GENETIC EPIDEMIOLOGY OF SUBCLINICAL CARDIOVASCULAR DISEASE AND OSTEOPOROSIS INDICES IN AFRICAN ANCESTRY FAMILIES

by

Allison L Kuipers

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This dissertation was presented

by

Allison L Kuipers

It was defended on

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and approved by

Clareann H Bunker, PhD
Associate Professor, Epidemiology
Graduate School of Public Health
University of Pittsburgh

Candace M Kammerer, PhD
Associate Professor, Human Genetics
Graduate School of Public Health
University of Pittsburgh

Iva Miljkovic, PhD
Assistant Professor, Epidemiology
Graduate School of Public Health
University of Pittsburgh

Kim C Sutton-Tyrrell, DrPH
Professor, Epidemiology
Graduate School of Public Health
University of Pittsburgh

Dissertation Advisor: Joseph M Zmuda, PhD
Associate Professor, Epidemiology and Human Genetics
Graduate School of Public Health
University of Pittsburgh

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Cardiovascular disease (CVD) is a major public health concern, especially in African ancestry populations, which have greater risk compared with Caucasians. Subclinical CVD measures provide information on the health of the vasculature and are predictive of future risk of clinical events. Vascular health indices have been associated with lower bone mineral density (BMD) and osteoporosis suggesting a potential common etiology. Intima-media thickness (IMT) and arterial diameter (adventitial diameter [AD] and lumen diameter [LD]) are subclinical CVD measures obtained by carotid ultrasound, whereas pulse pressure (PP) and pulse-wave velocity (PWV) are subclinical measures of arterial stiffness. The genetic influence on these subclinical CVD measures and in the link between CVD and osteoporosis has not been well defined in African ancestry populations. Therefore, we have estimated genetic heritability, genetic correlation of CVD and osteoporosis related traits, and performed univariate and bivariate genome-wide linkage analysis of these traits in 7 large, multigenerational families of African ancestry from the Caribbean island of Tobago. A total of 461 individuals aged ≥18 years were included in these analyses from probands and families who were recruited without regard to their health status. After removing the effects of covariates, subclinical CVD traits were all heritable and there was significant phenotypic and genetic correlation between CVD and osteoporosis related traits. The most promising evidence of linkage was detected for AD-BMD trait-pairs on chromosome 14 (max LOD=5.2) in bivariate analysis and for AD and LD on chromosome 11

(max LOD=4.1) in univariate analysis. The linkage regions contain several genes known to be involved in cardiovascular disease including the *ApoA1/C3/A4/A5* gene cluster, *IL18, BMP4,* and *ESR2*. Further studies of these regions may reveal novel insight into the genetic regulation of subclinical CVD and osteoporosis. These findings have public health significance because determining the genetic regulation of chronic disease may aid in risk prediction and, ultimately, minimize health disparities in African ancestry populations.

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PREFACE

There are many individuals needing acknowledgement for their contribution to this work and, without whom, there would be no project to present. First, I would like to acknowledge and thank my advisor and mentor of more than seven years, Dr. Joseph Zmuda. Thanks to his original interest in and commitment to a sophomore, undergraduate pre-med student, I was entrenched in the world of epidemiology and have been here ever since. His excitement for science and helping the 'little old lady with osteoporosis' got me interested in epidemiology and his willingness to fund a research project for an undergraduate student worker got me interested in genetics; the department of epidemiology hasn't been able to get rid of me since! Thank you, Dr. Zmuda, for always giving me substantial advice on project and for continuing to foster my desire for scientific inquisition.

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One of the most difficult parts of this project was cleaning the linkage analysis data and pedigree structures in the family study. This was quite a large undertaking and was completed in conjunction with a group of individuals the human genetics department. Specifically, the assistance from Ryan Minster, Amy Dressen and Hu Li was overwhelming and this project would be nowhere without their efforts and the expertise of our mentor Dr. Kammerer. I thank you all for your hard work. Also, I thank Dr. John Shaffer for his support and tolerance in contemplating any and all genetic questions that ever popped into my head!

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Lastly, I need to acknowledge and thank my friends and family whose support was critical in maintaining the drive and determination to finish this work. Thank you to my parents, sisters and, especially, my Aunt Elva, who passed away only a few months ago, for always believing in me and pushing me to achieve big goals. I love you all!

1.0 SPECIFIC AIMS

Cardiovascular disease (CVD) is the leading cause of death in the US. Major risk factors for CVD include hypertension, diabetes and dyslipidemia. Diet, obesity, exercise, tobacco use and alcohol consumption are among the most common, modifiable environmental factors affecting CVD risk; however, genetic factors are also well-recognized determinants of CVD. African ancestry individuals have greater risk of CVD compared to Caucasians. Yet, African ancestry individuals also have a more favorable lipid profile and in some studies less coronary atherosclerosis than Caucasians. African ancestry populations tend to have much higher blood pressure and prevalence of diabetes than Caucasians, although the increase in CVD risk appears to be independent of these factors.

Subclinical CVD can be assessed using multiple methods. Atherosclerosis and vascular adaptation can be assessed via carotid ultrasound scans yielding measures of carotid artery intima-media thickness (IMT), adventitial diameter (AD) and lumen diameter (LD). Pulse-wave velocity (PWV) is a non-invasive measure of arterial stiffening, or arteriosclerosis, which can be measured using automated tonometry devices. Pulse pressure (PP) is also a marker of arterial stiffness and reflects the difference between systolic and diastolic blood pressures. Each of these measures is correlated with traditional risk factors for CVD and is predictive of future CVD events and mortality, but the extent to which genetics influence these traits in African ancestry individuals is not well known. Most studies of subclinical CVD genetics have been primarily in

Caucasian populations, or in persons of admixed African descent, such as African Americans. However, given the difference in risk factor profiles and subsequent risk of disease in African ancestry populations, studies specifically characterizing the genetics of subclinical atherosclerosis in homogeneous African ancestry populations are warranted.

An association of osteoporosis related indices and cardiovascular disease has been well documented. For instance, bone mineral density has been inversely associated with subclinical and clinical CVD, albeit in Caucasian populations, even after adjusting for potential confounding factors, such as age, body size, dyslipidemia, diabetes and hypertension. The underlying physiologic link between these two disease processes is not completely understood; perhaps, these processes are connected through calcium or other bone metabolism pathways. For the most part, these observations have been epidemiologic or experimental. The potential for pleiotropic genetic effects on bone and the cardiovascular system has not been investigated. Also, there has been little investigation of this link in populations of African ancestry. Studies in African ancestry individuals could provide additional and unique insight on the CVD-osteoporosis relationship since African ancestry populations tend to have stronger bones but more adverse CVD than their Caucasian counterparts. The proposed dissertation research addressed the following specific aims:

AIM 1: To estimate the residual heritability of and perform genome-wide linkage analysis on common carotid artery traits (IMT, AD and LD). We quantified the genetic influence on these traits and identified genomic regions that may control their variance in 395 men and women from seven, large, Afro-Caribbean families from the island of Tobago.

AIM 2: To estimate the residual heritability of and perform genome-wide linkage analysis on PWV, brachial PP and blood pressure indices. We quantified the extent of genetic influence on these traits and identified genomic regions that may control their variance in 361 men and women from seven, large, Afro-Caribbean families from the island of Tobago.

AIM 3: To estimate the shared environmental and genetic effects between measures of subclinical CVD (IMT, AD and LD) and bone mineral density, bone geometry and bone strength in 461 men and women from seven, large, Afro-Caribbean families from the island of Tobago. Where appropriate, bivariate, genome-wide linkage analyses were used to identify genomic regions that may jointly influence the CVD and bone related traits.

2.0 INTRODUCTION

2.1 CARDIOVASCULAR DISEASE

Cardiovascular disease (CVD) is the leading cause of death in Westernized countries. In the United States in 2006, CVD affected 36.9% of the population[1] and 26.0% of deaths were attributed to diseases of the heart, while cerebrovascular disease was the underlying cause of 5.7% of all deaths[2]. For comparison, all types of malignancies were the cause of death in 23% of all deaths[2]. Currently, it is estimated that more than 1 of every 3 Americans has some type of prevalent CVD, including high blood pressure, coronary heart disease (CHD), heart failure (HF) or stroke[1]. There are marked differences in the prevalence of CVD by ethnicity. Heart disease is more prevalent in Whites than in Blacks (12.1% vs. 10.2%, respectively), however, hypertension (HT) and history of stroke are more prevalent in Blacks than in Whites (HT: 31.8% vs. 23.3%; stroke: 3.6% vs. 2.7%, respectively)[1]. Asian ethnicities have a lower prevalence of heart disease, HT and stroke than any other ethnic group[1]. While men have a greater CVD burden than women of all ages, this relationship appears to be ethnicity dependent. For example, CVD prevalence is higher in White men than in White women (38.1% vs. 34.4%, respectively) whereas CVD prevalence is greater in Black women than in Black men (46.9% vs. 44.6%, respectively)[1]. These differences in CVD are independent of potential confounders and are observed in different areas

of the world[3], suggesting there may be significant biologic differences in the pathophysiology of CVD between men and women and between different ethnicities.

Tobago is a Caribbean island off of the coast of Venezuela and is a part of the island nation of Trinidad and Tobago. The population of Tobago, with 51,000 inhabitants (1990 census[4]), has <3% admixture and is mostly of West African origin[5, 6]. Trinidad and Tobago has only in the last 50 years gone through the Epidemiologic Transition; wherein, the majority of public health burden comes from complex diseases such as CVD and cancers rather than infectious diseases and infant mortality[5]. Thus, the people of Tobago represent an African ancestry population with high risk for CVD that is newly representative of the Westernized world in lifestyle factors, but which is not greatly influenced by admixture.

2.1.1 Atherosclerosis

Atherosclerosis is characterized by the formation of plaques throughout the large arteries of the vasculature and is thought to occur via acute inflammatory responses to endothelial injury in the lining of the lumen[7]. These plaques primarily form in the intimal layer of the arterial wall and are composed of a lipid-rich core and a fibrous cap[8]. Initially, as a plaque grows, the artery attempts to keep the blood pressure and blood flow shear stress constant against the endothelium of the lumen; therefore, the vessel expands outward, increasing the adventitial diameter, but keeping the lumen diameter constant[8-11]. However, as atherosclerosis progresses and the plaques increase in size, blood flow through the lumen can become partially occuluded[8-11]. This is not the usual cause of death from atherosclerosis. Fatalities can occur when the plaque is generally unstable and is predisposed to rupture, at which time an acute inflammatory response is elicited from the contents of the plaque, including oxidized lipids, smooth muscle cells and other fibrous cellular

components, flowing into the blood stream. This results in the formation of a thrombosis, which can travel to the heart or brain leading to myocardial infarction or stroke, respectively.

There have been many risk factors associated with atherosclerosis ranging from lifestyle habits to biochemical measures, some of which are modifiable, while others are not. The three strongest risk factors for atherosclerosis are unmodifiable: age, sex and ethnicity[12-14]. The risk of atherosclerosis more than doubles from the age of 65 to the age of 85[15]. Most studies of cardiovascular risk have been conducted in White populations and show that the risk of atherosclerosis is greatest in men[12-15]; however, the relationship is reversed in Black populations where women have greater atherosclerotic risk[16-18]. Additionally, traditional risk factors for atherosclerosis are high total- or low-density lipoprotein (LDL)- cholesterol, low high-density lipoprotein (HDL)-cholesterol, high systolic blood pressure (SBP), cigarette smoking and diabetes[12].

A tool for risk prediction of coronary events, such as myocardial infarction (MI) or death due to coronary heart disease (CHD), was developed based on these risk factors and is able to identify persons at high 10-year risk for a coronary event - the Framingham Risk Score (FRS)[12]. However, the FRS is not able to completely identify each individual who will develop atherosclerosis. The observation of a meaningful number of events occurring in individuals with only intermediate 10-year risk has lead to years of research into potential risk factors that can further stratify those who would benefit from aggressive early intervention versus those for whom additional intervention is not necessary. Additional risk factors not assessed in the FRS, but that are associated with CVD include obesity[19], C-reactive protein (CRP)[7, 13], coronary artery calcium (CAC)[13, 17], carotid intima-media thickness (cIMT)[13, 17], ankle-brachial index (ABI)[13, 17], lipoprotein(a)[13, 17], homocysteine[13], leukocyte count[13], fasting glucose[13], periodontal disease[13],

metabolic syndrome[17], treadmill exercise response[17] and family history[17]. However, the clinical utility of the FRS in non-White populations is uncertain.

2.1.2 Arteriosclerosis

Arteriosclerosis is the progressive stiffening of arteries that occurs with age. Arterial stiffness generally disrupts the hemodynamics of the vasculature and is predictive of mortality, heart failure, myocardial infarction, stroke, coronary heart disease, dementia and target organ damage[20-24]. Increased vascular stiffness leads to faster pressure waves, which increases central pulse pressure (PP) via increased SBP and leads to isolated systolic hypertension[20]. If the arteries are very stiff, waves reflected at branching points of the vascular tree can return to the heart during systole instead of diastole, thereby increasing central PP, which can be quantified by the augmentation index (AIx)[20-22]. The early return of the pressure wave causes left ventricular hypertrophy leading to poor cardiac profusion and decreased cardiac output, and eventually heart failure[22, 24]. The endothelium of stiff arteries is characterized by a decrease of properly formed elastin molecules, an increase in collagen, disarrayed endothelial cells, fibrosis and infiltration by smooth muscle cells and inflammatory proteins[21, 24, 25]. The central elastic arteries incur the greatest degree of stiffening, while more peripheral arteries tend to be spared, although, they are stiffer than central arteries in healthy individuals and at young ages[20, 24]. While arteries stiffen with age, additional factors such as hypertension, salt intake[26], glucose regulation[27], neuroendocrine signaling[28] and matrix metalloproteinases are also contributing factors of arterial stiffening[20, 24]. Additionally, arterial stiffness tends to be greater in populations of African ancestry than in whites, even after adjusting for blood pressure and additional CVD risk factors[29, 30].

While direct characterization of arterial stiffness is possible via catheterization, the most common and appropriate way to assess arterial stiffness, non-invasively, is via PWV[21, 31], which will be described in more detail in Section 2.2.3. Additionally, waveform analysis, which measures AIx, is another indicator of arterial stiffness that can be obtained non-invasively using applanation tonometry[21, 32]. Elevated central PP and isolated systolic hypertension are also indicative of central arterial stiffening; however, neither they nor AIx are the preferred markers of arteriosclerosis because they are dependent on the speed of the wave[21]. Therefore, non-invasive measures of PWV are the most informative for assessing arterial stiffness in clinical practice and epidemiologic studies.

2.2 SUBCLINICAL CARDIOVASCULAR DISEASE

2.2.1 Carotid Intima-Media Thickness

Intima-media thickness is the distance from the lumen-intima interface to the media-adventitial interface and is, thus, the thickness of the intimal and medial layers of a vessel wall. In beginning stages of the CVD process, there is thickening of the vascular intima as oxidized lipids and inflammatory cells infiltrate the endothelial layer from the lumen[11]. Additionally, the gradual stiffening of the vasculature with age also results in thicker vessels from build up of collagen and fibrous material[24]. The most common location of IMT measurement for assessment of subclinical CVD is at the carotid artery. This location is easily viewable using non-invasive, ultrasound techniques[33-35].

Briefly, current B-mode ultrasonography techniques endorsed by the American Society of Echocardiography[33] include having the participant lie in a supine position, slightly hyperextend their neck and tilt their head approximately 45° away from the scanning probe. Then the sonographer starts with a transverse and longitudinal scan of the complete accessible carotid artery to assess the degree and presence of plaques within the vessel. The cIMT imaging proceeds with longitudinal assessment of the carotid segment to be measured. The images are captured during diastole then analyzed at a subsequent time. These methods allow visualization of the common carotid IMT, internal carotid IMT and the bifurcation IMT and are usually conducted on both the left and ride side of the neck[33-35].

It is possible to clearly visualize both the near and far walls of the common carotid artery, which provides a less variable cIMT measure because of the ability to use a mean measure, and is less dependent on the exact position of the ultrasound transducer[33]. However, there tends to be less plaque in this segment of the vessel and thus may not provide a complete description of underlying disease. Plaques form more readily in the carotid bulb and ICA. Unfortunately, these segments are much more difficult to visualize and only the far wall of the vessel can be accurately measured[33, 35]. This technological limitation increases the variation of any one measurement in these segments and means the measure is highly dependent on the placement of ultrasonographic transducer. Some studies choose to only assess common cIMT as it is highly reproducible and has lower variability[34], while others assess all carotid segments in order to have a broader description of the vessel thickness.

Each type of cIMT measurement appears to have a similar correlation with the amount of atherosclerosis within the entire body[36, 37]. Since ultrasound techniques cannot determine the intima from the media, it is not possible to determine if increased IMT is directly reflective of the

atherosclerotic process within the intima and not due to arteriosclerosis. However, there is a large body of evidence attesting to the observation that cIMT is associated with the level of atherosclerosis in the coronary arteries, the lower extremities and to arterial calcification[37]. Kablak-Ziembicka *et. al.* conducted a comprehensive study of the correlation and potential predictive value of cIMT with multi-level stenoses identified through invasive means[36]. They studied 415 patients with suspected coronary disease admitted to their clinic over a 6-month period. The participants underwent cIMT imaging for a mean aggregate measure of cIMT in the three carotid segments and ultrasonographic assessment of stenosis (defined as a focal plaque occluding >50% of the lumen) in the supra-aortic and iliac/femoral arteries, and angiography of the coronary and renal arteries. Patients were grouped according to number of arterial beds with significant lesions, which was then correlated with the cIMT measure. This study not only found that cIMT was highly correlated with number of arterial beds with significant stenosis ($r=0.751$, $P<0.001$), but in a multivariate model, cIMT was the single greatest predictor of the level of atherosclerosis.

Carotid IMT is also associated with traditional cardiovascular risk factors and predictive of future CVD events and mortality. Large, prospective studies of cIMT progression have been conducted in multiple cohorts including the Atherosclerosis Risk in Communities study[38, 39], the Carotid Atherosclerosis Progression Study[40], the Cardiovascular Health Study[41], the Malmö Diet and Cancer Study[42] and the Rotterdam Study[43]. Each study showed that increased cIMT measure, which differed between studies, was associated with a greater risk of myocardial infarction (MI), stroke or death, depending on outcome assessed. A meta analysis of these data in 2007 provided an overall hazard ratio of 1.26 (95% CI:1.21-1.30) and 1.32 (95% CI: 1.27-1.38) for the risk of MI and stroke, respectively, for every 1 standard deviation increase in common

carotid artery IMT after adjustment for age and sex[44]. These significant hazard ratios persisted after additional adjustment for traditional CVD risk factors, which were also significant covariates of IMT in these studies, such as BMI, systolic and diastolic BP, cholesterols, smoking and diabetes (MI HR[95% CI]: 1.17[1.13-1.22]; stroke HR[95% CI]: 1.23[1.18-1.28])[44]. In addition to these risk factors, IMT has also been shown to correlate with lipoprotein(a), oxidized LDL, homocysteine, C-reactive protein and metabolic syndrome, in some studies[45].

The difference in IMT between Black and White ethnicities has only been studied in smaller populations (n<1000). However, Blacks have consistently been shown to have thicker cIMT than Whites on average[46, 47]. There is evidence that this difference is only independent of other CVD risk factors at the common carotid artery segment, but not at the bifurcation and/or internal carotid artery, where most of prevalent plaques form[46, 47]. This is consistent with the general observation that Blacks have less heart disease than Whites for a given risk factor profile[1], and perhaps, the thickening at the common carotid artery is more reflective of vascular adaptation to hypertension than atherosclerosis.

2.2.2 Adventitial and Lumen Diameters

Arterial diameters are assessed during the carotid ultrasound scan with two measures: adventitial diameter (AD) and lumen diameter (LD). Adventitial diameter is the distance from the near-wall media-adventitia interface to the far-wall media-adventitia interface. Lumen diameter is the distance from the near-wall lumen-intima interface to the far-wall lumen-intima interface. Initially, as a plaque deposition occurs and hemodynamics of blood flow change with aging, the artery attempts to keep the blood pressure and blood shear stress constant against the endothelium of the lumen; therefore, the vessel expands outward, increasing the diameter, while

keeping the lumen a constant size[8-11, 23]. However, as atherosclerosis progresses and the plaques increase in size, the LD eventually narrows and the lumen becomes obstructed[8-11, 23]. Unlike cIMT, AD and LD can only be accurately assessed using the ultrasonography methods described above at the common carotid artery where the near-wall is more easily visible[33]. However, while some studies do report on the internal carotid artery AD, this measure should be interpreted cautiously[33].

Eigenbrodt *et. al.* showed that in healthy participants, arteries widen with age suggesting that arterial remodeling is a natural part of the aging process[48]. However, they also found the association to be much stronger in persons with prevalent CVD, suggesting that in the presence of cardiovascular risk factors and/or disease, this process occurs to a greater degree and, thus, arterial diameter is also reflective of adverse vascular health[48]. These observations are in accordance with a smaller study by Terry *et. al.*[49]. However, that study may have been invalid because they reported major findings from the internal carotid artery, which is known to be prone to measurement error[49]. Additionally, it has been shown that the AD is associated with prevalent MI and future cardiac events[50], although only a small portion of the information from AD is significantly associated with these events outside of the IMT measurement in older adults[51].

The carotid artery tends to be larger in men than in women, which may be explained through the positive association of vessel diameter with height. Vessels need to be larger in taller individuals to allow sufficient blood volume to flow through the vessels without increasing pressure[10, 11, 23]. Therefore, age, sex and height are variables that should be included in analyses of arterial diameter in order to assess the independence of any association from these main determinants. In addition to these factors, arterial diameter is also associated with CVD risk factors, such as obesity, systolic blood pressure, glucose, cholesterol, triglycerides, heart rate,

apolipoproteins, prevalent atherosclerotic plaques and Framingham Risk Score[52, 53]. Common carotid lumen diameter is greater in Blacks than in Whites, suggesting that there are less plaques, even after adjustment for confounding factors[53]. This also supports the observation that there is less heart disease in Blacks compared to Whites[1] with similar risk profiles.

2.2.3 Pulse-Wave Velocity

Direct methods of assessing arterial stiffness involve angiography to insert a catheter into the arterial segment of interest to simultaneously measure pressure and arterial diameter change[31]. However, these methods are very invasive and not generally applicable to large epidemiologic studies. Pulse-wave velocity is the most widely used and the most appropriate, accurate and valid, non-invasive measurement of arterial stiffness in epidemiologic studies[21]. This measure consists of monitoring the pulse-wave at different arterial beds throughout the body including the aorta, carotid, brachial, femoral and ankle arteries, and calculating how long it takes for the wave to travel from one location to the next. The gold standard for measurement of central stiffness is the carotid-femoral PWV (cfPWV)[21, 54]. This is because it is most reflective of central stiffening without influence from peripheral vessels and resembles the actual propagation of blood through the vasculature[21]. However, the measurement of the femoral artery pulse-wave can be physically difficult to obtain in persons with diabetes, metabolic syndrome or obesity[55]. Also, presence of central stenosis can alter the flow of the pulse-wave and result in erroneous time measurements[55]. A novel device from the Colin corporation measures PWV at the brachial and ankle (tibial) arteries and simultaneously measures blood pressure through the use of pressure cuffs on the arm and ankle[56]. Because this device does not obtain PWV measurements at the femoral artery, it is more appropriate for use in a wider variety of populations.

One technical consideration of the brachial-ankle PWV (baPWV) is that because it measures the pulse wave at the ankle, it is not only reflective of central stiffness[57], but of peripheral stiffness, as well, which is not usually of clinical interest[21]. However, a study by Sugawara *et. al.* indicates that the determination of baPWV by central stiffness (R=0.55) is similar to other PWV measures, suggesting the influence of peripheral artery stiffness doesn't diminish the utility of this measure[58]. Another study reported that in individuals with a moderate amount of stenosis in the peripheral arteries, as defined by an ABI<0.95, the baPWV measure may not be accurate because of the influence of the occluded vessels on the pulse-wave[59]. Overall, there is generally good reproducibility from the baPWV measures with inter-observer and intra-observer correlations of 0.98 and 0.87, respectively and corresponding coefficients of variations of 8.4% and 10.0%, respectively[56]. These are similar to reproducibility with other measures of PWV and are better than other measures of arterial stiffness such as AIx (coefficient of variation 13.0%)[54].

Brachial-ankle and other measures of PWV are associated with and predictive of future cardiovascular events and disease. Carotid-femoral PWV, which has been the most widely studied measure of PWV, has been shown to be predictive of cardiovascular mortality in hypertensives[60], end-stage renal disease patients[61, 62], persons with impaired glucose tolerance[63], the elderly[64-66] and in the general population[67, 68]. It is also predictive of future coronary heart disease events, such as MI, revascularization and angina pectoralis in hypertensives[69] and the elderly[64, 65], as well as with fatal strokes in hypertensives[70]. Brachial-ankle PWV has been associated with coronary artery disease[56, 71], presence of abdominal aortic calcification[72] and adds significantly to discriminating risk of future CVD as assessed by the FRS[73]. However, it is critical to note that the majority of studies using the baPWV measurement have been conducted

in Japanese populations where the Colin device was developed. Replication of these findings in other ethnic groups is crucial to determining the utility of baPWV in CVD-related risk prediction.

In addition to clinical CVD events, PWV has also been associated with CVD risk factors such as age, sex, hypertension, obesity, the metabolic syndrome, diabetes, dyslipidemia, smoking, homocysteine, C-reactive protein and race, in cross-sectional studies[21]. Pulse-wave velocity appears to be higher for men than women at younger ages[74], but after the menopausal transition, PWV in women greatly increases creating a quadratic curve in the relationship between PWV and age[73-75]. Pulse wave velocity also appears to be correlated with severity of hypertension, such that higher blood pressures are associated with higher PWV, especially in older individuals, as would be expected[76]. Obesity and the metabolic syndrome are conditions that have been repeatedly associated with increased PWV. It appears that central adiposity, particularly visceral fat, may have the strongest association with PWV[77] and this association as well as the association with the metabolic syndrome may be more evident in women than men[78-80]. Additionally, baPWV may be more informative than cfPWV as a measure of diabetic complication severity because it includes some information of the health of the peripheral, small artery, vasculature[81]. Measures of arterial stiffness tend to be worse in Blacks than in Whites, even after adjustment for hypertension and/or blood pressure[80, 82]. This suggests that there may be an underlying physiologic difference between races that cannot be explained by traditional CVD risk factor profiles alone.

2.2.4 Pulse Pressure

Pulse pressure (PP) is the difference of systolic blood pressure (SBP) and diastolic blood pressure (DBP). As arteries stiffen, SBP increases but DBP is unaffected, therefore, the gap between the two measures widens. Thus, PP is a measure of the gap between SBP and DBP. Naturally, PP increases due to the gradual arterial stiffness associated with age[83-85]. It was recognized in early epidemiologic studies that blood pressure measures, including PP, are predictive of future mortality and cardiovascular events[86-89]. The first measures of PP were taken at the brachial artery where SBP and DBP are traditionally measured. However, it has been more recently noted that brachial PP may not be the same as central PP[90] and, therefore, may not be as reflective of central arterial stiffness and predictive of cardiovascular outcomes.

Brachial PP is greater than central PP because of amplification of the pulse-wave in the peripheral arteries[91, 92]. As the pulse-wave travels away from the aorta, the systolic pressure increases due to the smaller, more muscular arteries with a greater number of branch points. This effect is seen more in younger individuals (<50 years), who can be 'diagnosed' with isolated systolic hypertension from brachial blood pressure measures because of the artifact of amplified pulse pressures[93]. This increase in PP is not due to increased resistance in stiff arteries, but, conversely, due to the normal hemodynamics of healthy arteries in the periphery[93]. The major factor associated with this amplification outside of physiologic features is heart rate[85, 94]. As the heart rate increases, the amplification in peripheral arteries is smaller and, therefore, peripheral artery blood pressure measures are more closely related to central measures. This explains that, with age, the brachial and central measures are more similar; also, men have less of an impact of the pressure amplification than women, because they have greater heart rate[85].

However, there are other noninvasive measures of PP that reflect central PP [95, 96]that are more applicable to clinical settings than the invasive, catheterization techniques of direct central PP acquisition[97]. The first is to use applanation tonometry at the carotid artery to determine SBP and DBP and calculate PP from that, since the carotid artery is elastic, like the aorta and central arteries, so it is not susceptible to pressure amplification. The second technique is to use applanation tonometry at the brachial or radial (peripheral) arteries and then use a standardized transfer function to equate that measure to the relative central PP[21, 95, 96, 98, 99]. While there has been debate to the generalizability of the transfer functions, they are generally accepted as an appropriate approximation for central PP[98, 99]. However, they do not exactly replicate the central values[100].

The important distinction, however, is whether this inherent inaccuracy in brachial PP measures is clinically relevant. In studies that tried to assess this issue, central PP was found to be a better predictor of future cardiovascular events[101, 102] and is a better marker of underlying disease[103, 104] than brachial PP. However, that is not to say that brachial PP does not have clinical use. Brachial PP has been associated with future mortality[89], CHD mortality[87], CHD events[86, 105], MI[88], left ventricular hypertrophy[101], decreased ejection fraction[101] and IMT[103, 104]. Although, in a small study of 180 end-stage renal disease patients, only central PP was associated all-cause and cardiovascular mortality[102]. Overall, most studies with large sample size conclude that, while central PP may be the best measure of cardiovascular risk, brachial PP is still a viable assessment tool when central PP is unavailable.

As expected, brachial PP is associated with traditional CVD risk factors. Pulse pressure increases with increasing age and tends to be greater in men than women[83-85, 106, 107]. By definition, it is also associated with essential hypertension[107]. In addition, it has shown to be

associated with BMI[106], diabetes[106, 107], hypercholesterolemia[106-108], smoking[107, 109] and prevalent CVD[107]. Along with age and sex, heart rate is the other, most important risk factor for increased PP[107]. Also, brachial PP was greater in African ancestry individuals compared to Whites in two very large multi-center studies[106, 110]. One of the studies in over 26,000 participants from America, found an interaction of ethnicity and age, sex, BMI and diabetes, such that, in African ancestry individuals, the effect of these CVD risk factors is multiplicatively worse than the effect seen in White individuals[106]. This suggests there may be an underlying physiologic difference in pressure regulation between ethnicities that is related to the differential rates of hypertension[1].

2.3 SUBCLINICAL CARDIOVASCULAR DISEASE GENETICS

2.3.1 Carotid Intima-Media Thickness Genetics

Heritability (h^2), or genetic heritability, is a measure of the potential genetic contributions to the variance in a trait. Residual heritability (h^2r) is a measure of the potential genetic contributions to the variance in a trait after the effects of environmental covariates are removed. Heritability in the broad sense is defined as the proportion of the total trait variance due to the genetic variance and is given by the following: $h^2_{broad} = \sigma^2_G / \sigma^2_T$ where h^2 is the heritability estimate, σ^2_G is the genetic variance and σ^2_T is the total trait variance[111]. The genetic variance can further be divided into additive and dominance effects, where additive genetic effects are those that are due to the number copies of an allele (0, 1 or 2) at any of the modeled polygenic loci. Dominance effects account for any shift from a strictly additive model; all single gene effects have an additive component but only those that deviate from the additive model have a dominance component.

Heritability is calculated using only additive genetic effects is called narrow sense heritability and is given by the following: $h^2_{narrow} = \sigma^2_A / \sigma^2_T$ where h^2 is the heritability estimate, σ^2_A is the additive genetic variance and σ^2_T is the total trait variance[111]. In both of these equations, it is important to note that because heritability is a proportion of the total trait variance, as that variance changes the estimate of heritability would also change[111]. Thus, heritability estimates are always population-specific. All methods for estimating heritability in large family studies can only estimate narrow sense heritability because they only model additive genetic variance[112].

Heritability of cIMT has been estimated in 17 studies[113-129] (Table 2.6.1). The reported estimates of residual heritability range from 0.16[126] to 0.92[129] with only one study reporting non-significant heritability[118]. Regardless of the population studied, covariates included for adjustment or the exact measure of common carotid artery IMT (ccaIMT) used, the majority of studies reported heritability estimates between 0.30 and 0.50. In studies that also reported estimates for other arterial segments[127, 128], ccaIMT had the highest h^2r with ICA and the bifurcation having lower heritability and a larger influence of environmental covariates. Therefore, there is consistent evidence that cIMT is at least partially heritable and warrants further genetic studies to identify potentially important genomic regulators of these traits.

The study that estimated a h^2r of 0.92[129] was the first heritability estimate published for cIMT in 1996 and has not yet been replicated. This high heritability estimate may have been inflated due to a low sample size, admixture and/or improper statistical methods. The study that found no significant evidence of heritability [118] was also based on a fairly small sample size of twin-pairs. Only two studies included African Americans[114, 125], and only one reported ethnic specific heritability[114]. Although based on a small sample size, the h^2r estimate for ccaIMT among 175 African Americans was 0.72 compared to 0.38 among 973 European Americans and

0.41 in the full sample. Therefore, it appears that cIMT is also heritable within different ethnic groups.

Since cIMT is heritable, many studies have been attempted to identify specific allelic variants contributing to inter-individual differences in these traits. Five genome-wide association or linkage studies have been performed for these traits[114, 120, 130-132], along with over 300[133] candidate gene association studies. The first genome-wide linkage analysis of cIMT was conducted in the Framingham Heart Study Offspring Cohort, a White sample recruited from the general population of Framingham, Massachusetts[132]. This study did not detect even suggestive evidence for linkage for ccaIMT; however, there was a significant linkage signal for the internal carotid artery IMT (icaIMT) on chromosome 12 at 161 cM.

A genome-wide association study using 100,000 single nucleotide polymorphisms (SNP) was also conducted in the Framingham Offspring Cohort[130]. Generalized estimating equations (GEE) and family-based association tests (FBAT) were used to control for the relatedness of individuals and perform linkage analyses. Also, this study analyzed many subclinical atherosclerosis traits including ccaIMT, icaIMT, ABI, stenosis and arterial calcification. The five most significant results for GEE and FBAT for ccaIMT were located on chromosomes 5 (no gene), 17 (*MYOID*), 18 (2 hits, no gene) and 20 (*PCSK2*). There were no significant or suggestive signals of linkage for ccaIMT. The five most significant results for GEE and FBAT for icaIMT were located on chromosomes 3 (no gene), 4 (no gene), 5 (*EFNA5*), 12 (no gene) and 17 (near *KRTAPI-1* and *KRTAPI-3*). There was also significant evidence for linkage on chromosomes 1 (max LOD = 4.23) and 12 (max LOD = 5.05).

The Diabetes Health Study[114] conducted linkage analysis of families affected with type 2 diabetes (T2D) in Forsythe County, NC including both European American (EA) and African

American (AA) participants. This study identified suggestive evidence of linkage on chromosomes 1 (EA only, 52 cM, LOD = 2.18), 13 (EA with T2D, 61 cM, LOD 2.27) and 21 (EA with T2D, 25 cM, LOD = 2.33). There were no linkage signals in the AA sample; however, there were only 72 African Americans included in that study.

The Northern Manhattan Study[120] conducted linkage analysis of cIMT traits in Caribbean Hispanics recruited from probands at high risk for cardiovascular events. This study examined ccaIMT, icaIMT, bifurcation IMT and an aggregate IMT measure. There was no evidence of linkage for ccaIMT or icaIMT. However, significant evidence for linkage with mean bifurcation IMT was found on chromosome 7 (7p14.3, 51 cM, LOD = 3.10) and suggestive evidence of linkage with mean aggregate IMT was found on chromosome 14 (14q31.1, 95 cM, LOD = 2.30).

Genetic association between SNPs from a targeted cardiovascular candidate gene array and aggregate mean cIMT was examined in the Study of Health Assessment and Risk in Ethnic Groups cohort [131]. This study included individuals of South Asian, Chinese and European Caucasian ancestry in Ontario. The array included ~50,000 SNPs that were chosen to encompass ~2100 cardiovascular candidate genes. The most consistent association with cIMT was for rs3791395 in *HDAC4* (chromosome 2). Additional genes with significant associations were *NPR1* (chromosome 1), *COL1A2* (chromosome 7), *SH3GL2* (chromosome 9), *CARKL* (chromosome 17) and *XRCC1* (chromosome 19).

There have also been 384 published candidate gene association reports as summarized in a meta-analysis published in 2010[133]. The most widely studied genes, as defined by being analyzed in >1 study and in a total of >5000 participants, were *APOE* (apolipoprotein E, chromosome 19), *ACE* (angiotensin I converting enzyme, chromosome 17), *MTHFR* (5, 10-methylenetetrahydrofolate reductase, chromosome 1), *NOS3* (nitric oxide synthase 3,

chromosome 7) and *ADD1* (adducin 1, chromosome 4). Only *APOE* demonstrated consistent association with cIMT in all subsets in meta-analyses. *APOE* has also been the most extensively studied candidate gene in Black populations.

2.3.2 Adventitial and Lumen Diameter Genetics

There has been little work published on the genetics of arterial diameter. The only study to estimate the heritability of arterial size was by North *et. al.*[122] in the Strong Heart Family Study among extended Native American families. The residual heritability of lumen diameter was 0.44, which was greater than the estimate for ccaIMT of 0.21. This observation suggests that carotid arterial diameters may be under greater genetic control than cIMT. However, neither this study nor any other, to our knowledge, has reported residual heritability estimates for AD.

Likewise, most genetic association studies have only analyzed minimum LD, since it is a surrogate for plaque severity. Polymorphisms in several candidate genes have been associated with LD, including apolipoprotein A-V (*APOA*5), microsomal triglyceride transfer protein large (*MTTP*)[134], and interleukin-6 (*IL-6*)[135]. Additionally, no association was detected between minimal LD and a candidate locus on chromosome 9p21[136, 137] or with the *ACE* gene[138]. Given the greater heritability of LD than IMT, there is a considerable need for additional research investigating the genetic determinants of carotid arterial diameters.

2.3.3 Pulse-Wave Velocity Genetics

The heritability of PWV has been reported in 7 studies[113, 119, 139-143] (Table 2.6.2). Significant residual heritabilities ranged from 0.19[141] to 0.54[142] and two studies reported no significant

heritability for carotid-brachial PWV (cbPWV)[139, 140]. Carotid-femoral PWV is the most often analyzed trait, however, other measures were reported. There have been no heritability estimates for baPWV reported. The only study that included Blacks did not assess heritability in stratified subgroups defined by ethnicity[142]. The overall estimates of aortic-radial and aortic-foot PWVs were adjusted for ethnicity (h^2r: 0.43 and 0.54, respectively), which doesn't take into account the population specific nature of heritability. Each study represents a different, and sometimes very discrete, population with different measures of PWV and covariate modeling.

In general, heritability estimates were similar between the different measures of PWV, except in studies that included carotid-brachial PWV[139, 140]. In both cases, cfPWV was reported to be significantly heritable (0.40). However, cbPWV had a heritability estimate <0.10 with large standard errors (>0.10); thus, cbPWV does not appear to be heritable in the populations assessed to date. These data raise the possibility that central aortic stiffness as assessed by cfPWV may be more genetically determined than PWV measures that represent a mix of both elastic, central and muscular, peripheral arteries. However, the sample in the study by Ge *et. al.* used aortic-radial and aortic-foot PWV measures, both of which are reflective of the periphery, and reported significant residual heritabilities for both measures[142].

There have also been three genome-wide studies of PWV variables[139, 140, 144]. A linkage analysis of cfPWV and cbPWV in the Framingham Offspring Study[139] identified four regions suggestive of linkage, but no significant signals. The suggestive peaks were on chromosomes 2 (94 cM, LOD = 2.46), 7 (29 cM, LOD = 2.5), 13 (108 cM, LOD = 2.10) and 15 (108 cM, LOD = 2.48).

A genome-wide association study based on 100,000 SNPs in the family-based Framingham Heart Study[140] reported significant genetic associations for cfPWV and cbPWV

using GEE and FBAT, even though the cbPWV was not heritable in their population. Significant genetic association with cfPWV was identified on chromosomes 12 (*USP15*, GEE only) and 4 (*ARHGAP24*, FBAT only). They also identified one genomic region with evidence for significant linkage on chromosome 2 (LOD = 3.04), as well as, three regions suggestive of linkage on chromosomes 4 (LOD = 2.17), 15 (LOD = 2.43) and 18 (LOD = 2.68). They did not identify any genomic region in even suggestive linkage with cbPWV.

A genome-wide association study of 500,000 SNPs and PWV in a family-based sample of 4221 individuals from the Mediterranean island of Sardinia[144] was also completed. The top 85 associations were tested for replication using an independent cohort of 1828 Sardinians and an additional sample of 831 Old-Order Amish. Consistent associations with two SNPs were observed in the Sardinians with one SNP in *COL4A1* (collagen type IV alpha 1, chromosome 13) and another in *MAGI1* (membrane associated guanylate kinase, chromosome 3). However, only the *COL4A1* SNP association was replicated in the Old-Order Amish.

Candidate gene association studies for arterial stiffness have focused on four major categories of genes, including the renin-angiotensin-aldosterone (RAA) pathway genes, matrix metalloproteinase (MMP) genes, endothelial cell-related (ECR) genes and inflammatory response genes[145, 146]. Gene products from the RAA and MMP categories, as well as, nitric-oxide (NO) ECR genes are directly involved in vessel wall elasticity and response. Variants in genes from the RAA pathways that have been associated with PWV including SNPs in the angiotensin II type 1 receptor (*AGTR1*)[147], the angiotensin converting enzyme (*ACE*)[148] and aldosterone synthase (*CYP11B2*)[149]. A genome-wide association study replicated an association between SNPs in the *CYP11B2* gene and arterial stiffness[150], providing stronger evidence for the involvement of this gene in arterial stiffness. SNPs in the *MMP3* and *MMP9* genes have been

associated with arterial stiffness indices, but only variation in *MMP9* has been associated with PWV[151]. The most widely studied NO gene is *NOS3* encoding endothelial nitric oxide synthase. Like *CYP11B2,* SNPs in *NOS3* have been widely replicated in candidate gene[152] and genome-wide[139] studies, affirming that *NOS3* is a strong candidate gene for arterial stiffness. Additional genes that may be involved in PWV variation identified from association studies include estrogen-related genes[153], part of the ECR group, and inflammatory genes such as interleukin-6 (*IL6*)[154] and others[155]. Additionally, the 9p21 locus, which has been associated with clinical cardiovascular disease[156, 157], has also been associated with PWV. However, there are no known genes in this region; thus, the mechanisms underlying the 9p21 association remain unclear.

2.3.4 Pulse Pressure Genetics

Pulse pressure heritability has been estimated in 19 studies[113, 139, 141, 150, 158-172] (Table 2.6.3). All but one[139] of these studies used traditional brachial PP instead of central PP measures. In all but one report[141], PP has been heritable with estimates ranging from 0.11[160]-0.54[170], suggesting there is good evidence that PP has some genetic component. The high estimate of heritability comes from a study of twin-pairs, which are known to give higher estimates of heritability due to the shared environmental factors. However, the greatest heritability estimate within a family study was still moderate at 0.49 in a group of Mexican American Families[158]. Yet, heritability estimates tended to be relatively low, between 0.2 and 0.4, in the majority of family studies. The one study reporting central PP had an estimated heritability of 0.35[139] and was in accordance with other brachial PP measures.

There are conflicting results of the magnitude of heritability for PP compared to other blood pressure traits. In some samples that report PP, SBP and DBP, PP has the greater

heritability than blood pressure traits[160, 164, 166, 168, 171], but in an equal amount of other studies, SBP has the greatest heritability[158, 159, 162, 170, 172]. Similar to other blood pressure traits, PP seems to be under low to moderate genetic control in most populations. There have been more studies that quantified the genetic effect of PP in populations of African descent[165-167, 170, 172] compared to other CVD traits for which few heritability studies have been conducted in non-White populations. This is likely due to the significantly greater prevalence of hypertension and blood pressure dysregulation in Blacks than in Whites[1]. These studies reported similar heritability as studies in the other, mainly White, populations (range h^2r: 0.13[172]-0.54[170]; max h^2r in families: 0.43[166]).

There have also been eight genome-wide studies of PP[139, 150, 160, 161, 164, 167, 169, 171]. These were all linkage studies in related individuals and only one studied used central PP measures[139]. There have been consistent findings for genomic regions suggestive of linkage with PP on chromosome 21[150, 167] (all 2.0<LOD<3.0). However, these are not very strong signals for linkage and the potentially causal genes have not been refined or discussed, as there are many genes under this peaks and none have been previously associated with PP. There has been significant linkage (LOD>3.0) identified on chromosomes 7 (max LOD = 3.30, 37 cM)[161], 11 (max LOD = 3.02, 17 cM)[164] and 18 (max LOD = 3.20, 75 cM)[167]. The region on chromosome 7 contains two previously studied cardiovascular-related genes: interleukin-6 (*IL6*) and neuropeptide Y (*NPY*). This linkage peak was identified in the Strong Heart Family Study in American Indian families[161] and may represent an ethnic group specific signal since it has never been replicated. Two regions further downstream on chromosome 7 have suggestive evidence of linkage in the Framingham Offspring cohort[139, 169], but do not overlap with each other or with the peak identified in the Strong Heart study.

The significant peak on chromosome 11 was identified in non-Hispanic White siblings from the GENOA study who were diagnosed with hypertension before the age of 60[164]. While this univariate linkage peak is also unique to this population, this study also reported significant bivariate linkage with PP and brain atrophy also in this region. This suggests that the region on chromosome 11 may play a greater role in determining PP in the brain and/or be related to early onset of essential hypertension since that is the sample studied. Nonetheless, there are genes under this peak that have been previously studied in a general cardiovascular context: insulin (*INS*), calcitonin gene-related peptide (*CALCA/CALCB*) and adrenomedullin (*ADM*).

The significant linkage peak identified on chromosome 18 was initially identified in a very large study in Black, Asian, Hispanic and White families (total n = 10,798)[167]. The significant signal was identified in the overall population, but not in any of the race-specific analyses, perhaps due to sample size constraints. Interestingly, a meta-analysis of linkage studies for PP that used these data as part of their report, found that the peak persisted through meta-analysis (rank p = 0.027)[173]. This was also the only peak in the meta-analysis containing a previously studied, candidate gene for PP: neural precursor cell expressed, developmentally down-regulated 4-like (*NEDD4L*). In addition, other CVD-related genes included: solute carrier family 14 members 1 and 2 (*SLC14A1/SLC14A2*), serpin peptidase inhibitor, clade B, member 2 (*SERPINB2*), mex3 homolog C (*RKHD2*) and melanocortin 4 receptor (*MC4R*). In addition to the peak on chromosome 18, the meta-analysis identified suggestive linkage peaks on chromosome 8 and 21 in the full cohort (rank p = 0.044 and 0.028, respectively), as well as, chromosome 22 and 10 in European Americans only (rank p = <0.001 and 0.031, respectively). Each of these peaks had been previously identified in one of the studies included in the meta-analysis except the peak on chromosome 10.

The report using central PP was conducted in the Framingham Offspring cohort[139]. They did not identify any genomic regions with significant linkage to central PP. The largest peak was on chromosome 15 (max LOD = 2.92, 122 cM), followed by chromosome 7 (max LOD = 2.85, 172 cM) and chromosome 9 (max LOD = 2.72, 160 cM). The region on chromosome 15 was also identified in a semi-overlapping cohort, the Framingham Heart Study, and contains many potentially causal genes including insulin-like growth factor 1 receptor (*IGF1RI*), myocyte enhancer factor 2A (*MEF2A*), chondroitin sulfate synthase 1 (*CHSY1*) and proprotein convertase subtilisin/kexin type 6 (*PACE4*). However, the other two regions are unique to this study and could suggest differential genetic determinants of central and brachial PP.

There have been over 80 published reports of candidate gene studies for general PP focusing on genes related to vessel wall structure, sympathetic nervous system, lipid metabolism, inflammation, NO metabolism and the rennin-angiotensin system. Genes related to the vessel structure with significant association with PP include fibrillin1 (*FBN1*: 15q21.1)[174] and plasminogen activator inhibitor (*SERPINE1*: 7q21.3-22)[175]. The sympathetic nervous system regulates hemodynamics and the beta2-adrenergic receptor, within that pathway, has also been associated with PP (*ADRB2*: 5q31-32). Lipoprotein genes with significant associations to PP include apolipoprotein B (*APOB*: 2p24-23)[176], apolipoprotein C-III (*APOC3*: 11q23.1-23.2) and lipoprotein lipase (*LPL*: 8p22)[177]. While the inflammation pathway is assumed to be important to all cardiovascular-related traits, the only gene associated with PP is TNF receptor superfamily, member 6 (*FAS*: 10q24.1)[155]. Both the NO and RAS pathways have been widely studied in relation to CVD genetics, including PP. Notably, the nitric oxide synthase 3 gene, which has been associated with many CVD traits, is associated with PP (*NOS3*: 7q36)[152]. The RAS pathway genes have been most consistently associated with PP including angiotensin II type 1 receptor

(*AT1R*: 3q24)[178] angiotensin II type 2 receptor (*AT2R*: 8p22)[179], angiotensin (*AGT*: 1q42-43)[178, 180] and angiotensin converting enzyme (*ACE*: 17q23.3)[64].

2.4 OSTEOPOROSIS AND CARDIOVASCULAR DISEASE

2.4.1 Epidemiology

Both CVD and osteoporosis (OP) risk and prevalence increase with age[181]. More recently, however, epidemiologic studies have demonstrated that this correlation is independent of age and other traditional risk factors[182]. Studies have focused on investigating the correlation of arterial calcification and calcified plaque with bone mineral density (BMD) and fractures in various populations including individuals with CKD and post-menopausal women, in addition to, general population samples of Whites, Mexican Americans and Japanese[183-188]. However, this relationship has not been adequately investigated in populations of African ancestry, even though there is a low risk of OP and high risk of CVD events compared with Caucasians. Additionally, correlation between arterial calcification and osteoprotegerin (OPG), a protein involved in bone metabolism, has been reported in epidemiologic studies[186]. There have also been reports on measures of CVD risk other than arterial calcification, such as PAD, arterial stiffness, intimal-medial thickening, dyslipidemia, diabetes and the metabolic syndrome, and their correlation with bone loss[186, 189-203]. Therefore, the relationship between CVD and OP appears to be more than just the association of both diseases with vascular calcification.

Arterial stiffness, as assessed by PWV, has been shown to be inversely associated with BMD in post-menopausal women[186, 192, 194, 195]. Three of these studies assessed the relationship in

Asian women from Japan[192, 195] or Korea[194], while the fourth was in Caucasian European women in London[186]. Each study was very small with sample sizes of less than 350 women. In each study, multivariate models included BMD at the lumbar spine, femur or total hip as a predictor of PWV (baPWV in the first three and cfPWV for the fourth) independent of other CVD risk factors, such as age, body size and blood pressure. Additionally, this correlation has also been reported in patients with chronic kidney disease in the US[204], Australia[205] and Japan[188]. However, vascular calcification is also very severe in these patients so this correlation may be confounded by the effect of calcification in stiffening vessels and its interference in accurate BMD assessment[205]. The extent to which the relationship between PWV and BMD exists in the general population and in African ethnicities is unknown.

Subclinical atherosclerosis, as assessed by IMT, has also been associated with low BMD[191, 193, 198, 206]. Two of the studies examined the correlation of cIMT and BMD in Mexican Americans, first in just women[198], then in men and women in a slightly different cohort[193]. In the 471 Mexican American women, cIMT was directly correlated with spine BMD, but not radius or hip BMD, after adjustment for age and BMI[198]. However, in the larger study of Mexican American men and women (n=870)[193], cIMT was directly correlated with hip BMD in younger women (<60 years), but inversely correlated with hip, spine and radius BMD in older women (>60 years). Similarly, in younger men there was a direct correlation between IMT and distal radius BMD, but an inverse correlation between cIMT and distal and mid radius BMDs. These findings suggest there may be sex and age affects on the relationship between IMT and BMD. Other smaller studies in post-menopausal Japanese (n=325)[191] and Moroccan (n=72)[206] women have also found correlations between cIMT and lumbar spine and proximal femur BMDs

independent of age and body size. Again, there have been no studies in individuals of African ancestry.

Additionally, atherosclerosis in the periphery, assessed by ABI, has also been reported to be directly correlated with BMD[189, 190, 197, 199]. Three of these studies were large (n>1000), population-based studies conducted in the Caucasian men and women in the US (n=1,778)[190], Caucasian and African American older men in the US (n=5,781)[199], and Chinese older men and women from Hong Kong (n=3,957)[189]. The fourth study was in 368 Italian post-menopausal women[197]. The study that investigated the association in older men, which was the largest study, found that men with PAD (ABI<0.9) had the greatest BMD loss at total hip, trochanter and femoral neck (p=0.02, 0.02, 0.001, respectively) after adjustment for many risk factors for CVD and osteoporosis. The next largest study of individuals in Hong Kong, only found a weak, positive correlation between ABI and total hip BMD (r=0.05, P<0.05) after adjustment for risk factors. Likewise, in the other study in the US, there was only a borderline association of PAD with greater bone loss at the total hip (P=0.05) in women only[190]. However, even in the smaller sample of post-menopausal, Italian women, femoral neck BMD was one of the strongest predictors of PAD after adjustment for additional risk factors. Given the conflicting results on the strength of this association in the general population, further research needs to be done. The largest study included African American individuals, but ethnicity was only adjusted for; thus, the association of PAD and BMD has not yet been addressed in a homogeneous African ancestry population.

2.4.2 Potential Mechanisms Linking Bone Metabolism with CVD

Each of the discussed measures of subclinical CVD have also been shown to be positively associated with serum OPG[186, 196, 207-210] and other markers of bone metabolism and turnover[208, 211], suggesting that there may be a role of bone homeostasis in CVD. Additionally, in certain clinical populations, such as individuals with chronic kidney disease[183] or diabetes[212], the elderly[213] and post-menopausal women[214], both CVD and OP have a higher risk of occurring concurrently in the same individual. This suggests that there may be an underlying shared pathology between the two diseases.

In 2003, Doherty *et al.* reported that calcified regions of the arteries contain cellular components that are also normal constituents of bone[215]. The calcified cells appear to be able to differentiate into osteoblasts, or bone forming cells that are otherwise only apparently in bone[216]. Also, there is bone formation and resorption, by an osteoclast-like cell from a phagocytotic lineage[217], within the calcified walls. Therefore, some have hypothesized that calcium deposition within the arteries occurs when the natural mechanism to inhibit this process is lost. In addition to the osteoblast and osteoclast-like cells, calcified plaques also contain other bone forming proteins such as bone morphogenetic-2, collagen, osteonectin, osteopontin, osteoclacin and osteoprotegerin (OPG)[215].

A major mechanism responsible for vascular calcium deposition is the OPG - receptor activator of nuclear factor kappa-β (RANK) - RANK-ligand (RANKL) pathway. In bone, RANKL is expressed on osteoblast cells and binds RANK on the surface of osteoclasts. This binding initiates osteoclast differentiation and activity. However, OPG is also expressed on osteoblast cells, including those in the vasculature. Osteoprotegerin blocks the binding of RANKL to RANK, leading to decreased bone resorption[218]. One of the earliest identifications for

a role of this pathway in vascular biology came from experiments in the OPG knockout mice, which develop vascular calcification in the renal and aortic arteries [219].

However, there are additional mechanisms that may explain a correlation between CVD and OP. It seems obvious that there may be dysregulation of calcium as vascular calcification tracks with bone mineral loss. A misappropriation of calcium to the vasculature, rather than to the skeleton could explain this link, although how and why is unknown[220]. Recently, a meta-analysis reported that use of calcium supplementation, without combined vitamin D therapy, in women with or at risk for osteoporosis increases risk of cardiovascular disease[221], which also may implicate calcium metabolism as a link between bone and CVD pathologies.

Also, there are characteristics common to both diseases, which may underlie the correlation, including chronic inflammation and sex steroids[222]. The increased risk of CVD and OP after the menopausal transition in women, provided the first suggestion that estrogen deficiency may be important in the like between CVD and OP[216]. Estrogens deficiency has long been associated with osteoporotic fractures, but it has more recently been shown that long-term exposure to estradiol may also lead to inflammation, insulin resistance and vascular calcification[203]. Oxidized lipids, which accumulate within the medial layer of arteries, are also a proposed link because they are known to increase CVD risk, as well as, slow bone formation[223]. Lastly, the regulation of the vascular endothelial lining is the first-line defense to CVD[224]. Endothelial function and tone are regulated by NO, which has recently been identified to be important in bone metabolism, although the exact mechanisms are unknown[225].

2.5 SUMMARY

Cardiovascular disease (CVD) is the leading cause of death in the US. Major risk factors for CVD include age, sex, ethnicity, hypertension, diabetes and dyslipidemia. Diet, obesity, exercise, tobacco use and alcohol consumption are among the most common, modifiable environmental factors affecting CVD risk; however, genetic factors are also well-recognized determinants of the disease process. African ancestry individuals have greater risk of CVD independent of traditional risk factors.

Subclinical cardiovascular disease can be assessed using multiple non-invasive methods. Carotid ultrasound scans can identify plaques and subclinical atherosclerotic thickening of the vessel wall, as well as, measure of arterial diameter. Pulse-wave velocity is a non-invasive measure of arterial stiffening, or arteriosclerosis, which can be measured using automated tonometry devices. Pulse pressure is also a measure of central arterial stiffening and reflects the difference between systolic and diastolic blood pressures. Each of these measures is correlated with traditional risk factors for CVD and is predictive of future CVD events and mortality.

In general, subclinical CVD traits tend to be heritable, with cIMT being the most widely studied measure in genetic studies. However, most studies of subclinical CVD genetics have been primarily in Caucasian populations, or in persons of admixed African descent, such as African Americans, rather than in homogeneous African samples. Given the difference in risk factor profiles and subsequent risk of disease in African ancestry populations, studies specifically characterizing the genetic contributions to subclinical atherosclerosis in homogeneous African ancestry populations are warranted.

The association of bone mineral density and cardiovascular disease has been well documented and is thought to reflect more than an age-related epiphenomenon. Bone density has

been inversely associated with subclinical and clinical CVD, mainly in white populations, even after adjusting for potential confounding factors, such as age, body size, dyslipidemia, diabetes and hypertension. However, there has been little investigation of this link in populations of African ancestry. Studies in African ancestry individuals could provide additional and unique insight on the bone – CVD relationship since African ancestry populations tend to have stronger bones but greater CVD risk than their Caucasian counterparts. While there has been some experimental research on potential mechanisms for this relationship in animal models, there has been little genetic research in humans.

Thus, the aims of this dissertation were threefold and conducted using extended pedigrees within a homogeneous, African ancestry sample in Tobago. First, we described CVD risk factor correlations with common carotid artery ultrasound traits (IMT, AD and LD). Additionally, we estimated their residual heritabilities and performed genome-wide linkage analysis of these traits after accounting for important covariates. Second, we described CVD risk factor correlations with arterial stiffness measures (PWV and PP), estimated their residual heritabilities and performed genome-wide linkage analysis of these traits. Lastly, we estimated the shared environmental and genetic effects on measures of subclinical CVD (IMT, AD and LD) and bone mineral density, bone geometry and bone strength. Where appropriate, bivariate, genome-wide linkage analyses were also used to identify potential chromosomal regions that contained genes with pleiotropic effects on CVD and bone related phenotypes.

2.6 TABLES

Table 2.1 Carotid Intima-Media Thickness Heritability Studies

Year	Author	Population	Sample N	Pedigree Type	Trait	h^2r Estimate
1996	Duggirala *et. al.*[129]	Mexican City low-income population	88	Siblings	CCA IMT ICA IMT	0.92* 0.86*
2002	Lange *et. al.*[125]	EA and AA Diabetics in Forsyth County, NC, USA (Diabetes Heart Study)	252	Siblings	CCA IMT	0.41*
2002	North *et. al.*[122]	American Indians (Strong Heart Family Study)	887	Extended families	CCA IMT	0.21*
2002	Xiang *et. al.*[116]	Latino families with one hypertensive parent	286	Nuclear families	CCA IMT	0.34*
2003	Fox *et. al.*[128]	EA offspring from the Framingham Heart Study	1886	Siblings	CCA IMT ICA IMT	0.38* 0.31*
2003	Swan *et. al.*[118]	Scottish population	264	Twins	Each wall individually	Range: 0.0-0.45
2004	Juo *et. al.*[127]	High-risk Caribbean Hispanics (Northern Manhattan Family Study)	440	Proband + 1st degree relatives	Total IMT CCA IMT Bif IMT ICA IMT	0.36* 0.39* 0.26* 0.12
2005	Kao *et. al.*[126]	Mexican Americans (San Antonio Family Heart Study)	620	Extended families	CCA IMT	0.16*
2005	Mayosi *et. al.*[124]	White British families with one hypertensive parent	854	Nuclear families	CCA IMT	0.24*
2005	Moskau *et. al.*[123]	German families with one parent at-risk for CVD	565	Nuclear families	CCA IMT	0.61*
2005	Sayed-Tabatabei *et. al.*[119]	Netherlander population (Erasmus Rucphen Family Study)	930	Extended families	CCA IMT	0.35*
2005	Wang *et. al.*[117]	Mexican American	274	Nuclear	CCA IMT	0.40*

Table 2.1 continued

		families with one parent with CHD		families		
2006	Pilia *et. al.*[113]	Population-based Sardians (SardiNIA study)	6049	Extended families	CCA IMT	0.19*
2008	Bowden *et. al.*[114]	EA and AA Diabetics in Forsyth County, NC, USA[a]	973 EA 175 AA	Siblings	CCA IMT (EA)	0.38* 0.72*
					CCA IMT (AA)	0.41*
					CCA IMT (All)	
2008	Rampersaud *et. al.*[121]	Old Order Amish families from Lancaster, PA, USA	478	Siblings	CCA IMT	0.29*
2008	Zhao *et. al.*[115]	Male Vietnam veteran twins (Twin Heart Study)	224	Twins	Aggregate carotid IMT	0.59*
2009	Sacco *et. al.*[120]	High-risk Caribbean Hispanics (Northern Manhattan Family Study)[b]	1390	Proband + 1st degree relatives	Total IMT	0.65*
					CCA IMT	0.56*
					Bif IMT	0.58*
					ICA IMT	0.47*

* Heritability significant at $P<0.05$

EA: European Americans; AA: African Americans; CHD: coronary heart disease; CCA: common carotid artery; ICA: internal carotid artery; Bif: bifurcation; IMT: intima-media thickness

[a] Extension of study by Lange *et. al.* (2002)

[b] Extension of study by Juo *et. al.* (2004)

Table 2.2 Pulse-Wave Velocity Heritability Studies

Year	Author	Population	Sample N	Pedigree Type	Trait	h^2r Estimate
2005	Mitchell *et. al.*[139]	EA offspring from the Framingham Heart Study	1480	Siblings	cfPWV	0.40*
2005	Sayed-Tabatabei *et. al.*[119]	Netherlander population (Erasmus Rucphen Family Study)	930	Extended families	cfPWV	0.26*
2006	Pilia *et. al.* [113]	Population-based Sardians (SardiNIA study)	6049	Extended families	cfPWV	0.23*
2007	Ge *et. al.*[142]	Population-based EA and AA twins (Georgia Cardiovascular Twin Study)	702	Twins	arPWV afPWV	0.43* 0.54*
2007	Levy *et. al.*[140]	EA population-based sample (Framingham Heart and Offspring Studies)[a]	644	Nuclear families	cfPWV cbPWV	0.43* 0.02
2008	Seidlerova *et. al.*[141]	Caucasian European population (European Project on Genes in Hypertension)	494	Nuclear families	cfPWV	0.18
2009	Cecelja *et. al.*[143]	Population-based Caucasian female twins (Twins UK cohort)	496	Twins	cfPWV	0.34*

* Heritability significant at $P<0.05$

EA: European American; AA: African American; cfPWV: carotid-femoral pulse-wave velocity; arPWV: aorto-radial pulse-wave velocity; afPWV: aorto-foot pulse-wave velocity; cbPWV: carotid-brachial pulse-wave velocity

[a] Extension of study by Mitchell *et. al.* (2005)

Table 2.3 Pulse Pressure Heritability Studies

Year	Author	Population	Sample N	Pedigree Type	Trait	h^2r Estimate
2001	Atwood *et. al.*[150]	Mexican Americans (San Antonio Family Heart Study)	1770	Extended families	bPP	0.21*
2002	Adeyemo *et. al.*[172]	Yorubans from population of Ibadan, Nigeria	1825	Extended families	bPP	0.13*
2003	Camp *et. al.*[171]	Families of proband with early coronary heart disease from Utah, USA	2444	Extended families	bPP	0.25*
2003	Snieder *et. al.*[170]	Population-based EA and AA twins (Georgia Cardiovascular Twin Study)	534	Twins	bPP	0.54*
2003	DeStefano *et. al.*[169]	EA population-based sample (Framingham Heart Study)	1584	Nuclear families	bPP	0.52*
2003	Fava *et. al.*[168]	Population from Malmö, Sweden (Macrovascular and Hemodynamic Genetics Study)	260	Siblings	dabPP obPP	0.53* 0.21
2005	Bielinski *et. al.*[167]	Black, Hispanic, Asian and White cohorts with hypertensive probands (Family Blood Pressure Program)	10798	Nuclear families	bPP	0.29*
2005	Bochud *et. al.*[166]	East African families from Seychelles islands with ≥ 2 siblings with hypertension	314	Nuclear families	dabPP obPP	0.54* 0.37*
2005	Hsu *et. al.*[165] (Reports results from three cohorts)	EA and AA Diabetics in Forsyth County, NC, USA (Diabetes Heart Study)	950	Siblings	bPP	0.37*
		Hispanic and AA families (Insulin Resistance and Atherosclerosis Family Study)	1856	Extended families	bPP	0.34*
		Population-based sample of EA in USA (NHLBI Family Heart	2761	Nuclear families	bPP	0.27*

Table 2.3 continued

		Study)				
2005	Mitchell *et. al.*[139]	Offspring from the Framingham Heart Study	1480	Siblings	cPP	0.35*
2005	Turner *et. al.*[164]	Non-Hispanic whites from Rochester, Mn, USA (Genetic Epidemiology Network of Arteriopathy)	488	Siblings	bPP	0.29*
2006	Pilia *et. al.*[113]	Population-based Sardians (SardiNIA study)	6049	Extended families	bPP	0.21*
2006	Scurrah *et. al.*[163]	Volunteer sample of Australians (Victorian Family Heart Study)	2911	Nuclear families	bPP	0.20*
2007	Van Rijn *et. al.*[162]	Netherlander population (Erasmus Rucphen Family Study)	1006	Extended families	bPP	0.24*
2008	Franceschini *et. al.*[161]	American Indians (Strong Heart Family Study)	1892	Extended families	bPP	0.25*
2008	Seidlerova *et. al.*[141]	Caucasian European population (European Project on Genes in Hypertension)	494	Nuclear families	bPP	0.02
2009	Aberg *et. al.*[160]	Population-based sample from Samoan Islands	1269	Extended families	bPP	0.11*
2010	Jelenkovic *et. al.*[159]	Caucasian population-based sample from the Greater Bilbao, Spain	1302	Nuclear families	bPP	0.14*
2010	Kochunov *et. al.*[158]	Mexican Americans (San Antonio Family Heart Study)[a]	357	Extended families	bPP	0.49*

* Heritability significant at $P<0.05$

EA: European American; AA: African American; bPP: brachial pulse pressure; dabPP: daytime ambulatory brachial pulse pressure; obPP: office visit brachial pulse pressure; cPP: central pulse pressure

[a] Extension of study by Atwood *et. al.* (2001)

3.0 MANUSCRIPT 1: HERITABILITY AND GENOME-WIDE LINKAGE ANALYSIS OF CAROTID ARTERY ULTRASOUND PHENOTYPES IN MULTIGENERATIONAL AFRO-CARIBBEAN FAMILIES

Allison L Kuipers, BS[1], Candace M Kammerer, PhD[2], Genevieve Woodard, PhD[1], Iva Miljkovic, MD, PhD[1], Amy S Dressen, MS[2], Hu Li, PhD, MS[1], Clareann H Bunker, PhD[1], Alan L Patrick, FRCP[3], Victor W Wheeler, MBBS, MRCOG[3], Anne B Newman, MD, MPH[1], Kim Sutton-Tyrrell, DrPH[1], Joseph M Zmuda, PhD[1,2]

[1]Department of Epidemiology, University of Pittsburgh, Pittsburgh, PA, USA

[2]Department of Human Genetics, University of Pittsburgh, Pittsburgh, PA, USA

[3]Tobago Health Studies Office, Scarborough, Tobago, Trinidad and Tobago

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3.1 ABSTRACT

Intima-media thickness (IMT) and arterial diameter (adventitial diameter [AD] and lumen diameter [LD]) predict cardiovascular disease and are determined in part by genetic factors. The genetic influence on these measures in African ancestry populations is not well defined. Therefore, we have estimated genetic heritability and performed genome-wide linkage analysis of carotid ultrasound traits in 7 large, multigenerational families of African ancestry from the Caribbean island of Tobago. Probands and families were recruited without regard to health status. A total of 395 individuals (mean family size 56; 2,392 relative pairs) aged ≥18 years, had an ultrasound scan of the common carotid artery. We estimated residual heritability and conducted multipoint quantitative trait linkage analyses using pedigree-based maximum likelihood methods. Significant covariates for all traits included age, sex, body size (BMI for IMT; height and waist circumference for diameter) and systolic blood pressure (treated hypertensives were excluded). After removing the effects of these covariates, residual heritabilities of mean IMT, max IMT, mean AD, max AD, mean LD and min LD were: 0.47±0.11, 0.35±0.10, 0.64±0.12, 0.62±0.13, 0.58±0.12 and 0.57±0.12, respectively (all $P<0.0001$). Significant evidence of linkage (LOD > 3.3) was detected for max AD, mean LD and min LD on chromosome 11 (region max LOD=4.09, 133cM: max AD). Suggestive evidence for linkage, LOD >2.0, was detected for mean AD, mean LD and min LD on chromosome 14 (region max LOD=2.50, 54cM: min LD) and for mean IMT and max IMT on chromosome 13 (region max LOD=2.70, 115cM: mean IMT). The linkage regions contain several genes known to be involved in cardiovascular disease including the *ApoA1/C3/A4/A5* gene cluster, *IL18,*

BMP4, ESR2 and *SMOC1*. Further studies of these regions may reveal novel insight into the genetic regulation of carotid ultrasound traits and atherosclerosis in African ancestry individuals.

3.2 INTRODUCTION

Cardiovascular disease (CVD) is a complex, multifactorial disease, which aggregates in families. It is the largest cause of mortality in Western societies[2, 3]. Factors influencing disease susceptibility include increased age, male gender, body mass index (BMI), hypertension, high LDL cholesterol and triglyceride levels, low HDL cholesterol levels, diabetes mellitus and family history, among others[8, 12-14]. Carotid intima-media thickness (IMT) is a non-invasive, reproducible measure of arterial wall response to blood flow and subclinical atherosclerosis[33]. It is strongly correlated to atherosclerosis in multiple arterial beds and predictive of subsequent stroke, myocardial infarction and mortality[33, 34, 44]. Additionally, larger adventitial diameter (AD) and smaller lumen diameter (LD) are also predictive of future cardiovascular risk[23, 51]. Each of these physiologic measures can be easily obtained for the carotid artery using ultrasound technologies[33].

Heritability estimates the genetic influence on a disease or phenotype using samples of related individuals such as families, siblings or twins[111, 226]. Many studies have reported statistically significant heritability of carotid IMT with estimates that range from 0.25-0.92[115-119, 121-129], with the majority of estimates between 0.30-0.40, after adjustment for various CVD risk factors. This suggests that there is a genetic component to carotid wall thickness. However, heritability estimates were derived from multiple samples with diverse ethnic backgrounds, including studies using mixed ethnicities. There have been no studies, to date, reporting on the

heritability of carotid artery phenotypes in a homogeneous, African ancestry population. Also, little is known about the heritability of other carotid ultrasound-derived measures of subclinical atherosclerosis, such as AD, although one study suggests that the heritability of AD may be greater than that of IMT[122].

Genome-wide linkage analyses have identified quantitative trait loci (QTL) for carotid IMT on chromosomes 2[117], 7[120], and 12[132]. A region on chromosome 12p has been replicated in an independent population-based cohort[130]. However, none of these linkage studies have been conducted in a collection of exclusively African ancestry families. Only a study by Bowden *et. al.* (2008) that included Caucasian and African American families found evidence of linkage (LOD=4.39) for calcified plaque in a region of chromosome 16p. However, this study found no evidence of linkage with IMT (all LOD<2.0) in the total sample or in the subset of African American families[114].

Additionally, many genome-wide association and candidate gene studies have found associations between subclinical atherosclerosis and the 9p21.3 region [136, 156, 157]. Other genes that have been studied in large populations with relation to carotid ultrasound traits in humans include *APOE, ACE, MTHFR, NOS3* and *ADD1* but only *APOE* has shown consistent and convincing evidence of association[133]. Therefore, we estimated the genetic heritability of carotid IMT, AD and LD in a well-characterized sample of large, multigenerational families of African ancestry from the Caribbean island of Tobago. We also used a genome-wide linkage panel of single nucleotide polymorphism (SNPs) to identify QTL peaks for carotid ultrasound phenotypes in these families.

3.3 MATERIALS AND METHODS

3.3.1 Study Sample

Participants for this analysis were from the Tobago Family Health Study. Briefly, 8 probands were recruited without regard to their medical history from a cohort study of bone mineral density and body composition on the Caribbean island of Tobago[4]. Probands were eligible if they had a spouse willing to participate and had at least six living offspring and/or siblings aged ≥18 years and who were residing in Tobago. All first-, second-, and third-degree relatives of the probands and their spouses were invited to participate. To date, we have recruited 471 individuals belonging to 7 large families. The families consisted of: 21, 26, 28, 49, 96, 98 and 153 individuals (mean = 67 individuals) and 4,206 relative pairs. The sample consists of 283 women and 188 men aged 18-103 years (mean age, 43 years).

An ancillary study in 2007 invited all participants to complete a carotid ultrasound scan. There were 415 participants who returned (88% of survivors) and completed the carotid scan. We have analyzed the data from ultrasound images from 395 of these individuals (152 men and 243 women) who form the basis for the current analyses. Written informed consent was obtained from each participant. The Tobago Division of Health and Social Services and the University of Pittsburgh Institutional Review Boards approved this study.

3.3.2 Carotid Ultrasound

The common carotid artery was imaged with B-mode ultrasonography using an Acuson Cypress portable ultrasound machine (Siemens Medical Solutions, Malvern, PA). Both the near and far

walls of the distal common carotid artery were captured for one centimeter proximal to the carotid bulb. Only the common carotid artery could be imaged with the portable technology. Intima-media thickness (IMT) was obtained using a semi-automated reading software system (AMS system; Dr. Thomas Gustavsson, Sweden). This system detects and traces lines, with reader input, between the lumen-intima and media-adventia borders across the 1 cm segment. Then, the software generates one thickness measurement per pixel across this area, for about 140 measures in total. Mean IMT measures correspond to the mean IMT across all pixels of both the near and far wall of the common carotid artery on both the right and left arteries. The mean max IMT measures the maximum thickness per wall, per side and then is recorded as the mean of those four maxima. Adventitial and lumen diameter measures were obtained from the same 1 cm region and correspond to the distance between near and far wall medial-adventitial borders and the distance between the near and fall wall luminal-medial borders, respectively. Both sides of each participant were averaged to obtain the mean AD or LD measure. Similar to IMT, the mean max AD corresponds to the mean of the largest diameter measure for each side of the participant, while the mean min LD corresponds to the mean of the smallest diameter measure for each side of the participant. All images were read centrally at the Department of Epidemiology's Ultrasound Research Laboratory (University of Pittsburgh, Pittsburgh, PA). Reproducibility analyses were conducted on 35 Tobago Family Health Study participants. The inter-sonographer intraclass correlation (ICC) was 0.97 for mean IMT and 0.95 for mean AD. Inter-reader ICC was 0.99 for mean IMT and mean AD.

3.3.3 Other Measures

Cardiovascular risk factors available for this analysis included age, sex, height, waist circumference, BMI, current smoking, alcohol intake, walking for exercise, diabetes, hypertension, menopausal status, parity, oral contraceptive use, serum lipid and lipoprotein concentrations, systolic blood pressure, use of anti-hypertensive medication and heart rate. Body weight was measured to the nearest 0.1 kg on a balance beam scale. Standing height was measured to the nearest 0.1 cm, without shoes, using a wall-mounted stadiometer. Waist circumference at the top of the hipbone was measured to the nearest 0.1 cm. Body mass index was calculated as weight in kg divided by standing height in meters^2.

Demographic, lifestyle and medical history variables were collected by trained clinic staff through administration of a questionnaire and interview. Race was based on self-report of grandparental ethnic origin. The Tobago population is predominately of West African origin with low admixture[6]. Smoking status was classified as either current or not (yes/no), and participants reporting smoking <100 cigarettes in their lifetime were considered non-smokers. Alcohol consumption was assessed by questionnaire and coded as having >1 drink per week (yes/no) because there was a very low prevalence of substantial alcohol intake. Physical activity was assessed by the number of minutes walked per week and participants were dichotomized into “not active” or “active” determined by a median split (≤25 minutes walked/week vs. >25 minutes walked/week, respectively). Participants were asked to bring current medications to their interview, and staff recorded each medication. Diabetes was defined as a fasting glucose level ≥126 mg/dl or current use of diabetes medication. Hypertension was defined as a seated diastolic blood pressure ≥90 mmHg, systolic pressure ≥140 mmHg and/or current use of anti-hypertensive

medication. For analyses including systolic blood pressure measures, we excluded individuals on anithypertensive medication (n=31).

Reproductive characteristics included menopausal status, parity, oral contraceptive (OC) use and hormone replacement therapy (HRT). Because only 5/283 women reported using HRT, this variable was not included in this analysis. Women were considered postmenopausal only if they had no menses for at least 12 months and were >40 years old, or if they had a hysterectomy or ovariectomy.

3.3.4 Lipid Measures

A fasting blood sample was collected at the time of interview. Serum samples were separated and stored at -80°C until time of assay. Lipid measures (LDL-c, HDL-c and triglycerides) were assessed in the Heinz Nutrition Laboratory at the University of Pittsburgh's Graduate School of Public Health, which has met the accuracy and precision standards of the Centers for Disease Control and Prevention and is CLIA certified. HLD-c was determined using the selective heparin/manganese chloride precipitation method. LDL-c was calculated by means of the Friedewald equation. Triglycerides were determined enzymatically using the procedure of Bucolo and David[227].

3.3.5 Genotyping and Multipoint Identity-by-Descent (IBD) Calculation

Genomic DNA was isolated from whole blood extracted by the salting out method and isolated by a Qiagen column procedure (Qiagen, Santa Clara, CA). Whole-genome genotyping by fluorescence-based methods was performed using the Infinium HumanLinkage-12 Genotyping

BeadChip (Illumina, San Diego, CA). After excluding single nucleotide polymorphisms (SNPs) with call rate <90%, Hardy-Weinberg equilibrium (P<0.001), minor allele frequency <0.05 or multipoint IBD calculation incompatibility, we retained 1512 autosomal SNPs and used the Markov chain Monte Carlo algorithm as implemented in the program Loki[228] to calculate multipoint IBD. The final SNP set had a median MAF of 0.325 with a median spacing of 1.92 cM based on the Kosambi mapping function[229].

3.3.6 Statistical Analysis

Variance components (VC) analysis determines the proportion of variation in a particular trait that is attributable to environmental and genetic factors. The Sequential Oligogenic Linkage Analysis Routines (SOLAR) program[112] employs VC methods to account for the genetic structure within the data using pedigree information. The VC methods within SOLAR not only control for this structure, but also estimate the proportion of the trait variation that is attributable to genetic, covariate (environmental) and error effects[111, 112, 230]. The model tested by SOLAR is as follows: $Y_i = \mu + g_i + \Sigma\beta_k z_{ik} + e_i$ where μ is the overall trait mean, g_i is the genetic component, $\Sigma\beta_k z_{ik}$ is the summation of all modeled covariate (environmental) effects and e_i is the residual unmodeled variation (error) in trait Y_i[112, 230].

All traits were assessed for non-normality and transformed as necessary. Outliers, defined as ≥4 SD from the mean, were removed for each trait; no more than 2 observations were removed from any ultrasound trait. To determine significant correlates of ultrasound traits, we tested each covariate separately using the VC framework in SOLAR. We first developed an age and sex adjusted model for each trait. Age and sex were forced into all subsequent models because they are established correlates of subclinical atherosclerosis. All potentially significant

covariates ($P<0.10$) were then assessed simultaneously for each trait. We required a p-value <0.05 for inclusion in our final models.

Maximum likelihood methods were used to simultaneously model the effect of additive genetics, or heritability (h^2), fixed covariate effects and error. Heritability reported herein is the residual heritability (h^2r), which is estimated as the proportion of phenotypic variation after the effects of covariates. We also used SOLAR to estimate the residual heritability (detailed in Section 3.2.1) and the variance attributable to the fixed covariate effects for each ultrasound trait.

To compare the effects of covariates across all traits, we calculated the percent difference in the ultrasound trait per unit increase in covariate. Percent differences were calculated as beta coefficient*unit/mean trait value*100. For continuous variables, the unit range was 1 SD, and for dichotomous variables, the unit range was 1.

Multipoint linkage analysis was used to identify genomic regions that were inherited with the ultrasound traits. Linkage is based on the sharing of genetic material between generations as identity-by-descent (IBD), that is, for any given genetic locus, a family pair can either have received one allele from a common ancestor or neither of the alleles from a common ancestor. Only siblings can have both of their alleles from the same ancestors (eg. the same allele from their father and the same allele from their mother).

Quantitative trait linkage analysis estimates the variance in a continuous trait attributable to a theoretical quantitative trait locus (QTL). Linkage tests the assumption that within a QTL responsible for a trait's variance, the traits are more similar between persons that share more of their genetic information at that locus IBD than those that do not. Multipoint IBD probabilities are used to assess IBD sharing along each chromosome. The significance of the theoretical QTL was tested with a likelihood ratio test at 1 cM intervals across each autosomal chromosome.

Maximum likelihood methods tested whether the model containing a parameter for the theoretical QTL was more likely than the model incorporating only polygenic effects. Logarithm of the odds (LOD) scores, computed as the log10 of the likelihood ratio, were used to assess the significance of the test. LOD score thresholds of 3.3 and 2.0 were considered to represent nominal genome-wide significant and suggestive evidence for QTLs, respectively[231]. Computational power previously limited the feasibility of these analyses for some pedigrees; however, using SOLAR[112, 232] this is now possible in extended, complicated pedigrees such as those from the Tobago Family Health Study, and is robust to type 1 error and has more power than traditional nuclear family linkage analyses[233, 234].

3.4 RESULTS

3.4.1 Family Study Characteristics

The characteristics of the Tobago Family Health Study participants are displayed in Table 3.6.1 for the whole sample, as well as, for men and women, separately. The sample is fairly young with a mean age of 42 years (range 18-86 years). This population is overweight on average, with a mean BMI of 28.4 kg/m^2. Women were significantly more overweight than men (BMI: 29.3 kg/m^2 versus 26.7 kg/m^2; P<0.0001). Even though women have a higher BMI than men, there was no significant difference in central adiposity, as assessed by waist circumference. As expected, men were significantly taller than women (177.1 cm vs. 166.4 cm, P<0.0001). They also had a much higher frequency of current smoking and alcohol consumption than women.

The prevalence of diabetes and hypertension were 8.3% and 26.6%, respectively. Women were more likely to be on antihypertensive medication than men (11.1% vs. 4.0%, P=0.012). Among individuals not on antihypertensive medication, women had lower systolic blood pressure (SBP) than men (114.1 mmHg vs. 125.7 mmHg, P<0.0001). LDL-c was ~10mg/dl greater in women than men (P=0.033), but there was no significant difference in HDL-c between sexes. Triglycerides were ~12mg/dl greater in men than women (P=0.019).

Characteristics of the carotid ultrasound traits are shown in Table 3.6.1 as well as in Figure 3.6.1. The carotid vessel walls were thin on average (mean IMT: 0.69 mm), but this was not unexpected in this relatively young population. IMT was similar in men and women overall and within 10-year age strata (Figure 3.6.1). IMT increased with age in both sexes. However, men had larger arterial diameters compared to women overall (P<0.0001 for AD and LD). Adventitial diameter and LD tracked similarly across 10-year age groups with women having much smaller diameters then men, especially in the younger age groups (not adjusted for height). Both diameter measures increased progressively across age groups in men. In contrast, arterial diameter measures were relatively stable in younger women but increased among women aged 50 years and older.

3.4.2 Environmental Correlates of Carotid Ultrasound Traits

Cardiovascular risk factors that were correlated with carotid ultrasound traits are described in Table 3.6.2. We first investigated the association between traditional risk factors and carotid ultrasound traits adjusted for age and sex. Factors of interest included BMI, height, waist circumference, current smoking, alcohol intake, walking, diabetes, hypertension, SBP, LDL-c, HDL-c and triglycerides. There were relatively few factors that were correlated with the carotid

traits in these families. The final models were similar for all traits including measures of age, sex, body size and SBP. Only age, sex, BMI and SBP were significant independent correlates of IMT. Height was not a significant correlate of IMT, but was significantly related to artery diameter. Only age, sex, height, waist circumference and SBP were significant independent correlates of AD and LD.

Age, BMI and SBP were positively correlated with IMT. For every five-year increase in age, IMT increased by 3-4%. For every standard deviation increase in BMI (6.4 kg/m^2), mean IMT increased by 1.4% and mean maximum IMT increased by 2.5%. For every standard deviation increase in SBP (21.8 mmHg), mean IMT increased 1.9% and mean maximum IMT increased by 3.4% (all $P<0.01$). Female sex was inversely correlated with IMT such that women had 1.9% thinner mean IMT ($P<0.05$), but sex was not significantly associated with maximum IMT measures.

Adventitial diameter was also correlated with age, sex and SBP in the same directions as IMT, although the associations with sex were not significant and the effect of age on increasing vessel diameter was not as strong (less than 1% per 5 year age increase versus 3-4% for IMT). Each standard deviation increase in height and waist circumference was associated with a 2% increase in AD ($P<0.01$). Lumen diameter had nearly the exact same covariate effect associations as AD, except that age was inversely associated with LD ($P<0.05$).

3.4.3 Variance Components of the Carotid Ultrasound Traits

The proportion of carotid ultrasound trait variance attributed to covariates and additive genetic effects are shown in Table 3.6.2. Covariates accounted for more of the variance in maximum and minimum traits compared with mean traits, and they also explained more than twice the variance

in IMT than in diameter traits (r^2: mean IMT = 0.552, mean max IMT = 0.564, mean AD = 0.242, mean max AD = 0.270, mean LD = 0.169, mean min LD = 0.182). There was significant residual heritability (h^2r) for each trait after adjusting for the effects of covariates ($P<0.0001$ for all). Heritabilities were higher for mean traits than for maximum or minimum traits, and they were larger for diameter traits than for IMT traits (h^2r: mean IMT = 0.467; mean max IMT = 0.349; mean AD = 0.641; mean max AD = 0.622; mean LD = 0.584; mean min LD = 0.573).

3.4.4 Linkage Analysis of Carotid Ultrasound Traits

Results of the linkage analysis of the ultrasound traits adjusted for the significant covariates are summarized in Table 3.6.3 and depicted in Figures 3.6.2 and 3.6.3. All peak LOD scores that were at least suggestive of linkage (LOD ≥ 2) with a carotid ultrasound trait are listed in Table 3.6.3. Evidence for significant linkage (LOD > 3.3) was identified around 134 cM on chromosome 11. The greatest LOD score was 4.09 for mean maximum AD. A nearly identical peak was identified for mean LD (LOD = 4.06) and mean minimum LD (LOD = 3.82). No other peak LOD scores were greater than 3.3. However, there were an additional seven, peak LOD scores ≥ 2.0. Mean AD also had a peak suggestive of linkage at the same region of chromosome 11 (LOD = 2.22). Mean AD, mean LD and mean minimum LD had peak LOD scores suggestive of linkage around 55 cM on chromosome 14 (LOD = 2.48, 2.17 and 2.50, respectively). Mean and mean maximum IMT had peak LOD scores suggestive of linkage around 114 cM on chromosome 13 (LOD = 2.70 and 2.18, respectively).

3.5 DISCUSSION

We examined carotid ultrasound phenotypes in a sample of multi-generational African ancestry families who represent a unique sample for whom these traits have not been described previously. We found that all carotid traits had significant residual heritability after accounting for important covariates including several major CVD risk factors. Residual heritability was greater for vessel diameters compared with IMT. We also identified a region on chromosome 11 in linkage with diameter measures in these families.

In accordance with previous reports in predominantly non-African ancestry families[115-119, 121-129], IMT was significantly heritable after adjustment for significant covariates. Covariates explained more than 50% of the variance in IMT, suggesting that while there were few significant covariates, they had a substantial influence on IMT. Covariates explained only ~20-40% of the variance in arterial diameter measures, but the estimates of heritability were larger then those for IMT. This suggests that the genetic component is more influential than the modeled covariates in determining arterial diameter.

Using a genome-wide panel of SNPs, we tested for linkage with carotid ultrasound traits. We identified a region in linkage with arterial diameter located at approximately 133 cM on chromosome 11. The peak LOD score was just over 4.0 for both maximum AD and mean LD. Adventitial diameter is thought to be an early adaptation to hemodynamic changes partially associated with atherosclerosis, whereas lumen diameter expands with AD initially, but then narrows in the later stages of atherosclerosis [10, 11]. This linkage region spans ~20 Mbp and contains 79 known genes. Interestingly, this genomic region contains the *ApoA1/C3/A4/A5* gene cluster and the *IL18* gene. The apolipoprotein gene cluster encodes apolipoproteins A and C, which aid in lipid transport [235]. Allelic variation in these genes has been associated with lipid

levels[236-244]. In particular, the associations between *ApoA5* and triglyceride levels[237, 238] and *ApoA1* and HDL cholesterol levels[243] have been replicated in multiple populations including genome-wide association studies[236]. Lipid profiles are notably more favorable in African ancestry populations than in Caucasians, yet their overall rates of CVD are higher[1, 3]. Further research on the apolipoprotein gene cluster and carotid phenotypes in these families may reveal important insight into the genetic regulation of subclinical atherosclerosis in African ancestry individuals.

The interleukin-18 (*IL18*) gene has been studied in multiple populations for association with clinical and subclinical CVD. Interleukin 18 is a proinflammatory cytokine[245] that has been associated with myocardial dysfunction and coronary events[246]. Genetic variation in the promoter of the *IL18* gene has been associated with serum IL18 levels, which were predictive of coronary events [247]. Genetic variation in *IL18* has also been associated with hypertension[248, 249], diabetes[250, 251], subclinical CVD[252] and coronary artery disease [247, 253, 254]. Given these previous findings, the *IL18* gene region is also a candidate locus of interest under our linkage peak. However, we cannot exclude other potentially important genes such as *TAGLN,* which controls in part vascular smooth muscle cell migration[255, 256]; *SCN2B*, which has been associated with atrial fibrillation[257]; *IL10RA*, which is involved in the inflammatory response in carotid plaques[258]; and *NNMT*, which may control homocysteine levels[259].

We also found suggestive evidence of linkage on chromosome 14 with arterial diameters. The max LOD was 2.50 for minimum LD at 54 cM on chromosome 14. This linkage peak spans a ~22 Mbp region of the genome and contains 137 known genes. There are no widely studied cardiovascular genes in the region so it is difficult to hypothesize the best genes for follow-up. A few genes had some evidence of involvement in the atherosclerotic process, including *BMP4,*

ESR2, *SMOC1*, *LGALS3* and *GCH1*. Bone morphogenetic protein 4 (*BMP4*) has been identified as a protein involved in vascular calcification[260] and vascular endothelial response to blood flow stress[261], and could be an interesting candidate gene for atherosclerosis. However, there are no previous genetic studies in humans to support a genetic association. Also, estrogen receptor 2 (*ESR2*) controls levels of circulating estrogens, which are associated with atherosclerosis[262]. Previously, genetic variation in this gene has been associated with subclinical CVD[153, 262]. *SMOC1*, a secreted calcium binding protein, was suggested by Sherva *et. al.,* who, in performing a linkage scan for pulse pressure in African Americans, also found a peak suggestive of linkage overlapping our peak on chromosome 14[263]. The genes *LGALS3* and *GCH1* could also be candidate genes for their control of lipids[264] and oxidative stress[265], respectively.

The only peak suggestive of linkage for IMT was on chromosome 13, spanned 7.5 Mbp, and contained 22 known genes. Of these 22 genes, only one has been associated with CVD (*SLC10A2).* SLC10A2 encodes a bile acid transporter that is expressed mainly in the ileum and the kidney[266]. There is evidence that disruption of the gene/protein can lead to primary bile acid malabsorption or familial hypertriglyceridemia resulting in changes in serum cholesterol levels[267, 268].

Many, previous genome-wide linkage scans for CVD traits have identified genomic regions that may have some control over CVD progression and outcomes. The most widely reported and replicated region is at chromosome 9p21.3[156, 157], which was not replicated in the current study. The region on chromosome 14 identified in this study has been identified in a genome-wide linkage scan (Sherva *et. al.*[263]) of pulse pressure/stroke volume ratio in the African American HyperGEN study. This index is related to short-term arterial diameter and may suggest that the region we identified on chromosome 14 may control short-term arterial response to

blood flow, rather than a change in arterial diameter due to atherosclerotic plaques. In fact, when our data was not adjusted for systolic blood pressure, the linkage signal on chromosome 14 reached a significant, maximum LOD of 3.8 (data not shown), but was only suggestive of linkage after complete adjustment (LOD = 2.5).

The carotid artery walls in our study sample were thin (mean = 0.69 mm), suggesting that the atherosclerotic burden in these families is low. Similar covariates were associated with IMT and arterial diameter traits, including age, sex, body size and systolic blood pressure. Many other risk factors that have been associated with CVD in other African ancestry populations[46, 47] were assessed, such as smoking, diabetes, dyslipidemia and other lifestyle factors, but none were significantly related to carotid ultrasound phenotypes. This apparent inconsistency in findings could reflect differences in the characteristics of the populations studied. For example, our sample was young and had relatively healthy arteries with little evidence of atherosclerosis.

In conclusion, carotid artery phenotypes including measures of IMT, AD and LD, are under significant genetic control in this sample of African ancestry individuals. Each trait was significantly associated with age, body size, and systolic blood pressure, but not related to other traditional cardiovascular disease risk factors such as smoking, dyslipidemia and diabetes. By using a moderately dense genome-wide SNP panel, we were able to identify a genomic region on chromosome 11 that is responsible for a significant proportion of the genetic variance in arterial diameter. This region contains multiple genes that may be involved in atherosclerotic disease process and may warrant further research.

3.6 TABLES AND FIGURES

Table 3.1 Characteristics* of the Afro-Caribbean Families

Trait	All (n=395)	Men (n=152)	Women (n=243)
Age (years)	42.1 ± 15.7	42.2 ± 15.4	42.1 ± 15.9
BMI (kg/m^2)	28.4 ± 6.3	26.8 ± 5.1‡	29.5 ± 6.7‡
Height (cm)	170.5 ± 8.3	177.1 ± 6.8	166.4 ± 6.3‡
Waist Circumference (cm)	89.7 ± 15.3	90.3 ± 12.9	89.4 ± 16.7
Current Smoker (%)	4.3	10.7‡	0.4‡
>1 Drink per week (%)	12.7	29.1‡	2.5‡
≥25 Min walk per week (%)	48.5	53.0	45.7
Diabetes (%)	8.3	6.4	9.4
Hypertension (%)	26.6	27.8	25.8
Hypertensive Medication (%)	8.4	4.0‡	11.1‡
Systolic Blood Pressure (mmHg)§	118.8 ± 21.8	125.7 ± 19.5	114.1 ± 22.1‡
Heart Rate (beats/min)	72.2 ± 12.1	66.7 ± 12.4‡	74.4 ± 11.4‡
LDL-c (mg/dl)	132.8 ± 40.1	126.9 ± 37.5‡	136.5 ± 41.3‡
HDL-c (mg/dl)	39.8 ± 12.3	40.7 ± 11.6	39.2 ± 12.7
Triglycerides (mg/dl)	88.9 ± 44.9	96.2 ± 54.1‡	84.5 ± 37.6‡
Menopausal (%)†	30.1	--	30.1
Parity (%)†	77.0	--	77.0
Oral Contraceptive (%)†	34.4	--	34.4
Mean carotid IMT (mm)	0.69 ± 0.15	0.70 ± 0.15	0.68 ± 0.14
Mean max carotid IMT (mm)	0.80 ± 0.17	0.81 ± 0.17	0.79 ± 0.16
Mean AD (mm)	7.23 ± 0.72	7.49 ± 0.68‡	7.07 ± 0.70‡
Mean max AD (mm)	7.53 ± 0.76	7.78 ± 0.74‡	7.36 ± 0.74‡
Mean LD (mm)	5.86 ± 0.64	6.09 ± 0.63‡	5.71 ± 0.61‡
Mean min LD (mm)	5.65 ± 0.64	5.89 ± 0.62	5.51 ± 0.61‡

*Characteristics are shown as mean ± SD or frequency (%)

§Excluding participants on antihypertensive medication

†Frequency in women only

‡Comparison by sex is statistically significant ($p<0.05$)

IMT: intima-media thickness; AD: adventitial diameter; LD: lumen diameter

Table 3.2 Significant Covariate Associations and Residual Heritability of Carotid Ultrasound Traits

Trait	Age (5 years)	Female Sex	BMI (6.3 kg/m^2)	Height (8.3 cm)	Waist Circumference (15.3 cm)	Systolic Blood Pressure (21.8 mmHg)	Proportion of Trait Variance	
							Covariate Effects (r^2)	Genetic Effects ($h^2r \pm SE$)[¥]
Mean IMT	2.6%[§]	-1.9%[†]	1.4%[‡]			1.9%[‡]	0.552	0.467±0.110
Mean Max IMT	4.1%[§]	-2.7%	2.5%[‡]			3.4%[§]	0.564	0.349±0.101
Mean AD	0.5%[‡]	-2.1%		2.0%[‡]	1.8%[§]	2.2%[§]	0.245	0.641±0.120
Mean Max AD	0.6%[§]	-2.0%		2.0%[‡]	1.9%[§]	2.1%[§]	0.270	0.622±0.127
Mean LD	-0.4%[†]	-2.1%		2.5%[‡]	2.1%[‡]	1.8%[‡]	0.169	0.584±0.123
Mean Min LD	-0.5%[†]	-2.2%		2.7%[‡]	2.1%[‡]	1.8%[‡]	0.182	0.573±0.120

Values shown by covariate depict the percent change in carotid ultrasound trait value for each unit change in covariate value. Unit values are 1 for dichotomous values and are shown in parentheses for continuous values. IMT: intima-media thickness; AD: adventitial diameter; LD: lumen diameter

[¥] All residual heritability estimates had a corresponding P<0.0001

[†] P<0.05

[‡] P<0.01

[§] P<0.0001

Table 3.3 Genome-Wide Linkage Results for Carotid Ultrasound Traits

Trait*	Peak LOD	QTL Location[§]				Genes in Region	
		Chr	cM (range)	Cytogenetic	Base Position (Mbp)	N	Potential Candidate Genes of Interest
Mean IMT	2.702	13	115 (105-119)	q32.3-q33.3	99.6-106.9	22	*SLC10A2*
Max IMT	2.179	13	113 (104-121)	q32.3-q33.3	99.6-107.1	22	*SLC10A2*
Mean AD	2.478	14	55 (45-66)	q22.1-q24.2	48.6-70.2	133	*BMP4*; *ESR2*
Mean AD	2.217	11	133 (131-136)	q23.1-q23.2	110.2-113.3	27	*IL18*
Max AD	4.089	11	134 (130-146)	q23.1-q23.3	110.2-117.4	55	*APOA1/C3/A4/A5* cluster; *IL18*
Mean LD	4.056	11	133 (128-149)	q22.3-q23.3	108.5-117.4	58	*APOA1/C3/A4/A5* cluster; *IL18*
Mean LD	2.174	14	54 (45-67)	q22.1-q24.2	48.6-70.7	137	*BMP4*; *ESR2*
Min LD	3.824	11	133 (128-152)	q22.3-q23.3	108.5-118.5	79	*APOA1/C3/A4/A5* cluster; *IL18*
Min LD	2.500	14	54 (45-65)	q22.1-q24.1	48.6-68.8	123	*BMP4*; *ESR2*

Only regions with a peak LOD ≥ 2.0 are shown.

IMT: intima media thickness; AD: adventitial diameter; LD: lumen diameter; Chr: chromosome

*All analyses were adjusted for covariates listed in Table 3.6.2 above

[§]Location defined by the position of the peak LOD±1.0 where peak LOD<3.0 and defined as LOD±2.0 where peak LOD≥3.0

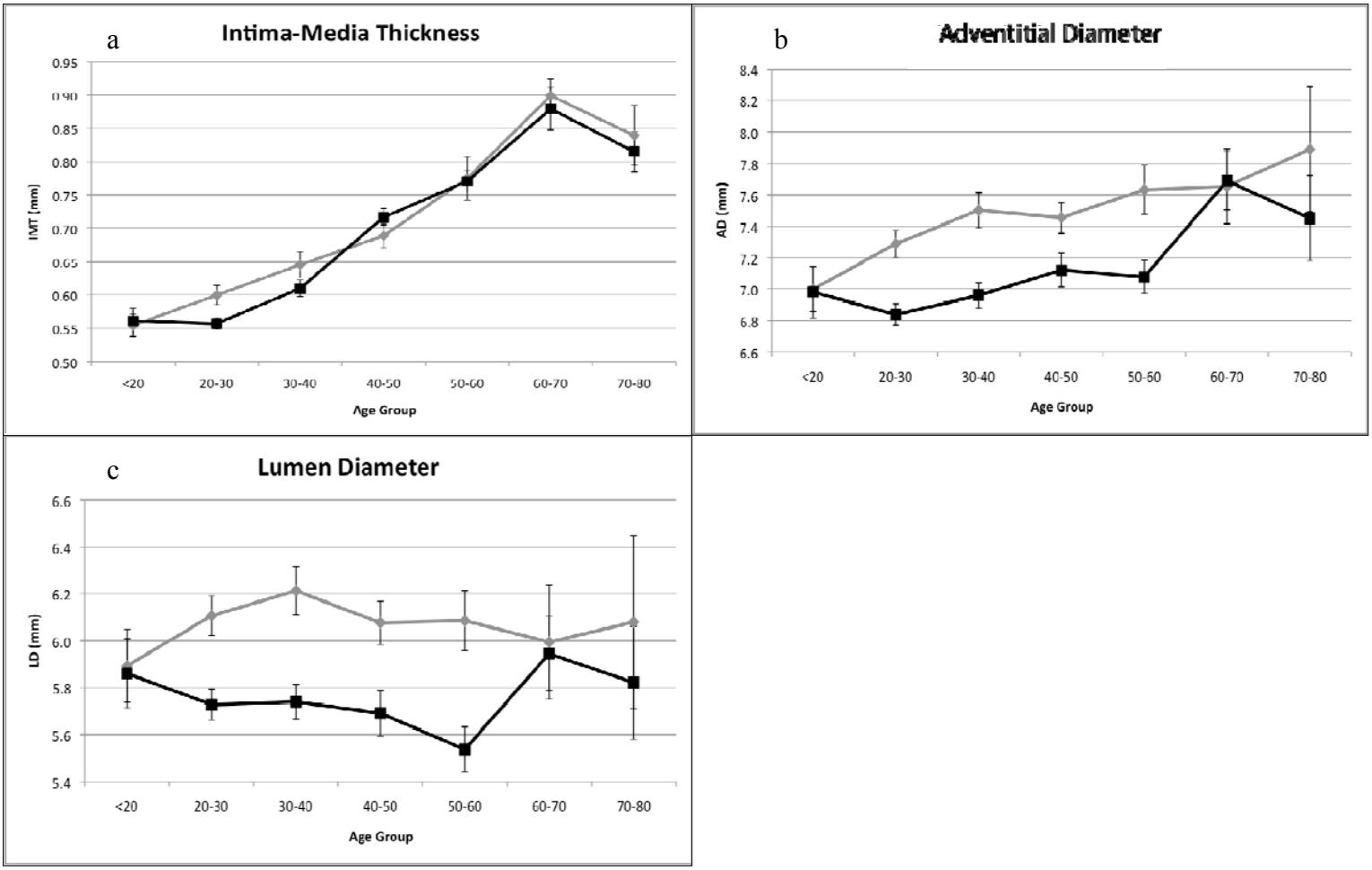

Figure 3.1 Carotid Ultrasound Traits by Age and Sex

Mean and standard errors of intima-media thickness (a), adventitial diameter (b) and lumen diameter (c) by 10-year age group for men (grey) and women (black), separately. IMT: intima-media thickness; AD: adventitial diameter; LD: lumen diameter

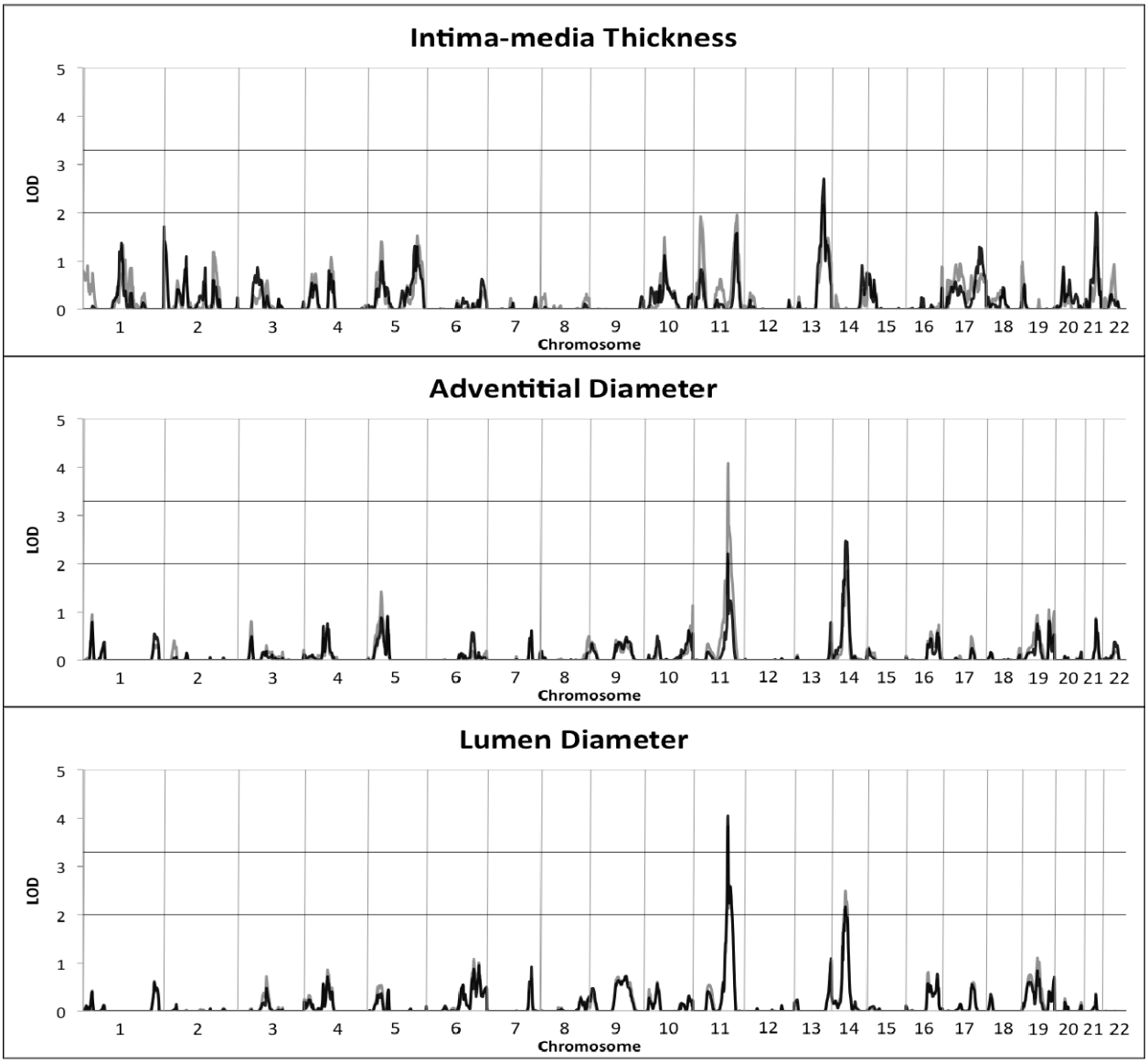

Figure 3.2 Genome-Wide Linkage LOD Scores for Carotid Ultrasound Traits

Multipoint LOD scores at each cM across the genome plotted for each ultrasound trait: intima-media thickness (a.), adventitial diameter (b.) and lumen diameter (c.). Dotted line at LOD=2.0 signifies suggestive evidence of linkage threshold. Dotted line at LOD=3.3 indicates significant evidence of linkage threshold. Black line=mean traits; Grey line=mean of the maximum (intima-media thickness and adventitial diameter) or minimum (lumen diameter) traits.

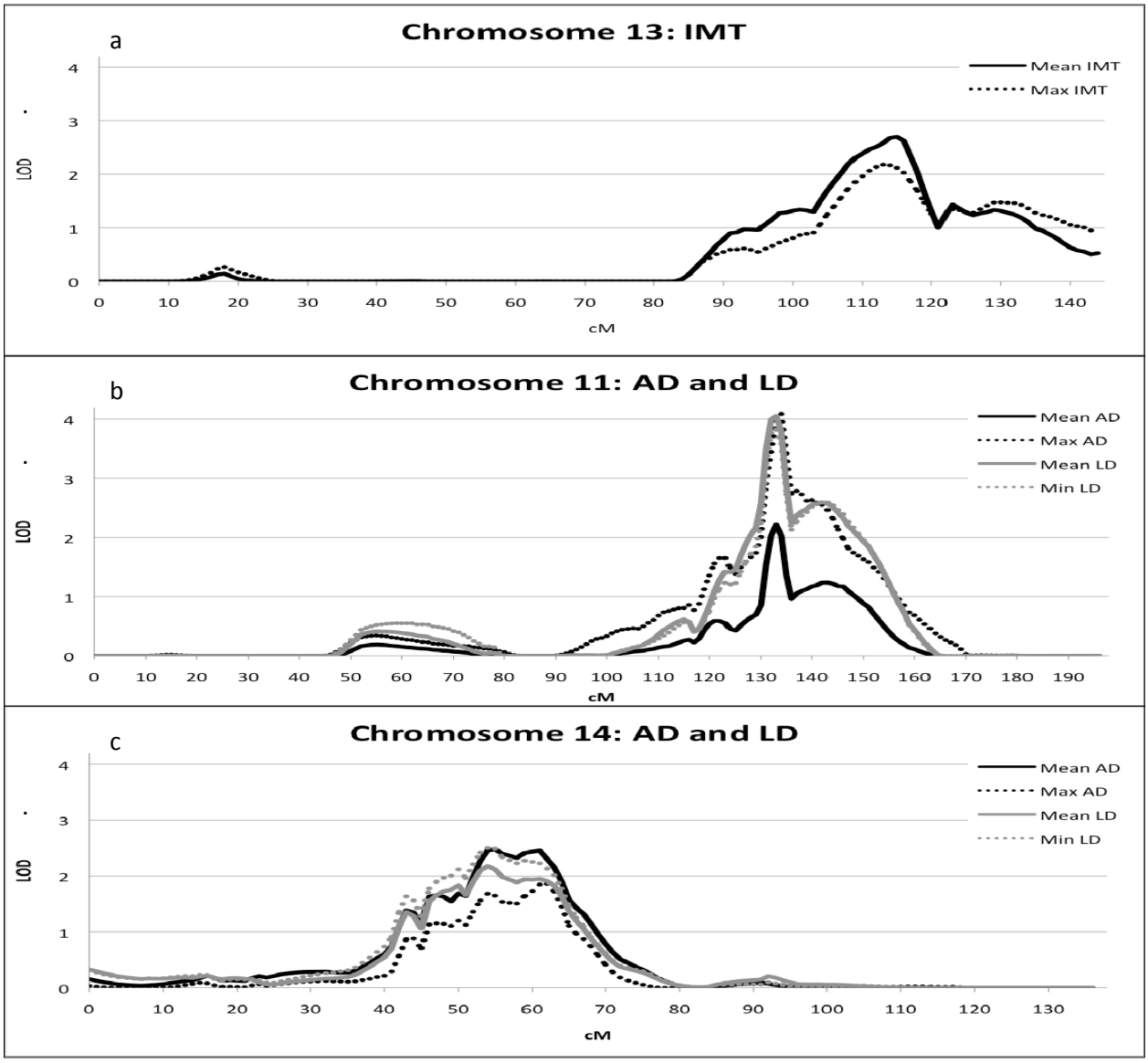

Figure 3.3 Chromosomes with Regions Suggestive of Linkage

Multipoint LOD scores at each cM across chromosome 13 (a.), 11 (b.) and 14 (c.) are plotted for IMT (a.) and diameter (b. and c.) traits. IMT: intima-media thickness; AD: adventitial diameter; LD: lumen diameter.

4.0 MANUSCRIPT 2: GENETIC EPIDEMIOLOGY OF ARTERIAL STIFFNESS IN MULTIGENERATIONAL AFRO-CARIBBEAN FAMILIES

Allison L Kuipers, BS[1], Candace M Kammerer, PhD[2], Genevieve Woodard, PhD[1], Iva Miljkovic, MD, PhD[1], Amy S Dressen, MS[2], Hu Li, PhD, MS[1], Clareann H Bunker, PhD[1], Alan L Patrick, FRCP[3], Victor W Wheeler, MBBS, MRCOG[3], Anne B Newman, MD, MPH[1], Kim Sutton-Tyrrell, DrPH[1], Joseph M Zmuda, PhD[1,2]

[1]Department of Epidemiology, University of Pittsburgh, Pittsburgh, PA, USA

[2]Department of Human Genetics, University of Pittsburgh, Pittsburgh, PA, USA

[3]Tobago Health Studies Office, Scarborough, Tobago, Trinidad and Tobago

Manuscript in preparation.

4.1 ABSTRACT

Pulse pressure (PP) and pulse-wave velocity (PWV) are measures of arterial stiffness that are predictive of cardiovascular disease events. Arterial stiffness is partially a result of hypertension, which is more prevalent in African ancestry populations than any other ethnicity. We have estimated the genetic heritability of and performed genome-wide linkage analysis of PP and PWV in 7 large, multigenerational families of African ancestry from the Caribbean island of Tobago. Probands and families were recruited without regard to health status. A total of 361 individuals (mean family size 52; 2,549 relative pairs) aged ≥18 years had non-invasive waveform analysis at the brachial and ankle (tibial) arteries to determine brachial systolic blood pressure (SBP) and diastolic blood pressure (DBP), brachial PP and brachial-ankle PWV. We estimated residual heritability and conducted multipoint quantitative trait linkage analyses using pedigree-based maximum likelihood methods. Significant covariates included age, sex, body size (BMI for SBP, DBP and PP; height and weight for PWV), heart rate, menopause (PP only) and diabetes (PWV only). Pulse pressure and PWV increased with age and were greater in men than women. Residual heritabilities of SBP, DBP, PP and PWV were: 0.29, 0.40, 0.27 and 0.24, respectively (all $P<0.05$). Suggestive evidence of linkage (LOD > 2.0) was detected for PP on chromosome 5 (max LOD=2.55, 35cM), replicating a previous genome-wide association study for subclinical cardiovascular disease. Further studies of this region may reveal novel insight into the genetic regulation of arterial stiffness.

4.2 INTRODUCTION

Cardiovascular disease (CVD) is the number one cause of death in the United States and it is estimated that more than 1 of every 3 Americans has some type of prevalent CVD, including hypertension, heart failure, coronary heart disease or stroke[1]. Identifying individuals at risk for CVD early in life is key to prevention and treatment efforts. With age, arteries gradually stiffen, which leads to faster pressure waves, thus, increasing systolic blood pressure and prevalence of isolated systolic hypertension[20]. Subclinical measures of arterial stiffness, such as pulse pressure (PP) and pulse-wave velocity (PWV), are used to assess risk of future clinical disease and document the natural progression of disease. These subclinical measures are related to blood pressure waveforms and can be measured using a single, automated applanation tonometry device[56].

Measures of arterial stiffness have been associated with traditional CVD risk factors[21] and future clinical events and mortality[67, 68]. However, heritability studies suggest that there is also a genetic influence on these traits (H^2r range: PP 0.13[172] to 0.50[169]; PWV 0.19[141] to 0.54[142]). Heritability studies of arterial stiffness measures have mostly been conducted in older, Caucasian adults [140, 141, 143, 164, 168, 169]. However, hypertension prevalence is significantly greater in individuals of African descent compared to Caucasians[1]. Therefore, the genetic determinants of subclinical CVD traits related to hypertension are of particular importance in African ancestry populations who are at increased risk of CVD. In the current study, we examined the clinical correlates and heritability of arterial stiffness and blood pressure measures in seven large, multigenerational families of African ancestry from the Caribbean island of Tobago. In addition, we conducted genome-wide linkage using single nucleotide polymorphisms (SNPs) to identify quantitative trait loci for arterial stiffness in these African ancestry families.

4.3 MATERIALS AND METHODS

4.3.1 Study Sample

Participants for this analysis were from the Tobago Family Health Study. Briefly, 8 probands were originally recruited without regard to their medical history from a cohort study of bone mineral density and body composition on the Caribbean island of Tobago[4]. Probands were eligible if they had a spouse willing to participate and had at least six living offspring and/or siblings aged ≥18 years and who were residing in Tobago. All first-, second-, and third-degree relatives of the probands and their spouses were invited to participate. Since 2003, 471 individuals belonging to 8 large families were recruited. Two families were later combined based on a discovered shared ancestry. The families consist of: 21, 26, 28, 49, 96, 98 and 153 individuals (mean = 67 individuals) and 4,206 relative pairs. The sample consists of 283 women and 188 men aged 18-103 years (mean age, 43 years).

An ancillary study in 2007 invited all participants to have their PWV and blood pressure measured using an automated tonometry machine. There were 392 participants who returned (82% of participants) and completed a PWV assessment. For this analysis, we excluded individuals on hypertensive medication (n=31) leaving a final sample of 361 individuals (145 males, 216 females). Written informed consent was obtained from each participant. The Tobago Division of Health and Social Services and the University of Pittsburgh Institutional Review Boards approved this study.

4.3.2 Arterial Stiffness and Blood Pressure Measurements

Arterial stiffness related traits were measured using a non-invasive, waveform analyzer (Colin-VP1000, Omron, Japan/WaveNexus, TX) as previously described[75]. Following 10 minutes of rest in a supine position, occlusion/monitoring cuffs are placed at standardized locations on the lightly clothed arm and bare ankle. Then, ECG electrodes are placed on the arm and ankle to visually monitor heart beat electric signals, while a phonocardiogram is placed on the left edge of the sternum to audibly detect heart sounds. The cuffs are connected to a plethysmographic sensor and an oscillometric pressure sensor, which record volume pressure waveforms and blood pressure, respectively. The volume pressure waveforms were collected at the arm (brachial artery) and ankle (tibial artery) over a total sampling time of 10 seconds with automatic gain analysis and quality adjustment. Blood pressures used for this study were means of the right and left side arteries and were measured by the oscillometric method using the above cuffs. Pulse pressure was calculated as systolic BP subtracted by diastolic BP.

Pulse-wave velocity was calculated as the (distance between arterial sites in cm) / (time between the foot of the waveforms in s). The distance is measured using a height based formula, rather than the physical distance on the outside of the participant, which was presented by Yamashina *et. al.* in 2002: path length from the heart to the brachium (Lb) =0.2195 x height of the patient (cm) - 2.0734; path length from the heart to ankle (La) = 0.8129 x height of the patient (cm) + 12.328[56]. This formula also accounts for the opposite direction of blood flow by subtracting the distance from the brachial artery to the heart. The actual baPWV, then, is calculated with: (La-Lb)/(time between brachial and ankle waveform). Mean baPWV was used in this analysis as the average between left and right baPWV measures.

4.3.3 Other Measures

Cardiovascular risk factors available for this analysis included age, sex, weight/height or BMI, current smoking, alcohol intake, walking for exercise, diabetes, menopausal status, parity, oral contraceptive use, lipoprotein concentrations and heart rate. Additionally, systolic BP was tested for association with baPWV only. Body weight was measured to the nearest 0.1 kg on a balance beam scale. Standing height was measured to the nearest 0.1 cm, without shoes, using a wall-mounted stadiometer. Waist circumference at the top of the hipbone was measured to the nearest 0.1 cm. Body mass index was calculated as weight in kilograms divided by standing height in meters^2.

Demographic, lifestyle and medical history variables were collected by trained clinic staff through administration of a questionnaire and interview. Race was based on self-report of grandparental ethnic origin. The Tobago population is predominately of West African origin with low admixture[6]. Smoking status was classified as either current or not (yes/no), and participants reporting ever smoking <100 cigarettes in their lifetime were considered non-smokers. Alcohol consumption was assessed by questionnaire and is coded based on having >1 drink per week (yes/no) because there was a very low prevalence of substantial alcohol intake. Physical activity was "not active" or "active" determined by a median split (≤25 minutes walked/week vs. >25 minutes walked/week, respectively). Participants were asked to bring current medications to their interview, and staff recorded each medication. Diabetes was defined as a fasting glucose level ≥126 mg/dl or current use of diabetes medication.

Reproductive characteristics included menopausal status, parity, oral contraceptive (OC) use, and hormone replacement therapy (HRT). Because only 5/283 women reported using HRT, this variable was not included in this analysis. Women were considered postmenopausal only if

they had no menses for at least 12 months and were >40 years old, or if they had a hysterectomy or ovariectomy.

4.3.4 Lipid Measurements

A fasting blood sample was collected at the time of interview. Serum samples were separated and stored at -80°C until time of assay. Lipid measures (LDL-c, HDL-c and triglycerides) were assessed in the Heinz Nutrition Laboratory at the University of Pittsburgh's Graduate School of Public Health, which has met the accuracy and precision standards of the Centers for Disease Control and Prevention and is CLIA certified. HLD-c was determined using the selective heparin/manganese chloride precipitation method. LDL-c was calculated by means of the Friedewald equation. Triglycerides were determined enzymatically using the procedure of Bucolo and David[227].

4.3.5 Genotyping and Multipoint Identity-By-Descent (IBD) Calculation

Genomic DNA was isolated from whole blood extracted by the salting out method and isolated by a Qiagen column procedure (Qiagen, Santa Clara, CA). Whole-genome genotyping by fluorescence-based methods was performed using the Infinium HumanLinkage-12 Genotyping BeadChip (Illumina, San Diego, CA). After excluding singly nucleotide polymorphisms (SNPs) with call rate <90%, Hardy-Weinberg equilibrium ($P<0.001$), minor allele frequency <0.05 or multipoint IBD calculation incompatibility, we retained 1512 autosomal SNPs and used the Markov chain Monte Carlo algorithm as implemented in the program Loki[228] to calculate

multipoint IBD. The final SNP set had a median MAF of 0.325 with a median spacing of 1.92 cM based on the Kosambi mapping function[229].

4.3.6 Statistical Analysis

All traits were assessed for non-normality and transformed as necessary. Outliers, defined as ≥4 SD from the mean, were removed; no more than 2 observations were removed from any trait. To determine significant correlates of each measure, we tested covariates separately in an age and sex adjusted model. Age and sex were forced into all models because they are known correlates of subclinical CVD. Because we analyzed family data, we used the variance component framework as implemented in the program SOLAR[112], which accounts for the non-independence between observations by using a familial correlation matrix. All potentially significant covariates ($P<0.10$) were then assessed simultaneously for each trait; we required a p-value <0.05 for inclusion in our final models.

Maximum likelihood methods were used to simultaneously model the effect of additive genetics, or heritability (h^2), fixed covariate effects and error. Heritability reported herein is the residual heritability (h^2r), which is estimated as the proportion of phenotypic variation after the effects of covariates have been removed.

To compare the effects of covariates across all traits, we calculated the percent difference in the ultrasound trait per unit increase in covariate. Percent differences were calculated as beta coefficient*unit/mean trait value*100. For continuous variables, the unit range was 1 SD, and for dichotomous variables, the unit range was 1.

Multipoint linkage analysis was performed using an extension of the variance components model described above. Maximum likelihood methods were used to estimate the

expected variance attributable to a theoretical QTL, based on the expected covariance between relatives, which were estimated using the multipoint IBD probabilities. The model containing the parameter for the theoretical QTL was then compared with models incorporating only polygenic effects using a likelihood ratio test. Logarithm of the odds (LOD) scores, computed as the $\log_{10}$ of the likelihood ratio, were used to assess the significance of the test. LOD score thresholds of 3.3 and 2.0 were considered to represent nominal genome-wide significant and suggestive evidence for QTLs, respectively[231].

4.4 RESULTS

4.4.1 Family Study Characteristics

Characteristics of the family members are reported in Table 4.6.1. On average, individuals were 41 years old (range 18-86 years). Women had significantly greater BMI than men; although the women weighed significantly less than the men, they were also significantly shorter ($P<0.05$ for all). Men were more likely to smoke and consume alcohol than women, although the prevalence of these activities was generally low. Diabetes prevalence was 8% and did not differ between men and women. Hypertension was significantly higher in men versus women (25.0% vs. 16.4%, $P<0.05$).

Blood pressure and arterial stiffness related measures are described in Table 4.6.1 and depicted in Figure 4.6.1. PP and baPWV measures were not suggestive of extensive arterial stiffness, especially in the younger age groups. Mean arterial stiffness measures were lower in women than in men ($P<0.05$). Pulse pressure increased with age and women had lower PP than

men at younger ages (age <50 years). However, PP was greater in women than men after age 50. Brachial-ankle PWV was similar in men and women across all ages. Both men and women appeared to have similar increases in baPWV across or 10-year age categories. Mean brachial SBP was 132.3 mmHg and mean brachial DBP was 76.1 mmHg. Women had lower blood pressure than men (P<0.05).

4.4.2 Environmental Correlates of Arterial Stiffness and Blood Pressure

Significant covariates for each trait are presented in Table 4.6.2. Age and sex were forced into all models; in addition, heart rate and BMI were significant predictors of most traits. For every 5-year increase in age, blood pressure-related traits increased by 0.2-3.6% in this population (P<0.05 for all). Women had 6.4%-11.5% lower values of every trait than men (P<0.01 for all). Every 12.3 beats/min (1 standard deviation) greater heart rate was associated with 3.1-3.9% increased arterial stiffness or blood pressure measure (P<0.01 for all). Body-mass index was related to all blood pressure measures except baPWV. A 6.1 kg/m^2 (1 standard deviation) greater BMI was associated with a 2.8-5.8% increased blood pressure measure (P<0.01 for all).

Pulse pressure was statistically significantly associated with menopausal status, such that post-menopausal women had 13.4% greater PP than pre-menopausal women and men (Figure 1a; P<0.0001). Brachial-ankle PWV was also associated with height, SBP and diabetes. Increased height was associated with lower baPWV and both increased SBP and prevalent diabetes were each associated with a ~7% increase in baPWV. Covariates explained 26 to 66% of the variance in the arterial stiffness and blood pressure traits.

4.4.3 Variance Components and Linkage Analysis of Arterial Stiffness and Blood Pressure

Genetic heritability estimates and results from genome-wide linkage analysis are presented in Table 4.6.3. After adjustment for covariates listed in Table 4.6.2, all traits had low, but significant, heritability ranging from 0.244 for baPWV to 0.399 for mean brachial DBP ($P<0.05$). Only one chromosomal region demonstrated suggestive evidence of linkage. The peak was located (maximum LOD=2.55) on chromosome 5 at 35 cM for PP. No other trait demonstrated suggestive evidence of linkage (LOD>2.0).

4.5 DISCUSSION

We conducted heritability and linkage analyses of blood pressure and arterial stiffness related measures in a homogenous, African ancestry pedigree sample. Blood pressure and arterial stiffness measures were significantly heritable with environmental factors accounting for a greater proportion of the variation in these traits. Nonetheless, our genome-wide analysis revealed suggestive evidence of linkage for pulse pressure on chromosome 5.

There are 15 genes underlying the linkage region for pulse pressure peak (range: chromosome 5, 28-41 cM, 10.9-21.2Mbp). Several genes in this region have been related to cardiovascular disease including dynein axonemal heavy chain 5 (*DNAH5*), family with sequence similarity 134, member B (*FAM134B*), ankylosis progressive mouse homolog (*ANKH*) and myosin X (*MYO10*). Our findings are consistent with a genome-wide association study that found SNPs in *DNAH5* to be associated with both ankle-brachial index, a measure of peripheral

arterial disease, and carotid IMT[130]. Others have found an interaction between alleles in *FAM134B* and *TNFRSF19* to be associated with risk of vascular dementia[269]. SNPs in *ANKH* have been associated with vascular, calcification[270, 271] and variants in *MYO10* have been associated with BMP-related angiogenesis[272]. Collectively, these findings suggest that the chromosome 5 region may harbor potentially important candidate genes for vascular physiology.

All measures of blood pressure and arterial stiffness increased with age. Previous population studies in mainly White participants, have reported that while SBP increases with age, DBP also decreases at later ages, resulting in increased PP[273, 274]. However, this was not observed in the present study. While PP did increase with age, it was not due to a decrease in DBP. Rather, DBP leveled off at older ages as SBP continued to rise, resulting in elevated PP in the oldest adults. We also found that women had significantly lower measures of blood pressure and arterial stiffness than men. This observation is consistent with reports in Whites[79, 83, 274] and Blacks[275]. Also, PP increased in women after the menopausal transition, eventually exceeding values in men, which is consistent with previous reports[274, 276]. The mean PP was >50 mmHg in individuals under the age of 40 in this population (mean PP: men, 58.1 mmHg; women, 51.0 mmHg), which is much higher than what is reported in populations of mostly White individuals, such as NHANES[273] and the Framingham Heart Study[274] with population-level pulse pressures under 40 mmHg in similarly aged individuals. Even in the Bogalusa Heart Study, which examined young (aged 20-38) Black and White individuals, the mean PP was 48 mmHg and 45 mmHg for men and women, respectively[275]. Another study that examined brachial PP in African Americans also reported lower PP than values in our sample at younger ages (age 30-39 PP=45 mmHg; age 40-49 PP=47 mmHg). However, among individuals over 60 years of age, PP was similar to values in our sample[110]. Therefore, this sample of Afro-Caribbean families has

markedly greater brachial PP under the age of 50 than has been seen in other populations, although measures in older adults are similar to other African American samples.

To our knowledge, this is the first study to present values for baPWV separately for men and women by age group in African ancestry individuals. Most previous studies were conducted in Asian populations[73, 74, 277]. In our study, baPWV increased with age and was lower in females than males. Menopausal status was not significantly associated with baPWV. A significant relationship between menopause and baPWV has been reported previously[73-75, 277]. The lack of association in our study may be due to limited statistical power to detect a difference, or due to ethnic differences in disease etiology. Measures of baPWV were greater in this African ancestry sample than in a report of >12,000 Japanese[74]. This is consistent with other reports, which have suggested that PWV is greater in African ancestry populations than other ethnic groups across all ages[29, 80, 82, 278, 279]. While, at younger ages, the baPWV measures from our study (age < 30 years) are more similar to measures reported in Japanese[280], they are much greater when compared to reports in older Japanese adults (age 60 years)[281]. Therefore, it appears that this African ancestry sample may have increased baPWV and possibly more arterial stiffness as assessed by baPWV, than in other cohorts, especially over the age of 30, which is similar to our observations for PP.

In addition to age and sex, heart rate and body size were both significant correlates of arterial stiffness. Body size was the strongest correlate of arterial stiffness measures consistent with previous findings of an adverse impact of adiposity on arterial stiffening[21, 77, 106]. However, other traditional factors associated with arterial stiffness measures, such as smoking[21, 107, 109], diabetes[21, 106, 107] and serum lipids[21, 106, 107] were not consistent correlates in our sample. The prevalence of smoking was very low in our families and likely limited our ability to detect any

relationship. Also, the impact of some risk factors on arterial stiffness may not have manifested yet in these relatively young and healthy families.

Blood pressure and arterial stiffness measures were significantly heritable in these African ancestry families. However, heritability of the arterial stiffness traits was lower in our families ($h^2r < 0.3$) compared to reports in other related individuals ($h^2r > 0.3$ usually). The lower heritability estimate in these families may indicate that environmental factors are more important than genetics in this group.

This study examined arterial stiffness related phenotypes across a large age range in African ancestry families. Measures of blood pressure and arterial stiffness were consistently related to age, sex, body size and heart rate. Values in our sample of families were more adverse than published values in other ethnic groups. We also found evidence for a low but statistically significant heritability of these traits, suggesting that genetic factors may not have a strong impact on blood pressure and arterial stiffness in these families. Although heritability was low, we found suggestive evidence of linkage to PP on chromosome 5. Further studies are needed to refine the potential association of the genes underlying this linkage peak to PP.

4.6 TABLES AND FIGURES

Table 4.1 Characteristics* of the Afro-Caribbean Family Members

Trait	All (n=361)	Men (n=145)	Women (n=216)
Age (years)	40.7 ± 15.6	41.5 ± 15.3	40.2 ± 15.7
BMI (kg/m^2)	28.0 ± 6.1	26.8 ± 5.1	28.9 ± 6.5‡
Weight (kg)	81.8 ± 18.6	84.1 ± 18.2	80.2 ± 18.8‡
Height (cm)	170.9 ± 8.2	177.1 ± 6.6	166.7 ± 6.2‡
Waist Circumference (cm)	88.5 ± 14.7	90.0 ± 12.8	87.5 ± 15.7
Current Smoker (%)	4.8	11.3	0.5‡
>1 Drink per week (%)	41.2	58.3	29.8‡
≥25 Min walk per week (%)	70.3	72.9	65.8
Diabetes (%)	7.6	6.7	8.2
Hypertensive¥ (%)	19.9	25.0	16.4‡
Heart Rate (beats/min)	72.5 ± 12.3	68.6 ± 12.2	75.1 ± 11.7‡
LDL-c (mg/dl)	130.8 ± 40.0	127.6 ± 36.1	133.0 ± 42.3
HDL-c (mg/dl)	40.0 ± 11.9	40.4 ± 11.1	39.8 ± 12.4
Triglycerides (mg/dl)	86.2 ± 42.6	93.1 ± 50.4	81.7 ± 36.1‡
Menopausal (%)†	15.2	*N/A*	15.2
Parity (%)†	44.6	*N/A*	44.6
Oral Contraceptive (%)†	20.3	*N/A*	20.3
Pulse Pressure (mmHg)	55.90 ± 10.90	57.42 ± 8.90	54.87 ± 11.97‡
Pulse-Wave Velocity (cm/s)	1368.44 ± 317.91	1416.59 ± 305.05	1335.41 ± 323.02‡
Mean Brachial SBP (mmHg)	132.32 ± 21.83	135.98 ± 19.23	129.86 ± 23.14‡
Mean Brachial DBP (mmHg)	76.05 ± 12.89	78.20 ± 12.08	74.62 ± 13.23‡

*Characteristics are shown as mean ± SD or frequency (%)

¥Based on systolic blood pressure ≥ 140 and/or diastolic blood pressure ≥ 90, individuals taking antihypertensive medication are excluded in all analyses

†Frequency in women only

‡Comparison by sex is statistically significant ($p<0.05$)

SBP: systolic blood pressure; DBP: diastolic blood pressure

Table 4.2 Significant Covariate Associations with Arterial Stiffness Indices

Trait	Age (5 yrs)	Female Sex	Heart Rate (12.3 bt/min)	BMI (6.1 kg/m^2)	Height (8.2 cm)	SBP (12.8 mmHg)	Menopause	Diabetes	Covariate Effects (r^2)
Pulse Pressure	0.9†	-11.5§	3.2‡	5.8§			13.4§		0.028
Pulse-Wave Velocity	3.6§	-7.2‡	3.8§		-2.5‡	7.4§		6.7†	0.662
Mean Brachial SBP	0.2§	-6.7§	3.1§	4.0§					0.303
Mean Brachial DBP	2.3§	-6.4§	3.9§	2.8‡					0.323

Values shown by covariate depict the percent change in trait value for each unit change in covariate value. Unit values are 1 for dichotomous values and are shown in parentheses for continuous values.

SBP: systolic blood pressure; DBP: diastolic blood pressure

$^{\dagger}P<0.05$

$^{\ddagger}P<0.01$

$^{\S}P<0.0001$

Table 4.3 Heritability and Results of Linkage Analysis of Arterial Stiffness Indices

Trait*	Genetic Effects ($h^2r \pm SE$)	Max LOD	Chromosome	cM (range)[§]
Pulse Pressure	$0.269 \pm 0.124^{\dagger}$	2.545	5	35 (28-41)
Pulse-Wave Velocity	$0.244 \pm 0.147^{\dagger}$	1.873	13	23 (13-33)
Mean Brachial SBP	$0.285 \pm 0.103^{\ddagger}$	1.628	16	95 (91-102)
Mean Brachial DBP	$0.399 \pm 0.103^{\ddagger}$	1.400	2	272 (265-qTer)

*All analyses were adjusted for covariates listed in Table 4.6.2 above

[§]Location defined by the position of the peak LOD±1.0

PWV: brachial-ankle pulse-wave velocity; SBP: systolic blood pressure; DBP: diastolic blood pressure

[†]$P<0.05$

[‡]$P<0.001$

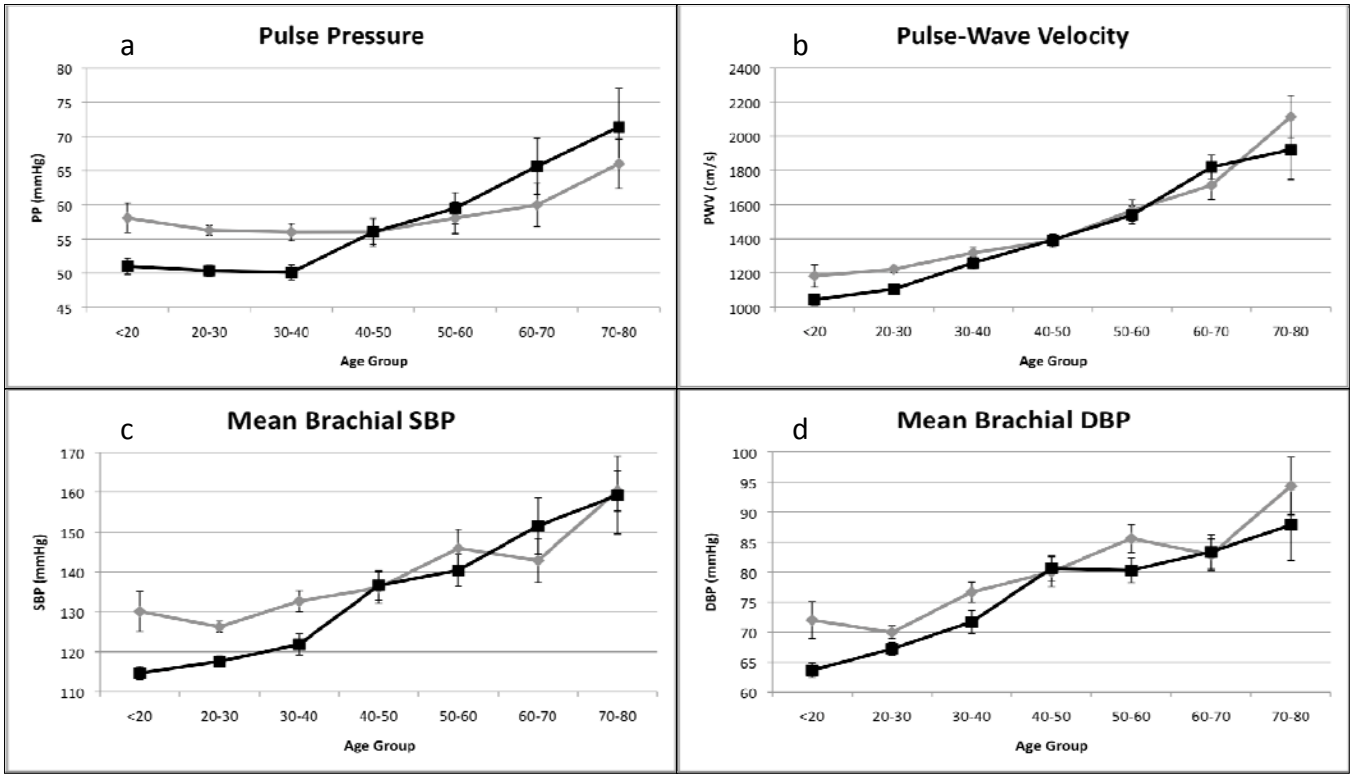

Figure 4.1 Arterial Stiffness and Blood Pressure Traits by Sex and 10-year Age Group

Mean and standard errors or prevalence of pulse pressure (a), brachial-ankle pulse-wave velocity (b), brachial systolic blood pressure (c) and brachial diastolic blood pressure (d) are plotted by 10-year age group for men (grey) and women (black), separately. PP: pulse pressure; PWV: brachial-ankle pulse-wave velocity; SBP: systolic blood pressure; DBP: diastolic blood pressure

5.0 MANUSCRIPT 3: GENETIC CORRELATION AND GENOME-WIDE LINKAGE ANALYSIS OF SUBCLINICAL CARDIOVASCULAR DISEASE AND INDICES OF OSTEOPOROSIS

Allison L Kuipers, BS[1], Candace M Kammerer, PhD[2], Genevieve Woodard, PhD[1], Iva Miljkovic, MD, PhD[1], Amy S Dressen, MS[2], Hu Li, PhD, MS[1], Clareann H Bunker, PhD[1], Alan L Patrick, FRCP[3], Victor W Wheeler, MBBS, MRCOG[3], Anne B Newman, MD, MPH[1], Kim Sutton-Tyrrell, DrPH[1], Joseph M Zmuda, PhD[1,2]

[1]Department of Epidemiology, University of Pittsburgh, Pittsburgh, PA, USA

[2]Department of Human Genetics, University of Pittsburgh, Pittsburgh, PA, USA

[3]Tobago Health Studies Office, Scarborough, Tobago, Trinidad and Tobago

Manuscript in preparation.

5.1 ABSTRACT

Both cardiovascular disease (CVD) and osteoporosis (OP) risk and prevalence increase with age. Subclinical measures of these diseases have been correlated such that adverse vascular health indices are associated with lower bone mineral density (BMD). In the current study, we estimated the phenotypic and genetic correlation between subclinical CVD measures and BMD. We also performed bivariate genome-wide linkage analysis of these traits. A total of 461 African ancestry individuals belonging to 7 large, multigenerational families were recruited without regard to health status (mean family size 66; 3,414 relative pairs). Participants underwent carotid ultrasound scans to determine adventitial diameter (AD) and intima-media thickness (IMT). Dual-energy X-ray absorptiometry and peripheral quantitative computed tomography were used to determine BMD. We determined genetic correlation and conducted bivariate, multipoint quantitative trait linkage analyses using pedigree-based maximum likelihood methods on models adjusted for age, sex, weight, height, menopausal status, current smoking, alcohol intake and walking for exercise. Significant evidence for genetic correlation was identified between AD and BMD (rhoG range -0.043 to -0.059; $P<0.05$). IMT was significantly genetically correlated with integral BMD in the lumbar spine (rhoG=-0.35, $P<0.05$) and borderline significantly correlated with trabecular BMD in the radius (rhoG=-0.33; $P<0.10$). Bivariate linkage analysis identified a region on chromosome 14 that may harbor genes with pleiotropic effects on AD and BMD (max LOD=5.2, 61 cM). This peak contains candidate genes, such as *BMP4* and *ESR2*, but needs to be refined to determine which genes may have pleiotropic effects on CVD and OP.

5.2 INTRODUCTION

Bone mineral density (BMD) has been inversely associated with subclinical and clinical CVD, even after adjusting for potential confounding factors[182, 183, 212-214]. However, the underlying physiologic link between these two disease processes is not completely understood. For the most part, the correlation between BMD and CVD has been observed in purely epidemiologic or experimental studies, and the potential for pleiotropic genetic effects have not been investigated.

Epidemiologic studies have mainly focused on investigating the correlation of arterial calcification and calcified plaque with bone mineral density (BMD) and fractures in various populations including individuals with CKD and post-menopausal women, in addition to, the general population of various ethnic groups including Whites, Mexican Americans and Japanese[183-188]. However, this relationship has not been adequately investigated in populations of African ancestry, even though there is a lower risk of OP and higher risk of any CVD event than in Caucasians. Subclinical atherosclerosis, as assessed by IMT, has been associated with low BMD, mainly at the lumbar spine and hip[191, 193, 198, 206]. To our knowledge, no studies have examined the correlation of other carotid ultrasound characteristics with BMD. Therefore, we assessed the heritability, phenotypic and genetic correlations between subclinical CVD, assessed by carotid ultrasound, and BMD in Afro-Caribbean families in Tobago. We also used bivariate, genome-wide linkage analysis to identify genomic regions that may have pleiotropic effects resulting in the coupling of CVD and bone phenotypes.

5.3 MATERIALS AND METHODS

5.3.1 Study Sample

Participants for this analysis were from the Tobago Family Health Study. Briefly, 8 probands were originally recruited without regard to their medical history from a cohort study of bone mineral density and body composition on the Caribbean island of Tobago[4]. Probands were eligible if they had a spouse willing to participate and had at least six living offspring and/or siblings aged ≥18 years and who were residing in Tobago. All first-, second-, and third-degree relatives of the probands and their spouses were invited to participate. In 2003-2004, we recruited 471 individuals belonging to 8 large families. Two families were later combined based on a discovered shared ancestry. The families consist of: 21, 26, 28, 49, 96, 98 and 153 individuals (mean = 67 individuals) and 4,206 relative pairs. The sample consists of 283 women and 188 men aged 18-103 years (mean age, 43 years).

An ancillary study in 2007 invited all family members to complete carotid ultrasound imaging. There were 415 participants who returned (88% of survivors) and completed the carotid scan, 395 of whom had useable data. In addition, bone mineral density was screened in 445 individuals at the initial baseline visit. We have analyzed the data from 461 individuals (184 men and 277 women) with ultrasound and/or BMD assessment, who form the basis of these analyses. Written informed consent was obtained from each participant. The Tobago Division of Health and Social Services and the University of Pittsburgh Institutional Review Boards approved this study.

5.3.2 Subclinical Cardiovascular Disease Traits

The common carotid artery was imaged with B-mode ultrasonography using an Acuson Cypress portable ultrasound machine (Siemens Medical Solutions, Malvern, PA). Both the near and far walls of the distal common carotid artery were captured for one centimeter proximal to the carotid bulb. Only the common carotid artery could be imaged with the portable technology. Intima-media thickness (IMT) was obtained using a semi-automated reading software system (AMS system; Dr. Thomas Gustavsson, Sweden). This system detects and traces lines, with reader input, between the lumen-intima and media-adventia borders across the 1 cm segment. Then, the software generates one thickness measurement per pixel across this area, for about 140 measures in total. Mean IMT measures correspond to the mean IMT across all pixels of both the near and far wall of the common carotid artery on both the right and left arteries. Adventitial diameter measures were obtained from the same 1 cm region and correspond to the distance between near and far wall medial-adventitial borders. Both sides of each participant were averaged to obtain the mean AD. All images were read centrally at the Department of Epidemiology's Ultrasound Research Laboratory (University of Pittsburgh, Pittsburgh, PA). Reproducibility analyses were conducted on 35 Tobago Family Health Study participants. The inter-sonographer intraclass correlation (ICC) was 0.97 for mean IMT and 0.95 for mean AD. Inter-reader ICC was 0.99 for mean IMT and mean AD.

5.3.3 Bone Mineral Density Measures

Measures of integral areal BMD at the spine and proximal femur were measured by dual energy X-ray absorptiometry (DXA) using a Hologic QDR-4500W densitometer (Hologic Inc., Bedford,

MA). Trabecular and cortical volumetric BMD at the non-dominant forearm and left tibia was measured by peripheral quantitative computed tomography (pQCT) using an XCT-2000 scanner (Stratec Medizintechnik, Pforzheim, Germany). Technicians followed standardized protocols for patient positioning and scanning. A scout view was obtained prior to the pQCT scan to define an anatomic reference line for the relative location of the subsequent scans (4% and 33% of the total length) at the radius and tibia. Tibia length was measured from the medial malleolus to the medial condyle of the tibia, and forearm length was measured from the olecranon to the ulna styloid process. A single axial slice of 2.5 mm thickness with a voxel size of 0.5 mm and a speed of 20 mm/s was taken at all locations. Image processing was performed using the Stratec software package (Version 5.5E). The short-term *in vivo* precision of the pQCT measurements for 15 subjects ranged from 0.65% (tibia cortical BMD) to 2.1% (tibial trabecular BMD).

5.3.4 Other Measurements

Covariates available for this analysis included age, sex, height, weight, current smoking, alcohol intake, walking for exercise, diabetes, hypertension, menopausal status, parity and lipoprotein concentrations. Body weight was measured to the nearest 0.1 kg on a balance beam scale. Standing height was measured to the nearest 0.1 cm, without shoes, using a wall-mounted stadiometer.

Demographic, lifestyle and medical history variables were collected by trained clinic staff through administration of a questionnaire and interview. Race was self-reported based on grandparental ethnic origin. The Tobago population is predominately of West African origin with low admixture[6]. Smoking status was classified as either current or not (yes/no), and participants reporting ever smoking <100 cigarettes in their lifetime were considered non-

smokers. Alcohol consumption was assessed by questionnaire and was coded based on having >1 drink per week (yes/no) because there was a very low prevalence of substantial alcohol intake. Physical activity was assessed by the number of minutes walked per week and participants were dichotomized into "not active" or "active" determined by a median split (≤25 minutes walked/week vs. >25 minutes walked/week, respectively). Participants were asked to bring current medications to their interview, and staff recorded each medication. Diabetes was defined as a fasting glucose level ≥126 mg/dl or current use of diabetes medication. Hypertension was defined as a seated diastolic blood pressure ≥90 mmHg, systolic pressure ≥140 mmHg and/or current use of anti-hypertensive medication.

5.3.5 Genotyping and Multipoint Identity-By-Descent (IBD) Calculation

Genomic DNA was isolated from whole blood extracted by the salting out method and isolated by a Qiagen column procedure (Qiagen, Santa Clara, CA). Whole-genome genotyping by fluorescence-based methods was performed using the Infinium HumanLinkage-12 Genotyping BeadChip (Illumina, San Diego, CA). The BeadChip included 6,090 single-nucleotide polymorphism (SNP) markers with an average spacing of 0.58 cM across the genome. After excluding SNPs with call rate <90%, Hardy-Weinberg equilibrium ($P<0.001$), minor allele frequency <0.05, we retained 5361 autosomal SNPs. From these SNPs, we selected a subset of 1512 SNPs, with a correlation <0.30 and ≥ 1 cM apart for multipoint IBD calculation because the Markov chain Monte Carlo algorithm as implemented in the program Loki[228] requires markers to be ≥1cm apart. For markers located at the same cM position on a chromosome, we selected the SNP that had the highest MAF. The final SNP set had a median MAF of 0.325 with a median spacing of 1.92 cM based on the Kosambi mapping function[229].

5.3.6 Statistical Analysis

All traits were assessed for non-normality and transformed as necessary. Outliers, defined as ≥4 SD from the mean, were removed for each trait; no more than 2 observations were removed from any trait. We adjusted each heritability, correlation and linkage model for age, sex, body weight, height, menopausal status, walking for exercise, current smoking and alcohol intake. We did not include adjustment for other conditions that have been suggested to be part of the physiologic mechanisms underlying the correlation between cardiovascular and bone related measures such as diabetes[212], hypertension[196] and dyslipidemia[223, 282]. We used SOLAR[112] to estimate the residual heritability and the variance attributable to the fixed covariate effects for each trait.

SOLAR was also used to determine the extent of genetic and environmental correlation between the variance components of CVD and BMD measures[112, 283]. When two traits have significant genetic correlation (ρ_G), then there is evidence that they are partially controlled by the same genetic variation. When two traits have significant environmental correlation (ρ_E), then there is evidence that they are partially controlled by the same environmental factors. Phenotypic correlation (ρ) between CVD and BMD measures was estimated from heritability, genetic correlation and environmental correlation estimates as: $\rho_{12} = \rho_G(\sqrt{h^2}_1)(\sqrt{h^2}_2) + \rho_E(1-\sqrt{h^2}_1)(1-\sqrt{h^2}_2)$. Pearson correlation coefficients were also estimated. The significance of the genetic correlation being different from zero or 1 (or -1) was then tested with a likelihood ratio test of the constrained and unconstrained models. Because the individual observations were not independent, we used variance component modeling.

Quantitative trait linkage analysis was used to identify genomic regions that were inherited with each of the traits. The significance of the theoretical QTL was tested with a likelihood ratio test at 1 cM intervals across each autosomal chromosome. Maximum likelihood

methods tested whether the model containing a parameter for the theoretical QTL was more likely than the model incorporating only polygenic effects. Logarithm of the odds (LOD) scores, computed as the log10 of the likelihood ratio, were used to assess the significance of the test. LOD score thresholds of 3.3 and 2.0 were considered to represent nominal genome-wide significant and suggestive evidence for QTLs, respectively[231]. Computational power used to be a hindrance to the feasibility of these analyses for some pedigrees; however, using SOLAR[112, 232] this is now possible in extended pedigrees such as those in the Tobago Family Health Study, and is robust to type 1 error and has more power than traditional nuclear family linkage analyses[233, 234].

For CVD and BMD trait-pairs with evidence of genetic correlation ($P<0.1$), we performed bivariate, whole-genome quantitative trait linkage analysis in order to identify genomic regions with potentially pleiotropic effects[283] with the same methods as were used in univariate analyses. However, intrinsic to bivariate linkage methodology, the likelihood ratio tests had 2 degrees of freedom rather than the 1 in univariate analyses. Therefore, the LOD scores between univariate and bivariate analyses are not directly comparable; a LOD of 3.0 in univariate analysis is closer to a LOD of 3.5 from bivariate analyses[283].

5.4 RESULTS

5.4.1 Family Study Characteristics

Participant characteristics are shown in Table 5.6.1. The family members ranged in age from 18 to 86 years (mean = 42.7 years). The sample was 60.1% female and 18.8% were post-

menopausal. Men and women were similar in body weight (mean = 82.3 kg), but men were taller than women resulting in women having a greater BMI than men. Smoking and drinking at least one alcoholic drink per week were significantly more common in men than women (11.6% versus 0.4% and 61.8% versus 29.0%, respectively). The frequency of walking for exercise was similar in men and women (mean 70.8%). Diabetes was present in 9% and hypertension was present in 28.0% of the sample. There was no difference in diabetes or hypertension by sex.

5.4.2 Variance Components of Univariate CVD and BMD Related Traits

All cardiovascular and BMD related traits were determined to be heritable after adjusting for age, sex, menopause, body weight, height, smoking, alcohol intake and physical activity (Table 5.6.2). Adventitial diameter had a residual heritability of 0.53 and IMT had a residual heritability of 0.44 ($P<0.001$ for both). Residual heritabilities of BMD at the femoral neck and lumbar spine were 0.54 and 0.59 ($P<0.001$ for both). Cortical BMD at the radius and tibia had the lowest residual heritabilities of any BMD trait (0.28 and 0.39, respectively; $P<0.001$ for both). In contrast, trabecular BMD had the greatest residual heritability (0.71 and 0.67 at the radius and tibia, respectively; $P<0.001$ for both). Covariates explained between 18% and 55% of the variance in cardiovascular and BMD related traits.

5.4.3 Univariate Linkage of CVD and BMD Related Traits

We initially performed univariate whole-genome linkage analysis of the CVD and BMD related phenotypes (data not shown; see Appendix: Table 7.1 and Figures 7.1 and 7.2). Analyses were adjusted for age, sex, menopause, body weight, height, smoking, alcohol intake and physical

activity. We identified significant linkage peaks for AD on chromosome 14 (max LOD = 4.71, 54 cM) and for radial trabecular BMD on chromosome 11 (max LOD = 3.44, 24 cM). A suggestive linkage peak was identified for IMT on chromosome 13 (max LOD = 2.36, 114 cM). Lumbar spine BMD also had a peak suggestive of linkage on chromosome 1 (max LOD = 2.53, 104 cM). Suggestive linkage peaks identified for cortical BMD differed by anatomical site. At the radius, a suggestive peak was identified on chromosome 12 (max LOD = 2.28, 80 cM) whereas at the tibia suggestive linkage peaks were identified on chromosomes 2 (max LOD = 2.62, 73 cM) and 19 (max LOD = 2.81, 42 cM). Despite significant heritabilities, there were no linkage peaks identified for femoral neck BMD or tibial trabecular BMD.

5.4.4 Correlation of CVD and BMD Related Traits

Correlations between CVD and BMD related traits are reported in Table 5.6.3. Overall, we found that with an increase in AD, BMD traits decreased significantly. AD was negatively correlated with all BMD traits, with phenotypic correlation coefficients around -0.2 ($P<0.05$). Only the correlation between AD and trabecular BMD at the tibia was not significant ($P>0.05$), even though the magnitude was similar to other trait-pairs. There was also significant genetic correlation between AD and all BMD traits except cortical BMD at the radius. Genetic correlation coefficients were generally high and ranged from -0.43 to -0.59 ($P<0.05$ for all). There was no significant phenotypic correlation between IMT and any of the BMD measures. However, there was evidence of some inverse genetic correlation between IMT and lumbar spine BMD and trabecular BMD at the radius (ρ_G = -0.35 and -0.33; $P<0.05$ and <0.1, respectively).

5.4.5 Bivariate Linkage of CVD and BMD Related Traits

We next performed bivariate, genome-wide linkage analysis for all CVD and BMD trait-pairs that had evidence of a genetic correlation (P<0.1) (Table 5.6.4 and Figures 5.6.1 and 5.6.2). We identified significant or suggestive bivariate linkage peaks for AD and BMD trait-pairs on chromosome 14 around 60 cM. The maximum LOD score was seen for AD and cortical BMD at the tibia (max LOD = 5.2). Other significant peaks were identified at the same region for femoral neck BMD, lumbar spine BMD and trabecular BMD at the radius and tibia (max LOD = 4.22, 3.55, 3.69 and 4.16, respectively). Additionally, we identified a peak suggestive of linkage on chromosome 11 around 24 cM for both AD and IMT with trabecular BMD at the radius (max LOD = 2.86 and 3.14, respectively). There was no suggestive linkage peak identified for the IMT and lumbar spine BMD trait-pair.

5.5 DISCUSSION

We examined the heritability and bivariate linkage of subclinical measures of CVD and BMD in large, multigenerational families of African ancestry. The individuals in these African ancestry families had generally healthy arteries and high BMD even though they tended to be overweight and had a high prevalence of hypertension. Measures of subclinical CVD and BMD were highly heritable, as has been reported previously in these families (Section 3.0 and Wang *et. al.*[284]). We found a significant, inverse phenotypic and genetic correlation between AD and measures of BMD, particularly for trabecular BMD. Bivariate genome-wide linkage analysis revealed evidence of linkage on chromosomes 14 for AD and cortical BMD at the tibia with a maximum

LOD of 5.2. To our knowledge, this is the first evidence to suggest that there may be a genetic basis for the link between indices of vascular and bone health.

The inverse phenotypic correlations suggest that persons with greater BMD (e.g., stronger bones) have smaller vessel diameters (e.g., healthier vasculature). This finding is consistent with previous reports of an association between vascular and bone health, in largely Caucasian ancestry populations. However, this is the first study to report a relationship with AD; the majority of previous reports have focused on arterial calcification as the marker of subclinical CVD[183-188]. In this cohort, IMT was not phenotypically correlated with BMD, in contrast to previous reports[191, 193, 198, 206]. The coefficients of correlation were negative for the most part; however, the magnitude of effect was small. This is likely due to the young and relatively healthy population that had very little atherosclerosis and, thus, had little variation in IMT measures.

There was also a significant, inverse genetic correlation between AD and BMD related traits. This observation suggests that there are genes that may cause an increase in AD and decrease in BMD, and/or vice versa. The strongest genetic correlations to AD were observed for trabecular BMD and lumbar spine, a mainly trabecular bone site, BMD. This observation may reflect the fact that trabecular BMD had the greatest heritability in these families. We also identified significant genetic correlation between IMT and lumbar spine BMD and trabecular BMD at the radius, but not for any of the cortical bone measures. A stronger genetic correlation between IMT and trabecular compared with cortical bone has not been demonstrated before.

Bivariate genome-wide linkage analysis revealed two linkage peaks with significant or suggestive evidence of linkage on chromosomes 11 and 14. The strongest evidence of linkage was identified on chromosome 14 for AD and cortical BMD at the tibia with a maximum LOD

of 5.2 at 61 cM. This linkage peak was originally seen in the univariate AD analyses, with a max LOD of 4.7 at 54 cM. Therefore, this peak in bivariate analyses is not a novel, genomic signal for AD and BMD, rather it is driven by the linkage with AD. Nonetheless, it is still of importance because it shows this region may have an impact on not only AD, but also on the etiology of the correlation of AD and BMD traits. The bivariate approach allowed identification of the importance of this region to the CVD-BMD relationship in these families, which is an advantage of the bivariate approach[283].

There are 153 known genes under the linkage peak on chromosome 14; yet, there are few that have been implicated in CVD and/or BMD regulation. The two strongest candidate genes are bone morphogenetic protein 4 (*BMP4*) and estrogen receptor 2 (*ESR2*). *BMP4* was first identified as an important inducer of bone formation[285]. However, it has also been shown to be partly responsible for the differentiation of vascular smooth muscle cells (VCMCs) into bone-forming, osteoblast-like cells[260, 286, 287]. Activation of BMP4 by RANKL in the adventitia of the vessel wall induces the VCMCs to differentiate into non-contractile cells with bone forming capacity[287], suggesting that there may be a role of *BMP4* in regulating vascular calcification. Additionally, *BMP4* may be involved in the vascular endothelial response to blood shear stress[261] and may also have a role in the inflammatory response of the arterial wall to lipids[288]. Therefore, *BMP4* may be a strong candidate gene for both AD size and BMD regulation. However, there was no significant association between SNPs in *BMP4* and vascular calcification in the only previous report to examine this association[289].

The *ESR2* gene controls the genomic response to estrogens, which are strongly associated with atherosclerosis[262] and BMD. Previously, genetic variation in this gene has been associated with subclinical CVD including IMT[262] and PWV[153]. Estrogen is also related to BMD, and

variation in *ESR2* has been associated with BMD[290], fractures[291] and osteoporosis[292]. *ESR2* genotypes may also aid in the prediction of clinical vascular disease risk beyond traditional risk factors[293]. There have been many *in vitro* studies of vascular calcification that show estrogens have a major role in its pathogenesis through regulation of cytokine and BMP activities[182, 216, 220, 294].

However, there are other genes in this chromosomal region that could be responsible for the bivariate linkage signal including protein kinase C, eta (*PRKCH*) and hypoxia inducible factor 1 α (*HIF1A*). Variants in *PRKCH* have been associated with multiple bone traits in a recent GWAS[295] and have also been associated with brain infarcts[296], suggesting it may play a role in both CVD and osteoporosis. *In vitro* studies have shown that *HIF1a* is involved in both bone regeneration[297] and angiogenesis[298]. In addition, there are numerous genes under the peak that have only been reported to be related to either CVD or OP, including *SLC8A3*, a known regulator of osteoblast function in bone[299, 300]; *LGALS3*, which may partly control serum lipids[264]; and *GCH1*, which is involved in oxidative stress[265].

Two previous genome-wide studies of arterial stiffness[263] and subclinical CVD[130] have identified this region on chromosome 14. The first study identified a suggestive linkage peak for PP/stroke-volume ratio in African Americans siblings, which overlaps with our peak[263]. Also, a genome-wide association study for coronary artery calcification in the Framingham Heart Study identified a significant association with a SNP in the drosophila NUMB homolog (*NUMB*) gene that resides at 72.8 MB within our identified genomic region on chromosome 14[130].

The other linkage peak identified in our bivariate analyses had suggestive evidence of bivariate linkage with a maximum LOD score of 3.14 on chromosome 11 at 24 cM for IMT and trabecular BMD at the radius. There was also suggestive evidence for linkage in this same region

for AD and trabecular BMD at the radius. Similar to the linkage peak on chromosome 14, this peak was also identified in the univariate linkage analysis, but for trabecular BMD at the radius. This suggests that this signal may not be a novel region with solely pleiotropic effects as the signal is driven by the linkage to BMD, but its reinforces its potential underlying genetic determination of variance in both traits or their correlation.

There are 93 genes within this linkage region with many potential candidates for osteoporosis, CVD or osteoporosis and CVD traits. The most plausible genes with impacts on both OP and CVD traits include adrenomedullin (*ADM*) and its related genes, calcitonin-related polypeptide α and β (*CALCA/B*), parathyroid hormone (*PTH*) and sex-determining region Y-box 6 (*SOX6*). Adrenomedullin is closely associated with calcitonin and the *CALCA/B* genes and is associated, *in vitro*, with osteoblast development[301], inflammation[302, 303] and vascular calcification[304-307]. Through epidemiologic studies, it has also been shown to be associated with diabetes[308], vascular calcification[309] and the correlation of adipose tissue with menopause[310]. Parathyroid hormone has a strong impact on bone metabolism[311], but it has also been shown to enhance expression of atherosclerotic factors[312]. Similarly, *SOX6* variants have been consistently related to BMD [313, 314] and with atherosclerotic plaques[315].

In conclusion, we have identified strong, inverse phenotypic correlations between AD and measures of BMD in large, multigenerational African ancestry families that appear to have a genetic, rather than environmental, basis. We have also identified genetic, but not phenotypic, correlation between measures of IMT and trabecular BMD. Bivariate genome-wide linkage analysis with a high-density SNP panel identified two genomic regions with potential pleiotropic, not co-incident, effects on these CVD and BMD related traits. This is the first study, to our knowledge, to document a correlation of AD with BMD and the first to test for genetic

correlation and bivariate linkage between these measures of subclinical CVD and osteoporosis. Our findings further support the hypothesis that there is an underlying shared etiology of CVD and osteoporosis. Further refinement of our linkage peaks may reveal genes that underlie this link.

5.6 TABLES AND FIGURES

Table 5.1 Characteristics of the Afro-Caribbean Family Members

Trait	All (n=461)	Men (n=184)	Women (n=277)
Age (years)	42.74 ± 16.6	42.93 ± 17.0	42.62 ± 16.4
Female Sex (%)	60.1	--	--
Post-menopausal (%)[†]	18.8	--	18.8
Weight (kg)	82.31 ± 18.4	84.03 ± 17.3	81.16 ± 19.0
Height (cm)	170.73 ± 8.6[$]	177.40 ± 7.1	166.32 ± 6.4
Body Mass Index (kg/m^2)	28.31 ± 6.3[$]	26.68 ± 4.9	29.38 ± 6.9
Current Smoker (%)	4.8[$]	11.6	0.4
>1 Drink per Week (%)	42.1[$]	61.8	29.0
Diabetes (%)	9.1	6.9	10.5
Hypertension (%)	28.0	30.1	26.6

* Characteristics are shown as mean ± SD or frequency (%)
[†] Frequency in women only
[$] Difference by sex significant (P<0.05)

Table 5.2 CVD and BMD Related Traits in Afro-Caribbean Families

Trait	Mean ± SD*	Genetic Effect ($h^2r \pm SE$)[¥]	Covariate Effects (r^2)
Mean AD (mm)	7.24 ± 0.7	0.530 ± 0.12	0.260
Mean IMT (mm)	0.69 ± 0.1	0.443 ± 0.11	0.551
Femoral Neck BMD (g/cm^2)	0.99 ± 0.2	0.542 ± 0.10	0.331
Lumbar Spine BMD (g/cm^2)	1.09 ± 0.2	0.590 ± 0.09	0.206
Radial Cortical BMD (g/cm^3)	1217.12 ± 27.5	0.278 ± 0.09	0.222
Tibial Cortical BMD (g/cm^3)	1183.30 ± 31.0	0.393 ± 0.10	0.178
Radial Trabecular BMD (g/cm^3)	221.59 ± 42.6	0.706 ± 0.10	0.275
Tibial Trabecular BMD (g/cm^3)	248.77 ± 35.8	0.674 ± 0.10	0.303

* Unadjusted mean and SD.

Genetic effect estimates are adjusted for age, sex, weight, height, menopausal status, current smoking, alcohol intake and walking for exercise

[¥] All residual heritability estimates had a corresponding $P<0.001$

IMT: intima-media thickness; AD: adventitial diameter; BMD: bone mineral density

Table 5.3 Correlation* Between CVD and BMD Related Traits

Trait	Adventitial Diameter				Intima-media Thickness			
	ρ_G	ρ_E	ρ_p	*r*	ρ_G	ρ_E	ρ_p	*r*
Femoral Neck BMD	**-0.502**	0.164	**-0.248**	**-0.183**	-0.232	0.082	-0.103	-0.078
Lumbar Spine BMD	**-0.589**	0.288	**-0.314**	**-0.197**	**-0.354**	0.242	-0.156	-0.051
Radial Cortical BMD	-0.241	-0.246	**-0.125**	**-0.223**	0.095	-0.053	0.027	-0.002
Tibial Cortical BMD	**-0.429**	-0.005	**-0.202**	**-0.197**	0.079	-0.019	0.029	0.002
Radial Trabecular BMD	**-0.537**	0.204	**-0.329**	**-0.215**	-0.328	0.195	-0.167	-0.081
Tibial Trabecular BMD	**-0.517**	**0.370**	-0.292	-0.130	-0.204	0.150	-0.098	-0.026

*All correlation analyses adjusted for covariates listed in Table 5.6.3

BMD: bone mineral density; ρ_G: genetic correlation; ρ_E: environmental correlation; ρ_P: phenotypic correlation (calculated); *r*: Pearson's correlation coefficient (estimated)

BOLD indicates coefficient is significant ($P<0.05$); ρ_P and *r* significance assessed by same variance components model outlined in methods section

UNDERLINE indicates ρ_G is suggestive ($P<0.10$) and will be included in bivariate linkage analysis along with all significant ρ_G trait-pairs

Table 5.4 Bivariate, Genome-Wide Linkage Results for CVD-BMD Traits

CVD Trait	BMD Trait	Max LOD*	QTL Location				Genes in Region	
			Chr	cM (range)§	Genomic (MBp)	Cytogenetic	N	Potential Candidates
AD	FN	4.22	14	61 (51-70)	53.8-73.6	q22.2-24.3	116	*BMP4*; *ESR2*
AD	LS	3.55	14	54 (46-70)	49.5-73.6	q22.1-24.3	153	*BMP4*; *ESR2*
AD	TCort	5.20	14	61 (51-66)	53.8-70.2	q22.2-24.2	97	*BMP4*; *ESR2*
AD	RTrab	2.86	11	23 (20-34)	8.0-18.7	p15.4-15.1	93	*ADM*; *SOX6*; *PTH*
AD	RTrab	3.69	14	54 (45-70)	48.6-73.6	q22.1-24.3	153	*BMP4*; *ESR2*
IMT	RTrab	3.14	11	24 (20-38)	8.0-20.0	p15.4-15.1	86	*ADM*; *SOX6*; *PTH*
AD	TTrab	4.16	14	54 (45-68)	48.6-72.0	q22.1-24.2	146	*BMP4*; *ESR2*

Only regions with a peak LOD ≥ 2.5 in bivariate analyses are shown

***** Analyses were adjusted for all covariates listed in Table 5.6.3

§ Range defined by the position of the peak LOD±1.0 where peak LOD<3.5 and defined as LOD±2.0 where peak LOD≥3.5

AD: adventitial diameter; IMT: intima media thickness; FN: femoral neck; LS: lumbar spine; TCort: tibia cortical; RTrab: radius trabecular; TTrab: tibia trabecular

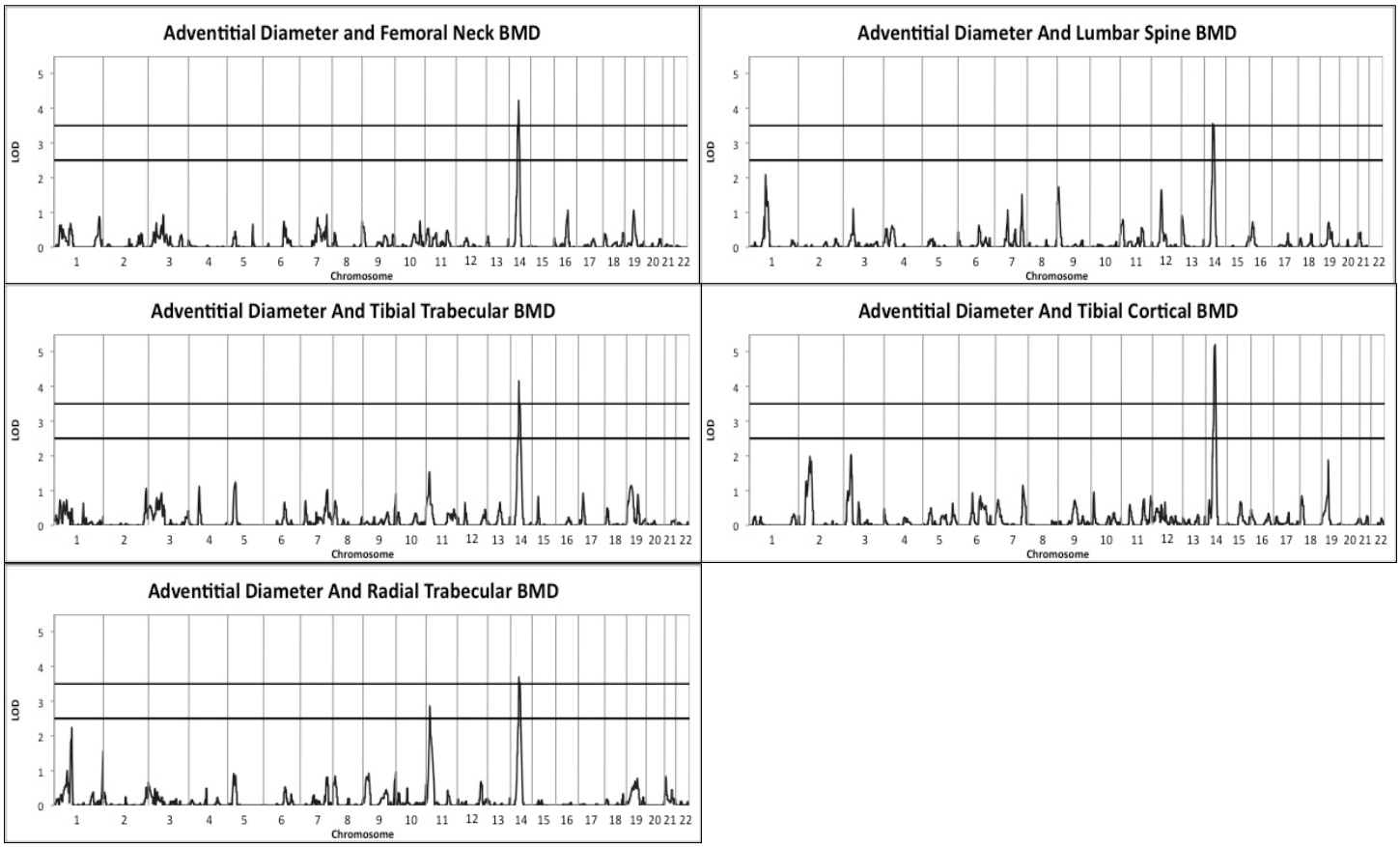

Figure 5.1 Bivariate, Genome-Wide Linkage Results for Adventitial Diameter and BMD Related Traits

LOD scores from bivariate, genome-wide linkage analysis of adventitial diameter and BMD trait pairs with significant genetic correlation are plotted by chromosome for each trait. Horizontal lines are drawn at LOD=2.5 and 3.5 to signify thresholds for suggestive and significant bivariate linkage, respectively. BMD: bone mineral density

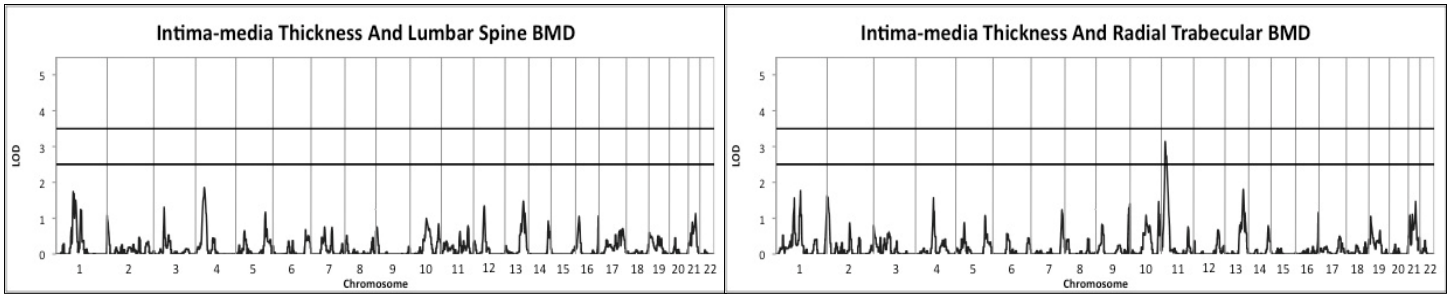

Figure 5.2 Bivariate, Genome-Wide Linkage Results of Intima-Media Thickness and BMD Related Traits

LOD scores from bivariate, genome-wide linkage analysis of intima-media thickness and BMD trait pairs with significant genetic correlation are plotted by chromosome for each trait. Horizontal lines are drawn at LOD=2.5 and 3.5 to signify thresholds for suggestive and significant bivariate linkage, respectively. BMD: bone mineral density

6.0 OVERALL CONCLUSIONS AND PUBLIC HEALTH SIGNIFICANCE

Currently, it is estimated that more than 1 of every 3 Americans has some type of prevalent CVD, and that prevalence is even greater in Blacks compared to Whites[1]. In the current study, we characterized the epidemiology and genetics of subclinical CVD in generally healthy, African ancestry families from the island of Tobago. Patterns of age- and sex-related differences in these measures were similar to previous reports in African ancestry populations, although measures of blood pressure and arterial stiffness may be more elevated in these families compared to African Americans. Carotid ultrasound measures were mainly associated with age, sex, body size and blood pressure. Measures of blood pressure and arterial stiffness were mainly determined by age, sex, body size and heart rate. Only pulse pressure was significantly related to menopausal status, and only pulse-wave velocity was associated with diabetes. These traditional CVD risk factors, along with smoking and lipid levels, were less predictive of subclinical CVD in this population than in previous reports. However, our sample was recruited from a very unique and geographically isolated island population, was relatively young, and characterized by different environmental risk factor profiles than Westernized societies. Thus, traditional CVD risk factors may be less strongly related to subclinical measures of CVD in our African ancestry families. Nonetheless, modifiable risk factors of subclinical CVD traits, such as excess body weight and blood pressure, were identified and should be a focus of efforts to improve CVD risk in the Tobago population.

We have also shown that there is a significant contribution of genetics to measures of subclinical CVD in these African ancestry families. Overall, measures of carotid ultrasound have a high proportion of variance due to genetics, compared to measures of blood pressure and arterial stiffness, which were less heritable. We conducted genome-wide univariate linkage analysis and identified genomic regions that may contribute to subclinical CVD measures, with the strongest linkage signal being on the q23 arm of chromosome 11 for adventitial diameter and lumen diameter. There was also a region identified on chromosome 14 q22-q24 with significant bivariate linkage for adventitial diameter and osteoporosis-related traits, as well as, univariate linkage to AD and LD. There are many potential candidate genes under these peaks including strong CVD-associated genes like the *APOA1/C3/A4/A5* gene cluster, *IL18* and *ESR2*. Further research will need to be done to refine these linkage peaks and identify the loci with univariate and/or pleiotropic effects on subclinical CVD and osteoporosis.

These findings are important because they describe CVD traits in a unique population of African ancestry individuals with very low admixture[6], which is unique compared to the United States and many Western European studies in Blacks. Also, the lifestyle factors for these individuals vary from most studies, which impacts the potential generalizability of our findings. While we did see a strong impact of some environmental factors, genetics determined a very large portion of the variance in subclinical CVD measures in these families.

We also found that body weight and blood pressure are the two strongest modifiable risk factors for subclinical CVD measures in these African ancestry families. These observations have significant public health importance as the rates of CVD continue to rise in Trinidad and Tobago[316]. We also determined that measures of subclinical CVD and osteoporosis are correlated both phenotypically and genetically. The genomic regions identified in these studies may provide

a starting place to refine the genetic determinants of CVD. Studying the genetic determinants of disease in ethnic minorities, such as these African ancestry families, can define ethnic differences in disease etiology, which will lead to ethnicity-specific therapeutic agents and risk prediction tools. Therefore, these data represent the critical first steps to ultimately understanding ethnic differences in disease progression and will hopefully aid in minimizing health disparities, which was, and continues to be, a major facet of the Healthy People 2010 and 2020 goals[317].

APPENDIX

SUPPLEMENTARY DATA

Table 7.1 Univariate, Genome-Wide Linkage Results for Cardiovascular and BMD Traits

Trait	Max LOD*	Chromosome	Location		
			cM (range)[§]	Genomic Mbp	Cytogenetic
Adventitial Diameter	4.71	14	54 (49-69)	52.9-73.4	q22.2-24.3
Intima-media Thickness	2.36	13	114 (104-121)	99.6-107.1	q32.3-33.3
Lumbar Spine BMD	2.53	1	104 (100-123)	64.0-90.3	p31.3-22.2
Radial Cortical BMD	2.28	12	80 (72-85)	52.9-67.6	q13.13-15
Tibial Cortical BMD	2.62	2	73 (56-84)	33.2-60.4	p22.3-16.1
Tibial Cortical BMD	2.81	19	42 (40-46)	11.3-15.6	p13.2-13.12
Radial Trabecular BMD	3.44	11	24 (15-50)	6.2-28.2	p15.4-14.1

All regions with a peak LOD $\geq$ 2.2 in univariate analyses are shown

*Analyses were adjusted for age, sex, weight, height, menopausal status, current smoking, alcohol intake and walking for exercise

[§] Range defined by the position of the peak LOD±1.0 where peak LOD<3.5 and defined as LOD±2.0 where peak LOD≥3.5

BMD: bone mineral density

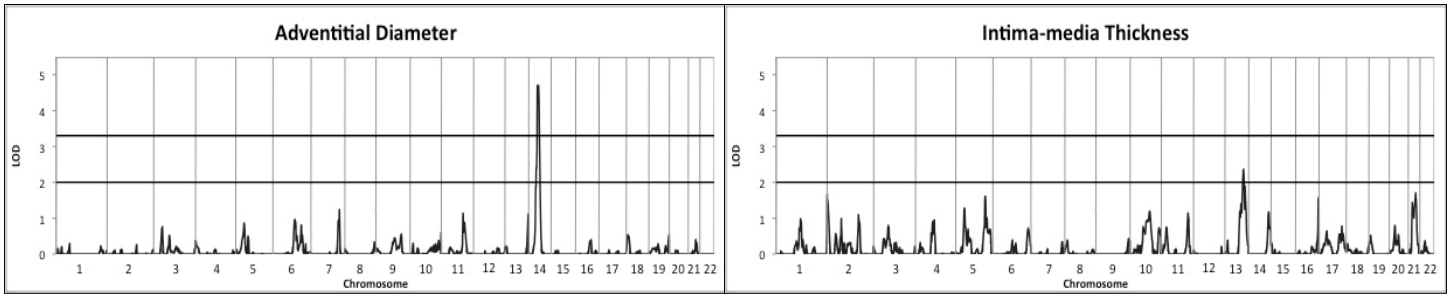

Figure 7.1 Univariate, Genome-Wide Linkage Results for Cardiovascular Traits

LOD scores from univariate, genome-wide linkage analysis of cardiovascular traits are plotted by chromosome for each trait. Horizontal lines are drawn at LOD=2.0 and 3.3 to signify thresholds for suggestive and significant univariate linkage, respectively.

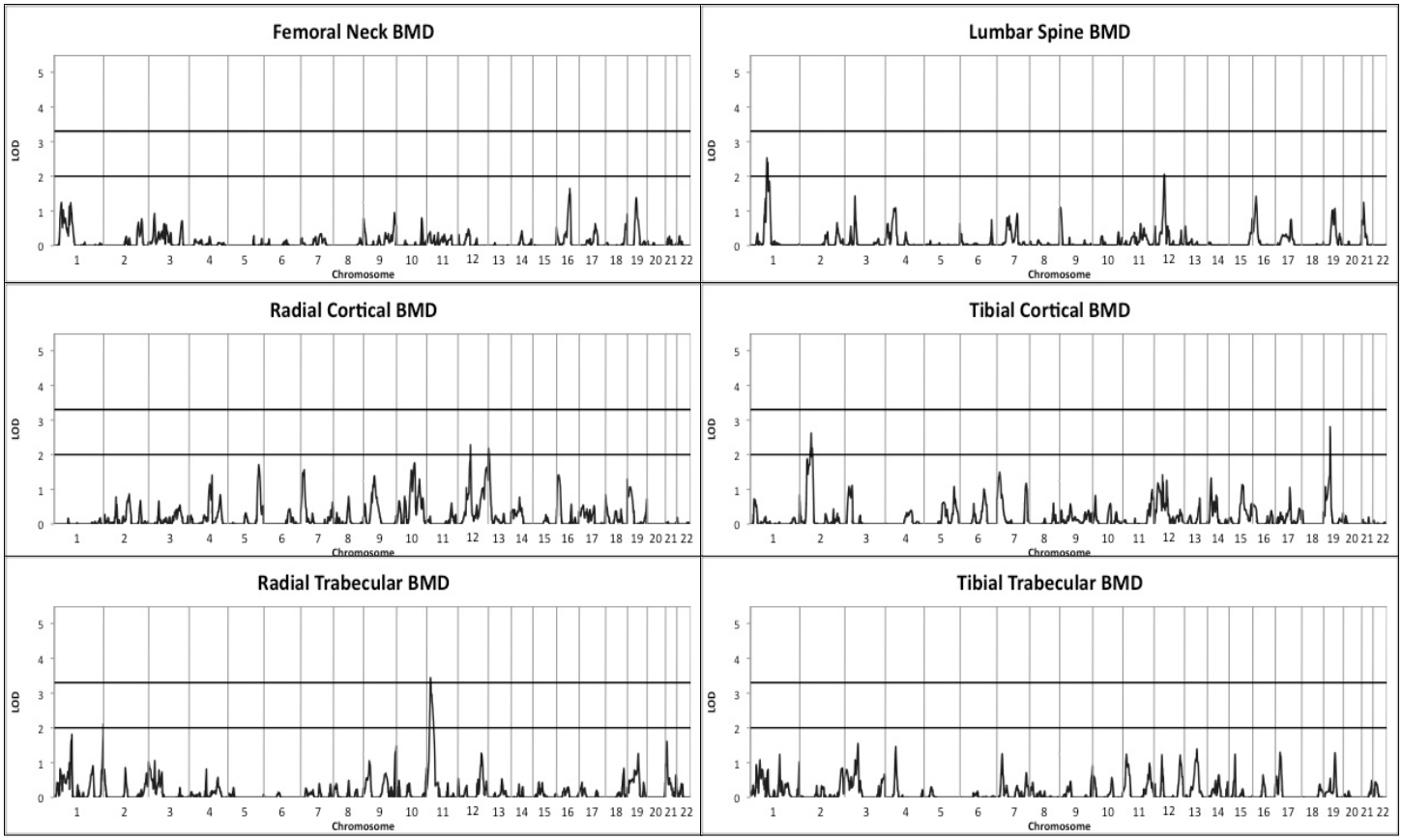

Figure 7.2 Univariate, Genome-Wide Linkage Results for BMD Related Traits

LOD scores from univariate, genome-wide linkage analysis of BMD related traits are plotted by chromosome for each trait. Horizontal lines are drawn at LOD=2.0 and 3.3 to signify thresholds for suggestive and significant univariate linkage, respectively. BMD: bone mineral density

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