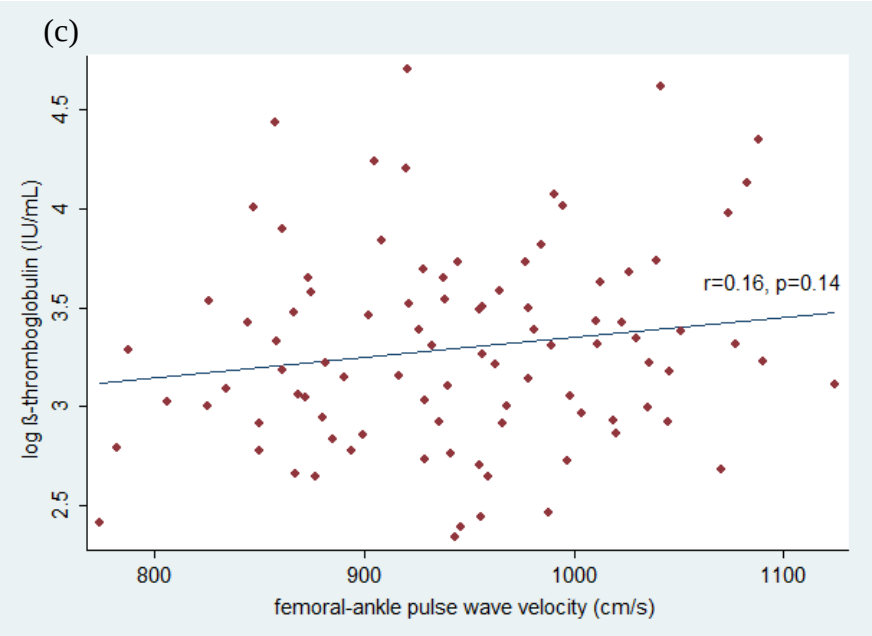


Figure 5.1 Scatterplots of Cumulative Pulse Wave Velocity Exposures vs. β-thromboglobulin for (a) cfPWV, (b) baPWV, and (c) faPWV

Table 5.3 Multiple Linear Regression Models for log β-thromboglobulin: Associations with Cumulative Pulse Wave Velocity Exposures

Variable	Parameter Estimate (SE)	P value
Model including Carotid-femoral PWV		
Carotid-femoral PWV (cm/s)	0.71 (0.36)	0.054
Leptin (ng/mL)	0.011 (0.005)	0.04
Model including Brachial-ankle PWV		
Brachial-ankle PWV (cm/s)	0.0010 (0.0005)	0.03
Leptin (ng/mL)	0.013 (0.005)	0.01

All independent variables are cumulative risk factor exposures over the course of the two year study (area under the curve divided by follow-up years).β-thromboglobulin and carotid-femoral PWV were log transformed. PWV=Pulse Wave Velocity

6.0 DISSERTATION DISCUSSION

6.1 SUMMARY OF FINDINGS

The objective of this dissertation was to examine the roles of circulating aldosterone and plasma β-TG in vascular remodeling and cardiometabolic risk in normotensive overweight and obese young adults. In our first study, we found that, among non-Hispanic whites, the T allele of SNP rs168753 in the gene encoding the main platelet thrombin receptor, was associated with having a higher mean carotid bulb IMT before but not after lifestyle modification. Higher plasma β-TG was not associated with any of the investigated allelic variants in genes encoding platelet membrane receptors but was correlated with greater CCA IMT. In addition, lower plasma β-TG measured one year after the conclusion of the intervention was correlated with lower BMI and greater weight loss during the study. This was the first study to show an association between carotid IMT and an allelic variant in the gene encoding PAR-1 and also the first study to show an association between weight loss and lower circulating platelet activity a considerable time after the weight loss intervention occurred. Given that recent genome-wide association studies of segment-specific carotid IMT have not detected significant SNPs near or within genes encoding platelet membrane receptors (222, 223), and given the nonsignificance of rs168753 as a determinant of carotid bulb IMT at the final study visit, it is probable that this SNP does not play a causal role in carotid atherosclerosis. However, it is known that platelet activation plays an

important role in early atherosclerosis and vascular remodeling. Activated platelets promote atherosclerosis through the actions of the numerous growth factors and cytokines they secrete (15). In obese individuals, platelets are larger and "angrier" than they are in normal weight individuals (141). This is thought to be due to obesity-related metabolic alterations, such as insulin resistance and elevated circulating leptin, which promote platelet activation and aggregation (15, 140, 230).

In our third study, we found that higher cfPWV and baPWV, assessed as cumulative exposures during the two year SAVE study, predicted higher plasma β-TG at the end of the study. Given the key roles that platelets play in thrombosis and atherosclerosis (127), these findings might also partly explain the association between higher arterial stiffness and increased risk of incident cardiovascular events (54). The small sample size of this study, however, limited our ability to adjust the associations of interest for multiple cardiovascular and metabolic risk factors. From these two studies together, it is evident that circulating platelet activity is correlated with the amount of excess weight and the extent of adverse vascular remodeling in young overweight/obese adults. Though the observational design and potential presence of unknown confounders prevent us from determining causality from these studies, their longitudinal design does enable us to suggest that weight loss reduces platelet activity and that platelet activity is promoted by stiffer arteries. In reality however, given the slow and complex process of vascular remodeling that occurs over years, it is likely that platelet activity is both a cause and consequence of atherosclerosis and arteriosclerosis (302).

Weight loss clearly improved cardiovascular risk profiles in SAVE trial participants, and a reduction in circulating platelet activity may be one pathway by which weight loss reduces CVD risk. Future studies should examine whether elevated platelet activity is a causative factor

in adverse vascular remodeling in apparently healthy overweight/obese young adults, for example by determining the long-term effects of low dose antiplatelet drugs on carotid IMT and PWV. These drugs have already been shown to lower carotid IMT progression in type 2 diabetics (164) and individuals with symptomatic peripheral arterial disease (163). In addition, future studies of lifestyle modification and other arterial "de-stiffening" strategies in overweight and obese adults should examine whether long-term, sustained reductions in arterial stiffness reduce thrombotic risk.

In our second study, we found that changes in serum aldosterone were associated with reductions in fasting insulin, HOMA-IR, CRP, leptin, heart rate, tonic cardiac sympathovagal balance, and increases in adiponectin, independent of dietary sodium reductions and weight loss. In addition, weight loss and reductions in intermuscular fat were associated with reduced serum aldosterone in individuals with MetS. Furthermore, associations between decreases in sodium excretion and decreases in blood pressure were more evident in individuals who had greater baseline metabolic dysfunction or who were of black race. This was the first longitudinal study in a large sample of overweight/obese otherwise healthy young adults to report associations between such a wide variety of obesity-related factors and circulating aldosterone. Given these findings, along with findings from animal studies indicating that MR antagonists improve adipose tissue function (117, 118), future clinical trials should address the effects of these drugs on adipose tissue structure and function and overall cardiometabolic health in overweight/obese adults. To date, there have been no such clinical trials. However, at least two randomized trials examining the impact of MR inhibition on vascular, metabolic, and inflammatory parameters are currently in the recruitment phase; one is recruiting adults with hypertension and metabolic syndrome and the other obese normotensive adults .

In conclusion, we found that (1) weight loss reduces circulating platelet activity, (2) higher arterial stiffness is prospectively associated with increased circulating platelet activity, and (3) increases in serum aldosterone are associated with adverse changes in a wide variety of obesity-related factors in normotensive overweight/obese young adults. Although these studies had several weaknesses, such as small sample sizes and lack of measurement of some potential confounders, they had numerous strengths, particularly the longitudinal design and lack of treatment-related confounding in all studies. Overall, this research clearly shows that elevated platelet activity and aldosterone play important roles in vascular remodeling and cardiometabolic health in overweight/obese young adults. These factors are likely to be useful targets for therapies to reduce cardiovascular and metabolic risk in this population.

6.2 PUBLIC HEALTH SIGNIFICANCE

Cardiovascular disease is the leading cause of death in the US and throughout the developed world (1). Overweight and obese individuals are at increased risk for cardiovascular disease compared to normal weight individuals (10, 304), and excess weight brings about adverse vascular changes long before the occurrence of clinical cardiovascular events (6-8, 304). Elevated aldosterone (12) and platelet activity (13-15) are two important factors linking obesity to declining vascular and metabolic health, which eventually can lead to hypertension, type 2 diabetes, and cardiovascular disease.

Recent nationally representative data from the Centers for Disease Control and Prevention show that 32% of American men and 35% of American women are obese (BMI > 30 kg/m^2) (305). The prevalence of abdominal obesity (waist circumference ≥ 102cm in men and ≥

88cm in women) is even higher, at 44% in men and 62% in women (305). Lifestyle modification must clearly continue to be recommended to overweight and obese individuals; as such changes produce marked improvements in cardiovascular and metabolic risk factors. Unfortunately, these changes are difficult for many overweight and obese individuals to initiate and maintain. Even among SAVE trial participants, who were highly motivated at study onset, there was substantial dropout, and among study completers average weight loss did not reach 10%, the goal of the intervention. Behavior modification programs that are both clinically effective and cost effective on a large scale remain elusive (306). Recently, several new and emerging tools and strategies for discussing weight and assisting overweight and obese patients in busy ambulatory settings have been shown to be useful, though more long-term research in this area is needed (306). Presently, it is clear that we must use all available and useful tools to reduce obesity and its comorbidities, including targeting obesity-related abnormalities such as elevated aldosterone and platelet activity. Only if we reduce obesity and its comorbidities in the population can we continue to see increases in quality of life and life expectancy in our society.

APPENDIX

SAVE TRIAL DATA COLLECTION TABLE

	Screening	Baseline	Intervention	3 mo.	6 mo.	12 mo.	24 mo.
Physical Measures							
Height	**X**						
Weight	**X**			**X**	**X**	**X**	**X**
Waist Circumference	**X**				**X**	**X**	**X**
Blood Pressure	**X**				**X**	**X**	**X**
Physical Measures Updates							
Weight *(If screening visit >4 weeks ago)*		**X**					
Waist Circ. *(If screening visit >4 weeks ago)*		**X**					
Blood Pressure		**X**					
Medications		**X**			**X**	**X**	**X**
Demographics							
Age	**X**						
Gender	**X**						
Race	**X**						
Marital Status	**X**						
Education	**X**						
SES	**X**						
Tobacco use	**X**				**X**	**X**	**X**
Alcohol use	**X**				**X**	**X**	**X**
Fasting Lab Values							
Glucose	**X**				**X**	**X**	**X**
Insulin	**X**				**X**	**X**	**X**
Total Chol.	**X**				**X**	**X**	**X**
HDL	**X**				**X**	**X**	**X**
LDL	**X**				**X**	**X**	**X**
ApoB	**X**				**X**	**X**	**X**
Triglycerides	**X**				**X**	**X**	**X**
Adiponectin	**X**				**X**	**X**	**X**
Leptin	**X**				**X**	**X**	**X**
Ghrelin	**X**				**X**	**X**	**X**
CRP	**X**				**X**	**X**	**X**

Aldosterone	X				X	X	X
Beta-thromboglobulin						X	X
DNA collection (buffy coat)	X						
24hr Urine							
Sodium		X		X	X	X	X
Creatinine		X		X	X	X	X
Potassium		X		X	X	X	X
Uric Acid		X			X	X	X
Food Intake							
DHQ – NCI Web Version		X				X	X
Physical Activity							
Modifiable Activity Questionnaire		X				X	X
Accelerometer (Actigraph)			X				X
7-Day Activity Diary			X				X
Intervention							
Weight Form			X				
Intervention Tracking Form			X				
Vascular Measures							
Aortic PWV (Complior)		X			X	X	X
Peripheral PWV (Omron)		X			X	X	X
Endothelial Function (FMD)		X			X		
CCA Diameter/IMT		X			X	X	X
Full IMT		X					X
Cardiac Indices (Omron)		X			X	X	X
Sympathetic/Parasympathetic Activity		X			X		
CT							
Abdominal and Thigh CT Scans		X				X	

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