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DNA Methylation of the AGTRI Promoter in a Hypertensive Population of Kenyans

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DNA METHYLATION OF THE *AGTR1* PROMOTER IN A HYPERTENSIVE POPULATION OF KENYANS

BY

Michael M. Roque

A Thesis

Submitted to the Honors College of the
University of South Alabama
in partial fulfillment of the
requirements for the degree of

Bachelor of Science

in

Biomedical Sciences

May 2024

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ABSTRACT

Michael M. Roque, B. S., University of South Alabama, May 2024. DNA methylation of the *AGTR1* promoter in a hypertensive population of Kenyans. Chair of Committee: Nancy A. Rice, Ph.D.

Cardiovascular disease (CVD), the leading cause of death worldwide, is rapidly increasing in low-and middle-income countries (LMICs), particularly those of Sub-Saharan Africa (SSA). Hypertension is the leading risk factor for CVD and is a multifactorial disease with no single genetic cause. Increasingly, evidence indicates that hypertension is predisposed by environmental regulation of genes through heritable, yet modifiable, epigenetic changes to DNA leading to changes in gene expression, e.g. methylation. While understanding the etiology of hypertension in LMICs is a global priority, few epigenetic studies exist from populations living in SSA. The renin-angiotensin system (RAS) is the primary hormonal pathway that regulates blood pressure through changes in salt and water retention. Previously, we have found a high prevalence of hypertension (55 % had systolic blood pressure (SBP) >130 mmHg) in a rural population of Kenyans that was not correlated with lifestyle or behavioral factors. As a result, this study investigates the hypothesis that epigenetic regulation, specifically DNA methylation of *AGTR1* promoter, as a result of exposure to household air pollution (HAP), results in an increased risk of high blood pressure in this population. Studies from our lab show there may be increased methylation in hypertensive versus normotensive

Kenyans (18.4 ± 2 and 12.92 ± 1.78, respectively) when four cytosine-phosphate guanine (CpG) sites of the *AGTR1* promoter were analyzed (n=34). Increased methylation in hypertensive versus normotensive Kenyans for CpG 3 was observed (44.1 ± 4.95 and 26.42 ± 6.68, respectively) (p=0.047). Additionally, when monetary spending on charcoal was analyzed as a proxy for HAP exposure, monthly spending on charcoal was increased for hypertensive versus normotensive Kenyans (552.94 ± 126.35 and 388.24 ± 136.62, respectively).

CHAPTER I

INTRODUCTION

Africa is subdivided into numerous lower- and middle-income countries (LMIC) and contains 11% of the world's population and 24% of the world's diseases (Seedat, 2007). Despite this, the continent has severe resource constraints with 3% of the world's healthcare workers and 1% of the world's financial resources. LMICs have concentrated most of their financial resources and human capital on fighting communicable diseases within the past few decades largely due to their acute health and socioeconomic effects (Dalal, Shona, *et al.*, 2011). Even with these efforts, in 2021 communicable diseases caused half of all the deaths in Sub-Saharan Africa (SSA). The worldwide focus on battling communicable disease mortality has led to a steadily increasing rise in the impact of non-communicable diseases (NCD). In 2000, NCD deaths accounted for 60.8% of mortality; in 2019, that percentage increased to 73.6% (World Health Organization, 2021). In SSA, the frequency of NCD is expected to stay the same or even increase beyond that of developed countries in the future. The rise in NCD deaths can also be explained by the patterns of mortality and population. This can be explained by the epidemiologic transition. The policy and efforts for communicable diseases have allowed the worldwide population to live longer. The major features of the transition include a decline in mortality and an increase in life expectancy (McKeown). The major shift is

that the leading causes of morbidity and mortality from communicable diseases have been replaced by NCD. Therefore, focus on NCD, including CVD, research, and treatment in SSA can play a vital role in the control and prevention of its burden.

1.1 Cardiovascular Disease

LMIC are significantly impacted by NCD. Forty-one million people die from NCD each year, accounting for 71% of deaths globally. Cardiovascular diseases account for the most NCD deaths worldwide, killing 17.9 million people each year (WHO). More than three-quarters of these deaths occur in lower- and middle-income countries, making CVD the biggest burden in LMIC.

Many trends associated with CVD differ between HIC and LMIC (Bowry *et al.*, 2015). WHO predicts that three times as many deaths from CVD in LMIC occur mostly with younger, working-age individuals (Bowry *et al.*, 2015). In contrast, the frequency of CVD deaths in HIC is statistically lower and occurs mostly in older individuals (>60 years old). In terms of controlling CVD, mortality rates from CVD in HIC dropped by 30-60% from 2000-2019 while in LMIC no improvement in lowering the rates of mortality was observed (Bray). The burden that CVD has worldwide, and specifically LMIC can be reduced with proper management that includes further research and treatment.

1.2 Risk Factors Associated with CVD

An increase in risk factors has contributed to the impact that CVD and other NCD have in LMIC. The lack of knowledge of the risk factors of various NCD, including CVD, has led to the lack of preventative care, an important role in controlling the CVD trends in HIC. The leading risk factors for CVD in urban regions of SSA have a strong association with lifestyle factors that are commonly associated with hypertension in the Western Hemisphere. This includes a lack of exercise and a diet high in salt and/or fats (World Heart Federation, 2007). A 2019 study of 1,182 individuals aged 18 to 75 years old living in SSA found that the prevalence of severe hypertension was double in urban areas (17.0%) in comparison to rural areas yet the frequency of untreated or undiagnosed severe hypertension was higher in rural areas of SSA (50.4% in rural areas versus 21.4% in urban areas) (Gaye *et al.*, 2019). Urban lifestyle risk factors for CVD, like tobacco smoking and a high caloric/salt intake, are also factors no seen in, traditional areas of Africa (World Heart Federation, 2007).

The efforts in reducing the burden of communicable diseases have allowed the SSA population to live longer. Since the population is living longer, the risk factor of age can contribute to the CVD burden. The economic development seen in rural areas has increased the exposure of possibly harmful lifestyle risk factors to the aging population. Even though rural populations are now exposed to many of the risk factors seen in urban areas, the frequency of these risk factors, compared to hypertension in rural areas of SSA is low (Hendriks *et al.*, 2012). A study conducted in 2012 of a cross-sectional survey across four countries in SSA, showed a prevalence of 21.4% for hypertension and only 4.6% for smoking in rural Kenya (Hendriks *et al.*, 2012). This trend exemplifies the

epidemiological transition that is happening in SSA. The populations in SSA continue to struggle to control the effects of both communicable and NCD, yet the current epidemiological transition indicates that CVD is still a burden in rural and urban areas (Dalal, Shona, *et al.*, 2011).

Previous studies have found an association between the presence of household air pollution (HAP) and elevated blood pressure (Williams, 2012). Air pollution is a health hazard largely affecting the world's poorest nations. In Kenya, around 80% of the population uses biomass fuels for cooking, and those affected by HAP are around 39 million (Williams, 2012). More research is showing a clinical link between air pollution and CVD, supporting the claim that CVD caused by air pollution is a critical health problem (Williams, 2012).

1.3 Hypertension

In 2017, the American College of Cardiology and the American Heart Association instituted a new set of guidelines regarding the categorization of hypertension. These categorizations will be used to organize the blood pressure data obtained for this study. Hypertension is currently defined as the following:

BLOOD PRESSURE CATEGORY	SYSTOLIC mm Hg (upper number)	and/or	DIASTOLIC mm Hg (lower number)
NORMAL	LESS THAN 120	and	LESS THAN 80
ELEVATED	120 – 129	and	LESS THAN 80
HIGH BLOOD PRESSURE (HYPERTENSION) STAGE 1	130 – 139	or	80 – 89
HIGH BLOOD PRESSURE (HYPERTENSION) STAGE 2	140 OR HIGHER	or	90 OR HIGHER
HYPERTENSIVE CRISIS (consult your doctor immediately)	HIGHER THAN 180	and/or	HIGHER THAN 120

Figure 1. Categories of blood pressure.

The leading risk factor for CVD is hypertension (World Heart Federation, 2019). Hypertension is a long-term state of high blood pressure, the force of blood against arterial walls, that can cause cardiovascular issues (Center for Disease Control and Prevention, 2021). Around 1.13 billion people were found to have hypertension in 2019, with around two-thirds living in LMIC (World Health Organization, 2021). While the Western Hemisphere experiences the lowest occurrence of hypertension at 18%, the African Region shows the largest frequency at 27% (World Health Organization, 2021). An important reason for support in this region is for the diagnosis and treatment of individuals living in these areas. Additionally, further research would provide more knowledge about CVD etiology and epidemiology, which would more clearly define the relationship between risk and hypertension prevalence in rural populations.

In comparison to the previous guidelines, the blood pressure values obtained for this study were lowered for hypertension and the pre-hypertensive stage was removed from the guidelines. These guidelines have increased the prevalence of people being defined as hypertensive due to lowered standards (Whelton *et al.*, 2017).

1.4 The Renin-Angiotensin Aldosterone System (RAS)

The renin-angiotensin system (RAS) is the main hormonal pathway that regulates blood pressure making it a particular area of interest. The RAS regulates blood pressure by controlling blood volume, sodium reabsorption, potassium secretion, water reabsorption, and vascular tone (Fountain, 2022). The hormonal pathway involves renin, angiotensin I-converting enzyme, angiotensinogen, and angiotensin II receptor type I (Figure 2). Each component of RAS is coded for by different genes. Renin is encoded by *REN*, angiotensin I-converting enzyme by *ACE*, angiotensinogen by *AGT*, and angiotensinogen II receptor type I by *AGTR1*. Because RAS is a critical pathway involved in blood pressure regulation, genes of the RAS are the most studied in association with hypertension.

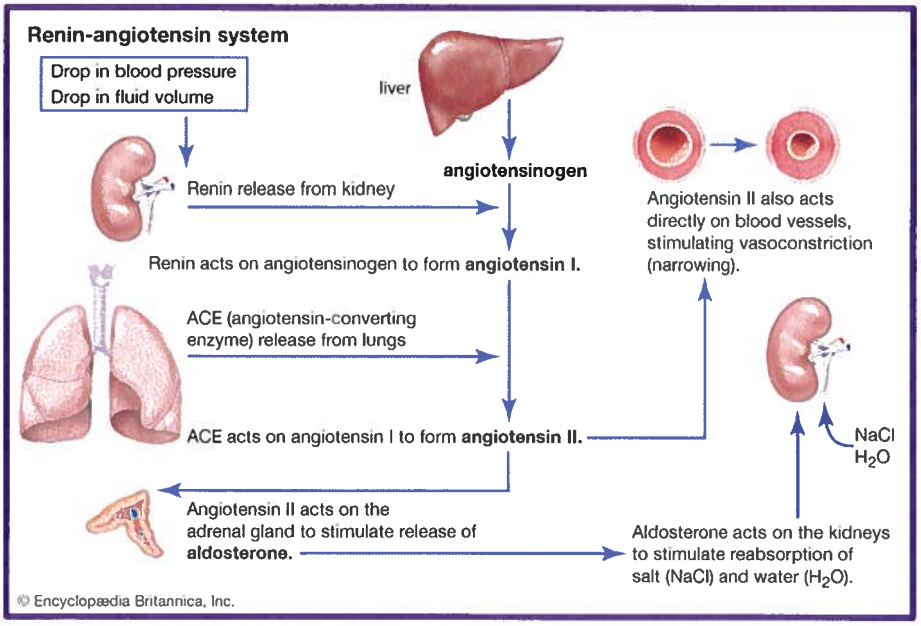

Figure 2. The Renin-Angiotensin System. Adopted from Encyclopedia Britannica. Angiotensinogen released from the liver is converted into angiotensin I by renin released from the kidney. Angiotensin I-converting enzyme (ACE) is responsible for converting angiotensin I into angiotensin II, a key effector protein that increases blood pressure. The binding of angiotensin II to angiotensin II receptor type I enables these blood pressure changes.

The study of the RAS is important in understanding the development of hypertension. More specifically, looking at the RAS and its epigenetic regulation can show how blood pressure is affected (Han et al., 2016). Epigenetics is the study of how your behaviors and environment can cause changes that affect the way your genes work and in contrast to genetic changes, epigenetic changes do not change DNA sequence but instead, change how your body reads DNA (CDC, 2022). Epigenetic changes affect the expression of genes in different ways. The epigenetic change of interest is DNA methylation which works by adding a methyl group to cytosine residues on the DNA

sequence. This methyl group affects gene expression by sterically blocking transcription factors from initiating transcription (Moore, 2012). In general, DNA methylation is implied to lower the transcription of genes while the absence of the chemical group allows the transcription of genes. Therefore, the methylation of genes associated with the RAS gene *AGTR1* impacts the presence of its respective protein and ultimately the regulation of blood pressure.

Dysregulation of angiotensin II receptor type 1 and the RAS pathway are known to result in hypertension (Marchesi *et al.,* 2008). Studying the methylation of the RAS can elucidate the relationship between methylation and the regulation of blood pressure. The promoter region of the RAS is where transcription can begin and a location where methylation can occur. Therefore, it is a particular location of interest. A previous study investigated the promoter region of the natriuretic peptide A gene (NPPA), which codes for atrial natriuretic peptide, another molecule that is important for regulating blood pressure (Li *et al.*, 2020). It was discovered that hypertensive participants showed lower methylation levels at the NPPA promoter in comparison to normotensive participants (Li *et al.*, 2020). These findings show that varying methylation levels of the promoter region of another gene involved in blood pressure regulation correlate to the prevalence of hypertension. Additionally, this study found that hypertension was linked to abnormal methylation levels of the promoter regions of both *ACE* and *AGTR1* (Li *et al.*, 2020). This study and its findings suggest that further investigation into blood pressure regulatory systems, like RAS, and methylation levels of *AGTR1* may elucidate knowledge on the epigenetic regulation of hypertension.

1.5 The *AGTR1* Gene

The *AGTR1* gene encodes for the angiotensin II receptor type 1 (AT1 receptor), a key receptor protein of the RAS. Angiotensin II, a regulatory molecule produced in the RAS, binds to the AT1 receptor. It then mediates vasoconstriction resulting in an increase in blood pressure and the release of aldosterone leading to water and sodium retention. The increase in water retention leads to further elevation of blood volume and ultimately pressure (Medline Plus). Because of the role the AT1 receptor has on blood pressure regulation, changes to the *AGTR1* gene can lead to drastic effects on blood pressure. In the context of this study, the DNA methylation of the promoter region of this gene is being explored to uncover the relationship between the prevalence of hypertension and the epigenetic regulation of *AGTR1*.

1.6 DNA Methylation

Historically, research linking disease to genetic changes investigated mutation in DNA sequences. Today, however, the emerging field of epigenetics allows researchers to study changes in gene expression and function that do not involve direct changes (point mutation, insertion, deletion, etc.) to the DNA sequence (Weinhold, 2017). Common epigenetic modifications include DNA methylation, histone acetylation, and nucleosome positioning and have been linked to numerous diseases including CVD (Jin *et al.*, 2011).

DNA methylation is the most researched epigenetic modification (Weinhold, 2017). In DNA methylation, methyl groups are transferred from the naturally occurring methyl donor, S-adenosyl methionine (SAM), to the C5 position of cytosine residues within DNA sequences (Ahuja I., 2017). DNA methyl transferases (DNMTs) most commonly methylate cytosines at cytosine-phosphate guanine (CpG) dinucleotides, the most significant location of methylation in mammals (Tost I., 2003). Approximately 80% of CpG sites are methylated in mammalian gene promoters (Janitz and Janitz, 2011).

CpG islands (CGIs) are defined as genomic areas of at least 500 base pairs that contain at least 55% of CpG dinucleotides (at least 55%) (Ahuja *et al.*, 2017). CGIs are found in approximately 40% of the gene promoters of mammals, and these sites are typically unmethylated (Janitz and Janitz, 2011). As a result, methylation of these CGIs can play a critical role in the expression of specific genes.

Specifically, DNA methylation downregulates transcription by sterically blocking the binding of transcription factors to DNA sequences (Jin *et al.*,2011). Therefore, methylation is found in the CGIs of many repressed genes. Additionally, DNA methylation influences other epigenetic modifications that typically increase gene expression (Jin *et al.*, 2011). For example, methylated CpG sites can attract methyl-binding proteins, obstructing transcription factors and further silencing gene expression (Javaid and Choi, 2017).

Epigenetic modifications can be influenced by lifestyle and environmental factors (Muka *et al.*, 2016). Understanding the regulation of pathophysiological processes and then identifying treatment targets is essential in the study of epigenetics (Muka *et al.*,

2016). Previous studies have shown that the expression of genes related to the biological features of CVD pathways, such as hypertension, inflammation, and atherosclerosis (hardening of the arteries) has been found to change with DNA methylation content (Zhong *et al.*, 2016). A 2014 study found hypermethylation of genomic sites within atherosclerotic aortas in comparison to healthy aortas (Zaina *et al.*, 2014). Additionally, a 2015 study analyzed 5 CpG sites along the *AGTR1* promoter (n=192) for 96 individuals with hypertension and 96 gender- and age-matched controls. Hypomethylation of one of these CpG sites in the *AGTR1* gene was suggested to increase the risk of hypertension (Fan, 2015).

Preliminary data from this laboratory has found hypermethylation in hypertensive versus normotensive Kenyans. The previous research analyzed 4 CpG sites of the *AGTR1* promoter (n=53). The analysis of the additional CpG sites will provide more data to better understand the role of DNA methylation in *AGTR1* expression as well as hypertension and CVD pathogenesis.

1.7 Primer Set Information

Prior research from this laboratory has analyzed the methylation percentages of 4 CpG sites using the Qiagen *AGTR1* primer set for 64 samples. In this study, the methylation patterns of 8 additional CpG sites in the promoter region of the *AGTR1* gene will be explored using a primer set known as J1 to amplify bp 4,834 to bp 4,972 (RefSeq:NG_008468). The amplicon length measures to be 266 nucleotides and has a guanine-cytosine content of 34.6 %. The forward primer had a length of 19 nucleotides, a

GC content of 52.6%, a melting temperature of 54.4°C. The reverse primer had a primer length of 28 nucleotides, a guanine-cytosine content of 42.9 %, and a melting temperature of 58.2°C. The J1 primer set contained eight CpG sites. The J1primer set was assessed using the NCBI Primer-BLAST tool and was given a score of 70.

Table 1. J1 Primer Set Information

J1 Primer Set							
Primer	Primer Length (Nt)	Primer GC Content (%)	Melting Temp (°C)	Primer Sequence	Amplicon Length (Nt)	Amplicon GC Content (%)	Target Area of *AGTR1* Gene (Nt)
Forward	19	52.6	54.4	GGGGTTAGGGATTAGG TGA	266	34.6	4,834-4,972
Reverse	28	42.9	58.2	CCTATCACTCCCTACTA CCTAAAATCCT			

1.8 Preliminary Studies

Preliminary results from this laboratory indicated hypertensive individuals had increased methylation versus normotensive individuals (total percent methylation = 4.78 ± 0.46 and 3.84 ± 0.45, respectively) when the average methylation levels across the first 4 CpG sites of the promoter region using the Qiagen *AGTR1* primer set (n=64). For these preliminary results, Qiagen's commercially available primer for the *AGTR1* promoter region was used to amplify the target region of the *AGTR1* promoter. In relation to this

study, this primer set bound upstream of the current study's target region and amplified four CpG sites (Figure 3).

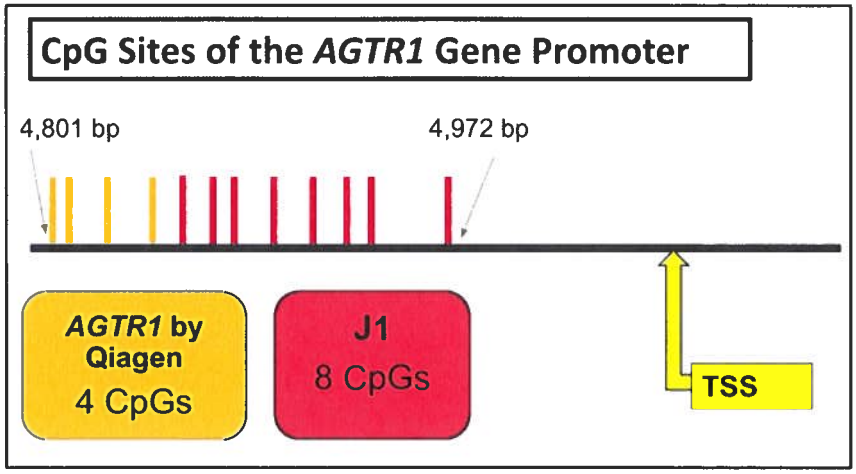

Figure 3. Schematic diagram of the CpG sites of the *AGTR1* promoter. The commercially available *AGTR1* primer set from Qiagen amplified 4 regions in yellow. The J1 primer set amplified 8 CpG islands in red is located between bp 4,801 and bp 4,972 on the AGTR1 gene, which corresponds to Chr3:148697672- 148,697,843 in the human genome.

1.9 Demographics of the Study Population

In this study, the country of Kenya will be used as a model LMIC to unravel the genetic epidemiology of high blood pressure. The East Sub-Saharan country of Kenya is composed of a total population of 54,027,490 individuals in 2022 (World Bank, 2022). The most recent data shows that in Kenya, there are 2.26 medical doctors per 10,000 individuals in a population. In contrast, there are 35.55 medical doctors per 10,000 individuals in the United States (World Health Organization, 2023). This study focuses on a population in Kasigau, Kenya. Kasigau is a region located in the Taita-Taveta County in the southeastern part of the country. Kasigau is characterized by large hills and

valleys and is part of the larger Taita Hills range. Mount Kasigau, part of this range, is surrounded by seven villages: Jora, Makwasinyi, Ngambenyi, Rukanga, Bungule, Kiteghe, Bughuta.

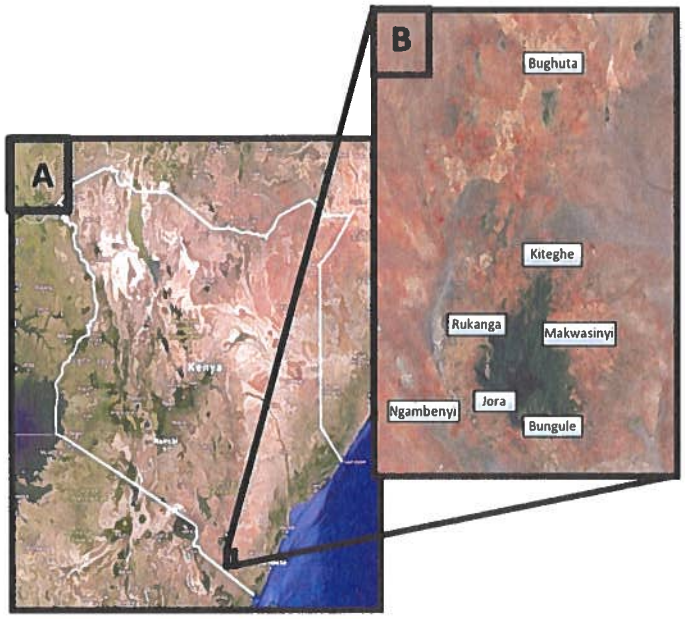

Figure 4. (A) Map of Kenya. (B) Mount Kasigau and the seven surrounding villages of the study population.

The Taita-Taveta County has a population of 340,671 persons (Taita-Taventa Health Services). In the county, the doctor-population ratio stands at 1:6,191, falling short by a lot, of the 1:600 standard set by the World Health Organization (WHO). In each of the seven villages, the Taita-Taveta Department of Health Services has a health center and/or a dispensary for medication.

Within this area of study, preliminary epidemiological studies of a pilot group of 159 individuals found that ~67% of the Kasigau population has EH (Williams, 2012)

(Figure 4). The blood pressures were categorized based on the American College of Cardiology's guidelines prior to 2017. Similar to other rural areas, common risk factors were not correlated to the prevalence of EH in this area (Williams, 2012). Therefore, this study aims to understand the relationship between hypertension prevalence in Kasigau and the DNA methylation of the promoter region of the RAS gene *AGTR1*.

CHAPTER II

HYPOTHESIS AND AIMS

Hypothesis: The Renin-Angiotensin System (RAS) Gene *AGTR1*, as a result of exposure to household air pollution (HAP), is hypomethylated in individuals with hypertension.

Aims:

- Quantify the DNA methylation of the *AGTR1* gene from a population living in Kasigau, Kenya.
- Investigate the correlation of the *AGTR1* gene methylation with the prevalence of hypertension.
- Compare spending habits on firewood and charcoal with corresponding blood pressures from a population living in Kasigau, Kenya.

CHAPTER III

MATERIALS AND METHODS

3.1 Consent and Compliance

For this project, human sample collection was approved by the University of South Alabama's Institutional Review Board, Kenya National Hospital-University of Nairobi-Ethics Review, the Taita-Taveta County Department of Health Services, and the Taita-Taveta chiefs and elders (Appendices A and B). All research participants read and agreed to the informed consent document provided by the Human Subjects Review Board (HSRB) (Williams, 2012). All researchers completed CITI Program Training involving ethical research prior to both sample collecting and research with human subjects and samples.

3.2 Study Population

For the seven villages in Kasigau, Kenya (Makwasinyi, Rukanga, Bughuta, Bungule, Ngambenyi, Jora), a cross-sectional population assessment was performed using the census data from this area. The area was divided into three sections: Rukanga, Bughuta, and Makwasinyi. Each section is composed of four individual villages with Rukanga making up 29%, Makwasinyi making 21% and Bughuta making up 50% of the

total population. Because hypertension onset occurs in older individuals, participants were 45-75 years of age. A random sample of 64 participants were selected (n=64), sample collection took place, and a questionnaire was completed regarding daily life practices in 2018. Over six years, 10 of the samples were not able to undergo analysis due to the lack of availability of these samples (n=54).

3.3 DNA Sample Collection and DNA Isolation

For each research participant, 2 mL saliva samples were collected using the Oragene-500 collection kit. Once the samples were collected, they were placed in liquid-tight sealed bags for shipping back to the United States for further processing. After DNA sample collection, each 2 mL saliva sample was divided into 500 µL aliquots in four different 1.5 mL microcentrifuge tubes. The aliquots were then stored at -20 °C for long-term storage.

Upon Genomic DNA Isolation, each 500 µL aliquots were incubated for two hours at 50°C in a dry bath. Subsequently, 20 µL DNA Genotek prepIT•L2P reagent was added to each sample aliquot. The reagent caused impurities to precipitate. The samples were then incubated on ice for 10 minutes and then centrifuged at room temperature at 15,000 x g for 5 minutes. After centrifugation, supernatants of each sample were pipetted into fresh 1.5 mL microcentrifuge tubes and the pellet of impurities was discarded. For each sample, 600 µL of RT 95%-100% ethanol was added to 500µL of supernatant. The addition of ethanol began DNA precipitation. Samples stood at RT for 10 minutes for full DNA precipitation. Samples were then centrifuged at RT at 15,000 x g for 2 minutes. The

pellet formed contained DNA and the supernatant was discarded. After the supernatant was discarded, 250 µL of 70% ethanol was added to each sample and then incubated at room temperature for 1 minute before the ethanol was removed. 100 µL of TE buffer solution was added to each sample for the hydration of DNA. Samples were then vortexed for 5 seconds and briefly centrifuged to bring the contents to the bottom of the microcentrifuge tube. Isolated DNA samples were stored in a freezer at -20°C.

3.4 DNA NanoDrop Spectroscopy

DNA concentration after the DNA isolation procedure was measured to ensure the presence of DNA and to assess the purity of the isolated genomic DNA. The DNA concentration and purity were measured using the ThermoFisher Scientific NanoDrop One spectrophotometer. The spectrophotometer contains preprogrammed applications for dsDNA, ssDNA, RNA , oligo DNA and oligo RNA. Post-genomic DNA isolation, bisulfite conversion and PCR amplification used the dsDNA, ssDNA, and dsDNA, respectively for concentration and purity analysis.

The NanoDrop One instrument was powered on and the desired application was selected. The hinged arm was gently lifted, and the pedestal was gently cleaned using distilled (DI) water. 1 µL blank of blanking solution, 1x TE buffer, was placed onto the pedestal and the arm was gently lowered. The blank was measured and the arm was lifted. After, the pedestal was cleaned gently with water and a Kimtech Kimwipe. Then, 1 µL of sample was placed onto the sample stage. The DNA concentration and A260:A280 ratio were documented. A260 and A280 values denote absorption values at a wavelength

of 260 nm and 280 nm, respectively. Nucleic acids have an absorption maximum at 260 nm and proteins have an absorption maximum at 280 nm. A260:A280 values 1.8 or above indicate low contamination in the sample were as low A260:A280 values indicate protein contamination in nucleic acids. After DNA Nanodrop Spectroscopy, the samples were stored at -20°C.

3.5 Bisulfite Conversion

After ensuring Genomic DNA isolation yielded sufficient DNA concentration and A260:A280 ratios, the samples underwent bisulfite conversion to convert the unmethylated cytosines into uracils. The Qiagen Epitect Fast Bisulfite Conversion Kit was used and carried out in two parts. For part 1, isolated DNA samples were removed from storage and thawed on ice. Bisulfite reactions were set up in 200 µL PCR tubes. Calculations using DNA concentrations for each sample were performed to ensure 1 µg of sample in each bisulfite reaction. The respective amount of volume was then pipetted into the PCR tubes. Then, calculations for RNase- free water performed based on the sample's volume of DNA that was previously added. Combined volume of DNA and RNase-free water totaled to 20µL for high-concentration samples and 40µL for low-concentration samples. The respective amount of water was added to each PCR tube.

85 µL of bisulfite solution and 35 µL of DNA protect buffer were added to each reaction PCR tube. The bisulfite solution was designed to be at optimal low pH for the conversion of unmethylated cytosines. The DNA protect buffer was used to prevent DNA fragmentation. The samples were then placed in the thermal cycler to undergo the

following cycle: 5 minutes at 95°C (denaturation step), 10 minutes at 60°C (incubation step), 5 minutes at 95 °C (denaturation step), 10 minutes at 60°C (incubation step), and an indefinite hold at 20°C.

For part 2, the PCR tubes were briefly centrifuged and transferred to clean 1.5 mL microcentrifuge tubes upon bisulfite conversion. 250 µL of 96-100 % of ethanol was added to each sample. The samples underwent pulse vortexing and subsequent centrifuge after the ethanol was added. The entire contents were placed into MinElute DNA spin columns and collection tubes. Contents were centrifuged for 1 min and the flow through was discarded. 500 µL of Buffer BW was added to each spin column followed by centrifugation for 1 min and discarding of the flow through. 500 µL of Buffer BD was placed into each spin column and incubated at room temperature for 15 minutes. Samples were centrifuged for 1 min and the flow through was discarded. 500 µL of Buffer BW was placed on each spin column and centrifuged for 1 minute followed by the discarding of the flow-through. This step was repeated once. 250 µL of 96-100% ethanol was added to each spin column, centrifuged for 1 minute, and flow through was discarded.

Spin columns were placed into new 2 mL collection tubes and centrifuged for 1 minute. To ensure the complete removal of the remaining flow-through, the tubes were incubated in a dry bath at 60°C with spin column lids open. The spin columns were placed into clean 2 mL collection tubes. 15 µL of Buffer EB was placed directly onto the center of each spin column membrane. The samples were incubated at room temperature for 1 minute. Samples were centrifuged for 1 minute to elute the DNA. The 15 µL of

each sample were pipetted and transferred into clean 1.5 mL microcentrifuge tubes and labelled, respectively. The samples were stored at -20°C.

3.6 PCR Amplification

After bisulfite conversion, amplification of the promoter region of the AGTR1 gene using the J1 primer set was carried out. The PCR amplification was completed using the PyroMark PCR Handbook Procedure. The reaction used the PyroMark PCR Master Mix, CoralLoad concentrate, the Qiagen J1 primer set, and 25 mM $MgCl_2$ (if required). The HotStarTaq DNA Polymerase is a modified form of the recombinant 94 kDa Taq DNA Polymerase from QIAGEN. It is in an inactive state with no activity at ambient temperature, preventing the formation of misprimed products at low temperatures. The PyroMark PCR Master Mix contains genomic DNA from a variety of species and bisulfite-converted DNA, allowing the production of PCR products that are highly suitable for pyrosequencing. PCR products amplified in the presence of CoralLoad Concentrate can be directly loaded onto an agarose gel without the need to add a gel loading buffer since it contains a gel loading reagent and two marker dyes that facilitate estimation of DNA migration distance and optimization of agarose gel run time. The reagents were thawed on ice and mixed through pulse vortexing and subsequent centrifugation to avoid localized concentrations of salt. The reaction was set up in 200 µL PCR tubes according to the following volumes/reaction: PyroMark PCR Master Mix – 12.5 µL, Coral Load Concentrate – 2.5 µL, J1 primer set – 2.5 µL. Before the reagents were transferred to the sample PCR tubes, a master mix of PyroMark PCR Master Mix,

Coral Load Concentrate and J1 primer set was made in a 1.5 mL microcentrifuge tube. Each respective value was multiplied by the number of samples to be amplified plus 1.5 to account for loss during the transfer of the solution. After the master mix was created, 17.5 µL of the mix was pipetted into the individual PCR tubes. Then, the amount of template DNA was calculated to have between 10-20 ng bisulfite-converted DNA and the respective amount of RNase-free water was calculated. After these calculations were made, the reagents were gently pipetted into the PCR tubes.

Unmethylated, 50% methylated/ 50% unmethylated, 75% methylated/ 25% unmethylated, and 100% methylated controls also underwent this reaction. All the controls started at atleast a DNA concentration of 10 ng/µL. Lastly, a no template DNA and no primer controls were used to compare against PCR-amplified genetic samples in the gel electrophoresis step. Once the PCR reagents were transferred, the PCR tubes were placed into a thermal cycler with a heated lid and underwent the following cycle: 15 minutes at 95°C (Initial PCR activation step- HotStarTaq DNA Polymerase is activated by this heating step), 30 s at 94°C (Denaturation), 30 s at 56°C (Annealing), 30 s at 72°C (Extension) with the last three steps repeating for 45 cycles. The last step is 10 minutes at 72°C for the final extension step and an indefinite hold at 10°C. Once the PCR reaction is complete, the PCR tubes were removed from the thermal cycler and then stored at -20°C for long term storage.

3.7 Gel Electrophoresis

To verify that the correct region of the promoter sequence was amplified, the samples underwent gel electrophoresis. To form a 1.2% gel, the respective mass of agarose was weighed out to equal 1.2% of the volume of 1x TBE buffer used. After the correct volume of 1x TBE buffer and mass of agarose were measured, they were placed in an Erlenmeyer flask. The flask was placed into the microwave for 1-2 minutes with periods of swirling every 20 seconds until all the solute was dissolved. After all solute had dissolved, the flask was carefully removed from the microwave and a Kimwipe was placed on the top of the flask to prevent gaseous TBE from escaping. Then, 5 µL of ethidium bromide was placed into the flask and swirled for even distribution. The flask was allowed to cool and poured into a gel electrophoresis rig with a comb placed at the top of the gel to help form the wells for loading.

To prepare the samples, the PCR-amplified samples were removed from the freezer and thawed on ice. A new PCR tube was used to prepare samples and 5 µL of sample, 5 µL of RNase-free water, and 2 µL of 6x loading dye were placed into their respective tubes. To ensure proper distribution of the reagents, the samples were pipetted throughout the solution. A quick centrifuge was carried out to ensure that all contents were at the bottom of the tube. The remaining PCR amplified samples were stored at -20 °C.

When the gel solidified, the comb was gently removed to reveal the wells, and 1x TBE buffer was poured until the fill line. The buffer covered the gel. In the first well, 10 µL of 100 bp DNA ladder was pipetted. In the remaining wells, all 12 µL of each sample was loaded into their respective wells. Once all the samples were loaded onto the gel, the

cover with the cathode and anode was placed onto the gel rig with the cathode located opposite to the wells. The ends of the cathode and anode were placed into the correct outlets on the voltmeter and the voltmeter was turned on. The gel ran at 121 volts for varying amounts of time. When using the rig that contained 20 wells, the gel ran for 40 minutes. When using the rig with 8 wells, the gel ran for 20 minutes because of its smaller size. A picture of each of the gels was taken using the LI-COR machine. The gel was loaded onto the sample area, the image was acquired, the grayscale was adjusted, and the image was printed. Lastly, the lanes were labeled with their respective sample.

3.8 Pyrosequencing

Once the sample amplification was verified using gel electrophoresis, the samples were sent for pyrosequencing, which yields quantitative analysis of methylation levels of the target region. Since the University of South Alabama does not have a pyrosequencing machine, the samples were sent to another facility for analysis. The Genetics Resources Core Facility (GRCF) at the Johns Hopkins School of Medicine performed pyrosequencing of samples and controls for the CpG sites that were amplified using the J1 primer set along the *AGTR1* promoter.

3.9 Amount Spent on Firewood and Charcoal

To investigate the role HAP exposure has on the prevalence of high blood pressure, the amount of biomass fuels was assessed for the study population. This was assessed using monetary expenditure on firewood and charcoal as a proxy for HAP

exposure. After pyrosequencing results were analyzed, a questionnaire with responses from the study's study population was used to determine the monetary expenditure individuals spent on firewood and charcoal per month. The questionnaire assessed how many Kenyan Shillings they spent in a month for both items. It was administered in 2018 alongside sample collection. Responses were the respective amount of Kenyan Shillings they spent per month for both firewood and charcoal.

3.10 Statistics

To elucidate the statistical differences between average percent methylation, monthly expenditure on firewood, and charcoal between normotensive/elevated (SBP<130) and hypertensive (SBP>130) individuals in this study, a Welch's t-test was performed. The Welch's t-test does not assume equal variances between the two groups and is considered more robust when sample sizes and variances are unequal between two groups. Since the degrees of freedom are calculated based on the sample sizes and variances of each group separately, it provides more accurate results when sample sizes and variances are unequal.

To assess the linear relationship between the CpG sites, a Pearson's correlation was performed across all 8 of the CpG sites. This analysis will quantify the strength and the direction of the linear association between the CpG sites. Finally, a multiple linear regression (MLR) analysis was used to explore the predictive ability of methylation of CpG 1-4 (independent variable) for SBP and DBP (continuous dependent variables). This

analysis will increase understanding of how DNA methylation levels at specific CpG sites relate to SBP and DBP.

CHAPTER IV

RESULTS

4.1 DNA Concentration

A NanoDrop spectrophotometer was used to determine genomic DNA concentration for the samples following DNA isolation, bisulfite conversion, and PCR amplification. Concentration and A260:A280 values were necessary for determining the presence of Genomic DNA without the presence of excess contaminants. Following isolation, DNA samples ranged in concentration from 6.2 ng/μL to 1403.4 ng/μL. Following bisulfite conversion, DNA samples ranged in concentration from 6.2 ng/μL to 73.5 ng/μL. Following PCR amplification, DNA samples ranged in concentration from 503.9 ng/μL to 1115.4 ng/μL.

4.2 Representative Gel for J1 Primer Set PCR Products

The J1 primer set was designed to amplify 266 bp. After PCR amplification, the products were verified using gel electrophoresis. 54 PCR products displayed bright, clear bands between 200 and 300 bp, closer to 300 bp. A 100 bp DNA ladder was used as a reference (Figure 6).

Six controls were amplified: 0%, 50%, 75%, 100% methylated controls, no DNA sample, and no primer control were run on a gel. The no DNA template control and the no primer control did not produce any bands on the gel. Because no bands were exhibited on these controls, no contamination was present within the samples during PCR amplification. The four other controls with varying levels of methylation produced a band of the desired size and were packaged along with the other genomic DNA samples to be pyrosequenced to serve as a standard for methylation percentages.

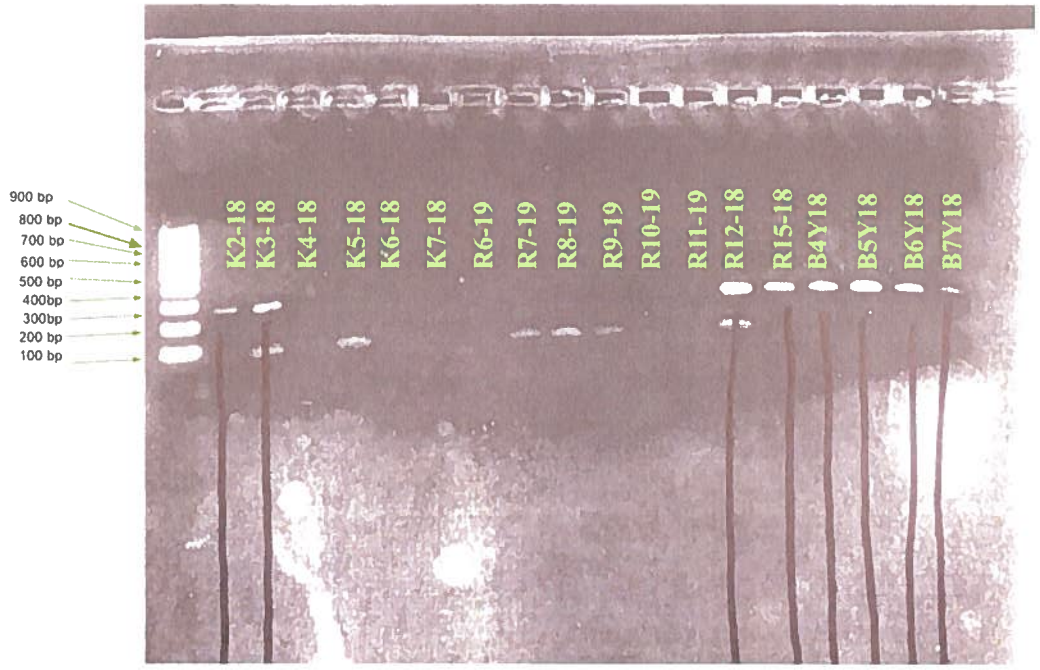

Figure 5. Representative gel electrophoresis image of PCR products amplified by J1.

4.3 Methylation Patterns of Kenyan Population

In this study, the J1 primer set was used to amplify four additional CpG sites downstream of the Qiagen *AGTR1* primer set named CpG 1, CpG 2, CpG 3, and CpG 4 on the *AGTR1* promoter region. Results from this study showed increased methylation in

hypertensive versus normotensive Kenyans based on SBP (total percent methylation = 38.33 ± 4.43 and 24.4 ± 5.29, respectively) when the average methylation levels of CpG 1-4 of the *AGTR1* promoter were analyzed (n=34). In normotensive/elevated individuals, the average systolic blood pressure (SBP) and diastolic blood pressure (DBP) were found to be 116.76 ± 2.22 and 81.12 ± 1.63, respectively. In hypertensive individuals, the average systolic blood pressure (SBP) and diastolic blood pressure (DBP) were found to be 150.35 ± 3.78 and 101.76 ± 2.79, respectively (n=34) (Figure 7).

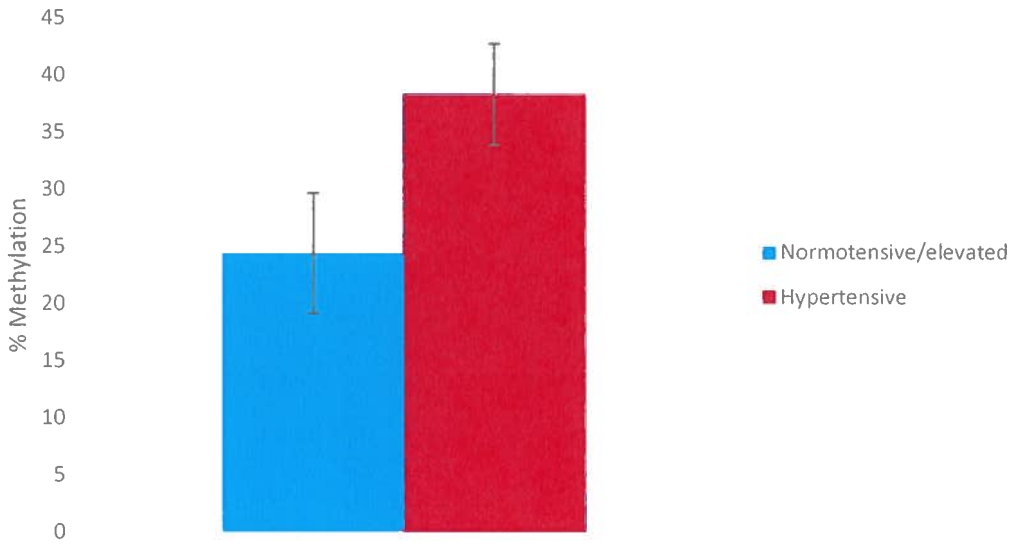

Figure 6. Average percent methylation for normotensive (SBP<130) and hypertensive (SBP>130) Kenyans across all CpG 1-4 (n=34)

Percent methylation at individual CpG sites at CpG 1-4 was compared for hypertensive and normotensive based on systolic blood pressure individuals. For hypertensive individuals, the CpG sites 1-4 displayed the following percent methylation:

30.5 ± 4.2, 36.4 ± 3.77, 44.1 ± 4.95, and 42.3 ± 5.6, respectively (n=34). For normotensive/elevated individuals, the CpG sites 1-4 displayed the following percent methylation: 20.83 ± 3.9, 24.5 ± 5.62, 26.42 ± 6.68, and 25.83 ± 5.89, respectively. Across all 4 CpG sites, the data showed increased methylation in hypertensive versus normotensive individuals. One statistically significant difference was found between percent methylation at CpG 3 for hypertensive versus normotensive individuals (p=0.0465) (Figure 8).

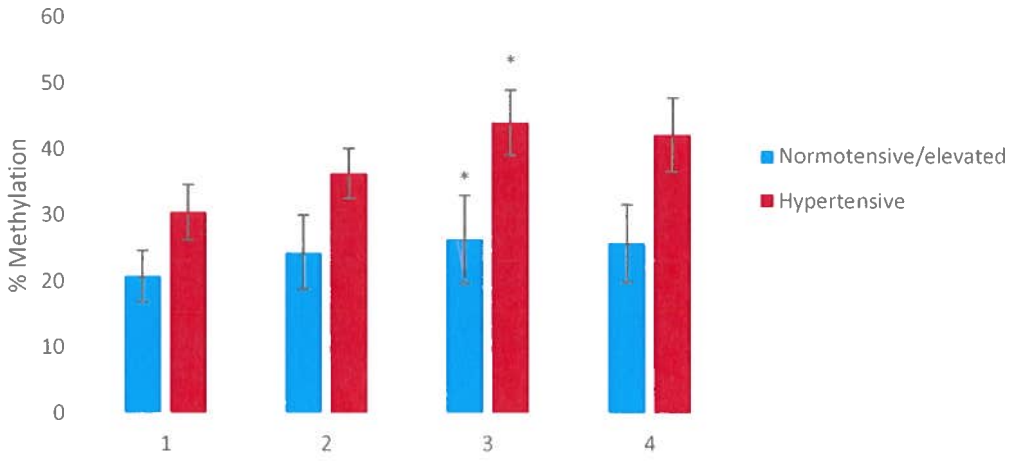

Figure 7. Percent methylation at each CpG 1-4 for normotensive (SBP<130) and hypertensive (SBP>130) Kenyans (n=34). Statistically significant difference at CpG 3.

The methylation data obtained from CpG 1-4 were combined with the previously obtained data from the first 4 CpG site: CpG Q1-Q4. Results from this analysis showed increased methylation in hypertensive versus normotensive Kenyans based on SBP (total

percent methylation = 18.4 ± 2 and 12.92 ± 1.78, respectively) when the average methylation levels of CpG Q1-4 of the *AGTR1* promoter were analyzed (n=34) (Figure 9).

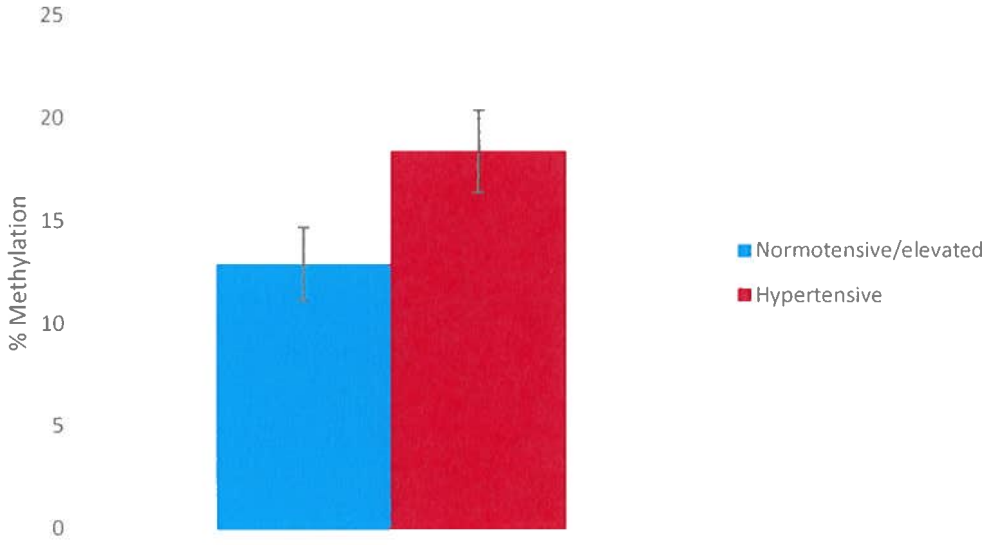

Figure 8. Combined average percent methylation for normotensive (SBP<130) and hypertensive (SBP>130) Kenyans across all CpG Q1-4 (n=34).

4.4 Monetary Spending of Firewood and Charcoal of a Kenyan Population

In efforts to uncover the relationship between exposure to HAP exposure and high blood pressure, the monetary expenditure on firewood and charcoal were used as proxies for HAP exposure. The average amount of money spent on firewood and charcoal per month was compared between normotensive/elevated and hypertensive individuals based on systolic blood pressure. This data was obtained from a questionnaire conducted in concurrence with the saliva sample collection. For hypertensive individuals, the average

amount spent on firewood and charcoal per month was found to be 2341.18 ± 649.61 and 552.94 ± 126.35 Kenyan Shillings, respectively. For normotensive/elevated individuals, the average amount spent on firewood and charcoal per month was found to be 1400 ± 215.23 and 388.24 ± 136.63 Kenyan Shillings, respectively. With the analysis on firewood spending, the data indicated that increased spending was seen in hypertensive versus normotensive Kenyans (Figure 9). There was a statistically significant difference between monetary spending on charcoal per month in hypertensive versus normotensive individuals (n=34) (Figure 10). For hypertensive individuals, there was an increased amount of monetary spending on charcoal per month in comparison to normotensive/elevated individuals.

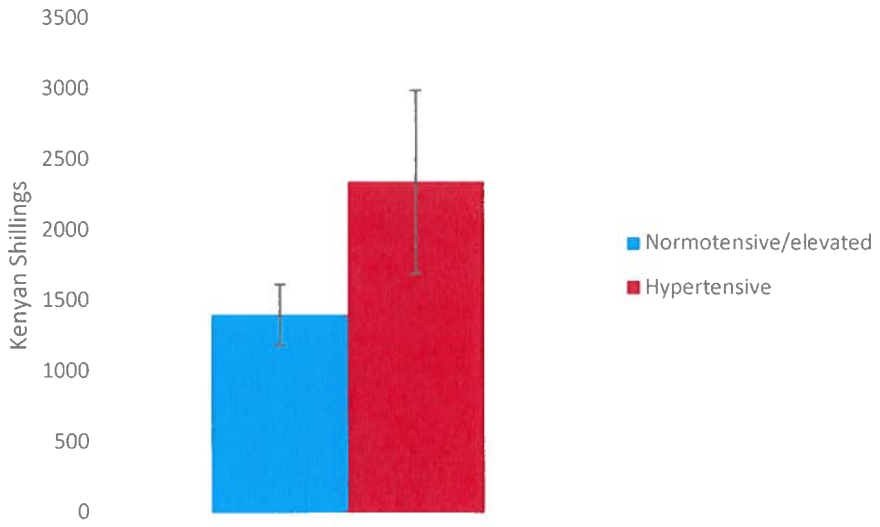

Figure 9. Average amount of Kenyan Shillings spent on firewood per month in hypertensive versus normotensive Kenyans

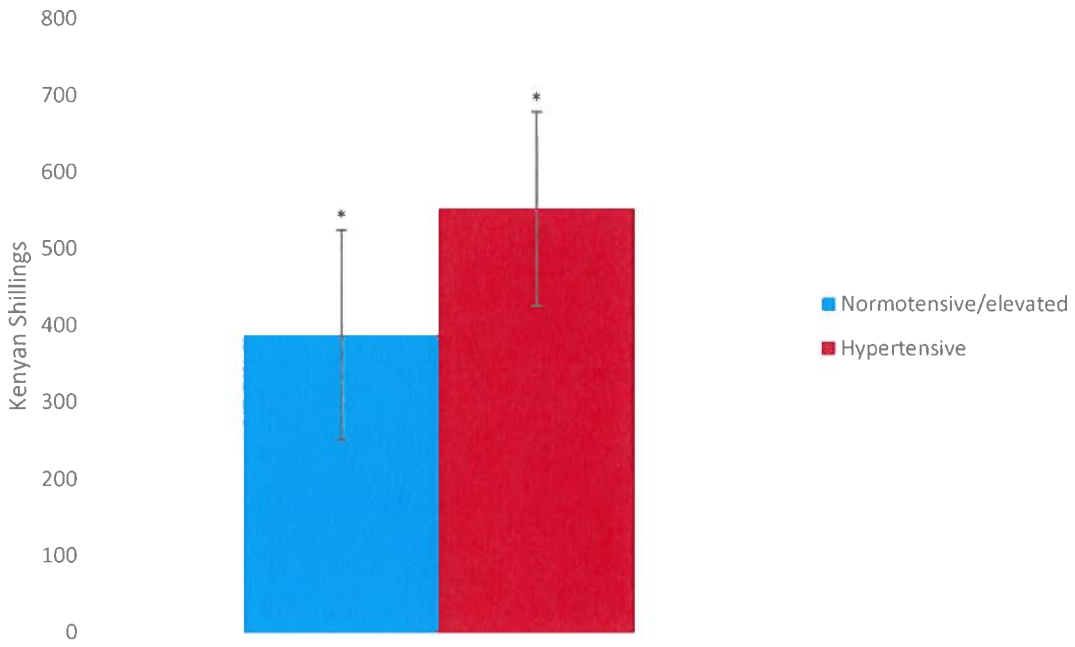

Figure 10. Average amount of Kenyan shillings spent on charcoal per month in hypertensive versus normotensive Kenyans. Statistically significant difference on charcoal spending habits.

4.5 Pearson's Correlation

The Pearson's correlation revealed nine statistically significant correlations between CpG sites Q2/Q3, CpG sites Q2/Q4, CpG sites Q3/Q4, CpG sites 1/2, CpG sites 1/3, CpG sites 1/4, CpG sites 2/3, CpG sites 2/4, and CpG sites 3/4. All statistically significant correlations were found to have positive, moderate to strong correlations with each other (Table 2).

Table 2. Pearson's correlation between eight CpG sites Q1-4. Statistical significance is indicated by an asterisk *.

Pearson's Correlation Between CpG Q1-4 (* = statistical significance)							
	Q1	**Q2**	**Q3**	**Q4**	**1**	**2**	**3**
Q1							
Q2	0.0187						
Q3	-0.104	**.438***					
Q4	0.219	**0.536***	**0.462***				
1	-0.056	0.138	0.183	0.271			
2	-0.047	-0.154	0.305	0.149	**0.743***		
3	0.0279	-0.0624	0.318	0.182	**0.841***	**0.969***	
4	0.0395	-0.203	0.211	0.203	**0.838***	**0.944***	**0.961***

4.6 Multiple Linear Regression

A multiple linear regression (MLR) was completed for the CpG sites1-4 and blood pressure. These results were found for both SBP and DBP (Tables 3 and 4). The p-value associated with each t-value represents the probability of observing such an extreme t-value (or more extreme) under the null hypothesis that the true coefficient for that predictor variable is zero. None of the CpG sites methylation had corresponding p-values less than 0.05. Therefore, the methylation levels for CpG 1-4 are not statistically significant in predicting SBP and DBP.

Table 3. MLR results for SBP

Multiple Linear Regression Results for SBP		
	t	P
CpG 1	-0.957	0.352
CpG 2	-1.25	0.228
CpG 3	1.335	0.199
CpG 4	0.295	0.771

Table 4. MLR results for DBP

Multiple Linear Regression Results for DBP		
	t	P
CpG 1	-1.468	0.16
CpG 2	-1.722	0.103
CpG 3	1.148	0.267
CpG 4	1.593	0.13

4.7 Pyroseqeuncing Quality Control

After 54 samples successfully underwent PCR amplification and produced bands at the desired size, they were sent for pyrosequencing at the Genetics Resources Core Facility (GRCF) at Johns Hopkins School of Medicine. Due to issues during pyrosequencing analysis, CpG 1-4 were the only sites to yield methylation data. Post-data acquisition, the sequence to analyze was shortened to only analyze the first CpG sites 1-4. In addition, minimum peak heights were changed to 15 and 7. In addition, 34 samples yielded a percent methylation value. Within the 34 samples, 17 of the individuals were hypertensive and 17 were normotensive/elevated based on systolic blood pressure.

CHAPTER V

DISCUSSION

Cardiovascular disease is emerging as the primary cause of global mortality and is a disease that disproportionately impacts LMIC. Hypertension, the leading risk factor for CVD was found to be a significant health burden affecting the Kenyan population based on previous studies in this laboratory. Previous studies have shown a high prevalence of hypertension in this population not statistically correlated with any typical risk factor or genetic polymorphisms known to increase predisposition to high BP (Williams, 2012). Even though CVD risk factors like hyperlipidemia, alcohol use, or obesity are emerging in LMICs, these factors do not appear to be the primary factors that drive the presence of hypertension. Air pollution is however a health hazard among the world's poorest populations. In Kenya, the official 2019 national census reports that 66.7% rely on solid biomass fuel, primarily firewood and charcoal, and those affected by household air pollution (HAP), are around 39 million (*Kenya National Bureau of Statistics*). Progressively, more research is showing a clinical link between air pollution and CVD reinforcing the fact that CVD caused by air pollution is a critical health problem worldwide. In the examination of this relationship, this study explores the methylation in the RAS gene *AGTR1* to see if exposure to HAP exposure can be correlated to the prevalence of high blood pressure.

Preliminary methylation analysis of the *AGTR1* promoter showed increased methylation in hypertensive versus normotensive/elevated individuals. In this project, methylation results for four CpG sites were obtained for 34 individuals. Primer set J1 was designed to amplify CpG 1-8 but was only successful in the amplification of CpG 1-4 directly downstream of the four CpG sites initially analyzed. The analysis of CpG 1-4 showed increased methylation in hypertensive versus normotensive/elevated individuals, with a statistically significant difference found between CpG 3 methylation for hypertensive versus normotensive individuals ($p=0.0465$).

Multiple factors were considered when trying to identify reasons for the poor quality received from the pyrosequencing analysis. Factors to consider are the number of samples, the quality of DNA content in each sample, and the previous design of the J1 primer set. Since sample collection, multiple factors have reduced the sample size from 64 to 34. Initially, a random sample of participants ($n=64$) was selected from various villages in Kasigau, Kenya. Saliva sample collection took place in 2018 in conjunction with the administering of a questionnaire regarding daily life practices. Due to the usage of DNA saliva samples since 2018, only 54 samples were able to be amplified in this project. Out of those 54 samples, 34 of the samples successfully underwent pyrosequencing, resulting in the analysis of 34 individuals in this project ($n=34$).

Genomic DNA concentrations varied between the 54 samples that underwent DNA isolation, bisulfite conversion, and PCR amplification. A factor that was considered in the quality of genomic DNA is that the samples were collected in 2018 and were divided into 500 μL aliquots since then. There is likely an increased chance that DNA

degradation has occurred to some extent in the last six years. These samples also have been used in multiple projects and have also been thawed and frozen on many occasions. This temperature change may also affect the possibility of degradation in the DNA.

High concentrations are necessary due to the harshness of bisulfite conversion. This "harshness" can be attributed to the chemical reactivity of sodium bisulfite with DNA and the extreme pH conditions that are required to promote the deamination of cytosine residues (Genereux, 2008). This DNA degradation could be a contributing factor for the variation in DNA concentrations, especially in low-concentration samples. In addition, this may be a source of downstream effects on the quality of methylation data received from pyrosequencing analysis.

Post-data acquisition, the data was shortened to only analyze CpG 1-4 and the minimum peak heights were to 15 and 7. Doing so, yielded average percent methylation data for CpG sites 1-4 for 34 of the 54 samples. Though methylation data for CpG 1-4 were obtained and analyzed, the quality of the percent methylation serves as a caveat and poses limitations on establishing definitive correlations. Therefore, additional rounds of testing are required to fully unravel the etiology and epidemiology of hypertension in LMIC. After it was determined that shortening the length of the amplicon yielded analyzable results, the GRCF suggested that the 266 bp amplicon length that the J1 primer set amplified may have been too long and insufficient pyrosequencing was impacted. Future directions may include the redesign of a new J1 primer set that only amplifies CpG 1-4 to reduce the chance of variability within the data. The data on CpG 1-

4 for 34 samples was analyzed and relationships were identified between methylation and the presence of hypertension.

The first analysis was the average percent methylation for hypertensive versus normotensive/elevated individuals based on SBP across CpG sites 1-4. Since no statistically relationship was found between average methylation across all four sites, a Welch's t-test analysis was carried out for each individual CpG site and respective blood pressures to see if any relationship existed. A statistically significant finding that CpG 3 indicated increased methylation in hypertensive versus normotensive/elevated patients was discovered (P=0.047). This analysis contributed the first set of pyrosequencing data for CpG 1-4, providing a broader view of methylation around the transcription start site of *AGTR1*. More analysis of a larger sample size and a redesigned primer set can reveal more specific correlations between methylation and blood pressure.

Further analysis of the 34 samples that underwent pyrosequencing across eight CpG sites showed interesting insights. A Pearson's correlation showed a strong positive correlation between CpG sites Q2/Q3, CpG sites Q2/Q4, CpG sites Q3/Q4, CpG sites 1/2, CpG sites 1/3, CpG sites 1/4, CpG sites 2/3, CpG sites 2/4, and CpG sites 3/4. Furthermore, the findings of the MLR showed no predictive ability for CpG 1-4 for both SBP and DBP. These results pose questions about how the observed correlation between methylation at different CpG sites may be related to hypertension. These findings may indicate new avenues of research to identify the use of CpG sites of *AGTR1* as indicators for hypertension.

In addition, the analysis of the cost habits on firewood and charcoal in a given month as proxies for HAP and the presence of hypertension was carried out. It was discovered that there was increased spending on firewood and charcoal per month for hypertensive versus normotensive individuals. Statistically significant differences were identified for the average amount spent on charcoal per month with increased spending for hypertensive versus normotensive Kenyans. This analysis was carried out for 34 individuals and introduces an avenue of research that can explore spending habits on biomass fuels as a factor for hypertension. An increased sample size would likely elucidate the relationship further.

The results of this study do not support the hypothesis that hypomethylation of the RAS gene *AGTR1* correlates with hypertension. The findings in this project indicated increased methylation in hypertensive versus normotensive individuals when total percent methylation of CpG sites were analyzed on average and individually. The Pearson's correlation and MLR results provide insight into the complex relationship that exists between methylation of the *AGTR1* gene and hypertension, and point to the importance that increased research is needed to elucidate the relationship in the future.

CHAPTER VI

CONCLUSIONS

- Hypomethylation of the *AGTR1* promoter was not observed.
- Overall, DNA methylation of the *AGTR1* promoter is elevated in hypertensive vs. normotensive/elevated individuals.
- CpG 3 methylation was statistically elevated in hypertensive versus normotensive/elevated individuals.
- No predictive relationship appears to exist between methylation at any CpG site and an increase in blood pressure.
- Increased expenditure of biomass fuels in hypertensive individuals may support the role that HAP exposure in the development of high blood pressure.

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APPENDICES

Appendix A: IRB Approval

irb@southalabama.edu

UNIVERSITY OF
SOUTH ALABAMA

TELEPHONE: (251) 460 6308
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INSTITUTIONAL REVIEW BOARD
September 17, 2019

Principal Investigator	Nancy Rice, PhD		
IRB # and Title	IRB PROTOCOL: 19.283 [1461110-2] Molecular Epidemiology of Cardiovascular Disease		
Status	APPROVED	Review Type	Full Committee Review
Approval Date	September 9, 2019	Submission Type	New Project
Initial Approval	September 9, 2019	Expiration Date	
Review Category	Full Board		

This panel, operating under the authority of the DHHS Office for Human Research and Protection, assurance number FWA 00001602, and IRB Database #00000286, has reviewed the submitted materials for the following:

1. *Protection of the rights and the welfare of human subjects involved*
2. *The methods used to secure and the appropriateness of informed consent*
3. *The risk and potential benefits to the subject*

The regulations require that the investigator not initiate any changes in the research without prior IRB approval, except where necessary to eliminate immediate hazards to the human subjects, and that **all problems involving risks and adverse events be reported to the IRB immediately!**

Subsequent supporting documents that have been approved will be stamped with an IRB approval and expiration date (if applicable) on every page. Copies of the supporting documents must be utilized with the current IRB approval stamp unless consent has been waived.

Notes:

EBR Full Board review was conducted and has determined that this study is minimal risk.

45 CFR 46.110 (3): Prospective collection of biological specimens for research purposes by noninvasive means.

Appendix B: Ethics Compliance

UNIVERSITY OF NAIROBI
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Ref: No.KNH/ERC/R/18

14th February, 2019

Prof. Nancy A. Rice
Principal Investigator
Department of Biology
Western Kentucky University
Bowling Green, KY, 42101-1080 USA
Email: nancy.rice@wku.edu

Dear Prof. Rice

Re: Approval of Annual Renewal – Investigating the Molecular Epidemiology of Hypertension in East Africa (P509/07/2016)

Refer to your communication dated January 21, 2019

This is to acknowledge receipt of your study progress report and hereby grant you annual extension approval for ethics research protocol **P509/07/2016**.

The approval dates are 23rd February 2019 -22nd February 2020

This approval is subject to compliance with the following requirements:

a) Only approved documents (informed consents, study instruments, advertising materials etc) will be used
b) All changes (amendments, deviations, violations etc) are submitted for review and approval by KNH-UON ERC before implementation
c) Death and life threatening problems and serious adverse events (SAE's) or unexpected adverse events whether related or unrelated to the study must be reported to the KNH-UON ERC within 72 hours of notification
d) Any changes, anticipated or otherwise that may increase the risks or affect safety or welfare of study participants and others or affect the integrity of the research must be reported to KNH-UON ERC within 72 hours
e) Submission of a request for renewal at least 60 days prior to expiry of the approval period (*Attach a comprehensive progress report in support of the renewal*)
f) Clearance for export of biological specimens must be obtained from KNH-UON ERC, this is a Research Committee for each batch of shipment

Prof. [illegible]

BIOGRAPHICAL SKETCH

Name of Author: Michael M. Roque

Undergraduate Schools Attended:

University of South Alabama, Mobile, Alabama