2010

Contributions of the atrial natriuretic peptide gene system to the relationship between pediatric body fat, free fatty acids, and blood pressure

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CONTRIBUTIONS OF THE ATRIAL NATRIURETIC PEPTIDE GENE SYSTEM TO THE RELATIONSHIP BETWEEN PEDIATRIC BODY FAT, FREE FATTY ACIDS, AND BLOOD PRESSURE

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A DISSERTATION

Submitted to the graduate faculty of The University of Alabama at Birmingham,
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

BIRMINGHAM, ALABAMA

2010

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ABSTRACT

Elevated blood pressure and nonesterified fatty acids (NEFA) in children can lead to increased chronic disease risk during adulthood. The atrial natriuretic peptide precursor A (NPPA) and natiuretic peptide receptor A (NPR1) genes encode for atrial natriuretic peptide (ANP) and its cell receptor, respectively, and may influence levels of blood pressure and NEFA. Additionally, it is unclear how body composition, physical activity, physical fitness, and racial genetic ancestry influence pediatric NEFA and blood pressure. In order to understand the associations among these factors, five investigations were performed. For experimental aim 1, the association between blood pressure and body composition measures was evaluated to determine which measure of body fat is most associated with blood pressure. The results indicate that increased waist circumference, a measure of central adiposity, is associated with pediatric hypertension. For experimental aim 2, physical fitness and physical activity were measured and their association with pediatric blood pressure evaluated. These findings indicate that low physical fitness, rather than activity, is associated with increased pediatric hypertension risk. Results from experimental aim 3 suggest that physical fitness varies by percent body fat, and that percent fat is a more reliable indicator of physical fitness level than body mass index. For experimental aim 4, results suggest that the relationship between fasting NEFA and hypertension status is dependent on racial genetic ancestry. For

experimental aim 5, results from the previous aims were used to develop statistical models to test the association of the NPPA (rs5063, rs5065, rs198358) and NPR1 (rs10082235) genes with fasting NEFA and blood pressure. Increased prevalence of hypertension was noted among carriers of the GA allele of rs5063. Carriers of the rs10082235 TT allele had higher fasting NEFA and a trend for increased hypertension. Additionally, higher rates of African racial ancestry were associated with hypertension risk, while greater Amerindian was associated with higher fasting NEFA. Overall, these results indicate that central adiposity may affect blood pressure, but that physical fitness is the main predictor of hypertension and elevated NEFA risk. They also suggest that a similar genetic/biological mechanism may control both blood pressure and NEFA levels.

ACKNOWLEDGMENTS

During my time as a student at UAB, so many individuals have endeavored to provide me with the educational and emotional support to complete this training experience. First and foremost, I would like to thank my mentor, Dr. Jose Fernandez, for his undying support and understanding that he has given me over the past several years. He provided me with a truly amazing research experience, and gave me the freedom to explore my own ideas and interests. To Dr. Heimburger, I would like to express my sincere appreciation for the constant encouragement he gave me even after leaving UAB. To my other committee members – Dr. Hunter, Dr. Deluca, and Dr. Beasley – you are not merely a group of professors assembled to evaluate a dissertation project. You are professors who have each spent countless hours training me during my time here, and I am grateful to have worked for such a remarkable group of professionals. I would also like to thank the members of the Americo research team – Paul, Alexandra, Michelle, Krista, Akilah, Lynae, and Yann – for all of the efforts required to complete this study, and for your friendship. I also thank the parent and participants of the Americo study for your participation. I am grateful to Kerry and Desiree for their patience while training a computer geek in the genetics lab. The Nutrition Sciences department staff, particularly Nancy, David, Paul, Maryellen, Cindy, and Bob, have also provided me with countless hours of assistance. I thank the CPCTP training program, not just for funding my training, but also for shaping my career goals over the years. To my parents – Kenny, Ann, James, and Milagros – you have all sacrificed to provide me with this and countless

other opportunities, and I can never repay you for such unconditional love. I also thank my Uncle Nikolai and Tia Gladys for their love, support, and often well-timed career advice. My brother, Scott, has always been my hero, and I thank him for bravely paving the way in higher education, and making it easier for me to follow. Most of all, I am eternally grateful to my wonderfully supportive husband, without whose love, patience, and Barnes and Noble trips I could not have completed this training.

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GENERAL DISCUSSION

INTRODUCTION

Circulating nonesterified fatty acids (NEFA) are considered a vital energy substrate for many organ systems, yet can produce detrimental metabolic affects when present in excess. NEFA levels affect multiple metabolic pathways and are suspected to play a role in the development of various chronic diseases including cancer, diabetes and cardiovascular disease.[1-4] However, less investigated is the relationship between NEFA and elevated blood pressure (hypertension). In particular, high blood pressure among children is predictive of adult hypertension. Hypertension is also a known risk factor for cardiovascular disease and some cancers, and epidemiological research supports a link between NEFA and hypertension.[5-8] Several hypotheses have been proposed to explain this relationship, including 1) NEFA-related impairment of endothelium-dependent vasodilation and nitric oxide production, and 2) NEFA induction of oxidant stress.[9] However, the relationship between NEFA and blood pressure levels may be partially explained by additional factors known to influence both variables, including body composition, physical fitness and diet, and genetic differences.

Several pediatric studies have investigated the contribution of body composition to hypertension risk and found that increased body fat is related to elevated blood pressure.[10-12] Interestingly, results are equivocal regarding which measures of adiposity (body mass index, total fat mass, central fat mass) are associated with pediatric blood pressure, possibly due to the way in which these variables are accounted for in statistical models.[13, 14] Such information is useful when evaluating the association between NEFA

and blood pressure since the rate of NEFA release from adipose tissue (lipolysis) varies according to fat depot.[15] Likewise, higher physical fitness is associated with lower blood pressure and NEFA levels, and greater adult physical fitness is associated with a significantly decreased hypertension incidence (hazard ratio: 0.63 [95% CI: 0.56-0.70]).[16, 17] Physical fitness may also be referred to as aerobic fitness, and is defined here as the circulatory system's ability to provide skeletal muscle with oxygen during sustained physical activity and calculated as the maximal oxygen consumption available (VO_2 max; $mL/kg^{-1}/min^{-1}$). Studies indicate that physical fitness may be lower among individuals with excess body fat, partly due to lower levels of physical activity and the increased mechanical load (i.e., weight) carried during activity. Both body composition and physical fitness levels are also found to differ among racial/ethnic groups, further complicating the relationship of these variables with blood pressure and NEFA.[18, 19] Hence, the association of adiposity and physical fitness to NEFA and blood pressure needs to be better elucidated to determine the appropriate targets for disease prevention.

Physiologically, blood pressure regulation and hence hypertension risk is controlled in part by the release or inhibition of atrial natriuretic peptides (ANP). Release of ANP into circulation results in lower blood pressure through natriuresis (salt exrection) and diuresis (fluid excretion).[20-22] ANP has long been known to influence blood pressure control, and was recently identified as a contributor to lipolysis (NEFA release) in human adipocytes and associated with NEFA levels.[23, 24] It is thus possible that genetic differences among the population could affect the release/inhibition of ANP and also contribute to the association between blood pressure and NEFA. In fact, polymorphisms (genetic variation between individuals) within the genes encoding both

ANP (NPPA gene) and its cell receptor (NPR1 gene) are associated with hypertension risk among adults.[25-28] Hence, the genetic polymorphisms that contribute to population differences in hypertension risk among adults may also influence NEFA levels, and could partly explain the relationship between these two variables. However, to date few studies have investigated the relationship of NPPA/NPR1 polymorphisms to pediatric blood pressure, and no studies have identified whether these genes contribute to differences in NEFA levels among adults or children.

Health-related risk factors present in children can increase the risk for chronic disease diagnosis as adults. Therefore, it is imperative to identify which physiological, environment, and genetic factors contribute to the NEFA/blood pressure relationship in children. As preventive strategies become increasingly necessary for overall health, understanding the relationships of body fat and physical fitness with increased NEFA and blood pressure is important. Additionally, the potential genetic contribution of NPPA/NPR1 should be considered as a mediating factor that influences both circulating NEFA and blood pressure to determine if associations between the two factors are partially attributable to this particular gene system.

Chronic Disease Incidence and NEFA

Rapid increases in cancer, type 2 diabetes and hypertension have occurred during the past two decades.[29-32] In particular, children have experienced some of the fastest gains in chronic disease risk factors such as obesity, insulin resistance and elevated blood pressure.[33, 34] The precise roles of traditional environmental factors such as diet, physical activity, and socioeconomic status, versus the contribution of and interaction with genetic factors in disease incidence continue to be debated. However, it is generally

acknowledged that both environment and genes can alter metabolic pathways that contribute to lipid accumulation and utilization. Through lipolysis, triacylglycerols (TAG) stored in adipose tissue are broken down into glycerol and NEFA, which are released into circulation. NEFA are then utilized by the heart, liver, and skeletal muscle as an energy substrate; however, if more NEFA is released than can be used by these organs and cannot be taken back up by the adipocyte, excess circulating NEFA results.[35-38]

Body composition, metabolism, and NEFA

Several factors are thought to influence the amount of NEFA released into circulation. Investigations into the association of body composition with NEFA levels have shown that fat free (i.e. lean) mass may contribute to circulating NEFA due to the metabolic demands of this tissue, which counts for approximately 25% of resting metabolic rate (RMR).[39, 40] Nielsen et al. has previously shown that RMR is a strong predictor of palmitate flux, an indicator of lipolysis, in men and women, and thus may influence NEFA concentrations.[41] However, excess fat mass is also associated with NEFA levels, and the amount of NEFA released via lipolysis varies according to the location of fat depots.[42] Obesity (excess total body fat), measured with the body mass index (weight[kg]/height[m^2]) may contribute to maximization of lipid stores in adipocytes, leading to greater total NEFA release. Obese individuals (BMI > 30) typically present with higher absolute fasting NEFA compared to normal weight (BMI 18.5-24.9) controls. However, after adjusting for total adiposity these differences often disappear in adults, and obese individuals have similar or lower adjusted NEFA levels.[3, 43-46] Additionally, Nielsen and colleagues have shown that there is no association

between total or compartmental measures of body fat with fasting NEFA when RMR is accounted for.[47] This suggests that when evaluating the association between NEFA and other factors, measures of RMR should be accounted for in the analysis. Despite these findings in the adult population, however, it is unknown whether RMR also plays a stronger role in NEFA circulation compared to fat free mass, total body fat or a specific fat depot among children.

In summary, elevated fasting NEFA levels are associated with an increased risk of chronic disease. Resting metabolic rate is strongly associated with NEFA levels among adults; however, few studies that investigate NEFA levels in children have included measures of RMR. Investigations into factors that contribute to elevated NEFA levels among children should therefore include measures of metabolic rate (RMR) as opposed to body composition.

Blood Pressure and NEFA

Circulating NEFA have long been recognized as an independent contributor to conditions such as obesity and type 2 diabetes in which higher rates of hypertension are noted.[48] Bulow and colleagues first noted a direct relationship between NEFA and blood pressure *in vivo* while stimulating lipolysis in pigs. The resulting acute increase in NEFA was accompanied by a significant rise in blood pressure.[49] An association between elevated NEFA, poor blood pressure control, and complications of cardiovascular disease has also been shown in humans.[50, 51] Central obesity, a factor associated with higher fasting NEFA, is thought to impair function of the rennin-angiotensin-aldosterone system and result in hypertension. Furthermore, a longitudinal study of 3000 normotensive men showed that those with higher baseline NEFA had a1.58 greater odds of developing

hypertension after controlling for other known risk factors.[52] Potential hypotheses for this relationship have included that NEFA could impair endothelium-dependent vasodilation and nitric oxide production, or induce oxidant stress.[53-56] However, these studies have not always controlled for body composition or physical fitness, two factors strongly associated with both blood pressure and NEFA levels.

Body Composition, NEFA, and Blood Pressure

Some studies have shown an association of BMI with both hypertension risk and fasting NEFA levels.[3, 57] However, BMI is a measure of both fat free and fat mass, and may not accurately represent differences in body fat levels of individuals.[58] As stated previously, BMI is no longer associated with fasting NEFA once the NEFA values are adjusted for total fat mass.[59] Total body weight and BMI have also been associated with hypertension in adults and children; however, studies in children are equivocal regarding whether measures of total fat (BMI; body weight) or abdominal fat (waist circumference) are a superior marker of hypertension risk.[60-66] Such studies are difficult in children since both blood pressure and body fat levels are positively correlated with height. Furthermore, changes in pediatric BMI may be more related to increases in fat free mass that fat mass, limiting BMI's utility as a proxy measure for pediatric adiposity.[67, 68] It is therefore crucial to determine which measure of adiposity is most associated with pediatric blood pressure in order to properly control for body fat when evaluating hypertension risk among children, and the NEFA/blood pressure relationship.

Body Composition and Physical Fitness

Physical (aerobic) fitness, defined here as the circulatory system's ability to provide skeletal muscle with oxygen during sustained physical activity and calculated as

the maximal oxygen consumption available (VO_2 max; $mL/kg^{-1}/min^{-1}$), has been associated with both NEFA and blood pressure levels.[69, 70] Physical fitness is considered an independent factor for morbidity and mortality in adults.[71-73] Unfortunately, few studies evaluating the NEFA and blood pressure relationship in children have controlled for the contributions of physical fitness. In particular, levels of physical fitness vary between racial/ethnic groups, with African-American adults and children consistently presenting with lower physical fitness compared to European-American controls.[74, 75] Studies that include Hispanic-American participants show their physical fitness levels to be comparable to or lower than those of European-American counterparts.[76-78] However, racial/ethnic differences identified in body composition could also contribute to these fitness differences, and hence, differences in blood pressure and NEFA levels. Increased body fat typically increases the mechanical "load" on the moving body; hence, an individual with greater body fat would present with lower physical fitness when $VO_{2\ max}$ is adjusted for body weight.[79, 80] However, simply adjusting VO_2 levels for total body weight may cause children with high lean mass and low body fat to present with artificially low physical fitness. Hence, considering the independent contributions of fat free mass and fat mass to fitness among children may provide a more accurate picture of the relationship between fitness, racial/ethnic group, and body composition. Additionally, recent investigations have indicated that differences in the oxygen carrying capacity of hemoglobin and altered mitochondrial function might also contribute to racial/ethnic fitness differences, thereby altering the body's response to changes in adiposity within racial/ethnic groups.[81] Since both body composition (through the influence on RMR) and physical fitness are considered predictors of NEFA levels and blood pressure, it is

necessary to determine if excess body fat independent of total weight influences pediatric physical fitness, and whether this relationship could influence the contribution of physical fitness to NEFA and/or blood pressure.

Genetic Contributions to NEFA and Blood Pressure

Several studies have investigated genetic contributions to blood pressure and hypertension risk.[82-85] In particular, the NPPA gene, encoding for atrial natriuretic peptide (ANP), and the NPR1 gene, encoding the ANP's cell receptor, may play a role in blood pressure regulation. Recently, ANP cell receptors were identified in adipose tissue, and this peptide was found to promote lipolysis, releasing NEFA into circulation.[86-88] Hence, it is also possible that the NPPA/NPR1 gene polymorphisms found to influence hypertension risk could also affect rates of lipolysis. Such a dual-mechanism would provide an additional explanation for the positive association between NEFA levels and blood pressure. Additionally, racial/ethnic differences have been identified in hypertension rates that persist after controlling for other biological/environmental parameters, suggesting that ancestral genetic background could influence blood pressure levels.[89]

ANP, Blood Pressure, and NEFA

Natriuretic peptides are a family of peptide hormones long associated with kidney function (via the rennin-angiotensin-aldosterone system) and hypertension (via myocardial relaxation).[90] Thus, ANP plays a critical role in blood pressure and cardiac regulation, and may also decrease secretion of inflammatory macrophage particles.[91] A great deal of literature concerning the structure, stimuli, and function of ANP as related to these systems has been published, and it was further noted that in conditions where serum

ANP is abnormally elevated, such as congestive heart failure, rapid body fat loss often occurs.[92] Hence, ANP was found to also influence NEFA levels, providing an additional mechanistic link between NEFA levels and blood pressure.[93, 94]

ANP and blood pressure. ANP is produced and stored in cardiomyocytes and is released into circulation when increased intravascular volume (i.e., increased blood pressure) leads to stretching of the ventricular and atrial walls of the heart.[95] ANP then works to decrease blood pressure by causing relaxation of aterioles (the terminal branches of arteries), inhibition of rennin and aldosterone secretion, and inhibition of sodium reabsorption via the kidneys' collecting ducts. Hence, ANP works as a counter-regulatory system to the rennin-angiotensin-aldosterone system. In a healthy individual, this natriuresis (sodium/water excretion) decreases blood volume and blood pressure.[21, 96, 97] However, the ANP regulatory system may not be effective in all circumstances:

1) If arteriole walls resist relaxation, or blood pressure is continually elevated due to other factors (dietary sodium intake, obesity, chronic stress), then a chronic elevation in circulating ANP could result, affecting any other cells with receptors for ANP.[21]
2) If cardiomyocytes are unable to secrete adequate ANP, then chronically elevated blood pressure (hypertension) would result. In fact, if ANP secretion is inhibited in mice either pharmacologically or through genetic mutation hypertension results.[98] Studies have also shown that in hypertensive mice without a viable ANP cell receptor, blood pressure is lowered if the receptor phenotype is rescued.[99]

The blood pressure/ANP relationship is therefore cyclical, with aberrations in either factor contributing to possible defects in the other. Hypertension represents a "threshold

point", at which higher levels of ANP are noted independent of other environmental or genetic factors.[100] This paradox was observed by Newton-Cheh et al when they investigated the relationship between fasting ANP and NPPA gene polymorphisms.[101] Fasting ANP levels varied according to NPPA polymorphism; however, within each genotype, hypertensive individuals presented with higher circulating ANP. Taking into account this relationship between blood pressure and ANP, it is possible that once an individual is diagnosed with hypertension, consistently higher ANP could also stimulate lipolysis and increase circulating NEFA in hypertensive individuals.

ANP and NEFA. Sarzani *et al.* and others identified receptors for ANP (NPR-A) on the surface of human fat cells, and exposure of these cells to ANP produced significant rates of lipolysis.[102, 103] The groups further found that unlike catecholamines and insulin, which activate/impair lipolysis through a cAMP-mediated process, ANP stimulates lipolysis by way of cGMP, suggesting a unique, independent pathway for lipolysis regulation.[104]

Infusion of ANP *in vivo* confirmed ANP's additional role as a basal lipolytic stimulus and during conditions that increase intravascular volume, such as exercise. Using a microdialysis probe inserted into SAT, Birkenfeld *et al.* found that infusing physiological doses of ANP into healthy adult males resulted in concomitant increases in extracellular NEFA and glycerol.[105, 106] Additionally, Moro et al. showed via microdialysis probe that physical activity in overweight men resulted in acute increases of circulating ANP, NEFA, and glycerol equivalent to the intensity of exercise.[107-109] However, these responses are blunted in obese compared to normal weight participants, suggesting a decreased responsiveness of ANP to activity in this group. This group also

noted a greater lipolytic response to ANP in women compared to men. Moro and colleagues further investigated ANP-stimulated lipolysis in obese women with polycystic ovary syndrome (PCOS), a condition associated with decreased catecholamine-stimulated lipolysis.[110] Compared to obese controls, these women had lower basal lipolysis and ANP-stimulated lipolysis. This body of literature collectively confirms that ANP release independently contributes to circulating NEFA levels and raises the possibility that lower ANP could result in decreased lipolysis on a per-adipocyte basis, contributing to obesity. However, this function was found to exist only in humans and non-human primates,[111] prohibiting genetic analysis in organisms such as rodents and fruit flies that would normally serve as models for fat regulation. This limitation underscores the importance of conducting genetic analysis in human participants to determine whether genetic differences in genes affecting ANP secretion or cellular uptake (i.e., NPPA/NPR1) could impact fasting NEFA.

NPPA/NPR1 Genes

To date, no work has been published evaluating the association between polymorphisms in the ANP regulatory system and lipolysis or fasting NEFA. However, several studies have been published noting association of polymorphisms with blood pressure, hypertension, and stroke among adults. These results are equivocal, perhaps due to differences in hypertensive status, central adiposity, and polymorphism frequency within the population.[112-115] The ANP gene natriuretic peptide precursor A (NPPA), located at chromosome 1p36 and composed of three exons and two introns, is associated with blood pressure and cardiovascular disease.[116-120] The type A natriuretic peptide receptor (NPR1) gene, located at chromosome 1q21-q22, is also associated with ANP

levels, hypertension and cardiovascular disease.[121-124] Polymorphisms of interest in this system include:

rs5063: The minor allele of this polymorphism (G664A) of the NPPA gene is associated with decreased blood pressure and lower incidence of hypertension. Studies report a MAF of 5% in most populations.[125, 126]

rs5065: The minor allele of this polymorphism (T2238C) of the NPPA gene is associated with circulating ANP and with higher rates of stroke, heart disease and hypertension. A possible gene – body mass interaction was also identified among children carrying the minor C allele who were above the 85th percentile (overweight) for body mass index. Studies report a minor allele frequency (MAF) of 6-10% for this polymorphism.[120, 127]

rs198358: This A/G polymorphism in the NPPA gene is also associated with circulating ANP and hypertension. It has a MAF of 19-35% depending on the study population and is in linkage disequilibrium (non-random association) with several NPPA gene SNPs. [128]

rs10082235: This polymorphism in the NPR1 gene has a MAF of 7-9% in European-American adults and 44-47% among African-American adults. Gene association studies have noted a correlation of differences in this polymorphism with carotid intima-media thickenss (a marker for cardiovascular disease risk) and stroke risk.[129]

It is possible that polymorphisms of the ANP regulatory system found to influence circulating ANP, hypertension risk, and cardiovascular disease could also influence circulating NEFA by mediating this pathway, and studies regarding this relationship in both adults and children are needed.

Genetic Admixture

Specific gene polymorphisms are thought to contribute to hypertension risk and elevated levels of circulating NEFA; however racial/ethnic differences in both factors may persist even after controlling for the polymorphisms. Specifically, African-American patients have higher rates of hypertension compared to European- and Hispanic-Americans.[130] Racial/ethnic differences in NEFA levels are less clear; some studies suggest that African-American children and adults have lower NEFA compared to European-Americans, while other studies indicate no differences between groups.[131, 132] Interestingly, one limitation in these analyses involves the use of self-identified "race" as a proxy for genetic differences. The unique history of the United States (US) has resulted in an "admixed" population, which has inherited genes from three parental populations: Europeans, Africans, and AmerIndian groups. These three population groups established residency in the US and comingled, resulting in a population with varied genetic ancestry.[133-135] For example, two adults could self-identify as African-American; however, adult 1 has African ancestry of approximately 80%, while adult 2 has an estimated African ancestry of 40%. Culturally, both participants are classified into the same racial/ethnic group; however, it is unknown whether these two adults will experiences differences in genetic susceptibility to disease. Hence, ethnicity alone is an imprecise measure of biological diversity and admixture mapping represents a method to represent biological diversity in studies separately from cultural factors. Such confounders could have influenced previous investigations in racial/ethnic differences in NEFA and blood pressure levels, and it is possible that use of this technique could clarify genetic differences in risk for elevated NEFA/blood pressure.

Experimental Aims

The following experimental aims were designed to investigate the multifactorial relationships that may exist between fasting NEFA, blood pressure levels, the NPPA/NPR1 genes, and racial genetic ancestry. Figure 2 presents the main analyses for each experimental aim.

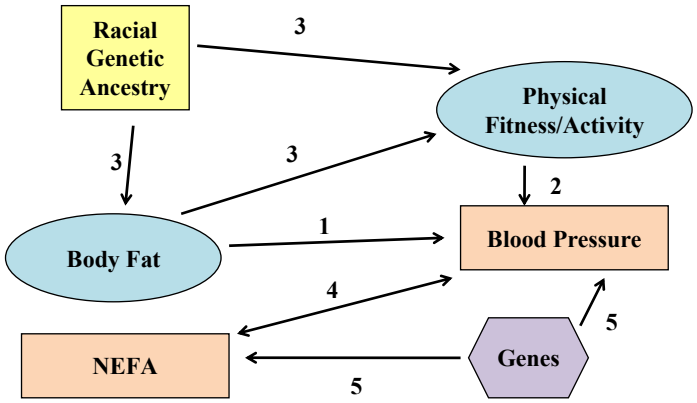

Figure 1. Outline of experimental aims

Experimental Aim 1

To determine which measure of body fat 1) is related to blood pressure in children after controlling for the multicolinearity with height and 2) hence would serve as the most appropriate covariate to investigate the blood pressure/NEFA relationship.

For this aim, we used anthropometric techniques and dual-energy x-ray absorptiometry (DXA) to measure BMI, waist circumference, and total fat mass and evaluate the association of each factor with blood pressure. In children, height can be highly associated with both body fat and blood pressure; hence, we use log-log regression to index each adiposity measure for height and also evaluate the association of the

following indexed measures with blood pressure: for BMI, the weight/height index; for waist circumference, the waist/height index, and for total fat mass, the fat mass index. When evaluating rates of hypertension, we noted a significantly greater percentage of African-Americans (16.3%) presenting with hypertension compared to European-American (5.1%) and Hispanic-American (2.7%) children. After adjusting for age, gender, socioeconomic status, diet, and physical activity, we found that greater BMI, waist circumference, and fat mass were associated with higher blood pressure in African-American and European-American children, but not Hispanic-American children. However, after indexing these measures with height, only waist circumference remained significantly associated with blood pressure. This study suggests that the waist/height index should be controlled for as a measure of adiposity when evaluating factors associated with pediatric blood pressure.

Experimental Aim 2

To investigate the associations of physical fitness and physical activity with blood pressure levels in European-American, African-American, and Hispanic-American children after controlling for age, sex, body composition, and genetic admixture.

For this aim, we evaluated blood pressure at four time points in a 24-hour period, and calculated the average systolic and diastolic blood pressure, as well as determining rates of normotension, prehypertension, and hypertension from blood pressure percentiles. We then measured physical fitness ($VO_{2\text{-}170}$) via indirect calorimetry and had the participants wear accelerometers for seven days to obtain estimates of minutes per day spent in moderate/vigorous physical activity. Due to the results of Experimental Aim 1, we computed the waist/height ratio using log-log regression to index waist

circumference (cm) for height (cm). We found that low physical fitness was associated with increased rates of hypertension and higher systolic blood pressure. However, no association of physical activity with blood pressure levels was noted. When both fitness and activity were included as covariates in statistical models, only physical fitness was associated with blood pressure. Additionally, African genetic admixture was positively associated with hypertension rates. These results suggest that physical fitness, rather than activity levels, should be measured when evaluating hypertension risk factors, and that individuals with higher levels of African racial ancestry are at increased risk for developing hypertension.

Experimental Aim 3

To evaluate the association of body fat and racial genetic ancestry with physical fitness levels among European-American, African-American, and Hispanic-American children accounting for lean mass, pubertal status, sex, physical activity, and genetic admixture.

We obtained submaximal measures physical fitness ($VO_{2\text{-}170}$) via an indirect calorimetry treadmill test and used accelerometers to estimate 7-day physical activity. Body mass index (BMI) was computed as weight (kg)/height (m^2). Fat mass was determined using dual-energy x-ray absorptiometry, and children classified into a low body fat group or a high body fat group based on their percent body fat. All children meeting the criteria for normal body fat levels (boys with < 25% and girls with < 30% body fat) were placed into the Low Body Fat Group (n = 164), whereas children exceeding those parameters were placed into the High Body Fat Group (n = 68). Genetic admixture estimates were obtained using 140 genetic markers informative for European,

African, and Amerindian ancestry. Using this information, we were able to compare the classification of children using BMI percentile (normal weight, overweight, obese) with versus percent body fat, and whether this difference impacted physical fitness levels. Approximately 12% of children classified as normal weight had high body fat, while approximately 49% of children classified as overweight and 9% classified as obese had low body fat levels. These differences impacted physical fitness test results, in that children classified as overweight but having low body fat actually presented with higher physical fitness than children considered both normal weight and overweight but who had high body fat. Additionally, African genetic admixture (but not Amerindian) was associated with lower fitness, and moderate/vigorous activity was associated with higher physical fitness. These results indicate that physical fitness in children with low body fat who are classified as overweight may be higher than in children considered normal weight but with high body fat, and that normal weight children with high adiposity would benefit from physical activity interventions designed to improve physical fitness.

Experimental Aim 4

To investigate the relationship between blood pressure and NEFA in European-American, African-American, and Hispanic-American children accounting for age, sex, body composition, physical fitness, and genetic admixture.

For this aim, we measured fasting NEFA and blood pressure levels in each participant. We then determined rates of normotension, prehypertension, and hypertension from blood pressure percentiles, and obtained estimates of African Amerindian, and European genetic admixture using 140 ancestry informative markers (AIMs). We found that among the total sample, there was no association of hypertension

with higher fasting NEFA. However, an interaction between genetic admixture and hypertension was associated with NEFA levels. Specifically, individuals in the lowest European admixture tertile did not experience an increase in fasting NEFA with blood pressure; however participants in the medium and high European admixture tertiles did present with elevated fasting NEFA in the presence of hypertension. These results suggest that genetic factors influencing the NEFA/hypertension relationship may differ depending on racial genetic ancestry.

Experimental Aim 5

To investigate the contributions of genetic polymorphisms in the NPPA and NPR1 genes to pediatric blood pressure and fasting NEFA.

For this aim, we used pyrosequencing to genotype four single nucleotide polymorphisms (SNPs) in the natriuretic peptide precursor A (NPPA) and natriuretic peptide receptor A (NPR1) genes: rs5063, rs5065, rs198358, and rs10082235. We also evaluated 140 AIMs to determine estimates of African, Amerindian and European genetic admixture. We then used four measurements of blood pressure to compute the average systolic and diastolic blood pressure, as well as incidence of prehypertension and hypertension. Finally, we measured fasting serum NEFA levels using "NEFA-C" reagents. The investigations completed for experimental aims 1-3 were used to determine which covariates were appropriate for the statistical models. We found an association of the rs5063 GA allele with decreased hypertension risk, and a trend for an association of the rs10082235 CT and TT alleles with higher rates of hypertension. Additionally the rs10082235 TT allele was associated with higher fasting NEFA levels. This suggests that polymorphisms in the NPPA/NPR1 genes may impact control of both

blood pressure and circulating NEFA levels. Additionally, we again noted that higher African genetic admixture was associated with hypertension risk, while greater Amerindian admixture was associated with higher fasting NEFA. This indicates that additional genetic factors associated with racial ancestry impact additional mechanisms involved in blood pressure and lipolytic control.

ADJUSTING ADIPOSITY AND BODY WEIGHT MEASUREMENTS FOR HEIGHT ALTERS THE RELATIONSHIP WITH BLOOD PRESSURE IN CHILDREN

by

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American Journal of Hypertension; in press

ABSTRACT

Adiposity measures are associated with increased pediatric blood pressure. However, this correlation can be confounded by the relationship of both variables to height. We evaluated whether adiposity and anthropometric measures were associated with pediatric blood pressure before and after adjusting each value for height. Participants included 281 African-American, European-American, and Hispanic-American children aged 7-12 years. Blood pressure percentiles were calculated according to pediatric guidelines using the average of four measurements. Total fat mass was determined using dual-energy x-ray absorptiometry. Socioeconomic status was calculated with the Hollingshead index. Adiposity measures were indexed for height using log-log regression analysis. Partial correlations identified measures associated with blood pressure. Linear regression was used to test the association of those measures with absolute blood pressure, while logistic regression was used to evaluate the odds for hypertension. More African-Americans (16.3%) presented with potential hypertension than European-American (5.1%) or Hispanic-American (2.7%) children. After adjusting for covariates, fat mass, body mass index, and waist circumference were positively significantly associated with absolute blood pressure and hypertension in African-American and European-American children ($P < 0.05$). When these measures were height-indexed, only waist remained significantly positively associated with hypertension risk in these two groups. No measures were significantly associated with blood pressure among Hispanic-American children. In this multiethnic pediatric population, waist circumference was the strongest significant adiposity predictor of hypertension risk among African-American and European-American children. Additional research is

needed to determine which environmental and genetic factors contribute to pediatric hypertension, particularly among Hispanic-American groups.

INTRODUCTION

Obesity and hypertension are associated with cardiovascular disease risk in adults, and rates of both conditions are increasing among the pediatric population.[1-4] In particular, elevated pediatric systolic blood pressure (SBP) predicts hypertension risk in adolescence and adulthood, and an association between anthropometric measurements and pediatric SBP has been noted.[5-7] Furthermore, ethnic/racial differences in obesity and hypertension risk have been identified, with African-American (AA) and Hispanic-American (HA) children at greater risk than their European-American (EA) counterparts.[3, 8]

Although a positive relationship between adiposity and blood pressure is generally accepted, these associations can be confounded by the relationship of height with both SBP and fat mass. Among adults, height does not play a significant role in determining blood pressure. However, in children, evidence suggests that both SBP and adiposity increase with pediatric height, and that the standard calculation of body mass index (dividing height by a power of 2) may be inadequate in children.[9-11] This multicollinearity of height with both variables could result in overestimation of the association of adiposity measures with SBP if height is simply included as a covariate in a regression model.[12] In fact, height-based measures such as body mass index (BMI) and percent body fat can overestimate total adiposity in taller children and underestimate adiposity among shorter children.[9, 13-15] Investigators have previously used allometric scaling (indexing) to adjust body size (weight) by body shape (height) by log-log regression analysis, thus removing the association between body weight and height.[16] Wells et al.[17] suggest indexing adiposity measures to height as preferable to using BMI or

percent body fat, since a height-adjusted adiposity index would provide an "independent measure of body fatness". We thus hypothesized that 1) total fat mass, BMI, and waist circumference would be associated with blood pressure in a pediatric multiethnic population, and 2) that the association of these body fat measures with pediatric blood pressure would decrease after those values were adjusted for height using log-log regression analysis. Since socioeconomic status, dietary intake, and physical activity levels are suggested to play a role in hypertension risk, these factors were also evaluated in the present study.

METHODS

Participants were 281 children aged 7-12 years and classified by parental report as African-American (n = 92), European-American (n = 116), or Hispanic-American (n = 73) measured between 2004-2008 for a cross-sectional study designed to investigate racial/ethnic differences in metabolic outcomes. . Approximately 95% of HA children were first generation immigrants from Central and South America. Children were recruited from the Birmingham, Alabama, area with community fliers/ presentations, and newspaper advertisements. Exclusion criteria included factors that could influence body composition, such as diagnosis of major medical conditions including hypertension, hyperlipedemia, and type 1 or type 2 diabetes. Children were also excluded if they had taken medications during the past 90 days known to affect body composition or blood pressure levels (glucocorticoid, antidepressant, or stimulant therapy). Only children with a pubertal status ≤ 3 (determined by physician exam) according to the criteria of Marshall and Tanner were included.[18, 19] The study was approved by the University of Alabama at

Birmingham (UAB) Institutional Review Board for Human Use, with children and parents providing informed consent prior to participation.

Protocol

Data were obtained during two study visits. During the first outpatient visit, body composition and anthropometric measurements were taken. Children wore an accelerometer for 7 days to record physical activity levels. During the second visit, participants were admitted to the UAB General Clinical Research Center at approximately 1730 hours for an overnight stay. Blood pressure was measured in the evening and morning. A 24-hour diet recall was administered at each visit using the triple-pass method.

Blood Pressure

Four blood pressure measurements were taken using an automated pediatric blood pressure cuff (Dinamap Pro 200, GE Medical Systems). Two measurements were obtained at approximately 1800 hours during the overnight visit. Two additional measurements were taken at approximately 0700 hours the following morning. Blood pressure was recorded after a minimum 10 minutes of seated rest. A five-minute rest separated the first and second measurements. There was no significant difference between evening and morning blood pressure measurements; thus, the four values were averaged to obtain a single systolic/diastolic measurement. Blood pressure percentile (BP%) was calculated using blood pressure tables developed by the National High Blood Pressure Education Program Working Group on High Blood Pressure in Children and Adolescents.[20] Children were classified as normotensive (systolic or diastolic BP% <

90th percentile), pre-hypertensive (BP% 90-94th percentile) or hypertensive (BP% ≥ 95th percentile).

Body Composition

Body weight was measured to the nearest 0.1 kg in light clothing without shoes (Scale-tronix 6702W; Scale-tronix, Carol Stream, IL). Height was determined without shoes using a mechanical stadiometer. Body mass index (weight kg / height m^2) was calculated from these values. Waist circumference was measured by the same registered dietitian using a flexible tape measure (Gulick II; Country Technology, Inc., Gays Mills, WI) as described by Lohman et al. and recorded to the nearest 0.1 cm.[21] The waist/height ratio was calculated from waist and height measurements.

Total fat mass was evaluated via dual energy x-ray absorptiometry (DXA) with a GE Lunar Prodigy densitometer (Lunar Radiation Corp., Madison, WI). Subjects were scanned in light clothing while lying flat on their backs with arms at their sides. DXA scans were analyzed with pediatric software enCORE 2002 version 6.10.029.

Socioeconomic Status

Socioeconomic status (SES) was measured with the Hollingshead 4-factor index of social class, which combines the educational attainment and occupational prestige for working parents in the child's family. Scores ranged from 8 to 66, with the higher scores indicating higher theoretical social status.[22]

Determination of Height-Indexed Fat Mass Values

To calculate height-indexed values, each body composition measure was regressed on height using log-log regression analysis. Variables were log transformed

with (log)height as the independent variable. Dependent variables included (log) fat mass, (log) body weight, and (log) waist. The value of the slope was used as the power of ^{p} (index = fat mass / height ^{p}) such that each index was confirmed as no longer correlated with height. Regression analyses were determined within each ethnic group, and by gender in each group. Calculations resulted in a fat mass index, weight/height index, and waist/height index. Regression slopes for the waist/height index indicated that it was appropriate to divide waist measurements by height with a power of one (waist / height^{1}) in all participants. Slope values for the fat mass and weight/height indices are shown in Table 1. Gender-specific slopes within each ethnic group were used to calculate each height-indexed value.

Diet Recalls and Physical Activity

Diet and physical activity levels were measured to control for possible effects on blood pressure. A registered dietitian administered two 24-hour diet recalls using the multi-pass method.[23] Data was entered into the Nutrition Data System for Research software version 2006 (Nutrition Coordinating Center, University of Minnesota, Minneapolis), and values from the two visits averaged for analysis. Physical activity levels were recorded for seven full days with a uniaxial ActiGraph accelerometer (GT1M – Standard Model 198-0100-02, ActiGraph LLC, Pensacola, FL). Epoch length was set at one minute and data expressed as counts per minute (counts min^{-1}). Daily counts were analyzed as average time (minutes/day) spent on moderate, hard, and very hard activities. Actigraph monitors have been shown to exhibit high inter-instrument reliability.[24]

Statistical Analysis

Descriptive statistics were analyzed between ethnic groups using analysis of variance with Tukey's post-hoc analysis. Average SBP between ethnic groups was also analyzed with analysis of covariance controlling for age, height, gender, and SES. The association of body mass and anthropometric measures with systolic blood pressure was assessed in multiple ways, and a modified Bonferroni procedure was utilized to adjust for multiple comparisons.[25] The relationships of fat mass, fat mass index, weight, BMI, weight/height index, waist, and waist/height index to BP% categorical classification were evaluated with partial correlations controlling for age, gender, and SES. Height was included as a covariate for non-indexed values.

Linear regression was used to test the association of each body composition and index measure with SBP in the total sample after adjusting for age, gender, and SES. We determined that we were adequately powered to repeat the analyses within racial/ethnic groups, which required a sample size n=43 for an alpha = 0.80 at a $P < 0.05$ per group. Height was included as a covariate for non-indexed values. A stronger relationship with body weight, cardiac outcomes, and adult hypertension is reported for systolic blood pressure (SBP); hence, when analyzing blood pressure as a continuous variable we utilized absolute SBP measures.[26-28] To improve normality of residuals, fat mass, weight, waist, and height were log-transformed in the models including those variables. Logistic regression was used to determine if body mass measures were associated with hypertension (BP% $\geq 95^{th}$ percentile) in the total sample. Analyses were not repeated within ethnic groups due to the small numbers of EA and HA children with hypertension.

All analyses were performed using SAS version 9.1 (SAS Institute, Inc., Cary, NC) with a significance level of $P < 0.05$.

RESULTS

Descriptive statistics are presented in Table 2. AA children had higher average SBP and a larger percentage of pre-hypertensive and hypertensive children compared to EA and HA (all at $P < 0.01$). HA children were shorter, had greater fat mass with a higher BMI percentile and waist circumference, and also consumed more calcium, potassium and magnesium than the other two groups ($P < 0.05$). There were no group differences in age or physical activity levels. All statistical models included dietary and physical activity variables as covariates, and these variables were evaluated for interaction effects with other covariates. However, none of these variables contributed to differences in blood pressure and hence were removed from subsequent analyses.

After adjusting for covariates, BP% category (normotensive, pre-hypertensive, hypertensive) was significantly associated with fat mass, BMI and waist circumference in the total sample. After indexing measures for height, only waist (waist/height index) remained associated with BP% category ($P < 0.05$; Table 3). . When analyzed within ethnic groups, only higher waist measurements (waist/height index) remained significantly associated with greater BP% category for AA and EA children (all at $P < 0.05$) when height-adjusted measures were used. After modified Bonferroni adjustment, BMI and waist/height index in the total sample and among EA children remained associated with BP% category. No measures of body mass were associated with BP% category in HA children.

Fat mass, BMI, and waist circumference were associated with increasing SBP levels after controlling for covariates in AA and EA children (all at $P < 0.05$; Table 4). However, substitution of height-adjusted indices for fat mass and weight in linear regression models removed this association. The waist/height index remained positively associated with SBP in AA ($P < 0.01$) and EA ($P = 0.02$). Across all models, African-American ethnicity was positively associated with hypertension risk (all at $P \leq 0.05$). Additionally, SES was associated with hypertension in AA only (all at $P < 0.05$).

Logistic regression revealed that fat mass (odds ratio [OR: 2.24 [95% CI: 1.05-4.81]) and BMI (OR: 1.17 [95% CI: 1.04-1.32]) were positively associated with hypertension (BP% category), while use of the fat mass and weight/height indices eliminated this association (Figure 1). However, waist circumference (OR: 1.07 [95% CI: 1.02-1.12]) remained associated with hypertension when using waist/height index in the analysis (OR: 2.45 [95% CI: 1.22-4.94]).

DISCUSSION

This study suggests that previously identified associations of anthropometric measures with blood pressure may be partially attributable to the confounding association of height with pediatric blood pressure. When indexed to remove the association with height, only waist circumference remained positively associated with blood pressure, suggesting that central adiposity may play a stronger role than total adiposity in pediatric hypertension.

Most previous studies have utilized anthropometric data alone as a proxy for body fatness. However, Maynard and colleagues found age-adjusted increases in pediatric BMI and BMI percentile are primarily attributable to increases in lean mass rather than

fat mass.[29] Brion et al. also proposed that the association of BMI with blood pressure could be due to changes in both lean and fat mass, and that BMI may account for the independent effects of both measures, minimizing its utility as a proxy for adiposity.[26] Our study benefited from a more precise measure of total body fat via DXA. In agreement with previous work, greater total fat predicted higher blood pressure, though after indexing for height total fat did not predict hypertension. BMI may be a useful clinical tool to identify obese children at risk for hypertension; however central, rather than total, body fat is related to SBP in our sample.

Consistent with other studies, however, central adiposity was associated with SBP in our sample even after indexing for height. Several previous studies suggest waist circumference is a superior predictor of blood pressure compared to BMI among normal weight and overweight children, though others have found waist circumference to be a reliable indicator of hypertension risk only among overweight/obese European children or among females.[30-36] Sample size did not allow for a comparison between weight categories in our population. We found no gender difference in the association between body composition measures and blood pressure; however, female children may have greater adiposity at a given height, and our use of gender-specific calculations to index body compositon values for height could have controlled for this. Further analysis could identify whether total central adiposity per se or a specific fat depot (i.e., subcutaneous versus intraabdominal fat) is associated with hypertension risk, and whether this association differs between normal weight and overweight children.

Although only 30% of participants were overweight as defined by a BMI $\geq 85^{th}$ percentile, our sample included a greater number of hypertensive children than the

current estimated prevalence among the general population. We oversampled AA and HA children relative to their percentage of the general population, which might have affected our results. However, power analysis confirmed an adequate sample size with three ethnic groups for these analyses, and our results using non-indexed measures were consistent with previous studies involving larger samples sizes.[5-7, 37] In Alabama, 34% of all children are considered overweight, compared to 33.3% of 6-11 year olds nationally, suggesting that our sample is representative regarding body fat indices.[38] Population-wide studies have estimated pediatric hypertension prevalence of 3-14% in normal-weight children, and as high as 11-30% among obese children.[3, 39-41] In our sample, approximately 5% of EA and 3% of HA children presented with hypertension; however, hypertension was identified in 16% of AA. Although this is a higher rate than reported in other studies, it is consistent with reports that residents of the Southeastern United States in general, and AA in particular, have worse health profiles compared to national averages even after relocation to another region.[42, 43] Hence, while greater than the national average, our AA hypertension rates may still be representative of Southeastern Region children, and emphasize the need for research and health interventions in this area.

There were ethnic differences in both the prevalence of and factors contributing to higher blood pressure. HA children accounted for 40% of overweight participants, yet despite having the highest levels of total body fat and waist circumference, presented with the lowest average SBP and hypertension rates, which could have impacted our findings. Previous studies completed within Latin-American countries that identified waist circumference or BMI as predictive of pediatric blood pressure studied populations

with higher rates of hypertension and obesity than found in our cohort, and did not index weight or waist measurement for height.[44-46] Studies investigating ethnic diversity in blood pressure have typically included only EA and AA, despite a lower prevalence of hypertension among HA compared to AA adults.[47] Such differences are likely caused by a combination of biological and environmental factors. For example, investigations into obesity-related diseases have identified alternate etiologies between ethnic groups for fat accumulation and insulin resistance development, as summarized by Goran.[48] Similarly, factors not measured here, such as higher rates of renal sodium excretion, decreased vascular resistance, and genetic polymorphisms could influence blood pressure more than central adiposity among HA in our cohort. Additionally, most HA in this study are first generation immigrants. Less acculturated, first-generation HA immigrants of Central- and South American origin have lower rates of hypertension compared to EA and AA adults regardless of body weight or waist circumference.[47, 49, 50] Morales et al. also reported higher blood pressure in second generation Mexican Americans compared to the first generation, inferring an environmental component to hypertension.[51] Hence, we might have seen higher SBP among our HA children had their families lived longer in the United States.[49, 50]

Conversely, almost one fifth of AA children in this study had elevated blood pressure despite having less body fat and a lower BMI percentile than HA children. AA ethnic/racial classification remained independently associated with hypertension risk in the total sample, and SES was associated with hypertension in AA only. Hence, additional genetic/epigenetic factors not captured in this study possibly contributed to a greater incidence of high blood pressure in this group.[52-54] Furthermore, AA children and

adults experience lower SES, greater stress and decreased access to health care, factors associated with elevated blood pressure.[55-57]

Our use of a multiethnic population allowed us to evaluate the unique contribution of adiposity measures to SBP within minority groups. Our study was limited in that most of our HA participants were first-generation immigrants claiming Central American ancestry, which could affect applicability of these results to Caribbean Hispanics or HA living in the United States for a longer period of time. Furthermore, individual hypertension is typically diagnosed from measurements obtained during multiple time points or from 24-hour ambulatory blood pressure monitoring. Our data was obtained during one overnight visit; however, such information is useful to evaluate population trends in hypertension risk. Additionally, gene-environment interactions we were unable to test for could possibly affect the study sample. We were unable to ascertain family history of hypertension from the data, and genetic differences could have contributed to higher rates of hypertension, particularly among AA children. Also, 70% of our study population was of normal weight as defined by a BMI at or below the 85th percentile, and chronic obesity could affect blood pressure differently in children over time. However, our use of a predominately normal weight sample allowed us to evaluate biological and environmental contributions to blood pressure that could have been overshadowed by the presence of significant obesity.

In conclusion, total fat mass and body weight are not associated with hypertension risk in healthy children after removing the association with height. Central adiposity, as measured by waist circumference, is associated with pediatric SBP in AA and EA, but not HA, children and might be a more accurate measure of hypertension risk in the clinic

setting for those groups. Additional studies could further elucidate how genetic, biological, and environmental factors interact to increase SBP in otherwise healthy children.

ACKNOWLEDGMENTS

The authors would like to thank Robert Petri and Alexandra Luzuriaga-McPherson for their assistance with data collection. This work was funded by the National Institutes of Health grants R01-DK067426, P30-DK56336, P60-DK079626, M01-RR-00032, and the National Cancer Institute Cancer Prevention and Control Training Program CA47888.

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Table 1. Slope values (95% CIs): regression of logFat Mass or logWeight (dependent variables) on logHeight (independent variable)

Group	Fat Mass			Weight		
	β	*95% CI*	*p*	β	*95% CI*	*p*
African-American	4.12	2.45, 5.79	<0.0001	2.73	2.30, 3.17	<0.0001
Boys (n=50)	4.25	1.90, 6.60	0.0007	2.77	2.21, 3.33	<0.0001
Girls (n=42)	3.72	1.46, 5.99	0.0019	2.67	1.97, 3.38	<0.0001
European-American	3.12	1.92, 4.32	<0.0001	2.45	2.13, 2.76	<0.0001
Boys (n=61)	4.19	2.43, 5.96	<0.0001	2.76	2.30, 3.22	<0.0001
Girls (n=55)	2.49	1.04, 3.94	0.0012	2.14	1.71, 2.58	<0.0001
Hispanic-American	3.80	2.69, 4.92	<0.0001	2.63	2.27,2.99	<0.0001
Boys (n=38)	5.19	3.61, 6.79	<0.0001	3.06	2.63, 3.49	<0.0001
Girls (n=35)	1.99	0.54, 3.43	0.0084	1.94	1.41, 2.48	<0.0001

Table 2. Baseline characteristics

Characteristic	AA (n=92)	EA (n=116)	HA (n=73)
Age (y)	9.6 (1.5)	9.7 (1.6)	9.3 (1.5)
Height (cm)	141.2 (10.2)[a]	140.3 (10.5)[a]	136.7 (10.7)[b]
Weight (kg)	37.4 (10.2)	35.5 (8.6)	36.6 (9.6)
BMI(kg/m2)	18.5 (3.2)[a,b]	17.9 (2.6)[a]	19.3 (2.7)[b]
BMI percentile	63.8 (27.3)[a]	59.7 (26.5)[a]	77.8 (18.2)[b]
Waist (cm)	62.9 (8.5)[a]	63.1 (7.6)[a]	67.2 (9.8)[b]
Fat Mass (kg)	8.1 (6.1)[a]	8.2 (5.1)[a]	10.3 (5.5)[b]
Lean Mass (kg)	27.1 (5.6)[a]	25.4 (5.0)[b]	23.9 (4.7)[b]
SBP	106.2 (10.9)[a]	102.2 (10.3)[b]	100.6 (9.1)[b]
BP% >95^{th,} n(%)	15 (16.3)[a]	6 (5.1)[b]	2 (2.7)[c]
BP% 90th > 95th, n(%)	8 (8.7)[a]	5 (4.3)[b]	3 (4.1)[b]
Sodium (gm)	3.4 (1.2)[a]	3.1 (0.9)[a]	3.2 (1.0)[a]
Calcium (mg)	740.5 (319.7)[a]	872.4 (318.2)[b]	985.9 (306.9)[c]
Magnesium (mg)	192.9 (537.3)[a]	210.3 (57.0)[a]	232.3 (63.8)[b]
Potassium (gm)	2.0 (0.6)[a]	2.1 (0.6)[a]	2.3 (0.6)[b]
Moderate/Vigorous activity(min/d)	59.7 (34.9)	60.8 (34.0)	52.1 (28.8)
Socioeconomic status	38.0 (10.7)[a]	49.2 (9.7)[b]	26.0 (12.6)[c]

BMI, body mass index; SBP, systolic blood pressure; BP%, blood pressure percentile
Data are mean ± SD or percentages. [a,b,c,] superscripts indicate significant differences (determined by ANOVA) between racial/ethnic groups at $P<0.05$, i.e. a≠b≠c.

Table 3. Separate partial correlation models evaluating association of systolic blood pressure with each body fat measure

	Total (n=281)	AA (n=92)	EA (n=116)	HA (n=73)
Fat Mass	0.12; p = 0.04	0.22; p = 0.05	0.24; p = 0.01	-0.04; p = 0.76
Fat Mass Index	0.11; p = 0.06	0.19; p = 0.08	0.13; p = 0.19	0.09; p = 0.50
Body Mass Index	0.22; p < 0.01	0.23; p = 0.04	0.26; p < 0.01	0.15; p = 0.23
Weight/Height Index	0.12; p = 0.05	0.24; p = 0.04	0.18; p = 0.07	-0.06; p = 0.64
Waist	0.14; p = 0.02	0.25; p = 0.03	0.23; p = 0.02	0.08; p = 0.51
Waist/height Index	0.14; p = 0.02	0.25; p = 0.03	0.22; p = 0.02	0.10; p = 0.42

Each correlation model adjusted for age, gender, height, and socioeconomic status. Analyses with index values not adjusted for height. AA, African-American; EA, European-American; HA, Hispanic-American.

Table 4. Linear regression models of SBP association with each body fat measure by race/ethnicity

Variable		Total Sample		AA		EA		HA	
		β	*p*	β	*p*	β	*p*	β	*p*
Model 1	Fat Mass	3.94	<0.01	<0.01	0.013	<0.01	<0.01	<0.01	0.78
Model 2	Fat Mass Index	0.04	0.99	17.3	0.10	0.78	0.30	-0.80	0.38
Model 3	BMI	0.92	<0.01	1.10	<0.01	1.11	<0.01	0.74	0.08
Model 4	Weight Height Index	0.67	0.63	--	--*	7.01	0.24	--	--*
Model 5	Waist	0.29	<0.01	0.56	<0.01	0.37	<0.01	0.07	0.61
Model 6	Waist Height Index	3.83	<0.01	8.00	<0.01	4.67	0.02	1.02	0.62

Covariates = age, height, gender, socioeconomic status. Analyses with index values not adjusted for height. Total Sample models also control for ethnicity with EA as reference group

*overall model not significant at P<0.05. AA, African-American; EA, European-American; HA, Hispanic-American; BMI, body mass index.

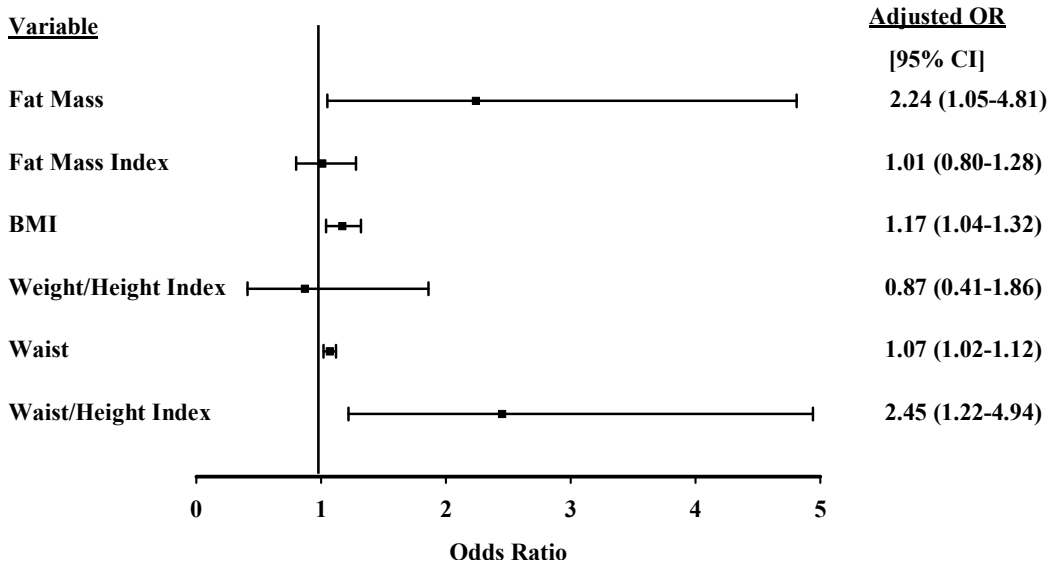

Figure 1. Adjusted odds ratio for risk of pre-hypertension (BP% 90-94th percentile) or hypertension (BP% $\geq 95^{th}$ percentile). Models adjusted for age, height, gender, ethnicity, and socioeconomic status. Analyses with index values not adjusted for height.

PHYSICAL FITNESS IS A STRONGER PREDICTOR THAN ACTIVITY LEVEL OF HYPERTENSION RISK IN A MULTIETHNIC PEDIATRIC POPULATION

by

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In preparation for *Hyp*

Format adapted for dissertation

ABSTRACT

Studies on the association of physical fitness and physical activity with pediatric hypertension risk are limited. We therefore evaluated whether fitness and activity were associated with blood pressure in 308 African-American, European-American, and Hispanic-American boys and girls aged 7-12 years. Blood pressure percentiles were calculated according to pediatric guidelines using the average of four measurements. Physical fitness was measured via indirect calorimetry, and accelerometry utilized to assess physical activity levels over 7 days. Estimates of African, Amerindian, and European genetic admixture were obtained through the evaluation of 140 ancestry informative markers (AIMs). Approximately 15% of participants were classified as having prehypertension or hypertension. After adjusting for age, female sex, waist ratio, African admixture, and Amerindian admixture, physical fitness, but not activity was inversely associated with systolic blood pressure ($r = -0.25$, $P < 0.01$). When evaluating the association of fitness and activity with hypertension incidence separately, only low physical fitness was associated with increased hypertension incidence (odds ratio: 2.63 [95% CI: 1.18 to 5.86]). Again, when both terms were included in the model, low physical fitness, but not activity, was associated with higher hypertension incidence (odds ratio: 3.04 [95% CI: 1.18 to 7.82]). Additionally African genetic admixture was positively associated with hypertension incidence in all models ($P < 0.01$). Physical activity may influence blood pressure levels through its effects on fitness or body compostion. However, physical fitness alone is associated with pediatric hypertension risk in the current investigation.

INTRODUCTION

The prevalence of hypertension has increased among children in recent decades, and several studies have noted an association of both increased physical fitness and physical activity with lower risk of developing hypertension among adults.[1-4] However, few studies have investigated these relationships among children. A negative association of physical activity with blood pressure has been noted in some, but not all, studies.[3, 5-7] This may be partly due to the use of questionnaires to measure pediatric activity levels. Self-reported physical activity data is reported to have a larger degree of measurement error compared to more objective measures of activity assessment, such as accelerometry.[8, 9] Use of a valid and reliable physical activity measure among children may allow for more precise assessment of the association between physical activity and pediatric hypertension risk

An association of decreased blood pressure with higher physical fitness has also been identified in adults and children.[4, 10] Physical fitness is typically measured in children as peak oxygen consumption ($VO_{2\text{-peak}}$) or submaximal oxygen consumption at a heart rate of 170 beats per minute ($VO_{2\text{-}170}$) during exercise. This value represents a measure of how well the body is able to transport and utilize oxygen during prolonged physical activity. Fitness is therefore influenced by multiple behavioral and genetic factors, including vigorous physical activity.[11, 12] However, it is unknown whether high levels of moderate physical activity alone are adequate to reduce the risk of pediatric hypertension, or if only vigorous activity levels that can improve physical fitness are sufficient for hypertension risk reduction. It is thus important to distinguish between the contributions of activity and fitness to pediatric hypertension risk to determine which

interventions may be most useful to prevent high blood pressure in children. We hypothesized, that similar to adults, fitness would present a stronger association than activity with blood pressure in a cross-sectional sample of 308 boys and girls. We evaluated the relationship of objective measures of physical fitness ($VO_{2\text{-}170}$) and activity (accelerometry), with both continuous measures of blood pressure and rates of prehypertension/hypertension.

METHODS

Subjects

Participants were 308 children (47.6% female) aged 7-12 years and identified by the parents/guardians as African-American (n = 106), European-American (n = 120), or Hispanic-American (n = 82). Children were recruited from the Birmingham, Alabama area via fliers, newspaper advertisements, and community presentations to study the effects of genetic and environmental parameters on racial/ethnic differences in metabolic outcomes. Exclusion criteria included diagnoses of type1 or type 2 diabetes, any glucose or lipid disorders, or use of any medication known to affect body composition or metabolism. Only children with a pubertal status ≤ 3 (determined by physician exam) according to the criteria of Marshall and Tanner were included.[13-15] The study was approved by the University of Alabama at Birmingham (UAB) Institutional Review Board for Human Use, and each child and parent provided informed consent prior to participation.

Protocol

All data was collected during visits to the UAB General Clinical Research Center (GCRC) and the Department of Nutrition Sciences between 2004 and 2008. Data were

obtained at two study visits no more than 30 days apart. During the first outpatient visit, pubertal status and body composition were measured and physical fitness assessed. Children then wore an accelerometer for 7 days to measure physical activity. At the second visit, participants were admitted to the UAB GCRC at approximately 1730 hours for an overnight stay, with blood pressure measurements obtained in the evening and morning.

Blood pressure

Four blood pressure measurements were taken using an automated pediatric blood pressure cuff (Dinamap Pro 200, GE Medical Systems). Two measurements were obtained at approximately 1800 hours during the overnight visit. Two additional measurements were taken at approximately 0700 hours the following morning. Blood pressure was recorded after a minimum 10 minutes of seated rest. A five-minute rest separated the first and second measurements. There was no significant difference in the classification of hypertension between evening and morning blood pressure measurements; thus, the four values were averaged to obtain a single systolic/diastolic measurement. Blood pressure percentile (BP%) was calculated using blood pressure tables developed by the National High Blood Pressure Education Program Working Group on High Blood Pressure in Children and Adolescents.[16] Children were classified as normotensive (systolic or diastolic BP% < 90^{th} percentile), pre-hypertensive (BP% 90-94^{th} percentile) or hypertensive (BP% $\geq 95^{th}$ percentile).

Body Composition

Body weight was measured to the nearest 0.1 kg in light clothing without shoes (Scale-tronix 6702W; Scale-tronix, Carol Stream, IL). Height was determined without

shoes using a mechanical stadiometer. Body mass index (BMI; weight kg / height m^2) was calculated from these values, and BMI percentile determined using age- and sex-specific CDC growth charts (http://apps.nccd.cdc.gov/dnpabmi/). Waist circumference was measured by the same registered dietitian using a flexible tape measure (Gulick II; Country Technology, Inc., Gays Mills, WI) as described by Lohman et al. and recorded to the nearest 0.1 cm.[17] We previously determined that the waist/height index was the body composition measure most associated with blood pressure and computed this measure as previously described.[18]

Physical Fitness

$VO_{2\text{-}170}$ was determined by indirect calorimetry on a treadmill with participants in the post-prandial state for a minimum of 3 hours. Children were given the opportunity to familiarize themselves with the treadmill prior to beginning the test. Participants then walked on a flat incline 2.5 mph for 4 minutes. Speed was then increased to 3 mph for the remainder of the test, with the incline increasing 2% every 2 minutes until the child reached a heart rate greater than 170 beats/minute. Heart rate (HR) was monitored with the Polar Vantage XL HR monitor (Polar Beat, Port Washington, NY). Volumes of O_2 and CO_2 were measured continuously using open circuit spirometry until recording the VO_2 level at a heart rate of 170 beats per minutes using a Max-II metabolic testing system (PHYSIO-DYNE, Quogue, NY). Data were expressed as $VO_{2\text{-}170}$ adjusted for body weight ($mL \cdot kg^{-1} \cdot min^{-1}$).

Physical Activity

Children wore the MTI Actigraph accelerometer (GT1M, ActiGraph Health Services, Pensacola, FL) for 7 days to objectively measure physical activity levels.

Epoch length was set at one minute and data expressed as counts per minute (counts min^{-1}). Children wore the monitor on an elastic belt at the waist over the right hip, with removal only occurring during activities such as sleeping, bathing and swimming. Actigraph monitors have been shown to exhibit a high degree of inter-instrument reliability.[19] Daily counts per minute were summed and analyzed as the average time spent in moderate and vigorous physical activity (MVPA).

Genotyping

Genotyping of the ancestry informative markers (AIMs) for the measurement of genetic admixture was performed at Prevention Genetics (www.preventiongenetics.org) using the Chemicon Amplifuor SNPs Genotyping System coupled with ArrayTape technology (www.global-array.com). A panel of 140 ancestry informative markers was used to estimate the genetic admixture proportion of each subject. Molecular techniques for the allelic identification and methodology for genetic admixture application have been described elsewhere and information regarding marker sequences, experimental details, and parental population allele frequencies has been submitted to dbSNP (http://www.ncbi.nlm.nih.gov/SNP/) under the handle PSU-ANTH.[20] The ancestry estimates from AIMs was translated into estimates of African, European, and Native American admixture for each subject using maximum likelihood estimation based on the ML algorithm described by Hanis et al.[21, 22] In brief, the maximum likelihood method estimates the proportion of genetic ancestry for an individual, using a range of proportions from 0 to 1 and identifies the most probable value of admixture based on the observed genotypes.

Socioeconomic Status

Socioeconomic status (SES) was measured with the Hollingshead 4-factor index of social class, which combines the educational attainment and occupational prestige for the number of working parents in the child's family.[23] Scores ranged from 8 to 66, with the higher scores indicating higher theoretical social status.

Statistical Analysis

Patient characteristics were analyzed by blood pressure category (normotensive, prehypertensive, hypertensive) using analysis of variance with Tukey's post-hoc test. The association of physical fitness and physical activity with continuous blood pressure was investigated using Partial Pearson correlations adjusted for age, female sex, waist ratio, African admixture, and Amerindian admixture. A stronger relationship with body weight, cardiac outcomes, and adult hypertension is reported for pediatric systolic blood pressure (SBP); hence, when analyzing blood pressure as a continuous variable we utilized absolute SBP measures. Additionally, the sum total of the three admixture estimates is equal to 1; therefore, European admixture was used as the reference group and only African and Amerindian admixture were included in all statistical models.

Quartiles of physical fitness and activity were computed (quartile 1 = lowest level of fitness or physical activity), and the proportion of participants with prehypertension or hypertension determined by fitness and activity level. Logistic regression was used to evaluate the association of fitness and activity with incident hypertensionrisk. Four models were analyzed with odds ratios (OR) and 95% confidence intervals (CI) computed for each variable. The first model included participant characteristics and body

composition, including age, female sex, waist ratio, SES, African admixture, and Amerindian admixture. The second model included physical fitness, testing the association of low fitness (quartile 1 versus others) with hypertension incidence. The third model tested the association of low physical activity (quartile 1 versus others) with hypertension incidence. The fourth model included both physical fitness and physical activity. All analyses were performed using SAS version 9.2 (SAS Institute, Inc., Cary, NC), with significance determined at $P < 0.05$.

RESULTS

Patient characteristics are presented in Table 1. There were no significant differences in sex, pubertal status, or body fat among participants by blood pressure category. Children with hypertension had higher levels of African admixture compared to children classified as normotensive ($P < 0.05$). Both systolic and diastolic blood pressure were significantly higher in the prehypertensive and hypertensive groups ($P < 0.01$), while there was a trend for lower physical activity levels in these participants compared to children classified as normotensive ($P = 0.057$). When prevalence of prehypertension and hypertension was computed by quartiles of physical fitness, 24.6% of participants in the lowest quartile of fitness had prehypertension or hypertension, compared to only 10%, 13.6%, and 6.4% of participants in fitness quartiles 2, 3, and 4, respectively (Figure 1a). However, when prehypertension and hypertension incidence were compared among physical activity quartiles, there were no differences in proportions among quartiles (Figure 1b).

When the relationships between physical fitness and activity with SBP were evaluated, physical fitness was negatively associated with SBP ($r = -0.25$, $P < 0.01$;

Figure 2). However, no association of physical activity with SBP was observed (r = -0.03, $P = 0.71$). The results of each logistic regression model are presented in Table 2. African admixture was positively associated with hypertension incidence in all models ($P < 0.01$). After adjusting for covariates, low physical fitness was associated with increased hypertension risk (OR 2.63, [95% CI 1.18,5.86]); however, there was no significant association of low physical activity with hypertension incidence. When both terms were included in the model, low fitness remained associated with increased hypertension risk (OR 3.04, [95% CI 1.18,7.82]), while no association of low activity with hypertension was noted.

DISCUSSION

The results of this cross-sectional investigation suggest that physical fitness, rather than physical activity level, is inversely associated with pediatric blood pressure and hypertension risk. Despite the increasing prevalence of increased blood pressure among children, few studies have evaluated factors outside of body composition that could impact hypertension risk. Those studies that have investigated the association of physical fitness or activity with pediatric hypertension have often evaluated pediatric blood pressure as a secondary outcome of cardiovascular risk or metabolic syndrome analyses.[10, 24] Additionally, less precise measures of both fitness and activity may be used, which can impact study findings. Our use of accelerometry to measure physical activity may have minimized the measurement error that can often result when using self-report questionnaires. However, the average amount of daily physical activity for most participants in this study was below the recommended average of 60 minutes a day for children(http://www.cdc.gov/physicalactivity/everyone/guidelines/index.html) and it is

possible that an association of activity with blood pressure might have been observed if the children had been more physically active.

Additionally, many of our participants had low levels of vigorous physical activity, and we were unable to independently test the association of vigorous activity levels with blood pressure or fitness. Vigorous activity is associated with increases in physical fitness levels; hence vigorous physical activity could be associated with blood pressure independently or through its effects on physical fitness levels.[2, 3, 12] Investigations that include children with higher levels of physical activity as measured by accelerometry could elucidate the potential interactions between activity, fitness, and pediatric hypertension.

Other studies that have shown an association of physical activity with blood pressure among children have reported the association as part of a weight loss intervention, making it difficult to determine whether improvements in blood pressure were due to the physical activity or its effects on body composition. Farpour-Lambert *et al.* reported significant improvements in blood pressure levels among children involved in a 3-month physical activity intervention.[25] However, the children also experienced improvements in anthropometric measures, and it is unclear whether physiological improvements in blood pressure control were due to the effects of increased activity or decreased body fat. We have previously identified an association of higher waist circumference with hypertension risk and controlled for this measure in the current study.[18] However, no association of body composition with blood pressure was found among our participants. It is possible that changes in central adiposity over time or physical activity measured at multiple time points in a longitudinal study-design could

impact blood pressure levels. Interestingly, when Carnethon and colleagues evaluated the association of both physical fitness and adiposity with hypertension risk in adults over a 20-year period after adjusting for BMI, they noted an association of increased physical activity with lower hypertension risk.[3] However, this association was no longer significant when physical fitness was included in the models. Consistent with our findings, they noted an independent association of fitness with decreased hypertension risk even after controlling for physical activity levels. It is thus possible that physical activity interventions to prevent hypertension may be most effective if the activity level is sufficient to improve physical fitness. Longitudinal investigations among children are warranted to determine if physical activity over time influences hypertension risk, or if similar to the findings of Carnethon et al, physical fitness is more strongly associated with lower blood pressure.[3]

We also noted a positive association of African genetic admixture with increased hypertension risk, which is consistent with studies showing a higher prevalence of elevated blood pressure among African-American compared to European-American adults and children.[1, 26] Additionally, genetic polymorphisms are associated with differences in physical fitness levels among individuals; hence, the relationship between blood pressure and fitness could be mediated by a shared genetic/biological pathway.[11] The use of racial/ethnic group to classify study participants may confound the actual associations of environmental factors with chronic disease risk, as race/ethnicity represents a social (rather than biological) construct that is typically based on unique cultural and behavioral practices that could affect metabolic pathways and disease risk independent of self-classified race. In the Americas, European and African immigrants

comingled with the Amerindian indigenous population to produce an admixed population. Hence individuals that may classify themselves within the same racial/ethnic background may share similar cultural experiences that impact factors such as physical activity, yet these individuals may have greatly differing levels of ancestral genetic background that could influence chronic disease risk.[27] Our use of genetic admixture allowed us to evaluate the association of biological factors with blood pressure independent of environmental factors such as physical activity and SES that may be influenced by culture. We were most likely unable to account for all of the genetic factors that influence blood pressure and fitness. However, the fact that racial ancestry remained associated with hypertension risk even after controlling for other environmental factors suggests that additional studies are needed to understand the racial disparities in hypertension prevalence.

Our study benefited from the use of more precise measurements of physical activity and physical fitness than used in many studies that evaluate factors associated with pediatric hypertension. We were limited by the use of four blood pressure measurements obtained during a single 24-hour period. Hypertension is typically diagnosed from measurements obtained during multiple time points or from 24-hour ambulatory blood pressure monitoring. However, multiple measurements obtained during one day can be useful to evaluate population trends in hypertension risk. Additionally, we lacked information on family history of hypertension risk, which could be a surrogate measure of genetic risk for high blood pressure, though the measurement of genetic admixture allowed us to control for the independent effects of racial ancestry on hypertension risk in a multiethnic population.

In summary, greater physical fitness is associated with decreased hypertension risk among boys and girls ages 7-12. Longitudinal pediatric studies are needed to confirm these associations and evaluate the potential interactive effects of fitness and activity on blood pressure; however, our results suggest that physical activity interventions may have a greater impact on blood pressure levels if the activity levels are sufficient to improve pediatric physical fitness.

ACKNOWLEDGMENTS

The authors would like to thank Robert Petri and Alexandra Luzuriaga-McPherson for their assistance with data collection. This work was funded by the National Institutes of Health grants R01-DK067426, P30-DK56336, P60-DK079626, M01-RR-00032, and the National Cancer Institute Cancer Prevention and Control Training Program CA47888.

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Table 1. Population characteristics

Variable	Total Sample	Normotensive	Prehypertensive	Hypertensive
n	308	263	19	26
Age (years)	9.6 ± 1.6	9.6 ± 1.6	9.0 ± 1.6	9.2 ± 1.4
Sex (n, % female)	147 (47.6)	124 (47.2)	10 (52.6)	13 (50.0)
Tanner Stage	1.5 ± 0.7	1.4 ± 0.7	1.6 ± 0.8	1.4 ± 0.8
Height (cm)	139.5 ± 10.6	139.8 ± 10.6	137.5 ± 9.4	137.6 ± 12.1
Weight (kg)	36.7 ± 9.6	36.6 ± 9.2	36.5 ± 9.4	36.9 ± 13.0
BMI	18.6 ± 3.0	18.5 ± 2.9	19.0 ± 2.5	19.0 ± 3.9
BMI percentile	66.5 ± 26.0	65.5 ± 26.1	78.4 ± 18.9	67.0 ± 28.5
Waist (cm)	64.5 ± 9.0	64.4 ± 8.7	64.8 ± 7.2	64.3 ± 12.3
Genetic Admixture:				
European (%)	53.1 ± 37.6	54.8 ± 37.5	50.9 ± 38.0	38.5 ± 36.2
African (%)	28.9 ± 37.5	25.9 ± 36.3[a]	37.4 ± 40.2[ab]	51.7 ± 38.7[b]
Amerindian (%)	17.9 ± 25.3	19.3 ± 26.1	11.7 ± 20.5	9.8 ± 17.8
SES	38.9 ± 14.6	39.1 ± 14.7	32.8 ± 13.1	40.4 ± 13.6
Systolic BP (mmHg)	103.3 ± 10.6	100.6 ± 8.6[a]	113.6 ± 7.7[b]	122.2 ± 5.8[c]
Diastolic BP (mmHg)	60.1 ± 6.5	58.9 ± 5.8 [a]	63.5 ± 6.2 [b]	68.8 ± 6.9 [c]
$VO_{2\text{-}170}$ (ml/kg/min)	29.6 ± 6.0	30.0 ± 6.0	27.8 ± 6.9	27.1 ± 5.2[†]
MVPA (min/d)	59.2 ± 34.2	58.8 ± 34.4	54.6 ± 27.7	62.7 ± 36.9

[a,b,c] superscripts indicate group difference at $P < 0.05$; † = trend for significance at $P = 0.057$; BMI = body mass index (weight kg/height m^2); RMR = resting metabolic rate; SES = socioeconomic status; MVPA = moderate/vigorous physical activity

Table 2. Logistic regression testing associations of physical fitness and activity with hypertension risk (odds ratio [95% confidence intervals])

	Model 1	Model 2	Model 3	Model 4
Age	**0.79[0.64,0.98]**	0.83[0.64,1.07]	0.85[0.65,1.10]	0.86[0.63,1.17]
Female	1.15[0.59,2.24]	1.66[0.76,3.65]	1.20[0.54,2.64]	1.74[0.70,4.29]
WaistRatio	1.78[0.93,3.41]	1.21[0.57,2.55]	1.65[0.77,3.56]	1.21[0.52,2.84]
SES	1.01[0.98,1.04]	1.01[0.98,1.04]	1.01[0.98,1.05]	1.02[0.98,1.05]
Af. Adm.	**5.71[2.29,14.19]**	**4.63[1.66,12.88]**	**6.08[2.06,17.95]**	**6.40[1.97,20.83]**
Low Fit		**2.63[1.18,5.86]**	--	**3.04[1.18,7.82]**
Low Act			0.71[0.26,1.90]	0.58[0.19,1.80]

Bolded values significant at $P < 0.05$; SES = socioeconomic status; Af. Adm. = African admixture; Low Fit = low physical fitness; Low Act = low physical activity

Figure 1. Percentage of participants with hypertension by fitness and activity quartiles (white bars = normotension; gray bars = prehypertension; black bars = hypertension).

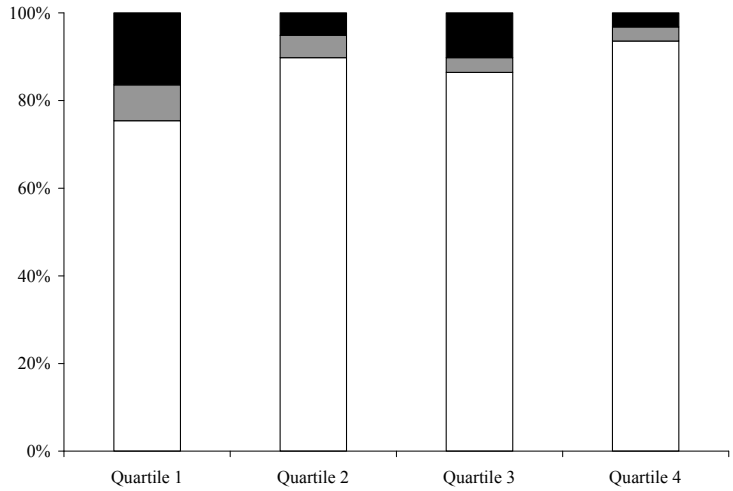


Figure 1a. Hypertension by physical fitness quartile

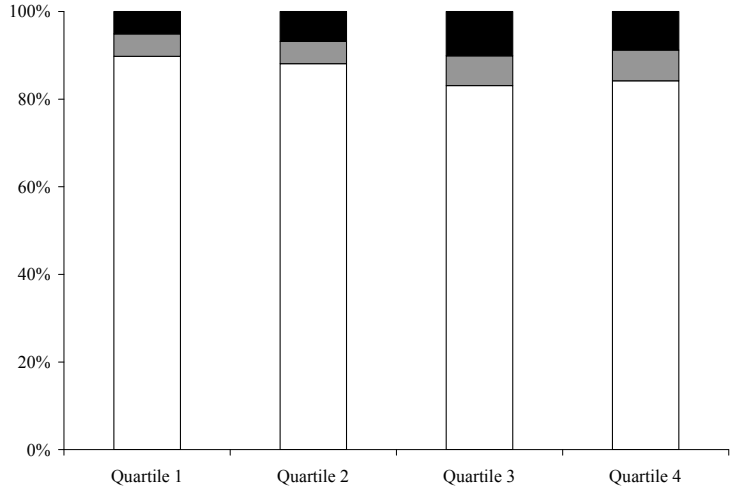
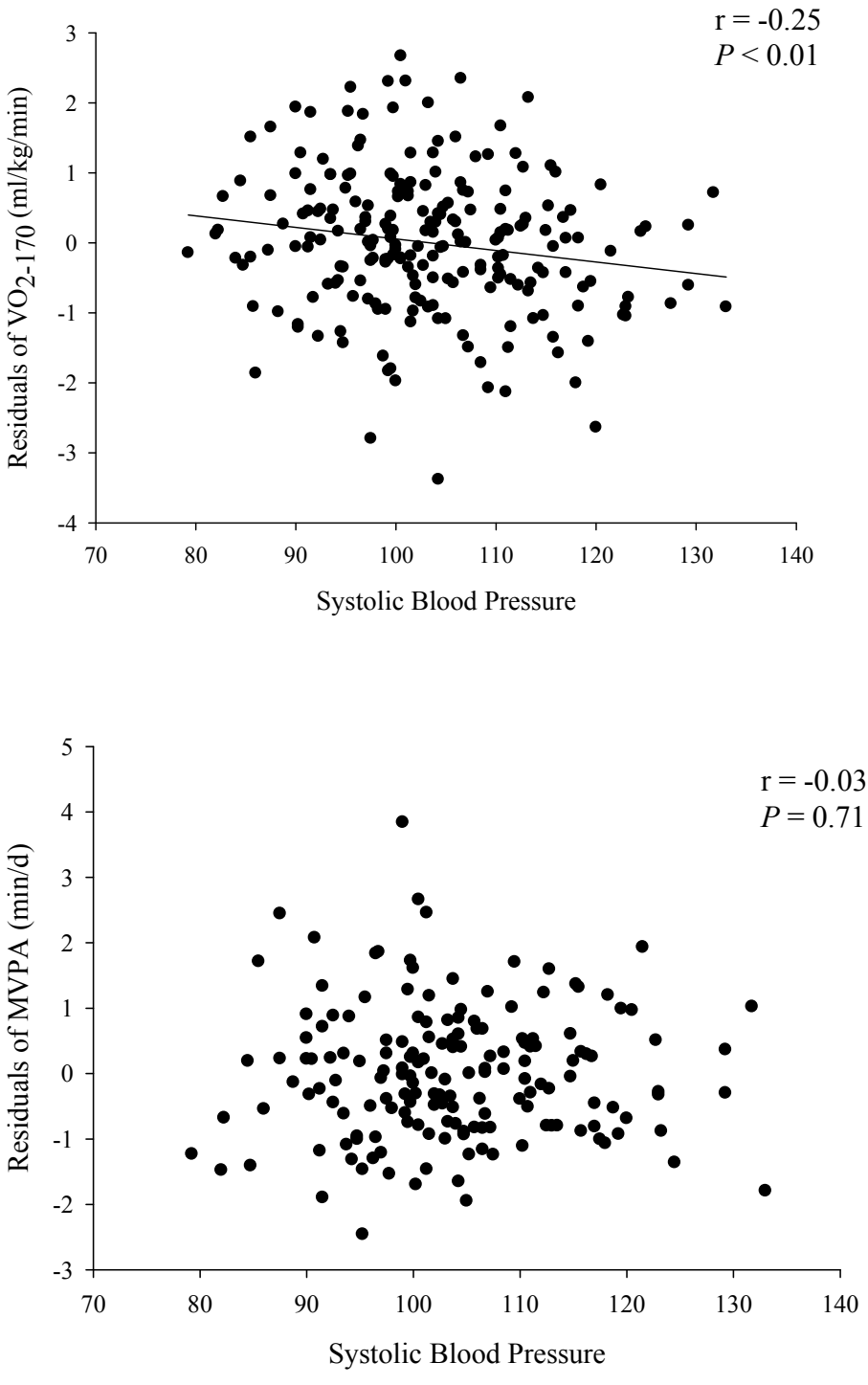


Figure 1b. Hypertension by physical activity quartile

Figure 2. Parital Pearson correlations of physical fitness and activity variables with systolic blood pressure (mmHg)



MVPA = moderate/vigorous physical activity; models adjusted for age, sex, waist ratio, African and Amerindian admixture

BODY FAT AND RACIAL GENETIC ANCESTRY INFLUENCE PHYSICAL FITNESS LEVELS IN A MULTIETHNIC PEDIATRIC POPULATION

by

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In preparation for *Int J Ped Obes*

Format adapted for dissertation

ABSTRACT

Low physical fitness is associated with increased chronic disease risk factors in children. Fitness is often lower in individuals considered overweight/obese, and among African-American children. We evaluated the relationship of physical fitness with more precise measures of body fat and racial ancestry in a sample of 232 children self-identified as AA (n=75), EA (n=90), or HA (n=67) aged 7-12 years. Physical fitness was measured via a submaximal indirect calorimetry treadmill test ($VO_{2\text{-}170}$). Body mass index (BMI) was computed as weight (kg)/height (m^2). Fat mass was determined using dual-energy x-ray absorptiometry, and children classified into a low body fat group or a high body fat group based on their percent body fat. Genetic admixture estimates were obtained using 140 genetic markers informative for European, African, and Amerindian ancestry. Physical activity was measured via accelerometry for seven days. The percentage of children with low versus high body fat was compared by BMI category (normal weight, overweight, obese). Analysis of covariance and linear regression models were used to evaluate the associations of body composition, genetic admixture, and physical activity with physical fitness after controlling for pubertal status, sex, lean mass, and fat mass. Among children classified as normal weight, 12% actually had high levels of body fat, while 49% of overweight and 9% of obese participants had low body fat and higher fitness levels than those with high body fat classified as overweight. Regression analysis also showed a positive association of physical activity and a negative association of African admixture with physical fitness. These results suggest that physical fitness in children with low body fat who are classified as overweight may be higher than in children considered normal weight but with high body fat, and indicated that physical activity and genetic admixture are significantly associated with fitness in children.

INTRODUCTION

High physical fitness has been associated with decreased risk for several chronic diseases in both children and adolescents.[1-3] Higher body weight is associated with lower physical fitness due to independent contributions of lean and fat mass, with greater fat mass being negatively associated with fitness.[4, 5] Pediatric studies often use gender- and age-adjusted body mass index (BMI; weight [kg]/height [m^2]) percentiles (%) when designing interventions to increase pediatric physical fitness, in which a BMI $\geq 85^{th}$ % is considered overweight, and BMI $\geq 95^{th}$ % categorized as obese.[6] However, a higher BMI is often due to greater amounts of both lean and fat mass, and the actual contribution of excess adiposity to physical fitness may be masked if BMI cut points are used to estimate excess adiposity.[7, 8] Hence, more precise measures of pediatric body fat are needed to accurately assess the relationship between physical fitness and adiposity.

Recent investigations have also identified racial/ethnic differences in physical fitness, as well as in the relationship between BMI and body fat.[5, 9-12,] Racial/ethnic differences in fitness as measured by maximum oxygen consumption ($VO_{2\text{-max}}$) among African-American (AA) and European-American (EA) children and adults are well documented, with a lower $VO_{2\text{-max}}$ in AA regardless of body weight or training status.[9, 10] Other studies have indicated that Hispanic-American (HA) children may present with physical fitness levels similar to or lower than that of EA children.[11, 12] Although the etiology of these racial/ethnic differences is not completely understood, they may be influenced in part by cultural and behavioral differences in the habits and perceptions related to factors such as physical activity.

It was also recently reported that the traditional cut points for overweight and obese classification may represent different levels of body fat at the same BMI or BMI percentile in AA and HA children and adults.[13, 14] For example, an AA child at the 85th% for BMI may have low body fat levels, while an HA child may present with excess body fat at a BMI < 85th%. These observed racial/ethnic differences in fat mass accrual may respond to cultural, behavioral and genetic factors. When exploring biological determinants of health, genetic admixture has been used to explain individual variability in metabolic outcomes.[15-18] Use of genetic admixture in pediatric fitness studies could allow for more accurate analyses of which factors are associated with higher physical fitness. We analyzed a multiethnic cohort of 232 boys and girls aged 7-12 to determine whether physical fitness levels differed depending on classification by BMI or percent body fat, and whether genetic admixture influences physical fitness levels independent of body fat.

METHODS

Subjects

Two-hundred and thirty-two children self-identified as AA (n=75), EA (n=90), or HA (n=67) aged 7-12 years were recruited from the Birmingham, Alabama area to investigate genetic and environmental contributions to racial/ethnic differences in metabolic outcomes. The children were pubertal stage ≤ 3 as assessed by a qualified pediatrician according to the criteria of Marshall and Tanner.[19-21] Exclusion criteria included diagnoses of type1 or type 2 diabetes, any glucose or lipid disorders, or use of any medication known to affect body composition or metabolism. Before participating in the study, the nature, purpose, and potential risks were carefully explained to the parents

and children. The children and parents provided informed consent to the protocol, which was approved by the Institutional Review Board for human subjects at the University of Alabama at Birmingham (UAB).

Protocol

All measurements for this analysis were performed at the UAB Department of Nutrition Sciences (DNS) between 2004 and 2008. For this protocol, participants arrived at the DNS at 1600h. A physician conducted a complete medical history and physical exam, during which pubertal status was assessed. Body composition, physical fitness measurements, and demographic information including socioeconomic status were also obtained. The children then wore an accelerometer for seven days to capture physical activity data.

Body Composition

Body weight was measured to the nearest 0.1 kg in light clothing without shoes (Scale-tronix 6702W; Scale-tronix, Carol Stream, IL). Height was determined without shoes using a mechanical stadiometer. Body mass index (BMI; weight kg / height m^2) was calculated from these values, and BMI percentile determined using age- and sex-specific CDC growth charts (http://apps.nccd.cdc.gov/dnpabmi/).

Total fat mass and lean mass were evaluated via dual energy x-ray absorptiometry (DXA) with a GE Lunar Prodigy densitometer (Lunar Radiation Corp., Madison, WI). Subjects were scanned in light clothing while lying flat on their backs with arms at their sides. DXA scans were analyzed with pediatric software enCORE 2002 version 6.10.029. Children were separated into two body fat groups according to the criteria established by Williams *et al* for a multiethnic population [22]. All children meeting the criteria for

normal body fat levels (boys with < 25% and girls with < 30% body fat) were placed into the Low Body Fat Group (n = 164), whereas children exceeding those parameters were placed into the High Body Fat Group (n = 68).

Physical Fitness

$VO_{2\text{-}170}$ was determined by indirect calorimetry on a treadmill with participants in the post-prandial state for a minimum of 3 hours. Children were given the opportunity to familiarize themselves with the treadmill prior to beginning the test. Participants then walked on a flat incline 2.5 mph for 4 minutes. Speed was then increased to 3 mph for the remainder of the test, with the incline increasing 2% every 2 minutes until the child reached a heart rate greater than 170 beats/minute. Heart rate (HR) was monitored with the Polar Vantage XL HR monitor (Polar Beat, Port Washington, NY). Volumes of O_2 and CO_2 were measured continuously using open circuit spirometry until recording the VO_2 level at a heart rate of 170 beats per minutes using a Max-II metabolic testing system (PHYSIO-DYNE, Quogue, NY). Data were analyzed as described by Gutin *et al*. [23] and expressed as absolute $VO_{2\text{-}170}$ (L/min) and $VO_{2\text{-}170}$ adjusted for body weight ($mL \cdot kg^{-1} \cdot min^{-1}$). Additionally, it is possible that adjusted $VO_{2\text{-}170}$ by body weight mathematically penalizes heavier individuals who may have more lean mass; hence we also present $VO_{2\text{-}170}$ adjusted by total fat-free mass ($mL \cdot kgFFM^{-1} \cdot min^{-1}$).

Physical Activity

Children wore the MTI Actigraph accelerometer (GT1M, ActiGraph Health Services, Pensacola, FL) for 7 days to objectively measure physical activity levels. Epoch length was set at one minute and data expressed as counts per minute (counts min^{-1}). Children wore the monitor on an elastic belt at the waist over the right hip, with

removal only occurring during activities such as sleeping, bathing and swimming. Actigraph monitors have been shown to exhibit a high degree of inter-instrument reliability.[24] Daily counts per minute were summed and analyzed as the average time spent in moderate and vigorous physical activity (MVPA).

Genetic admixture

Genotyping of the ancestry informative markers (AIMs) for the measurement of genetic admixture was performed at Prevention Genetics (www.preventiongenetics.org) using the Chemicon Amplifuor SNPs Genotyping System coupled with ArrayTape technology (www.global-array.com). A panel of 140 ancestry informative markers was used to estimate the genetic admixture proportion of each subject. Molecular techniques for the allelic identification and methodology for genetic admixture application have been described elsewhere and information regarding marker sequences, experimental details, and parental population allele frequencies has been submitted to dbSNP (http://www.ncbi.nlm.nih.gov/SNP/) under the handle PSU-ANTH.[16] The ancestry estimates from AIMs was translated into estimates of African, European, and Native American admixture for each subject using maximum likelihood estimation based on the ML algorithm described by Hanis et al.[15, 25] In brief, the maximum likelihood method estimates the proportion of genetic ancestry for an individual, using a range of proportions from 0 to 1 and identifies the most probable value of admixture based on the observed genotypes.

Socioeconomic Status

Socioeconomic status (SES) was measured with the Hollingshead 4-factor index of social class, which combines the educational attainment and occupational prestige for

the number of working parents in the child's family.[26] Scores ranged from 8 to 66, with the higher score indicating higher theoretical social status.

Statistical Analysis

Differences in descriptive statistics between low and high body fat groups were explored with paired *t*-tests. The percentage of children within each BMI category (normal weight, overweight, obese) who had low body fat versus high body fat were also computed. Analysis of covariance (ANCOVA) was then used to evaluate the difference in physical fitness within each BMI category for low- versus high-body fat groups. ANCOVA analysis was not completed for participants in the obese group due to small sample size in the obese/high fat group. Models evaluating differences in $VO_{2\text{-}170}$ were controlled for lean mass, fat mass, pubertal status (Tanner stage), sex, MVPA, SES, and genetic admixture. Models evaluating differences in $VO_{2\text{-}170}$/kg body weight and $VO_{2\text{-}170}$/kg lean mass were controlled for pubertal status (Tanner stage), sex, MVPA, SES, and genetic admixture. The sum total of the three admixture estimates is equal to 1; therefore, European admixture was used as the reference group and only African and American admixture were included in the models. Linear regression was then utilized to analyze the association of body composition variables and genetic admixture in the total sample and within body fat groups after also controlling for the above covariates. All analyses were performed using SAS statistical software version 9.2 (SAS Institute, Cary, NC, 2002) with a significance level established at $P < 0.05$.

RESULTS

Table 1 presents the study population characteristics by body fat group. The high fat group present with a greater percentage of Amerindian admixture and slightly higher

pubertal stage ($P < 0.05$). Children in this group were also taller, had more fat mass and lean mass, and participated in less physical activity compared to the low fat group (all at $P < 0.01$). When analyzed by BMI percentile categories, approximately 12% of children classified as normal weight had high body fat (Figure 1). Conversely, approximately 49% of children classified as overweight and 9% classified as obese had low body fat levels.

Results of ANCOVA models by BMI category and body fat group are presented in Table 2. Participants at normal weight but with high body fat presented with higher $VO_{2\text{-}170}$ but lower $VO_{2\text{-}170}$/kg than those with low body fat ($P < 0.05$). There was no difference between the two groups in $VO_{2\text{-}170}$/kg lean mass. Similarly, participants classified as overweight but with low body fat had a higher $VO_{2\text{-}170}$/kg compared to those with high body fat. There was also a trend for greater $VO_{2\text{-}170}$/kg lean mass in participants classified as overweight but with low body fat.

When linear regression analysis was used to evaluate the association of covariates with $VO_{2\text{-}170}$, higher African Admixture was associated with lower physical fitness, while increased lean mass and MVPA were associated with higher fitness (both $P < 0.01$). Total fat mass and female sex were negatively associated with physical fitness only when MVPA was not included in the model (data not shown). When analyzed by body fat group, African admixture, lean mass and MVPA remained associated with $VO_{2\text{-}170}$ ($P < 0.01$) in participants with low body fat; however, only African admixture and lean mass were associated with $VO_{2\text{-}170}$ in the high fat group ($P < 0.05$). Results were consistent when physical fitness was analyzed as $VO_{2\text{-}170}$/kg of body weight and lean mass. Since MVPA was associated with physical fitness in the low fat but not the high fat group, a fat

mass x MVPA interaction was tested for in the total sample. However, no significant interaction was identified.

DISCUSSION

This investigation was conducted to evaluate the associations of body fat and racial genetic ancestry with pediatric physical fitness in boys and girls. We found that when BMI is used as a surrogate measure for excess adiposity in children, approximately 18% of participants with low levels of body fat were classified as overweight or obese, while 26% of participants with high body fat were classified as normal weight. Children with low body fat who were classified as overweight had greater physical fitness compared to those classified as overweight who had high body fat. Additionally, African admixture was associated with lower physical fitness and MVPA associated with higher fitness independent of additional covariates. However, gender and body fat were only associated with fitness when MVPA was not considered.

Several studies in children and adults have suggested that BMI may be an imprecise measure of body fat, which is of concern when evaluating the effect of adiposity on physical fitness.[13, 14] Romero-Corral and colleagues found that BMI was a poor indicator of percent body fat among men and women.[27] Similarly, Flegal et al. noted that less than half of children and adolescents who were classified as overweight actually had high levels of body fat.[14] This is consistent with our finding that half of the children categorized as overweight in this study actually had low body fat. Interestingly, when physical fitness in these two groups was compared, those with low body fat had higher fitness levels than children with high body fat. Children classified as low fat/overweight also had higher physical fitness than those who were classified as high

fat/normal weight. This suggests that when interventions are designed to improve physical fitness in children considered overweight/obese, estimation of body fat percent may be a more accurate indicator to determine which children would benefit from the intervention.

We also noted an association of African genetic admixture with lower physical fitness among the total sample and when analyzed within body fat groups. Previous studies that have used the racial/ethnic group classifications of African-American and European-American have reported lower physical fitness in African-American children and adults.[11, 28] Roy *et al* attributed ethnic differences in $VO_{2\text{-max}}$ between AA and EA women to reduced ability of the cardiovascular system to deliver oxygen to muscle and reduced mitochondrial function as measured by prolonged ADP time constants (measure of post-exercise recovery time).[9] This work suggests contributions to fitness aberrations by both lower oxygen carrying capacity and muscle oxidative aerobic capacity, and complements other findings of reduction in type I oxidative skeletal muscle fibers among AA women.[9, 29] Hence, it is likely that variations in fitness within our sample are at least partially attributable to inherent differences in muscle physiology that could interact with genetic background.

Race/ethnicity represents a social (rather than biological) construct that is typically based on unique cultural and behavioral practices that could affect metabolic pathways and disease risk independent of self-classified race. Many individuals in the United States, however, exhibit racial ancestry influenced by European, African, and Amerindian parental populations.[18, 30] The estimation of ancestral genetic proportions through genetic admixture elucidates biological rather than environmental variance

within individuals, and may therefore be a more appropriate measure of ancestral contributions to disease risk than self-identified racial/ethnic classification. Lohman et al. reported a fat mass x racial/ethnic group (African-American versus other) interaction effect on physical fitness in a multiethnic sample of eighth-grade girls.[5] Similarly, when we included race/ethnic group as a covariate in our model in place of genetic admixture, we noted a fat mass x racial/ethnic group interaction (data not shown). However, this interaction was not present when genetic admixture was included in the models. It is possible that the pubertal transition may have impacted adiposity in the eighth-grade girls that in turn influenced physical fitness levels. However, unmeasured environmental factors associated with racial/ethnic group, rather than African racial ancestry itself, could also account for the interaction with fat mass seen with racial/ethnic group but not genetic admixture estimates.

We did not detect an association of Amerindian admixture with physical fitness, though participants in the high body fat group had higher estimates of Amerindian ancestry. Among our participants, Amerindian ancestry was highest in those self-classified as Hispanic-American. Current literature regarding fitness levels in HA compared to EA and AA youth is equivocal. Shaibi and colleagues evaluated fitness differences by VO_2-$_{peak}$ in 73 early pubertal and pubertal normal- and overweight boys and girls. Both AA and HA groups had significantly lower fitness than EA children and adolescents.[11] Conversely, Treuth *et al* observed no differences in fitness between EA and HA girls.[12] Additional investigations are warranted to determine how Amerindian admixture and Hispanic-American culture may influence physical fitness levels.

We also noted that body fat and sex were only associated with physical fitness when physical activity (MVPA) was not included in statistical models. Participants with high body fat were significantly less physically active, suggesting that physical activity may mediate the relationship between adiposity and fitness. Investigations among adults have reported higher physical fitness in men compared to women, possibly due to a combination of physical activity, body composition, and hormonal differences.[31] Among children, physical fitness is also higher in boys compared to girls.[32] However, in early pubertal children this difference is less likely due to hormonal differences compared to physical activity levels. Other studies are in agreement that increased physical activity is associated with higher physical fitness levels.[5, 33]

Our study benefited from measurement of racial genetic ancestry in a multiethnic population with both boys and girls, and an increased precision of body composition measures using DXA. This study was limited by the use of a sample of HA children predominately of Central- and South American origin with higher levels of Amerindian ancestry. Hence, these results may not be applicable to HA children with origins outside those regions. We also determined aerobic fitness levels from a submaximal measure ($VO_{2\text{-}170}$), which may have lower validity than $VO_{2\text{-}peak}$ or $VO_{2\text{-}max}$ values. However, studies that have previously utilized $VO_{2\text{-}170}$ as a measure of pediatric physical fitness report similar outcomes to those that measure $VO_{2\text{-}peak}$ or $VO_{2\text{-}max}$.

In conclusion, these study findings suggest that body fat may be a more accurate way than BMI to classify children as normal weight or overweight when evaluating physical fitness. Additionally, use of genetic admixture confirmed the association of African ancestry with lower physical fitness that has been suggested in other studies.

ACKNOWLEDGMENTS

This work was funded by the National Institutes of Health grants R01-DK067426, P30-DK56336, P60-DK079626, M01-RR-00032, and the National Cancer Institute Cancer Prevention and Control Training Program CA47888.

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Table 1. Descriptive characteristics by body fat group (mean ± Std or n, %).

	Total Sample (n=232)	Low Body Fat (n=164)	High Body Fat (n=68)
Female Sex (n,%)	108 (46.6)	77 (46.7)	31 (46.3)
Race(AA/EA/HA)	75/90/67	58/73/33	17/17/34*
Tanner stage 3 (n,%)	32 (14.0)	20 (12.1)	11 (16.4)†
Age	9.6 ± 1.5	9.5 ± 1.6	9.9 ± 1.4
Height (cm)	139.5 ± 10.1	138.4 ± 10.2	142.2 ± 9.7*
Weight (kg)	36.7 ± 9.0	33.4 ± 6.4	44.7 ± 9.7*
BMI (kg/m^2)	18.6 ± 2.9	17.3 ± 1.6	21.9 ± 2.8*
BMI percentile	66.7 ± 25.4	57.8 ± 24.7	88.6 ± 8.2*
Fat-free mass (kg)	25.8 ± 5.1	25.3 ± 4.9	27.2 ± 5.3*
Fat mass (kg)	8.8 ± 5.4	6.1 ± 2.3	15.3 ± 5.1*
Body fat (%)	23.2 ± 9.0	18.5 ± 5.6	34.2 ± 5.5*
MVPA (min/day)	59.4 ± 34.1	66.2 ± 36.3	44.2 ± 23.2*
SES	38.8 ± 14.8	41.6 ± 14.0	33.2 ± 14.7*
Genetic Admixture:			
European	53.9 ± 37.3	55.9 ± 39.0	48.7 ± 32.9
African	27.4 ± 36.8	31.2 ± 38.7	19.3 ± 30.5†
Amerindian	18.7 ± 25.3	13.0 ± 20.7	31.9 ± 30.1*
$VO_{2\text{-}170}$ (L/min)	1.08 ± 0.30	1.04 ± 0.29	1.17 ± 0.32*
$VO_{2\text{-}170}$ (ml/kg/min)	29.68 ± 6.04	31.13 ± 5.86	26.33 ± 5.13*
$VO_{2\text{-}170}$ (ml/lean/min)	41.63 ± 7.29	41.14 ± 7.07	42.89 ± 7.82

† = $P < 0.05$; * = $P < 0.01$ for significant body fat group difference; BMI = Body Mass Index; MVPA = Moderate/Vigorous Physical Activity; SES = Socioeconomic Status

Table 2. Adjusted means ± SE of physical fitness by BMI percentile category and body fat group*

	Normal Weight		Overweight	
	Low Fat	High Fat	Low Fat	High Fat
n	92	11	28	29
$VO_{2\text{-}170}$ (L/min)	1.04±0.02	1.14±0.06*	1.16±0.06	1.12±0.05
$VO_{2\text{-}170}$ (ml/kg/min)	31.44±0.51	28.79±1.12†	32.70±1.02	26.16±0.82*
$VO_{2\text{-}170}$ (ml/lean/min)	41.39±0.60	44.13±1.76	45.33±1.45	42.50±1.35†

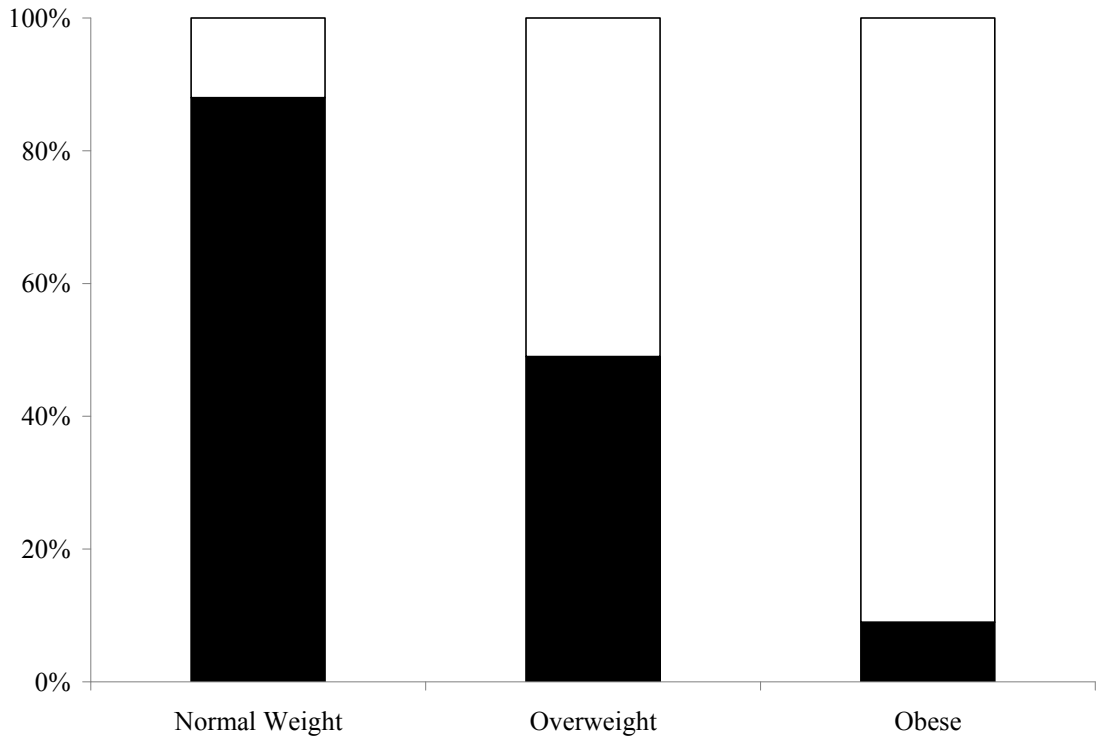
*Means adjusted for lean mass, fat mass, sex, pubertal status, moderate/vigorous physical activity, African and Amerindian admixture; SE = standard error; BMI = body mass index; * = $P < 0.05$; † = $P < 0.08$ trend for significance

Table 3. Regression models predicting physical fitness ($VO_{2\text{-}170}$; L/min).

	Total Sample		Low Body Fat		High Body Fat	
	β	*P*	β	*P*	β	*P*
Fat free mass	0.04	<0.01	0.88	<0.01	0.05	<0.01
Fat mass	-0.01	0.09	<0.01	0.89	-0.01	0.13
Gender (female)	-26.06	0.35	-0.04	0.20	16.12	0.81
Pubertal Status	-82.30	0.06	-0.01	0.84	-142.31	0.13
African Admixture	-213.06	<0.01	-0.03	<0.01	-238.10	0.04
Amerindian Admixture	113.90	0.10	0.01	0.20	162.66	0.24
MVPA	1.10	<0.01	0.05	<0.01	0.72	0.58
Socioeconomic status	-0.81	0.47	-0.08	0.03	1.65	0.50

MVPA = moderate/vigorous physical activity (min/d)

Figure 1. Percentage of children classified at low-fat and high-fat by BMI percentile category



Black bars = low fat group; white bars = high fat group

BRIEF REPORT: GENETIC ADMIXTURE INFLUENCES THE RELATIONSHIP BETWEEN PEDIATRIC HYPERTENSION AND NONESTERIFIED FATTY ACIDS

by

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In preparation for *J App Physiol*

Format adapted for dissertation

ABSTRACT

A positive association has been identified between nonesterifed fatty acids (NEFA) and blood pressure levels among adults. Studies suggest that individuals with hypertension have higher fasting NEFA levels compared to normotensive individuals. However, few studies have investigated this association among children or included participants from racial/ethnic minority groups. The objective of this study was to evaluate the association of fasting NEFA levels with hypertension prevalence among a multiethnic pediatric samples aged 7-12 years. Blood pressure was measured during the evening and morning of an overnight stay, and hypertension prevalence determined using calculated blood pressure percentiles. Fasting NEFA levels were measured with a colorimetric assay, and genetic admixture determined using 140 ancestry informative markers. Tertiles of European admixture were then computed and linear regression models used to evaluate the association of hypertension and racial ancestry with NEFA levels. Average NEFA did not differ by level of European admixture or hypertension status in the total sample. However, an interactive effect of admixture and hypertension on NEFA was identified, such that individuals with hypertension who were in the lowest tertile for European admixture presented with significantly lower fasting NEFA compared to individuals with hypertension and higher levels of European ancestry ($P < 0.02$). These results suggest that factors associated with racial ancestry impact the association between NEFA levels and hypertension.

INTRODUCTION

Recent investigations have indicated that elevated levels of nonesterified fatty acids (NEFA) are associated with increased risk for hypertension.[1, 2] Significant acute increases in systolic and diastolic blood pressure have also been observed during intravenous lipid infusions among adults.[3, 4] Additionally, studies in adults have shown that obese individuals with hypertension may have higher baseline fasting NEFA levels compared to normotensive individuals.[5] Collectively, this research suggests that hypertension status should be considered when investigating risk factors for elevated NEFA. However, few of these studies have been completed among children, and it is unknown whether a similar association of hypertension with elevated fasting NEFA is present among this group.

Racial/ethnic differences in hypertension risk have also been noted, with a higher risk of hypertension reported in African-American (AA) versus European-American (EA) and Hispanic-American (HA) adults and children.[6, 7] Although race/ethnicity is considered a social construct, biological ancestry underlying populations has been evaluated as an important factor influencing disease risk among populations. In the United States, European, African, and Amerindian parental populations intermingled to produce an admixed population, increasing the complexity of disentangling biological and cultural contributions to the etiology of elevated blood pressure.[8] The use of genetic admixture to estimate racial ancestry allows investigators to identify biological contributors to disease risk among individuals of mixed racial/ethnic ancestry without assignment of racial/ethnic categories that may also include cultural population differences.[9, 10] This approach can be especially useful when evaluating factors

associated with hypertension, a disease process of complex etiology. The objective of this study, therefore, was to evaluate the association of hypertension prevalence and racial genetic ancestry with NEFA levels among a multiethnic, pediatric population.

METHODS

Subjects

Participants were 293 children (48.5% female) aged 7-12 years and identified by the parents/guardians as African-American (n = 98), European-American (n = 116), or Hispanic-American (n = 79). Children were recruited from the Birmingham, Alabama area via fliers, newspaper advertisements, and community presentations to study the effects of genetic and environmental parameters on racial/ethnic differences in metabolic outcomes. Exclusion criteria included diagnoses of type1 or type 2 diabetes, any glucose or lipid disorders, or use of any medication known to affect body composition or metabolism. Only children with a pubertal status ≤ 3 (determined by physician exam) according to the criteria of Marshall and Tanner were included.[11, 12] The study was approved by the University of Alabama at Birmingham (UAB) Institutional Review Board for Human Use, and each child and parent provided informed consent prior to participation.

Protocol

All data was collected during visits to the UAB General Clinical Research Center (GCRC) and the Department of Nutrition Sciences between 2004 and 2008. Data were obtained at two study visits that were no more than 30 days apart. During the first outpatient visit, pubertal status and body composition were measured and physical fitness assessed. At the second visit, participants were admitted to the UAB GCRC at

approximately 1730 hours for an overnight stay. All participants consumed the same in-patient meal, and received only water or noncaloric, decaffeinated beverages after 2000 hours. Approximately 30 minutes after waking the following morning, resting metabolic rate (RMR) was measured and fasting blood samples obtained.

Blood Pressure

Four blood pressure measurements were taken using an automated pediatric blood pressure cuff (Dinamap Pro 200, GE Medical Systems). Two measurements were obtained at approximately 1800 hours during the overnight visit. Two additional measurements were taken at approximately 0700 hours the following morning. Blood pressure was recorded after a minimum 10 minutes of seated rest. A five-minute rest separated the first and second measurements. There was no significant difference between evening and morning blood pressure measurements; thus, the four values were averaged to obtain a single systolic/diastolic measurement. Blood pressure percentile (BP%) was calculated using blood pressure tables developed by the National High Blood Pressure Education Program Working Group on High Blood Pressure in Children and Adolescents.[13] Children were classified as normotensive (systolic or diastolic BP% < 90^{th} percentile), pre-hypertensive (BP% 90-94^{th} percentile) or hypertensive (BP% $\geq 95^{th}$ percentile).

Body Composition

Body weight was measured to the nearest 0.1 kg in light clothing without shoes (Scale-tronix 6702W; Scale-tronix, Carol Stream, IL). Height was determined without shoes using a mechanical stadiometer. Body mass index (BMI; weight kg / height m^2) was calculated from these values, and BMI percentile determined using age- and sex-

specific CDC growth charts (http://apps.nccd.cdc.gov/dnpabmi/). Total fat mass and trunk fat mass were evaluated via dual energy x-ray absorptiometry (DXA) with a GE Lunar Prodigy densitometer (Lunar Radiation Corp., Madison, WI). Subjects were scanned in light clothing while lying flat on their backs with arms at their sides. DXA scans were analyzed with pediatric software enCORE 2002 version 6.10.029.

Resting Metabolic Rate

Baseline energy expenditure is strongly associated with fasting NEFA levels.[14] Hence, resting metabolic rate (RMR) was measured in the morning following the overnight visit at approximately 0700 h immediately after awakening. Measurements were obtained with the participant lying supine on a bed in a quiet, well-ventilated room with the head enclosed in a plexiglass canopy. Participants were instructed not to sleep and remain quiet and still, breathing normally. A computerized, open-circuit, indirect calorimetry system with a ventilated canopy (Delta Trac II; Sensor Medics, Yorba Linda, CA) was used to measure RMR. After resting for fifteen minutes, one-minute average intervals of oxygen uptake (VO_2) and carbon dioxide production (CO_2) were measured continuously for thirty minutes. The last 20 minutes of measurement were used for analysis.

Physical Fitness

$VO_{2\text{-}170}$ was determined by indirect calorimetry on a treadmill with participants in the post-prandial state for a minimum of 3 hours. Children were given the opportunity to familiarize themselves with the treadmill prior to beginning the test. Participants then walked on a flat incline 2.5 mph for 4 minutes. Speed was then increased to 3 mph for the remainder of the test, with the incline increasing 2% every 2 minutes until the child

reached a heart rate greater than 170 beats/minute. Heart rate (HR) was monitored with the Polar Vantage XL HR monitor (Polar Beat, Port Washington, NY). Volumes of O_2 and CO_2 were measured continuously using open circuit spirometry until recording the VO_2 level at a heart rate of 170 beats per minutes using a Max-II metabolic testing system (PHYSIO-DYNE, Quogue, NY). Data were expressed as $VO_{2\text{-}170}$ adjusted for body weight ($mL \cdot kg^{-1} \cdot min^{-1}$).

Laboratory Analysis

Fasting blood samples were analyzed by the Metabolism Core Laboratory of the UAB Clinical Nutrition Research Center for concentrations of NEFA and insulin. NEFA were assayed using "NEFA-C" reagents (Wako Diagnostics, Richmond, VA; mean intra-assay coefficient of variation (CV) 3.89%; mean inter-assay CV 5.87% at NEFA concentration of 0.433 mEq/l). Minimum assay sensitivity was 0.0014 mEq/l. Additionally, fasting insulin was shown to be inversely associated with fasting NEFA levels.[15] Insulin was therefore assayed in duplicate 100-µl aliquots using reagents from Millipore Corporation (St. Charles, MO; sensitivity 3.35 µIU/ml; mean intra-assay CV 3.49%; mean inter-assay CV 5.57%).

Genotyping

Genotyping of the ancestry informative markers (AIMs) for the measurement of genetic admixture was performed at Prevention Genetics using the Chemicon Amplifuor SNPs Genotyping System coupled with ArrayTape technology (www.global-array.com). A panel of 140 ancestry informative markers was used to estimate the genetic admixture proportion of each subject. Molecular techniques for the allelic identification and methodology for genetic admixture application have been described elsewhere and

information regarding marker sequences, experimental details, and parental population allele frequencies has been submitted to dbSNP (http://www.ncbi.nlm.nih.gov/SNP/) under the handle PSU-ANTH.[16] The ancestry estimates from AIMs was translated into estimates of African, European, and Native American admixture for each subject using maximum likelihood estimation based on the ML algorithm described by Hanis et al.[10, 17] In brief, the maximum likelihood method estimates the proportion of genetic ancestry for an individual, using a range of proportions from 0 to 1 and identifies the most probable value of admixture based on the observed genotypes.

Statistical Analysis

Tertiles of European admixture were computed based on the range of European admixture in the sample. Descriptive characteristics by tertile of European admixture were then analyzed using analysis of variance with Tukey's post-hoc analysis. Pearson correlations were used in exploratory analyses to determine which measures of body fat were associated with fasting NEFA levels, and it was determined that trunk fat mass would be used as a covariate in statistical models (data not shown). Analysis of covariance (ANCOVA) with Tukey's post-hoc test was used to evaluate the association of BP category (dummy coded as hypertension = 1 versus normotension and prehypertension = 0) and genetic admixture with fasting NEFA levels after controlling for age, female sex, trunk fat mass, RMR, fasting insulin, physical fitness ($VO_{2\text{-}170}$ (ml/kg/min)), and SES. Additionally, an interaction term between European admixture and BP category was created and added to the statistical model. Participants in the lowest category of European admixture who were in the BP category of hypertenstion were coded as (1), with all other participants coded as (0). The finally model was evaluated for

normality of residuals and no log-transformations of data required. All analyses were completed using SAS version 9.2 (SAS Institute, Inc., Cary, NC) with significance determined at $P < 0.05$.

RESULTS

Descriptive characteristics for the total sample and by tertiles of European admixture are presented in Table 1. The range of European admixture for each tertile was as follows: for Tertile 1, 0-20%; for Tertile 2, 21-92%, and for Tertile 3, 93-100%. Compared to Tertile 3, participants in Tertile 2 presented with higher measures of body composition and fat mass as measured by BMI, BMI percentile, total fat mass, and trunk fat mass (all at $P < 0.05$). Participants in Tertile 3 had significantly higher SES scores ($P < 0.05$), greater European admixture, and lower African and Amerindian ancestry compared to the other groups (all at $P < 0.05$). No differences were noted among groups in fasting NEFA levels or average systolic blood pressure; however, a significantly higher percentage of children in Tertile 1 were classified as having hypertension ($P < 0.05$).

Figure 1 shows the mean fasting NEFA level by tertiles of admixture and BP category. No difference in NEFA levels by tertile was noted for children classified as normotensive. Children in Tertiles 3 who were classified as normotensive had a mean fasting NEFA level of 0.47 ± 0.22 mEq/l, compared to mean levels of 0.48 ± 0.19 mEq/l for children in both Tertiles 1 and 2. However, children in Tertile 1 who were classified as hypertensive had significantly lower fasting NEFA (0.44 ± 0.19) compared to children categorized with hypertension in Tertile 2 (0.59 ± 0.17) and Tertile 3 (0.60 ± 0.27.

When the independent associations of European admixture and hypertension with NEFA were tested, neither variable was significantly associated with fasting NEFA after controlling for covariates (data not shown). However, a significant effect of hypertension x low European admixture was noted (Table 2), such that lower fasting NEFA was identified in individuals with low European admixture and hypertension compared to children with hypertension in the other tertiles (overall model: $F = 4.76$, $r^2 = 0.20$, $P < 0.001$).

DISCUSSION

Higher NEFA levels have been reported among adults with hypertension.[1] However, when we evaluated this relationship among a group of EA, AA, and HA children, we identified an association of hypertension prevalence with low fasting NEFA only among individuals in the low tertile (< 20%) of European admixture. To our knowledge, this is the first report of an interactive effect of hypertension and racial ancestry on fasting NEFA levels. Bulow and colleagues first identified an association of acute increases in blood pressure with lipid infusions in pigs.[18] Since that time, studies have reported a similar association of acute blood pressure increase with lipid infusion among EA and AA adults.[3, 4, 19] However, most studies to report higher NEFA levels among individuals with hypertension have included participants of primarily European ancestry. Hence, it is possible that individuals with less European ancestry may not differ from other groups in the response to acute increases in circulating NEFA levels, while differing substantially in the metabolic response of adipose tissue to chronically elevated blood pressure.

We were unable in the present investigation to elucidate the mechanisms involved in hypertension development that may differentially impact fasting NEFA levels among individuals with differing levels of racial ancestry. However, increased insulin secretion is associated with decreases in adipocyte lipolysis and subsequent decreases in acute NEFA levels.[20] Gower et al. have previously reported that lower European ancestry is associated with decreased sensitivity to insulin, resulting in higher fasting insulin levels.[21] Participants with low European admixture in this study did present with higher fasting insulin levels compared to those with the highest levels of European admixture. Hence, it is possible that the inhibitory effects of higher insulin levels on fasting NEFA may account for a portion of the difference noted among participants by admixture tertile. Studies of the NEFA/blood pressure association with insulin are limited. Jensen et al. reported suppression of NEFA levels with physiological increases in insulin levels among adults with upper-body obesity.[22] Conversely, Egan and colleagues identified an inability of insulin to suppress NEFA levels among abdominally obese, hypertensive individuals.[5] Further studies are thus needed to confirm our findings and investigate the mechanisms which may influence the NEFA/blood pressure relationship among children.

Our study benefited from the use of a multiethnic population and estimations of genetic admixture, which allowed us to identify differences in NEFA levels among hypertensive individuals with low European ancestry. Our results were limited by the use of four blood pressure measurements taken at two time points as a marker for hypertension. Hypertension is commonly diagnosed using blood pressure measurements obtained during multiple time points, or with 24-hour ambulatory blood pressure monitoring. However, blood pressure measurements obtained over only a few time

points may be useful in identifying population trends in hypertension risk. Additionally, it is possible that genetic factors not controlled for with racial ancestry could influence the NEFA/blood pressure associations identified here.

In summary, hypertension is associated with no change in fasting NEFA levels compared to participants without hypertension among individuals with low European ancestry. However, higher NEFA levels were observed among individuals with medium/high levels of European ancestry, suggesting that divergent physiological mechanisms in lipolytic control are influenced by factors associated with racial ancestry.

ACKNOWLEDGMENTS

This work was funded by the National Institutes of Health grants R01-DK067426, P30-DK56336, P60-DK079626, M01-RR-00032, and the National Cancer Institute Cancer Prevention and Control Training Program CA47888.

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Table 1. Baseline characteristics in total sample and by tertiles of European admixture (mean ± SD; n(%))

Variable	Total Sample	Tertile 1	Tertile 2	Tertile 3
n	293	96	102	95
Age (years)	9.6 ± 1.6	9.6 ± 1.5	9.4 ± 1.6	9.7 ± 1.7
Sex (n, % female)	142 (48.5)	41 (42.7) [a]	57 (55.9) [b]	44 (46.3) [a]
Tanner Stage	1.5 ± 0.7	1.4 ± 0.7	1.6 ± 0.8	1.5 ± 0.8
Height (cm)	139.6 ± 10.6	140.3 ± 10.1	138.2 ± 11.0	140.5 ± 10.6
Weight (kg)	36.6 ± 9.6	36.8 ± 9.3	37.3 ± 10.2	35.8 ± 9.1
BMI	18.6 ± 3.0	18.5 ± 3.0[ab]	19.3 ± 3.2[a]	17.9 ± 2.6[b]
BMI percentile	65.8 ± 26.3	64.4 ± 27.1[ab]	72.9 ± 23.5[a]	60.0 ± 26.8[b]
Lean Mass (kg)	25.6 ± 5.2	26.7 ± 5.3	25.0 ± 5.3	25.5 ± 4.9
Fat Mass (kg)	8.9 ± 5.7	7.8 ± 5.4[a]	10.2 ± 6.1[b]	8.4 ± 5.4[ab]
Trunk Fat Mass (kg)	3.7 ± 2.8	3.1 ± 2.6 [a]	4.4 ± 3.1 [b]	3.3 ± 2.5 [a]
$VO_{2\text{-}170}$ (ml/kg/min)	29.7 ± 6.1	28.6 ± 6.0	30.0 ± 5.7	30.3 ± 6.7
RMR (kcal/day)	1192.0 ± 235.9	1190.4 ± 207.1	1195.2 ± 241.2	1190.6 ± 254.3
Insulin (mEq/l)	12.8 ± 5.6	13.8 ± 6.8[a]	13.0 ± 6.5[ab]	11.0 ± 4.3[b]
Genetic Admixture:				
European (%)	53.3 ± 37.7	11.5 ± 5.6[a]	51.1 ± 24.0[b]	97.4 ± 2.6[c]
African (%)	28.9 ± 37.5	72.8 ± 28.2 [a]	14.9 ± 23.5 [b]	1.0 ± 1.0 [c]
Amerindian (%)	17.8 ± 25.2	15.7 ± 26.5 [a]	34.1 ± 25.6 [b]	2.2 ± 2.4 [c]
SES	12.7 ± 6.1	34.7 ± 11.1[a]	32.4 ± 15.2[a]	49.3 ± 9.7[b]
NEFA (mEq/l)	0.5 ± 0.2	0.5 ± 0.2	0.5 ± 0.2	0.5 ± 0.2
SBP (mmHg)	0.5 ± 0.2	105.5 ± 11.1	102.5 ± 9.5	102.4 ± 11.0
HTN (n, %)	24 (8.2)	14 (14.6)[a]	6 (5.9) [b]	4 (4.2) [b]

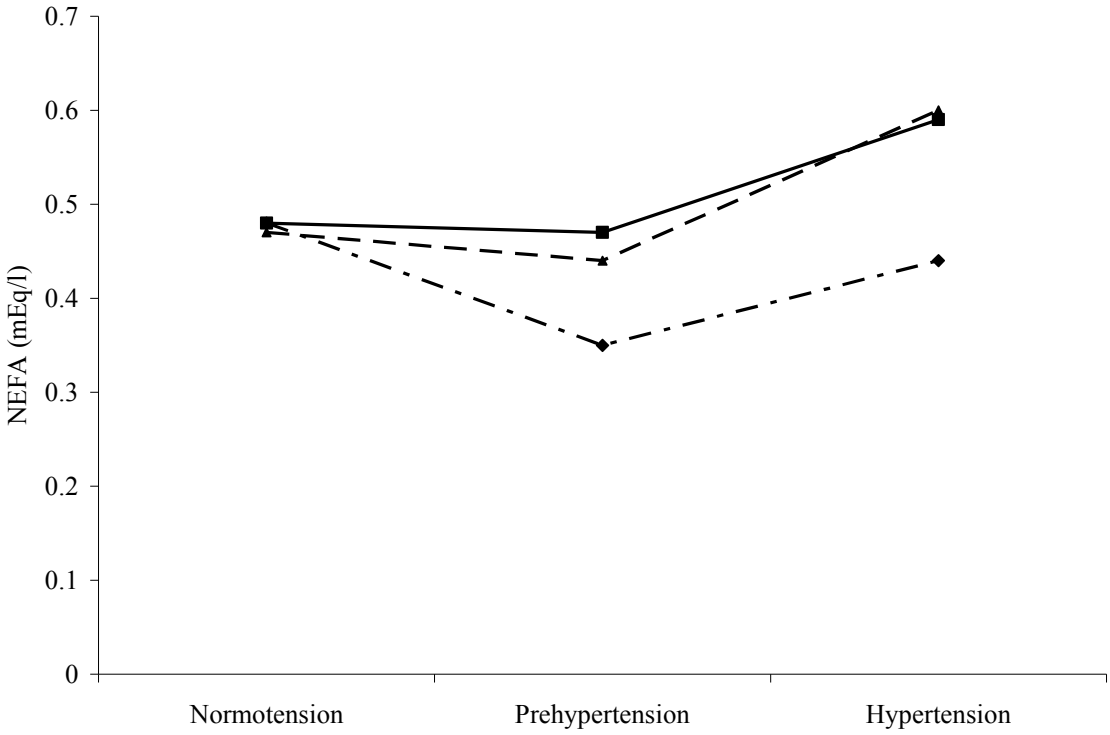
[a,b,c] superscripts indicate group difference at $P < 0.05$; BMI = body mass index (weight kg/height m^2); RMR = resting metabolic rate; SES = socioeconomic status; NEFA = nonesterified fatty acids; SBP = systolic blood pressure; HTN = hypertension

Table 2. Association of study variables with NEFA levels

Variable	*F*-value	*P* value
Age	5.56	0.02
Sex (female)	1.79	0.18
Trunk Fat	4.25	0.04
Resting Metabolic Rate	15.82	<0.01
Fasting Insulin	4.07	0.05
$VO_{2\text{-}170}$/kg	2.35	0.13
Socioeconomic Status	1.54	0.22
Low European Admixture	1.23	0.27
HTN	6.33	0.01
HTN * Low European Admixture	6.00	0.02

HTN = hypertension. Resting metabolic rate, fasting insulin, $VO_{2\text{-}170}$/kg, and HTN*admixture were inversely associated with fasting NEFA.

Figure 2. Mean NEFA (mEq/l) by European admixture tertile and blood pressure category



Dashed/dotted line = low European admixture (tertile 1); dashed line = medium European admixture (tertile 2); Solid line = high European Admixture (tertile 3)

VARIATION IN THE NPPA GENE IS ASSOCIATED WITH PEDIATRIC HYPERTENSION RISK

by

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In preparation for *J Hyp*

Format adapted for dissertation

ABSTRACT

Hypertension prevalence is increasing among children, potentially increasing later risk for stroke and cardiovascular disease. Previous studies have identified an association between the natriuretic peptide precursor A (NPPA) and the atrial natriuretic peptide A receptor (NPR1) genes with hypertension and cardiovascular function in adults, but few studies have investigated these relationships among children. We studied 308 boys and girls ages 7-12 identified as African-American, European-American, or Hispanic-American to determine whether four polymorphisms – rs5063, rs5065, rs198358, and rs10082235 – in the NPPA/NPR1 genes were associated with pediatric blood pressure. Blood pressure percentiles were calculated according to pediatric guidelines using the average of four measurements. Physical fitness was measured via indirect calorimetry. Total fat mass was determined using dual-energy x-ray absorptiometry, and socioeconomic status was calculated with the Hollingshead index. Higher rates of prehypertension and hypertension were noted with the rs5063 GG genotype and the rs10082235 CT and TT genotypes. However, after controlling for age, sex, waist, physical fitness, socioeconomic status, and African and Amerindian genetic admixture, only rs5063 remained associated with blood pressure levels, with presence of the GA genotype indicating lower pediatric blood pressure ($P = 0.0043$). We also noted a significant association of greater African genetic admixture with higher pediatric blood pressure levels ($P = 0.0068$). These results suggest that polymorphisms in the NPPA/NPR1 genes may influence blood pressure even during childhood, although additional biological factors associated with African ancestry may also influence hypertension risk.

INTRODUCTION

Hypertension is considered an independent risk factor for stroke and cardiovascular disease. Rates of pediatric hypertension have increased over the past two decades, and high blood pressure during childhood is associated with increased risk for adult hypertension.[1, 2] Although pediatric hypertension has been increasingly studied in recent years, little is known about which factors may predispose a child to elevated blood pressure. Obesity is considered one risk factor for pediatric hypertension, and our group previously identified an association between greater waist circumference measurements and high blood pressure in a multiethnic pediatric population.[3, 4] Additionally, low physical fitness, as measured by indirect calorimetry, has been associated with increased blood pressure.[5, 6] However, the etiology of hypertension is multifactorial, with both genetic and environmental factors influencing blood pressure levels.

Investigations among adult populations have identified an association of atrial natriuretic peptide (ANP) levels with hypertension risk.[7, 8] ANP assists with blood pressure control by exerting influence on the rennin-angiotensin-aldosterone system via natriuresis (salt excretion) and diuresis (fluid excretion).[8-10] It is therefore plausible that genes associated with ANP function could influence susceptibility to hypertension. Several studies have identified associations of single nucleotide polymorphisms (SNPs) in the genes that encode for NPA (NPPA) and the ANP cell receptor (NPR1) with hypertension in the adult population.[11-14] However, only one study evaluated the association of a genetic polymorphism in the NPPA gene (rs5063) with hypertension prevalence in a sample of 115 Eastern European children.[15] Additionally, few studies have investigated the association of genetic polymorphisms in these genes with blood

pressure levels among African-American or Hispanic-American children, despite the fact that minority groups present with higher rates of hypertension compared to European-Americans. We therefore evaluated the association of polymorlphisms in the NPPA (rs5063, rs5065, rs198358) and NPR1 (rs10082235) genes with risk for elevated blood pressure in a multiethnic pediatric sample after controlling for other factors, including waist circumference and physical fitness, known to affect blood pressure levels.

METHODS

Subjects

Participants were 308 children (47.6% female) aged 7-12 years and identified by the parents/guardians as African-American (n = 106), European-American (n = 120), or Hispanic-American (n = 82). Children were recruited from the Birmingham, Alabama area via fliers, newspaper advertisements, and community presentations to study the effects of genetic and environmental parameters on racial/ethnic differences in metabolic outcomes. Exclusion criteria included diagnoses of type1 or type 2 diabetes, any glucose or lipid disorders, or use of any medication known to affect body composition or metabolism. Only children with a pubertal status ≤ 3 (determined by physician exam) according to the criteria of Marshall and Tanner were included.[16, 17] The study was approved by the University of Alabama at Birmingham (UAB) Institutional Review Board for Human Use, and each child and parent provided informed consent prior to participation.

Protocol

All data was collected during visits to the UAB General Clinical Research Center (GCRC) and the Department of Nutrition Sciences between 2004 and 2008. Data were

obtained at two study visits that were no more than 30 days apart. During the first outpatient visit, pubertal status and body composition were measured and physical fitness assessed. At the second visit, participants were admitted to the UAB GCRC at approximately 1730 hours for an overnight stay, with blood pressure measurements obtained in the evening and morning.

Blood pressure

Four blood pressure measurements were taken using an automated pediatric blood pressure cuff (Dinamap Pro 200, GE Medical Systems). Two measurements were obtained at approximately 1800 hours during the overnight visit. Two additional measurements were taken at approximately 0700 hours the following morning. Blood pressure was recorded after a minimum 10 minutes of seated rest. A five-minute rest separated the first and second measurements. There was no significant difference in the classification of hypertension between evening and morning blood pressure measurements; thus, the four values were averaged to obtain a single systolic/diastolic measurement. Blood pressure percentile (BP%) was calculated using blood pressure tables developed by the National High Blood Pressure Education Program Working Group on High Blood Pressure in Children and Adolescents.[18] Children were classified as normotensive (systolic or diastolic BP% < 90^{th} percentile), pre-hypertensive (BP% 90-94^{th} percentile) or hypertensive (BP% $\geq 95^{th}$ percentile).

Body Composition

Body weight was measured to the nearest 0.1 kg in light clothing without shoes (Scale-tronix 6702W; Scale-tronix, Carol Stream, IL). Height was determined without shoes using a mechanical stadiometer. Body mass index (BMI; weight kg / height m^2)

was calculated from these values, and BMI percentile determined using age- and sex-specific CDC growth charts (http://apps.nccd.cdc.gov/dnpabmi/). Waist circumference was measured by the same registered dietitian using a flexible tape measure (Gulick II; Country Technology, Inc., Gays Mills, WI) as described by Lohman et al. and recorded to the nearest 0.1 cm.[19] We previously determined that the waist/height index was the body composition measure most associated with blood pressure and computed this measure as previously described.[4]

Physical Fitness

$VO_{2\text{-}170}$ was determined by indirect calorimetry on a treadmill with participants in the post-prandial state for a minimum of 3 hours. Children were given the opportunity to familiarize themselves with the treadmill prior to beginning the test. Participants then exercised at 2.5 mph for 4 minutes and 3 mph thereafter. The incline was increased by 2% every 2 minutes after the first 4 minutes. Heart rate (HR) was monitored with the Polar Vantage XL HR monitor (Polar Beat, Port Washington, NY). Volumes of O_2 and CO_2 were measured continuously using open circuit spirometry until recording the VO_2 level at a heart rate of 170 beats per minutes using a Max-II metabolic testing system (PHYSIO-DYNE, Quogue, NY). Data were expressed as $VO_{2\text{-}170}$ adjusted for body weight ($mL \cdot kg^{-1} \cdot min^{-1}$).

Genotyping

Genotyping of the NPPA/NPR1 SNPs was performed at the UAB Genomics Core Laboratory via pyrosequencing. Each DNA sample was amplified using PCR with pooled biotinylated primers. PCR products were then captured with streptavin-coated beads and hybridized to sequence-specific primers and reactions run using the Biotage

96HS PSQ Pyrosequencing Machine (Biotage AB, Charlotte, NC). Forward (F), reverse (R), and sequencing (S) primers were designed for the following SNPs: for rs5063 (F) 5'ACTGGCATTCCAGCTCCTAGGTC'3, (R) 3'TGGCCCTACCTTGAAATCCATC'5, (S) CCCATGTACAATGCC; for rs5065 (F) 5'CTTGTCCTCCCTGGCTGTTATC'3, (R) 3'AGGATGGGCACACTCATACATG'5, (S) CTGTGTTCTCTTTGCAGTAC; for rs198358 (F) 5'GGATGGATGCAGGAGCTGAAC'3, (R) 3'CTTGCTTTTGGTTTTGCAAGAAGAG'5, (S) CGACCACTGATGGAAC; and for rs10082235 (F) 5'CTTGCAGGCCAGATAGGAAGC'3, (R) 3' GGCAGGAACAAGGCTCATTC'5, (S) AATCATGCCCATGTTAG. Following the pyrosequencing process, genotype results were reviewed and confirmed by two separate investigators, with a minimum of 96.3% genotyping completion rate for all SNPs.

Genotyping of the ancestry informative markers (AIMs) for the measurement of genetic admixture was performed at Prevention Genetics (www.preventiongenetics.org) using the Chemicon Amplifuor SNPs Genotyping System coupled with ArrayTape technology (www.global-array.com). A panel of 140 ancestry informative markers was used to estimate the genetic admixture proportion of each subject. Molecular techniques for the allelic identification and methodology for genetic admixture application have been described elsewhere and information regarding marker sequences, experimental details, and parental population allele frequencies has been submitted to dbSNP (http://www.ncbi.nlm.nih.gov/SNP/) under the handle PSU-ANTH.[20] The ancestry estimates from AIMs was translated into estimates of African, European, and Native American admixture for each subject using maximum likelihood estimation based on the ML algorithm described by Hanis et al.[21, 22] In brief, the maximum likelihood method

estimates the proportion of genetic ancestry for an individual, using a range of proportions from 0 to 1 and identifies the most probable value of admixture based on the observed genotypes.

Socioeconomic Status

Socioeconomic status (SES) was measured with the Hollingshead 4-factor index of social class, which combines the educational attainment and occupational prestige for the number of working parents in the child's family.[23] Scores ranged from 8 to 66, with the higher scores indicating higher theoretical social status.

Statistical Analysis

All analyses were performed using SAS version 9.2 (SAS Institute, Inc., Cary, NC). Allele frequencies were computed and Hardy-Weinberg equilibrium (HWE) test performed using Haploview version 4.1 (Broad Institute, Cambridge, MA). Our sample included participants from separate racial/ethnic backgrounds, and allele frequency is known to differ by population background; hence, HWE was calculated within each racial/ethnic group. Participant characteristics were analyzed by blood pressure category (normotensive, prehypertensive, hypertensive) with analysis of variance with tukey's post-hoc test for group differences. A stronger relationship with body weight, cardiac outcomes, and adult hypertension is reported for pediatric systolic blood pressure (SBP); hence, when analyzing blood pressure as a continuous variable we utilized absolute SBP measures.[3, 24] Additionally, the sum total of the three admixture estimates is equal to 1; therefore, European admixture was used as the reference group and only African and American admixture were included in the models. The frequency of prehypertension and hypertension within each genetic polymorphism was compared using chi-square analysis

or Fisher's exact test. Linear regression was used to evaluate the association of each genotype separately with systolic blood pressure using additive, dominant, and recessive models. All models were controlled for age, sex, waist/height ratio, $VO_{2\text{-}170/kg}$, Amerindian and African genetic admixture, and SES. To account for multiple hypothesis testing we utilized the PROC MULTTEST procedure in SAS, which adjusts model *p*-values for multiple comparisons. We further tested for interactive effects of genotype with physical fitness, waist circumference, and percent body fat in each model; however, no interactions were identified and results are presented here without interactions terms in the models.

RESULTS

Characteristics of the total population and by BP category are summarized in Table 1. Children with hypertension had significantly greater levels of African genetic admixture, as well as higher systolic and diastolic blood pressures compared to the groups classified as normotensive and prehypertensive (all at $P < 0.05$). There was also a trend for children with hypertension to have lower physical fitness as expressed by $VO_{2\text{-}170/kg}$ ($P = 0.057$). There was no significant difference by BP category in body composition measures.

Table 2 presents the minor allele frequency for each SNP studied. When the SNPs were investigated within each racial/ethnic group, all SNPs were in HWE among each racial/ethnic group (African-American, European-American, Hispanic-American; $P > 0.05$). The population frequencies found in our study are equivalent to those reported among other populations similar to those studied here (http://hapmap.ncbi.nlm.nih.gov/cgi-perl/gbrowse/hapmap27_B36/).

When the frequencies of prehypertension and hypertension were evaluated for each SNP, no hypertension was noted among carriers of the rs5063 heterozygous allele (GA; figure 1a). Additionally, there was a trend for participants with the rs10082235 homozygous minor allele (TT) to present with higher rates of prehypertension and hypertension (figure 1b). Table 2 presents the results of the four linear regression models. Results of the recessive models are presented for rs5065, rs198358, and rs5065, while results of the dominant model are presented for rs5063 (testing GG versus GA alleles). After controlling for age, female sex, waist ratio, $VO_{2\text{-}170/kg}$, socioeconomic status, African admixture, and Amerindian admixture, only the rs5063 heterozygous allele (GA) was associated with lower systolic blood pressure.

DISCUSSION

Genetic polymorphisms of the NPPA and NPR1 genes have been associated with hypertension risk in the adult population, primarily among individuals of European ancestry.[11, 14, 25] When we evaluated the relationship between blood pressure and four polymorphisms associated with these genes (rs5063, rs5065, rs198358, rs10082235) among a multiethnic pediatric population, lower rates of prehypertension and hypertension were noted among participants with the rs5063 heterozygous allele (GA). Additionally, a trend for greater prehypertension and hypertension prevalence was noted with the rs10082235 heterozygous (CT) and homozygous minor (TT) alleles. After controlling for body composition and physical fitness, only the heterozygous allele of rs5063 (GA) was associated with lower blood pressure levels. Zorc-Pleskovic et al. previously identified a genotype*obesity interaction for pediatric hypertension risk; we thus investigated the potential interactive effects for hypertension of each genotype with

waist circumference, percent body fat, and physical fitness.[15] However, no interactions were detected among this population.

Consistent with our findings, several previous investigations among adults have noted an association of the rs5063 GA and AA alleles with lower hypertension risk. Conen et al. found that presence of the rs5063 minor allele (A) associated with decreased hypertension risk in over 18,000 women of European ancestry.[11] Additional studies among populations of European and Asian ancestry have also found an association of rs5063 with hypertension and cardiovascular disease risk, though not all studies have confirmed this association.[13, 26, 27] Newton-Cheh and colleagues, however, were unable to confirm an association between rs5063 and ANP levels, suggesting that additional mechanistic studies are needed to confirm how allelic differences in rs5063 could impact blood pressure.[14]

We also noted greater levels of African genetic ancestry among participants with hypertension (table 1), and greater African admixture was associated with higher blood pressure even after controlling for additional biological and environmental factors (data not shown). This finding is consistent with literature that identifies higher rates of hypertension among African-American adults.[28] However, race/ethnicity represents a social (rather than biological) construct that is typically based on unique cultural and behavioral practices that could affect metabolic pathways and disease risk independent of self-classified race. Many individuals in the United States exhibit racial ancestry influenced by European, African, and Amerindian parental populations. Use of genetic admixture elucidates biological rather than environmental variance within individuals, and may therefore be a more appropriate measure of ancestral contributions to disease

risk.[29, 30] Our findings suggest that additional genetic factors associated with African ancestry that were not studied here could contribute to the higher prevalence of hypertension seen among the African-American population, and among individuals with mixed racial ancestry that includes African heritage.

This study benefited from the use of genetic admixture analysis, which allowed us to study a multiethnic cohort and is one of the first to include Hispanic-Americans in the investigation of NPPA/NPR1 genotypes and blood pressure levels. It is also one of the first studies to investigate this relationship among children. Our sample was limited in that individual hypertension is typically diagnosed from measurements obtained during multiple time points or from 24-hour ambulatory blood pressure monitoring. Our data was obtained during one overnight visit; however, such information is useful to evaluate population trends in hypertension risk. Also, approximately 70% of our population was of normal weight as defined by a BMI at or below the 85th percentile (http://www.cdc.gov/healthyweight/assessing/bmi/childrens_bmi/about_childrens_bmi.html), which along with a smaller sample size could have impaired our ability to detect gene*body fat interactions that could influence blood pressure levels. However, we were able to measure factors such as body composition and physical fitness that have previously not been accounted for in larger candidate gene and genome-wide association studies, but that are associated with hypertension risk in both adults and children.

In summary, the rs5063 A allele was associated with hypertension prevalence and higher systolic blood pressure levels in a multiethnic, pediatric sample of both boys and girls. Future studies are warranted to confirm this association and to elucidate the

mechanism by which this genetic polymorphism could affect ANP production and influence hypertension risk.

ACKNOWLEDGMENTS

The authors would like to thank Robert Petri and Alexandra Luzuriaga-McPherson for their assistance with data collection, as well as Kerry Lok and the UAB Genomics Core Laboratory for genotyping assistance. This work was funded by the National Institutes of Health grants R01-DK067426, P30-DK56336, P60-DK079626, M01-RR-00032, and the National Cancer Institute Cancer Prevention and Control Training Program CA47888.

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Table 1. Population characteristics

Variable	Total Sample	Normotensive	Prehypertensive	Hypertensive
n	308	263	19	26
Age (years)	9.6 ± 1.6	9.6 ± 1.6	9.0 ± 1.6	9.2 ± 1.4
Sex (n, % female)	147 (47.6)	124 (47.2)	10 (52.6)	13 (50.0)
Tanner Stage	1.5 ± 0.7	1.4 ± 0.7	1.6 ± 0.8	1.4 ± 0.8
Height (cm)	139.5 ± 10.6	139.8 ± 10.6	137.5 ± 9.4	137.6 ± 12.1
Weight (kg)	36.7 ± 9.6	36.6 ± 9.2	36.5 ± 9.4	36.9 ± 13.0
BMI	18.6 ± 3.0	18.5 ± 2.9	19.0 ± 2.5	19.0 ± 3.9
BMI percentile	66.5 ± 26.0	65.5 ± 26.1	78.4 ± 18.9	67.0 ± 28.5
Waist (cm)	64.5 ± 9.0	64.4 ± 8.7	64.8 ± 7.2	64.3 ± 12.3
$VO_{2\text{-}170}$ (ml/kg/min)	29.6 ± 6.0	30.0 ± 6.0	27.8 ± 6.9	27.1 ± 5.2†
Genetic Admixture:				
European (%)	53.1 ± 37.6	54.8 ± 37.5	50.9 ± 38.0	38.5 ± 36.2
African (%)	28.9 ± 37.5	25.9 ± 36.3^{a}	37.4 ± 40.2^{ab}	51.7 ± 38.7^{b}
Amerindian (%)	17.9 ± 25.3	19.3 ± 26.1	11.7 ± 20.5	9.8 ± 17.8
SES	38.9 ± 14.6	39.1 ± 14.7	32.8 ± 13.1	40.4 ± 13.6
Systolic BP (mmHg)	103.3 ± 10.6	100.6 ± 8.6^{a}	113.6 ± 7.7^{b}	122.2 ± 5.8^{c}
Diastolic BP (mmHg)	60.1 ± 6.5	58.9 ± 5.8 ^{a}	63.5 ± 6.2 ^{b}	68.8 ± 6.9 ^{c}

^{a,b,c} superscripts indicate group difference at $P < 0.05$; † = trend for significance at $P = 0.057$; BMI = body mass index (weight kg/height m^2); RMR = resting metabolic rate; SES = socioeconomic status

Table 2. Genetic polymorphisms studied

Polymorphism	Location	Minor allele	MAF	% Genotyped
NPPA:				
rs5063	Exon 1 (*Met*32*Val*)	A	0.042	98.7%
rs5065	Exon 3 (*Arg*152*Ter*)	C	0.233	98.7%
rs198358	3' UTR	G	0.329	96.3%
NPR1:				
rs10082235	Intron 1	T	0.215	98.3%

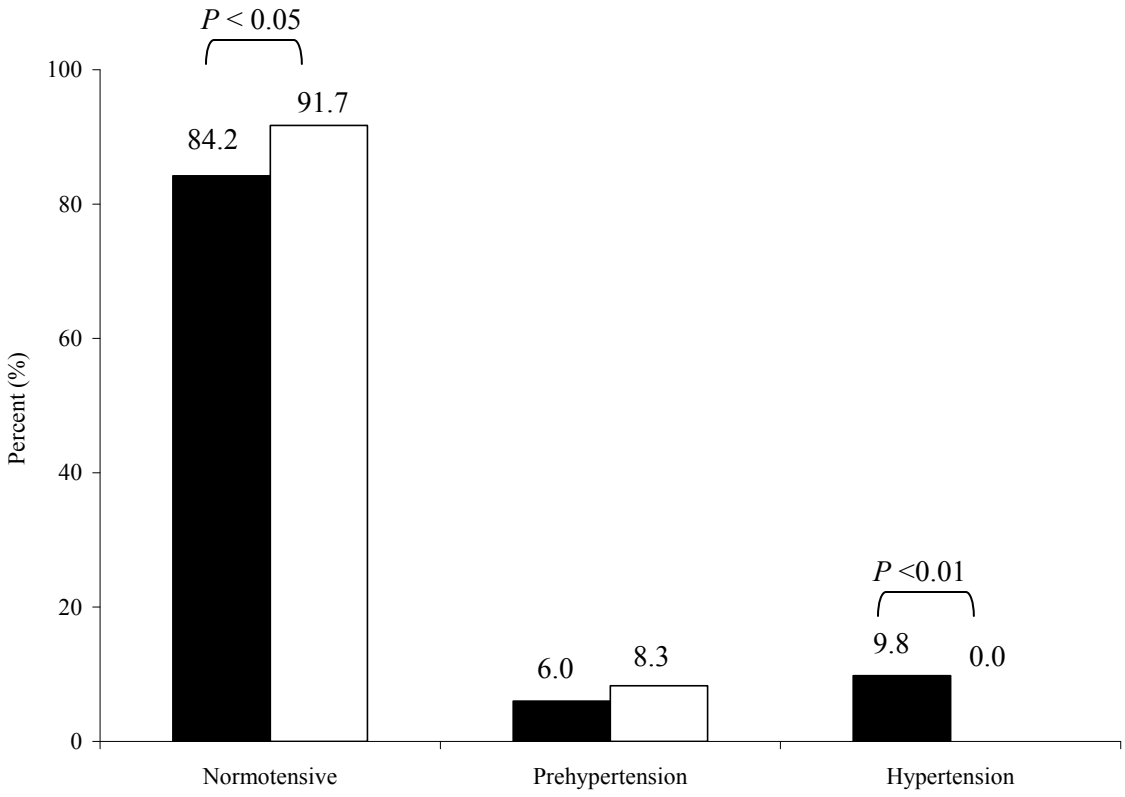
MAF = minor allele frequency

Table 3. Association of genetic polymorphisms with systolic blood pressure*

Polymorphism	ß	*P*
rs5063	-6.53	0.0043
rs5065	3.11	0.2647
rs198358	-2.2	0.3190
rs10082235	-3.04	0.3358

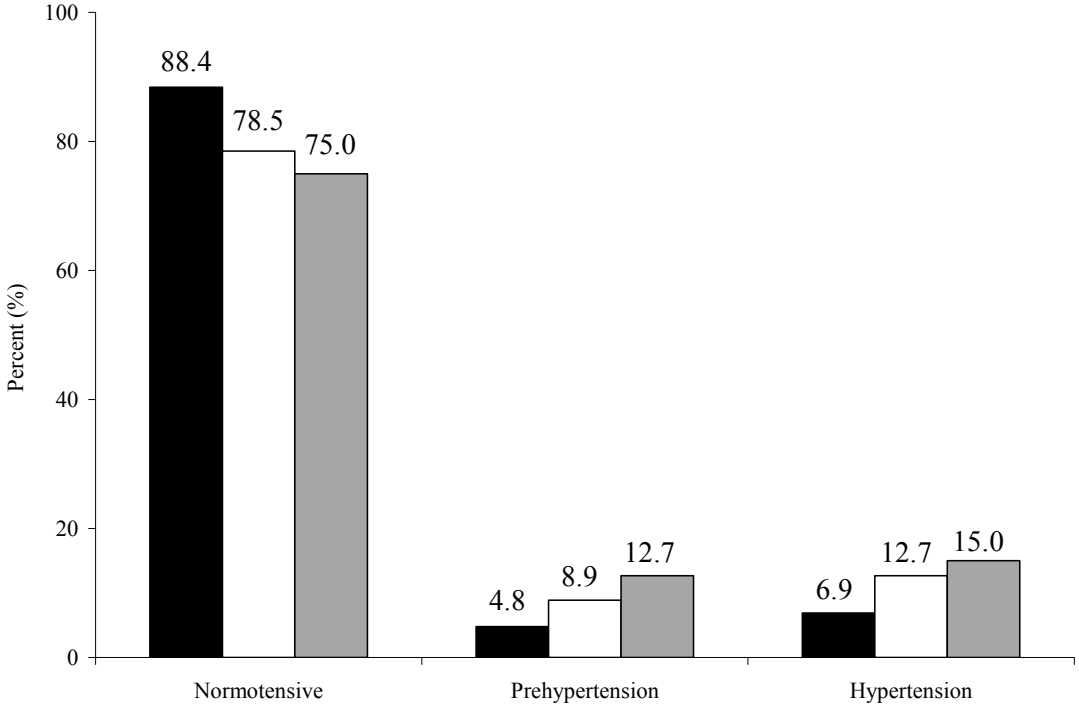
*Each model adjusted for age, female sex, waist ratio, $VO_{2\text{-}170/kg}$, socioeconomic status, African admixture, and Amerindian admixture

Figure 1a. Hypertension prevalence by rs5063 genotype



Black bars = GG; white bars = GA

Figure 1b. Hypertension prevalence by rs10082235 genotype*



Black bars = CC; white bars = CT; gray bars = TT; *Trend for significant difference in hypertension rates by genotype at P = 0.0750

VARIATION IN THE NPR1 GENE IS ASSOCIATED WITH NONESTERIFIED FATTY ACIDS

by

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In preparation for *Obesity*

Format adapted for dissertation

ABSTRACT

Elevated nonesterified fatty acids (NEFA) are associated with metabolic disease risk in children. Atrial natriuretic peptide is known to influence NEFA levels; thus genetic polymorphisms in the natriuretic peptide precursor A (NPPA) or the atrial natriuretic peptide A receptor (NPR1) genes could be associated with NEFA levels. We evaluated the association of fasting NEFA with four polymorphisms – rs5063, rs5065, rs198358, and rs10082235 – in the NPPA/NPR1 genes among a cohort of 293 boys and girls identified as European-American, African-American or Hispanic American. Fasting NEFA and insulin were measured following an overnight fast. Resting metabolic rate (RMR) and physical fitness were determined via indirect calorimetry, and body composition was measured using dual-energy x-ray absorptiometry. Socioeconomic status was calculated with the Hollingshead index. Linear regression analysis was used to test the association of each polymorphism with fasting NEFA. After controlling for age, sex, RMR, physical fitness, trunk fat mass, fasting insulin, genetic admixture, and socioeconomic status, the homozygous minor allele (TT) of rs10082235 was associated with higher NEFA levels ($P = 0.03$). Additionally, higher Amerindian admixture was associated with higher NEFA, while RMR and physical fitness were inversely associated with NEFA levels (all at $P < 0.05$). These results are the first to suggest that polymorphisms in the NPR1 gene may influence fasting NEFA levels.

INTRODUCTION

Both fasting and day-long elevations of nonesterified fatty acids (NEFA) have been associated with increased risk for metabolic syndrome, cardiovascular disease, and certain cancers.[1, 2] Several factors are associated with alterations in NEFA levels among children and adults, including sex, metabolic rate, and physical activity.[3-5] In recent years, atrial natriuretic peptide (ANP) has also been shown to stimulate human adipose tissue lipolysis and hence affect levels of NEFA.[6] This takes place through an insulin-independent, cGMP-dependent process, in which ANP stimulates receptors (NPR-A) on the adipocyte plasma membrane that increase intracellular cGMP levels and result in activation of adipocyte perilipin and hormone sensitive lipase, as described by Lafontan *et al*.[7, 8] The process is of particular interest in children, since this group is experiencing rapidly increasing rates of hypertension, hyperlipedemia, and metabolic syndrome.[9-11] Identifying the factors that influence ANP action on adipocytes could provide further understanding of the processes that affect chronic disease risk among this population.

Blood pressure levels and cardiovascular function are also influenced by ANP,[12, 13] and genetic polymorphisms found in both the natriuretic peptide precursor A (NPPA) gene and the atrial natriuretic peptide receptor-A (NPR1) gene have been associated with hypertension/stroke risk factors in children and adults.[14, 15] It is therefore possible that NPPA and NPR1 gene polymorphisms are also associated with NEFA levels. Souren and colleagues estimated the heritability of fasting NEFA to be approximately 37%; however, little is known about which genetic factors may be associated with NEFA.[16] Identifying these associations in large-scale genetic studies may be further complicated by the lack of precise measures for other factors associated with NEFA levels, such as resting metabolic

rate, physical fitness, body composition, and racial genetic ancestry. This study was thus conducted in a multiethnic cohort of boys and girls ages 7-12 to determine whether genetic factors previously associated with blood pressure and/or cardiovascular function are also associated with fasting NEFA levels. Specifically, we tested the hypothesis that polymorphisms in the NPPA (rs5063, rs5065, rs198358) and NPR1 (rs10082235) genes would be associated with fasting NEFA levels measured during an in-patient visit at which more precise measures of additional factors that influence NEFA were also obtained.

METHODS

Subjects

Participants were 293 children (48.5% female) aged 7-12 years and identified by the parents/guardians as African-American (n = 98), European-American (n = 116), or Hispanic-American (n = 79). Children were recruited from the Birmingham, Alabama area via fliers, newspaper advertisements, and community presentations to study the effects of genetic and environmental parameters on racial/ethnic differences in metabolic outcomes. Exclusion criteria included diagnoses of type1 or type 2 diabetes, any glucose or lipid disorders, or use of any medication known to affect body composition or metabolism. Only children with a pubertal status ≤ 3 (determined by physician exam) according to the criteria of Marshall and Tanner were included.[17, 18] The study was approved by the University of Alabama at Birmingham (UAB) Institutional Review Board for Human Use, and each child and parent provided informed consent prior to participation.

Protocol

All data was collected during visits to the UAB General Clinical Research Center (GCRC) and the Department of Nutrition Sciences between 2004 and 2008. Data were obtained at two study visits that were no more than 30 days apart. During the first outpatient visit, pubertal status and body composition were measured and physical fitness assessed. At the second visit, participants were admitted to the UAB GCRC at approximately 1730 hours for an overnight stay. All participants consumed the same in-patient meal, and received only water or noncaloric, decaffeinated beverages after 2000 hours. Approximately 30 minutes after waking the following morning, resting metabolic rate (RMR) was measured and fasting blood samples obtained.

Body Composition

Body weight was measured to the nearest 0.1 kg in light clothing without shoes (Scale-tronix 6702W; Scale-tronix, Carol Stream, IL). Height was determined without shoes using a mechanical stadiometer. Body mass index (BMI; weight kg / height m^2) was calculated from these values, and BMI percentile determined using age- and sex-specific CDC growth charts (http://apps.nccd.cdc.gov/dnpabmi/). Waist circumference was measured by the same registered dietitian using a flexible tape measure (Gulick II; Country Technology, Inc., Gays Mills, WI) as described by Lohman et al. and recorded to the nearest 0.1 cm.[19]

Total fat mass and trunk fat mass were evaluated via dual energy x-ray absorptiometry (DXA) with a GE Lunar Prodigy densitometer (Lunar Radiation Corp., Madison, WI). Subjects were scanned in light clothing while lying flat on their backs with

arms at their sides. DXA scans were analyzed with pediatric software enCORE 2002 version 6.10.029.

Resting Metabolic Rate

Baseline energy expenditure is strongly associated with fasting NEFA levels.[4] Hence, resting metabolic rate (RMR) was measured in the morning following the overnight visit at approximately 0700 h immediately after awakening. Measurements were obtained with the participant lying supine on a bed in a quiet, well-ventilated room with the head enclosed in a plexiglass canopy. Participants were instructed not to sleep and remain quiet and still, breathing normally. A computerized, open-circuit, indirect calorimetry system with a ventilated canopy (Delta Trac II; Sensor Medics, Yorba Linda, CA) was used to measure RMR. After resting for fifteen minutes, one-minute average intervals of oxygen uptake (VO_2) and carbon dioxide production (CO_2) were measured continuously for thirty minutes. The last 20 minutes of measurement were used for analysis.

Physical Fitness

$VO_{2\text{-}170}$ was determined by indirect calorimetry on a treadmill with participants in the post-prandial state for a minimum of 3 hours. Children were given the opportunity to familiarize themselves with the treadmill prior to beginning the test. Participants then walked on a flat incline 2.5 mph for 4 minutes. Speed was then increased to 3 mph for the remainder of the test, with the incline increasing 2% every 2 minutes until the child reached a heart rate greater than 170 beats/minute. Heart rate (HR) was monitored with the Polar Vantage XL HR monitor (Polar Beat, Port Washington, NY). Volumes of O_2 and CO_2 were measured continuously using open circuit spirometry until recording the

VO_2 level at a heart rate of 170 beats per minutes using a Max-II metabolic testing system (PHYSIO-DYNE, Quogue, NY). Data were expressed as $VO_{2\text{-}170}$ adjusted for body weight ($mL \cdot kg^{-1} \cdot min^{-1}$).

Laboratory Analysis

Fasting blood samples were analyzed by the Metabolism Core Laboratory of the UAB Clinical Nutrition Research Center for concentrations of NEFA and insulin. NEFA were assayed using "NEFA-C" reagents (Wako Diagnostics, Richmond, VA; mean intra-assay coefficient of variation (CV) 3.89%; mean inter-assay CV 5.87% at NEFA concentration of 0.433 mEq/l). Minimum assay sensitivity was 0.0014 mEq/l. Additionally, fasting insulin was shown to be inversely associated with fasting NEFA levels.[20] Insulin was therefore assayed in duplicate 100-µl aliquots using reagents from Millipore Corporation (St. Charles, MO; sensitivity 3.35 µIU/ml; mean intra-assay CV 3.49%; mean inter-assay CV 5.57%).

Genotyping

Genotyping of the NPPA/NPR1 SNPs was performed at the UAB Genomics Core Laboratory via pyrosequencing. Each DNA sample was amplified using PCR with pooled biotinylated primers. PCR products were then captured with streptavin-coated beads and hybridized to sequence-specific primers and reactions run using the Biotage 96HS PSQ Pyrosequencing Machine (Biotage AB, Charlotte, NC). Forward (F), reverse (R), and sequencing (S) primers were designed for the following SNPs: for rs5063 (F) 5'ACTGGCATTCCAGCTCCTAGGTC'3, (R) 3'TGGCCCTACCTTGAAATCCATC'5, (S) CCCATGTACAATGCC; for rs5065 (F) 5'CTTGTCCTCCCTGGCTGTTATC'3, (R) 3'AGGATGGGCACACTCATACATG'5, (S) CTGTGTTCTCTTTGCAGTAC; for

rs198358 (F) 5'GGATGGATGCAGGAGCTGAAC'3, (R) 3'CTTGCTTTTGGTTTTGCAAGAAGAG'5, (S) CGACCACTGATGGAAC; and for rs10082235 (F) 5'CTTGCAGGCCAGATAGGAAGC'3, (R) 3' GGCAGGAACAAGGCTCATTC'5, (S) AATCATGCCCATGTTAG. Following the pyrosequencing process, genotype results were reviewed and confirmed by two separate investigators, with a minimum of 96.3% genotyping completion rate for all SNPs.

Genotyping of the ancestry informative markers (AIMs) for the measurement of genetic admixture was performed at Prevention Genetics (www.preventiongenetics.org) using the Chemicon Amplifuor SNPs Genotyping System coupled with ArrayTape technology (www.global-array.com). A panel of 140 ancestry informative markers was used to estimate the genetic admixture proportion of each subject. Molecular techniques for the allelic identification and methodology for genetic admixture application have been described elsewhere and information regarding marker sequences, experimental details, and parental population allele frequencies has been submitted to dbSNP (http://www.ncbi.nlm.nih.gov/SNP/) under the handle PSU-ANTH.[21] The ancestry estimates from AIMs was translated into estimates of African, European, and Native American admixture for each subject using maximum likelihood estimation based on the ML algorithm described by Hanis et al.[22] In brief, the maximum likelihood method estimates the proportion of genetic ancestry for an individual, using a range of proportions from 0 to 1 and identifies the most probable value of admixture based on the observed genotypes.

Socioeconomic Status

Socioeconomic status (SES) was measured with the Hollingshead 4-factor index of social class, which combines the educational attainment and occupational prestige for the number of working parents in the child's family.[23] Scores ranged from 8 to 66, with the higher scores indicating higher theoretical social status.

Statistical Analysis

All analyses were performed using SAS version 9.2 (SAS Institute, Inc., Cary, NC). Allele frequencies were computed and Hardy-Weinberg equilibrium (HWE) test performed using Haploview version 4.1 (Broad Institute, Cambridge, MA). Our sample included participants from separate racial/ethnic backgrounds, and allele frequency is known to differ by population background; hence, HWE was calculated within each racial/ethnic group. Additionally, the sum total of the three admixture estimates is equal to 1; therefore, European admixture was used as the reference group and only African and American admixture were included in the models. Participant characteristics were analyzed by NEFA tertile with analysis of variance with tukey's post-hoc test for group differences. Additive models for the association of each genotype with fasting NEFA were tested using analysis of covariance with least square means *p* values after adjusting for age, sex, trunk fat mass, RMR, fasting insulin, $VO_{2\text{-}170/kg}$, Amerindian and African genetic admixture, and SES. Linear regression was used to evaluate the association of each genotype separately with fasting NEFA using additive, dominant, and recessive models. All models were controlled for the above covariates, and continuous variables log transformed to obtain normality of residuals. To account for multiple hypothesis

testing we utilized the PROC MULTTEST procedure in SAS. Interaction terms were evaluated in models where the genotype was significantly associated with fasting NEFA.

RESULTS

Participant characteristics for the total sample and by NEFA tertile are presented in Table 1. Mean age of the participants was 9.6 ± 1.6 years with a BMI percentile of 65.8 ± 26.3%. Children with the highest fasting NEFA levels (tertile 3) presented with lower physical fitness ($VO_{2\text{-}170/kg}$) and RMR ($P < 0.05$). There were no significant differences in body composition among NEFA tertile groups.

Table 2 presents the minor allele frequency for each SNP studied. All SNPs were in HWE among each racial/ethnic group ($P > 0.05$). When analyzing mean differences in NEFA levels by genotype, fasting NEFA were significantly higher in participants with the rs10082235 TT minor allele (Figure 1). Additionally, linear regression results identified an association of the rs10082235 homozygous minor allele (TT; recessive model) with higher fasting NEFA ($P = 0.03$; Table 3). No other SNPs were associated with fasting NEFA levels; however, higher NEFA levels were also positively associated with greater Amerindian admixture ($P = 0.01$) and SES ($P < 0.01$). Higher RMR and physical fitness levels were associated with lower fasting NEFA (both at $P < 0.01$). No interaction between this SNP and any of the factors included in the model were identified.

DISCUSSION

Secretion of ANP has been shown to influence hypertension and cardiovascular risk, and more recently adipose tissue lipolysis.[12, 24, 25] Polymorphisms in the genes that

encode for ANP (NPPA gene) and its cell receptor (NPR1 gene) have previously been associated with blood pressure and/or cardiovascular function.[14, 15] It is therefore possible that these same polymorphisms could be associated with circulating NEFA levels. When we investigated the relationship between these SNPs and fasting NEFA in a multiethnic pediatric population, we found that the homozygous minor allele (TT) of rs10082235, a SNP in the NPR1 receptor gene, was associated with higher fasting NEFA. We also identified a positive association of Amerindian genetic admixture with NEFA, and consistent with previous studies, found an inverse association of fasting NEFA with RMR and fasting insulin levels. However, the relationship of rs10082235 with NEFA remained significant even after controlling for these factors.

To our knowledge, this is the first investigation into the relationship between NPPA/NPR1 genetic polymorphisms and NEFA levels. Sarzani et al. previously identified receptors (NPR-A) for ANP on the surface of human adipocytes, and it was found that exposure of these cells to ANP produced significant rates of lipolysis.[26, 27] Additional studies found that unlike catecholamines and insulin, which activate/impair lipolysis through a cAMP-mediated process, ANP stimulates lipolysis by way of cGMP, suggesting a unique, independent pathway for lipolysis regulation.[6] Levels of ANP were not measured for the present investigation; however, previous work has indicated that genetic polymorphisms in the NPR1 gene alter NPR-A receptor gene expression.[28] Hence, it is possible that allelic differences in rs10082235 could impact the number or effectiveness of NPR1 adipocyte receptors, with presence of the minor allele enhancing the efficiency of ANP action on adipocytes. Interestingly, a recent genome-wide association study (GWAS) among a multiethnic adult population showed an association

of the rs10082235 homozygous minor allele with decreased intima-mediated thickness (IMT), a marker of cardiovascular disease risk.[15] Additional investigations are needed to determine how rs10082235 allelic differences may alter gene expression of the NPR-A receptor, and how this change may impact adipocyte lipolysis and circulating NEFA levels.

Both NEFA and ANP levels among adults have been shown to differ between men and women. Even though our children were early pubertal as evidenced by a Tanner stage ≤ 3, we still detected sex differences in fasting NEFA, with higher levels among girls. However, these differences were attenuated when physical fitness was added to the model, suggesting that among children sex differences in fasting NEFA may be partially attributable to levels of fitness. Interestingly, Moro and colleagues have also shown that physical activity among adults results in acute increases in lipolysis and circulating NEFA.[29, 30] Physical activity influences fitness levels; however, it is unknown whether these variants in the NPPA/NPR1 genes could affect the impact of physical activity on fat utilization during exercise. Future investigations could determine whether adipocyte response to exercise varies by NPPA/NPR1 genotype, and whether this relationship could ultimately influence body fat levels or weight loss during physical activity interventions.

In addition to the association with the NPR1 polymorphism, a positive association between Amerindian genetic admixture and fasting NEFA was also noted. Racial/ethnic differences have been previously identified for fasting NEFA and several of the study covariates measured here, including RMR and fasting insulin levels.[20, 31] However, race/ethnicity represents a social (rather than biological) construct that is typically based on unique cultural and behavioral practices that could affect metabolic pathways and

disease risk independent of self-classified race. European and African immigrants to the American continent intermingled with the indigenous Amerindian groups, resulting in the present day admixed population.[32] Hence, individuals who identify culturally with one racial/ethnic group may present with genetic markers indicating that their recent ancestry includes influences from multiple racial/ethnic groups. Use of genetic admixture elucidates biological rather than environmental variance within individuals, and may be a more appropriate measure of ancestral contributions to disease risk.[33, 34] Our results suggest that individuals with greater levels of Amerindian admixture could present with high fasting NEFA levels independent of body fat or metabolic rate. Higher fasting NEFA are also associated with greater risk for nonalcoholic fatty liver disease, and children with high levels of Amerindian ancestry are noted to have increased risk for this condition.[35] Elucidation of the biological mechanisms related to higher fasting NEFA among individuals with greater Amerindian genetic ancestry could thus provide information regarding the increased risk for certain diseases experienced by these individuals.

This study benefited from the use of robust measures of body composition and RMR, factors previously shown to affect fasting NEFA, that are often not precisely measured in larger genome studies. When these variables were considered, only the polymorphism of the NPR1 receptor was associated with fasting NEFA. However, previous investigations of the association of NPPA with hypertension have indicated small effect sizes (association) with this gene,[14] and our sample size may have prevented us from detecting smaller contributions of these SNPs to the fasting NEFA levels. Additionally, we were unable to measure NEFA during physical activity, and it is

possible that effects of these SNPs could be detected during acute physiological increases of ANP. We were also unable to directly measure fasting ANP; however, previous investigations have indicated that circulating ANP levels and cell receptor activity differ with allelic differences in the NPPA and NPR1 genes.[28, 36]

In conclusion, this study among a multiethnic sample of boys and girls is one of the first to show an association of rs10082235, a SNP in the NPR1 gene, with fasting NEFA levels. This suggests that genetic polymorphisms associated with the atrial natriuretic peptide gene system could impact adipocyte lipolysis and circulating NEFA, thereby influencing risk for chronic diseases that are impacted by differences in NEFA.

ACKNOWLEDGMENTS

The authors would like to thank Robert Petri and Alexandra Luzuriaga-McPherson for their assistance with data collection. We would also like to thank the Nutrition Obesity Research Center Genetics Core and the UAB Genomics Core Laboratory for genotyping of the NPPA/NPR1 polymorphisms. This work was funded by the National Institutes of Health grants R01-DK067426, P30-DK56336, P60-DK079626, M01-RR-00032, and the National Cancer Institute Cancer Prevention and Control Training Program CA47888.

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Table 1. Baseline characteristics in total sample and by NEFA tertiles (mean ± SD; n(%))

Variable	Total Sample	Tertile 1	Tertile 2	Tertile 3
n	293	97	98	98
Age (years)	9.6 ± 1.6	9.4 ± 1.6	9.7 ± 1.6	9.6 ± 1.6
Sex (n, % female)	142 (48.5)	36 (37.1)	47 (48.0)	59 (60.2)
Tanner Stage	1.5 ± 0.7	1.4 ± 0.7	1.6 ± 0.8	1.5 ± 0.8
Height (cm)	139.6 ± 10.6	138.7 ± 10.7	141.2 ± 10.2	138.8 ± 10.6
Weight (kg)	36.6 ± 9.6	36.0 ± 8.9	38.1 ± 10.4	35.7 ± 9.3
BMI	18.6 ± 3.0	18.5 ± 2.7	18.9 ± 3.4	18.3 ± 3.0
BMI percentile	65.8 ± 26.3	67.4 ± 25.3	66.3 ± 26.6	63.8 ± 26.9
Lean Mass (kg)	25.6 ± 5.2	25.9 ± 5.1	26.3 ± 5.5	24.7 ± 4.8
Fat Mass (kg)	8.9 ± 5.7	8.1 ± 5.0	9.9 ± 6.4	8.7 ± 5.6
Trunk Fat Mass (kg)	3.7 ± 2.8	3.2 ± 2.4	4.1 ± 3.2	3.6 ± 2.8
$VO_{2\text{-}170}$ (ml/kg/min)	29.7 ± 6.1	31.0 ± 7.0[a]	29.5 ± 5.7[ab]	28.3 ± 5.2[b]
RMR (kcal/day)	1192.0 ± 235.9	1228.5 ± 239.3[a]	1202.1 ± 225.1[ab]	1144.4 ± 237.3[b]
Insulin (mEq/l)	12.8 ± 5.6	12.8 ± 5.6	13.4 ± 6.6	11.9 ± 6.0
Genetic Admixture:				
European (%)	53.3 ± 37.7	56.5 ± 38.1	50.0 ± 37.4	53.4 ± 37.8
African (%)	28.9 ± 37.5	26.5 ± 36.4	31.2 ± 38.5	29.0 ± 37.8
Amerindian (%)	17.8 ± 25.2	17.0 ± 24.4	18.8 ± 26.1	17.7 ± 25.4
SES	12.7 ± 6.1	37.8 ± 14.7	38.3 ± 14.6	40.4 ± 14.1
NEFA (mEq/l)	0.5 ± 0.2	0.3 ± 0.1[a]	0.5 ± 0.1[b]	0.7 ± 0.1[c]

[a,b,c] superscripts indicate group difference at $P < 0.05$; NEFA = nonesterified fatty acids; BMI = body mass index (weight kg/height m^2); RMR = resting metabolic rate; SES = socioeconomic status

Table 2. Genetic polymorphisms studied

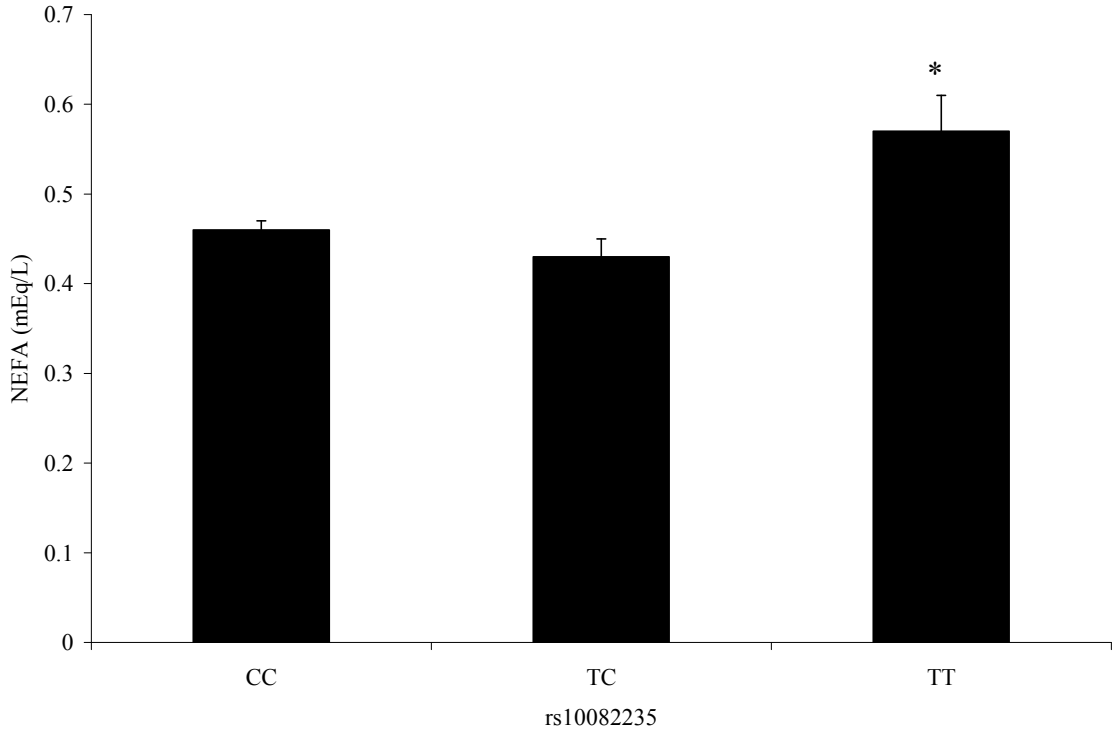
Polymorphism	Location	Minor allele	MAF	% Genotyped
NPPA:				
rs5063	Exon 1 (*Met*32*Val*)	A	0.042	98.7%
rs5065	Exon 3 (*Arg*152*Ter*)	C	0.233	98.7%
rs198358	3' UTR	G	0.329	96.3%
NPR1:				
rs10082235	Intron 1	T	0.215	98.3%

MAF = minor allele frequency

Table 3. Linear regression association of rs10082235 minor allele with NEFA levels

Variable	ß	*P* value
Age	0.59	<0.01
Sex (female)	0.12	0.06
Trunk Fat	0.10	0.13
Resting Metabolic Rate	-0.66	<0.01
Fasting Insulin	-0.23	<0.01
$VO_{2\text{-}170}$/kg	-0.29	0.08
Amerindian Admixture	0.38	0.01
African Admixture	0.09	0.35
Socioeconomic Status	0.23	<0.01
rs10082235 (TT)	0.30	0.03

Figure 1. Least square means NEFA by genotype



NEFA = nonesterified fatty acids; * = significant difference $P < 0.05$ (means adjusted for age, sex, trunk fat mass, resting metabolic rate, insulin, $VO_{2\text{-}170/kg}$, Amerindian admixture, African admixture, socioeconomic status)

GENERAL DISCUSSION

Hypertension and elevated NEFA levels are each independently related to increased risk for cardiovascular disease, stroke, and certain cancers.[3, 136-138] The etiology of abnormal blood pressure appears to be multifactorial and individuals may be symptomatic during childhood. The present project identified complex physiological relationships that provide insights into the understanding of mechanisms associated with risk for hypertension and elevated fasting NEFA levels, in particular:

1) Excess total and central adiposity are associated with elevated blood pressure.[144] However, among children the relationship of each factor with height increases the difficulty of evaluating these associations in pediatric studies. We identified waist circumference as the measure most associated with blood pressure that should therefore be controlled for in statistical models evaluating the association of NPPA/NPR1 with hypertension risk.

2) Physical fitness and physical activity are also each associated with hypertension risk and NEFA levels among adults.[145, 146] However, among children it is unclear whether fitness or activity plays a stronger role in blood pressure control. We identified physical fitness as the factor associated with pediatric blood pressure.

3) Physical fitness and excess body fat are also inversely associated in children.[147] However, few pediatric studies control for this relationship when evaluating factors that influence pediatric fitness. We found that body fat, rather than BMI percentile, was more

strongly associated with fitness level, and potentially indirectly influences fasting NEFA levels and hypertension risk.

4) An association has been noted between elevated blood pressure and higher fasting NEFA levels among adults.[148] We found that this association is not present among children with low European racial ancestry.

A general discussion of these issues is therefore warranted, along with a review of the relationship of ANP and NPPA/NPR1 with blood pressure and NEFA.

Body composition and blood pressure

In investigations for aim 1, we found that waist circumference adjusted for height was the body composition measure associated with blood pressure among children. Several previous studies have found an association of pediatric hypertension with BMI, percent body fat, total fat mass, and waist circumference. These findings would suggest that any excess accumulation of adiposity is detrimental to blood pressure control. However, Zhang et al. found a stronger association of waist circumference with blood pressure among adults, while Watts and colleagues noted a similar relationship in children.[149-151] Other investigations found no difference in the association of BMI or waist circumference with pediatric blood pressure.[152-154]

One reason for this discrepancy could be the dual association of pediatric height with body fat and blood pressure. Children generally experience a linear increase in both body weight and blood pressure with increases in height. Thus, including both height and body weight in a statistical model could result in a spurious association of body weight with hypertension risk. One way to avoid this issue is to adjust body weight for height, as with BMI.[155-157] However, Maynard et al. reported that increases in BMI and BMI

percentile among children are more strongly associated with fat free mass than fat mass. Similar limitations have been noted in the use of percent body fat among children.[67] Therefore, the use of allometric scaling, to adjust body size (weight) by body shape (height) through log-log regression analysis[158-160] allowed us to evaluate the independent association of each body fat measure with pediatric blood pressure, leading to association of waist circumference and blood pressure in this study.

The mechanism in which greater waist circumference could contribute to hypertension risk remains to be elucidated. However, increased levels of macrophages, white blood cells involved in immune response, have been noted among adults with central obesity. The presence of excess macrophages is associated with decreased flexibility of the vessels and worsening kidney function, two factors that could impair blood pressure control.[161-163] It remains to be seen, though whether this condition could significantly impair blood pressure control among children. Additionally, it is unclear whether total abdominal adiposity contributes to hypertension risk, or whether a specific fat depot (i.e., subcutaneous versus intraabdominal fat) is responsible for this association. Some studies note an association between intraabdominal fat and blood pressures, though Jensen *et al.* and others propose that subcutaneous fat might play a greater role in disease risk.[164-166] We were unable to test for these relationships in the present study. Interestingly, though, we noted high rates of hypertension among AA compared to HA children, even though HA participants presented with a higher waist circumference. It is therefore possible that additional genetic and environmental factors that we did not control for play a stronger role in pediatric hypertension risk that adiposity levels.

Physical fitness and blood pressure

One environmental factor that may have also been associated with blood pressure levels is physical fitness/activity,[167, 168] and for aim 2 we investigated the association of each of these factors with hypertension risk. We found that physical fitness alone was inversely associated with pediatric blood pressure. This observation is interesting since physical fitness and physical activity represent two distinct, but related, concepts. Physical activity is a measure of the amount and intensity of movement performed by an individual over a given time period. Physical activity can be subdivided into minutes per day spent at a moderate versus vigorous activity level. Activity levels are thus considered an environmental measure that can affect disease risk, although the propensity to perform various amounts or intensities of activity may have a genetic component. Conversely, physical fitness is a measure of the body's oxygen-carrying capacity during maximum exercise effort, and is thus considered a biological measure that is influenced by genetics and environment. Fitness and activity are interrelated in that adequate levels of physical activity are positively associated with fitness.[169, 170] However, individuals with high levels of overall activity will not necessarily present with high physical fitness.[171] To improve physical fitness, sufficient vigorous activity is required.[172] This association of higher physical activity with greater fitness was also noted among our study participants, though again not all participants who were highly active also had high physical fitness (Figure 1). Unfortunately, our participants overall participated in very low levels of vigorous physical activity, and we were unable to test the association of vigorous activity alone with blood pressure. However, other studies have shown that vigorous activity may only have a secondary association with blood pressure through its

positive effects on physical fitness, suggesting that physical fitness is factor actually associated with pediatric blood pressure.[173]

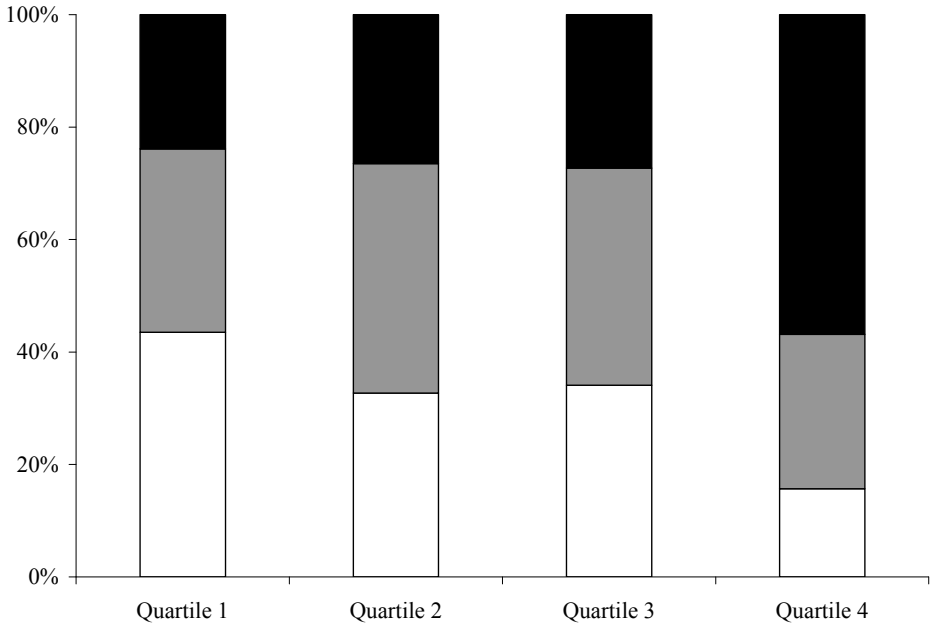


Figure 1. Proportion of participants in physical fitness tertiles by physical activity quartile. Quartile 1 = lowest activity level; quartile 4 = highest activity level. White bars = low fitness; gray bars = medium fitness; black bars = high fitness

We were unable to determine from this study precisely how physical fitness level may affect hypertension risk. However, other investigators have found that greater fitness is associated with decreased inflammation and increased flexibility of the vascular walls, both factors which could improve blood pressure control.[174]

Body composition and physical fitness

An association of both body fat and physical fitness with blood pressure is present in our study population. Hence in aim 3 of this study we investigated the relationship of these two factors with each other. Central adiposity is most associated with blood pressure; however, total body fat can impact the results of physical fitness levels in

children. Higher body weight in general places a greater mechanical work load on the body, and studies have shown that when objective measures of physical fitness, such as $VO_{2\text{-}170}$, are adjusted for total body weight they are lower among individuals classified as overweight or obese.[175, 176] However, increased lean mass is associated with increased fitness, and BMI or BMI percentile is usually the method by which individuals are evaluated as normal weight, overweight, and obese. As discussed earlier pediatric BMI is a poor indicator of excess body fat.[67] Standardized cut-points for excess pediatric body fat have not been established; however, Williams et al. found that among a multiethnic cohort chronic disease risk factors were greatest among boys with over 25% body fat and girls with over 30% fat.[177] We found striking differences in body fat levels among children by BMI category, with over half of children classified as overweight having low body fat levels. Most interestingly, children with low body fat presented with greater physical fitness independent of which BMI category they were placed in. Previous investigations have found that physical fitness is impaired among individuals with less oxygen-carrying capacity in the blood.[178] It is also possible that the presence of excess body fat results not just in increased mechanical load, but also impacts vascular reactivity during stress (i.e., exercise), which would impair the ability to improve physical fitness. We were unable to detect a stronger association of waist circumference versus total body fat with physical fitness, though, and it remains unclear whether these two factors may impact blood pressure through separate or interactive mechanisms.

Blood pressure and NEFA

We also evaluated whether there was an association between NEFA and blood pressure levels. Few studies have investigated the relationship of blood pressure with

circulating NEFA among children. An association of higher fasting NEFA with hypertension is present among adults, and studies of animals and human adults have found that blood pressure increases during intravenous lipid infusion.[179, 180] It has been proposed that prolonged elevated NEFA contributes to endothelial dysfunction in the vascular walls, which could impair blood pressure control.[181] We thus hypothesized that when evaluating the association of genetic variants with fasting NEFA, we would need to include hypertension incidence as a covariate in statistical models. However, these studies have typically included small samples sizes and predominately European-American participants. Using a multiethnic cohort, we identified an interaction between hypertension and racial ancestry, indicating that fasting NEFA levels were indeed higher among children with hypertension who also had medium-high levels of European ancestry. However, among children with the lowest levels of European ancestry there was no change in fasting NEFA levels with hypertension incidence. It is unclear from the current literature why we identified this association within our study population. Goran *et al.* has previously discussed the possibility that disease etiologies could differ between racial/ethnic groups.[182] This finding highlights the importance of considering genetic background when investigating risk factors for chronic diseases.

Genetic admixture and disease risk

Throughout the current study, independent associations of racial ancestry with blood pressure, physical fitness, body composition, and NEFA were identified. As previously described, racial ancestry is estimated using genetic admixture analysis.[183] However, most literature investigating chronic disease risk classifies individuals by racial/ethnic group. For the current investigation, these groups would include European-

American, African-American, and Hispanic-American classification. Use of these categories in research can confound the true association between biological factors that influence disease risk versus cultural/environmental factors associated with racial/ethnic groups (i.e. attitudes about physical fitness, activity and body size). Additionally, such classification prevents the inclusion of recently-admixed individuals in research studies. Most studies require that a participant's parents and grandparents all claim to be from the same racial/ethnic group in order for the participant to be included as a study subject. As the number of individuals born into multicultural families increases, research studies will need to include these individuals. Genetic admixture analysis represents one way in which the genetic contributions of racial ancestry can be explored independent of socioenvironmental confounders. Our data supports that, compared to racial/ethnic classification, utilization of genetic admixture is a more appropriate approach when evaluating biological contributions to racial disparities in hypertension risk.

NPPA and NPR1 genes

Using the results of the initial study aims to determine model covariates, we evaluated the association of NPPA/NPR1 genes with blood pressure and fasting NEFA. This study is among the first to evaluate these associations within the pediatric population, and suggest that the ANP system may indeed play a role in disease risk even at a young age.

NPPA/NPR1 and blood pressure

Several candidate gene studies and genome-wide association studies (GWAS) have tested for genetic associations with blood pressure among adults, with equivocal results.[184-187] We noted a trend for increased prehypertension/hypertension incidence

with the "T" allele of rs10082235 in the NPR1 cell receptor gene, which has previously only been studied in the context of cardiovascular disease GWAS.[188] Studies have shown that genetic polymorphisms in the NPR1 gene can decrease production of natriuretic peptide receptor A, though however additional studies are need to determine whether this particular SNP has the same effect on cell receptor number.[189] However, our finding that the "A" allele of rs5063 in the NPPA gene is associated with decreased hypertension risk among children is consistent with the literature, and the among the first to identify this association in children. Of note, none of our participants were homozygous for the "A" allele of rs5063, which is consistent with the allele frequencies published for European, African, and Amerindian populations (http://hapmap.ncbi.nlm.nih.gov/cgi-perl/gbrowse/hapmap27_B36/#search). Interestingly, the A/A homozygous genotype has been identified in other studies as protective against hypertension, stroke, and cardiovascular risk factors.[190, 191] However, not all studies have found an association of this allele with blood pressure.[192, 193] Among our participants, no individuals with the G/A allele presented with hypertension, yet prehypertension was present among individuals with this genotype, suggesting that additional genetic and environmental factors measured here may interact with this gene to influence ANP levels. Additionally, studies are equivocal on whether allelic differences in rs5063 are associated with alterations in ANP secretion.[194, 195] It is possible that this SNP is in linkage disequilibrium (non-random association; i.e., inherited together) with another genetic polymorphism that actually affects ANP production or secretion. However, the consistent association of rs5063 with hypertension across studies suggest that additional

investigations beyond the current study are needed to determine the true relationship of this polymorphism with blood pressure.

NPPA/NPR1 and NEFA

To date, no investigations have evaluated the association with NPPA/NPR1 genetic polymorphisms with NEFA levels. As described in the introduction, ANP secretion results in an increase in circulating NEFA among adults.[196-198] Hence, genetic polymorphisms in the genes affecting this system could impact fasting NEFA. We found that the homozygous TT allele of rs10082235, a SNP in the NPR1 cell receptor gene, is associated with higher fasting NEFA levels. Interestingly, a trend for was also noted for an association of this allele with hypertension risk. We are unable to determine from the present investigation why a polymorphism in the cell receptor, but not the gene encoding for the peptide itself, was associated with NEFA levels. Moro et al. showed that adipocytes exhibit rapid response to ANP-stimulation of lipolysis during exercise, a condition that stimulates atrial stretch and promotes release of ANP into circulation.[199] The number of receptors available on the cell surface, rather the overall amount of ANP available, may therefore be most predictive of the adipocyte response to ANP secretion. Once secreted, however, ANP has a half-life of approximately 2-4 minutes, and it is also possible that had we measured NEFA levels during physical activity we would have seen an association with NPPA genetic polymorphisms.[200] Additional studies are thus needed to determine precisely how this allele influences NPR1 gene function, and whether the association of NEFA with NPPA/NPR1 genes varies during conditions of fasting versus exercise.

Taken together, these findings suggest that NPPA/NPR1 genetic polymorphisms may impact circulating NEFA levels and blood pressure controls. The NPPA/NPR1 genes may therefore indirectly impact the risk for diseases associated with increased NEFA and blood pressure, including hypertension, stroke, cardiovascular disease, and kidney cancer. However, future investigations should also measure plasma ANP levels to determine whether genetic polymorphisms in the NPPA gene are in fact associated with circulating ANP, which we were unable to do in the present investigation. Additionally, polymorphisms in the gene that encodes for the receptor responsible for clearance of ANP from the cell may also impact ANP levels.[201-203] Polymorphisms in this gene, however, are not well-studied and hence were not included in this investigation.

Summary

Atrial natriuretic peptide, i.e., ANP, has been shown to directly influence levels of blood pressure and NEFA, and genetic polymorphisms in the genes that encode for ANP and its cell receptor have been implicated in hypertension risk. However, additional factors such as body composition, physical fitness and activity, and racial genetic ancestry may also influence blood pressure and NEFA. Our results from experimental aim 1 suggest that central adiposity rather than total adiposity *per se* is associated with pediatric hypertension risk. Results from experimental aim 2 suggest that physical fitness, rather than physical activity, is associated with hypertension risk and should be measured when considering factors that impact blood control. In experimental aim 3, findings indicate that physical fitness varies more among differing levels of adiposity rather than total body weight. Experimental aim 4 results suggest that the association between hypertension and increased fasting NEFA may differ by racial genetic ancestry.

Results from experimental aim 5 suggest that genetic polymorphisms in the ANP cell receptor may influence NEFA and blood pressure levels, while a polymorphism in the NPPA gene associated with hypertension risk in adults may also influence blood pressure control in children. Results of all aims suggest a contribution of genetic factors associated with racial genetic ancestry to both blood pressure levels and fasting NEFA.

Figure 2 presents a summary of the associations identified in this study. As noted in Figure 2a, Physical fitness is inversely associated with high blood pressure and high fasting NEFA. Fitness itself is influenced by levels of total body fat and physical activity, and may thus serve as a mediator between these factors and NEFA/blood pressure. Though not tested in this study, physical activity is also shown to be inversely associated with total body fat and waist circumference,[204] and may also indirectly influence blood pressure levels in this manner. Waist circumference is positively associated with high blood pressure, though neither body fat measure is directly associated with fasting NEFA levels. Additionally, there is no direct association of NEFA and blood pressure levels. Polymorphisms in the NPPA and NPR1 gene are associated with blood pressure levels, while only rs10082235 in the NPR1 gene is associated with fasting NEFA levels.

Figure 2b includes the association of racial genetic ancestry with the study variables. Amerindian genetic admixture is positively associated with fasting NEFA, while African Admixture is 1) negatively associated with physical fitness, total body fat, and waist circumference, and 2) positively associated with high blood pressure. Additionally, there is an interactive effect of low European genetic admixture with hypertension, and among

individuals with greater European genetic admixture hypertension is associated with higher fasting NEFA.

In summary, this study further elucidates which factors are associated with increased risk for elevated fasting NEFA and blood pressure. The results suggest that genetic factors play a role in the development of both conditions. They also suggest that among children who are not morbidly obese, physical fitness may play a greater role than body fat in risk for hypertension and high fasting NEFA levels.

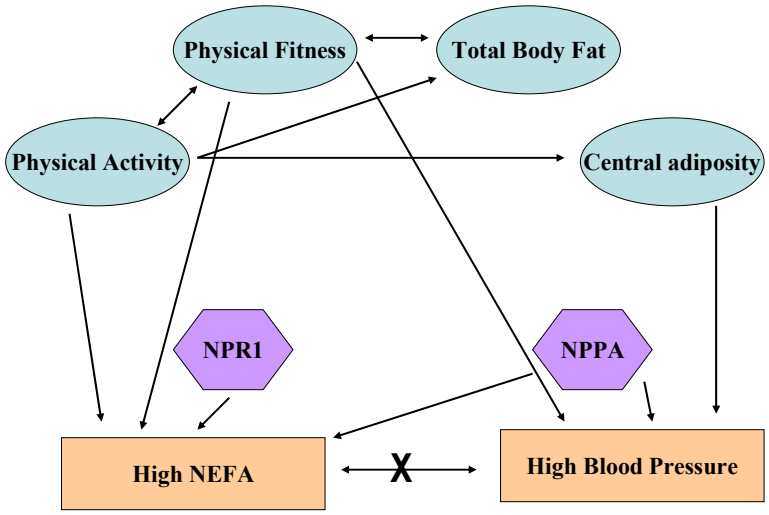


Figure 2a.

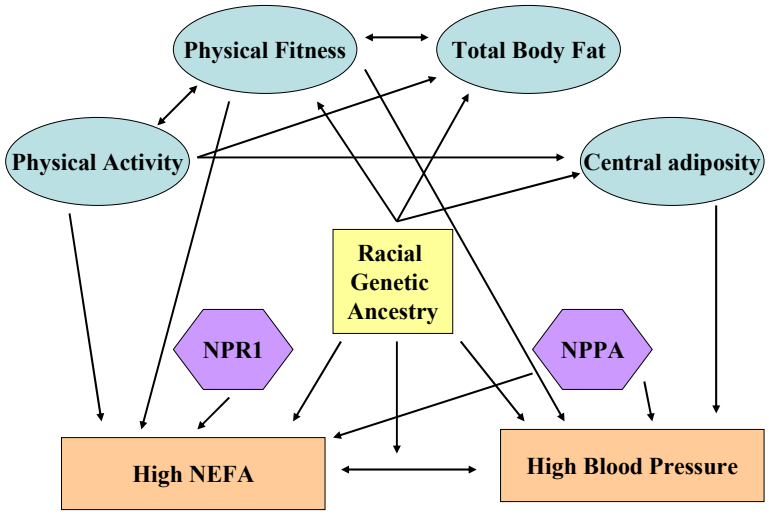


Figure 2b.

Figure 2. Association of study variables with fasting NEFA and blood pressure

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APPENDIX A

LIST OF ANCESTRY INFORMATIVE MARKERS

ANCESTRY INFORMATIVE MARKERS

rs6003	rs2118350	rs2542481	rs638169
rs2814778	rs16834947	rs2695	rs7163836
rs2752	rs9809114	rs1980888	rs2228478
rs140864	rs9847307	rs1327805	rs292932
rs905595	rs1112828	rs2789823	rs764679
rs2340727	rs719776	rs10756789	rs17822931
rs12737539	rs1403454	rs7854707	rs12598094
rs2806424	rs1229984	rs1891760	rs2549156
rs1780349	rs12647878	rs2207782	rs2816
rs1008984	rs2877967	rs1594335	rs717962
rs832173	rs3340	rs2776937	rs1074075
rs723822	rs3309	rs11004105	rs1003645
rs1506069	rs1461227	rs1800498	rs345162
rs725416	rs16891982	rs594689	rs3760281
rs725667	rs463717	rs1042602	rs7211872
rs2225251	rs1881826	rs1487214	rs1058115
rs10752631	rs1935946	rs624328	rs8072587
rs7531501	rs2077681	rs12277775	rs1369290
rs3287	rs951554	rs174548	rs386569
rs1435090	rs222541	rs726391	rs2216595
rs1526028	rs1044498	rs2078588	rs12459172
rs1861498	rs3123101	rs717091	rs602662
rs1320149	rs2763	rs3782972	rs7246566
rs2045431	rs2161	rs9542741	rs718092
rs2292549	rs1320892	rs10483251	rs2426512
rs2284708	rs2341823	rs911041	rs11467165
rs3827760	rs2396676	rs11160369	rs16383
rs12692384	rs7795585	rs8007610	rs878825
rs2710663	rs7786541	rs4646	rs2157257
rs4676223	rs344461	rs2862	rs732381
rs17203	rs2671110	rs2351254	rs1986586
rs768324	rs4717627	rs724729	rs1415878
rs938431	rs285	rs275183	rs2188457
rs1316579	rs983271	rs734780	MID377
rs1465648	rs1373302	rs12439722	
rs1352158	rs1808089	rs3751631	

APPENDIX B

IRB APPROVAL FORMS

Project Revision/Amendment Form

(PLEASE TYPE: In MS Word, highlight the shaded, underlined box and replace with your text; double-click checkboxes to check/uncheck.)

- Federal regulations require IRB approval before implementing proposed changes.
- Change means any change, in content or form, to the protocol, consent form, or any supportive materials (such as the Investigator's Brochure, questionnaires, surveys, advertisements, etc.).
- Complete this form and attach the changed research documents.

Today's Date: *12/2/2008*

1. Contact Information

Principal Investigator's Name: *Fernandez, Jose* BlazerID: *jose@uab.edu* E-mail: *jose@uab.edu*
Contact Person's Name: *Paul Zuckerman* BlazerID: *dannyz* E-mail: *dannyz@uab.edu*
Telephone: *4-4386* Fax: *4-7049*
Campus Address: *Webb 409*

2. Protocol Identification

Protocol Title: *Admixture Mapping for Racial/Ethnic Insulin Complex Outcomes (AMERICO)*
IRB Protocol Number: F040109007____

Current Status of Project (check only one):

- ☒ Currently in Progress (Number of participants entered: *320*)
- ☐ Study has not yet begun (No participants entered)
- ☐ Closed to participant enrollment (remains active)—
  Number of participants on therapy/intervention:____
  Number of participants in long-term follow-up only:____
- ☐ Closed to participant enrollment (data analysis only)—
  Total number of participants enrolled:____

DEC - 2 2008

This submission changes the status of this study in the following manner (check all that apply):

- ☐ Protocol Revision
- ☐ Protocol Amendment
- ☐ Study Closed to participant entry
- ☐ Study Closure
- ☐ Other, (specify) ____
- ☐ Revised Consent Form
- ☐ Addendum (new) consent form
- ☐ Enrollment temporarily suspended by sponsor
- ☒ Change in protocol personnel

3. Reason for change

Briefly describe, and explain the reason for, the change. If normal, healthy controls are included, describe in detail how this change will affect those participants.
Include a copy of the protocol and any other documents affected by this change (e.g., consent form, questionnaire) with all the changes highlighted.
We are adding Amanda Willig as a co-investigator for the purpose of completing her doctoral dissertation using data acquired from the AMERICO study. The title of her dissertation is "Contributions of the Atrial Natriuretic Peptide Gene System to the Relationship between Pediatric Body Fat, Fatty Acids, and Blood Pressure". This research is an integral part of the original AMERICO concept and requires no additional specimens. Ms Willig has not started any analysis pending IRB approval.

4. Does this change revise or add a genetic or storage of samples component?

☐Yes ☒No

If yes, please see the Guidebook to assist you in revising or preparing your submission, or call the IRB office at 934-3789.

5. Does the change affect subject participation (e.g., procedures, risks, costs, location of services, etc.)?

☐Yes ☒No

If yes, Fiscal Approval Process (FAP)-designated units complete a FAP submission and send to fap@uab.edu. For more on the UAB FAP, see www.uab.edu/ohr.

6. Does the change affect the consent document(s)?

☐Yes ☒No

If yes, briefly discuss the changes.

Will any participants need to be reconsented as a result of the changes? ☐Yes ☒No
If yes, when will participants be reconsented?____

Signature of Principal Investigator Jose R. Fernandez Date 12-02-08
Dec 8-2008

☒Approved Expedited ☐To Convened IRB

Chair or Vice-Chair Date
Dec 4, 2008

OMB No. 0990-0263
Approved for use through 1/31/2012

Protection of Human Subjects
Assurance Identification/IRB Certification/Declaration of Exemption
(Common Rule)

Policy: Research activities involving human subjects may not be conducted or supported by the Departments and Agencies adopting the Common Rule (56FR28003, June 18, 1991) unless the activities are exempt from or approved in accordance with the Common Rule. See section 101(b) of the Common Rule for exemptions. Institutions submitting applications or proposals for support must submit certification of appropriate Institutional Review Board (IRB) review and approval to the Department or Agency in accordance with the Common Rule.

Institutions must have an assurance of compliance that applies to the research to be conducted and should submit certification of IRB review and approval with each application or proposal unless otherwise advised by the Department or Agency.

1. Request Type	2. Type of Mechanism	3. Name of Federal Department or Agency and, if known, Application or Proposal Identification No.
[ ] ORIGINAL [X] CONTINUATION [ ] EXEMPTION	[ ] GRANT [ ] CONTRACT [ ] FELLOWSHIP [ ] COOPERATIVE AGREEMENT [ ] OTHER:_____	
4. Title of Application or Activity Admixture Mapping for Insulin Complex Outcomes (AMERICO STUDY)		5. Name of Principal Investigator, Program Director, Fellow, or Other FERNANDEZ, JOSE R

6. Assurance Status of this Project *(Respond to one of the following)*

[X] This Assurance, on file with Department of Health and Human Services, covers this activity:
Assurance Identification No. FWA00005960, the expiration date 01/23/2012 IRB Registration No. IRB00000196

[ ] This Assurance, on file with *(agency/dept)*_____, covers this activity.
Assurance No._____, the expiration date_____ IRB Registration/Identification No._____*(if applicable)*

[ ] No assurance has been filed for this institution. This institution declares that it will provide an Assurance and Certification of IRB review and approval upon request.

[ ] Exemption Status: Human subjects are involved, but this activity qualifies for exemption under Section 101(b), paragraph_____.

7. Certification of IRB Review (Respond to one of the following IF you have an Assurance on file)

[X] This activity has been reviewed and approved by the IRB in accordance with the Common Rule and any other governing regulations.
by: [X] Full IRB Review on (date of IRB meeting) 7/29/2009 or [ ] Expedited Review on (date) _____
[ ] If less than one year approval, provide expiration date _____

[ ] This activity contains multiple projects, some of which have not been reviewed. The IRB has granted approval on condition that all projects covered by the Common Rule will be reviewed and approved before they are initiated and that appropriate further certification will be submitted.

8. Comments
Protocol subject to Annual continuing review.

Title F040109007
Admixture Mapping for Insulin Complex Outcomes (AMERICO STUDY)

IRB Approval Issued: 7/31/09

9. The official signing below certifies that the information provided above is correct and that, as required, future reviews will be performed until study closure and certification will be provided.	10. Name and Address of Institution University of Alabama at Birmingham 701 20th Street South Birmingham, AL 35294
11. Phone No. *(with area code)* (205) 934-3789 12. Fax No. *(with area code)* (205) 934-1301 13. Email: smoore@uab.edu	
14. Name of Official Albert Oberman, M.D., MPH	15. Title Vice Chair, IRB
16. Signature Albert Oberman, MD, MPH	17. Date 7/31/09

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THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Review Board for Human Use

MEMORANDUM

TO: Jose R. Fernandez, PhD
Principal Investigator
FAX: 4-7049 (Zuckerman)

FROM: Denise Ball Denise Ball/te
On behalf of IRB 01

DATE: July 7, 2010

RE: F040109007
Admixture Mapping for Insulin Complex Outcomes (AMERICO STUDY)

The IRB 01 met on **July 7, 2010** and approved the protocol referenced above. The approval form is enclosed. **This approval will expire and no longer be valid on July 7, 2011.**

Please note the following as related to this review:

- Each time you submit renewal materials, you should include a memorandum that confirms or reassigns the Children's Risk Level (CRL). If you propose a change to the CRL, you will need to explain what changes in the research warrant the change.

- The IRB noted this protocol was permanently closed to enrollment and may qualify, in the future, for expedited review under Category 8. If the research-related follow-up requires radiographic procedures such as X-rays, the protocol will not qualify for expedited review. Expedited review would be appropriate where
  (i) the research is permanently closed to the enrollment of new subjects; and
  (ii) all subjects have completed all research-related interventions; and
  (iii) the research remains active only for long-term follow-up of subjects; or
  where no subjects have been enrolled and no additional risks have been identified; or
  where the remaining research activities are limited to data analysis.
  A change in the protocol may result in convened IRB review being required. To apply for expedited review, please refer to Section 10 of the IRB Guidebook.

- The UAB online course about informed consent is the only option approved for continuing IRB training credit in 2009-2010. That course must be completed by all protocol staff before December 31, 2010. For complete details, please see http://www.uab.edu/irb/2009.

TC/clp

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OMB No. 0990-0263
Approved for use through 1/31/2012

Protection of Human Subjects
Assurance Identification/IRB Certification/Declaration of Exemption
(Common Rule)

Policy: Research activities involving human subjects may not be conducted or supported by the Departments and Agencies adopting the Common Rule (56FR28003, June 18, 1991) unless the activities are exempt from or approved in accordance with the Common Rule. See section 101(b) of the Common Rule for exemptions. Institutions submitting applications or proposals for support must submit certification of appropriate Institutional Review Board (IRB) review and approval to the Department or Agency in accordance with the Common Rule. Institutions must have an assurance of compliance that applies to the research to be conducted and should submit certification of IRB review and approval with each application or proposal unless otherwise advised by the Department or Agency.

1. Request Type	2. Type of Mechanism	3. Name of Federal Department or Agency and, if known, Application or Proposal Identification No.
[ ] ORIGINAL [X] CONTINUATION [ ] EXEMPTION	[ ] GRANT [ ] CONTRACT [ ] FELLOWSHIP [ ] COOPERATIVE AGREEMENT [ ] OTHER:_____	
4. Title of Application or Activity Admixture Mapping for Insulin Complex Outcomes (AMERICO STUDY)		5. Name of Principal Investigator, Program Director, Fellow, or Other FERNANDEZ, JOSE R

6. Assurance Status of this Project *(Respond to one of the following)*

[X] This Assurance, on file with Department of Health and Human Services, covers this activity:
Assurance Identification No. FWA00005960, the expiration date 10/26/2010 IRB Registration No. IRB00000196

[ ] This Assurance, on file with *(agency/dept)*_____, covers this activity.
Assurance No._____, the expiration date_____ IRB Registration/Identification No._____*(if applicable)*

[ ] No assurance has been filed for this institution. This institution declares that it will provide an Assurance and Certification of IRB review and approval upon request.

[ ] Exemption Status: Human subjects are involved, but this activity qualifies for exemption under Section 101(b), paragraph_____.

7. Certification of IRB Review (Respond to one of the following IF you have an Assurance on file)

[X] This activity has been reviewed and approved by the IRB in accordance with the Common Rule and any other governing regulations.
by: [X] Full IRB Review on (date of IRB meeting) 7/7/2010 or [ ] Expedited Review on (date) _____
[ ] If less than one year approval, provide expiration date _____

[ ] This activity contains multiple projects, some of which have not been reviewed. The IRB has granted approval on condition that all projects covered by the Common Rule will be reviewed and approved before they are initiated and that appropriate further certification will be submitted.

8. Comments	Title F040109007
Protocol subject to Annual continuing review.	Admixture Mapping for Insulin Complex Outcomes (AMERICO STUDY)

IRB Approval Issued: 07-07-10

9. The official signing below certifies that the information provided above is correct and that, as required, future reviews will be performed until study closure and certification will be provided.	10. Name and Address of Institution University of Alabama at Birmingham 701 20th Street South Birmingham, AL 35294
11. Phone No. *(with area code)* (205) 934-3789 12. Fax No. *(with area code)* (205) 934-1301 13. Email: smoore@uab.edu	
14. Name of Official Albert Oberman, M.D., MPH	15. Title Vice Chair, IRB
16. Signature Albert Oberman, MD, MPH/db	17. Date 07-07-10

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THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

MEMORANDUM

TO: Jose R. Fernandez, M.D. **FAX:** 4-7050
Principal Investigator

FROM: Amanda Murphy, RN, CIP
On behalf of IRB 02

DATE: December 16, 2004

RE: F040109007
Admixture Mapping for Insulin Complex Outcomes (AMERICO STUDY)

The IRB 02 met on December 15, 2004 and approved with limited modifications the above-referenced protocol. Please make the additions and /or corrections below:

1. Please add to the Risks and Discomforts section of the consent form that there is a risk of claustrophobia associated with undergoing the CT scan.

Please note that this protocol was approved with limited modifications and the IRB has requested changes to the informed consent form. The IRB office must review these changes before issuing formal approval of the protocol and consent form. However, since the changes to the consent form are minor, you may continue to use the existing consent form to enroll participants until December 23, 2004, and you may continue to follow currently enrolled participants. Please submit the revised consent form at your earliest convenience. You may not use the existing consent form after December 23, 2004.

Upon receipt of the following, the IRB Office will issue formal approval of this protocol. **Please note that you will need to provide:**

1) One copy of the revised consent form with the revisions highlighted, and
2) One copy of the revised consent form for the IRB approval stamp.

itb