Association of DNA Copy Number and Structural Variation with Racial Disparities in Childhood Obesity

by

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A dissertation submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Doctor of Philosophy

Auburn, Alabama
May 1, 2021

Keywords: nutrition, copy number variation,
DNA, genetics, childhood obesity

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Abstract

Obesity has become a major public health concern throughout the world. Alabama is 6th highest ranked with obesity in the United States. The development of obesity can be influenced by several factors, such as diet, environment, and genetics. Genomic variability among individuals is largely due to copy number variations (CNVs). Recent genome-wide association studies (GWAS) have successfully identified quite a few loci containing CNV related to obesity. Genomic diversity influences the mechanisms of expression in a variety of genders and ethnicities. Chapter 1 reviews the current literature on the relationships between obesity and the CNV of several loci. The subsequent chapters detail the connection between DNA copy number and structural variation (CNV) and childhood obesity in European American (EA) and African American (AA) elementary school children in Alabama. Chapter 2 evaluates the association between *AMY1* copy number and obesity measurements as well as racial disparities between the two ethnic groups. Our findings suggest that overweight/obese children have a low *AMY1* copy number and the effect is more prominent in AA children. Chapter 3 describes the relationship between the copy number of the *11q11* gene and obesity measurements. A significant inverse association between obesity measurements and *11q11* copy number was observed. EA children have a stronger association between low *11q11* copy number and obesity compared to AA children. The last chapter illustrates the connection between telomere length ratio, blood pressure, and childhood obesity. Our results demonstrated that AA children have high telomere to the single copy gene (T/S) ratio compared to EA children. The high T/S ratio is negatively associated with diastolic pressure.

Acknowledgments

I would like to thank my committee members for their time, interest and helpful guidance that made my Ph.D. work possible. I would also like to thank my friends and extended Auburn family for all their love and encouragement. For my loving, supportive husband whose support during the doctoral journey is so appreciated. And most of all for my mom and dad, who have raised me to believe I will succeed, no matter my chosen field of endeavor. Thank you for supporting me in all my pursuits.

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List of Abbreviations

CNV	copy number variations
GWAS	genome-wide association studies
FoSTeS	fork stalling and template switching
MMBIR	microhomology-mediated break-induced replication
OR	Olfactory receptor
GPCR	G-protein-coupled receptor
AMY1	salivary amylase 1
CYP	Cytochromes p450 superfamily
CLZ	chlorzoxazone
WHO	World Health Organization
AA	African American
EA	European American
NW	normal weight
OW	overweight
OB	obese
LCNV	low copy number variants
HCNV	high copy number variants
NHANES	National Health and Nutrition Examination Survey
SNP	single nucleotide polymorphism
BMI	body mass index
cAMP	cyclic adenosine monophosphate
CDC	Centers for Disease Control and Prevention

NEGR1	neuronal growth regulator 1
WC	waist circumference
WHtR	waist circumference adjusted height
NAFLD	nonalcoholic fatty liver disease
PCR	polymerase chain reaction

Chapter 1

DNA Copy Number and Structural Variation (CNV) Contributions to Adult and Childhood Obesity

Abstract

In recent years, obesity has reached epidemic proportions globally and has become a major public health concern. The development of obesity is likely caused by several behavioral, environmental, and genetic factors. Genomic variability among individuals is largely due to copy number variations (CNVs). Recent genome-wide association studies (GWAS) have successfully identified a number of loci containing CNV related to obesity. These obesity related CNVs are informative to the diagnosis and treatment of genomic diseases. A more comprehensive classification of CNVs may provide the basis for determining how genomic diversity impacts the mechanisms of expression for obesity in children and adults of a variety of genders and ethnicities. In this review, we summarize current knowledge on the relationship between obesity and the CNV of several genomic regions, with an emphasis on genes at the following loci: 11q11, 1p21.1, 10q11.22, 10q26.3, 16q12.2, 16p12.3 and 4q25.

Introduction

According to the National Health and Nutrition Examination Survey (NHANES), the prevalence of obesity in the United States is around 39.8 percent in adults and 18.5 percent in adolescents [1]. Obesity is a risk factor for some of the leading causes of mortality, including type 2 diabetes, heart disease, certain types of cancers, and COVID-19 [2]. What makes obesity different from other risk factors is that it is preventable [3]. The development of obesity is likely caused by several dietary, metabolic, and genetic factors [4]. Genetic variation plays a major role in determining the susceptibility or resistance an individual may have to the environment. Factors that contribute to this environment include reduced energy expenditure, access to fresh fruits and vegetables, increased high-calorie food intake, and socioeconomic status [5]. Previous analysis of the human genome points to single nucleotide polymorphisms (SNPs) to be the primary source of human genetic variation. However, current research shows copy-number variants (CNVs) are responsible for a large extent of structural change in the genome of mammals [6]. A CNV is a DNA segment spanning one kilobase (kb) or larger that has a variable number of copies among individuals in a population when compared to a reference genome [7]. Variations can be deletions or duplications [8]. Some CNVs do not appear to influence phenotype; however, several have been conclusively linked with disease. Through identifying the CNV and associated proteins increasing susceptibility to obesity, recent studies have determined pathways involved in obesity pathogenesis. Also, there is evidence that interaction with other genetic or environmental factors may influence whether CNVs affect phenotypes [9]. For example, exposure to environmental mutagens, such as radiation, can lead to inherited genetic predispositions [10].

The mechanism of fork stalling and template switching (FoSTeS) and microhomology-mediated break-induced replication (MMBIR) model can also lead to genomic rearrangements of single exons, duplication or triplication of individual genes providing an innovative perspective for exploring gene and genome evolution [11]. The CNV represents a source of genetic diversity in human health, disease, and evolution [12, 13]. The genome-wide association studies (GWAS) have identified several loci associated with obesity [14]. D'Angelo *et al.* described the copy number variants of genes and genomic regions in obesity associated with developmental delay, intellectual disabilities (ID), and malformative features [15]. However, there is still more to be studied regarding the association of CNVs and obesity. In this article, we review the literature on the relationships between obesity and the CNV at the following loci: 11q11, 1p21.1, 10q11.22, 10q26.3, 16q12.2, 16p12.3, and 4q25 (Table 1).

The Chromosome *11q11* Locus

It has been hypothesized that the sense of smell, like the taste, may play a significant role in the pathogenesis of obesity. A potential link between olfactory receptors and obesity has been proposed based on the observation of altered olfactory function in morbidly obese patients. Richardson et al. reported that adult patients with a body mass index (BMI) greater than 45 were more likely to have olfactory dysfunction when compared with subjects with BMI of less than 45 [16]. The ability to detect chemicals in the environment is a sensory system dependent on olfactory receptors. A chemical compound has an odor when it is sufficiently volatile to be transported to the olfactory system [17]. Olfactory receptor (OR) neurons project their axon directly to the olfactory bulb in the brain. The central olfactory system is separated from the epithelium by the cribriform plate of the ethmoid bone. The cribriform plate, which supports the olfactory bulb, is perforated by a grouping of holes, known as the olfactory foramina, creating a channel for the olfactory nerves [18]. The first step in odor transduction begins when odorous ligands activate ORs on the olfactory epithelium [19]. The epithelium contains millions of ORs that are capable of binding with odor molecules. However, an odor molecule will not bind to just any OR. It must bind to the OR specifically designed to identify that molecule [20]. Since ORs belong to the G-protein-coupled receptor (GPCR) superfamily, the transduction of chemical information into electrical impulses involves signal amplification by G-protein-coupled activation of adenylyl cyclase. This facilitates a rise in cyclic AMP (cAMP), and subsequently, the opening of cyclic nucleotide-activated, nonselective cation channels. The cell membrane of the olfactory neuron becomes depolarized from the influx of cations through these channels. Ultimately, these results in an increase in the frequency of action potentials that travel down the

axons to the glomeruli, which are spherical structures located in the outer part of the olfactory bulb [17]. OR genes have been identified on several chromosomes. With around 400 functional genes, they encompass one of the largest gene families in mammalian genomes [21].

The chromosome *11q11* locus, spanning approximately 80 kb, exclusively covers three olfactory receptor genes, all belonging to the OLR family 4: OR4P4, OR4S2, and OR4C6 [22]. In addition to functional ORs, the gene family also contains a large number of pseudogenes, which no longer encode ORs due to loss of function mutations occurred during evolution [23]. Earlier studies have identified variation in the copy number of the *11q11* locus in association with obesity (Table 1, Fig. 1). Copy number at this locus can range from 0 to 8 and appears to vary with gender, age, and ethnicity. León-Mimila et al. reported a lower *11q11* copy number in Mexican children aged 6-12 years were significantly associated with lower obesity risk in children, but not in adult subjects [24]. A hereditary factor has been observed in children of German descent. Family-based GWAS have demonstrated a lower *11q11* copy number in obese children compared to those of normal weight [22]. Zhang et al. observed a significant increase in the risk of obesity in Chinese children with deletions at the *11q11* locus. Interestingly, a cumulative effect was observed when these subjects also had deletions at two other loci related to obesity: 10q11.22 and 4q25. This suggests a robust collective association between increased CNV at-risk alleles and childhood obesity [25].

In addition to GWAS, *11q11* has been studied in individual case studies. Obesity is associated with a variety of liver abnormalities collectively known as nonalcoholic fatty liver disease (NAFLD) that is characterized by an accumulation of triglycerides in the liver (steatosis). This can range from simple steatosis to its inflammatory counterpart non-alcoholic steatohepatitis (NASH) [26]. NAFLD is associated with cardiometabolic syndrome (CMS),

increasing the risk of insulin resistance, impaired glucose tolerance, hypertension, dyslipidemia, and central adiposity [27]. The *11q11* region has been shown to have one copy number less in adult patients with NAFLD when compared to controls, who had a copy number around 2 [26].

The variation in the copy number appears to be influenced by several environmental factors, including stress levels, circadian rhythms, and diet [28]. Dietary habits have been shown to directly influence the copy number of certain obesity-related genes, Falchi et al. was the first study to identify the genetic link between carbohydrate metabolism and obesity [29]. Specifically, individuals consuming greater amounts of starch have been shown to have higher levels of both salivary amylase protein and serum amylase compared to those consuming less starch. Recent research has indicated that the expression of the salivary amylase 1 (*AMY1*) gene on chromosome 1p21.1 is upregulated by a high starch diet [30, 31]. Salivary amylase, as well as pancreatic amylase, is secreted during the first steps of starch digestion, which continues after passing from the oral cavity into the stomach. These enzymes are specifically responsible for the hydrolysis of α-1,4 glycosidic bond, which yield oligosaccharides such as maltose and maltotriose during glycolysis [32]. Thus, high *AMY1* copy number and salivary amylase activity are both favorable for more efficient dietary starch digestion [33, 34]. A significant negative association of *AMY1* copy number with body mass index and obesity was first reported by Falchi et al [29] (Fig. 1). *AMY1* copy number is also found to be associated with serum salivary amylase enzyme levels and gene expression [29].

AMY1 is one of the most variable loci in copy number in the human genome, ranging from two to twenty copies [32]. Interestingly, the correlation of *AMY1* copy number with obesity seems to be significant in the pediatric populations of various ethnicities. A recent study from our lab found a negative correlation between its copy number and BMI in pediatric population of both African American and European American ancestry in Alabama. Further, the negative relationship of *AMY1* copy number with obesity measurements was stronger in African

American children than in European American children [35]. An Italian population known to rely on a high starch diet consisting of primarily complex carbohydrates demonstrated a significant correlation as well. A lower *AMY1* copy number was associated with increased BMI in pediatric boys [36]. In a study done in Mexican children, having less than a specific number of copies increased the risk of obesity. An *AMY1* copy number less than 6 correlated to a higher risk of obesity compared to those with at least 6 *AMY1* copy numbers [24]. Similar studies have shown this correlation between being not only significant in pediatrics but also adult populations. A lower *AMY1* copy number was associated with a higher BMI in both European and Asian adults [29]. In contrast, other studies observed differences in gender. In a Finnish study, Viljakainen et al. found no difference in *AMY1* copy numbers between healthy individuals and individuals with a history of childhood-onset obesity. However, obese men had a higher copy number compared to obese females. Further, *AMY1* copy number correlated significantly with whole body fat percent and BMI only in obese females [34].

The genetic variation of the individual influences not only influences phenotype but also the microbiome composition. Previous studies have focused on the effect the gut microbiome may have on specific genotypes. Recent evidence supports the idea that gene copy number also varies. It has been established that *AMY1* copy number is a genetic factor associated with microbiome composition and function. In a month-long diet intervention study, Poole et al. not only showed that diet standardization was a catalyst for gut microbiome convergence, but also that *AMY1* copy number correlated with the composition and function of oral and gut microbiomes [37]. The microbiomes of individuals with low-*AMY1* copy numbers had enhanced the capacity to metabolize complex carbohydrates. Interestingly, high-*AMY1* copy number subjects had higher levels of salivary Porphyromonas. Gut microbiota of those individuals had a

more considerable amount of resistant starch-degrading microbes and produced higher levels of short-chain fatty acids [37]. In a parallel study, León-Mimila et al. explored possible correlations between *AMY1* copy number and several identified gut microbiota genera. The abundance of Prevotella was positively correlated to *AMY1* copy number in the adult population. Prevotella copri, specifically, was two times higher in adults with at least 10 copies of *AMY1* compared to those with less than 4 copies [24].

NPY4R and Appetite Control

In addition to *AMY1*, current research has uncovered another obesity-related gene involved in digestion. A CNV region located on chromosome 10q11.22 is known to cover the Neuropeptide Y Receptor type 4 (*NPY4R*) gene, also known as *PPYR1* [22]. *NPY4R* encodes the NPY receptor that responds to the pancreatic polypeptide (PP), which has been shown to be an effective appetite inhibitor [38] (Figure 1). There are four genes in the NPY receptor family in humans, all of which are expressed in the hypothalamic region of the brain that is involved in appetite control and energy metabolism. Pancreatic PP-cells release PP postprandially in proportion to caloric intake to self-regulate pancreatic secretion activities [38].

The relationship between CNV of the *NPY4R* gene and BMI, waist circumference, and dietary intake has been evaluated in recent studies. Zhang et al. identified 10q11.22 to be significantly associated with obesity in a pediatric population in China. The deletion of 10q11.22 was linked to a higher BMI and waist to height ratio. The risk of obesity increased even further among the 10q11.22 deletion carriers who had meat-based diets, which could indicate a multiplicative interaction (MI) between deletions of 10q11.22 and a preference for meat-based meals [25]. It has been shown that protein, dietary fat, and to some degree, glucose stimulates PP production [39]. The intake of meat regularly may stimulate PP secretion, which would subsequently regulate the intake of food via *PPYR1*. However, in subjects with 10q11.22 deletions, PP cannot function normally. As a result, the 10q11.22 deletion carriers with meat-based diets might be more prone to developing obesity due to a pathologic inability to control food intake. In addition to a meat-based diet, there was a significant association between subjects with the 10q11.22 deletion and a preference for sweet foods. This could, in part, be due to glucose consumption stimulating the release of PP [25].

Another study in Chinese subjects found that deletions at *NPY4R* were not only significantly associated with higher BMI in pediatrics, but also in the elderly [40]. In contrast, Sun et al. found no copy number changes at *NPY4R* in Chinese obese or healthy controls [41]. Other studies have provided evidence of differences in both ethnicity and age group. Aerts et al. performed a mutation screen for variants in the *NPY4R* coding region in 2 groups: obese Belgian children and adolescents and lean White adults. The CNV analysis demonstrated a significantly higher frequency of *NPY4R* containing 10q11.22 CNV loss in the obese Belgian pediatric population. Furthermore, a CNV gain in this region was more prevalent in the lean White adult population [42]. Gender differences were observed in a community of Swedish adults. A positive correlation was found between *NPY4R* copy number and BMI, as well as waist circumference, in Swedish women. Each additional copy of *NPY4R* correlated with a 2.6 kg/m2 increase in BMI and a 5.67 cm increase in waist circumference. The same findings were not seen in Swedish adult men [38].

CYP2E1 and Lipid Metabolism

The ability of certain endogenous or exogenous factors to influence the activity level of genes has been shown to have a significant role in human health [43]. In addition to *NPY4R*, a region at the 10q26.3 locus comprises a superfamily of functional genes that are also inducible by several endogenous factors. The Cytochromes p450 superfamily (CYP) encodes enzymes and proteins that catalyze many reactions involving drug metabolism and synthesis of steroids, cholesterol, and lipids [44]. CYP proteins can be located in several different compartments of the cell [43]. In addition to their localization in the Golgi apparatus and plasma membrane, several different forms have been detected as part of the endoplasmic reticulum membrane, known as microsomes, and in the mitochondria [45]. Different types of signals are required for targeting of P450 proteins to ER and mitochondria. Microsomal P450 is inserted into the rough-surfaced portion of the ER membrane through a non-cleavable hydrophobic signal sequence [46]. CYPs that play a major role in endogenous metabolisms are found mostly in mitochondria [47]. In contrast to microsomal P450s, mitochondrial P450s are comprised of cleavable amphipathic presequences that are important for targeting of the precursor protein (preprotein) to the mitochondria post-translationally [48].

Of particular interest is Cytochrome P450 family 2 subfamily E member 1 (*CYP2E1*). *CYP2E1* is important in signaling pathways related to diabetes and obesity, such as ω-1 hydroxylation of fatty acids like arachidonic acid. *CYP2E1* also plays a role in the propanediol pathway, an alternative pathway of gluconeogenesis, which offers a way to generate glucose during starvation via hydroxylation of acetone, hydroxyacetone, and pyruvate [44]. One of the most distinguishing features that set *CYP2E1* apart is its inducibility by a large variety of substrates [47]. Endogenous factors that induce *CYP2E1* expression levels include obesity,

diabetes, fasting, and chronic alcohol ingestion [49]. The fluctuation of *CYP2E1* levels and expression might alter the balance, and subsequently, TG metabolism.

The expression of CYPs manifests in the liver, as well as other tissues, such as the brain, kidneys, and lungs. The first evidence of *CYP2E1* localization within the liver was in 1997. Avadhani et al. discovered the presence of the *CYP2E1* protein located in the inner membrane of rat liver mitochondria [50]. Hepatic *CYP2E1* activity has been reported to be upregulated in individuals with severe obesity, defined as a BMI higher than 35 kg/m2 [51]. This finding can offer a potential explanation for why morbid obesity is often associated with fatty liver disease (steatosis) [52]. The degree of severity may range from steatosis alone to steatohepatitis (accompanying inflammation of the liver) with advanced fibrosis [53]. Emery et al. assessed the activity of *CYP2E1* by rate of chlorzoxazone (CLZ) clearance, which has been used extensively to evaluate *CYP2E1* activity. Both the total and unbound oral CLZ clearance (Clu/F) was approximately three times higher in morbidly obese subjects compared with controls. One year after the morbidly obese subjects underwent gastroplasty, the total oral CLZ clearance and Clu/F declined by 46 percent and 35 percent, respectively. A positive association between the degree of steatosis and *CYP2E1* activity before and after surgery suggests that increases in *CYP2E1* activity may be related to the hepatic pathology of the liver resulting from morbid obesity (Figure 1). Furthermore, the upregulation of *CYP2E1* activity may accelerate liver injury when significant steatosis is present [52].

The CNV of *CYP2E1* can influence activity by altering gene expression [54]. There is evidence showing that individuals have a copy number range of 1-4. Over 96 percent of individuals have two copies of the *CYP2E1* gene. 3 percent of people have 3 copies (tri-copy), and less than 2 percent of the population has either 1 copy (haplotype) or 4 copies (tetratype)

[54]. Yang et al. reported association results in White and African American groups that indicated CNV at 10q26.3 might be a common variant for obesity across different ethnicities. The CNV found on *CYP2E1* showed a strong positive association with both BMI and body fat mass in three independent populations: an unrelated sample of White subjects, a family-based sample of White subjects, and an unrelated sample of African American subjects [55].

One of the most severe lipid metabolism disorders related to obesity is Hypertriglyceridemia (HTG). HTG is characterized primarily by levels of plasma triglyceride higher than 1.7 mmol/L. When the level of plasma TGs is higher than 10 mmol/L, genetic factors are thought to play a dominant role in HTG [56]. Interestingly, individuals with *CYP2E1* tri-copy and tetratype often show obesity and HTG phenotypes.

Fat Mass and Obesity Associated (*FTO*) gene and Shift from Brown to White Adipose Cells

Another gene heavily involved with lipids is alpha-ketoglutarate dependent dioxygenase (*FTO*), which is located at the 16q12.2 chromosomal region. *FTO* was first identified through GWAS as the obesity-susceptibility gene [57]. *FTO* functions as an RNA and single-strand DNA demethylase that mediates oxidative demethylation of several different RNA species, including N6-methyladenosine (m6A) in mRNA transcripts. *FTO* is highly expressed in the adrenal glands and forebrain, specifically the hypothalamus and pituitary regions [58]. In doing so, *FTO* plays the role of regulator for adipogenesis and energy homeostasis (Figure 1). This contributes to the regulation of body size and body fat accumulation, specifically the differentiation into white or brown fat cells [59-61]. It is uncertain whether variations associated with obesity affect the function of the *FTO* gene directly, or modify the expression of adjacent genes, such as IRX3 [62]. A pathogenic intronic *FTO* variation (rs1421085) interrupts the sequence for ARID5B binding that has remained essentially unchanged throughout evolution. Without ARID5B binding, the two genes distal to *FTO* (IRX3 and IRX5) will be overexpressed, which alters the differentiation of pre-adipocytes and shifts from brown to white fat cells. This alteration results in a loss of mitochondrial thermogenesis and increased lipid storage [59]. This type of IRX-dependent shift from consuming energy to storing it in the form of adipocytes may offer the protection of body fat under conditions where energy supply is limited. This type of defense mechanism is pertinent for lean children in order to protect body weight [63].

Recent research suggests IRX3 may be the main mediator of obesity risk in children who are carriers of the *FTO* risk variant. González-Herrera et al. observed differences in CNV of the *FTO* gene that was associated with overweight status in Mexican-Mayan pediatric boys. Mexican Mayans from Yucatán have been reported to have a much higher prevalence of

overweight and obesity than the national average. When comparing *FTO* risk-allele carriers to non-risk-allele carriers, adipocyte-specific expression of both IRX3 and IRX5 was increased only in lean children. Overweight boys showed higher average CNV than boys with normal weight [64]. Interestingly, Mexican children from central and northern Mexico differ from those indicating a lack of *FTO* rs1421085 association with obesity [65].

Other Obesity-related Loci

In addition to the previously mentioned chromosomal regions, there are loci in which the biological function is not completely understood. Among these regions is 16p12.3, a 21-kb deletion located roughly 50 kb upstream of the gene G protein-coupled receptor, family C, group 5, member B (*GPRC5b*). The protein encoded by this gene is a member of the type-3 G protein-coupled receptor family. Research suggests the function of this protein may facilitate the cellular effects of retinoic acid on the G protein signal transduction cascade. The direct correlation between *GPRC5b* and the molecular biology of obesity phenotypes is largely undiscovered [66]. Regarding the GPCR5 CNV, it has been previously suggested that the effect of the deletion of CNV is ethnic-specific, as it has been significantly associated with obesity in Europeans, who have a higher CNV deletion frequency. The same association was not found in Chinese populations who have a lower CNV deletion frequency. The *GPCR5* CNV deletion frequency observed in the Mexican people by León-Mimila et al. was consistent with the previous findings in Chinese individuals. The copy number was not associated with obesity [24]. Other loci with an incomplete understanding contain CNV deletions that are correlated with a higher risk of childhood obesity. The *4q25* locus may be mediated by long-range regulators such as enhancers or repressors. CNVs may exert their effects on genes as far as 1 Mb away. Little is known about the biological function of this chromosomal region [25].

Conclusions

The research summarized in this article (Table 1) may not only assist in understanding how genetic factors respond to diet but also how gene-diet interactions intricately operate on a cellular level. CNV in obesity-related genes may be able to contribute to the diagnosis and treatment of genomic diseases. A more comprehensive classification of CNVs may provide the basis for determining how genomic diversity impacts the mechanisms of expression for obesity in children and adults of a variety of ethnicities.

Table 1. Summary of studies on the relation between DNA CNVs and obesity.

Locus	Gene	CNV	Function(s)	Region of Activity	Effect(s)
11q11	OR4P4, OR4S2, OR4C6 *Olfactory Receptor Family 4*	0-8	Specifically recognizes odorous molecules in the 1st step of odor transduction	Expressed in the olfactory bulb glomeruli of the brain	↓ CN =↓ obesity in Mexican children [24] ↓ CN = ↑ obesity in German children [22] ↓ CN = ↑ obesity in Chinese children [25] ↓ CN = ↑ obesity related to NASH in Malaysian adults [26]
1p21.1	AMY1 *Salivary Amylase 1*	2-20	Aids in hydrolysis of α-1,4 glycosidic linkages in starch metabolism	Expressed at high levels in salivary glands	↓ CN = ↑ obesity in EA and AA children [35] ↓ CN = ↑ BMI in Italian pediatric boys [36] CN < 6 = ↑ obesity in Mexican children [24] ↓ CN = ↑ obesity in European and Asian adults [29] ↑ CN = ↑ BMI and BF% in obese Finnish women [25] ↑ CN = ↑ obesity and *Porphyromonas* in Mexican adults [37]
10q11.22	NPY4R (PPYR1) *Neuropeptide Y Receptor Y4*	2-8	Y4 receptor responds to PP (appetite inhibitor)	Expressed in the hypothalamic region of the brain involved in appetite control and energy metabolism	↓ CN = ↑ BMI and WHtR in Chinese children [25] ↓ CN = ↑ BMI in Chinese elderly [40]

					↓ CN = ↑ obesity in Belgian children [42] ↑ CN = ↓ obesity in White adults [42] ↑ CN = ↑ body weight in Swedish women [38]
10q26.3	CYP2E1 *Cytochrome p450 2E1*	1-4	Involved in cholesterol and lipid synthesis Induced by fasting, diabetes and obesity	Primarily expressed in the mitochondria of hepatic cells	↑ CN = ↑ BMI and body fat in EA adults [55] ↑ CN = ↑ BMI and body fat in AA adults [55]
16q12.2	FTO *Alpha-ketoglutarate Dependent Dioxygenase*	0-2	Involved in the regulation of thermogenesis, adipogenesis, fat mass, body weight and the control of adipocyte differentiation into brown or white fat cells.	Expressed in adrenal glands and brain, specifically the hypothalamus and pituitary gland.	↑ CN = ↑ obesity in Mexican-Mayan pediatric boys [64] ↓ CN = ↑ obesity in Chinese children [25]
16p12.3	GPRC5b *G protein-coupled receptor, family C, group 5, member b*	0-2	Facilitates the cellular effects of retinoic acid on the G-protein signal transduction cascade	Not Available	↓ CN = ↑ BMI and body fat in EA adults [66]
4q25	Intergenic region	-	Modifier of rare ion channel mutation associated with familial atrial fibrillation	Cardiac ion channel	↓ CN = ↑ obesity in Chinese children [25]

Figure 1

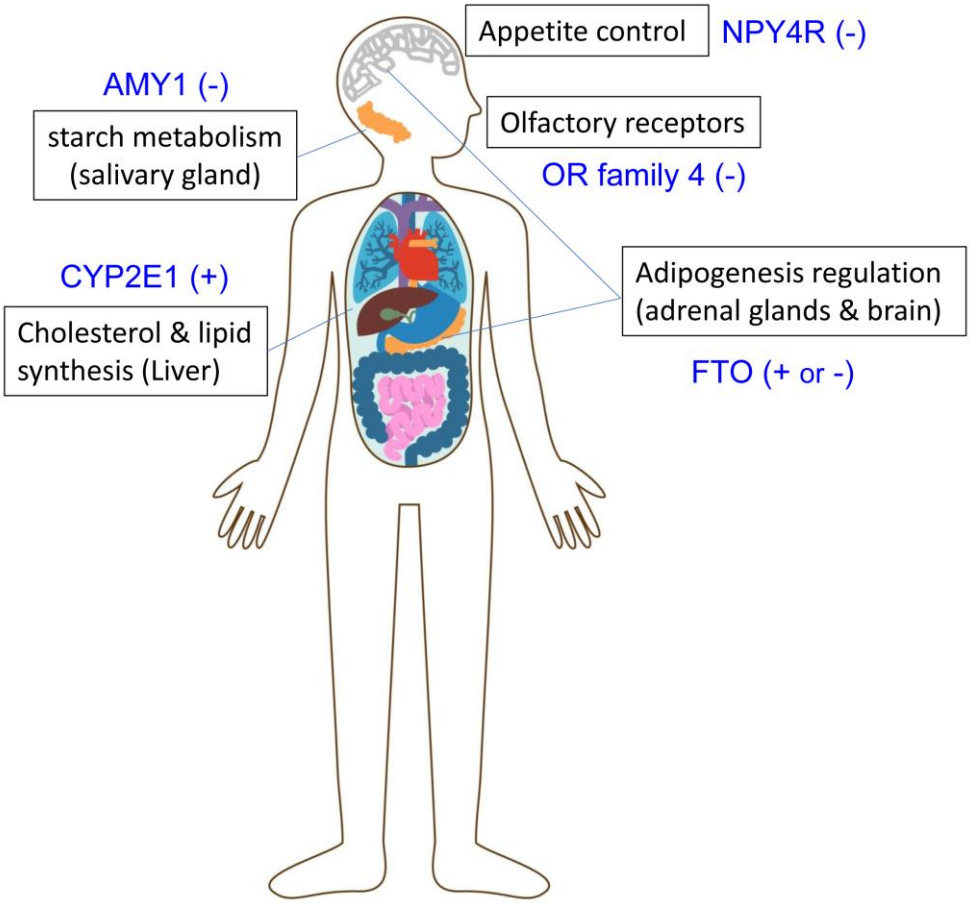

Figure 1. Copy number variation (CNV), physiological pathways and obesity. The gene name and pathway information are labeled in the diagram. (+) positive correlation between CN and BMI; (-) negative correlation between CN and BMI.

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Chapter 2

Association of Salivary Amylase (*AMY1*) Gene Copy Number with Obesity in Alabama Elementary School Children

Abstract

Salivary amylase (*AMY1*) is the most abundant enzyme in human saliva, responsible for the hydrolysis of α-1,4 glycosidic linkages that aids in the digestion of starch. Recently studies have shown that the copy number of *AMY1* is associated with obesity; however, the data varies with location. One-third of children are overweight/obese in Alabama. In this study, we aim to determine the relationship between the copy number of *AMY1* gene and obesity measurements in children from Alabama. One hundred twenty-seven children aged between 6 to 10 years participated in this study. Anthropometric measurements were measured using WHO recommendations. Genomic DNA was extracted from saliva, and the copy number of the *AMY1* gene was estimated by digital PCR. The association between *AMY1* copy number and obesity measurements was analyzed by linear regression. The mean *AMY1* copy number significantly decreased in overweight/obese (6.21 ± 1.48) compared to normal weight (7.97 ± 2.35) children. *AMY1* copy number inversely associated with the obesity measurements. African Americans had a stronger association between low *AMY1* copy number and obesity compared to white/European Americans. Our findings suggest that overweight/obese children have a low *AMY1* copy number and the effect is more prominent in African Americans.

Introduction

Obesity is a major health problem throughout the world [1]. Around 12.7 million children and adolescents are obese in United States [2]. Alabama is 6th highest ranked with obesity in United States, and 35% of children are overweight and obese [3]. The major factors contributing to the increase in prevalence of childhood obesity might be environmental conditions [4], sedentary activities [5], socioeconomic status [6], and food availability [7]; in addition to these, genetic factors could also be involved [8]. However, the genetic factors contributing to childhood obesity in elementary school children from Alabama has been poorly studied.

Copy number variations (CNV) contribute novel insights to the genetic heritability of human diseases such as autism [9], type 2 diabetes [10], and obesity [11]. A segment of DNA expressed in a different copy number among individuals compared to a reference genome is defined as CNV [12]. In early-onset obesity, the first study reported the deletion within chromosome 16p11.2 [13], which has been particularly well studied since; deletions of this type are associated with obesity and duplications are associated with an underweight phenotype [14]. Previous studies have identified variation in the copy number of the candidate regions near the neuronal growth regulator 1 (NEGR1) locus [15], chromosome 10q11.22 [16], *11q11* [17], and 10q26.3 [18] with obesity. The salivary and pancreatic amylases (*AMY1* and AMY2) are secreted enzymes responsible for the hydrolysis of a-1,4 glycosidic linkages that aids in digestion of dietary starch [19]. The salivary amylase (*AMY1*) is the most abundant enzyme in human saliva, accounting for 40% to 50% of total salivary protein [20,21]. The CNV of *AMY1* ranges from two to 20 [14,22]. The copy number of *AMY1* is dependent upon the dietary habits. Specifically, individuals consuming higher levels of starch have a greater amount of *AMY1* compared to those consuming less starch [19,22]. The levels of salivary amylase protein and serum amylase is

found to be correlated with the copy number [19,23]. Increased secretion of salivary amylase protein and copy number helps in the starch digestion. This suggests a genetic link between carbohydrate metabolism and obesity. The variation in the copy number is not only dependent upon the diet but also environmental factors, including stress levels and circadian rhythms [24–26]. In European and Asian adults, it has been shown that *AMY1* copy number is associated with obesity [23]; higher BMI is associated with lower gene copy number. Another study in Finland, Viljakainen et al. found no difference in *AMY1* copy numbers between healthy subjects and subjects with history of childhood-onset obesity, but obese men had higher copy number compared to females [27]. In children from an Italian school, BMI was negatively associated with *AMY1* copy number only in boys [28]. However, in Mexican children, all normal weight children had a *AMY1* copy number greater than 10 [29]. The results vary with location. The objective of this study was to evaluate the association between *AMY1* copy number and obesity measurements as well as racial disparity between white/European Americans and African Americans in elementary school children from Alabama. On the basis of the other studies, we hypothesized that the *AMY1* copy number would be lower in overweight/obese children compared to normal weight children and that there may be differences depending upon the race and ethnicity.

Materials and Methods

Participants Around 127 children aged 6–10 (6.93 ± 1.79) years were recruited from Lee County and Macon County, Alabama by posting flyers. Children with major health disorders such as diabetes or cardiovascular disease based on an initial phone survey with the parents were excluded. The parents brought their child to Auburn University to participate in this study. Written consent was obtained from the parents and participants. The study was approved by the Auburn University Institutional Review Board.

Anthropometric Measurements All the anthropometric measurements were carried out using WHO recommendations. The body weight was measured without shoes and light clothing using a Tanita digital scale to the nearest 4 ounces. The height was measured on a calibrated scale attached to a stadiometer to the nearest 0.1 cm [30]. The Body Mass Index (BMI) was calculated to determine the body fat and approximate the weight and height of the participants. As growth occurs until the age of 20, and not all the growth is related to body fat, BMI z-scores were calculated utilizing a SPSS macro based on WHO growth reference 2007 data adjusted for age and sex [31]. The Centers for Diseases Control and Prevention (CDC) standard for classification in children are: underweight (85th to ≤ 95th percentile), and obese (>95th percentile) [32]. The recruited participants were classified as normal weight and overweight/obese based upon their percentile range. The waist circumference was determined to the nearest 0.1 cm using flexible non-elastic tape at the midpoint between the lowest ribs and the iliac crest. The z-scores for waist circumference (WC) and waist:height ratio (WHtR) were calculated using the R macro package developed by Sharma et al., based onLMS [Lambda (L) for the skew, Mu (M) for the median, and Sigma (S) for the generalized coefficient of variation] tables from NHANES III [33].

AMY1 Gene Copy Number Saliva was collected from children using DNA GenoTek Saliva Collection Kit (Ontario, Canada). Genomic DNA from the saliva was extracted using the PrepIT.L2P method (DNA GenoTek, Ontario, Canada), according to the manufacturers protocol. The copy number of *AMY1* gene was estimated by digital PCR (QuantStudio™ 3D Digital PCR) containing two TaqMan assays, one for *AMY1* (Hs07226361_cn, FAM-labeled) and second, specific for the reference gene (RNase P, VIC labeled) (Life Technologies, Carlsbad, CA, USA). In brief, 14.5 µL of TaqMan PCR reaction mixture was prepared by adding 7.25 µL of QuantStudio™ 3D Digital PCR Master Mix, 0.725 µL of 20× *AMY1*, 0.725 µL of 20× RNase P, and 6 µL of diluted DNA (10 ng/µL). This reaction mixture was loaded into the QuantStudio™ 3D Digital PCR Chip, which has 20000 mini-chambers. PCR was performed using the ProFlex™ 2× Flat PCR System with the following cycling conditions. Initial denaturation at 96 ◦C for 10 min, 39 cycles of 60 ◦C for 2 min and 98 ◦C for 30 sec, followed by one cycle of 60 ◦C incubation for 2 min, and then 4 ◦C hold. The chip was scanned in QuantStudio™ 3D Digital PCR instrument, and subsequent analysis was performed using the QuantStudio 3D Analysis Suite Software. Hap Map sample NA18956 (Coriell Institute, Camden, NJ, USA) was used as a calibrator sample, as this sample was consistently reported to have six copies of *AMY1* by several independent methods [14,19,34].

Statistical Analysis Data are expressed as mean ± standard deviation. Statistical analyses were performed using SPSS (version 24, IBM, Armonk, NY, USA). Independent sample t-test was used to assess the difference between the mean values of two groups. Non-parametric median test was used to analyze the difference between the median values of two groups. Linear regression analysis was used to investigate the association of *AMY1* copy number with BMI z-score, waist circumference z-score (WC z-score), and waist circumference adjusted height z-

score (WHtR z-score). When considering interaction, a p value <0.05 was considered statistically significant. The standardized β-coefficient value was used to quantify the association.

Results

This study cohort consisted of 127 participants (76 normal weight (NW) and 51 overweight/obese (OW/OB) children) aged between 6 to 10 years. Table 1 shows the general characteristics in the study population. The mean age of children was not statistically different between the groups. However, as expected, all anthropometric characteristics were significantly greater in OW/OB children when compared to NW children. The BMI (Kg/m2) of OW/OB (21.16 ± 3.15) children was significantly ($p < 0.00001$) greater compared to NW (16.03 ± 1.56) subjects. Likewise, the waist circumference of OW/OB subjects (71.06 ± 8.67) was significantly greater ($p < 0.0001$) compared to NW (60.28 ± 5.26) subjects.

Table 1. General characteristics of the study population.

	All	Normal Weight (NW)	Overweight/Obese (OW/OB)	*p* Value
Sex (N)	127	76	51	
Male	69	43	26	
Female	58	33	25	
Race (N)	127	76	51	
EA	68	41	27	
AA	59	35	24	
Age (years)	6.93 ± 1.79	6.80 ± 1.94	7.26 ± 1.52	
Height (cm)	131.95 ± 11.44	130.05 ± 11.07	134.80 ± 11.50	0.02
Weight (Kg)	71.05 ± 24.17	60.72 ±14.37	86.44 ± 27.54	0.00001
BMI (Kg/m^2)	18.09 ± 3.43	16.03 ± 1.56	21.16 ± 3.15	0.00001
BMI z-score	0.77 ± 1.22	−0.03 ± 0.78	1.99 ± 0.59	0.00001
Waist circumference (cm)	64.61 ± 8.62	60.28 ± 5.26	71.06± 8.67	0.00001

Table 2 shows the descriptive analysis of *AMY1* copy number in the study population. The copy number of *AMY1* ranged from 2.03 to 16.25 with a median of 6.93. In NW subjects, the *AMY1* copy numbers were in the range of 2.03 to 16.25 with a median of 7.835. In the OW/OB subjects, the *AMY1* copy numbers ranged from 2.95 to 10.46 with a median of 5.89. A

race specific descriptive analysis of *AMY1* copy number is shown in Table 3. The *AMY1* copy number in white/European Americans (EA) ranged from 2.03 to 15.16, and in African American (AA) children it was from 2.95 to 16.25.

Table 2. Descriptive analysis of *AMY1* copy number variations (CNV) in study population.

Groups	Mean	N	Standard Deviation	Median	Minimum	Maximum	Range
NW	7.9650	76	2.34632	7.8350	2.03	16.25	14.22
OW/OB	6.2076	51	1.48473	5.8900	2.95	10.46	7.51
Total	7.2593	127	2.21353	6.9300	2.03	16.25	14.22

Table 3. Race specific descriptive analysis of *AMY1* CNV.

Groups	Mean	N	Standard Deviation	Median	Minimum	Maximum	Range
White/European American (EA)	7.1684	68	2.07064	6.8950	2.03	15.16	13.13
African American (AA)	7.3641	59	2.38129	6.9400	2.95	16.25	13.30
Total	7.2593	127	2.21353	6.9300	2.03	16.25	14.22

Figure 1A shows the distribution of *AMY1* copy number in NW and OW/OB children. The median copy number of *AMY1* in OW/OB (5.89) is significantly lower ($p < 0.0001$) compared to NW (7.83) children (Figure 1B). Figure 2A shows the race specific distribution of *AMY1* copy number in the study population. The *AMY1* copy number in EA (6.89) and AA (6.94) are not statistically significant as shown in Figure 2B.

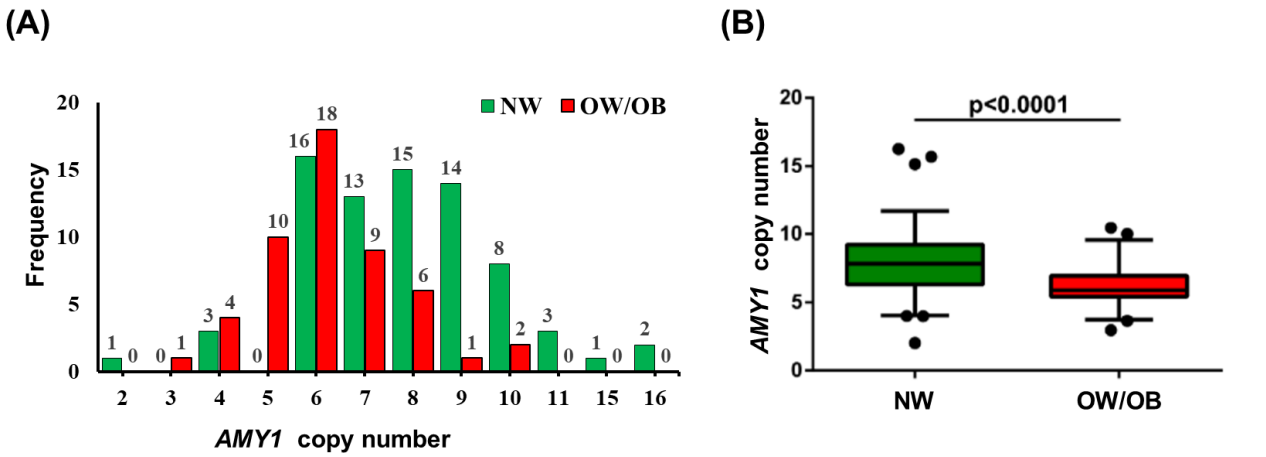

Figure 1. The *AMY1* copy number in the study population. (**A**) Distribution of *AMY1* copy number in the study population. The copy number of *AMY1* was rounded to nearest integer. (**B**) Mean *AMY1* copy number in normal weight (NW) and overweight/obese (OW/OB) participants ($p < 0.0001$).

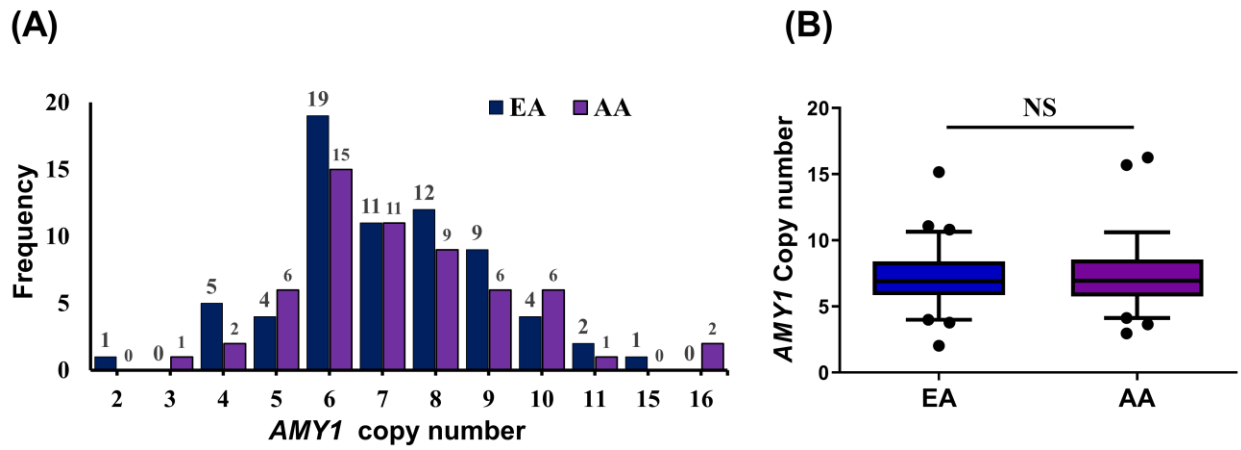

Figure 2. The *AMY1* copy number in White/European American (EA) and African American (AA) children. (**A**) Distribution of *AMY1* copy number in European American and African American children. The copy number of *AMY1* was rounded to nearest integer. (**B**) Mean *AMY1* copy number in European American and African American. NS—No significance.

We next analyzed the association between *AMY1* copy number and BMI z-score, waist circumference z-score (WC z-score), and waist:height ratio z-score (WHtR z-score) (Figure 3). Linear regression analysis showed a significant inverse association between *AMY1* copy number and BMI z-score in the whole study population (β co-efficient; −0.369, $p < 2.0 \times 10-5$) (Figure 3A). Similarly, a significant inverse association trend was observed between *AMY1* copy number and both WC z-score (β co-efficient; −0.341, $p < 8.6 \times 10-5$) and WHtR z-score (β co-efficient; −0.282, $p < 0.001$) (Figure 3B,C).

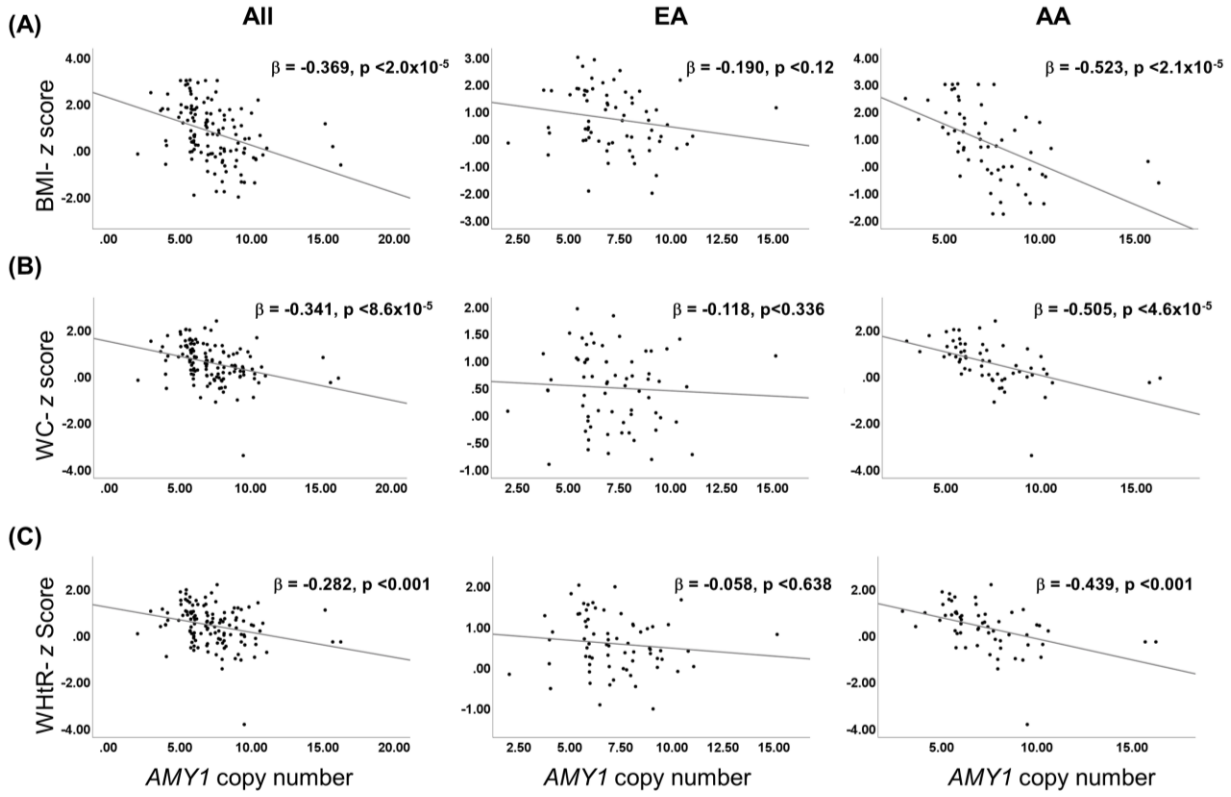

Figure 3. Relationship between *AMY1* copy number and obesity measurements. Association of *AMY1* copy number with (**A**) BMI z-score, (**B**) WC z-score, and (**C**) WHtR z-score in European American, African American, and study population. Standardized β-coefficient value was used to quantify the association. When considering interaction, a p value <0.05 is significant.

Race specific linear regression analysis between *AMY1* copy number and BMI z-score showed that AA (β co-efficient; −0.523, $p < 2.1 \times 10-5$) had a greater significant inverse association compared to EA (β co-efficient; −0.190, $p < 0.12$) (Figure 3A). AA also had a greater significant inverse association between *AMY1* copy number and both WC z-score (β co-efficient; −0.505, $p < 4.6 \times 10-5$) and WHtR Nutrients 2019, 11, 1379 6 of 9 z-score (β co-efficient; −0.439, $p < 0.001$) compared to EA (β co-efficient; −0.118, $p < 0.336$ and β co-efficient; −0.058, $p < 0.638$, respectively) (Figure 3B,C).

Discussion

This present study explored the association between the obesity measurements and *AMY1* copy number in elementary school children aged 6–10 years old with normal weight and overweight/obese. The median copy number of *AMY1* was less in obese children compared to normal weight children. We also found that the *AMY1* copy number was negatively associated with the obesity measures such as BMI z-score, waist circumference z-score, and waist circumference adjusted height z-score. These results correspond to another study reported with Mexican children, which suggested that normal weight participants had an *AMY1* copy number greater than 10 and a higher copy number reduced the risk of obesity [29]. Increased BMI was also found to be associated with a low *AMY1* copy number and decreased level of salivary amylase in European and Asian adults [23]. A genetic link between obesity and carbohydrate metabolism has been reported [23]. Patients with obesity, type 1 and 2 diabetes, and metabolic syndrome have been shown to have lower serum salivary amylase levels [35]. The copy number of serum and salivary *AMY1* and AMY2 is lower in patients with metabolic syndrome [23,27,36]. Normal weight adults with high *AMY1* copy number have shown improvements in the glycemic control [37].

We also assessed the impact of race and found that the *AMY1* copy number was negatively associated with the obesity measures more significantly in African Americans compared to European Americans from the similar age range and region. However, there is no difference in the mean *AMY1* copy number between EA and AA as shown in Figure 2B. In another study including European American and African American children, a genome-wide study revealed a difference in some of the CNVs and showed the genetic vulnerability of common childhood obesity in the participants [38]. Viljakainen et al., in a study based in

Finland, showed that there was no difference in *AMY1* copy number between healthy and obese participants aged 15–25 years but only the obese women had a lower copy number compared to healthy participants [27]. Therefore, a large discrepancy exists in people from different locations and within a specific population. The difference is mainly due to adaptation to different dietary habits and environmental factors. Several nutrition education programs are working to reduce and prevent obesity by promoting a healthy diet, limiting sweetened beverages, and increasing physical activity in schools in Alabama [39,40]. The limitation of this study is the small number of participants on which the results are based. The dietary habits, environmental factors, socioeconomic status, and parental influences that might influence copy number are also not included.

In summary, we found the *AMY1* copy number was significantly decreased in obese compared to normal weight children. A significant inverse association between obesity measurements and *AMY1* copy number was observed. African Americans have a stronger association between low *AMY1* copy number and obesity compared to European Americans.

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Chapter 3

Association of *11q11* Gene Copy Number with Obesity in Alabama Elementary School Children

Abstract

The *11q11* locus encompasses the olfactory receptor family of genes (OR4P4, OR4S2, OR4C6). These genes are responsible for specifically recognizing odorous molecules in the first step of odor transduction. Recent studies have shown that the copy number of *11q11* is associated with obesity; however, the data varies depending on location. One-third of children are overweight/obese in Alabama. In this study, we aim to determine the relationship between the copy number of *11q11* gene and obesity measurements in school-age children from Alabama. One hundred twenty-seven children aged between 6 to 10 years participated in this study. Anthropometric measurements were assessed using WHO recommendations. Genomic DNA was extracted from saliva, and the copy number of the *11q11* gene was measured by digital PCR. The association between *11q11* copy number and obesity measurements was analyzed by linear regression. The *11q11* CNV descriptive analysis data showed a significant increase ($p<0.025$) in *11q11* mean CNV in girls (1.724±0.09) compared to boys (1.473±0.07). We found significant difference ($p<0.017$) in gender specific percentage proportion of both low copy number variants (LCNV) and high copy number variants (HCNV). The girls had higher *11q11* CNV due to increased HCNV in OW/OB participants. AA participants (1.716±0.09) showed a significant increase ($p<0.031$) in the *11q11* copy number compared to EA participants (1.476±0.07). We analyzed the proportion of participants in NW and OW/OB group in EA and AA participants and found that AA participants had a higher mean *11q11* CNV than EA. European American OW/OB boys had a stronger association between low *11q11* copy number and obesity compared to African American OW/OB boys. *11q11* copy number was inversely associated with the obesity measurements. Our findings suggest that overweight/obese children have a low *11q11* copy number and the effect is more prevalent in European Americans.

Introduction

In recent years, obesity has reached epidemic proportions worldwide and has become a major public health concern. According to the National Health and Nutrition Examination Survey (NHANES), approximately 39.8 percent of adults and 18.5 percent in adolescents are obese in the United States. Alabama is ranked 6th highest [1, 2]. Obesity is a major risk factor for some of the leading causes of mortality, including type 2 diabetes, heart disease, certain types of cancers, and COVID-19 [3]. However, obesity differs from other risk factors because it is preventable [4]. It is probable that the development of obesity is caused by several dietary, metabolic, and genetic factors [5]. Genetic variation plays a significant role in determining the susceptibility an individual may have to the environment. Factors that contribute to this environment include energy expenditure, access to fresh fruits and vegetables, high-calorie food intake, and socioeconomic status [6]. The genetic factors contributing to obesity in elementary school children from Alabama has not been studied extensively. Previous analysis of the human genome indicates that the primary source of human genetic variation may be single nucleotide polymorphisms (SNPs). However, current research shows copy-number variants (CNVs) are responsible for a considerable amount of structural change in the genome of mammals [7]. A CNV is a DNA segment spanning at least one kilobase (kb) in length that has a variable number of copies among individuals in a population when compared to a reference genome [8]. Recent studies have determined pathways involved in obesity pathogenesis by identifying the CNV and associated proteins related to the susceptibility of obesity. The CNV represents a source of genetic diversity in human health, disease, and evolution [9, 10]. Genome-wide association studies (GWAS) have identified several loci associated with obesity [11]. Previous studies have

identified CNV in regions near the Salivary Amylase 1 (*AMY1*) locus [12], chromosome 10q11.22 [9], and 10q26.3 [13] with obesity.

It has been the assumption that the sense of smell may play a significant role in the pathogenesis of obesity. A possible link between olfactory receptors and obesity has been proposed. This theory is based on the observation of altered olfactory function in morbidly obese patients. Richardson et al. discovered olfactory dysfunction was more probable in adult patients with a body mass index (BMI) greater than 45 compared with subjects with BMI of less than 45 [14]. The ability to smell is a sensory system dependent on olfactory receptors. A chemical compound has an odor when it is volatile enough to be transported to the olfactory system [15]. Olfactory receptor (OR) neurons project their axon directly to the olfactory bulb in the brain. The central olfactory system is separated from the epithelium by the cribriform plate of the ethmoid bone. Olfactory nerves innervate the cribriform plate through a grouping of holes, or channels, called the olfactory foramina [16]. The first step in odor transduction is initiated when ORs on the olfactory epithelium are activated by odorous ligands [17]. Odor molecules must bind to the OR specifically designed to identify that molecule [18]. ORs are part of the G-protein-coupled receptor (GPCR) superfamily, which means the transduction of chemical information into electrical impulses involves signal amplification by G-protein-coupled activation of adenylyl cyclase. This enables cyclic AMP (cAMP) to rise, facilitating the opening of cyclic nucleotide-activated, nonselective cation channels. The cell membrane of the olfactory neuron then becomes depolarized from the influx of cations through these channels. Ultimately, the frequency of action potentials that travel down the axons to the glomeruli increases [19]. ORs encompass one of the largest gene families in mammalian genomes and have been detected on several chromosomes [20].

The chromosome *11q11* locus spans approximately 80 kb [21]. In addition to a large number of pseudogenes, *11q11* covers three functional olfactory receptor genes, all belonging to the OLR family 4: OR4P4, OR4S2, and OR4C6 [22]. Earlier studies have identified variation in the copy number of the *11q11* locus in association with obesity [23-25]. Copy number at this locus can range from 0 to 8 and appears to vary with gender, age, and ethnicity. A hereditary factor has been observed in children of German descent [21]. Family-based GWAS have discovered a lower *11q11* copy number in obese children compared to those of normal weight [22]. León-Mimila et al. reported a lower *11q11* copy number in Mexican children aged 6-12 years were significantly associated with lower obesity risk in children, but not in adult subjects [24]. Zhang et al. observed a significant increase in the risk of obesity in Chinese children that had deletions at the *11q11* locus. Interestingly, a cumulative effect was observed when this was accompanied by deletions at two other loci related to obesity: 10q11.22 and 4q25. This indicates a strong collective association between loci with increased CNV and childhood obesity [25].

The purpose of this study was to evaluate the association between *11q11* copy number and obesity measurements as well as racial differences between white/European American and African American school-aged children from Alabama. Based on the other studies, we hypothesized that the *11q11* copy number would be lower in overweight/obese children compared to normal weight children and that there may be differences depending upon the gender and ethnicity.

Materials and Methods

Study participants The cross-sectional study consisted of 127 children including both boys and girls. Initial survey by phone or email was conducted to the parents of interested participants to find out the existence of major health problems such as diabetes, cardiovascular disease or taking long-term medications were excluded from the study. The inclusion criteria were the participants should be in the age group of 6-10 years and belong to either White/European American or African American ethnicity. The children were brought to Auburn University to participate and written consent was collected from the parents and participants. Auburn University Institutional Review Board approval was obtained.

Anthropometric measurement of the participants Participants' anthropometric measurements were recorded as per WHO guidelines the details are described in our earlier publication by Venkatapoorna et al. [12]. Children were categorized as underweight if their BMI percentile is <5, normal weight if the percentile is between ≥ 5 to ≤ 85, overweight between >85 to ≤95, and obese if the percentile is >95 according to the standardized growth curves developed by Centers for Disease Control and Prevention (CDC) [26].

11q11 copy number measurement by 3D digital PCR The participants provided saliva samples in the DNA GenoTek Saliva Collection kit (Ontario, Canada). Isolation of genomic DNA from salivary samples was performed with the PrepIT-L2P method (DNA GenoTek, Ontario). Briefly, the saliva in the DNA GenoTek kit was mixed well by inversion of the tube for five seconds, incubated in the water bath at 50°C for a minimum of 1 h to release the DNA and inactivate the nucleases. PrepIT-L2P (20 µl) was added to the saliva sample, incubated in ice for 10 min, and centrifuged (15000 x g) for 5 min. The supernatant containing the DNA was separated, mixed with absolute alcohol (600 µl), and the DNA precipitate formed was left to sit

at room temperature (RT) for 10 min. The samples were centrifuged for 2 min, and the pellet containing DNA was separated. The pellet was washed with 250 µl of 70% ethanol, centrifuged (15000 x g) at RT for 1 min, and thoroughly removed the ethanol. TE buffer (75 µl) was added to the pellet, the DNA sample left at RT overnight for complete rehydration and used for 3D digital PCR analysis.

The copy number of *11q11* gene was analyzed using digital PCR (Quantstudio 3D Digital PCR). For performing this reaction mix was prepared with two TaqMan assays, *11q11* (Hs03802074_cn, FAM-labeled; amplicon length 109) and a reference gene (RNase P, VIC labeled) along with 3D digital PCR master mix and diluted DNA (10 ng/µl). From the reaction mix, 14.5µl was loaded into the QuantStudio 3D Digital PCR chip with the help of a chip loader. Chips were loaded into the ProFlex 2X Flat PCR system with the PCR cycling condition of initial denaturation at 96°C for 10 min, followed by 39 cycles at 60°C for 2 min and 98°C for 30 sec, one cycle at 60°C incubation for 2 min and infinite hold at 4°C. Once PCR was completed, the chips were scanned using the QuantStudio 3D Digital PCR system and data analyzed using QuantStudio 3D Analysis Suite software. The copy numbers of 11 q11 in the samples were calculated using the reference gene RNAaseP.

Statistical Analysis The statistical analysis was performed with SPSS 25 software (IBM, Armonk, NY, USA). The graphs were prepared with GraphPad Prism V 8.2.0 for t-test and one-way ANOVA. The data are expressed as Mean ± SEM. The independent sample t-test was used to assess differences between the two groups set a p-value <0.05 as considerably statistically significant. Based on the median of the copy number, the low copy number variant (LCNV) and high copy number variant (HCNV) groups were separated. Categorical variables were determined by calculating the frequencies and calculated as percentage. The statistical variation

between groups was derived with online Medcalc software's comparison of proportion calculator (https://www.medcalc.org/calc/comparison_of_proportions.php).

Results

The study consisted of 127 participants, including 68 EA and 59 AA provided their salivary samples for *11q11* gene analysis. The number of participants with low and high copy number variants in different categories and their corresponding percentages are provided in Table 1. We used the same study participant's salivary samples in our previous study and the details of the anthropometric data are presented in our published article. The anthropometric measurements showed significant differences in OW/OB groups compared to the NW group in participants' weight, BMI, BMI z-score, and waist circumference [12].

The *11q11* CNV descriptive analysis data (Figure 1A) in boys and girls participants showed a significant increase ($p<0.025$) in *11q11* mean CNV in girls (1.724±0.09) compared to boys (1.473±0.07). The *11q11* CNV ranged from zero to four and the distribution of copy number in boys and girls are provided in Figure 1B. We analyzed the percentage of participant proportion in NW and OW/OB groups among boys and girls. Even though the reduced proportion of participants was observed in the NW girls (43%) group compared to NW boys (57%), it was not statistically significant (Figure 1C). However, we found significant difference ($p<0.017$) gender specific in percentage proportion of both low copy number variants (LCNV) and high copy number variants (HCNV). Boys have 61% with LCNV and 39% have HCNV in contrast to girls as shown in Figure 1D. Figure 1F shows that the NW (37%) and OW/OB (33%) girls have significant decreased proportion of participants with LCNV compared to boys. However, in the HCNV group, OW/OB girls (63%) had significant ($p<0.009$) greater participant proportion compared to OW/OB boys (37%). These results suggest that the girls have higher *11q11* CNV due to increased HCNV in OW/OB participants.

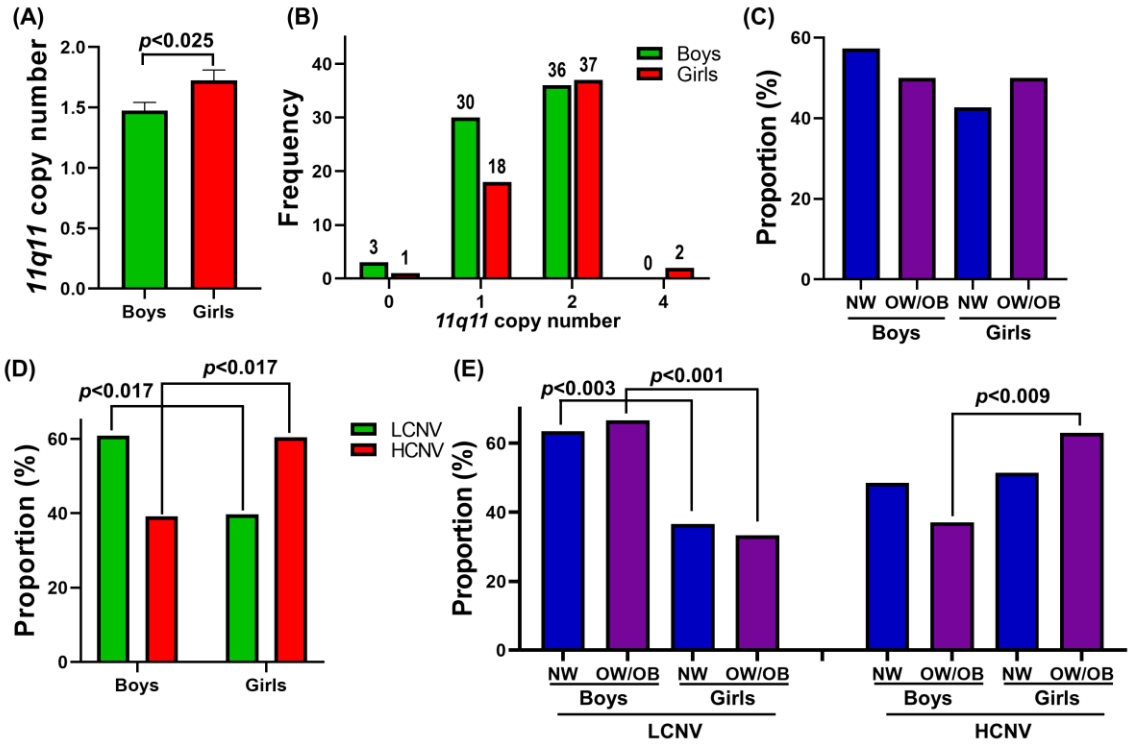

Figure 1. The *11q11* copy number in boys and girls. (**A**) *11q11* CNV descriptive analysis in boys and girls. The copy number of *11q11* was rounded to nearest integer. (**B**) Distribution of copy number in boys and girls. (**C**) Percentage of participant proportion in NW and OW/OB groups among boys and girls. (**D**) Gender specific percentage of participant proportion of both LCNV and HCNV. (**F**) Percentage of participant proportion in NW and OW/OB groups among boys and girls with LCNV and HCNV.

Similarly, we analyzed the differences in the mean *11q11* CNV between white/European American (EA) and African American (AA) participants. AA participants (1.716±0.09) showed a significant increase ($p<0.031$) in the *11q11* copy number compared to EA participants (1.476±0.07). The race specific frequency distribution of CNV is shown in Figure 2B, and two of

the AA participants have four *11q11* CNV, whereas EA did not. We analyzed the proportion of participants in NW and OW/OB group in EA and AA participants and found no significant difference as shown in Figure 2C. In addition, no significant difference was found with LCNV and HCNV between NW and OW/OB group in EA and AA participants (Figures 2D and 2E). This suggests that AA participants have increased mean *11q11* CNV than EA, but there is no significant difference in CNV between NW and OW/OB group.

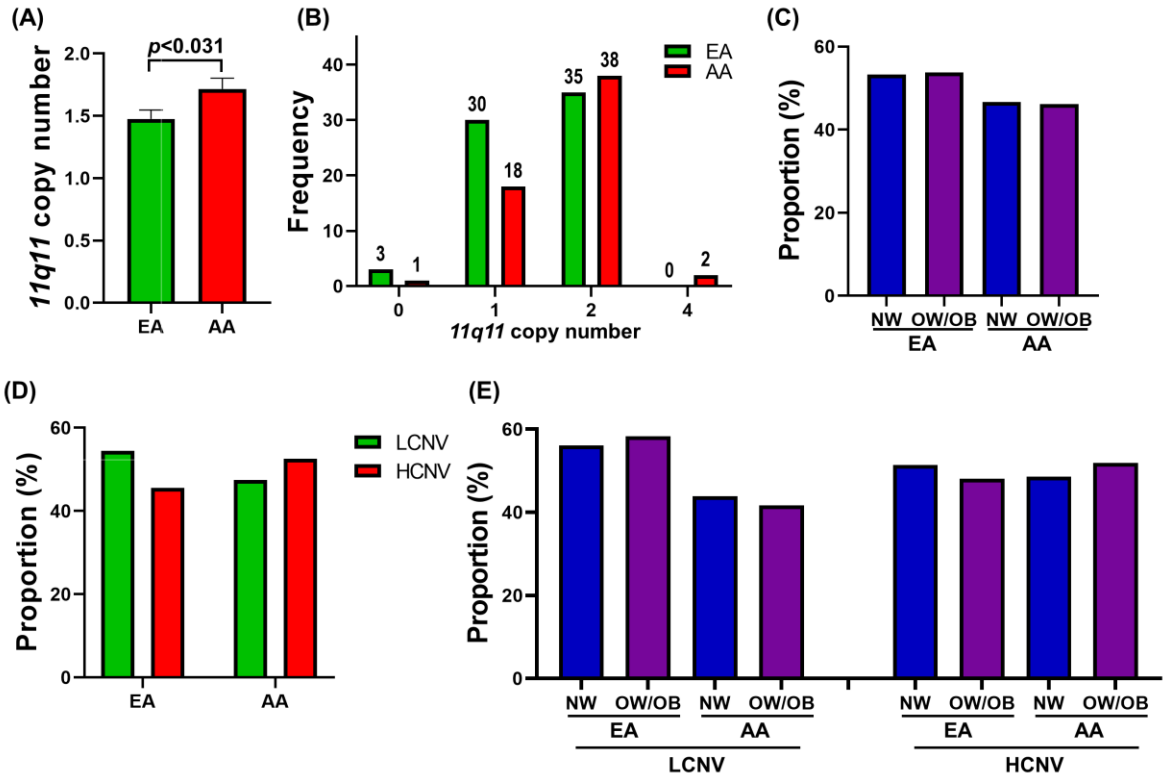

Figure 2. The *11q11* mean copy number in White/European American (EA) and African American (AA) children. (**A**) Distribution of *11q11* copy number in EA and AA. The copy number of *11q11* was rounded to nearest integer. (**B**) The race specific frequency distribution of CNV. (**C**) Percentage of participant proportion in NW and OW/OB groups among EA and AA. (**D**) The proportion of participants with LCNV and HCNV group in EA and AA participants. (**E**) Percentage of participant proportion in NW and OW/OB groups among EA and AA with LCNV and HCNV.

Figure 3A shows the mean *11q11* CNV among boys and girls in EA and AA. There was no significant difference observed in boys and girls among the races. The gender specific frequency distribution in EA and AA are shown in Figures 3B and 3C. Increased number of African American girls had 2 and 4 *11q11* copy number compared to EA participants. Figure 3D showed the proportion difference between LCNV and HCNV in NW and OW/OB group, gender and race specific. In EA, the OW/OB girls (7%) have significantly decreased proportion of participants with LCNV ($p<0.0001$) compared to the corresponding OW/OB boys (44%). The HCNV in EA was significantly increased ($p<0.03$) in OW/OB girls (33%) compared to OW/OB boys (15%). In AA, there was no significant difference in the LCNV and HCNV between OW/OB boys and girls. The comparison of percent proportion between the race showed a significant increase in LCNV ($p<0.003$) in OW/OB boys of EA (44%) compared to OW/OB boys of AA (17%). But, the OW/OB girls of EA (7%) have significantly decreased LCNV ($p<0.017$) than OW/OB girls of AA (25%). The results suggest that the increase in the *11q11* CNV in girls is mainly due to the increase in HCNV in EA OW/OB participants.

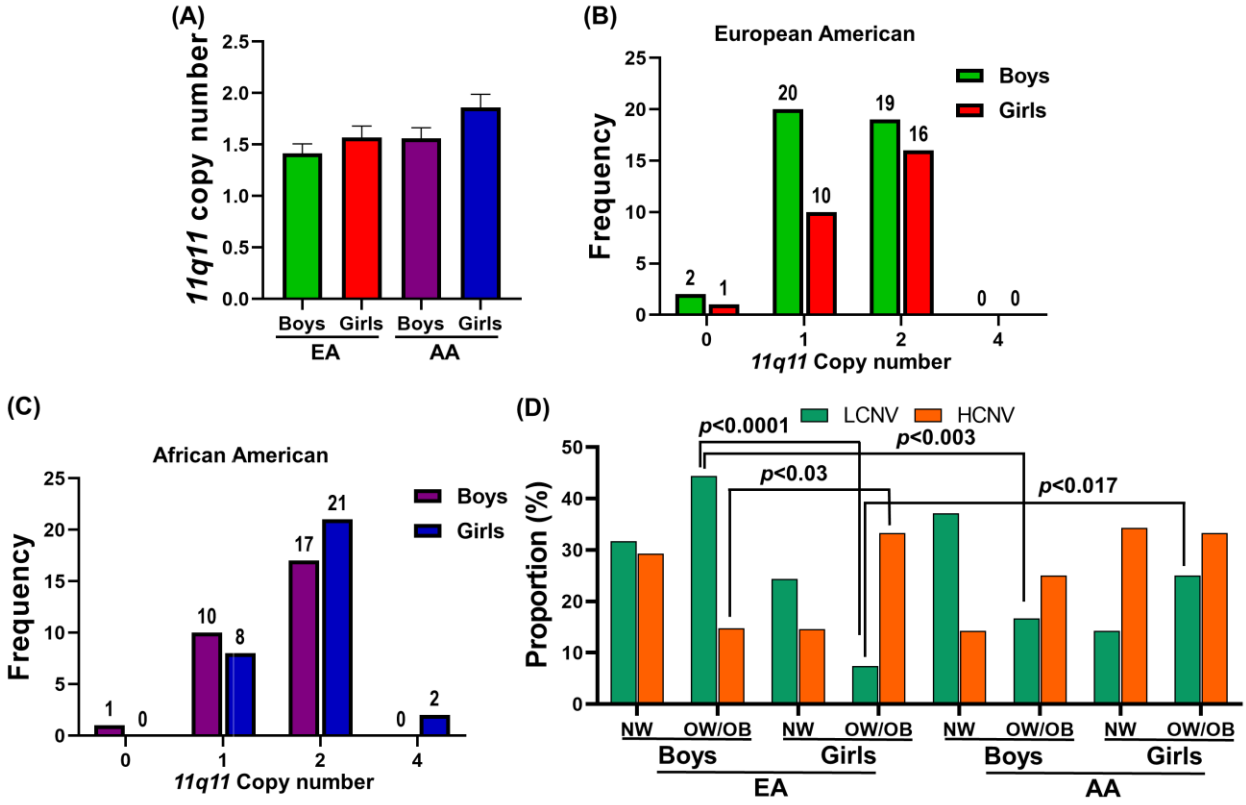

Figure 3. The *11q11* mean copy number analysis. (**A**) Mean *11q11* CNV among boys and girls in EA and AA. The copy number of *11q11* was rounded to nearest integer. (**B)** Gender specific frequency distribution in EA and **(C)** AA. (**D**) Proportion difference between LCNV and HCNV in NW and OW/OB group, gender and race specific.

Discussion

The present study examined the association between the obesity measurements and 11q11 copy number in elementary school children in Alabama aged 6–10 years old with normal weight and overweight/obese. The median copy number of *11q11* was higher in girls compared to boys. We found that the *11q11* copy number was negatively associated with the obesity measures, such as weight, BMI, BMI z-score, and waist circumference. Interestingly, these results correspond to another study in children of German descent, which suggested that a genetic link between obesity and *11q11* copy number. A family-based genome-wide study revealed a lower *11q11* copy number in obese children compared to those of normal weight [21]. Increased BMI was also found to be associated with a low *11q11* copy number in Chinese children. Zhang *et al.* observed a significant increase in the risk of obesity in Chinese children that had deletions at the *11q11* locus [23]. In addition, a cumulative effect was seen in Chinese children when this was accompanied by deletions at two other loci related to obesity: 10q11.22 and 4q25. This indicates a strong cumulative association between loci with increased CNV and childhood obesity [23]. In contrast, León-Mimila *et al.* reported a positive association in Mexican children aged 6-12 years [22]. A lower *11q11* copy number was significantly associated with lower obesity risk in children. The same association was not seen in adult subjects [22]. Therefore, a large discrepancy exists in people of different ethnicities and within a specific population.

In addition to GWAS, 11q11 has been analyzed in individual case studies. Obesity is associated with a variety of liver abnormalities collectively known as nonalcoholic fatty liver disease (NAFLD) that is characterized by an accumulation of triglycerides in the liver (steatosis)

[25]. NAFLD is associated with cardiometabolic syndrome (CMS), increasing the risk of insulin resistance, impaired glucose tolerance, hypertension, dyslipidemia, and central adiposity [26]. The *11q11* region has been shown to have one copy number less in adult patients with NAFLD when compared to healthy adults, who had a copy number around 2 [23]. We also assessed the impact of race and found that the *11q11* copy number was negatively associated with the obesity measures more significantly in European American boys compared to African American boys from the similar age range and region [24].

There are a few limitations to this study worth noting. The participants on which the results are based were recruited from only two counties in Alabama. A broader geographical range and number of participants would be ideal for a more indicative sample. In addition, the dietary habits, environmental factors, socioeconomic status, and parental influences that might influence copy number are not included. Several nutrition education programs are working to reduce and prevent obesity by educating children on healthy diet and the importance of fresh fruits and vegetables in schools across the state of Alabama [27].

In summary, we found the 11q11 copy number was significantly lower in obese compared to normal weight children. A significant inverse association between obesity measurements and 11q11 copy number was observed. European Americans have a stronger association between low 11q11 copy number and obesity compared to African Americans.

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Chapter 4

Telomere Length as a Biomarker for Race-Related Health Disparities

Abstract

Disparities between the races have been well documented in health and disease in the USA. Recent studies show that telomere length, a marker of aging, is associated with obesity and obesity-related diseases, such as heart disease and diabetes. The current study aimed to evaluate the connection between telomere length ratio, blood pressure, and childhood obesity. The telomere length ratio was measured in 127 children from both European American (EA) and African American (AA) children, aged 6–10 years old. AA children had a significantly high relative telomere to the single copy gene (T/S) ratio compared to EA children. There was no significant difference in the T/S ratio be-tween normal weight (NW) and overweight/obese (OW/OB) groups of either race. Blood pressure was significantly elevated in AA children with respect to EA children. Hierarchical regression analysis adjusted for race, gender, and age expressed a significant relationship between the T/S ratio and diastolic pressure. Low T/S ratio participants showed a significant increase in systolic pressure, while a high T/S ratio group showed an increase in diastolic pressure and heart rate of AA children. In conclusion, our findings show that AA children have high T/S ratio compared to EA children. The high T/S ratio is negatively associated with diastolic pressure.

Introduction

Telomeres are present at the end of the chromosomes as shielding caps and protect the degradation targeted by the chromosome's cellular DNA damage. Telomeres consist of a repeated number of tandem sequences, TTAGGG, incorporated with a protein complex [1]. Telomere shows a substantial variation among individuals from birth and decreases during aging.

Obesity is a chronic disease in children and adults, developed in recent decades, and increases comorbidities in adulthood [2]. Worldwide obesity prevalence increased rapidly due to its high impact among children and adults [3]. Hence, it is imperative to treat young children to avoid obesity-related difficulties during their adulthood. The literature reveals that the correlation between obesity and telomere length have shown ambiguous results in adults. Some reports indicate a direct or inverse relationship be-tween obesity and telomere length [4–7], but it differs in children. There is no significant difference in the telomere length reported between normal weight obese Caucasian children, but the obese adults had a shorter telomere length than the normal weight adults [8]. Very few studies discussed the correlation between telomere length and obesity due to lifestyle changes, suggesting that telomere length is either protected or extended while maintaining or reducing weight [4,5,9]. Even though overall telomere length is shorter in the obese state [10], studies conducted in older adults did not show any relationship between telomere length and obesity [5,7]. All individuals vary in telomere length with its reflection at birth [11], and the difference is observed throughout life [12,13]. Oxidative stress is a process that increases the shortening of telomeres during each cell division in somatic cells [14,15]. Inflammation is an additional factor responsible for telomere reduction in leukocytes by increasing hematopoietic stem cells [16]. Obesity is the foremost independent risk factor for increasing aging and it is associated with metabolic diseases, such as hypertension, diabetes,

cardiovascular disease, and cancer [17–21]. Even though the causal directions of these relationships persist to be defined [22,23], shorter telomeres are a well-established biomarker for various age-related conditions [24].

Recent studies have shown that telomere length, a marker of cellular aging, is used in studies of race-based health disparities and it is sensitive to effects of social stress [25–28]. Notably, African Americans have high levels of poor health, and it is expected for African Americans to have shorter telomeres than European Americans (EAs). Interestingly, recent findings show that African Americans (AAs) have longer telomere length in contrast to the expectations [29–33].

In industrialization, an age-related rise in pulse pressure is used as a phenotype of biological aging of the vasculature. In almost 95% of the hypertensive cases, hypertension is unknown [34]. Telomere attrition has been linked through hypertension and, during aging, it has been found in cells such as vascular endothelial cells, smooth muscle cells, and cardiomyocytes [35]. Therefore, in the current study, we examined the relationship between telomere length and blood pressure among EA and AA children. The relative telomere length to a single copy gene (T/S ratio) was calculated by com-paring the telomeric DNA (T) level with the single copy (S) hemoglobin subunit γ one gene in salivary genomic DNA by quantitative real-time PCR. First, we tested whether the mean T/S ratio differed across the overall participants' races or weight category and low and high T/S ratio groups. Second, we tested the relationship between blood pressure and heart rate with low and high T/S ratios.

Materials and Methods

Study Subjects The Auburn University Institutional Review Board for the Protection of Human Subjects in Research approved this study. The written consent was obtained from the participants and their parents before sample collection. By distributing the study flyer in the Lee and Macon counties in Alabama, participants were recruited from schools, after-school programs, through friends, and via participant referrals. Participants were recruited between ages 6–10 years, and a phone survey was conducted before recruiting to exclude the participants with health conditions such as diabetes and cardiovascular disease.

Anthropometric Measurements and Saliva Collection The participants' body weight and height were measured, as described in our previous study [36]. The body mass index (BMI) percentile range was calculated according to the Centers for Diseases Control and Prevention (CDC), and the participants were grouped into normal weight and overweight/obese [37]. Saliva collection and isolation of salivary DNA was performed, as previously discussed [36]. The DNA isolated was used to measure the telomere length.

Blood Pressure Measurement Measurement of blood pressure (BP) is known as an integral part of clinical ex-aminations. The "gold standard" method was used to record the BP in participants as auscultatory, with an aneroid non-mercury manometer connected to a suitable cuff for the children. The systolic and diastolic pressure, along with heart rate, was recorded.

Telomere Length Measurement The telomere length was measured as previously described in Cawthon et al. (2002), using a QuantStudio 3 real-time polymerase chain reaction (PCR) system [38]. The qPCR reaction mixture was performed with a volume of 20 µl using the following reagents. Assays were performed using 96-well plates (Thermo Fisher Scientific, Waltham, MA, USA) and the qPCR mix contained SYBR green PCR master mix (which

included ROX passive dye), telomere primers (Tel 1, 5'-GGT TTT TGA GGG TGA GGG TGA GGG TGA GGG TGA GGG T-3' and Tel 2, 5'-TCC CGA CTA TCC CTA TCC CTA TCC CTA TCC CTA TCC CTA-3') (Integrated DNA Technologies, Inc., Coralville, IA, USA), and RNase free water to adjust the reaction volume. With this qPCR mix, a template DNA was added to perform the PCR. Simultaneously, single-copy gene qPCR was performed by preparing the qPCR mix of SYBR green PCR master mix with ROX passive dye, single copy number gene primers (hbg 1, 5'-GCT TCT GAC ACA ACT GTG TTC ACT AGC -3' and hbg 2, 5'-CAC CAA CTT CAT CCA CGT TCA CC-3') (Integrated DNA Technologies, Inc., Coralville, IA, USA), and RNase free water, along with template DNA. The telomere PCR and single-copy gene PCR was performed in separate 96-well plates matching the same well position of telomere PCR in the first plate and single-copy gene PCR on the second plate. The plates were prepared with six-point reference HeLa DNA (New England BioLabs, Ipswich, MA, USA) from the highest concentration of 150 ng/mL three-fold dilution in duplicates to make a stand-ard curve. There were two negative control wells and four positive control wells (equivalent concentration of sample DNA template) on every plate. Plates were sealed with transparent optical adhesive covers (Thermo Fisher Scientific, Waltham, MA, USA) and loaded in the QuantStudio 3 real-time PCR system. The reaction was carried out using the following cycling profile of initiation for 10 min at 95 °C, followed by 40 cycles of denaturation at 95 °C for 20 s, annealing at 52 °C for 20 s and extension at 72 °C for 45 s with signal acquisition; and then a melt curve was performed.

For each run of qPCR, amplification curves and melt curves were inspected to evaluate the sample and qPCR run quality, along with the absence of amplification in negative controls. Before calculating the T/S values, a coefficient variation of an inter and intra assay was

examined. The relative measure of telomere length and T/S ratio in each sample was calculated in a separate excel sheet using the following formula:

$\Delta Ct = CtTel - Cthbg$

ΔCt of control $= CtTel - Ctcontrol$

$\Delta\Delta Ct = \Delta Ct - \Delta Ct$ of control

Relative $T/S = 2^{-\Delta\Delta Ct}$

The low and high T/S ratio groups were separated based on the median T/S ratio. The low T/S ratio group denotes that the T/S ratio values were less than the median. On the other hand, the participant's T/S ratio was higher than the median, named a high T/S ratio group.

Statistical Analysis All the data in the graphical representation was expressed as mean ± SEM, $p < 0.05$ considered as significant. The descriptive and hierarchical regression analyses were performed using the Statistical Package for Social Sciences (SPSS, version 25, IBM, Armonk, NY, USA). We used hierarchical regression analysis, introducing co-variates such as race, age, and gender to predict the dependent variables. A two tailed Students t-test was used to calculate the mean difference between the two groups. The graphs were prepared using Graphpad Prism version 8 (San Diego, CA, USA). The proportion graphs were prepared using the number of participants in each category of study. The statistically significant difference in proportion graphs was calculated using the online MedCalc software website (Ostend, Belgium).

Results

The anthropometric measurements, telomere length ratio, and blood pressure for low and high T/S ratio groups are listed in Table 1 from the 127 participants, aged 6–10 years. There was no significant difference observed between the low and high T/S ratio of age, height, weight, and BMI. The T/S ratio did not show a significant difference be-tween EA and AA children, normal weight (NW) and overweight/obese (OW/OB) groups. Systolic, diastolic pressure and pulse were among the low and high T/S ratio groups not showing significant changes. The mean T/S ratio of all participants was 1.06 ± 0.04, with a median of 1.066. The mean T/S ratio of EA participants was 0.907 ± 0.04 (median 0.805) and AA participants was 1.23 ± 0.07 (median 1.216). The mean T/S ratios of NW and OW/OB participants were 1.07 ± 0.04 and 1.04 ± 0.07, respectively.

Table 1. General characteristics of the study population.

	Low T/S Ratio (64)	**High T/S Ratio (63)**	***p* Value**
Age (year)	8.40 ± 0.19	8.19 ± 0.18	$p < 0.427$
Height (cm)	133.01 ± 1.42	130.89 ± 1.45	$p < 0.297$
Weight (kg)	32.39 ± 1.34	32.07 ± 1.42	$p < 0.868$
BMI (kg/m^2)	17.98 ± 0.41	18.21 ± 0.45	$p < 0.696$
T/S Ratio			
All	0.739 ± 0.02	1.38 ± 0.05	$p < 0.0001$
EA	0.724 ± 0.02	1.26 ± 0.04	$p < 0.0001$
AA	0.776 ± 0.06	1.44 ± 0.08	$p < 0.0001$
NW	0.750 ± 0.03	1.35 ± 0.05	$p < 0.0001$
OW/OB	0.726 ± 0.04	1.43 ± 0.11	$p < 0.0001$
Blood Pressure			
Systolic Pressure (mmHg)	100.52 ± 1.31	101.61 ± 1.72	$p < 0.610$
Diastolic Pressure (mmHg)	66.05 ± 1.24	67.38 ± 1.40	$p < 0.477$
Heart rate (BPM)	84.52 ± 1.58	84.51 ± 1.57	$p < 0.997$

European American (EA); African American (AA); normal weight (NW); overweight/obese (OW/OB); telomere length/ single copy gene (T/S ratio); values are expressed as mean ± SEM.

To determine the difference in the telomere length ratio between races, the calcu-lated T/S ratio was analyzed by the t-test. AA participants showed a significantly ($p < 0.0001$) higher T/S ratio compared to EA participants (Figure 1a). The telomere length ratios among races for NW and OW/OB groups were also compared. The NW and OW/OB participants did not show any significant differences in either EA participants (NW-0.922 ± 0.05; OW/OB-0.883 ± 0.05) or AA participants (NW-1.235 ± 0.07; OW/OB-1.22 ± 0.14) (Figure 1b,c).

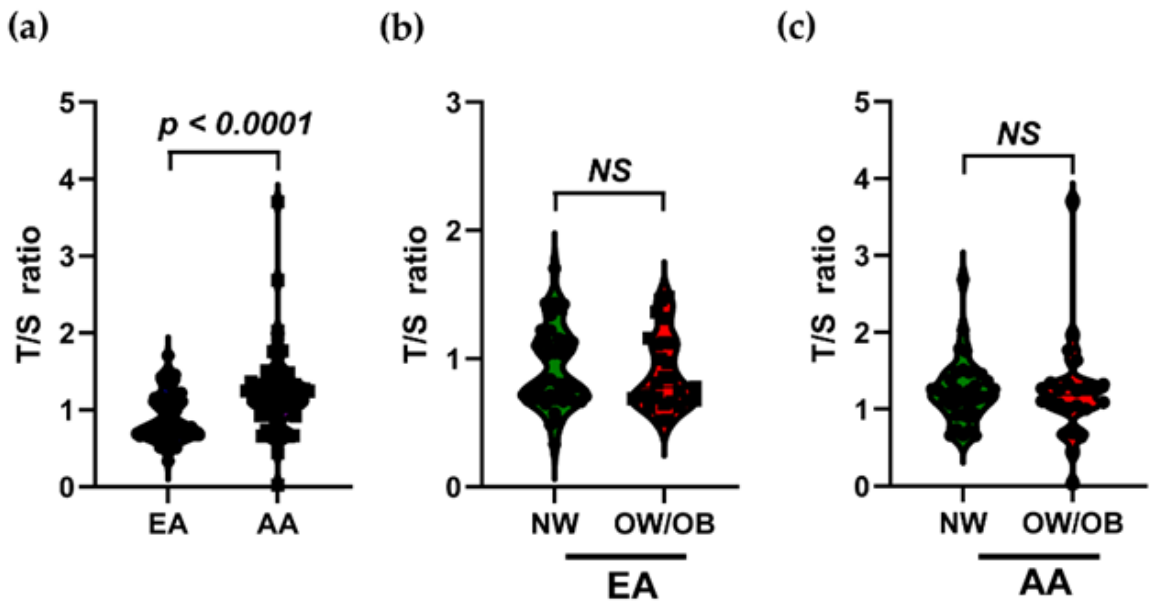

Figure 1. The violin plot with data points shows the distribution of the T/S ratio in EA and AA children. (a) The mean T/S ratio is compared between EA and EA children. The T/S ratio of NW and OW/OB children is compared in EA (b) and AA children (c). Values are expressed as mean ± SEM. European American (EA); African American (AA); normal weight (NW); overweight/obese (OW/OB); telomere length/ single copy gene (T/S ratio); Not significant (NS).

Based on the median T/S ratio, the study group was divided into two: low and high T/S ratios. The proportion of the low T/S ratio participants was significantly higher in the EA group (70.31%) than the AA group (29.69%). On the contrary, a greater proportion of high T/S ratio participants were found in the AA group (63.49%) in comparison to the EA group (36.51%), as shown in Figure 2a. The proportion of participants with low T/S ratios in both NW and OW/OB groups of the AA participants decreased significantly ($p < 0.01$) compared to EA participants of NW and OW/OB groups. However, the proportion of high T/S ratios in both NW and OW/OB groups of AA participants significantly ($p < 0.05$) increased in comparison to corresponding EA participants (Figure 2b). This result suggests that the T/S ratio in AA participants significantly increased compared to EA participants, but there was no difference between the NW and OW/OB groups with EA or AA children.

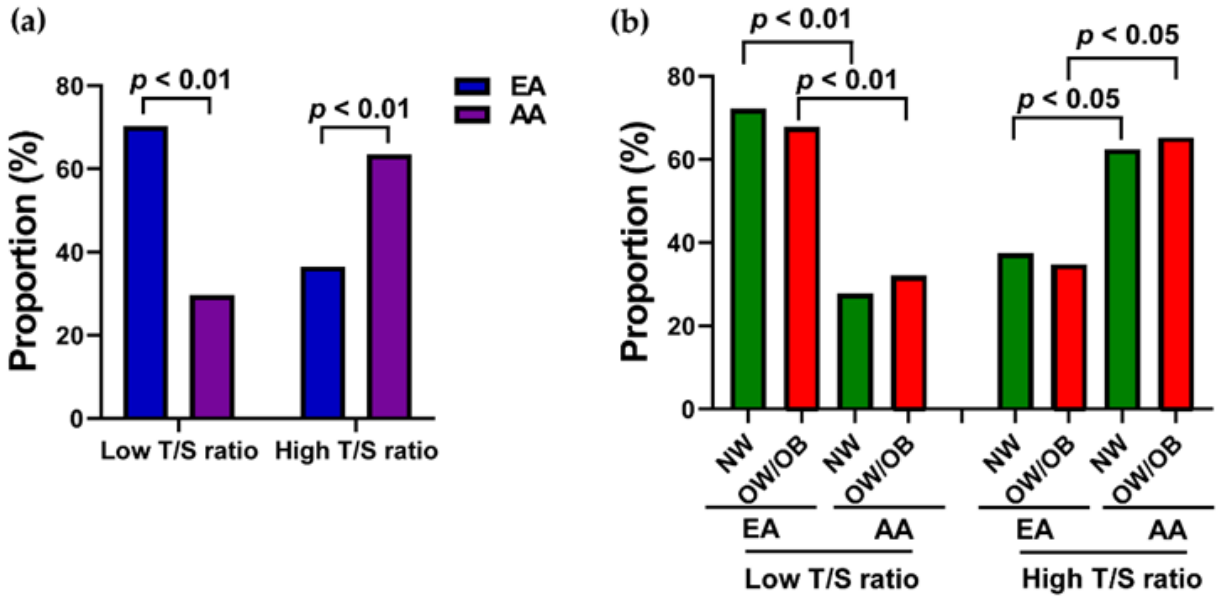

Figure 2. Proportion of participants with low and high T/S ratio groups. (**a**) The proportion difference participants with low and high T/S ratios in EA and AA children. (**b**) The proportion difference among NW and OW/OB children of EA and AA participants with low and high T/S ratios.

Next, the difference in the circulatory pressure between race, NW, and OW/OB participants was determined. The systolic blood pressure ($p < 0.004$) and diastolic pressure ($p < 0.002$) among AA participants significantly increased compared to the EA group (Figure 3a,b). However, there was no difference observed between AA and EA participants' pulse rates (Figure 3c). The OW/OB participants of both the EA and AA groups had significantly higher systolic blood pressure compared to NW participants. However, the diastolic pressure and pulse rate among NW and OW/OB groups of EA and AA participants did not show significant changes (Figure 4a,b). This suggests that the systolic and diastolic pressure increased in the AA participants compared to EA participants but only the systolic pressure in OW/OB was greater than the NW group of EA and AA participants.

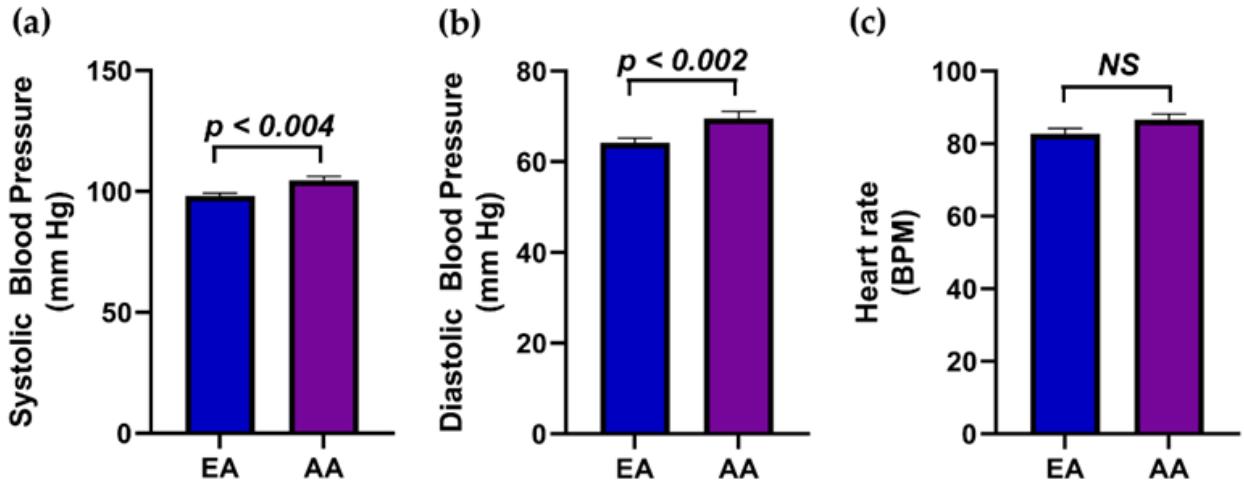

Figure 3. The blood pressure of EA and AA participants. The difference in the (**a**) systolic pressure, (**b**) diastolic pressure, and (**c**) heart rate between EA and AA children. Values are expressed as mean ± SEM.

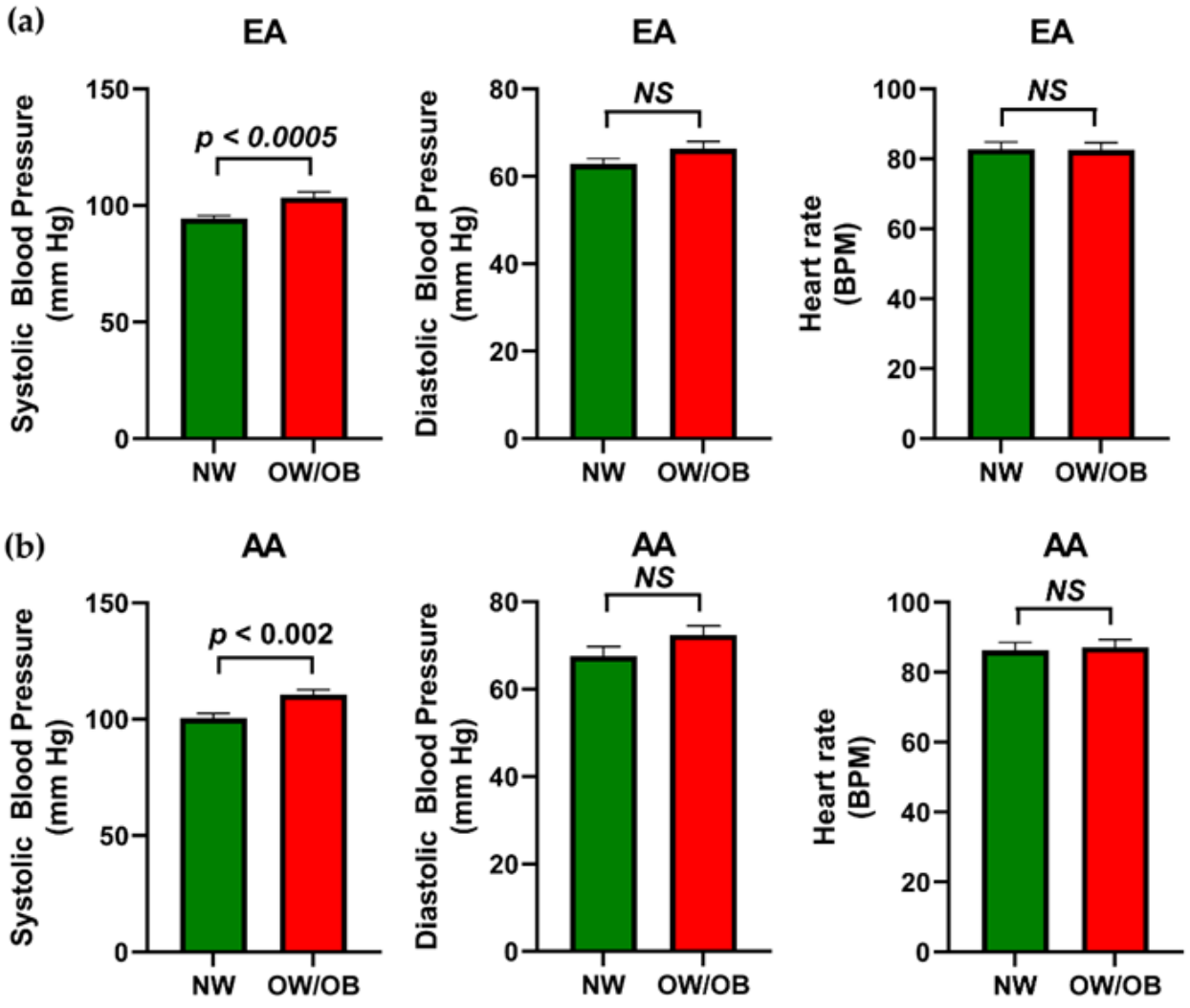

Figure 4. Comparison of blood pressure in NW and OW/OB children of EA and AA participants. The bar graphs shows the differences in the blood pressure in the NW and OW/OB groups of (**a**) EA participants and (**b**) AA participants. Values are expressed as mean ± SEM.

The differences in the blood pressure and heart rate of the low and high T/S ratio participants were determined between EA and AA groups. The systolic pressure was significantly increased ($p < 0.004$) in AA participants compared to EA participants with low T/S ratios. However, there was no difference in diastolic pressure and heart rate with low T/S ratios (Figure 5a). AA participants with a high T/S ratio observed significantly increased diastolic pressure ($p < 0.01$) and heart rate ($p < 0.01$) compared to the EA group. Nevertheless, systolic blood pressure did not significantly change in high T/S ratio participants (Figure 5b). This explains that the AA children with low T/S ratios had higher systolic pressure and those with high T/S ratios had higher diastolic pressure and heart rate compared to EA children.

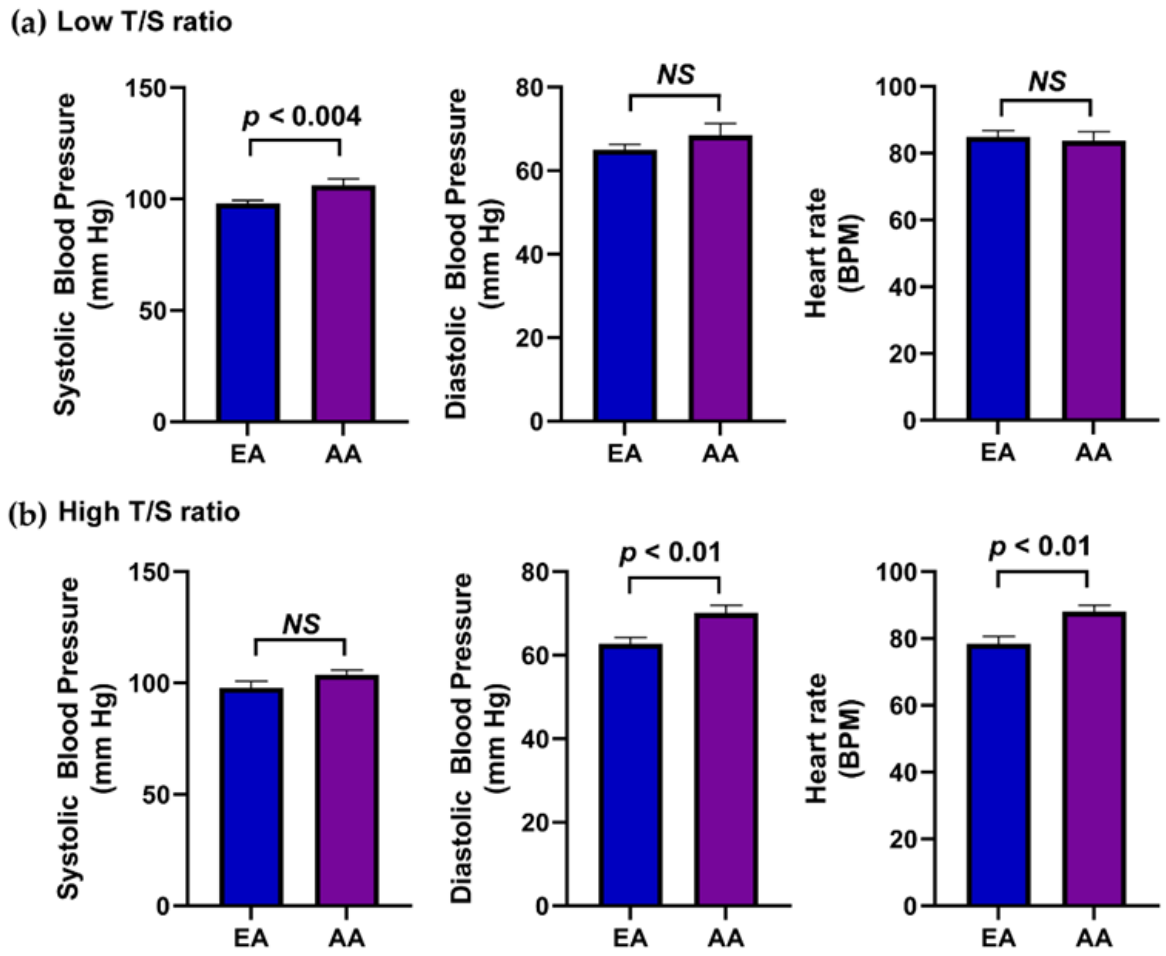

Figure 5. Comparison of blood pressure in EA and AA children with low and high T/S ratios. Bar graphs shows the differences in blood pressure in EA and AA participants with (**a**) the low T/S ratio and (**b**) the high T/S ratio. Values are expressed as mean ± SEM.

The relationship between the T/S ratio and circulatory pressure for unadjusted and adjusted hierarchical regression was analyzed. Race, gender, and age of the participants were entered to adjust for covariates. Both adjusted and unadjusted hierarchical regression was performed with all participants for low and high T/S ratio groups between T/S ratios, systolic, diastolic pressure, and heart rate. Even though systolic pressure showed positive and diastolic pressure, it showed a negative correlation with the T/S ratio. There was no significant difference observed in the β-coefficient of unadjusted regression analysis. After adjusting for race, gender, and age, diastolic pressure showed a significant negative correlation ($\beta = -0.327$; $p < 0.049$) with high T/S ratio participants (Table 2). This suggests that children with high T/S ratios are negatively correlated with diastolic pressure.

Table 2. The relationship between T/S ratio and blood pressure by hierarchical regression analysis (Adjusted for race, gender, and age).

Parameters	Unadjusted						Adjusted					
	B	SE	B-Coefficient	95% Confidence Interval for b		*p*-Value	B	SE	B-Coefficient	95% Confidence Interval for b		*p*-Value
				Lower Bound	Upper Bound					Lower Bound	Upper Bound	
All participants												
Systolic Pressure	0.005	0.004	0.136	−0.003	0.014	0.242	0.003	0.004	0.079	−0.006	0.011	0.497
Diastolic Pressure	−0.005	0.005	−0.113	−0.015	0.005	0.333	−0.007	0.005	−0.162	−0.016	0.002	0.143
Pulse	0.000	0.003	0.012	−0.006	0.007	0.891	−0.002	0.003	−0.057	−0.008	0.004	0.508
Low T/S ratio participants												
Systolic Pressure	0.000	0.003	0.017	−0.006	0.006	0.923	−0.001	0.004	−0.051	−0.008	0.006	0.800
Diastolic Pressure	−0.003	0.003	−0.135	−0.009	0.004	0.444	−0.001	0.003	−0.077	−0.008	0.005	0.679
Pulse	−0.001	0.002	−0.093	−0.005	0.002	0.474	−0.001	0.002	−0.090	−0.005	0.003	0.495
High T/S ratio participants												
Systolic Pressure	0.007	0.005	0.251	−0.002	0.017	0.122	0.006	0.005	0.205	−0.004	0.016	0.228
Diastolic Pressure	−0.010	0.006	−0.259	−0.021	0.002	0.109	−0.012	0.006	−0.327	−0.024	0.000	**0.049**
Pulse	0.003	0.004	0.085	−0.006	0.011	0.508	−0.001	0.005	−0.034	−0.010	0.008	0.809

Unstandardized coefficient (B); standard error (SE). The bold number shows the significant.

Discussion

The current study includes 68 EA and 59 AA (total 127) children with an age range of 6–10 years. The study population was divided into low and high T/S ratios based on the median. We did not observe differences in the T/S ratio according to age, height, weight, or weight category. The results agree with previous studies in children and adult populations [39,40]. This may be due to the narrow range of ages and sample sizes used in the study. The mean T/S ratio in the AA children increased compared to the EA children. Similar results were found by Rewak et al., with significantly longer leucocyte telomere length (LTL) in Blacks than Whites at birth and adulthood [41]. Several studies showed longer LTL on average in AA participants [29–33]. In adulthood, shorter leukocyte telomeres are correlated with BMI in women [42] and waist-hip ratios in both genders [13]. However, previous studies on childhood obesity and telomere length showed an inconclusive result. The NW and OW/OB children in both EA and AA participants did not show any significant changes in the mean T/S ratio. These results are in correlation with Zannolli et al., where the leukocyte telomere length did not show any difference between obese and non-obese Italian children [8]. The participants were separated based on the T/S ratio's median into low and high T/S ratio groups. In the high T/S ratio group, the proportion of the AA children was more compared to EA children, and the proportion of participants of NW and OW/OB children in the AA group showed higher numbers. This result clearly correlates with the previous results published by Rewak et al. on the childhood telomere length [41].

Systolic and diastolic pressure was significantly increased in AA children com-pared to EA children. Globalization increases systolic blood pressure throughout life [43,44]. Diastolic blood pressure is also increased in early life, but it tends to decrease in aged persons [45]. Arterial aging has predominantly increased the stiffness of central elastic arteries, which is the

factor that regulates pulse pressure. The factors that increase the biological aging of vessel formation, including essential hypertension [46], other diseases, such as diabetes [47], and higher amounts of salt intake [48], independently increase the arterial stiffness. Connecting all these processes proposes that aortic blood pressure might show a phenotype of aging and is one of the major risk factors for cardiovascular disease [49–51].

The systolic pressure of the OW/OB children of both EA and AA races was significantly increased than in NW children. The overall T/S ratio was high in AA participants, and the diastolic blood pressure and heart rate also increased in the same participant group. Our results are consistent with earlier cross-sectional findings of race/ethnicity differences in telomere length at different age groups. In particular, Rewak et al. showed a longer telomere length in the AA population [41]. The study conducted with NHANES data suggests that cardiovascular health is associated with smaller leucocyte telomere lengths and race/ethnicity [52]. Numerous studies have consistently reported that more prevalence of hypertension in AA participants than EA participants is the primary reason for increased cardiovascular disease occurrence in the AA community [53]. Based on the median of the T/S ratio, significantly increased diastolic pressure and heart rate was observed in the high T/S ratio of AA participants. The shorter telomere lengths are correlated with hypertension in adults [54]. We found that systolic pressure was only significantly increased in AA children with a low T/S ratio. Additionally, our results suggest that the characteristic is only a significant analyst of telomere length in adulthood and not in childhood. Aydos and Tukun (2007) describe no correlation between systolic pressure, diastolic pressure, BMI, and telomere length [55]. However, in this study, the hierarchical regression analysis, after adjusting with covariates such as race, gender, and age, indicates that children with high T/S ratios are negatively correlated with diastolic pressure.

Some limitations need to be acknowledged. We used a real-time PCR method to measure the telomere length ratio with higher assay variability in general than the traditional telomere restriction fragment method [56,57]. The plate-to-plate variability effect was minimized using the corresponding well layout of the telomere primer and single-copy gene [9]. The results should also be considered preliminary due to the small sample size in the study.

Conclusions

In this study, we explored the connection between telomere length ratio, blood pressure and childhood obesity. Our results demonstrated that AA children have a greater mean T/S ratio and systolic and diastolic pressure compared to EA children. The OW/OB children of both EA and AA groups have higher systolic pressure com-pared to NW children. AA children with low T/S ratios have increased systolic pressure and children with high T/S ratios have increased diastolic pressure and heart rate than EA children. The children with high T/S ratio are negatively correlated with diastolic pressure. This study validates the use of non-invasive salivary measurement of telomere length and addresses the gap between the T/S ratio and blood pressure among children.

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