GENETIC POLYMORPHISMS ASSOCIATED WITH HYPERTENSION IN THE ETHNIC POPULATIONS OF CALABAR AND UYO, NIGERIA

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ABSTRACT

Hypertension is a public health challenge due to its high prevalence, and is a major risk factor for cardiovascular diseases. Hypertension is a complex disease resulting from an interaction of genes and environmental factors. Inconsistent association between polymorphisms of the renin angiotensin aldosterone, the atrial natriuretic peptide systems and hypertension has been reported among various ethnic groups, but not for the Efiks and Ibibios in south-south Nigeria. This study was designed to determine the frequency of gene polymorphisms of these two systems and their association with hypertension in Calabar and Uyo, Nigeria.

A population-based case control design was used. A total of 1224 participants, 612 each of patients and controls were randomly recruited from hypertension clinics and the general population. Genotyping of the M235T allele of the angiotensinogen, Insertion/Deletion allele (I/D) of the angiotensinogen converting enzyme, A1166C allele of the angiotensin II type I receptor and C664G allele of the atrial natriuretic peptide genes to identify variants was performed using polymerase chain reaction and restriction enzyme digestion. The Hardy-Weinberg equation was used to calculate the allele and genotype frequencies. Plasma angiotensinogen levels were measured by Enzyme Linked Immunosorbent Assay. Hypertensinogenic factors such as age, familial history, physical exercise and drinking were assessed using questionnaires. Descriptive statistics, chi-square, multiple regression analysis and odds ratio were used to analyze the data.

The frequency of the genotypes M235M, M235T, T235T of the M235T allele for the Efiks were 0.4, 7.7, 92 % in patients and 0, 6, 94 % in controls; for the Ibibios were 0.5, 1.2, 87 % in patients and 0, 7, 93 % in controls. The I/D genotypes II, ID, DD frequencies for the Efiks were 11, 44, 46 % in patients and 16, 45, 39 % in controls; for the Ibibios were 11, 40, 49 % in patients and 13, 49, 38 % in controls. The frequency of the A1166C carriers was 1 % while 99 % of the study population had the wild type A1166A genotype for the A1166C allele. Only the CC genotype was observed for the C664G allele. These frequencies did not conform to the Hardy-Weinberg assumptions. There were no significant differences between the genotype frequencies of patients and controls. Plasma

angiotensinogen values were significantly higher in the patients with M235T allele than in the controls. Age was a positive predictor for systolic blood pressure (SBP, $r = 0.60$) in patients and diastolic blood pressure (DBP, $r = 0.56$) in controls. Other hypertensinogenic variables were not predictors for SBP and DBP in the population ($p < 0.05$). The Insertion/Deletion allele was a risk factor for hypertension, (O.R = 1.15).

A high frequency was observed for the M235T allele and the Insertion/Deletion allele, which was associated with an increased risk for hypertension. The lack of association between the alleles of the M235T, A1166C and the C664G and hypertension suggests that other loci or environmental factors are involved in the disease outcome.

Keywords: Polymorphism, Hypertension, Allele and genotype, Efiks and Ibibios
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CERTIFICATION

I certify that this work was carried out by Mrs. M.E. Kooffreh in the Department of Zoology University of Ibadan and in the Virology Unit of the International Institute of Tropical Agriculture, Ibadan.

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DEDICATION

This work is dedicated to:

The one who loved me and gave himself for me,

My husband, Esien Ita Kooffreh, whose support has been overwhelming,

My daughter Mary Esien Kooffreh who tried to grow up on her own and be a good girl, the years mummy was not there for her.

And the boys, Joseph, Emmanuel, David and Paul Kooffreh

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ABBREVIATIONS

AGT: Angiotensinogen

EH: Essential hypertension

RAAS: Renin-Angiotensin-Aldosterone System

ANP: Atrial Natriuretic Peptide

ACE: Angiotensin Converting Enzyme

AT1R: Angiotensin II Type I receptor

ELISA: Enzyme Linked Immunosorbent Assay

BP: Blood Pressure

BMI: Body Mass Index

MM: Wild type allele for angiotensinogen with methionine at position 235

MT: Heterozygote allele for angiotensinogen with a threonine substitution at position 235

TT: Recessive homozygous allele for angiotensinogen with threonine replacing methionine at position 235

II: Insertion allele of the angiotensin converting enzyme gene

ID: Insertion/Deletion heterozygote of the angiotensin converting enzyme gene

DD: Deletion allele of the angiotensin converting enzyme gene

AA: Wild type allele for the angiotensin II type I receptor gene with adenine at Position 1166

AC: heterozygous allele for the angiotensin II type I receptor gene with a cytosine Substitution at 1166

CC: Recessive homozygous allele for the angiotensin II type I receptor gene with a cytosine replacing adenine at position 1166

CC: Wild type allele for the atrial natriuretic peptide gene with cytosine at position 664

CG: Heterozygous allele for the atrial natriuretic peptide gene with guanine substitution at position 664

GG: Recessive minor allele for the atrial natriuretic peptide gene with guanine Replacing cytosine at position 664

IITA: International Institute for Tropical Agriculture

CHAPTER ONE

INTRODUCTION

Hypertension is a multifactorial disorder that results from an interaction of many risk genes such as molecular variants of the angiotensinogen gene, angiotensin converting enzyme gene, angiotensin II receptor I gene and the corin gene (Cooper *et al.*, 2000; Hilgers *et al.*, 1999; Dries *et al.*, 2005; Sethi *et al.*, 2003), and environmental factors such as obesity, body mass index (BMI), dietary salt intake, alcohol consumption, stress and high-density lipid (HDL) – cholesterol levels. Genes determine approximately 20 to 60% of the variability in blood pressure in different populations (Sethi *et al*., 2003; Cooper *et al.*, 2000).

Hypertension is defined as high arterial blood pressure and it is generally agreed that blood pressure above 140/90 mm Hg is hypertension (Kadiri, 2000). Hypertension is a condition in which an increase in resistance to blood flow causes the blood to exert excessive pressure against the walls of the blood vessels. The heart must therefore work harder to pump blood through the narrowed blood vessels. If the condition persists, damage to the heart and blood vessels tend to increase the risk for stroke, heart attack, kidney or heart failure (Akinkugbe, 2000). Essential hypertension is high blood pressure for which no medical cause can be found and it accounts for more than 90% of cases of hypertension. The remaining 5-10% of hypertension also referred to secondary hypertension is due to other conditions that affect the kidneys, arteries, heart and the endocrine systems. Hypertension affects about one quarter of the adult population in industrialized countries and it contributes significantly to morbidity from stroke, heart failure, coronary heart disease and chronic kidney failure (Angius *et al.*, 2002).

Blood pressure is usually classified based on the systolic and diastolic blood pressure. Systolic pressure is the blood pressure in the vessels when the heart contracts or during heart beat. Diastolic pressure is the pressure in the vessels when the heart relaxes or between heart beats. Based on the recommendation of the seventh report of the Joint National Committee of Prevention, Detection, Evaluation and Treatment of High blood Pressure (JNC VII), the classification of blood pressure (expressed in mm Hg) for adults aged 18 or older is as follows: Normal - systolic lower than 120, diastolic lower than 80. Prehypertension - systolic 120-139, diastolic 80-90. Stage 1 hypertension – systolic 140-159, diastolic 90-99. Stage 2 hypertension - systolic equals or more than 160, diastolic equals or more than 100. The JNC VII report emphasizes that patients with prehypertension are at risk for progression to hypertension and that lifestyle modifications are important preventive strategies (Sharma and Kortas, 2008). These classifications are based on the average of an individual's blood pressure readings taken on two occasions. Diagnosis of hypertension is on the basis of chronic or persistent high blood pressure, it requires three separate sphygmomanometer measurements a week apart. A diagnosis for essential hypertension is made usually after all other forms of hypertension have been excluded using biochemical and clinical investigations.

Mild to moderate essential hypertension is usually asymptomatic. Signs and symptoms associated with severe hypertension in adults and children include headache, drowsiness, fatigue, vision disorders, nosebleeds and facial paralysis, nausea and

vomiting. In infants and neonates, symptoms include failure to thrive, difficulty in breathing and seizures. (Rodriguez *et al.*, 2010). Some other signs and symptoms suggest that hypertension is as a result of disorders in hormone regulation. These symptoms are specific to the disorder. An example is hypertension in pregnancy which is one of the symptoms of pre-eclampsia that usually progresses to eclampsia, a life threatening condition characterized by seizures.

Although no direct cause has been identified as being responsible for essential hypertension, several factors have been shown to predispose individuals to high blood pressure. These include sedentary lifestyle, smoking, obesity, salt sensitivity, alcohol intake and vitamin D deficiency. The risk for essential hypertension also increases with age, some inherited genetic mutations, a family history of hypertension, an overactive sympathetic nervous system and increase in the levels of renin. Low birth weight has also been included as risk factor for adult essential hypertension. Secondary hypertension is usually due to an identifiable cause which if treated properly reduces the elevated blood pressure. Many other conditions also cause secondary hypertension, they include: hormonal imbalances, kidney disease, preeclampsia during pregnancy, certain prescriptions and illegal drugs such as decongestants and corticosteroids as well as some nutritional substances such as caffeine (McCrindle, 2010).

Investigations into the molecular genetics of human hypertension is aimed at identifying the loci involved, detecting gene variants of these loci and associating them with intermediate phenotypes for proper estimation of their quantitative effect on blood pressure and interactions with principal environmental factors (Corvol and Jeunemaitre, 1997; Zhu *et al.*, 2003; 2005). Evidence for a genetic influence on blood pressure comes from various sources. Twin studies have shown a greater concordance of blood pressures in monozygotic than dizygotic twins (Feinleib *et al.*, 1977), and population studies show greater similarity in blood pressure within families than between families (Longini *et al.*, 1984). The latter observation is not attributable to only a shared environment since adoption studies demonstrate greater concordance of blood pressure among biological siblings than adoptive siblings living in the same household (Biron *et al.*, 1976). Furthermore, single genes can have major effects on blood pressure, accounting for the rare Mendelian forms of high and low blood pressure (Lifton *et al.*, 2001). Although identifiable single-gene mutations account for only a small percentage of hypertension cases, the study of these rare disorders may clarify pathophysiologic mechanisms that predispose to more common forms of hypertension and may suggest novel therapeutic approaches (Lifton *et al.*, 2001). Mutations in 10 genes that cause Mendelian forms of human hypertension and 9 genes that cause hypotension have been described to date, as reviewed by Lifton and colleagues (Lifton *et al.*, 2001; Wilson *et al.*, 2001). In most cases, hypertension results from a complex interaction of genetic, environmental, and demographic factors. Improved techniques of genetic analysis, especially genome-wide linkage analysis, have enabled a search for genes that contribute to the development of essential hypertension in the population.

The application of these techniques has found statistically significant linkage of blood pressure to several chromosomal regions, including regions linked to familial combined hyperlipidemia (Hsueh *et al.*, 2000; Kristjansson *et al.*, 2002; Hunt *et al.*, 2002). These findings suggest that there are many genetic loci, each with small effects on blood pressure in the general population. Overall, however, identifiable single-gene

causes of hypertension are uncommon, consistent with a multifactorial cause of essential hypertension (Oparil *et al.*, 2003). The candidate gene approach typically compares the prevalence of hypertension or the level of blood pressure among individuals of contrasting genotypes at candidate loci in pathways known to be involved in blood pressure regulation.

The most promising findings of such studies relate to genes of the renin–angiotensin–aldosterone system, such as the M235T variant in the angiotensinogen gene, which has been associated with increased circulating angiotensinogen levels and blood pressure in many distinct populations (Jeunemaitre *et al.*, 1992b; Corvol *et al.*, 1999; Staessen *et al.*, 1999), and a common variant in the angiotensin-converting enzyme (*ACE*) gene that has been associated in some studies with blood pressure variation in men (Fornage *et al.*, 1998; O'Donnel *et al.*, 1998). However, these variants seem to only modestly affect blood pressure, and other candidate genes have not shown consistent and reproducible associations with blood pressure or hypertension in larger populations (Lifton *et al.*, 2001); thus, demonstration of common genetic causes of hypertension in the general population remains elusive (Corvol *et al.*, 1999; Niu *et al.*, 1998; Luft, 2000).

1.1 Gene polymorphisms under consideration

The *AGT* gene belongs to the serpin (serine protease inhibitor) super family (Corvol and Jeunemaitre 1997). The human AGT cDNA is 1,455 nucleotides long and codes for a 485-amino acid protein (Kageyama *et al.*, 1984). The *AGT* gene contains five exons and four introns which span 13kb. *In situ* hybridization studies indicate that the human *AGT* gene is located on chromosome 1q42-43 (Isa *et al.*, 1990; Gaillard –Sanchez *et al.*, 1990). The human AGT protein is a globular glycoprotein with a molecular mass of 55-65 kDa, depending on the state of glycosylation. (Corvol and Jeunemaitre, 1997). A high molecular mass form of AGT protein is present in human plasma. In addition to the liver, the brain, large arteries, kidney and adipose tissues are all established sites of AGT synthesis (Dzau *et al.*, 1987).

The renin-angiotensin (R-A) system is a powerful pressure system which influences salt and water homeostasis. Angiotensinogen (AGT) is a key component of this system, it is cleaved by renin to yield angiotensinogen 1 (AGT 1), which is cleaved by angiotensinogen converting enzyme (ACE) to yield angiotensinogen II (AGT II), responsible for carrying out a range of functions that include i) prompting the constriction of blood vessels causing a rise in blood pressure, ii) ensuring the release of aldosterone by the adrenal cortex which acts on the tubules causing absorption of more water and salt from urine. Blood volume increases so does blood pressure. Potassium ions are excreted from the tubules in exchange for sodium iii) mediates the release of antidiuretic hormone from the pituitary that enhances the reabsorption of water, it also increases an individual's appetite for salt and stimulates the sensation of thirst (Caulfield *et al.*, 1994). The M235T polymorphism was associated with a 10% - 30% increase in plasma AGT. Chronic increases in plasma AGT concentration may slightly increase blood pressure and facilitate hypertension (Corvol and Jeunemaitre, 1997).

The measurement of angiotensinogen concentration has proved to be a convenient method for monitoring the activity of R-A system in human populations since it

circulates at relatively constant level. AGT is usually measured by an enzymatic assay for Ang 1 after its complete hydrolysis by excess renin. Direct immunoassays, using polyclonal and monoclonal antibodies against AGT, that measure both intact AGT and its inactive C-terminal part, residual AGT have also been developed (Genain *et al.*, 1984; Katsurada *et al.*, 2007). Cooper *et al.* (1999) that higher angiotensinogen levels were indicative of higher blood pressure. Simple and accurate methods to measure human angiotensinogen are available but very expensive. This study used one of the sandwich ELISA methods used by International Institute for Tropical Agricultural-IITA, Ibadan to detect plant viruses, the reagents are cheaper and if successful could be used to measure human angiotensinogen in plasma and also determine its concentration in the tropics to reduce cost.

Angiotensin Converting Enzyme (ACE), a key enzyme in the renin angiotensin-aldosterone pathway, is found in the kidneys. It catalyzes the conversion of angiotensin I to a physiologically active angiotensin II that controls fluid electrolyte balance and systemic blood pressure (Wang *et al.*, 2000). *ACE* gene is mapped to chromosome 17q23. The insertion/deletion (I/D) polymorphism was discovered in 1990 and is characterized by the presence of (insertion) or absence (deletion) of a 287- AluYa5 element inside intron
16 producing three genotypes: II homozygote, ID heterozygote, DD homozygote (Rigat *et al.*, 1990). Though the polymorphism is located in a non- coding region of the *ACE* gene, several investigators (Sakuma *et al.*, 2004; Salem, 2008; Tsezou *et al.*, 2008; Ramachandran *et al.*, 2008; Sameer *et al.*, 2010) have observed that the polymorphism is not silent but the DD homozygote is associated with increased activity of ACE in the serum and several diseases including hypertension.

The angiotensin II protein is a well known vasoconstrictor that exerts most of its influence through the angiotensin II type 1 receptor (AT_1R). Angiotensin II type 1 receptor (AT_1R) is a membrane-bound G protein coupled- receptor that mediates the effects of angiotensin II (De Gaspora *et al.*, 2000). The highly polymorphic human AT_1R gene is 55kb long having five exons and four introns, A1166C polymorphism is a single base substitution of adenine for cytosine at position 1166 in the 3^1 untranslated regions of the gene located on chromosome 13. The A allele is the larger fragment that lacks the restriction enzyme while the smaller fragment from the C allele has the restriction enzyme site. The physiological significance of the polymorphism is uncertain because data on the function of the *AT1R* polymorphism is limited. Thus the mechanism responsible for the association of hypertension status with A1166C polymorphism has remained largely unknown and the amino acid sequence of the receptor is not altered. It is however thought to affect mRNA stability and transcription and is in linkage disequilibrium with some other polymorphism. It is also associated with some diseases (Bonnardeaux *et al.*, 1994; van Geel *et al.*, 2000; Stankovic *et al.*, 2003; La pierre *et al.*, 2006).

In addition to the kidney, the heart plays an important role in regulating salt and water balance. This is mediated by a cardiac hormone referred to as the atrial natriuretic peptide (ANP) or factor (ANF). This is a potent natriuretic and vasorelaxant hormone that is mainly secreted by cardiomyocytes and plays a role in cardiovascular homeostasis in

opposition to the RAAS. When blood sodium and blood pressure levels increase, ANP secreted from the heart bind to its receptors in the kidney and blood vessels, promotes the excretion of large amounts of salt in urine, thereby lowering blood volume and also relaxing blood vessels. The heart and kidney are thus involved in maintaining a fine balance of electrolytes and body fluid. (Fig 1.1) The ANP is a 28 amino acid peptide in humans that assumes a hairpin structure by virtue of a cystein bridge that links residues 7 and 23. The *ANP* gene is located on chromosome 1q21. Several nucleotide polymorphisms have been identified in the ANP gene. One of them is the –C664G polymorphism located in the promoter region. Rubattu *et al.* (2006) reported that the –C664G polymorphism is responsible for the down regulation of ANP gene transcription; it is associated with left ventricular hypertrophy in Italians. The –C664G has been reported to be monomorphic among the Chinese and no other SNPs are in linkage disequilibrum with the –C664G polymorphism (Xue *et al.,* 2008).

1.2 Justification

Genetic variations of genes encoding components of the renin-angiotensin-aldosterone system have been associated with susceptibility to hypertension making them strong candidate genes for investigating the genetic basis of hypertension. In addition to the RAAS, the natriuretic peptide system also affects blood pressure directly through its vasodilatory and natriuretic activities and indirectly inhibiting the RAAS. This has generated considerable interest in the role of ANP in the development of hypertension.

Some researchers have reported the frequency of the M235T and the T174M alleles of the angiotensinogen gene in western Nigeria. Most of the studies carried out so far in Nigeria have been concentrated in individuals and population in the South Western part of the country. Little is found in literature on the genetics of hypertension in other geographical and ethnic areas of Nigeria, and its genetic epidemiology, especially since variations in the dietary intake, culture (way of life) etc exist in these places. In Blacks, differences have been observed in the expression of risk factors such as level of circulating angiotensinogen, urbanization, dietary factors and gene variants for hypertension depending on the environment (Cooper *et al.,* 1998; Cooper *et al.,* 2005; Mufunda *et al.,* 2006).

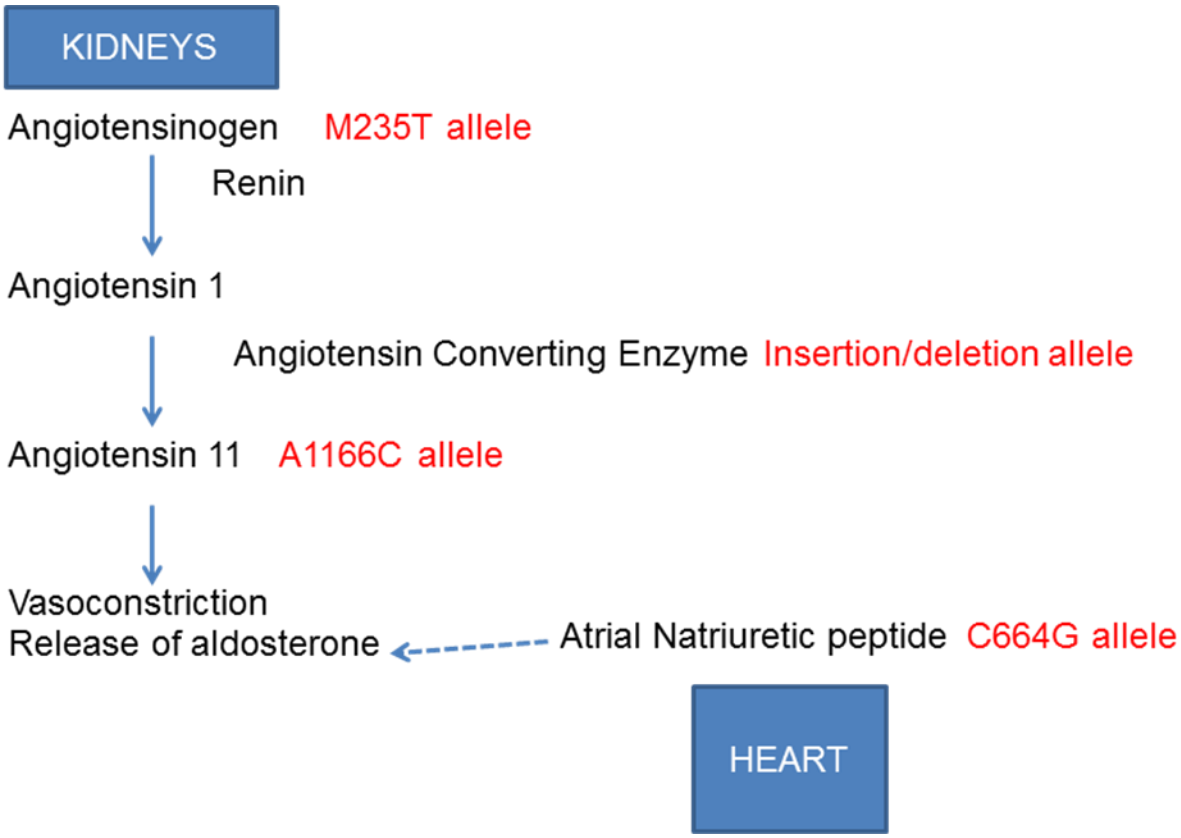


Fig 1.1 The Renin Angiotensin Aldosterone and the Atrial Natriuretic peptide systems

Apart from the M235T allele, there are no published data on other alleles in Nigeria. This study will provide baseline data for these areas and adds to the knowledge on the genetic basis of hypertension among these ethnic groups. The M235T polymorphism is associated with a 10-30% increase in plasma angiotensinogen. Chronic increases in plasma AGT concentration is thought to increase blood pressure and facilitate hypertension. Simple and accurate methods are available but very expensive. This study used one of the sandwich ELISA methods used by the International Institute for Tropical Agriculture Ibadan to test plant specimens for viruses, to measure the levels of angiotensinogen in human plasma and determine its concentration in relation to the presence of the T235 allele in the study population.

1.3 Hypothesis

There is an association between the mutant variant of the genes for angiotensinogen; angiotensin converting enzyme; angiotensin II type I receptor; atrial natriuretic peptide (with possible interactions of environmental factors) and hypertension in the study population.

1.4 Objectives

The main goal of this research was to improve our knowledge of the genetic epidemiology of hypertension in Calabar and Uyo. Specific objectives are:

1. To genotype the study population for:
   a) M235T allele of the angiotensinogen gene
   b) The A1166C allele of the angiotensin 11 type 1 receptor gene,
   c) The insertion deletion polymorphism of the angiotensin converting enzyme gene and
   d) The C664G allele of the atrial natriuretic peptide gene
2. To determine the association of these polymorphisms with hypertension status in a sample population of individuals living within the cities of Calabar and Uyo.
3. To relate the angiotensinogen levels in the plasma of hypertensives and controls to the M235T allele and to hypertension.

CHAPTER TWO
LITERATURE REVIEW

2.1 Pathophysiology of hypertension

Hypertension results in a compromise of the pathophysiological mechanisms. The mechanisms associated with secondary hypertension are fully understood but those associated with essential hypertension are still unclear. However it is known that cardiac output is raised at the onset of the disease with total peripheral resistance (TPR) normal, as the disease progresses, cardiac output drops to normal with an increase in total peripheral resistance. Three theories have been put forward to explain this phenomenon:

1 Atrial natriuretic factor is secreted to promote the excretion of sodium by the kidney resulting in an increase in the total peripheral resistance.

2 Overproduction of sodium-retaining hormones and vasoconstrictors; long-term high sodium intake leading to vasoconstriction and retention of sodium and water; inadequate dietary intake of potassium and calcium. The result is an increase in blood volume that leads to hypertension.

3 Increased sympathetic nervous system activity, perhaps related to heightened exposure or response to psychosocial stress.

Structural and functional abnormalities in the cardiac vasculature, including endothelial dysfunction, increased oxidative stress, vascular remodeling have been implicated in the development of hypertension, it is still unclear whether endothelial changes precede disease development or if such changes are due to chronic elevated blood pressure(Oparil *et al.*, 2003) Figure 2.1.

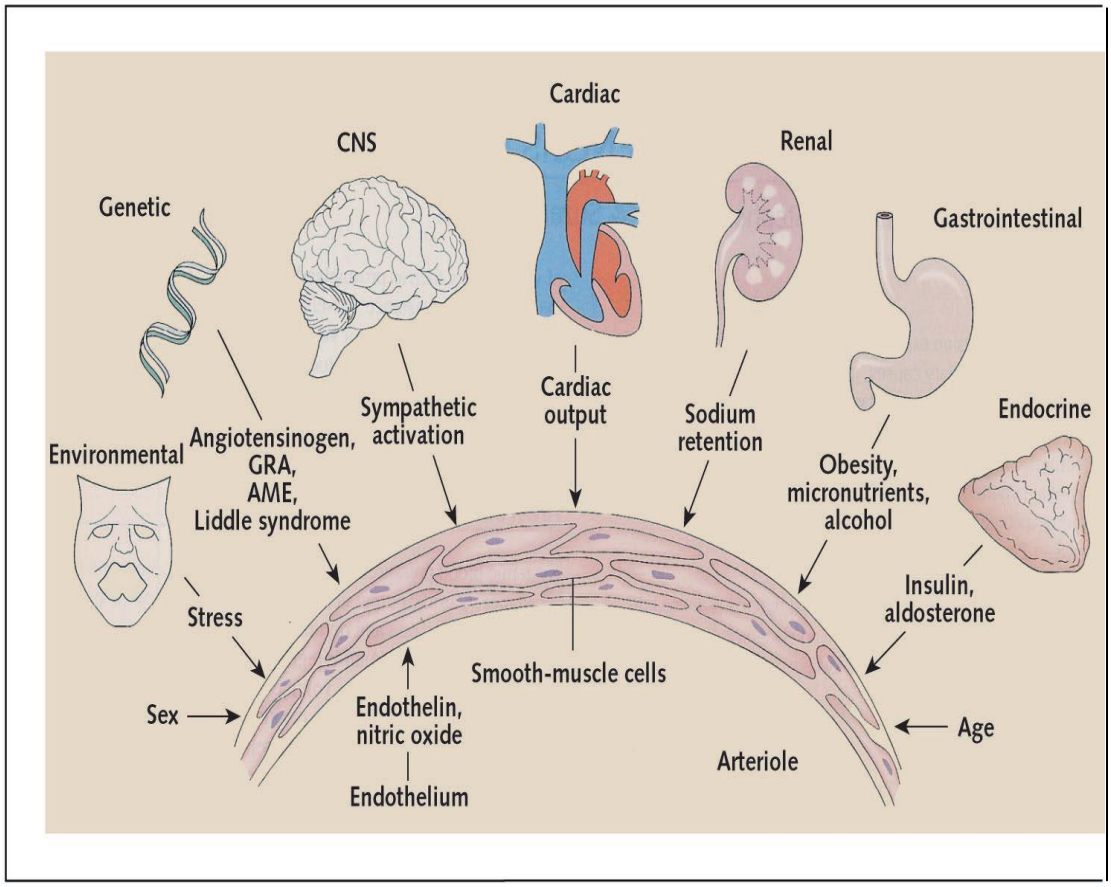


Fig 2.1 Pathophysiologic mechanisms of hypertension (Oparil *et al.*, 2003)

2.2 Epidemiology of hypertension

High blood pressure or hypertension `a silent killer' condition is now the most common chronic condition affecting 20-30% of the adult population in the U.S. An estimated 63.3 million (31.0%) US adults currently have a BP exceeding 140/90mm Hg and the prevalence is higher for blacks than for other racial/ethnic subgroups. (Giles *et al.*, 2007). National health surveys in various countries have reported a prevalence of 22% in Canada, of which 16% is controlled, 26.3% in Egypt of which 8% is controlled, 13.6% in China of which 3% is controlled (Sharma and Kortas, 2008). Ideal data on hypertension prevalence and incidence based on large population-based surveillance that use standardized and validated protocols are lacking for most countries in sub-Saharan

Africa. The aggregate of prevalence data published in the 1990s excluded highly selected groups in Africa with either very low or very high prevalence of 5-20%.

In these studies, hypertension prevalence increased in a graded fashion with the established biological and psychosocial determinants of raised BP.

Cooper *et al.*, (1997) showed that hypertension was more common among the urban poor (17%) than among rural dwellers (7%). It was also substantially more prevalent among salaried suburban workers (26%). The effects of a family history of hypertension, alcohol intake, physical inactivity and advancing age in the prevalence of hypertension were similar to those established for developed nations although their relative importance as a cause of hypertension was different, though less evaluated in Africa. More recent data, such as those from Tanzania, Ghana, Nigeria, Egypt and South Africa suggest that hypertension prevalence (using a partition value of 140/90mm Hg) is on the rise in Africa and commonly exceeds 20 - 25% in rural areas and is over 30% in urban and semi urban areas (Addo *et al.*, 2007).

In a study of two linked cross sectional population- based surveys of a middle - income urban district and a relatively prosperous rural area in Tanzania, Edward *et al.* (2000) reported an age standardized hypertension prevalence of 37.3% and 39.1% among men and women respectively in the urban district, 26.3% and 27.4% among men and women respectively in the rural area. In a cross sectional study of adults age 18 years and older in four rural communities in the Ga district of Ghana, Addo *et al.* (2006) observed a hypertension prevalence of 25.4%. In particular, the age adjusted odds ratio for developing hypertension for overweight and for obesity were 5.8 and 6.9 respectively. Burkett (2006) reported a prevalence of 32.8% in a survey of volunteers in two villages in the Volta region of Ghana, more than 1 in 4 adults (28.7%) have high B.P with an even higher prevalence in semi urban areas (32.9%). Studies suggest that hypertension rises with age in both men and women, in adults as well as children and the prevalence tends to be higher in the urban than rural areas, though some populations in Africa still show relatively low hypertension prevalence (Kaufman *et al.*, 1999; Cooper *et al.*, 2005).

Nigeria has a population of about 130 million people (National Census, 2006) and is the largest Black nation in the world. The crude prevalence of hypertension has been documented as 11.2% (based on blood pressure threshold of 160/95 mm Hg), with an age-adjusted ratio of 9.3%. This number translates into 4.33 million Nigerian hypertensives aged ≥15 years according to the National Census figures (Akinkugbe, 2000). However, according to the current definition of hypertension from the seventh Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure (JNC 7) guidelines (JNC 7, 2003) many more Nigerians (20–25%) would be classified as hypertensive. In a study carried out by the International Collaborative Study of Hypertension in Blacks (ICSHIB), the age-adjusted prevalence of hypertension in Nigeria was 14.5%. Based on gender, the prevalence of hypertension was 14.7% for men and 14.3% for women (Cooper *et al.*, 1997). Ulasi *et al.* (2011) reported a prevalence of 42.2% among market traders at Ogbete in Enugu, Nigeria. Major target-organ complications of hypertension, such as left ventricular hypertrophy (Opadijo *et al.*, 2003), diastolic dysfunction (Ike and Onwubere, 2003), congestive heart failure (Falase *et al.*, 1983) ischemic heart disease (Falase *et al.*, 1974) and renal failure (Akinkugbe, 1992) are well documented in Nigeria. Nigerians are particularly susceptible to

hypertension and its complications such as disabling and fatal strokes which remain a major cause of morbidity and mortality (Osuntokun *et al.*, 1979; Iyalomhe *et al.*, 2008).

In a study of cardiovascular diseases in multiple centers in Nigeria (Ekere *et al.*, 2005) hypertension was ranked first. Hypertension and its complications constitute <25% of emergency medical admissions in urban hospitals in the country (Iyalomhe *et al.*, 2008). It is the medical illness most frequently diagnosed in elderly Nigerians (Bella *et al.*, 1993). Ogunniyi *et al.* (2001) studied 613 elderly Nigerians (398 women and 215 men) aged 65–110 years in a cohort at Ibadan. They found that cardiovascular disease was the most common condition in this cohort, and hypertension (27.8%) was the most frequent diagnosis. Bella *et al.* (1993) also reported a similar figure. Ogah (2006) observed that hypertension was also the most common condition associated with dementia in Nigeria. It was the most common condition in senior executives (Okojie *et al.*, 2000) and army recruits (Awoyemi *et al.*, 2001).

Two autopsy studies showed that hypertension is the most common cause of sudden unexpected natural death. Amakiri *et al.* (1997) studied 876 consecutive coroners' autopsies at Ibadan and found that the most common cause of sudden natural death was cardiovascular disease, and complications of hypertension accounted for most cases. This finding was corroborated by Aligbe *et al.* (1997) 3% of hypertensive Nigerians die each year. The population-attributable risk has been established at 7% (Kaufman *et al.*, 1996). Low economic status was implicated in the development of hypertension among Nigerian adults (Adedoyin *et al.*, 2005).

2.3 Association studies in hypertension

RAAS (renin–angiotensin–aldosterone system)

The human *AGT* gene (coding for angiotensinogen), an AGT precursor of the system, encodes a hepatic protein cleaved by renin and digested further by angiotensin-converting enzyme - ACE to generate the physiologically active angiotensin II (Ang II). Ang II via presynaptic type 1 Angiotensin II receptors - AT1Rs, potentiates the release of noradrenaline (norepinephrine). This peptide, together with aldosterone, which is generated in the adrenal zona glomerulosa by *CYP11B2*, maintains the circulating plasma volume that, in turn, through stimulation of cardiopulmonary and arterial mechanoreceptors, may influence sympathetic tone and increase heart rate variability (Grassi, 2001). Due to the important role of RAAS in the regulation of water and sodium balance (Naber and Siffert, 2004), numerous studies have investigated the relationship between RAAS and EH (Caulfield *et al.*, 1995; Atwood *et al.*, 1997; Brand *et al.*, 1998; Salem, 2008; Badaruddoza *et al.*, 2009; Sameer *et al.*, 2010). Two molecular variants of the angiotensinogen gene, (M235T and T174M) were found to correlate with high levels of angiotensinogen in the plasma as well as hypertension (Sethi *et al.*, 2003). These two variants are at some distance from both the cleavage sites for the promoter region, it is assumed that they are important in angiotensinogen transcription and messenger RNA stability (Corvol and Jeunemaitre, 1997).

The *AGT* gene variant M235T is associated with higher circulating AGT levels and EH in several, but not all, populations (Jeunemaitre *et al.*, 1992c; Atwood *et al.*, 1997; Brand *et al.*, 1998; Vasku *et al.*, 2002). Support for linkage of M235T locus to essential hypertension was shown in studies on populations of European ancestry (Caulfield *et al.*, 1994). Results from Japanese studies also show an association between

the angiotensinogen gene M235T variant and essential hypertension (Hata *et al.,* 1994). Other studies have reported no association of the angiotensinogen variant with hypertension in African Americans and African Caribbeans (Caulfield *et al.,* 1995; Rotimi *et al.,* 1996; Sethi *et al.,* 2001). The *AGT* M235T and the promoter G-6A polymorphisms showed association with essential hypertension in Tibetan women. No association could be detected for polymorphisms in *ACE* and *AT1R* with essential hypertension (Sun *et al*., 2004).

The *AGT* M235T allele was demonstrated to be in linkage disequilibrium with allelic variants in the *AGT* promoter region (G-6A and A-20C), which may affect the basal rate of AGT transcription and could account for phenotypic variation in plasma AGT concentrations (Jeunemaitre *et al.,* 1997; Inoue *et al.*, 1997; Ishigami *et al.*, 1997) . In Nigerians, Rotimi *et al.* (1997) reported a significant association between the presence of the M235T allele and high mean AGT concentration which was also significantly related to hypertension status, though Cooper *et al.* (1999) reported a high level of plasma angiotensinogen but low hypertension status in Igbo-Ora, Nigeria.

The *ACE* gene was also implicated in the aetiology of hypertension. The gene-coding area carries an insertion/deletion - ID polymorphism within intron 16. Several studies have associated the *ACE* I/D polymorphism with elevation in blood pressure in the Japanese and other ethnic groups (Morise *et al.,* 1994; Tobina *et al.,* 2006 and Das *et al.,* 2008) Some studies have shown that this polymorphism is strongly associated with increased blood pressure in males (O'Donnell *et al.,* 1998; Fornage *et al.,* 1998; Sunder-Plassmann *et al.,* 2002); however, a negative association was also detected in some linkage and association studies (Jeunemaitre *et al.*, 1992a; Sugiyama *et al.,* 1999). Gupta *et al.* (2009) reported a negative association between the *ACE* polymorphism and hypertension in a rural population in India. The relationship between essential hypertension and the genes encoding for *AGT*, *ACE* as well as *AT1R* was studied in 173 hypertensive individuals and 193 normotensive Chinese Tibetan individuals. The M235T and the G-6A polymorphisms showed association with essential hypertension in Tibetan women. However no associations were observed for polymorphism of the *ACE* and *ATIR* with essential hypertension. (Sun *et al.,* 2004).

The relationship between *ACE* and environmental factors predisposing to EH has been investigated in 1099 subjects from one Mongolian population. The study reported evidence for an interaction between the *ACE* DD (deletion/deletion) and ID polymorphism and cigarette smoking, alcohol drinking and BMI -body mass index (Xu *et al.,* 2004). The *ACE2* gene, a homologue of *ACE*, has been discovered (Donoghue et *al.,* 2000), and appears to be a negative regulator of ACE in the heart (Eriksson *et al.,* 2002). A case-control study investigating four single nucleotide polymorphisms - SNPs of *ACE2* and EH provided no evidence for an association in an Anglo–Celtic Australian population (Benjafield *et al.,* 2004). In this study, the 152 hypertensive subjects studied were the offspring of parents who both had hypertension, and similarly the 193 normotensive subjects were from normotensive parents over the age of 50 years. However, the data indicate little support for *ACE2* in genetic pre-disposition to EH (Benjafield *et al.,* 2004). As RAAS plays important roles in the regulation of water and sodium balance, the α-adducin Gly460Trp variation is also believed to induce significant differences in the activity of the Na^+/K^+ ATPase which, in the renal proximal tubule, affects sodium reabsorption (Lalouel *et al*, 2001). One study in a Chinese Han population (479 subjects

from 125 nuclear families) revealed that *ACE* ID, α-adducin Gly460Trp and CYP11B2 -344C/T polymorphisms interact to influence SBP (systolic BP; $P<0.05$), suggesting that these genes might indeed predispose to hypertension, especially in an ecogenetic context characterized by high salt intake (Wang *et al.*, 2004).

The A1166C polymorphism is associated with prevalent hypertension and increased aortic stiffness (Benetos *et al.*, 1996; Wang *et al.*, 1997; Danser and Schunket, 2000). This polymorphism has also been associated with other diseases such as left ventricular hypertrophy (Takami *et al.*, 1998) pregnancy induced hypertension; early coronary disease and excessive vasoconstriction (Alvarez *et al.*, 1998; van Geel *et al.*, 1998; 2000). Stankovic *et al.* (2003) reported a significant association between this polymorphism and hypertension in males but not females. The frequency of the C1166 allele was high among hypertensives (Rubattu *et al.*, 2004; Dzida *et al.*, 2001). Some other studies have also reported a negative association between the $A^{1166}C$ polymorphism and hypertension (Tiret *et al.*, 1998; Takami *et al.*, 1998 and Kikuya *et al.*, 2003). These variations were attributed to ethnic differences in the various populations (Agachan *et al.*, 2003, Kikuya *et al.*, 2003).

Rubattu *et al.* (2007) found that young Italian men heterozygous for the G allele mutation of the *ANP* gene had an increased risk for an early onset of the disease. When compared with homozygous G individuals, carriers of the-664G mutation also had an increased left ventricular mass index in a study among a highly homogenous population of Caucasian patients (Rubattu *et al.*, 2006). The C664G polymorphism showed a borderline association with hypertension in Japanese subjects (Kato *et al.*, 2000). Hu *et al.* (2007) genotyped 1186 individuals from the Matsu area in Taiwan, 35 years and above, no difference was observed between the allele frequency of patients and controls. Zhang *et al.* (2006) also reported no association between the –C664G mutant and hypertension. Kato *et al.* (2002) did not observe any association between this polymorphism and stroke.

2.4 Mendelian forms of hypertension

Molecular genetic studies have identified mutations in eight nuclear genes and one mitochondrial gene that cause Mendelian forms of hypertension (Table 2.1). They include *CYP11B1*/*CYP11B2* -genes encoding steroid 11β hydroxylase/aldosterone synthase on chromosome 8p in glucocorticoid remediable aldosteronism - GRA (Lifton *et al.*, 1992),

SCNN1B and *SCNN1G* are genes encoding for β and γ subunits of epithelial Na^+ channel - ENaC respectively found on chromosome 16p in Liddle's syndrome (Shimkets *et al.*, 1994; Hansson *et al.*, 1995; Tamura *et al.*, 1996; Melander *et al.*, 1998), *HSD11B2* gene encoding 11 β-hydroxysteroid dehydrogenase 2 - 11 β HSD2 on chromosome 16q in the syndrome of apparent mineralocorticoid excess - AME (Mune *et al.*, 1995; Stewart *et al.*, 1996), *NR3C2* gene encoding mineralocorticoid receptor - MR on chromosome 4q in pregnancy induced hypertension (Geller *et al.*, 2000). Also mutant genes of the serine/threonine protein kinases, *WNK1* (lysine) protein kinase 1 on chromosome 12p and *WNK4* on chromosome 17q, cause pseudohypoaldosteronism type II also known as familial hyperkalaemia and hypertension - FHH or Gordon's syndrome (Wilson *et al.*, 2001). Extensive studies of FHH by Mayan *et al.* (2004) have found that affected subjects with *WNK4* Q565E mutations have hypercalciuria accompanied by lower serum calcium

levels supporting a mechanism of renal calcium wasting. Together with the observation that *WNK4* regulates the renal outer medullary potassium channel, as well as epithelial chloride/base exchange and the sodium/potassium/chloride co-transporter, an interaction between *WNK4* and a calcium channel transporter was suggested (Mayan *et al.*, 2004). Most of these disorders are due to defective genes acting in the same physiological pathway in the kidney, altering the net renal reabsorption of salt (Lifton *et al.*, 2001).

Table 2.1 Causative mutation for Mendelian forms of hypertension

Monogenic syndrome	Causative gene	Characteristics of mutations	Enzyme function	Mode of Inheritance	Chromosome
GRA	*CYP11B1* and *CYP11B2*	Fusion gene arising from unequal crossover	Increasing	AD	8p
Pseudo-aldosteronism (Liddle's syndrome)	*SCNN1B* and *SCNN1G*	Truncation mutations in C-terminal region and missense mutations	Increasing	AD	16p
FHH (Gordon's syndrome)	*WNK1* and *WNK4*	Deletion and missense mutations	Increasing	AD	12p and 17q
AME	*HSD11B2*	Missense and deletion mutations	Decreasing	AR	16q
Hypertension exacerbated in pregnancy	*NR3C2*	Missense mutation	Increasing	AD	4q
HTNB	Unknown	Unknown	Unknown	AD	12p
Hypertension, hypercholesterolaemia and hypomagnesaemia	*MT-TI*	Missense mutation	Mit. dysfunction	Mit.	Mit.

HSD11B2, gene encoding 11βHSD2; Mit., mitochondrial; *MT-TI*, mitochondrially encoded $tRNA^{Ile}$; *NR3C2*, gene encoding MR; *SCNN1B* and *SCNN1G*, genes encoding β and γ subunits of epithelial sodium channel respectively.
GRA – Glucocorticoid remediable aldosteronism, HTNB – Hypertension and brachydactyly, AME – Syndrome of apparent mineralocoticoid excess, FHH – Familial Hyperkalaemia and Hypertension

(Gong and Hubner, 2006)

Hypertension and dyslipidaemia cluster more often than expected for the risk of many common cardiovascular diseases, i.e. myocardial infarction, congestive heart failure and stroke (Stergiou and Salgami, 2004). A cluster of metabolic defects caused by mutation in a mitochondrial tRNA was identified in one large Caucasian kindred by Wilson *et al* (2004). The kindred featured a cluster of hypertension, hypercholesterolaemia and hypomagnesaemia. Direct sequencing and single-strand conformational polymorphism (SSCP) analysis of the entire mitochondrial genome were performed. A novel mutation conferring a uridine→cytidine transition was identified at nucleotide 4291 of the mitochondrial $tRNA^{Ile}$- *MT-TI* gene. The mutation occurs immediately 5´ to the $tRNA^{Ile}$ anticodon. Uridine at this position is one of the most conserved bases. Biochemical studies with anticodon stem–loop analogues of tRNA have been performed and indicate that substitution of cytidine for uridine at this position markedly impairs ribosome binding (Ashraf *et al.*, 1999). Thus Wilson *et al.* (2004) speculated that complexity can arise from a single mutation because of the combined effects of reduced penetrance and pleiotropy which underlines the value of studying very large kindreds. Another Mendelian form of hypertension, hypertension and brachydactyly - *HTNB* has been mapped to a defined chromosomal region $12p^{12}$ (Schuster *et al.*, 1996), but the molecular basis of the underlying defect is still not clear. This genetic region nearly overlaps with a later whole-genome-scan linkage analysis for essential hypertension in a large Chinese pedigree (Gong *et al.*, 2003), which indicates the susceptibility genes for essential hypertension may reside on chromosome $12p^{12}$.

Even though these rare syndromes with Mendelian inheritance only account for a small fraction of the pathological BP variation in the general population, they provide insight into the pathophysiology of hypertension. The identification of the molecular mechanisms of BP variation also has implications for the development and use of antihypertensive treatments that need not be restricted only to individuals with Mendelian forms of hypertension (Lifton *et al.*, 2001).

2.5 Genes acting on pathways for BP regulation outside the RAAS

An ever-expanding repertoire of genes outside of the RAAS has been tested for involvement in the genetic basis of essential hypertension (Table 2.2). A number of

Table 2.2 Summary of studies of Candidate genes acting on pathways for BP regulation outside the RAAS.

Candidate genes	Molecular basis in EH	Polymorphisms	Association with EH
GRK4	Desensitization of G-protein-coupled receptors, including the D_1 receptor in proximal tubules	R65L and A142V	No
HSD3B1	The biosynthesis of steroid hormones, including aldosterone	A486V	Yes
		Leu^{338} T→C	No
*PTP1*B	Regulating insulin signalling via receptor dephosphorylation		No
	Regulating insulin signalling via receptor dephosphorylation	1484insG	No
SLC9A3	Regulation of sodium reabsorption in the proximal tubule	G1579A, G1709A, G1867A, C1945T, A2041G and C2405T	No
SCNN1B	A key determinant of sodium homoeostasis	βENaC G589S and βENaC i12-17CT	Yes
SCNN1G	A key determinant of sodium homoeostasis	γENaC V546l	No
GREB1	Depressor effect through the improvement of endothelial dysfunction and modulation of sympathetic nerve activation	-13945A→T and 45718A→G	Yes in men
HPCAL1	Protection of neurons against calcium-induced death stimuli in co-operation with neuronal apoptosis inhibitory protein	IMS-JST 126186 A→ C	Yes in women
BDKRB1	Activate the arachidonic acid nitric oxide cascade	SNPs in NT 026437, 76646507, 76623594 and 76647595	Yes in American Caucasians
*BDKRB*2	Activate the arachidonic acid nitric oxide cascade and affect the insulin-dependent glucose transport/utilization	-58C/T	Yes in African Americans
		SNP in NT 026437 76648043	Yes in American Caucasians
CAT	Reduce smooth muscle cell contraction and proliferation induced by endothelia, Ang II and a-adrenoreceptor agonists	-844 A/G and -262 T/C	Yes in Greek Caucasians

AD, autosomal dominant; AR: autosomal recessive; *HSD11B2*, gene encoding 11β HSD2; Mit., mitochondrial; *MT-TI*, mitochondrially encoded $tRNA^{Ile}$; MOI, mode of inheritance; *NR3C2*, gene encoding MR; *SCNN1B* and *SCNN1G*, genes encoding β and γ subunits of ENaC respectively; EH, essential hypertension.

(Gong and Hubner, 2006)

studies have shown a correlation between hyperinsulinaemia, insulin resistance and hypertension (Wang *et al.*, 2004). Speirs *et al.* (2004) tested several novel potential candidates, namely, gene encoding G-protein-coupled-receptor kinase 4 - *GRK4*, gene encoding 3β-hydroxysteroid dehydrogenase/isomerase 1 - *HSD3B1* and gene encoding protein phosphatase 1B - *PTP1B* genes in 168 Caucasian EH patients and 312 normotensive controls. The regulation of sodium excretion by the kidney is of paramount importance for homoeostasis of the extracellular fluid volume and thereby of arterial BP. GRK4 was implicated in human hypertension by desensitization of G-protein-coupled receptors, including the dopamine 1 - D_1 receptor (Jose *et al.*, 1998; Sanada *et al.*, 1999; Jose *et al.*, 2003). In humans with EH, there is a decrease in the responsiveness of the D_1 receptor in proximal tubules due to the uncoupling of the D_1 receptor from its G-protein–effector enzyme complex (Sanada *et al.*, 1999; Jose *et al.*, 2003). 3β - Hydroxysteroid dehydrogenase/isomerase 1 plays a role in the biosynthesis of steroid hormones, including aldosterone (Simard *et al.*, 1996).

It has been proposed that allelic variations in the *HSD3B1* gene could lead to elevated plasma aldosterone, resulting in an increased intravascular volume and hypertension (Azizi *et al.*, 1997). PTP1B negatively regulates insulin signaling via receptor dephosphorylation (Hashimoto and Goldstein, 1992). However, no association between variants in *HSD3B1* and *PTP1B* genes and hypertension could be detected (Speirs *et al.*, 2004). In contrast, the V allele of the A486V variant of *GRK4* showed association with elevated BP (P=0.02 for EH). Zhu *et al.* (2004) studied the relationship between the *SLC9A3* gene coding for sodium/hydrogen exchanger 3 - NHE and essential hypertension in 399 subjects of African or Afro–Caribbean origin (68% with essential hypertension) and 292 subjects Caucasian origin (50% with essential hypertension), trying to examine the relationship with hypertension and biochemical indices of sodium balance. Six variants were identified in total. NHE3 is a member of an increasing number of NHEs responsible for transport of sodium and hydrogen ions across the proximal tubule (Orlowski and Grinstein, 1997). Moreover, animal studies highlight that \this class of genes has potential importance in the control of BP (Schulthies *et al.*, 1998a and 1998b; Aldred *et al.*, 2000) however, no association between the variants was detected in EH patients from either African and Afro–Caribbean origin or Caucasian origin (Zhu *et al.*, 2004). Gain-of-function mutations in the β- and γ-subunits of ENaC cause the monogenic form of hypertension known as Liddle's syndrome (Shimkets *et al.*, 1994; Hansson *et al.*, 1995; Tamura *et al.*, 1996). A recent investigation in a Finnish population (Hannila- Handelberg *et al*, 2005) has shown a higher prevalence of three *ENaC* variants (βENaC G589S, βENaC i12-17CT and γENaC V546I) in 347 hypertensive patients compared with 175 normotensive individuals and 301 randomly chosen blood donors (P<0.01). When frequencies of the individual gene variants in the hypertensive patients were compared with those in the other two groups combined, only the frequency of the βENaC i12-17CT variant was significantly higher among the hypertensive patients than in the other two groups (P=0.001), whereas there was no significant difference in the prevalence of βENaC G589S and γENaC V546I variants

between the hypertensive and control groups. Patients carrying the three variant alleles also had an increased urinary potassium excretion rate in relation to their renin levels (P=0.034). However, no change in activity of the two ENaC amino acid variants was detected when they were expressed in *Xenopus* oocytes compared with wild-type *ENaC* (Hannila- Handelberg *et al.*, 2005).

Chromosome 2p24-p25 has been shown to be linked with hypertension in several studies (Zhu *et al.*, 2001; Angius *et al.*, 2002; Laivuori *et al.*, 2003). A study was thus carried out in a Japanese general population investigating the association of polymorphisms in this region with BP. Forty-seven polymorphisms in 14 genes in the region between D2S2278 and D2S168 and in the region just outside of these two markers (between nucleotides 8845292–11946689) were genotyped in 1880 individuals, 796 of whom were hypertensive and 1084 normotensive (Kamide *et al.*, 2005). Multivariate logistic regression analysis with adjustment for age, BMI, hyperlipidaemia, diabetes mellitus, smoking, drinking and antihypertensive medication identified 11 SNPs in three genes that were associated with hypertension using a dominant or recessive model (P<0.05). From them, only one SNP in the *HPCAL1* gene coding for hippocalcin-like 1 in women (P=0.003) and two SNPs in the *GREB1* gene coding for gene regulated by oestrogen in breast cancer 1 in men (P=0.008) had a significant association with susceptibility to hypertension and BP modulation (Kamide *et al.*, 2005). SBP in women with the AA+AC genotype of the positively associated SNP IMS-JST126186 in the *HPCAL1* gene was 16.7 mmHg higher that that with the CC genotype (P=0.003). HPCAL1 shares 94% amino acid identity with hippocalcin, which functions as a neuronal calcium sensor and possesses a Ca^{2+}/myristoyl switch allowing it to translocate to the membrane (Mercer *et al.*, 2000). The SBP in men with GG+GC genotypes of IMS-JST149391 in the *GREB1* gene was 9.2 mmHg higher than in the men with the CC genotype (P=0.008), and was 9.2 mmHg higher in men with the AA+AG genotype of IMS-JST 149390 in the *GREB1* gene than in those with the GG genotype (P=0.008). The two SNPs in the *GREB1* gene were in tight linkage disequilibrium. GREB1 was identified as a direct target gene of oestrogen receptor α - ERα and is evolutionarily conserved compared with mouse genome (Rae *et al.*, 2005; Lin *et al.*, 2004).

Oestrogen has depression effects through the improvement of endothelial dysfunction (Yen *et al.*, 2004) and modulation of sympathetic nerve activation (Brandin *et al.*, 2004) in animal experiments. Oestrogen insufficiency may be related to postmenopausal hypertension (Dubey *et al.*, 2002). Genetic variation in ERα has been associated with coronary artery wall atherosclerosis and stroke (Shearman *et al.*, 2003; 2005). ERs are required for normal vascular physiology in males (Mendelsohn *et al.*, 2003) and oestrogen has direct vasodilator properties in men, as it does in women, but the relevance of this remains to be understood. Thus the authors concluded that GREB1 might play a role in BP regulation (Kamide *et al.*, 2005).

Genetic heterogeneity may exist in different populations for the genesis of hypertension. One association was assessed between the SNPs in the promoter region of the *CAT* gene (coding for catalase) and EH in Greek Caucasians and

African–Americans. An association was found with the specific genotype combination of CAT-844 homozygous AA together with CAT-262 CT or TT in Caucasians only (100 hypertensive and 93 normotensive subjects; *P*=0.0339), whereas no association was observed in African–Americans -129 hypertensive and 98 normotensive subjects (Zhou *et al.*, 2005). The role of oxidative stress in hypertension has been tested in a number of studies (Trouyz and Schiffin, 2004). Catalase, an enzyme H_2O_2 into water and oxygen, has been shown to reduce smooth muscle cell contraction and proliferation induced by endothelia, Ang II and α-adrenoreceptor agonists (Wassman *et al.*, 2004). Experimental studies have shown a protective role of higher catalase expression levels in hypertensive animal models (Uddin *et al.*, 2003; Yang *et al.*, 2003). Similarly, one SNP in the *BDKRB2* gene coding for bradykinin receptor B_2 and three SNPs in the *BDKRB1* gene coding for bradykinin receptor B_1 were associated with hypertension in American Caucasians (*n*=220; *P* values were between 0.026 and 0.0004).

One SNP in the promoter region of *BDKRB2* gene was associated with hypertension in African–Americans [*n*=218; *P*=0.044] (Cui *et al.*, 2005). Bradykinin has a variety of vasoactive and metabolic effects, including vasodilatation via interaction with components of the arachidonic acid cascade (Merkus *et al.*, 2002). Bradykinin enhances insulin-independent glucose transport through B_1 and B_2 receptors. Genetic variations of these receptors may alter the functional capacity of bradykinin, which may in turn alter an individual susceptibility to hypertension. The results described above suggest that individual SNPs may not be as important as the interaction among several SNPs. The genetic factors that contribute to hypertension are likely to be different among different ethnic populations. Further studies of association in a large number of genes in different pathways will be required to identify the possible interaction among genes and the full array of genetic factors causing hypertension.

Epidemiological studies show that the risk of cardiovascular disease mortality and morbidity is much higher in hypertensive patients compared with normotensive people. Whether the genetic variants causing hypertension have an additive effect on pathways related to the risk for developing end-organ damage has been studied by Fabris *et al.* (2005) who examined variants from AGT M235T, *ACE* ID, *AT_1R* A1166C and *CYP11B2* -344C/T in 86 hypertension patients with renal insufficiency and 172 hypertensive patients without renal damage matched for age and hypertension. The cohort was followed for 2 years and investigated whether these variants may act synergistically to confer an increased risk for renal failure in hypertensive patients. *AGT* TT/*AT_1R* AC (*P*=0.0018) and *CYP11B2* CC/*ACE* DD (*P*=0.0012) showed a positive interaction in the development of renal insufficiency among hypertensive patients, and the association of *AGT* MM/*AT_1R* AA (*P*=0.04) and *AGT* MM/*AT_1R* AA/*CYP11B2* TT (*P*=0.04) or *AGT* MM/*AT_1R* AA/*CYP11B2* TC (*P*=0.03) combinations were associated with a reduced risk of renal failure.

Fabris *et al.* (2005) then concluded that, in patients with essential hypertension, an unfavourable allelic pattern of components of the RAAS may contribute to the increased risk for the development of renal failure.

Lipocalin-type prostaglandin D synthase - L-PGDS is a secretory protein of the lipocalin superfamily, which synthesizes prostaglandin D_2 - PGD_2 from prostaglandin H_2 - PGH_2 (Urade *et al.*, 1985). Patients with hypertension exhibited a higher level of L-PGDS in serum and urine (Hirawa *et al.*, 2002). A group of Japanese researchers investigated the association between its variants and the severity of carotid arteriosclerosis in 782 EH patients. The study by Hirawa *et al.* (2002) suggested that the 4111A→C polymorphism in the *PTGDS* gene (coding for L-PGDS) is associated with the severity of carotid arteriosclerosis (P=0.002) and inversely correlated with increased high-density lipoprotein - HDL cholesterol (P<0.001) in Japanese hypertensive patients. However, the other variants had no relationship with the phenotype studied. The functional mechanism of the 4111A/C in L-PGDS remains unknown (Miwa *et al.*, 2004).

A small cross-sectional study in 140 normotensive subjects was carried out to ascertain the relationship between the polymorphism of the *GSTM1* gene coding for glutathione S-transferase – GST M1, BP level and exposure to cigarette smoking. For analysis, the combination of genotypes, sex and smoking behaviour were used as qualitative variables, and age, BMI and heart rate were used as covariates. The combination 'present-GSTT1 (GST theta 1), null-GSTM1' genotypes odds ratio - OR, 0.001; 95% CI, 0.00–0.439; P=0.025], heart rate (OR, 1.065; 95% CI, 1.018–1.114; P=0.006) and interaction between BMI and combination of 'present-GSTT1, null-GSTM1' genotypes (OR, 1.319; 95% CI, 1.058–1.644; P=0.014) was detected to be associated with SBP. The results suggested that the *GSTM1* gene is one of the candidate genes altering baseline SBP in normotensive individuals when the age, sex and smoking behaviour were considered (Saadat and Dadbine- Pour, 2005).

The GSTs are involved in the detoxification of many toxic compounds of different chemical structures in cigarette smoke (Ketterer *et al.*, 1992), whereas cigarette smoking is one of the major risk factors to cardiovascular diseases (Kannel *et al.*, 1990).

2.6 Mitochondrial genome mutations and Essential Hypertension

DeStefano *et al.* (2001) studied maternal and paternal effects in the development of human essential hypertension in American Caucasians, Greek Caucasians and African–Americans. They observed that, among parents with known hypertensive status, the proportion of affected mothers was significantly higher than the proportion of affected fathers in all three ethnic groups (DeStefano *et al.*, 2001). The fraction of patients with EH potentially due to mtDNA (mitochondrial DNA) mutation involvement was estimated at 55% [95% CI, 45–65%] (Sun *et al.*, 2003). A complete sequencing of the mitochondrial genome from 20 hypertensive probands in African–American (n=10) and Caucasian families (n=10) was carried out. A total of 297 bp exchanges were identified, including 24 in rRNA genes, 15 in tRNA genes and 46 amino acid substitutions, with the remainder involving the non-coding regions (Schwartz *et al.*, 2004). Several of these have been associated with cardiovascular and renal pathologies in previous studies (Watson *et al.*, 2001; Khogali *et al.*, 2001). Among them, an A10398G mutation in the *MT-ND3* gene (coding for mitochondrially encoded

NADH dehydrogenase 3), identified in 12 hypertensive individuals of both ethnic groups, had been shown to occur with increased frequency in African–Americans with EH associated with end-stage renal disease (Watson *et al.*, 2001). UCPs (uncoupling proteins) are inner mitochondrial-membrane-associated proteins and act as proton channels or transporters. Although mitochondria uses energy derived from fuel combustion to create a proton electrochemical gradient across the mitochondrial inner membrane, UCPs uncouple proton entry in the mitochondrial matrix from ATP synthesis (Boss *et al.*, 2000).

The functional polymorphism (-866 G/A) in the UCP2 promoter has been reported to be associated with obesity in an analysis of 340 obese and 256 never-obese middle-aged Caucasian subjects (P=0.007) (Esterbauer *et al.*, 2001). Another association study between this polymorphism, obesity, and hypertension as well as Type II diabetes mellitus was carried out in a Japanese population with 342 Type II diabetic patients (among them 158 patients complicated with hypertension), 156 hypertensive patients without diabetes mellitus and 134 control subjects. The polymorphism was detected to be significantly associated with hypertension (frequency of A allele, 51.8% in hypertensives compared with 46.6% in normotensives; P<0.05), but was not associated with obesity in the Japanese population, which is in contrast with the significant association with obesity in Caucasians (Ji *et al.*, 2004).

Mitochondrial coupling factor 6, an essential component of mitochondrial ATP synthase, suppresses the synthesis of prostacyclin in vascular endothelial cells (Knowles *et al.*, 1971). The role of the gene was studied in spontaneous hypertensive rats - SHRs (Osanai *et al.*, 2001). *In vivo*, the peptide circulates in the rat vascular system, and its gene expression and plasma concentration are higher in SHRs than in normotensive controls. Functional analysis suggests it acts as a potent endogenous vasoconstrictor in the fashion of a circulating hormone c). Circulating coupling factor 6 is elevated in human hypertensive patients (n=30) compared with normotensive subjects (n=27; P<0.01) and was increased after salt loading in hypertensive patients. The percentage changes in plasma coupling factor 6 level after salt restriction and loading were positively correlated with those in mean BP (r=0.57; P<0.01) and negatively correlated with those in plasma nitric oxide level [r=-0.51; P<0.05] (Osanai *et al.*, 2001; 2003).

The elevated circulating coupling factor 6 in SHRs and human hypertension patients indicates that it is involved in the regulation of arterial BP in physiological and pathological conditions (Osanai *et al.*, 2001; 2003). All of the above studies suggest that essential hypertension may not be only polygenic, but may also a ‘polygenomic’ disorder.

2.7 Genome-wide linkage analysis

Genome-wide linkage analysis predicts that multiple chromosomal regions may play a role in the development of human EH; however, lack of consistency across studies makes it difficult to draw any general conclusion for the genetic cause of human EH (Gong *et al.*, 2003; Angius *et al.*, 2002; Harrap *et al.*, 2003). An investigation focusing on only SBP and DBP (diastolic BP) in 1109 white female dizygotic twin pairs has been carried out (de Lange *et al.*, 2004). No

significant linkage with BP could be detected in this study, but several suggestive linkage regions were replicated and one novel suggestive linkage region for SBP on chromosome 11p was detected (de Lange *et al.*, 2004). Significant linkage for longitudinal SBP from the Framingham Heart study was detected on chromosome 1q. In this study (James *et al.*, 2003), the SBP for each individual was modelled as a function of age using a mixed modelling methodology; it was thus the best linear unbiased predictor of the individual's deviation from the population rate of change in SBP for each year of age whilst controlling for gender, BMI and hypertension treatment. Two previous linkage studies of hypertension had found peak LOD (logarithm of the odds) scores in the same region (Hunt *et al.*, 2002). Similarly, linkage on chromosomes 12q, 15q and 17q for mean SBP and linkage for both SBP slope and curvature on chromosome 20q were detected in the other study of the data from the Framingham Heart study (Jacobs *et al.*, 2003).

The linkage analysis for age at diagnosis of hypertension and early-onset hypertension in the HyperGEN (Hypertension Genetic Epidemiology Network) cohort of different populations was carried out (Wilk *et al.*, 2004). Several suggestive linkage loci were detected and some of them have been reported to be linked to hypertension and BP in previous studies. These encouraging results suggest that linkage can be replicated from other studies and, therefore, new genetic factors with moderate-to-large effects can potentially be discovered. Considering the power of the individual studies, two genome-scan meta-analysis for hypertension were carried out individually (Liu *et al.*, 2004; Koivukoski *et al.*, 2004). Interestingly, the previous meta-analysis with different populations (Kristjansson *et al.*, 2002; Perola *et al.*, 2000; Ranade *et al.*, 2003) failed to detect significant linkage to hypertension, only several regions with suggestive linkage were identified, including chromosomes 2p, 5q, 6q, 8p, 9p, 9q and 11q. From them, only regions on chromosomes 5q, 6q and 11q had $P<0.05$ (Liu *et al.*, 2004). Controversially, meta-analysis of genome-wide scans for hypertension and blood pressure in Caucasians (Hunt *et al.*, 2002; Kardia *et al.*, 2003; Von Wowern *et al.*, 2003) had significant linkage on chromosomes 2p12-q22 and 3p14-q12(Koivukoski *et al.*, 2004). The results strongly suggest a population difference in the common phenotype. The mixed populations probably have a considerable degree of genetic heterogeneity, which is one of the main reasons why pooling of the results in different populations in the meta-analysis did not enhance the signals. However, pooling of the results in Caucasians possesses a smaller degree of genetic heterogeneity.

One admixture mapping for hypertension loci with genome-scan markers was carried out in African–Americans (Zhu *et al.*, 2005) using individuals from Nigeria as African ancestral population and European–Americans for the estimates of allele frequencies for European ancestors. The distribution of marker-location-specific for African ancestry was shifted upwards in hypertensive cases compared with normotensive controls, and the markers were located on chromosome 6q24 and 21q21.

CHAPTER THREE
MATERIALS AND METHODS

3.1 Study

A population based association – case control – study was used to determine the frequency of M235T allele, ACE I/D allele, AT1R allele, ANP gene and its relationship with the hypertension status in two populations in Akwa Ibom and Cross River States. The study also measured angiotensinogen levels in the plasma using the enzyme linked immunosorbent assay to relate it to the M235T allele and hypertension.

3.2 Study Area

Cross River and Akwa Ibom states are sister states, both are part of the old Calabar kingdom Fig 3.1. Cross River State is a coastal state in South-South Nigeria, named after Cross River which passes through the state. It is located in the Niger Delta. Calabar is the state capital Fig 3.2. The state occupies 20,156sq kilometers. It shares boundaries with Benue state to the north, Enugu and Abia states to the west, to the east by Cameroon republic and to the south by Akwa Ibom and the Atlantic Ocean. The three major languages are Efik, Ejagham, and Bekwara. These different groups though distinct bear striking similarities in their cultures. Official population figures stand at 2,888,966 (National Population Census, 2006).

Akwa Ibom State is also located in the coastal South-South of the country, lying between latitudes $4^{o}321$ and $5^{o}331$ north, and longitudes $7^{o}251$ and $8^{o}251$ east. Uyo is the state capital Fig 3.3. The state is bordered on the east by Cross River state, on the west by Rivers and Abia states, on the south by the Atlantic Ocean. Akwa Ibom has a population of 3,920,208 million according to the 2006 population census estimates. Along with English, the main languages here are Ibibio, Annang, Oron, Ibeno and Eket.

3.3 Subject and Enrolment

A total sample population of 1,224 adult men (497) and women (727) from different ethnic groups were included in this study. The participants were grouped based on their ethnicity, but not on the basis of location. Of this number, 612 were patients attending the hypertension clinics in the University of Calabar Teaching Hospital, Calabar, the University of Uyo Teaching Hospital, Uyo and the General Hospital, Calabar. The other 612 were individuals whose blood pressure was below 140/90mmHg, who were not taking hypertensive drugs and not below the age of twenty from the same population. These individuals served as the control group.

Inclusion criteria: All patients were individuals whose BP were consistently above 140/90 mmHg or were taking hypertensive medications. Controls were individuals whose BP were consistently below 140/90 mmHg and were not taking hypertensive medications.

Exclusion criteria: females in the population using oral contraceptives were excluded from the study population.

3.4 Collection of samples

Venous blood (3ml) was collected from each participant after they had given informed consent, into bottles containing anticoagulant EDTA Some participants refused blood collection from the upper arm; blood was obtained from thumb pricks and blotted

onto a chromatography (Whatman, no 3) paper, allowed to dry at room temperature and preserved in plastic bags prior to DNA extraction.. Plasma was obtained by centrifugation of blood samples. Blood and plasma was kept frozen in the freezer/cold room and transported by air to the Department of Zoology University of Ibadan (in coolers with ice packs) where samples were kept at -70^{o}. Samples were then transferred on wet ice to freezers in the International Institute of Tropical Agriculture, Ibadan for subsequent lab investigations. DNA was extracted from blood for genotyping of the polymorphism.

3.5 Ethical Approval

Subjects included in the study gave informed consent and ethical approval for the study was obtained from the joint UI/UCH ethical review committee and each of the health establishment concerned- University Teaching Hospital Calabar, University of Uyo Teaching Hospital and the General Hospital, Calabar.

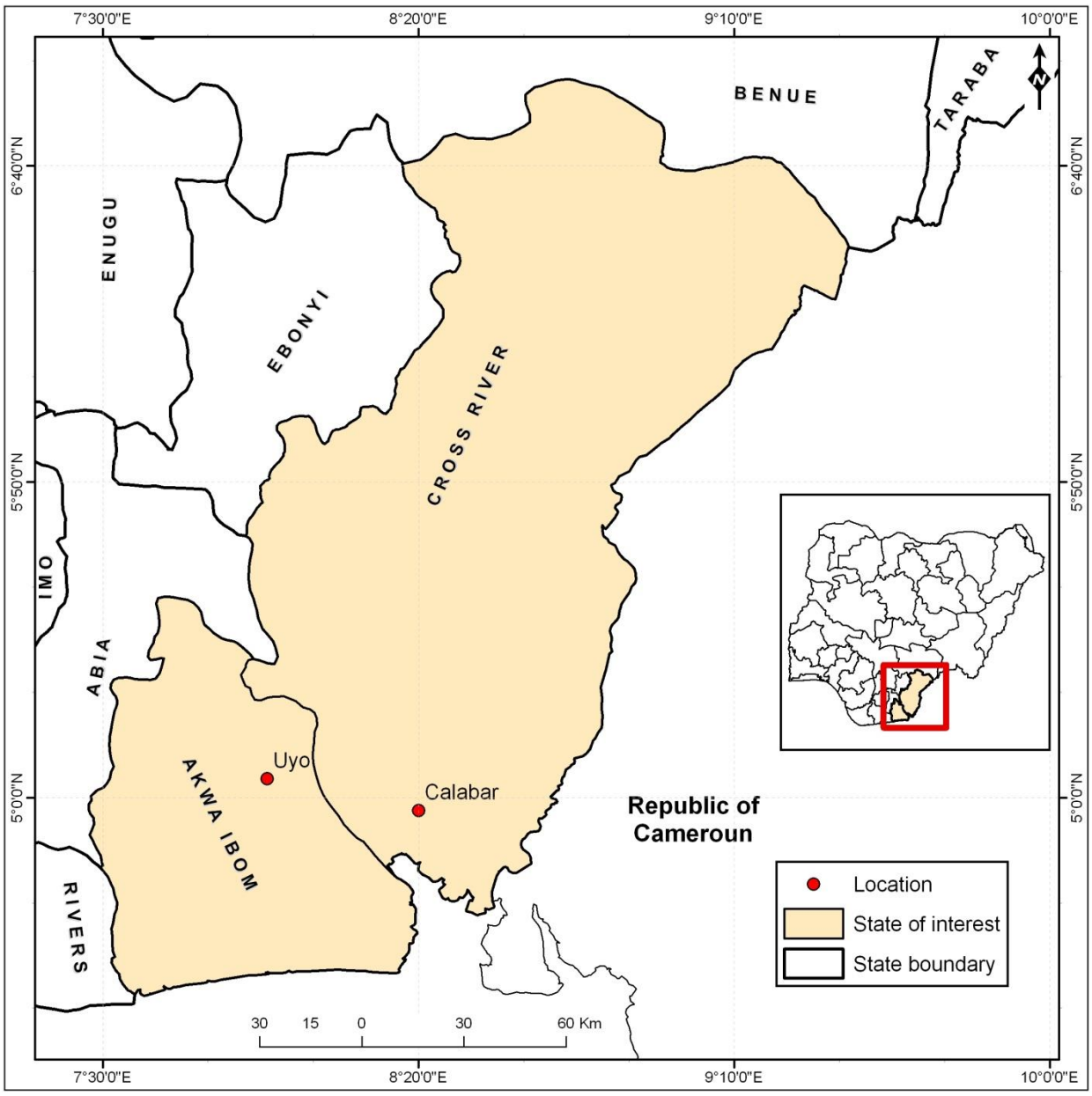


Fig 3.1 Map showing towns where samples were collected in south- south Nigeria

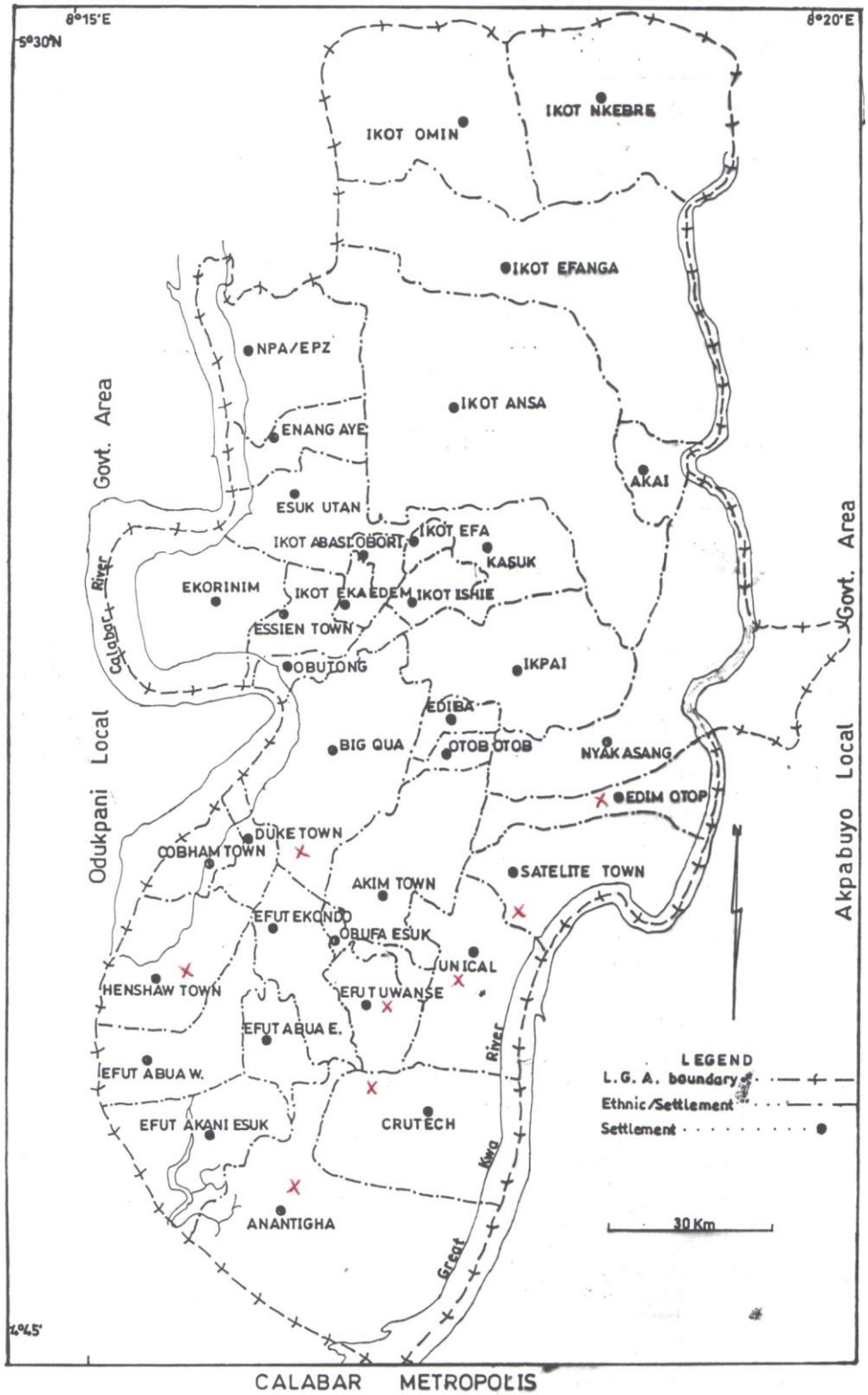
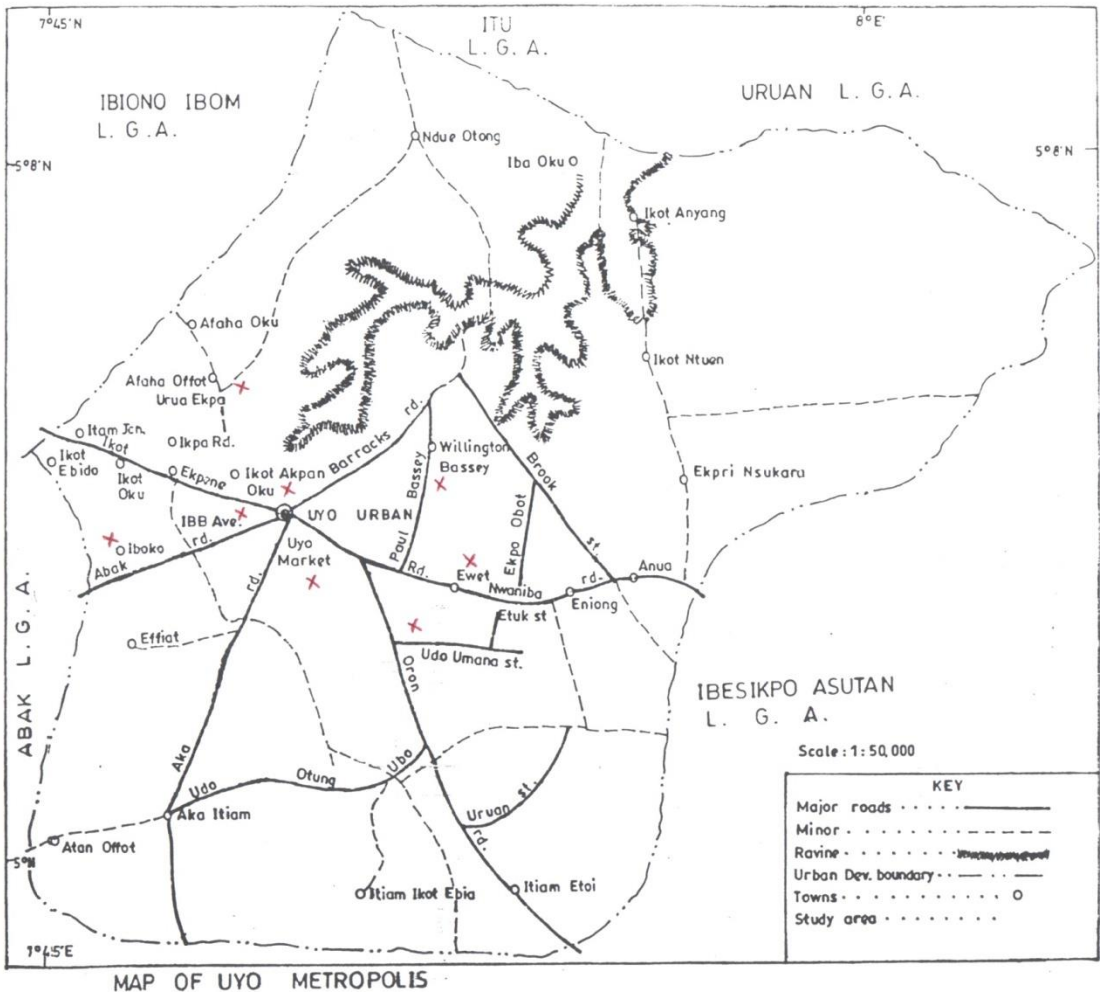


Fig 3.2 Map showing sampling sites in Calabar city



MAP OF UYO METROPOLIS

Fig 3.3 Map showing sampling sites in Uyo city.

3.6 Questionnaire

Questionnaires were developed in English to assist in acquiring information on family history for hypertension and socio-demographic data, age, sex, dietary habits, physical activity, smoking habits and alcohol consumption (which could predispose an individual to hypertension). Informed consent was translated into Efik and Ibibio dialects to assist in explaining the research to the participants.

3.7 Height Measurement

The wall in the collection centre was calibrated in meters. Individuals stood without foot or head wear facing the investigator, looking straight ahead and the investigator placed a ruler on top of the head of the individual and the reading in meters was recorded.

3.8 Weight Measurement

Using the conventional weight scale, weight was measured in kilograms.

3.9 Blood Pressure (BP) Measurement

Readings was taken using a sphygmomanometer in millimeters of mercury by certified medical personnel for the patients in the clinics and a certified nurse for the controls in the general population. Systolic and diastolic BP values were recorded. Before taking the measurement, the respondent was advised to sit quietly for 5 mins, with the legs uncrossed and the right hand free from clothing. The right hand was placed on the table with the palm facing upwards. The appropriate cuff size was selected and the cuff wrapped and fastened securely. The cuff was kept at the same level as the heart during measurement. The upper reading, the systolic blood pressure (SBP) and lower reading the diastolic blood pressure (DBP) recorded, the first and second reading were taken twice and the average of the two used for the analysis.

3.10 DNA Extraction

DNA was extracted according to Dellaporta, (1983) with some modifications. Packed red blood cell after plasma had been removed (150µl) was put into 1.5ml eppendorf tube placed on a rack. Extraction buffer (350µl) was then added to the tube. Sodium dodecyl sulphate -SDS (40µl of the 20% solution) was added and the tubes were inverted 3-4 times before they were incubated in a water bath at 65^{o}C for 10 mins. The tubes were brought out of the incubator and left on the bench to cool to room temperature. Potassium acetate (160µl of the 5M solution) was added to the tubes. The tubes were inverted 2-3 times and centrifuged at 10,000rcf for 10 mins. About 400µl of the supernatant was carefully transferred into new eppendorf tubes and 200µl of cold isopropanol was then added. The tubes were inverted gently 5-6 times to precipitate DNA. The tubes were kept on ice or stored in the fridge at 4^{o}C for 15-20 mins.

The tubes were then centrifuged at 10,000 rcf for 10 mins to sediment the DNA. The supernatant was decanted gently in order to ensure the pellet was not disturbed, 500µl of cold ethanol was added to the pellet to wash the DNA, then centrifuged at 10,000rcf for 5-10 mins. The ethanol was decanted and the DNA was air-dried at room temperature until no trace of alcohol was seen in the tubes.

Modifications to the original plant DNA extraction method originally developed by Dellaporta, (1983) are: 500µl of extraction buffer was added to 50mg of ground leaves of a plant; Plant samples are usually vortexed before incubation in the water bath and after the addition of 5M potassium acetate and 33µl of sodium dodecyl sulphate was added to the mixture of the ground leaf and extraction buffer.

The DNA was re – suspended in 50µl of Tris-EDTA – T.E buffer and stored in the freezer as stock solution. An aliquot of the DNA stock solution was run on agarose gel electrophoresis to check the quantity of DNA. Each sample of genomic DNA (5µl) was mixed with 2ul of loading dye and transferred into the wells of the gel. A voltage of 110V was applied for about 45 mins. The samples were scored faint, +, 2+, 3+ depending on the intensity of the bands to indicate the amount of DNA extracted. Plate 3.2

3.11 Filter paper extraction of DNA

This was done according to Bereczky *et al.* (2005). Pieces (1-2) of the filter paper about 5mm in diameter were cut using a sterile blade for each samples. These pieces were placed in an eppendorf tube, soaked in 65µl of T.E buffer. The tube was incubated at 50^{o}C for 15 mins in a water bath. The pieces were pressed gently at the bottom of the tube several times using a new pipette tip for each sample.

The eppendorf tubes were heated again for 15mins at 97^{o}C to elute the DNA. The liquid condensing on the lid and the walls of the tube were removed by centrifuging for 2-3 secs.The DNA extract that is the supernatant was kept at -20^{o}C before use.

3.12 Preparation of agarose gel

The ends of the plastic tray supplied with the electrophoresis apparatus was sealed with adhesive tape. 100ml of 1× TAE was measured and put into a bottle; 1.5g of agarose powder was added and heated in the microwave oven for 5 min to dissolve the agarose. The bottle was then allowed to cool to about 60^{o}C; 5µl of ethidium bromide was added. When checking for DNA quality, 0.8g of agarose and 0.8ul of ethidium bromide was used. The agarose was poured into the tray and allowed to set at room temperature (30 – 45 mins). The comb and tape were removed and the gel was mounted in the electrophoresis tank.1× TAE buffer was added enough to cover the gel.

The samples of DNA were mixed with loading dye and slowly loaded into the wells of the submerged gel. For PCR, the buffer already contained the dye. The amplicons were loaded straight into the wells. A voltage of 110V was applied for 30 – 45 mins until the bromophenol blue had migrated an appropriate distance from the gel. The current was turned off and the gel was examined under ultraviolet light.

3.13 Polymarase Chain Reaction - PCR:

Stock DNA samples were diluted 1/10, 1/50, 1/100 depending on the amount of DNA in the sample checked after extraction. A 1/10 dilution was done for all filter paper extracted DNA stock solution. Samples that were not amplified in the gel at the first PCR were repeated.

M235T allele of the angiotensinogen gene

Genomic DNA (2µl) was amplified in a 15µl reaction mix containing Go Taq® green master mix (Promega) 7.5µl, upstream and downstream oligonucleotide primers 0.30µl each and 4.9µl of nuclease-free water.

Cycling conditions: Below is the primer sequence used for these experiments, an initial denaturation for 10 mins at 95^{o}C was followed by 35 cycles of 1 mins at 94^{o}C, 1 mins at 56^{o} C and 1 min 30 secs at 72^{o} C and a final elongation of 10 mins at 72^{o}C. Primer sequence (Procopciuc *et al*, 2002)

5' CAGGGTGCTGTCCACACTGGACCCC 3'
3' CCGTTTGTGCAGGGCCTGGCTCTCT 5'

3.14 Enzymatic digestion

The specific mismatch incorporated into the antisense primer creates a Tth 111l site if the T235 variant is present fig 2.2. The presence of a cytosine at position 704; GACN↓NNGTC, the enzyme cuts to give a 141 and a 24 bp fragment. For the 235M variant, presence of thymine at position 704; GATNNNGTC, the enzyme does not cut the fragment leaving a 165 bp fragment with no restriction fragments (Basak *et al*, 2008). A cocktail of 0.25ul of the Tth111I enzyme, 1.5ul of the 10 x buffers and 3ul of sterile water was added to 10ul of the PCR product. The enzyme digestion was performed in a final volume of 14.75ul at 65^{o}C for 2 hours. The digested products were separated on 3% agarose gel stained with 1.2ul of ethidium bromide for 30 mins at 125V.

ENZYMATIC DIGESTION
M235T
25-Mer MBI sense: (5'-CCG TT GTG CAG GGC CTG GCT CTC T-3') and
25-Mer MBI antisense: (5'-CAG GGT GCT GTC CAC ACT GGA CCC C-3').
T GGC ACC CTG GCC TCT CTC TAT CTG GGA GCC TTG GAC CAC ACA GCT GAC AGG CTA CAG GCA ATC CTG
GGT GTT CCT TGG AAG GAC AAG AAC TGC ACC TC CGG CTG GAT GCG CAC AAG GTC CTG TCT GCC CTG
CAG GCT GTA CAG GGC CTG CTA GTG GCC CAG GGC AGG GCT GAT AGC CAG GCC CAG CTG CTG CTG T***CC***↓
AC(/T)G GTG GTG GGC GTG TTC ACA GCC CCA GGC CTG CAC CTG AAG CAG CCG TT GTG CAG GGC CTG GCT
CTC TAT ACC CCT GTG GTC CTC CCA CGC TCT CTG GAC TTC ACA GAA CTG GAT GTT GCT GCT GAG A*AG ATT*
*GAC AGG TTC ATG CAG GCT G*TG ACA GGA TGG AAG ACT GGC TGC TC CT***G ATG↓GG***(AG)→***(GT)C***C AGT GTG
GAC AGC ACC CTG

Fig 3.2 The sequencing of the region which contains T174M and M235T polymorphisms.
(Basak *et al., 2*008). The italic letters mark the sequence corresponding to the primers used for T174M, the underlined letters- for M235T primers, and the bold letters represent the restrictions sites.

This PCR cocktail was used for the ACE I/D allele. Genomic DNA (2µl) was amplified in a 12.5µl reaction mix containing Promega flexi green buffer 2.5µl, dNTPs 0.25µl, upstream and downstream oligonucleotide primers 0.25µl each, magnesium chloride 0.75µl, 6.44µl of nuclease-free water and Taq DNA polymerase 0.06µl.

Cycling conditions: An initial denaturation for 5 mins at 94^{o}C was followed by 30 cycles of 45 secs at 94^{o}C, 45 secs at 56^{o} C and 45 secs at 72^{o}C and a final elongation of 10 mins at 72^{o}C.

(I/D) POLYMORPHISM OF THE ACE GENE: Primer sequence
Sense 5'- CTG GAG AGC CAC TCC CAT CCT TTC T -3'
Antisense 3'- GAC GTC GCC ATC ACA TTC GTC AGA T -5'

For the angiotensin 11 type 1 receptor gene and atrial natruiretic peptide gene that require RFLP digestion to identify alleles, Genomic DNA (4µl) was amplified in a 25µl PCR reaction mix containing Promega flexi green buffer 5µl, dNTPs 0.5µl, upstream and downstream oligonucleotide primers 0.5µl each, magnesium chloride 1.5µl, 12.88µl of nuclease-free water and Taq DNA polymerase 0.06µl.

AT_1R gene: Cycling conditions include an initial denaturation at 94^oC for 2 mins, followed by 40 cycles of a further denaturation at 94^oC for 1 min, annealing at 60^oC for 1 min, extension 72^oC for 2mins, and a final extension of 72^oC for 10 mins.
A1166C Polymorphism of the Angiotensin 11 Type 1 Receptor primer sequence
5' - AAT GCT TGT AGC CAA AGT CAC CT- 3'
5' - GGC TTT GCT TTG TCT TGT TG -3'

A cocktail of 0.25µl of the Dde1 enzyme, 1µl of the 10 x buffer D; 0.1µl of acetyl BSA and 8.5µl of sterile water was added to 10µl of the PCR product. The enzyme digestion was performed in a final volume of 19.85µl at 37^oC for 4 hours. For the Rsa1 enzyme, the same concentration was used in preparing the cocktail but Rsa1 enzyme and 10x buffer E were substituted. The digested products were separated on 2% agarose gel stained with 10µl of ethidium bromide for 30 mins at 125V.

ANP gene: Cycling conditions include an initial denaturation at 95^oC for 3 mins, followed by 35 cycles of a further denaturation at 94^oC for 20 secs, annealing at 60^oC for 30 secs, extension 72^oC for 30 secs, and a final extension at 72^oC for 5 mins.
$C^{664}G$ Polymorphism of the Atrial Natriuretic Peptide Gene primer sequence
5' – AAC AGC AAC GGA AGA AAT GA -3'
5' – ATC CAA CCC CCA AAT AGA AGT A-3'

3.15 Protein A sandwich Enzyme Linked Immunosorbent Assay (ELISA) for the detection of plasma angiotensinogen.

Principle: The protein A sandwich ELISA procedure uses protein A to increase the sensitivity and specificity of the test by controlling the orientation of antibodies. The plate surface is coated with protein A followed by the trapping antibodies (polyclonal antibodies to angiotensinogen). Application of protein A increases the proportion of appropriately aligned antibody molecules. The plasma samples are then added, followed by the secondary antibody that is identical to the primary antibody (also polyclonal antibodies to angiotensinogen). The detecting agent is protein A conjugated to a marker enzyme (alkaline phosphatase). The protein A will only bind to the secondary antibody if the antibody is in the correct orientation. The substrate is added. Subjective analysis of ELISA results is achieved by quantifying the amount of light absorbed by the substrate. For p-nitrophenyl phosphate substrate, the substrate is exposed to light with a wavelength of 405nm and the absorbance is determined by measuring the amount of light that passes through the substrate. This is quantified as the absorbance value (O.D.)

Method: Protein A was diluted 1 in 1000µl of coating buffer, 100µl of diluted protein A solution was added to each well of the ELISA plate. The plate was covered and incubated for 2 hours at 37ºC. The plate was washed vigorously with wash fluid PBS-Tween using a wash bottle. Each well was filled with PBS- Tween and left for three mins; the fluid was removed from the wells by snapping. The wash step was repeated three times and the plate was tapped dry on absorbent paper.

Polyclonal angiotensinogen antibodies were diluted 1 in 1000µl of PBS-Tween, 100µl of diluted polyclonal anti AGT was added to each well. The plate was covered and incubated for 2 hours at 37ºC. The plate was removed and the wash procedure was repeated. Plasma samples were diluted 1 in 10µl in distilled water,

100µl of diluted sample was added to each well, and the plate was covered and kept in the fridge overnight. The plate was removed from the fridge and the wash procedure was repeated. Blank wells contained distilled water.

Polyclonal angiotensinogen antibodies were diluted 1 in 1000µl of PBS-Tween, 100µl of diluted polyclonal anti AGT was again added to each well. The plate was covered and incubated for 2 hours at 37ºC (the antigen will be sandwiched between the antibodies). The plate was removed and the wash procedure was repeated.

Protein A alkaline phosphatase conjugate was diluted 1 in 15000µl of conjugate buffer, 100µl of diluted conjugate was added to the wells of the ELISA plate. The plate was covered and incubated for 2 hours at 37ºC. The wash procedure was repeated.

p-Nitrophenyl phosphate substrate was diluted 0.01g in 10000µl of substrate buffer; 100µl of diluted substrate was added to each well. The plate was covered and incubated in the dark for 1 hour at room temperature. The bottom of the plate was wiped with absorbent paper and inserted into the microplate reader. The absorbance values were measured at 405nm and recorded after 1hour, 3 hours and overnight.

3.16 Data Management.

The Statistical Package for Social Sciences - SPSS for windows® Version 16.0 was used to statistically analyze the data obtained. Descriptive statistics was used to analyze all variables studied which include marital status, occupation, snack consumption, smoking practices, alcohol consumption and the genotypic frequencies of the four polymorphisms in the study population. Genotypic frequencies in control and hypertensive groups were compared by chi-square analysis. Continuous variables were compared between hypertensives and controls by independent t test.

The effect of AGT genotype on BP was analyzed using general linear model. Influence of the gene variants on continuous variable was analyzed using one way ANOVA. Multiple regression analysis was also carried out using SBP or DBP as dependent variable, then sex, age, BMI and other variables were used as independent variables. P >0.05 was considered statistically significant. The linkage tables were plotted using Haploview version 4.

CHAPTER FOUR
RESULTS

A sample population of 1224 individuals was genotyped to determine the frequencies of the M235T variant of the angiotensinogen gene, I/D allele of the ACE gene, $A^{1166}C$ of the AT1R gene and the C664G allele of the ANP gene and associate these alleles with hypertension status. Polymerase chain reaction and enzymatic digestion was performed on the 612 control and 612 patient samples collected from Uyo and Calabar to determine the frequency of the gene variants and its relationship with hypertension status. Protein A sandwich ELISA was also used to determine the concentration of angiotensinogen in the plasma samples of 300 patients and 300 controls in relation to the M235T allele of the AGT gene.

4.1 Agarose gel electrophoresis results

After DNA extraction, the amount of DNA in the sample were quantified by checking the intensity of the bands on agarose gel and scored as faint, +, 2+ and 3+. Serial dilutions were done accordingly as 1 in 10, 1 in 50 and 1 in 100 dilution of T.E buffer for PCR. Plate 4.1

Angiotensinogen gene polymorphism: The normal individual M235M gives an undigested 165bp, a mutant individual M235T gives two fragments of 141bp and 24bp. Recessive individual T235T gives a 141bp fragment. However agarose gel allows the visualization of a 165bp fragment for M235M, a 141bp fragment for T235T, a 165bp and 141bp for the M235T individuals respectively.
(Plate 4.2 and 4.3).

Angiotensin converting enzyme gene polymorphism: Agarose gel allows the visualization of a 490bp band for a homozygous individual with the insertion (I) allele and a 190bp band for a homozygous individual with the deletion (D) allele. The heterozygous individual was identified by the presence of the 190bp and the 490bp PCR products (Plate 4.4).

Angiotensin II type 1 receptor gene polymorphism: The Dde1 enzyme cuts the PCR product into two pieces, 600bp and 250bp in the A variant. An additional Dde1 recognition site is created in the C variant at nucleotide 1166 located within the 250bp fragment. The homozygous CC individual produces three bands (600bp, 140bp and 110bp long). The homozygous AA individual produces two bands (600bp and 250bp long). The heterozygous individual produces four bands 600bp, 250bp, 140bp and 110bp long (Lapierre *et al.*, 2006).The homozygous CC individual was not observed (Plate 4.5 and 4.6).

Atrial natriuretic peptide gene polymorphism: The Rsa1 enzyme cuts the PCR product into two pieces (134bp and 23bp). The common allele individual gives an undigested 157bp; the minor allele carrier gives two fragments of 134bp and157bp. Minor allele individual gives a 134bp fragment. However agarose gel does not allow the visualization of the 23bp fragment for a minor allele individual, (Kato *et al*, 2000). However, in the study population, only individuals with the 157bp product for the common allele were observed (Plate 4.7 and 4.8).

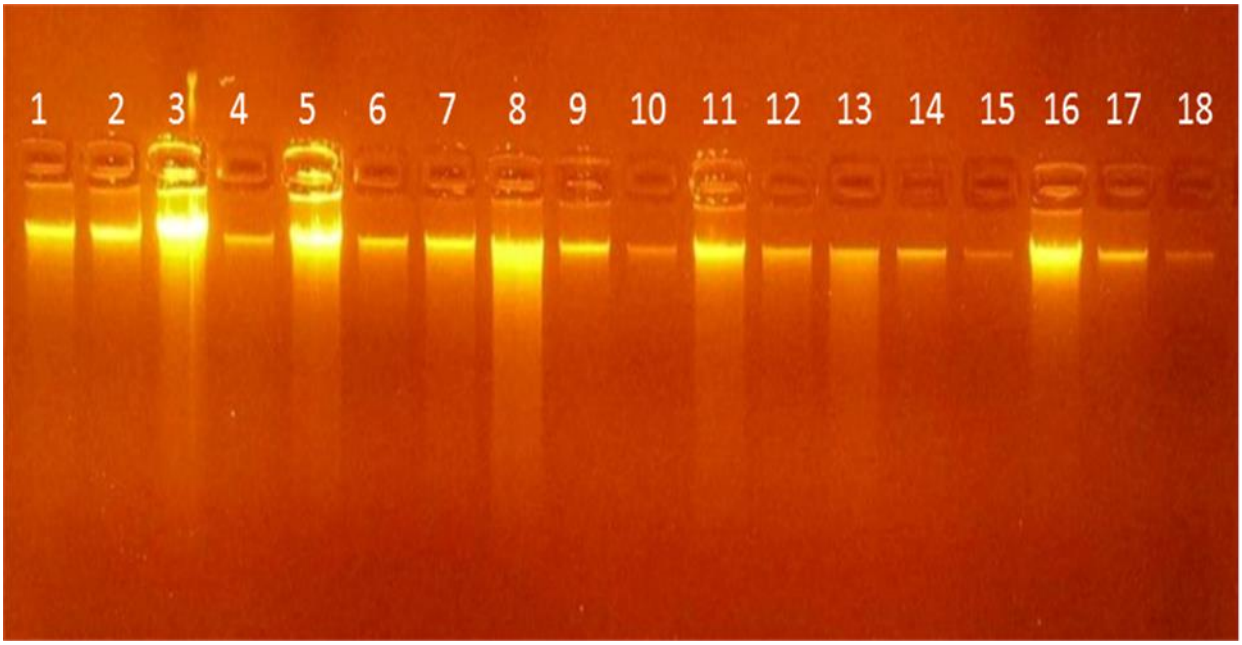


Plate 4.1 Gel electrophoresis showing DNA quality

Legend: The amount of DNA was scored in
Lanes 3,5,8,11 and 16 as 3+
Lanes 1,2,7 and 13 as 2+
Lanes 4,6,9,12,14 and 17 as +
Lanes 10,15 and 18 as faint

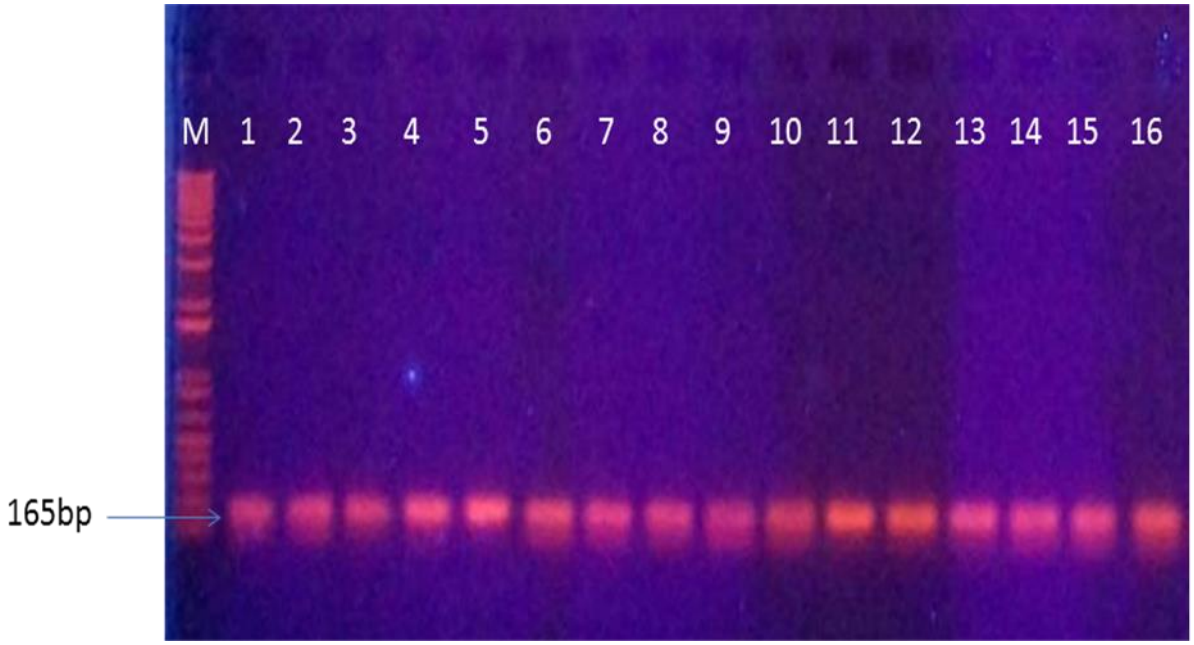


Plate 4.2 Gel electrophoresis showing 165 bp PCR product after amplification of the angiotensinogen gene

Legend:
Lane M is the 1kb plus DNA ladder
Lanes 1-16 contain the amplified PCR product

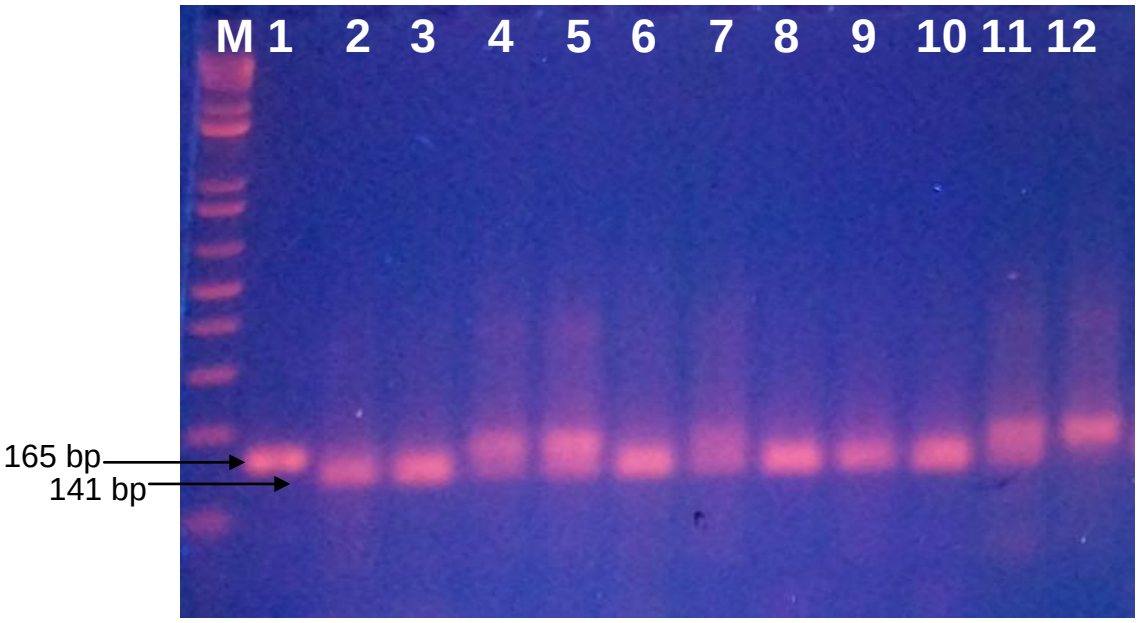


Plate 4.3 Agarose gel electrophoresis showing the amplification of the 165 bp fragment after enzymatic digestion with the Tth 111l restriction endonuclease enzyme.

Legend:
Lane M is a 1kb plus DNA ladder
Lane 1 is a 165 bp PCR product
Lanes 2, 3, 6, 8, 9 and 10 are 141 bp digested fragments showing recessive homozygous individuals TT
Lanes 4, 5, 7 and 11 are 165 and 141 bp fragments showing heterozygous individuals MT
Lane 12 is a 165 bp undigested fragment showing a normal wild type MM

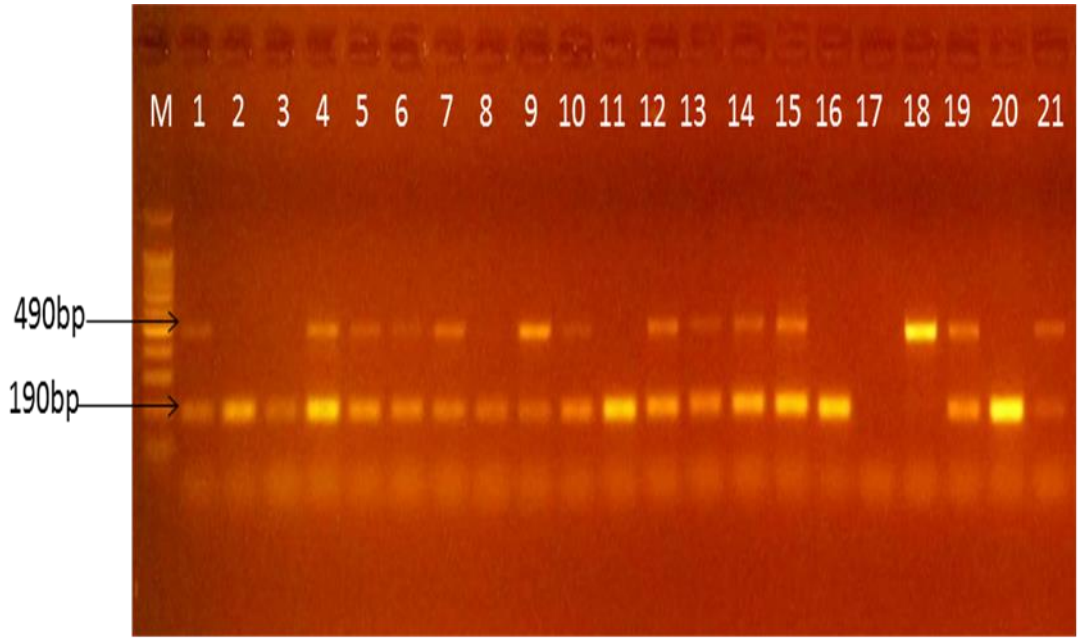


Plate 4.4 Agarose gel showing the amplification of the insertion/deletion of the angiotensin converting enzyme gene

Legend:
Lane M is the 100bp DNA ladder
Lanes 1, 4, 5, 6, 7, 9, 10, 12, 13, 14,15,19 and 21 are the 490bp and 190bp PCR products showing heterozygous individuals I/D
Lanes 2, 3, 8, 11, 16 and 20 are the 190bp PCR product showing individuals that have a homozygous deletion I/I
Lane 18 is the 490bp PCR product showing an individual with homozygous insertion D/D
Lane 17 did not amplify and the PCR had to be repeated

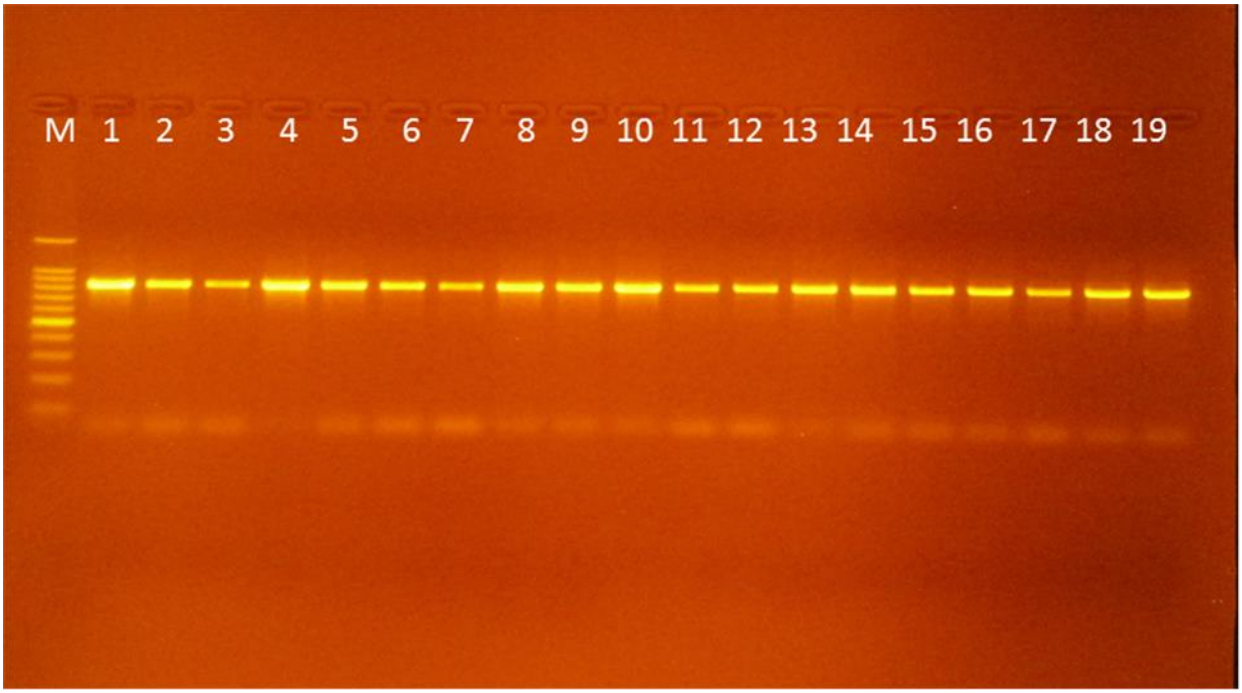


Plate 4.5 Agarose gel electrophoresis showing the 850bp PCR product of the Angiotensin II type I receptor gene.

Legend
Lane M is the 100bp DNA ladder
Lane 1-19 are the amplified PCR product

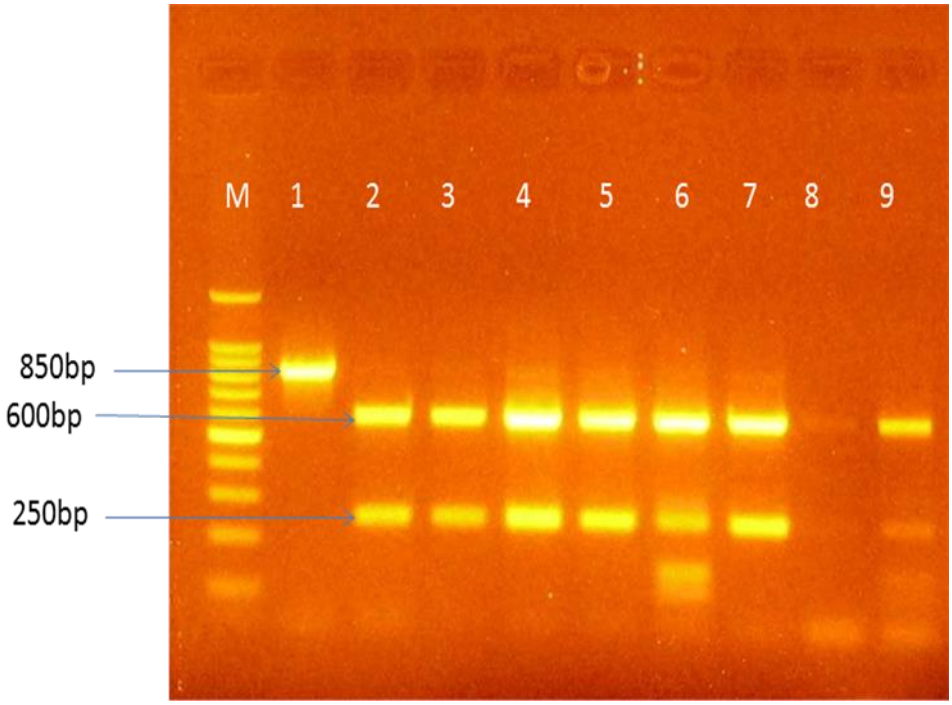


Plate 4.6 Agarose gel electrophoresis showing digestion of the 850bp PCR product by the Dde1 restriction endonuclease.

Legend
Lane M is the 100bp DNA ladder
Lane 1 is the PCR product
Lanes 2, 3, 4, 5 and 7 are the homozygous AA individuals
Lanes 6 and 7 are the heterozygous AC individuals
Lane 8 was faint and the digestion was repeated

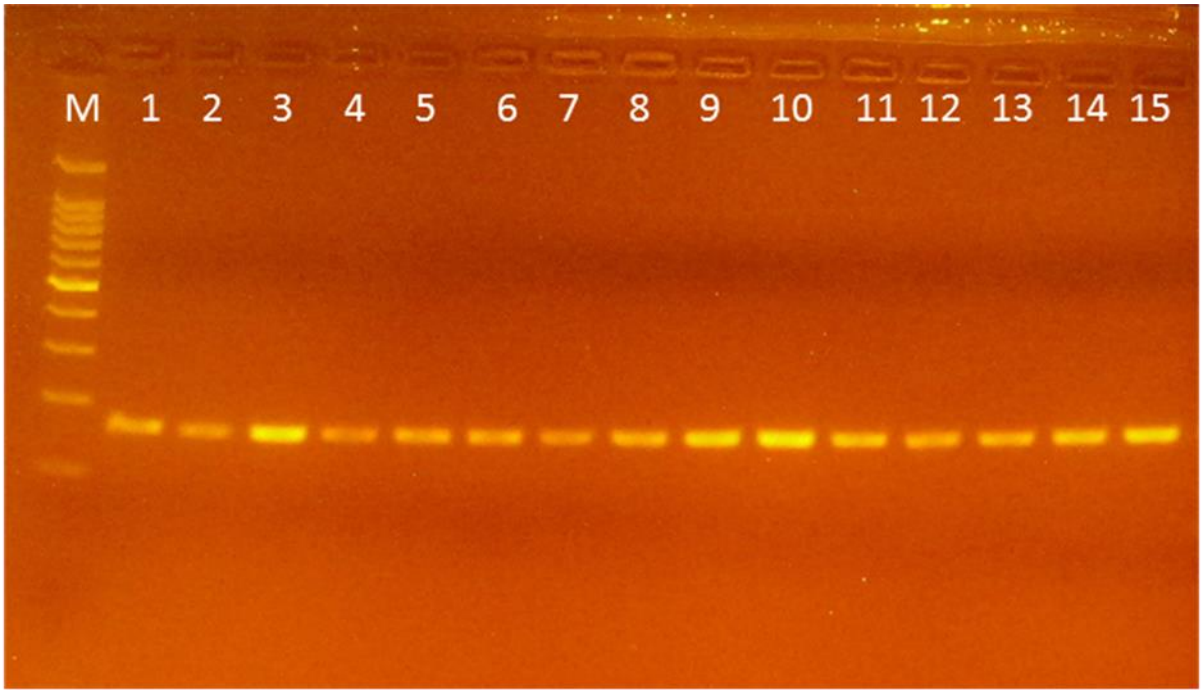


Plate 4.7 Agarose gel electrophoresis showing 157 bp PCR product of the atrial natriuretic peptide gene.

Legend
Lane M is the 100bp DNA ladder
Lane 1-15 are the amplified PCR product

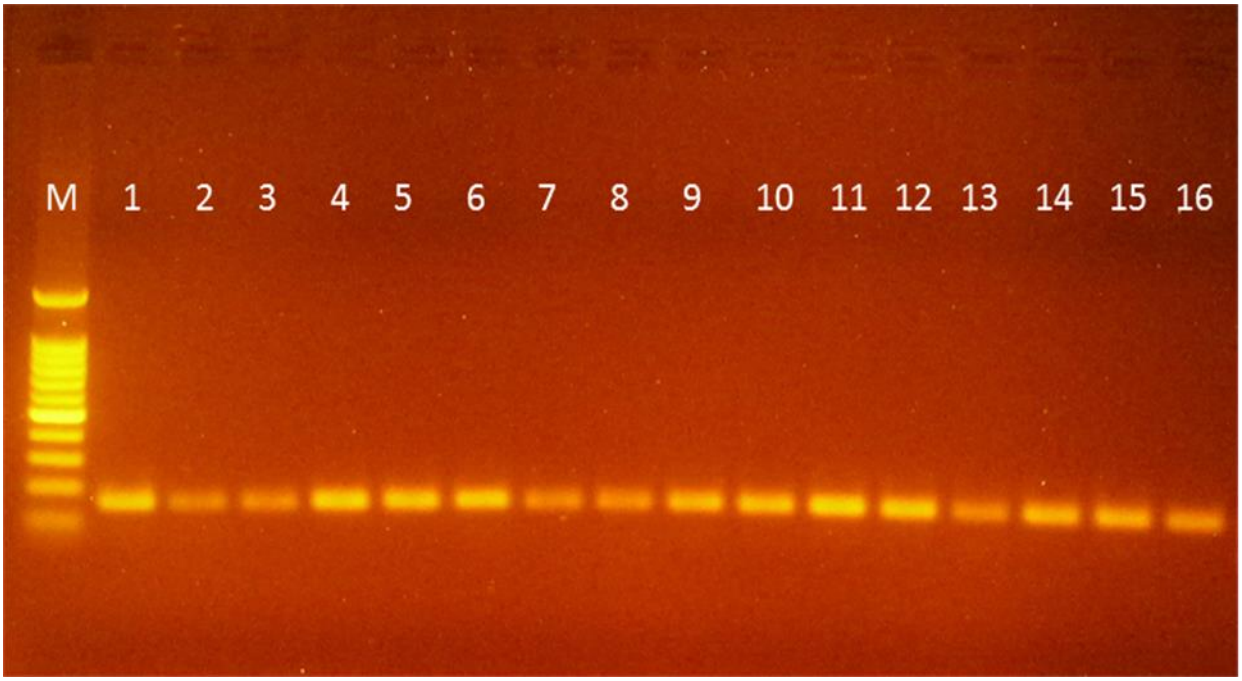


Plate 4.8 Agarose gel electrophoresis showing the 157bp product after enzymatic digestion with Rsa1 restriction endonuclease.

Legend
Lane M is the 100bp DNA ladder
Lane 1-16 are the undigested product

4. 2 Demographic Data

There were a total of 1,224 subjects recruited into the study, consisting of 612 hypertensives (225 males and 387 females) and 612 normotensives (272 males and 340 females) fig 4.1 and as a consequence more females were observed with TT for AGT and I/D and D/D genotype for the ACE, fig 4.2 - 4.5 shows the distribution of the polymorphisms by gender in the study population. The Efiks and the Ibibios (34.2; 32.4% respectively, n=612) were the main ethnic groups among the patients. The Ibibios (37.1%, n=612) were the predominant ethnic group among the controls. Fig 4.6-4.10 shows the ethnic groups and the distribution of the genes by ethnic group in the study population. The major ethnic groups had a higher frequency I/D variant among the controls and D/D genotype among the patients for the ACE. Most ethnic groups showed a high frequency of the TT genotype. Table 4.1 shows the genotype and allele frequencies of the polymorphisms among the major ethnic groups in Calabar and Uyo.

4.3 Genotypic frequencies

The prevalence of AGT mutation was 88.4% for hypertensives and 92.2% for controls (homozygous mutation). 10.9% hypertensives and 7.5% control for the heterozygous mutation. The wild type allele was prevalent at 0.3% and 0.7% for patients and controls respectively. For the I/D allele of the ACE gene, the deletion was 45% and 39% (homozygous),the carriers of the deletion were 43% and 49% in the patient and control population, while the insertion allele was 12% in both control and patient populations. For the ATIR allele, 99% of the study population had the wild type allele and 1% was heterozygous carriers of the mutation. Only the wild type gene was observed for the ANP allele in the study population Table 4.2.

Among the Efiks which are the predominant ethnic group in Calabar town, the genotype distribution of the M235T allele was 1, 16, 192 and 0, 10, 163 for the MM, MT, TT, genotypes among patients and control groups. The frequency of the 235T allele was 0.97. For the ACE polymorphism, the genotype frequency was 22, 91, 96 and 27, 78, 68 for the II, ID, DD genotypes among patients and controls. The frequency of the D allele was 0.70. For angiotensin 11 type 1 receptor gene, the genotype frequencies were 208, 1 and 174, 1 for the AA and AC genotypes. The CC genotype was not observed in this population. The frequency of the C allele is 0.003 in the controls and 0.002 in the patients.

Among the Ibibios which also happens to be the predominant ethnic group in Uyo town. The genotype distribution of the M235T allele was 1, 24, 173 and 0, 16, 211 for the MM, MT, TT genotypes in patient and controls. The frequency of the 235T allele was 0.97. For the ACE polymorphism, the genotype frequencies were 22, 80, 96 and 30, 112, 85 for II, ID, DD frequencies with the D allele frequency as 0.70. For the angiotensin 11 type 1 receptor gene polymorphism, the genotype frequencies were 197, 1 and 286, 3 for the AA and AC genotypes, the CC genotype was also not observed in this group. The C allele frequency was 0.005 and 0.003 in the control and patient population Table 4.1.

There were no significant differences between the genotype frequencies of hypertensive and the control groups by $\chi 2$ analysis for all the polymorphisms under consideration in this study. When continuous variables were compared between hypertensive and control groups, significant differences existed between the age, BMI, systolic and diastolic blood pressure of controls and patients.

4.4 Blood Pressure:

For patients the mean diastolic blood pressure was 93.25 ±13.768, the mean systolic blood pressure was 161.14 ±23.247. For the controls, the mean systolic blood pressure was 116.76 ±9.19; the mean diastolic blood pressure was 72.181 ±8.41. Table 4.2 According to the JNC classification on hypertension, 265 patients had stage one hypertension and 347 patients had stage two hypertension, for the systolic BP measurement.

From the diastolic BP measurement, 366 patients were grouped into the stage 1 category and 246 patients had stage 2 hypertension. For the systolic BP measurement in controls, 350 were classified into the prehypertension group while 262 were classified as normal. For the diastolic BP measurement, 279 controls were classified into the prehypertension group and 333 controls as normal. Table 4.3

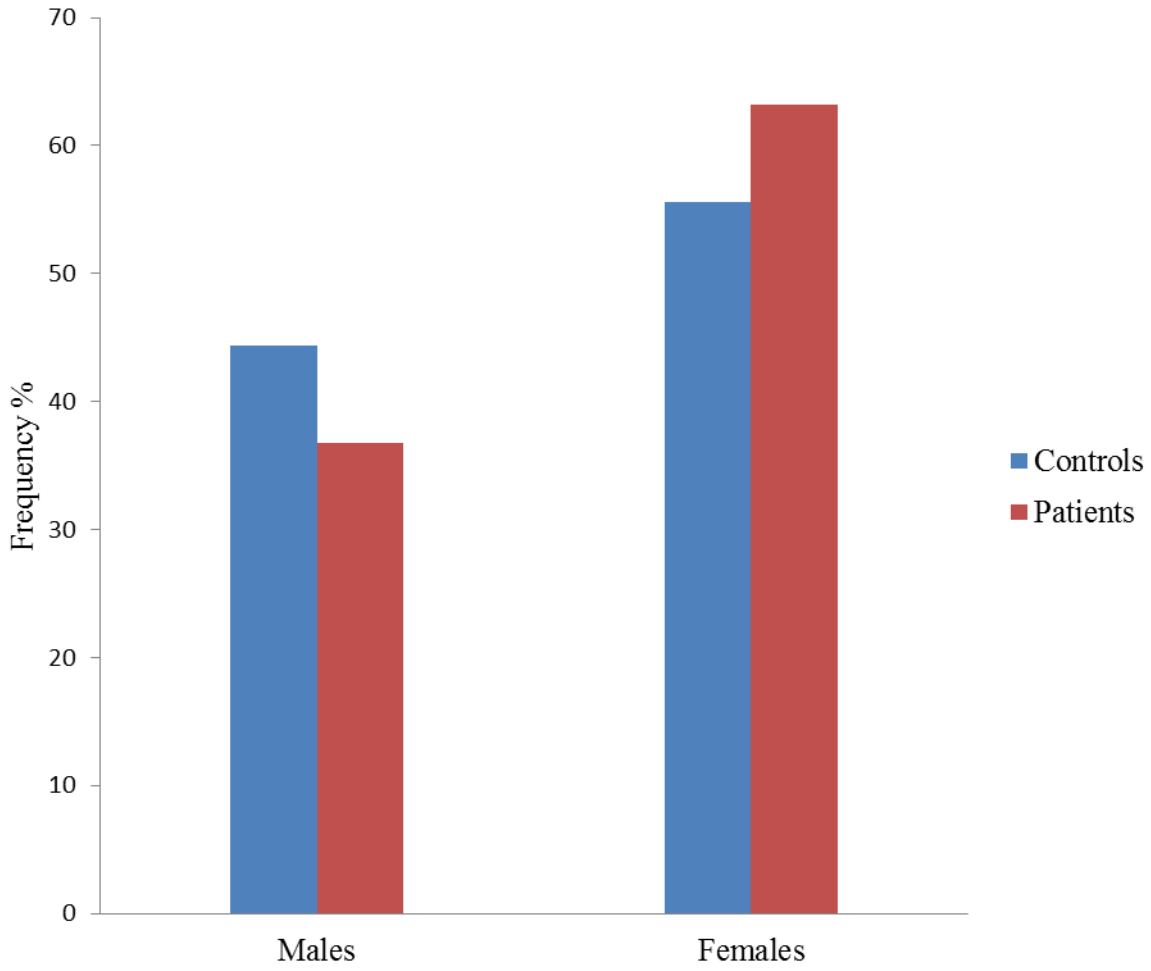


Fig 4.1 Distribution by gender in the control and patient groups

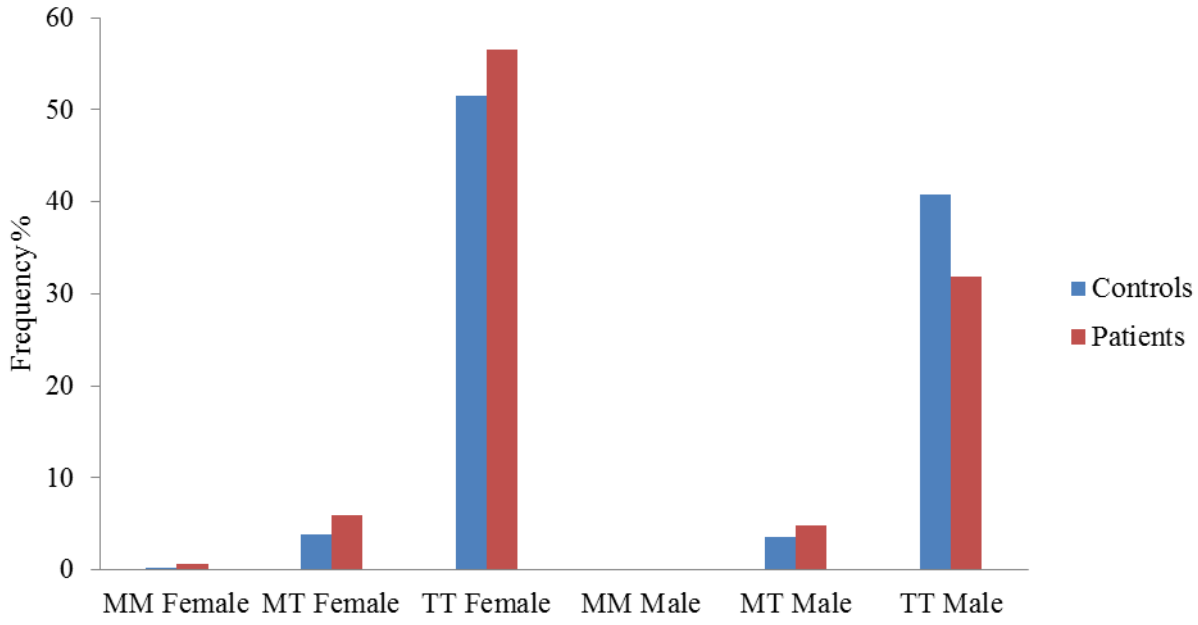


Fig 4.2 Distribution of the AGT M235T polymorphism by gender among the patient and control population

Legend:
MM is the wild type individual
MT is the heterozygous mutant individual
TT is the homozygous recessive mutant individual

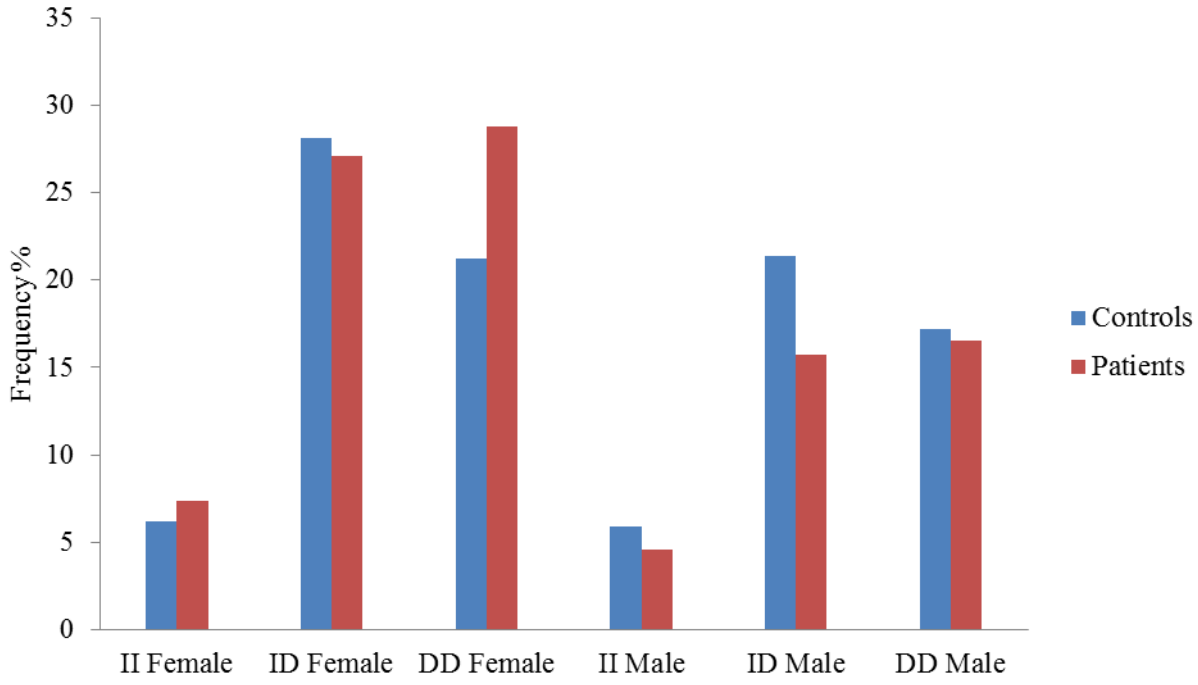


Fig 4.3 The ACE I/D allele distribution by gender in the control and patient population

Legend:
II is the wild type insertion individual
ID is the heterozygous deletion individual
DD is the homozygous recessive deletion individual

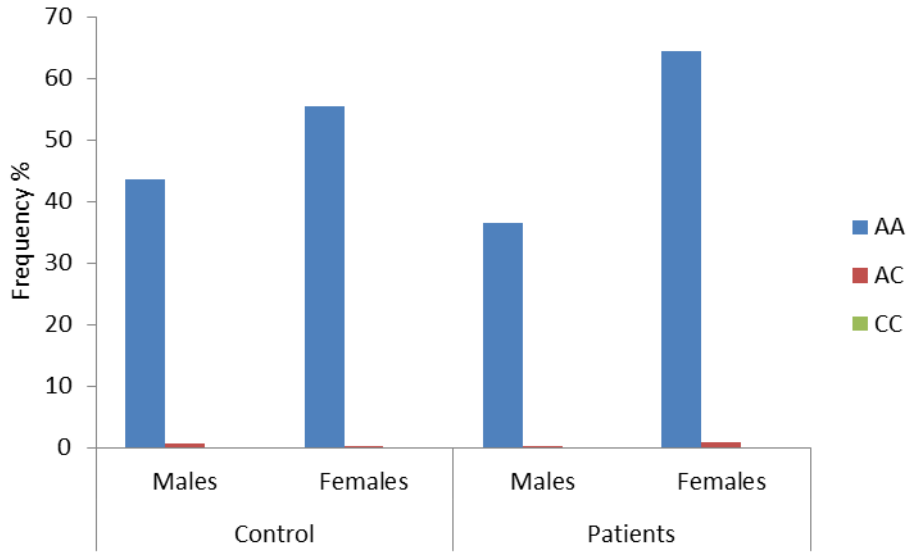


Fig 4.4 The ATIR allele distribution by gender in the control and patient population

Legend:
AA is the homozygous wild type individual
AC is the heterozygous mutant individual
CC is the homozygous recessive mutant individual

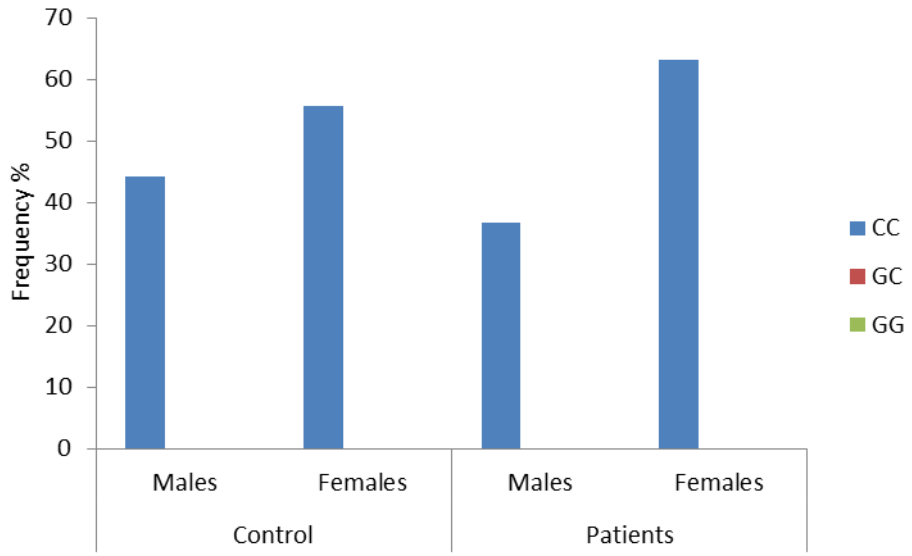


Fig 4.5 The ANP allele distribution by gender among the control and patient population

Legend:
CC is the wild type homozygous individual
GC is the heterozygous mutant individual
GG is the homozygous recessive individual

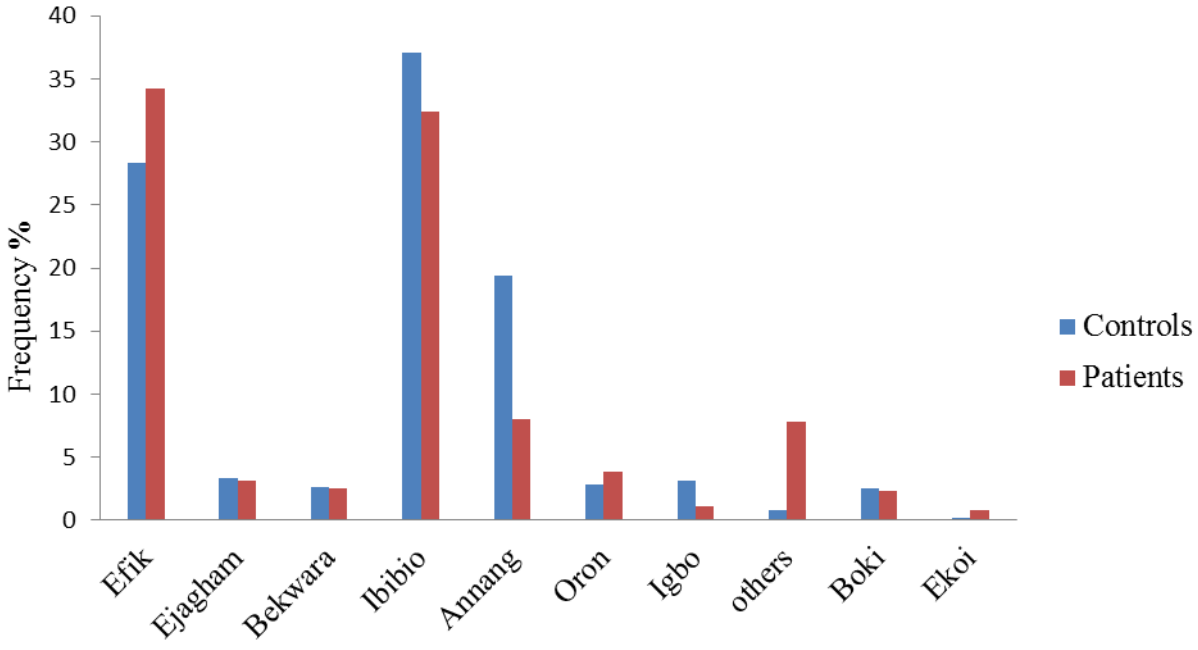


Fig 4.6 Ethnic distribution among the Control and Patient groups.

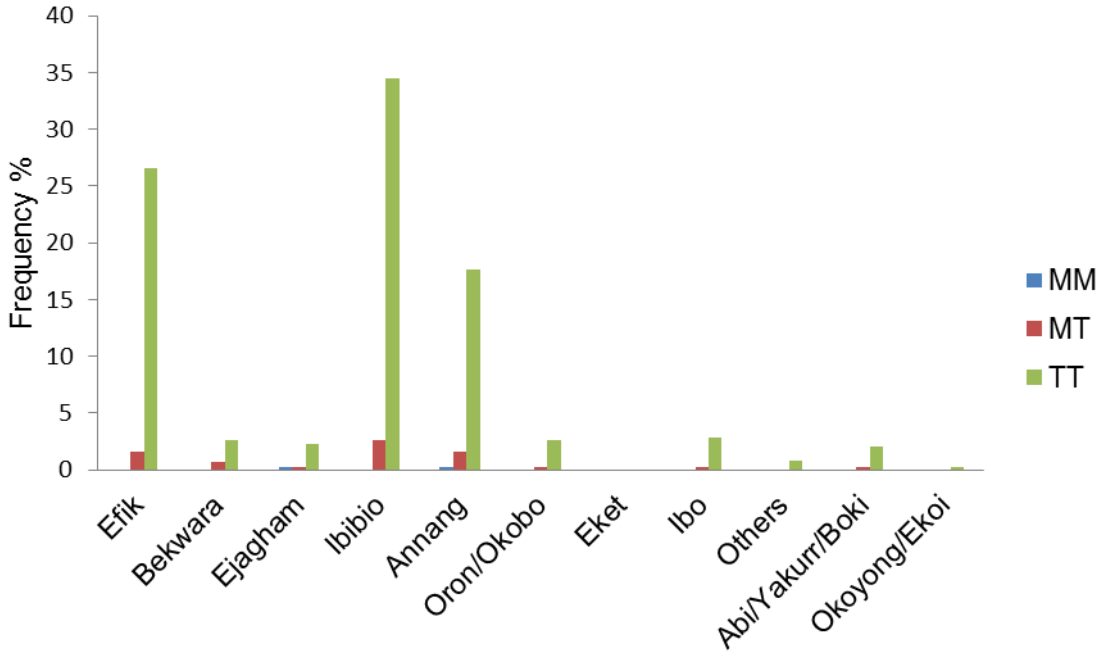


Fig 4.7 Distribution of the AGT M235T polymorphism among the Ethnic groups in the control population.

Legend:
MM are the homozygous dominant individuals
MT are the heterozygous mutant individuals
TT are the homozygous recessive individuals

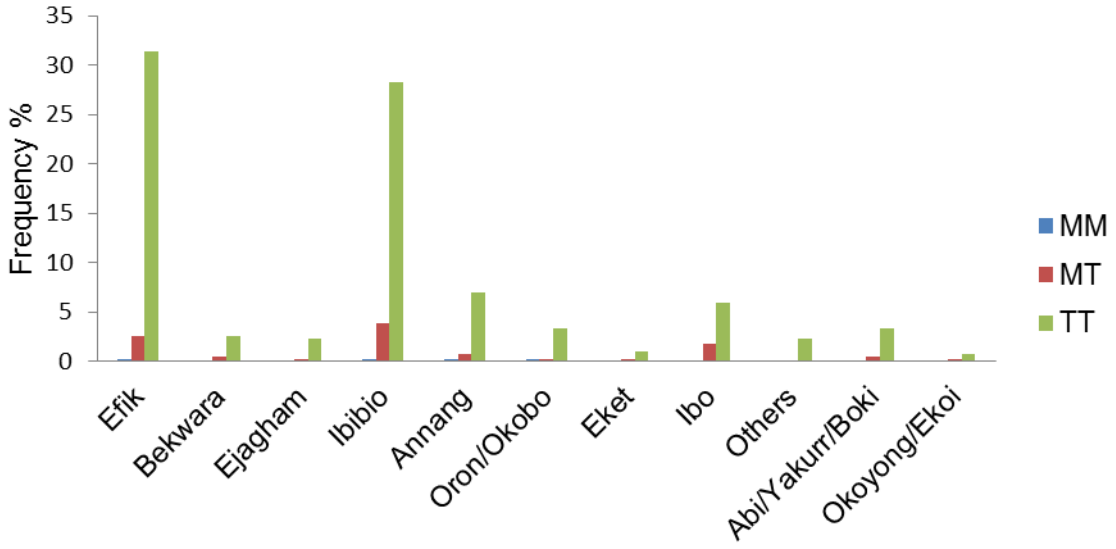


Fig 4.8 Distribution of the AGT M235T polymorphism among the Ethnic groups in the Patient population.

Legend:
MM are the homozygous dominant individuals
MT are the heterozygous mutant individuals
TT are the homozygous recessive individuals

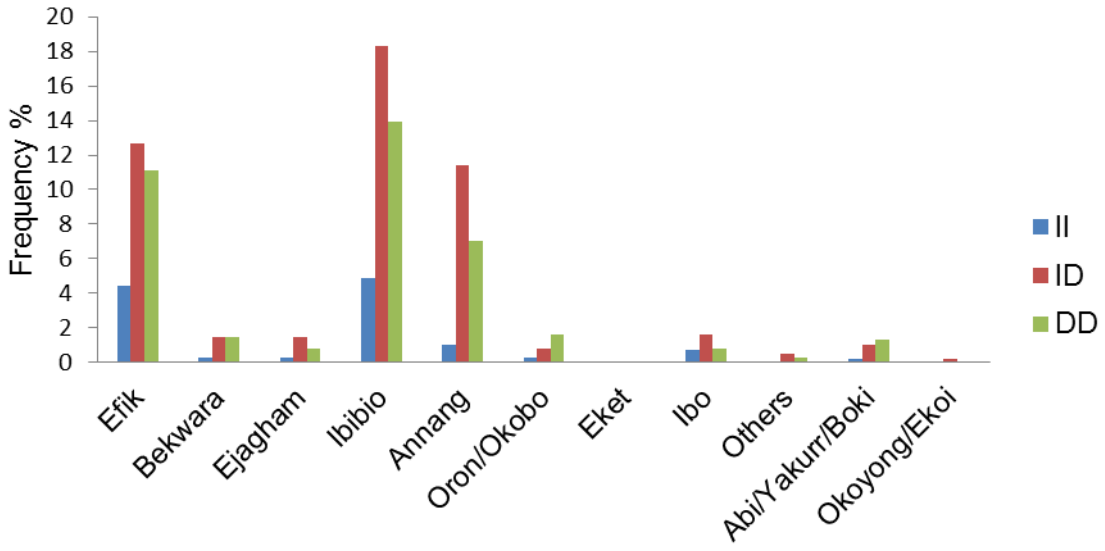


Fig 4.9 Distribution of the ACE I/D polymorphism among the Ethnic groups in the Control population.

Legend:
II are the homozygous insertion individuals
ID are the heterozygous individuals
DD are the homozygous deletion individuals

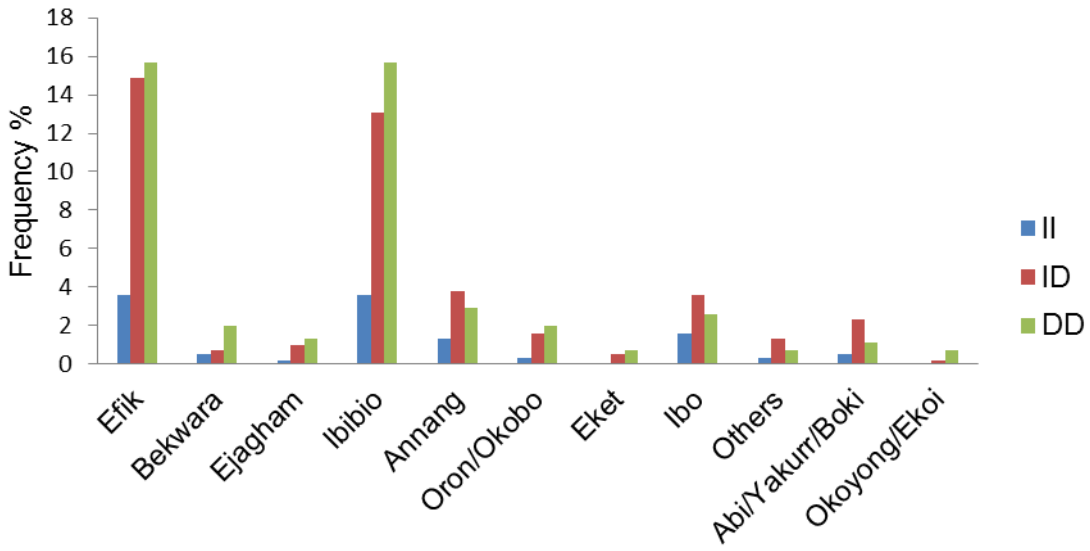


Fig 4.10 Distribution of the ACE I/D polymorphism among the Ethnic groups in the Patient population.

Legend:
II are the homozygous insertion individuals
ID are the heterozygous individuals
DD are the homozygous deletion individuals

Table 4.1 Genotype and allele frequencies of the polymorphisms among the major ethnic group in Calabar and Uyo.

		AGT					ACE					AT1R				
		MM	MT	TT	M	T	II	ID	DD	I	D	AA	AC	CC	A	C
Efiks	Patients	1	16	192	0.04	0.96	22	91	96	0.3	0.7	208	1	0	0.998	0.002
	Controls	0	10	163	0.03	0.97	27	78	68	0.4	0.6	174	1	0	0.997	0.003
Ibibios	Patients	1	24	173	0.07	0.93	22	80	96	0.3	0.7	197	1	0	0.997	0.003
	Controls	0	16	211	0.04	0.96	30	112	85	0.3	0.7	286	3	0	0.995	0.005

Table 4.2 Genotype and allele frequency of the RAAS and ANP polymorphisms in the patient and control population.

Groups	N	Genotype Frequencies			Allele Frequencies		
M235T		MM	MT	TT	M	T	Odds Ratio
Hypertensives observed	612	4	67	541	75	1149	
		0.01	0.11	0.88	0.06	0.94	
Hardy-Weinberg predicts expected		0.004	0.113	0.884			
Controls observed	612	2	45	565	49	1175	
		0.003	0.075	0.922	0.04	0.96	
Hardy-Weinberg predicts expected		0.002	0.077	0.921			O.R=0.65,95%CI(0.396-1.074)
I/D		II	ID	DD	I	D	
Hypertensives observed	612	73	262	277	408	816	
					0.33		
		0.12	0.43	0.45	0.67		
Hardy-Weinberg predicts expected		0.11	0.44	0.45			
Controls observed	612	74	303	235	451	773	
					0.37		
		0.122	0.490	0.388	0.63		
Hardy-Weinberg predicts expected		0.137	0.466	0.397			O.R=1.15,95%CI(0.923-1.456)
AT1R		AA	AC	CC	A	C	
Hypertensive observed	612	605	7	0	1217	7	
					0.994		
		0.99	0.01	0	0.006		
Hardy-Weinberg predicts		0.99	0.01	0			

expected								
Controls	observed	612	606	6	0	1218		6
							0.99	
			0.99	0.01	0		0.01	
Hardy-Weinberg predicts								
expected			0.98	0.02	0			
ANP			CC	CG	GG			
Hypertensives	observed	612	612	0	0			
Controls	observed	612	612	0	0			

Table 4.3 Classification of the study population according to the JNC V11 classification of blood pressure.

Groups	Blood pressure	No of Individuals	Groups according to JNC Classification	Mean Blood pressure
Patients	Systolic	265	Stage 1 hypertension	
		347	Stage 2 hypertension	161.11 ± 23.26
	Diastolic	366	Stage 1 hypertension	
		246	Stage 2 hypertension	93.25 ± 13.77
Control	Systolic	350	Prehypertension	
		262	Normal	116.25 ± 9.22
	Diastolic	279	Prehypertension	
		333	Normal	72.48 ± 8.37

4.5 Age:

The hypertensive subjects ranged from 24 to 90 years old with a mean age of 51.2 years. Among the patient group, 463 (75.7) persons were more than 40 years of age and 149 (24.3) patients were less than 40 years. Normotensives ranged from 20 to 73 years old with a mean age of 31.9 years, 498 (81.3%) controls were less than 40 years of age while 114 (18.6%) controls were above forty years Fig 4.11.

4.6 Knowledge of and family history of hypertension:

Among the hypertensive group, about 71% were fully aware of their hypertension status, 29% were not aware of their status Fig 4.12. 330 persons (53.9%) reported they do not have a positive history of hypertension.163 persons (26.6%) have a positive history of hypertension. 119 (19.4%) persons have no idea. None of the normotensives had hypertension although 22.7% had a positive family history of hypertension. 26.6% reported a negative family history of hypertension, 50.7% had no idea if there was any history of familial hypertension.

4.7 Smoking and alcohol consumption status:

603 (98.5%) of patients were non smokers, 594 (97.1%) of normotensives were non smokers. 434 (70.9%) of patients do not consume alcohol, 19.1% consume very little alcohol occasionally. 339 (55.4%) of controls do not consume alcohol, 201 (32.8) consume alcohol occasionally. 77 (11.8%) persons consumed alcohol on a regular basis. Figure 4.13-fig 4.16

4.8 Salt consumption:

Salt intake was normal in 509 (83.2%) patients, 103 (16.8%) consume extra salt. controls whose salt intake was considered normal was 467(76%); the remaining 145 (24%) take more salt than normal. Fig 4.17- fig 4.19

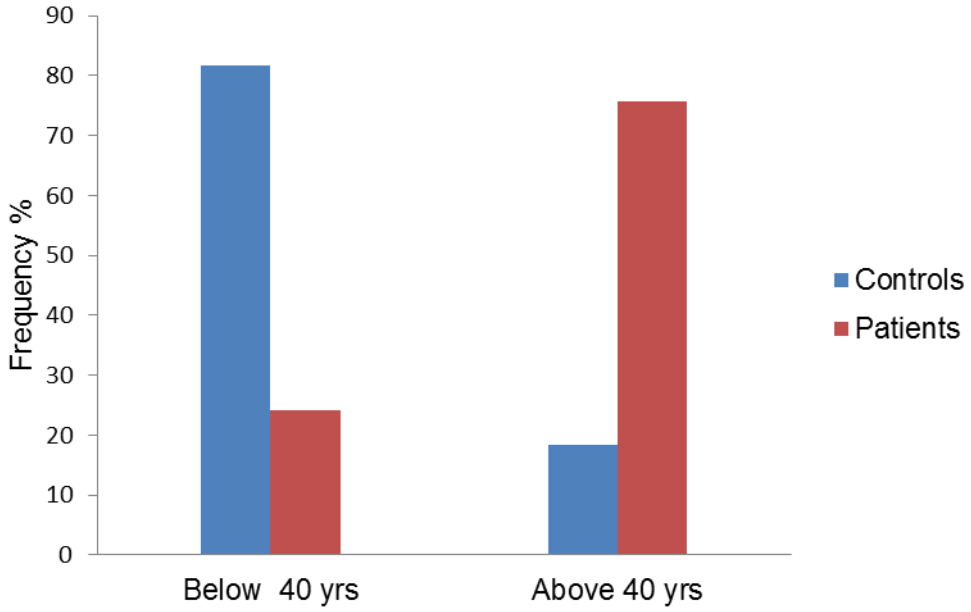


Fig 4.11 Age distribution of patient and control groups

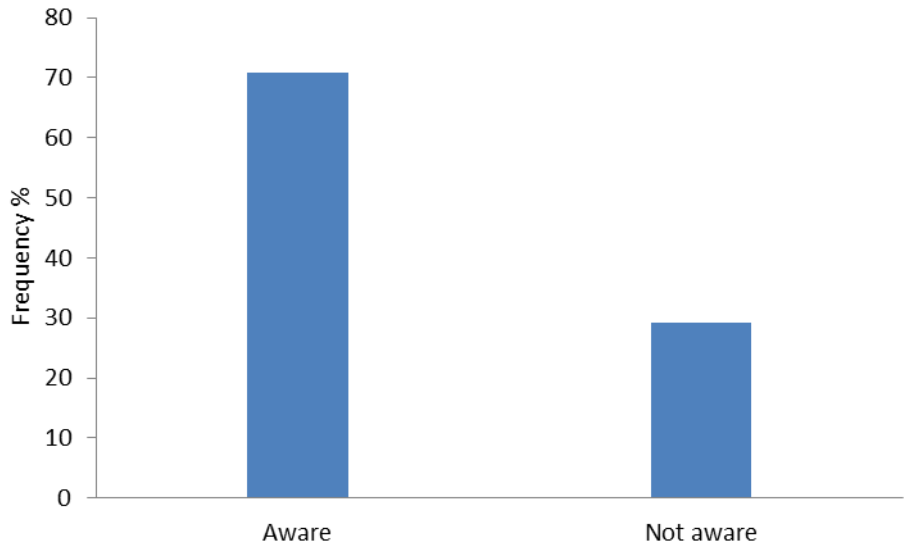


Fig 4.12 Knowledge of hypertension status among patients

Legend
71% were aware of their hypertension status

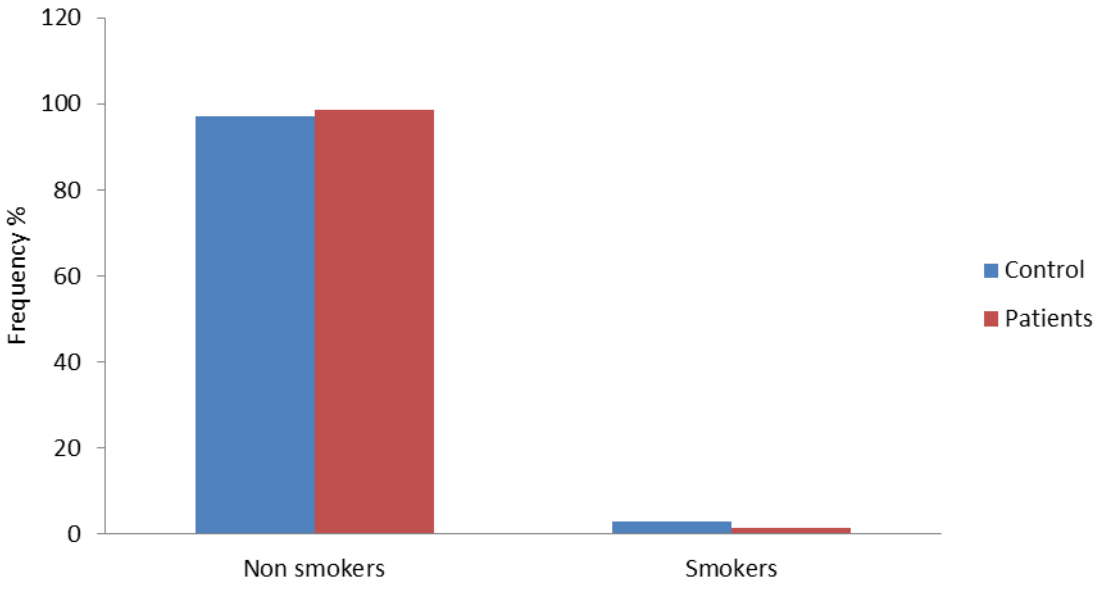


Fig 4.13 Smoking status among control and patient group

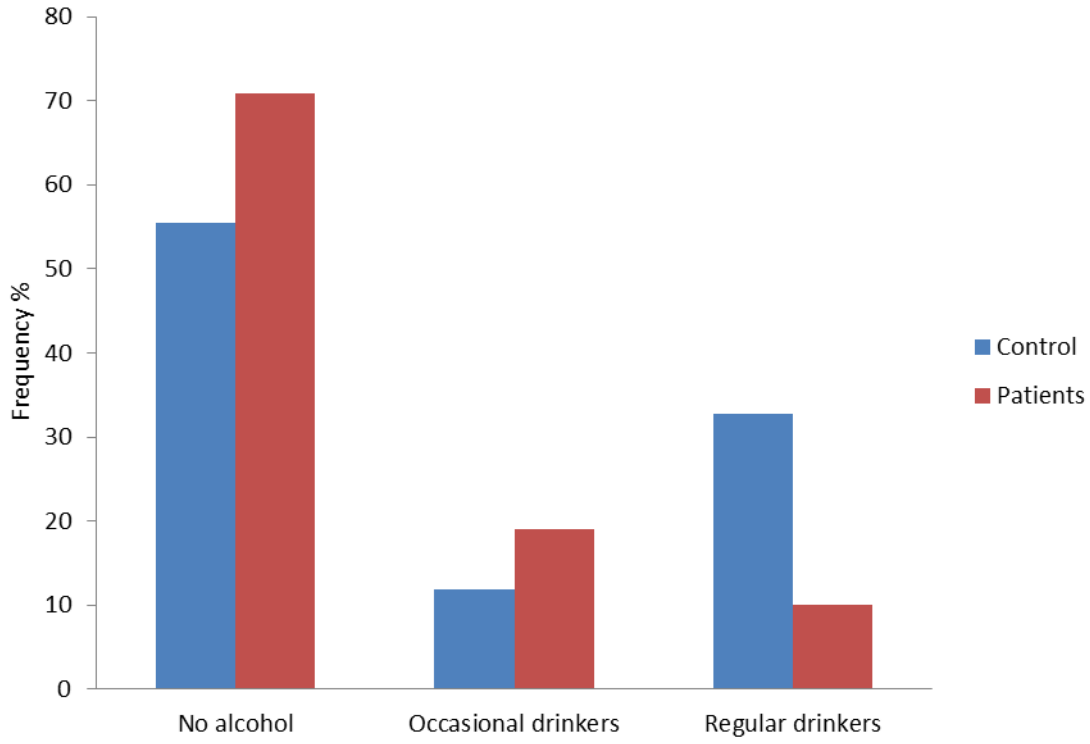


Fig 4.14 Alcohol consumption among control and patient groups

Legend:
Individuals under no alcohol response do not consume alcohol
Individuals under the occasional drinkers responses consume alcohol once in a while
Individuals under the regular drinkers responses are regular consumers of alcohol

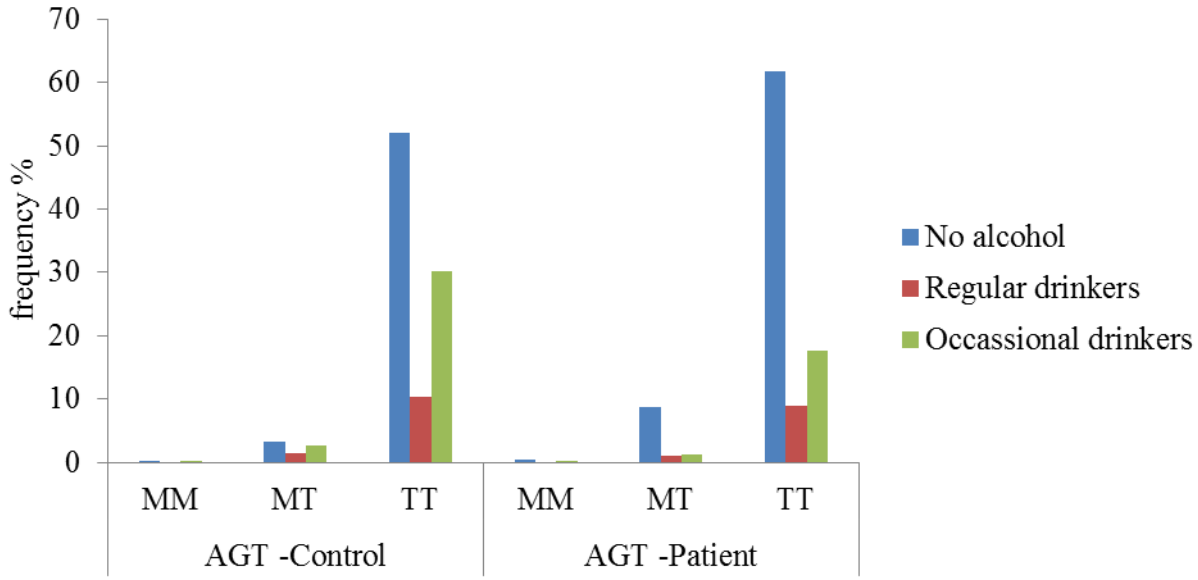


Fig 4.15 Distribution of the AGT M235T polymorphism and alcohol consumption in the study population.

Legend:
MM are the homozygous dominant individuals
MT are the heterozygous mutant individuals
TT are the homozygous recessive individuals

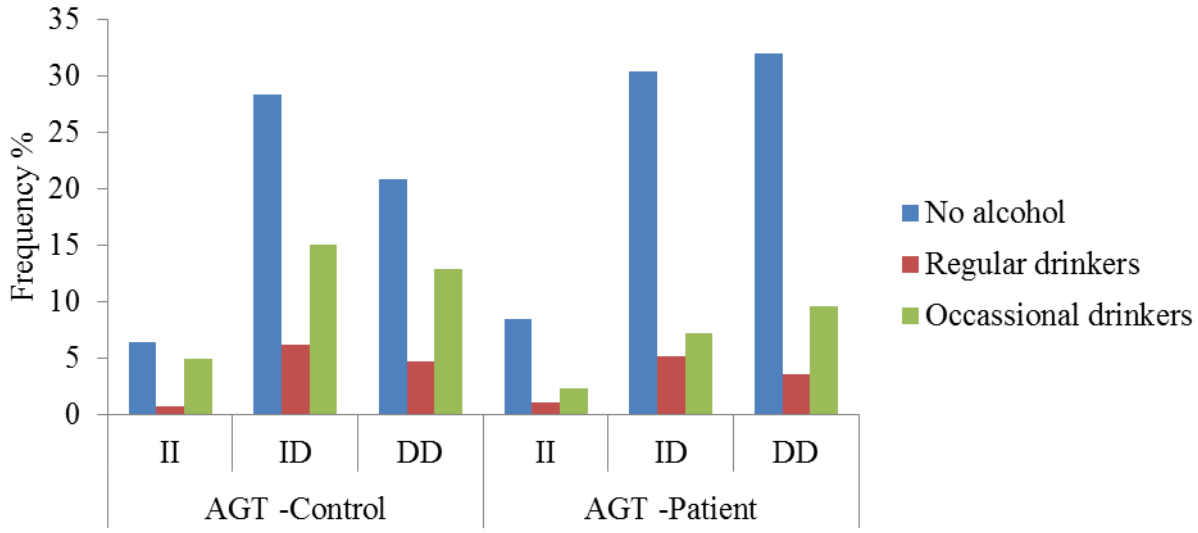


Fig 4.16 Distribution of the ACE I/D polymorphism and alcohol consumption in the study population.

Legend:
II are the homozygous insertion individuals
ID are the heterozygous individuals
DD are the homozygous deletion individuals

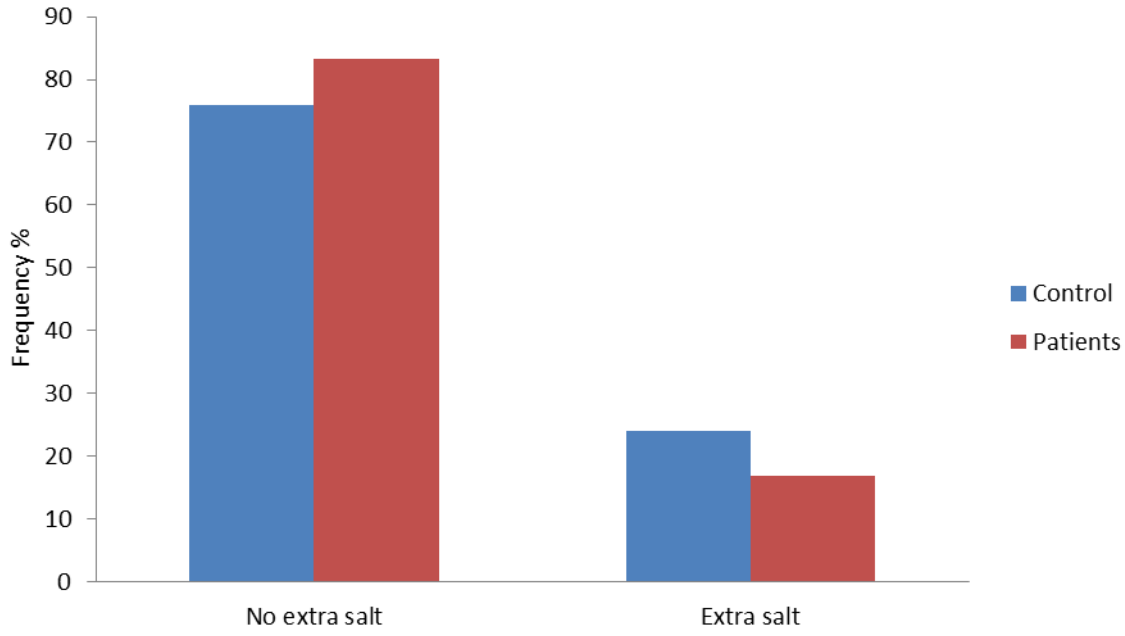


Fig 4.17 Salt intake among control and patient groups

Legend:
No extra means subjects do not add extra salt to their diet
Extra means subjects add extra salt to their diet

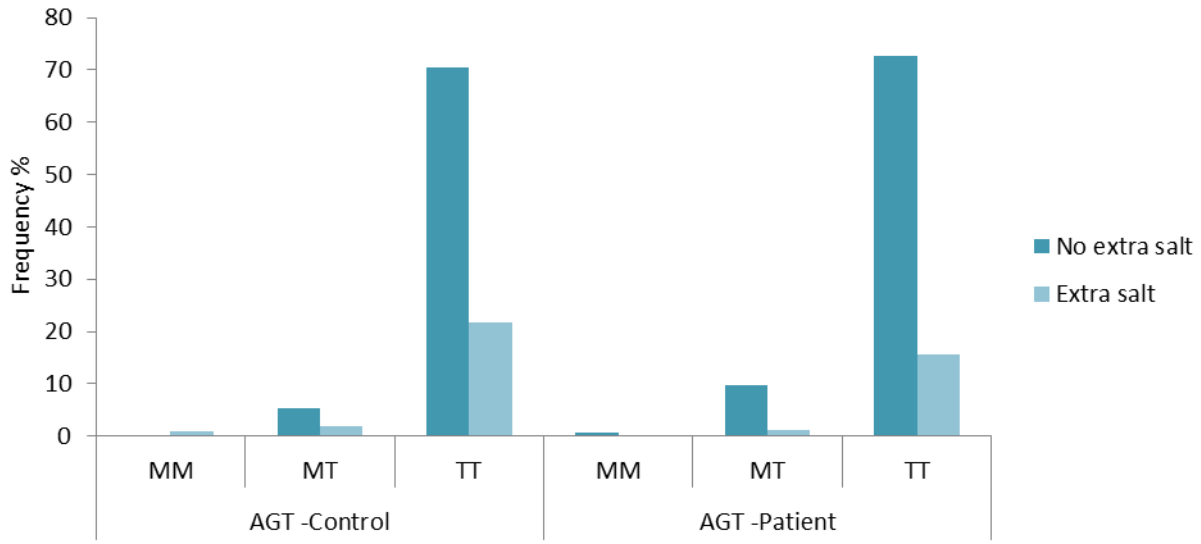


Fig 4.18 Distribution of the AGT M235T polymorphism and salt intake among the study population.

Legend:
The TT individuals that consume no extra salt were the predominant group among the patients and the controls.
MM are the homozygous dominant individuals
MT are the heterozygous mutant individuals
TT are the homozygous recessive individuals

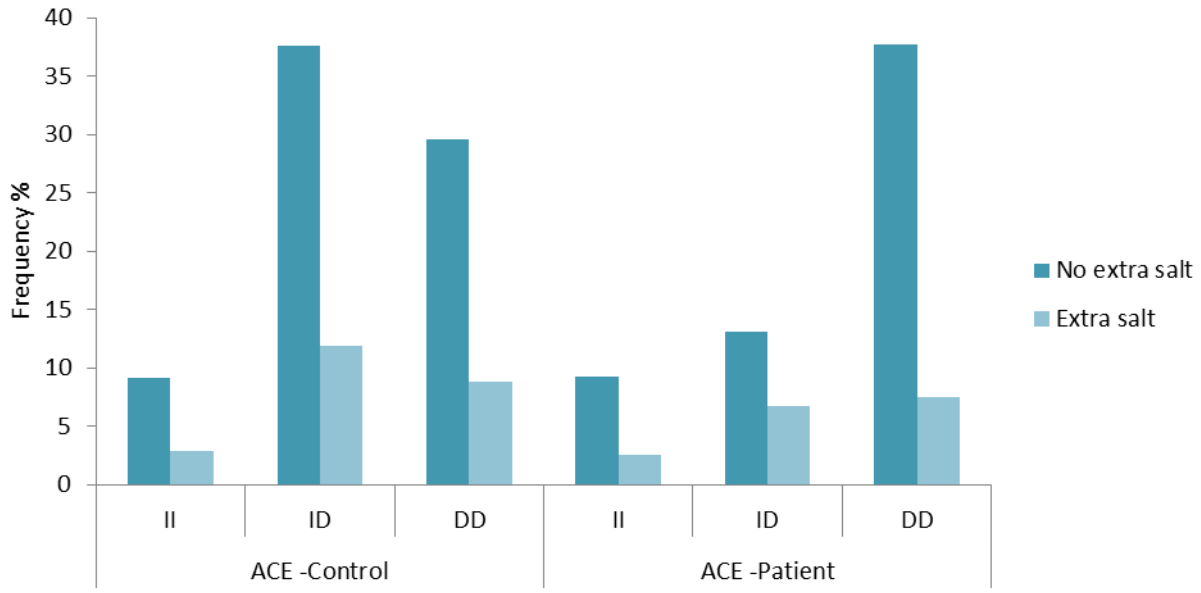


Fig 4.19 Distribution of the ACE I/D polymorphism and salt intake among the study population.

Legend:

The I/D individuals that consume no extra salt were the predominant group among the controls and the D/D were predominant among the patients.

II are the homozygous insertion individuals

ID are the heterozygous individuals

DD are the homozygous deletion individuals

4.9 Educational Attainment

Among the patient group, 245(40%) individuals attended only primary school, 167(27.3%) individuals attended secondary, 101(16.5) individuals attended tertiary institution and 99(16.2) individuals had no form of formal education. Among the control group, individuals who attended primary schools were 139(22.7%); secondary schools were 246(40.2%); tertiary institutions were 201(32.8%) and individuals that had no formal education were 26(4.2%) Fig 4.20- 4.22.

4.10 Body mass index

In the patient population, BMI below 24.9kg/m^2 was observed in 234 persons (31.54%), BMI between 25 – 30kg/m^2 was observed in 193 persons (38.23%) and BMI above 30kg/m^2 was found in 185 persons (30.23%). In the controls, BMI above 30kg/m^2 was found in 80 persons (12.79%), BMI between 25 – 30kg/m^2 was found in 121 persons (20%) and a BMI below 24.9kg/m^2 was observed in 411 persons (67.10). Fig 4.23

4.11 Exercise

33.2% controls do not carry out any form of exercise, 296(48.4%) controls indicated strolls to be the main exercise usually carried out. In the patient group, 293(47.9) do not carry out any form of exercise, 281(45.9) indicated strolls to be the main form of exercise Fig 4.24-Fig 4.27.

4.12 Marital status

In the control group, 294 (48%) persons were married and 316 (51.6%) persons were singles. For the patients, 523 (85.5%) persons were married and 54 (8.8%) were singles. There was no divorcee among controls but 1 among patients. There were 2 (0.3) widows among controls and 34 (5.5) widows among patients Fig 4.28.

4.13 Visits to fast food joints

547 patients do not go to fast food joints at all except for 65 persons. 45.9% mentioned strolling as their exercise type, 47.9% of patients did not practice in any form of exercise Fig 4.29.

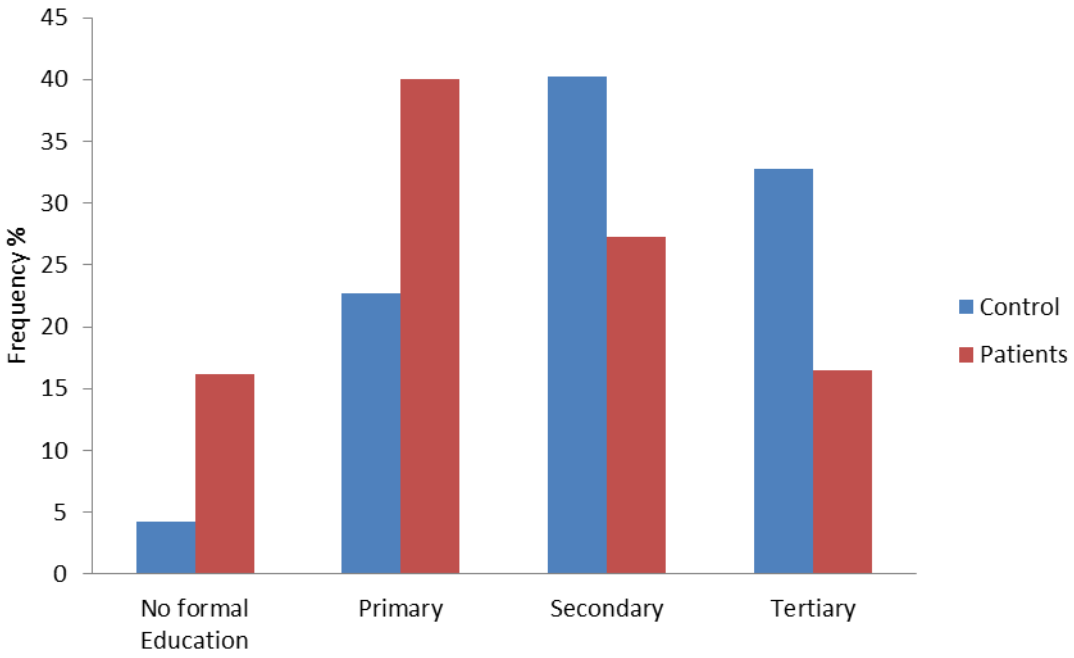


Fig 4.20 Educational levels among patient and control groups.

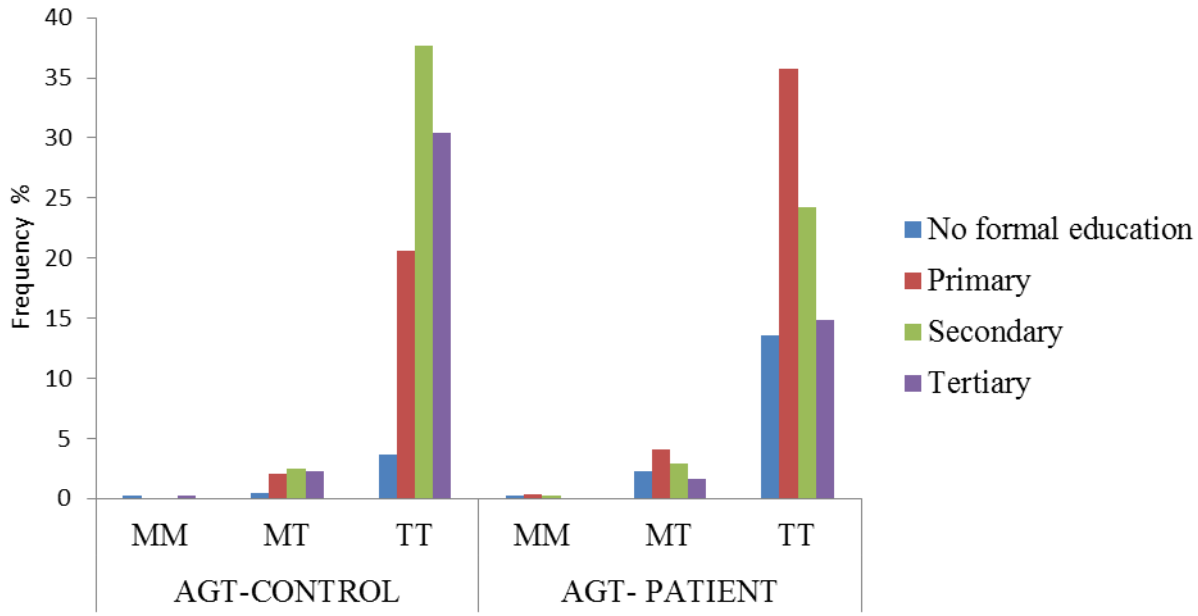


Fig 4.21 Distribution of the AGT M235T polymorphism and educational levels among the study population.

Legend:
MM are the homozygous dominant individuals
MT are the heterozygous mutant individuals
TT are the homozygous recessive individuals

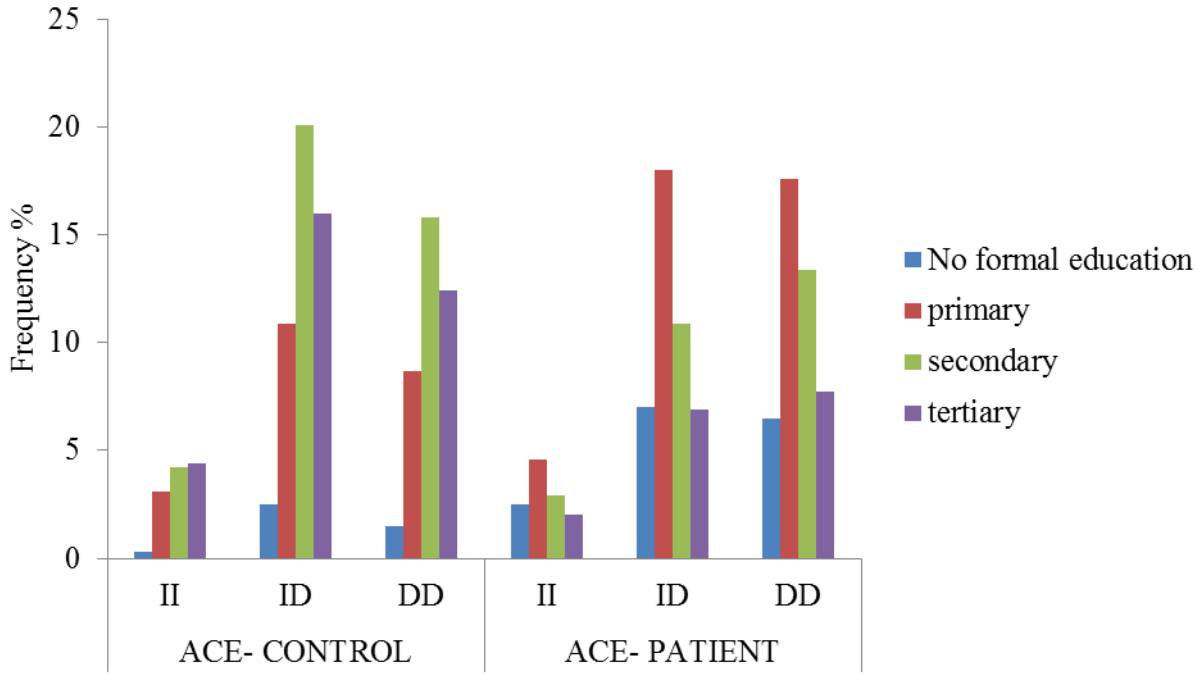


Fig 4.22 Distribution of the ACE I/D polymorphism and educational levels among the study population.

Legend:
II are the homozygous insertion individuals
ID are the heterozygous individuals
DD are the homozygous deletion individuals

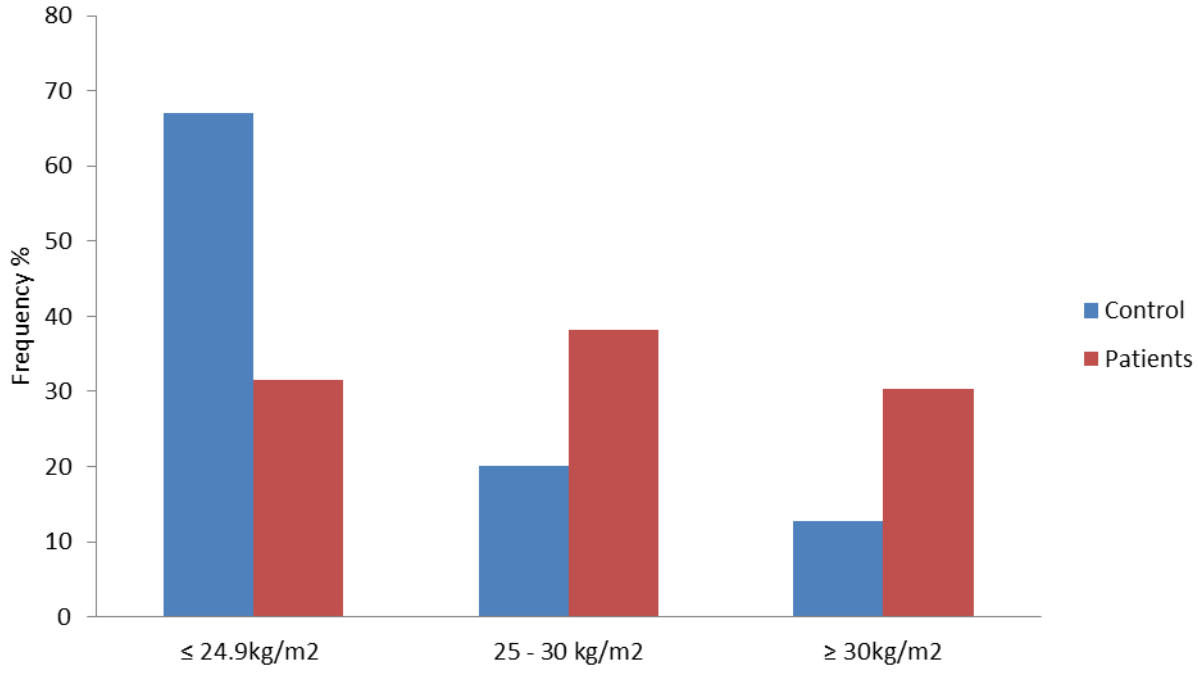


Fig 4.23 Body Mass Index observed among control and patient groups.

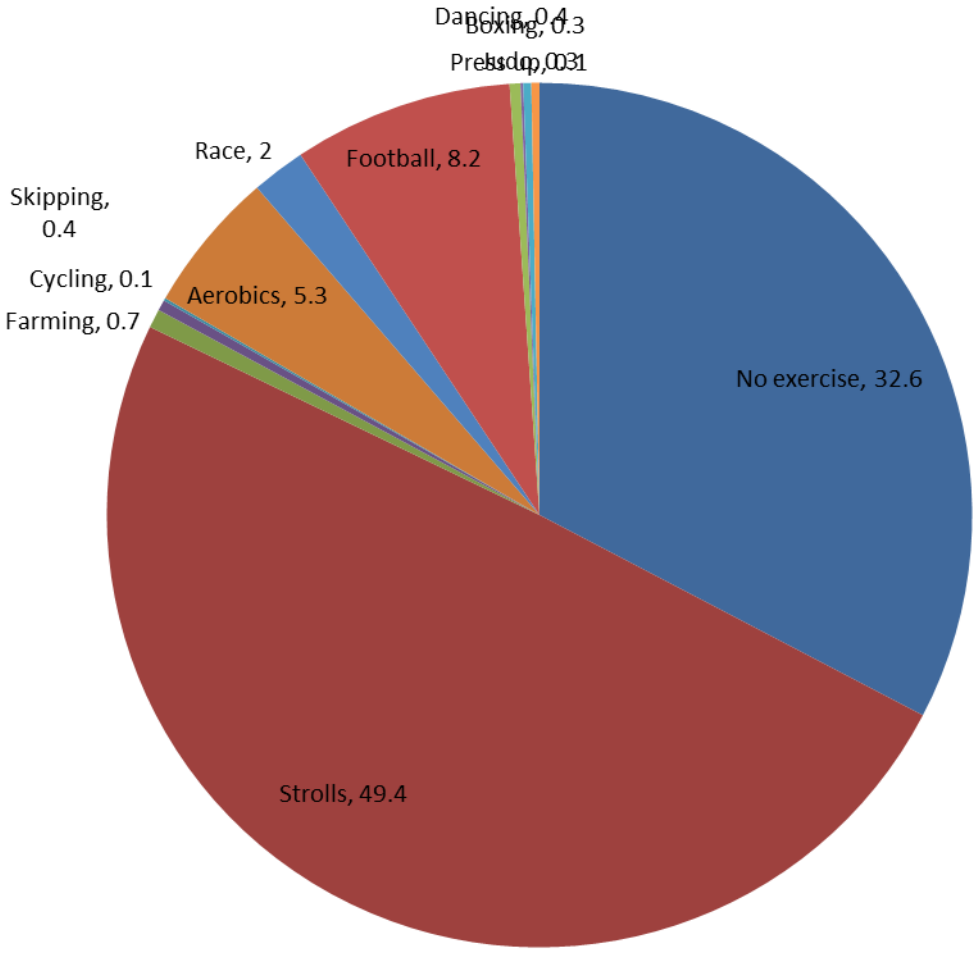


Fig 4.24 Exercise types observed among control group

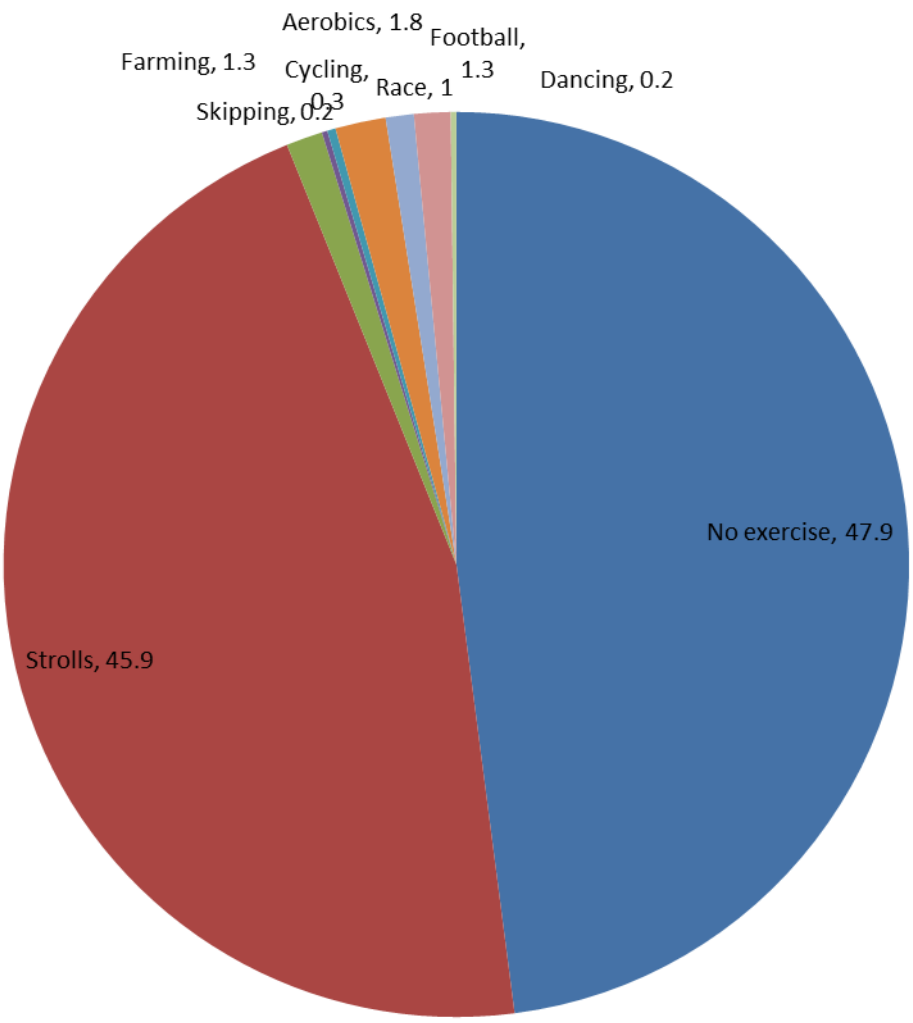


Fig 4.25 Exercise types observed among patient group

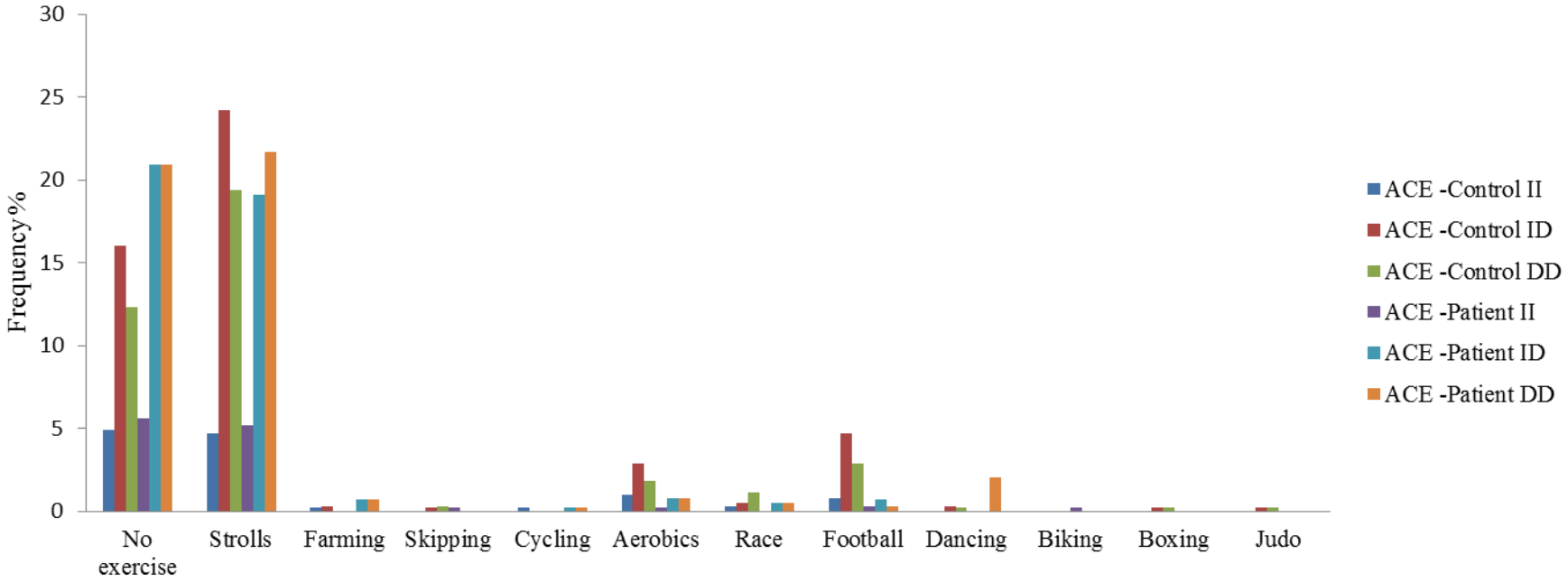


Fig 4.26 Distribution of the ACE I/D polymorphism and exercise types among the study population.

Legend:
II are the homozygous insertion individuals
ID are the heterozygous individuals
DD are the homozygous deletion individuals

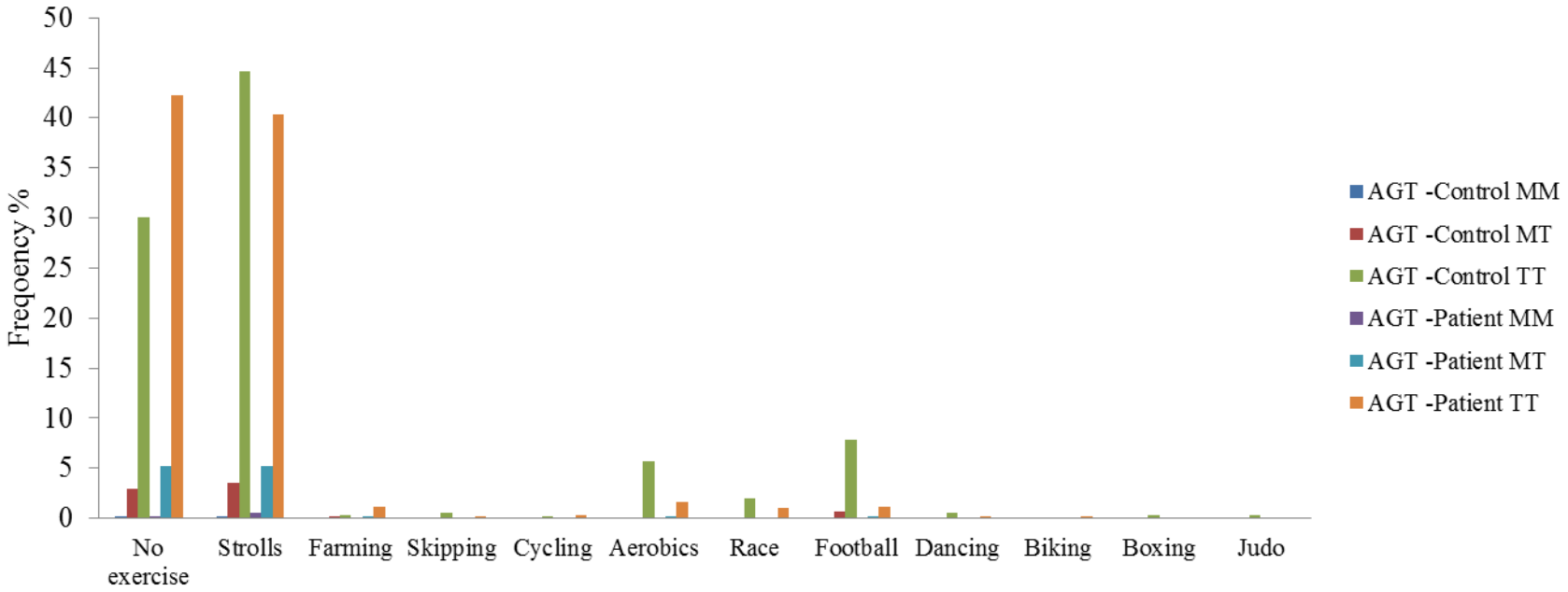


Fig 4.27 Distribution of the AGT M235T polymorphism and exercise types among the study population.
Legend:
MM are the homozygous dominant individuals
MT are the heterozygous mutant individuals
TT are the homozygous recessive individuals

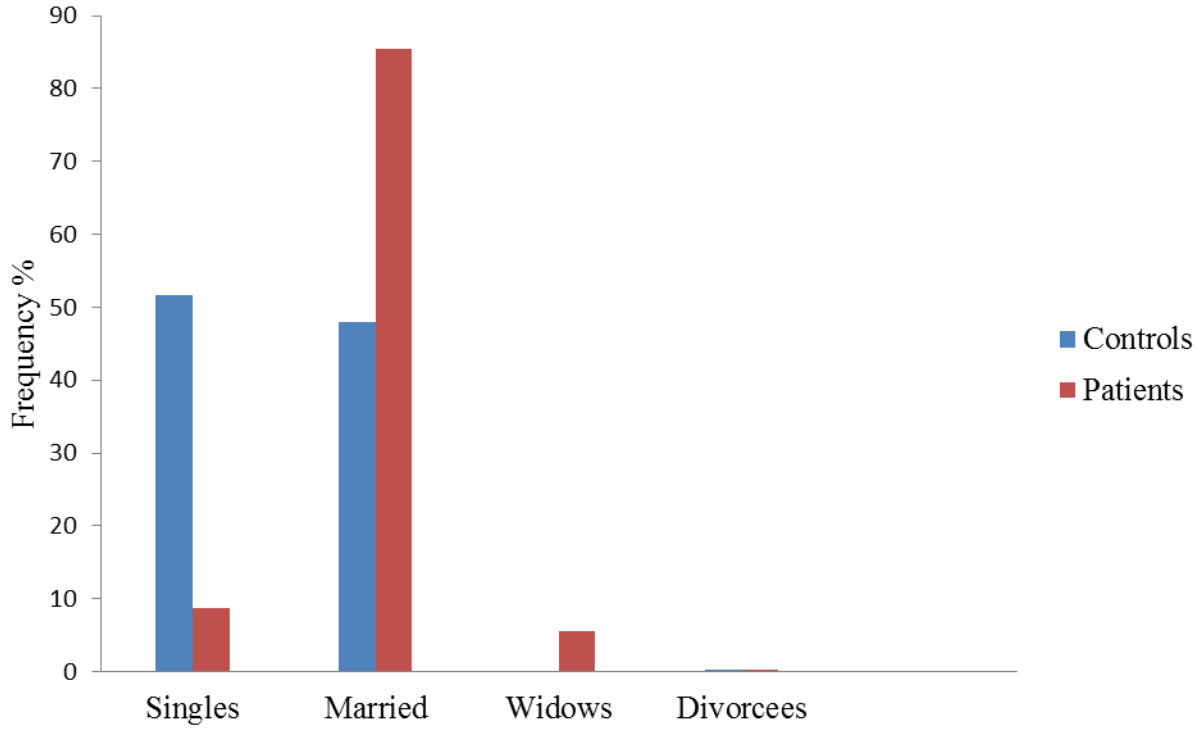


Fig 4.28 Marital status of control and patient groups

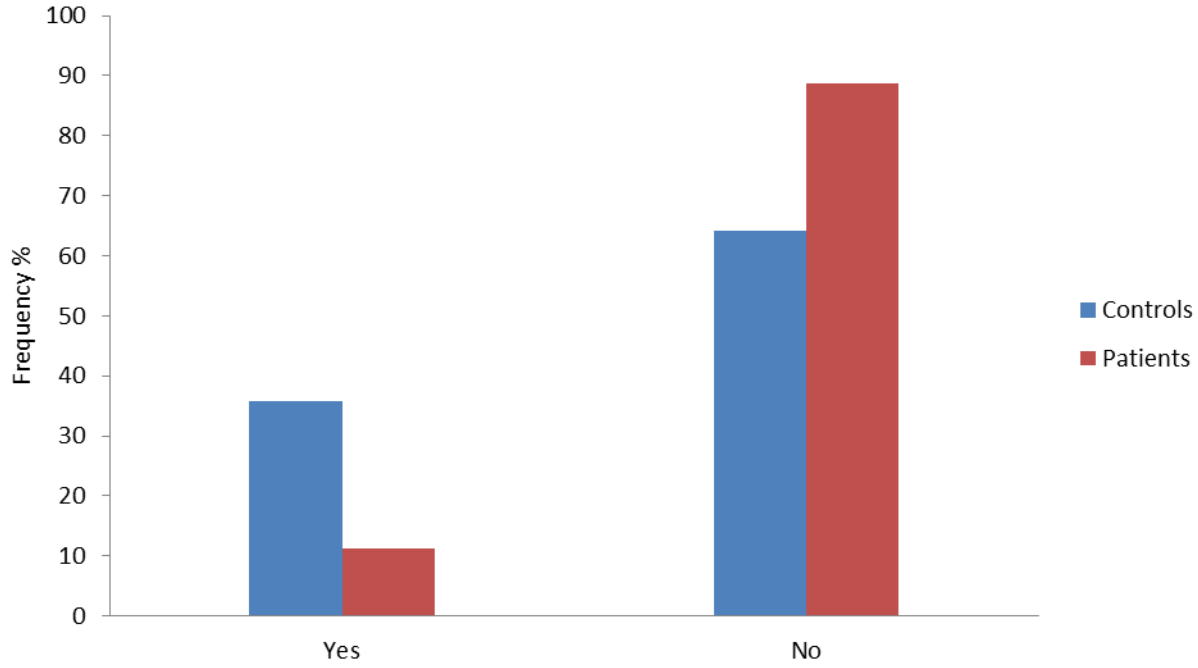


Fig 4.29 Frequency of visits to fast food joints in the control and patient group

Legend:
Individuals who give a yes response visit the fast food joints at least once a week
Individuals who give a no response do not go to the fast food joints at all

4.14 Occupation

Among the patients, the main occupational groups were Pensioners13.6%, Self employed/Business 13.9%, Civil/Public servant 11.4%, Farmers 10%, Traders 7%. Among the controls, the main occupational groups were Academics 29.7%, Self employed/Business 17.2%, Traders 11.9, Civil/Public servant 11.9%, Domestic workers 11.8%. Fig 4.26 -fig 4.29

4.15 Regression analysis

By multiple regression analysis, age was the predictor for SBP and DBP in the hypertensive group r=0.604 SBP, r=0.594 DBP, $p \leq 0.05$. Age was a predictor for DBP in the control group r=0.542, gender was a predictor for SBP in the control group. Gender, body mass index, AGT genotype, ANP genotype, ACE genotype and other independent variables were not predictors for SBP and DBP in the hypertensive group. The influence of AGT genotype on continuous variable was compared using 1 way ANOVA. There was significant differences in the age and systolic in the control group and the systolic in the patient group but no significance differences between the continuous variation of other groups. When blood pressure and other variables using general linear model ANOVA with no adjustments age was significant for the MT and TT variables. See tables in appendix.

4.16 Hardy-Weinberg Theory

The observed frequencies of the polymorphism are not equal to those frequencies predicted by hardy-Weinberg which means there are evolutionary mechanisms influencing the loci under consideration. Thus the population does not meet the assumption of the hardy-Weinberg theory Table 4.2.

4.17 Measurement of plasma angiotensinogen using sandwich ELISA

In the control group, the mean O.D value for plasma angiotensinogen was 0.53 in individuals with the M235T variant; 0.49 in individuals with the T235T variant. There was only one individual with the M235M variant (O.D value 0.28). In the patients, the mean O.D value was 0.71 in individuals with the M235T variant; 0.66 in individuals with the T235T variant. There was also only one individual with the M235M variant (O.D value 0.41) among the patient group. Several dilutions of the purified protein did not yield any results, thus the actual concentration of the protein in the samples could not be determined Table 4.4.

4.18 **Analysis of linkage disequilibrium**

Linkage disequilibrium (LD) was calculated between all possible pairs of the four polymorphisms. The was Linkage disequilibrium between the polymorphisms of the C664G and the M235T D'=1.00; r^2= 0.86 in the control group Table 4.5. In the patient group, Linkage was also observed between C664G and I/D polymorphism D'=0.99; r^2= 0.39 Table 4.6. All other comparison between alleles of the polymorphisms have negative correlation values.

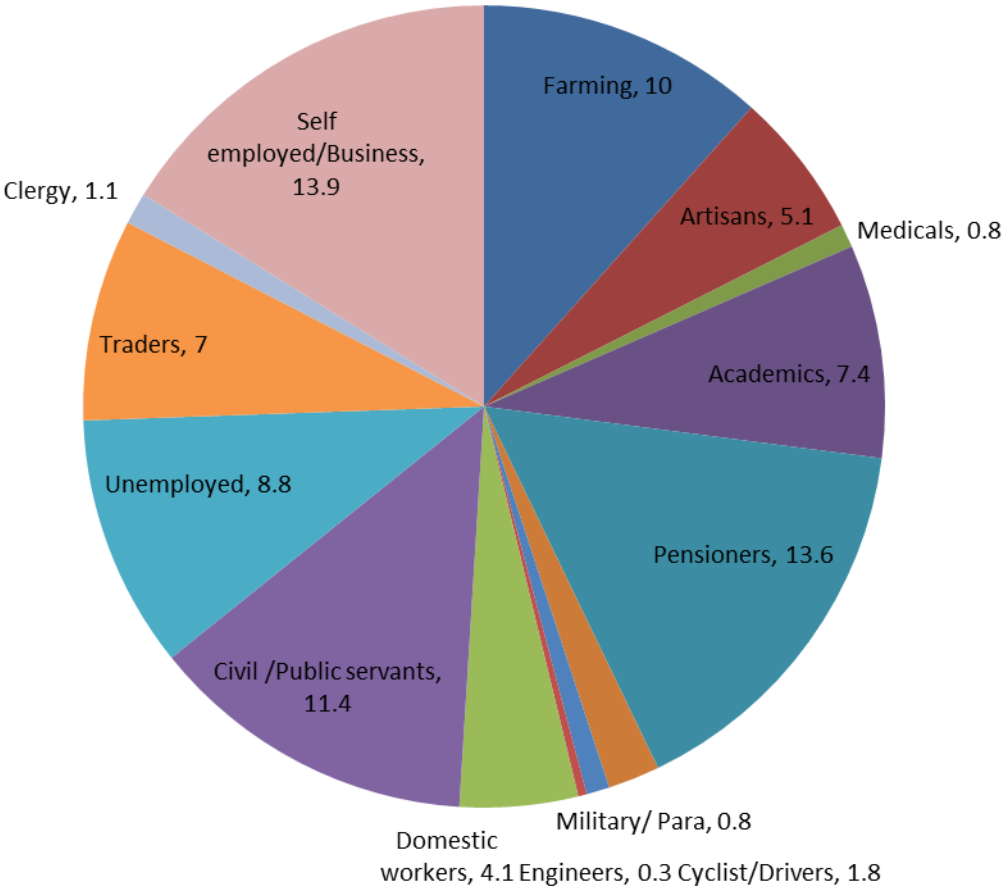


Fig 4.30 Occupational groupings for patient population

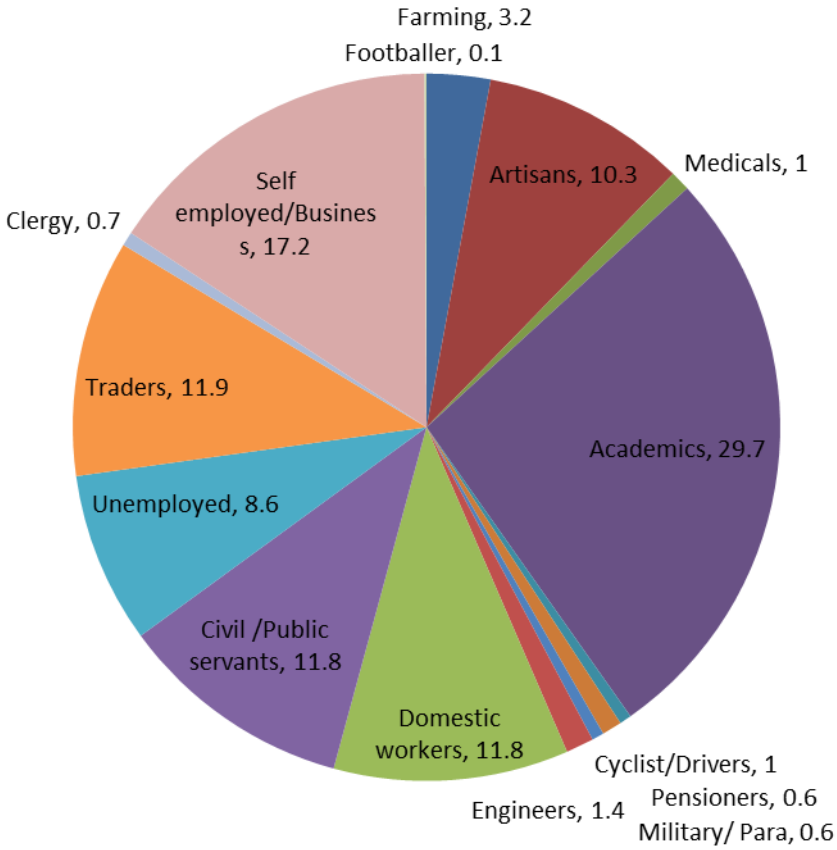


Fig 4.31 Occupational grouping for control population.

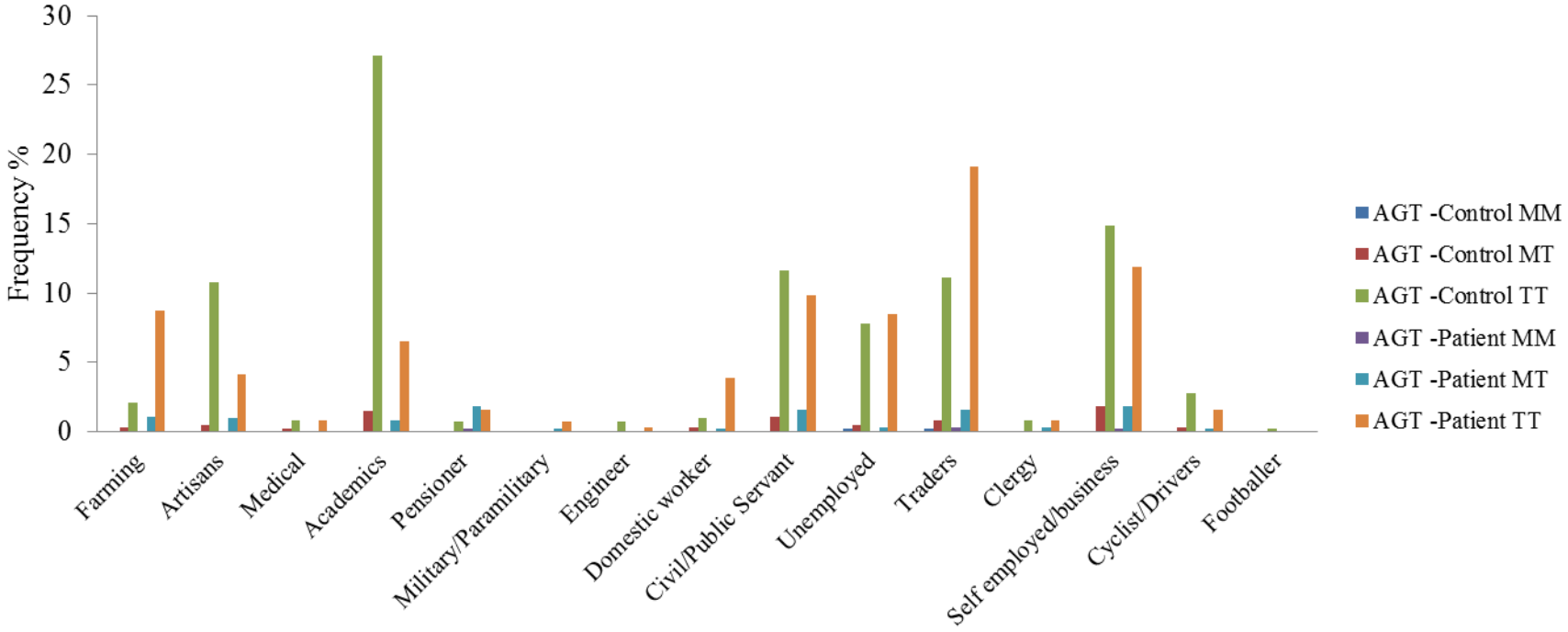


Fig 4.32 Distribution of the AGT M235T polymorphism and occupation among the study population

Legend:
MM are the homozygous dominant individuals
MT are the heterozygous mutant individuals
TT are the homozygous recessive individuals

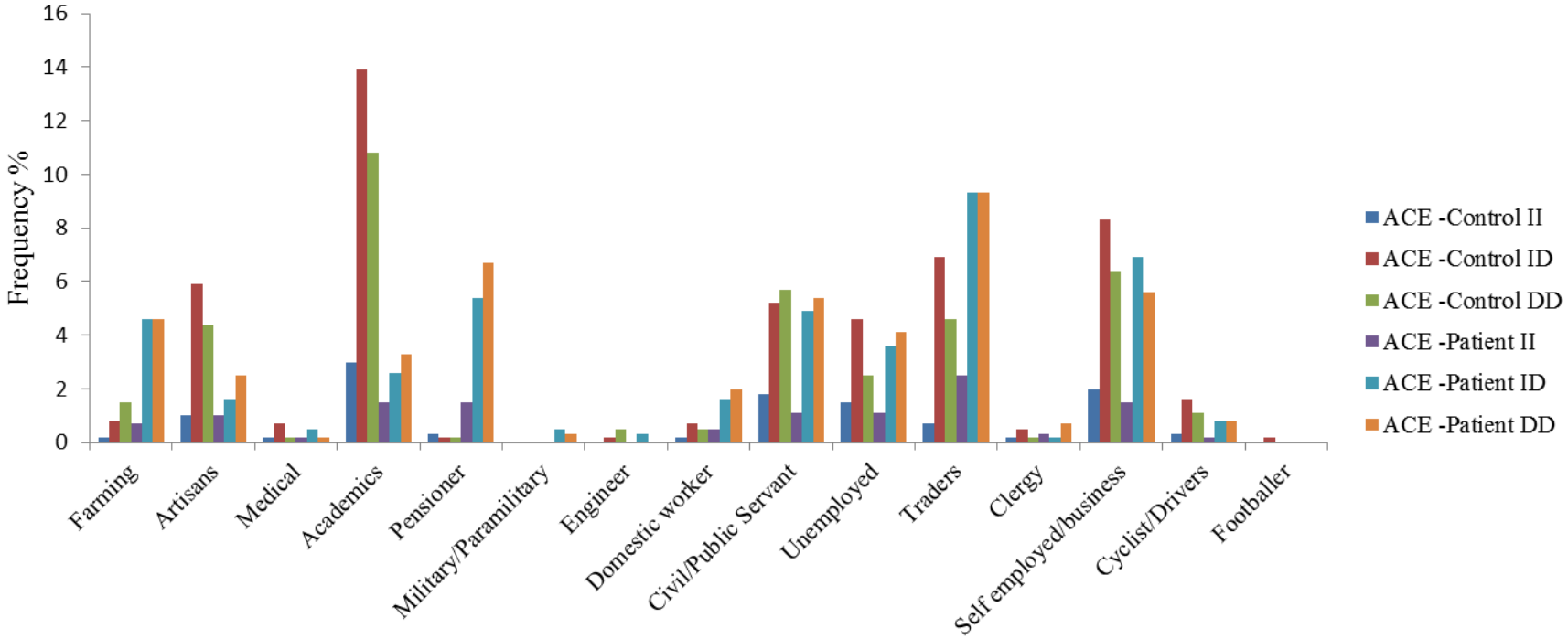


Fig 4.33 Distribution of the ACE I/D polymorphism and occupation among the study population

Legend:
II are the homozygous insertion individuals
ID are the heterozygous individuals
DD are the homozygous deletion individuals

Table 4.4 Characteristics of the individuals used in the protein A sandwich ELISA for the measurement of plasma angiotensinogen

		Controls	Patients
O.D values for ELISA	Ihr	0.16 ± 0.06	0.17 ± 0.05
	3Hrs	0.26 ± 0.12	0.38 ± 0.20
	Overnight	0.50 ± 0.21	0.66 ± 0.33
Genotype	TT	0.49 ± 0.20	0.66 ± 0.33
	MT	0.53 ± 0.27	0.71 ± 0.33
	MM	0.28	0.41
Age		34.56 ± 10.27	53.45 ± 14.14
BMI		23.05 ± 6.51	26.31 ± 5.78
Systolic Blood pressure		115.60 ± 9.63	163 ± 23.87
Diastolic blood pressure		71.39 ± 8.50	94.87 ± 13.59

Table 4.5 Linkage disequilibrium matrix for controls

Row	Marker1	Marker2	Mutual Information	Multi-Allelic D	Multi-Allelic D'	Multi-Allelic R	Multi-Allelic RSquare	ChiSquare df	ChiSquare value	ChiSquare p-value
1	C664G	A1166C	0.0000	0.0000	0.0000	0.0000	0.0000	1	0.0	1.0000
2	C664G	I_D	0.3461	0.1258	1.0000	-0.5799	0.3362	1	411.6	0.0000
3	C664G	M235T	0.8051	0.2312	1.0000	0.9275	0.8602	1	1052.9	0.0000
4	A1166C	I_D	0.3380	0.1240	1.0000	-0.5714	0.3265	1	399.6	0.0000
5	A1166C	M235T	0.7786	0.2278	1.0000	-0.9139	0.8352	1	1022.3	0.0000
6	I_D	M235T	0.3071	0.1164	1.0000	-0.5378	0.2892	1	354.0	0.0000

Table 4.6 Linkage disequilibrium matrix for patients

Row	Marker1	Marker2	Mutual Information	Multi-Allelic D	Multi-Allelic D'	Multi-Allelic R	Multi-Allelic RSquare	ChiSquare df	ChiSquare value	ChiSquare p-value
1	C664G	A1166C	0.9549	0.2471	1.0000	-0.9886	0.9774	1	1196.3166	0.0000
1	C664G	I_D	0.3836	0.1420	0.9900	0.6278	0.3941	1	482.3452	0.0000
1	C664G	M235T	0.0000	0.0000	0.0000	0.0000	0.0000	1	0.0000	1.0000
2	A1166C	I_D	0.3759	0.1403	0.9897	-0.6204	0.3849	1	471.1052	0.0000
1	A1166C	M235T	0.7323	0.2208	1.0000	-0.8885	0.7894	1	964.6408	0.0000
3	I_D	M235T	0.3175	0.1262	0.9862	-0.5615	0.3153	1	385.3202	0.0000

CHAPTER FIVE
DISCUSSION

Genetic variations of genes encoding components of the renin-angiotensin-aldosterone-systems (RAAS) have been associated with susceptibility to hypertension making them strong candidate genes for investigating the genetic basis of hypertension. In addition to the RAAS, the natriuretic peptide system also affects blood pressure directly through its vasodilatory and natriuretic activities and indirectly by inhibiting the RAAS. This has also generated interest in the role of ANP in the development of hypertension. The renin angiotensin aldosterone system plays a major role in blood pressure regulation and angiotensinogen (AGT), a key substrate in this pathway has been an attractive candidate gene for the study of hypertension by many investigators (Inoue *et al.*, 1997; Jain *et al.*, 2002; Markovic *et al.*, 2005).

The T235 mutation was over 90% prevalent in the study population (0.94 for hypertensives and 0.96 for controls) which was very high, this is consistent with literature. Nigerians (specifically among the Yorubas) have been reported to have a high frequency of this molecular variant, between 80-93%. The frequency of this allele is also high in Africans and African Americans, about 0.92 (Rotimi *et al.*, 1994; 1997). The frequency of the T235 mutation among hypertensive Malaysians was reported as 0.45 and 0.75 among the Japanese (Say *et al.*, 2005; Nishiuma *et al.*, 1995).

In the present study, it was found that the frequency of the M235T polymorphism of the AGT gene though very high was not associated with hypertension, the odds ratio for hypertension in this study was 0.65 (95% CI, 0.396 to 1.074) which means in effect that the M234T allele is not a positive predictor for hypertension in the study population. An explanation for this observation could be differences in genetic background. The participants were not selected according to family history of hypertension. A more heterogenous family background could dilute the genetic component and emphasize the importance of enviromental factors.

Results of association studies between angiotensinogen and hypertension have been conflicting; some studies have indicated that the M to T amino acid substitution at position 235 was associated with hypertension in several ethnic groups including Caucasian and Japanese populations (Hata *et al.*, 1994; Matinez *et al.*, 2002, Pereira *et al.*, 2003). Jeunemaitre *et al.* (1992) were the first to report the linkage of the molecular variant M235T with hypertension in Whites/Caucasians. Subsequent studies among Whites/Caucasians supported an association (Schmidt *et al.*, 1997). Early Chinese and Taiwanese studies had also reported positive associations with hypertension (Niu *et al.*, 1999; Chiang *et al.*, 1997). Studies in the Japanese population found a positive association between hypertension and the M235T variant with an odds ratio of 2.67 (Hata *et al.*, 1994; Nishiuma *et al.*, 1995).

Markovic *et al.* (2005) reported a significant association of nucleotide base substitutions at position 6, 29, 793, and 776 in the promoter region of the angiotensinogen gene with hypertension in African-Americans and Caucasians.

Kunz *et al.* (1997) in a meta analysis performed to examine the association of the 235T allele and hypertension in 5493 White patients showed an odds ratio of 1.20 (95% CI, 1.11 to 1.29), the odds increased to 1.42 (95% CI, 1.25 to 1.61) in subjects with a positive history of hypertension. Pereira *et al.* (2003) reported an odds ratio of 1.33 (95% CI, 1-04 to 1.70) for hypertension in individuals with the TT genotype.

Most females (both in controls and patients) were of the T235T genotype. A study had indicated that homozygosity for the Thr 235 allele predicted risk for hypertension in women and not in men (Sethi *et al.*, 2001). Other studies provided no evidence for association with essential hypertension while stratifying for sex (Hegele *et al.*, 1994; Fornage *et al.*, 1995). Periera *et al.* (2003) also reported no association between gender and T allele in a cross sectional study involving 647 females and 776 males though the study was not confined to essential hypertensive patients only. Lindan *et al.* (2010) observed a significant association of the TT genotype of the AGT polymorphism with essential hypertension in Han Chinese, an odds ratio of 1.54 (95% CI, 1.16 -2.03). Say *et al.* (2005) reported a high odds ratio for the TT genotype of the AGT allele with hypertension among Malaysian subjects 1.98 (95% CI, 1.46 to 2.67). The T235T variant was also reported to be associated with preeclampsia (Ward *et al.*, 1993; Procopciuc *et al.*, 2002). In Han Chinese women, maternal AGT allele was reported to have no effect on the risk of pregnancy-induced hypertension (PIH) but fetal 235T allele was significantly associated with PIH in the women. Fetuses with the TT genotype have a protective effect against PIH in the study population (Xiang *et al*, 2011).

Other studies on White/Caucasians did not support any association of the M235T variant with hypertension (Fornage *et al.*, 1995; Hegele *et al.*, 1997). Some Japanese studies did not find any association between the M235T and hypertension (Morise *et al.*, 1995; Iwai *et al.*, 1995). Association studies in Africans and African-Americans also found a negative association(Rotimi *et al.*, 1994; Caulfield *et al.*, 1995; Wu *et al.*, 2003).

The mechanism by which the molecular variant M235T allele of the AGT gene is related to hypertension is poorly understood. Although the AGT 235T allele was found to be in complete linkage disequilibrium with a guanine to adenosine transition at -6bp upstream of the initiation site of transcription (Inoue *et al.*, 1997). *In vitro* test of promoter activity and DNA binding studies with nuclear proteins show that this nucleotide substitution affects the basal transcription rate of the gene in various cell lines thereby affecting the AGT T235 variant, increased plasma AGT levels that might lead to increased blood pressure (Jeunemaitre *et al.*, 1992c).

The T allele was associated with increased plasma AGT (Bloem *et al.*, 1997, Rotimi *et al.*, 1997). Elevation in plasma levels of angiotensinogen has been associated with hypertension. Well developed assays to measure this protein are few and expensive. Previous research carried out usually measure renin using the Plasma Renin Activity (PRA) test involving a radioimmuno precipitation method. The present study used a sandwich ELISA method to assess the levels of plasma angiotensinogen in the participants. The mean O.D values for plasma angiotensinogen was significantly higher in the patients than the controls with the mutant T allele implying an association with hypertension. There is need to further improve this technique which might be a useful tool in measuring angiotensinogen for diagnostic purposes. The protein A sandwich ELISA was sensitive enough to identify the presence of the angiotensinogen in the plasma of patients and controls, the O.D values indicate the presence of higher concentration of angiotensinogen in the patients than in the control which is in line with literature (Jeunemaitre *et al.*, 1992 and Corvol *et al.*, 1999). Corvol and Jeunemaitre (1997) reported that the M235T allele was associated with a 10-30% increase in plasma angiotensinogen which is able to increase blood pressure,

thus facilitating hypertension. The limitation of this study was the fact that the assay could not pick up the standard angiotensinogen dilutions to enable a proper interpretation of the concentration of angiotensinogen in each plasma sample. More work still needs to be carried out in this area.

The ACE genotype frequencies were 73(12%), 262(43%) and 277(45%) for the II, ID, DD respectively in the patient group. The ACE alleles were 85(12%), 341(49%), 270(45%) for the II, ID, DD respectively in the control group. A higher frequency of the ID allele was observed in controls of which 208(61%) were females. Among the major ethnic groups residing in the two towns, the D allele frequency was 70% and the I allele was 30% which was high compared to literature. Rotimi *et al.* (1997) reported the frequency of the D allele among African Americans as 63% while Morshed *et al.* (2002) reported 69.3% for the D allele in hypertensives and 45.7% in controls. He also observed a higher frequency of the I allele in the controls (54.2%) than the hypertensives (50%). Wang *et al.* (2005) reported the D allele frequency as 40.8% which is lower than what was obtained by O'Donnel *et al.* (1998) in European samples (55.3%). Kario *et al.* (1996) reported a frequency of 34% for Japanese individuals. Dankova *et al.* (2009) reported 0.53 frequency for the mutant D allele in Slovak subjects and 0.447 in Romany subjects. A frequency of 52.9% for patients and 56.3% for controls was reported for the D allele in a Lebanese diabetic cohort by Chmaisse *et al.* (2009). Ismail *et al.* (2004) reported a significantly higher frequency (0.55) of the ACE II genotype in the hypertensive group than in the control group of the same age but no overall significant differences were observed between the II, ID, DD ACE genotypes. The D allele has been associated with hypertension in some studies in White American and Japanese men but not in women (O'Donnel *et al.*, 1998; Katsuya *et al.*, 1998). Sagnella *et al.* (1999) however observed a significant association between the D allele and hypertension in women of African descent. Many studies have failed to establish an association between the D allele and hypertension (Hsieh *et al.*, 2000). A strong association of the I allele was found in an Australian population with familial hypertension (Zee *et al.*, 1992). The conflicting results of I/D polymorphism of the ACE gene in hypertension has been attributed to gender and ethnic differences (Ramachandran *et al.*, 2008).

In this study, the I/D allele of the ACE gene is associated with an increased risk for hypertension with an odds ratio of 1.15(95% CI, 0.924 -1.456). Ji *et al.* (2010) observed a higher odds ratio of 1.61(95% CI, 1.32 – 1.98) for the ACE gene among the Han Chinese population. Sagnella *et al.* (1999) reported an odds ratio of 1.65 (95% CI, 1.04- 2.64) in women of African descent (OR=2.54; 95%CI, 1.38 -4.65) but not in men of African descent (OR=0.79; 95% CI, 0.36 – 1.72). Bhavani *et al.* (2004) reported a significant association of the ACE I/D allele with hypertension in men with age adjusted odds ratio of 2.25 (95% CI, 1.14 – 4.42) and 2.20 (95% CI, 1.22 – 3.80) for DD and ID respectively. In women there was no significant association of ACE genotype with hypertension, age adjusted odds ratio being 1.20 (95% CI, 0.38 – 3.92) and 0.44 (95% CI, 0.17 – 1.06) respectively for the DD and ID genotypes. Das *et al.* (2008) observed that the odds of being hypertensive in a population of Asian Indians of Calcutta was 7.48 (95%CI, 1.75 – 30.190) in the DD homozygous individual suggesting a very strong association of the ACE polymorphism with essential hypertension in Asian Indians. Sameer *et al.* (2010) observed a strong association between the ACE polymorphism and hypertension among the peoples of Kashmir, India.

World distribution of the D allele according to Salem (2008) suggest that the I/D polymorphism in the human ACE gene is of African origin. The allele is believed to have moved out of Africa with Paleolithic (second part of the stone age that began about 750,000 to 500,000 BC and lasted until the end of the ice age about 8,500BC) migrations 100.000 years ago. The ACE I/D polymorphism is due to an insertion of a 287bp AluYa5 element into intron 16 of the gene (Rigat *et al.*, 1992). This insertion is believed to have occurred a few million years ago during the evolution of primates (Jurka, 2004). Although an insertion or a deletion event is implied in the I/D polymorphism, only an insertion event occurred. This makes the D allele without an insertion the ancestral state of the gene. Primate specific Alu elements have been reported to be the most abundant transposable elements in the human genome making up more than 10% (Batzer and Deininger, 2002). The mechanism by which D allele leads to blood pressure elevation is not clearly documented in literature.
It has been observed that there were differences in the response of different ethnic groups to ACE blockers and diuretics in the treatment of hypertension (Douglas *et al.*, 2003; Wright *et al.*, 2005). Whites were observed to have better response to ACE blockers than Blacks (those of African ancestry) while the reverse was the case with diuretics ACE blockers or inhibitors are a type of drug commonly used to treat hypertension (Gard, 2010). These drugs reduce the activity of angiotensin converting enzyme, thus reducing the concentration or levels of angiotensin II facilitating a reduction in hypertension (Johnson *et al.*, 2008). Exner *et al.* (2001) found in a one year therapy with the ACE inhibitor enalapril, that it was associated with significant reductions in blood pressure in White patients but not among black patients, using cardiovascular consequences such as fatal and non fatal myocardial infarction and heart failure as end points. The D allele of the ACE I/D polymorphism is associated with increased ACE activity which is associated with increased incidence of cardiovascular disease and a resistance to ACE inhibitor therapy. The molecular mechanisms are not clearly explained in literature (Wright *et al.*, 2005).

In Caucasians and Asians, the *ACE* polymorphism has been observed to produce higher levels of protein in the blood, meaning that if these patients require treatment for high blood pressure, they should not respond to ACE inhibitors. However, African Americans have the same gene variant but this does not increase the blood protein levels in this population, thus ACE inhibitors should work in this population. This is conflicting with what is generally reported among blacks. The reason for this difference is unclear (Mellen and Herring, 2005).

Mc Donagh *et al*. (2011) further explained that the plasma soluble form of ACE is not involved in the cleavage of angiotensin I to angiotensin II and that ACE inhibitors usually target tissue bound ACE that are actually involve in angiotensin I conversion thereby resulting in the down regulation of angiotensin II that subsequently lowers blood pressure. However Gainer *et al,* (2001) reported that bradykinin which is a potent vasodilator contributes to the effect of angiotensin-converting enzyme ACE inhibition in humans. Decreased production of bradykinin or decreased vasodilation in response to bradykinin is thought to play a role in hypertension and also in the decreased antihypertensive response to ACE inhibition in Blacks (Gainer *et al*., 2001)

The CC allele of the AT1R gene was not observed in this study population. 99% of the population had the AA wild type gene. This is in contrast to what is reported in some literature. A high prevalence of the CC genotype was observed in Chinese hypertensives than controls (Ono *et al.*, 2003). In a sample of Swedish twins, Iliadou *et al.* (2002) did not observe any association between the ACE I/D

polymorphism or AT1R $A^{1166}C$ polymorphism and blood pressure. Schmidt *et al.* (1997) also did not detect any association between the A1166C allele and hypertension but a decreased prevalence of C allele was observed among hypertensives. Tiret *et al.* (1998) reported a higher prevalence of the C allele among female hypertensives than controls but no such observation among men. A higher prevalence of the CC genotype was observed in Chinese hypertensives than controls (Jiang *et al.*, 2001).

Generally large interethnic differences in the frequencies of genotype polymorphisms of the RAS exist in different populations. To explain ethnic differences observed in allele frequency in different study populations, Lynch *et al.* (2008) reported that there are differences in linkage disequilibrium for different loci among various populations, if such differences exist in regions where these variants are found and such variants are not causal but are in linkage disequilibrium with the putative locus, this might explain the inconsistency in results across population. Among the Efiks and the Ibibios, the major ethnic group in this study population, the frequency of the A allele was 99.7 and 0.3 for C allele, the average C allele frequency reported in the Chinese population is 0.11. In another study, the homozygous A1166 allele frequency was 92.8 among the studied subjects. The frequency of homozygous A allele was significantly higher in the hypertensives than the normotensive subjects (97.5% and 80% respectively) with a higher frequency among male patients (Farrag *et al.*, 2011).

Farrag *et al.* (2011) proposed that the A allele may be a predisposing factor for essential hypertension in Egyptians. Zhenni *et al*. (2001) observed only two genotypes AA and AC of the AT1IR polymorphism, but reported a higher frequency of the A allele among the patients than the controls. Lee and Kim (2003) observed 96% and 6% for the A and C allele of the angiotensin 11 type 1 receptor polymorphism in Korea. Hahntow *et al.* (2010) found the A allele to be associated with high blood pressure. They also reported that the A allele showing an association was not totally out of place given the fact that this locus had no major impact on hypertension phenotype as suggested by genome wide studies (Wu *et al.*, 2006; Rice *et al.*, 2006 and Newton-Cheh *et al.*, 2009). Other studies (Stankovic *et al.*, 2003; Ono *et al.*, 2003) have reported the C allele instead to be associated with hypertension.

The -C664G mutant of the atrial natriuretic peptide gene was not observed in this population. The wild type C664C allele of this polymorphism was present in both patient and control groups. This implies that this mutation has not been introduced into this population. however Rubattu *et al.* (2006) observed a 97.4% of the C664C allele and 2.6% of the C664G allele among hypertensive patients in Milan, Spain. Though the C664C allele frequency was high, the C664G allele was associated with left ventricular mass index in hypertension. Rubattu *et al.* (2007) also found that young men heterozygous for the G allele had an increased risk for an early onset of hypertension. The C664G, G1837A AND T2238C of the ANP gene has been investigated in association with hypertension and found to be monomorphic among the Chinese (Xue *et al.*, 2008). More research needs to be carried out to confirm these results.

Among the ethnic groups of the sample population, the Efiks were 384 persons and the Ibibio's were 487 persons, the Efiks and Ibibios made up the main ethnic groups in this study as is logical since the study was conducted in Calabar and Uyo.

Most patients were married and advanced in age (above 40 years). Increase in age is thought to increase blood pressure because the arteries become hardened, less

active, kidney function decreases and the body does not process salt as well as before (Lloyd-Jones *et al.*, 2005). It was observed that most individuals below the age of 40 years were normotensives.

Awareness of hypertension status was about 70%. The remaining 30% consisted of individuals who were first timers at the clinic; individuals from the population whose blood pressure readings were discovered to have increased and had to register at the hypertension clinic for proper follow up.

Tobacco smoking has been shown to increase blood pressure, blood pressure was observed to decrease in smokers who did not smoke for a week (John *et al.*, 2006). Smoking and alcohol consumption was low in both populations. The hypertensive had been educated by their doctors not to consume alcohol. The reason for abstinence among controls was due mainly to their religious beliefs. The possibility exist that participants did not give a truthful answer because drinking is culturally frowned at.

Strolls were the most frequent exercise on a regular basis reportedly carried out by both patients and controls, though 47.9% of patients did not perform any form of exercise (this group was made up mainly of the very elderly, above 60 years of age). Lack of exercise makes it easier to become overweight and increases the chances of high blood pressure. Exercise is an important treatment for hypertension, only people with severe uncontrolled hypertension are advised not to carry out any exercise. People whose hypertension are less severe or controlled by drugs are advised to participate in exercise as a way of managing their blood pressure.

Salt intake was considered 'normal' for persons who do not consume raw salt except for the quantity used in food preparation. Those classified into high salt intake group actually consume raw salt that is use it to drink garri, eat dry fish and add extra salt to their food when they feel the salt content is not enough. Salt metabolism and its effect on hypertension are extremely complex. Primary or essential hypertension and age-related increases in blood are almost absent in populations where individual consumption of salt (sodium chloride) is less than 50mmol per day but mainly observed in population in which people consume more than a 100mmol of salt per day (Kaplan, 2006). Although individual salt intake in most populations throughout the world exceeds 100mmol per day, most people remain normotensive. This implies that salt intake that exceeds 50 - 100mmol per day is necessary but not sufficient for an individual to develop the disease, other factors come into play.

Weinberger *et al.* (2001) carried out a study and concluded that it is not the hypertension produced by salt that is the most important cause of health problems; instead it is whether the individual is salt sensitive (salt sensitivity is an increase in blood pressure in response to a higher salt intake than that in a baseline diet (Morris *et al.*, 1999). For salt-sensitive individuals, the risk of dying from cardiovascular problems is increased with high dietary salt whether they are hypertensive or not. Iwamoto *et al.* (2004) explained how excess salt affects high blood pressure. The key substance is a hormone ouabain secreted by the adrenals which in turn affects two proteins that together regulate the sodium-calcium content of the smooth muscle cells of arteries. Excess salt intake stimulates the secretion of excess ouabain; this upsets the balance by disabling the sodium pump and causes sodium to accumulate in the artery cell. The excess sodium causes the Na - Ca2+ exchanger protein to bring in more calcium to replace the sodium and this in turn triggers artery constriction and hypertension. Another explanation for salt as predisposing factor for hypertension is that the human kidneys usually retains sodium and excrete potassium. Prehistoric

human who consumed a poor sodium and potassium rich diet were favoured by this mechanism. In this case sodium excretion is negligible and potassium excretion is high thus the need for potassium rich foods. This mechanism does not favour our modern day sodium- rich and potassium- poor diet. The end result of the failure of the kidney to adapt to this diet is an excess of sodium and a deficit of potassium in hypertensive patients. (Adrogué and Madias, 2007).

Fast foods have become popular in the western world due to their taste, convenience and affordability. Fast foods have gained popularity among Nigerians though it is common among the rich because it is expensive. Fast foods contain high amount of saturated fats, trans fats, salt and in some cases sugars. Chronic consumptions of high amounts of fast foods for a long time increases a person's risk for adverse health conditions such as hypertension, atherosclerosis and heart failure. In this study, patients (89%) did not go to fast food joints, the reason being that doctors had advised against it. Even among controls, visit to fast food joints was still very low. The people of these areas still prefer to eat their local dishes of vegetables, root tuber and fresh sea foods. But this trend is likely to change in the near future with more fast food joints opening up and the people, youths to be precise, embracing the western way of living.

A higher patient number do not consume snacks because they were advised by the doctors in the clinics to reduce intake of snacks for health reasons.

Most controls in the study had a BMI less than or equal to 67.10%. In the patient population, overweight 38.23% and obesity 30.23% reach a moderate prevalence among patients. Hypertension has been reported to be strongly correlated with BMI. Weight gain in adulthood is seen as an important risk factor for hypertension (Jafar *et al.*, 2006). Humayan *et al.* (2009) observed a high trend of hypertension with increasing BMI among Pakistanis, with a high incidence among females whose weight was above normal that is less than 24.9 kg/m^2. Positive associations between body mass index and blood pressure have also been documented in cross sectional studies in different Asian population. Ethnic differences existed in the association between BMI and hypertension and in optimal mi cutoffs for overweight Chinese, Indonesians and Vietnamese adults. (Stamler *et al.*, 1978; Stevens *et al.*, 2002; 2008; Bell *et al.*, 2002; Tuan *et al.*, 2009)

All the hypertensinogenic factors considered did not contribute significantly to the disease when regression analysis was carried out.

Potential risk factors for hypertension are not only genetic variables but also psychological and environmental influences. Hypertension is therefore the end result of various events that develop gradually over many years. Studies have demonstrated a negative association of cardiovascular disease mortality and morbidity as measured by education and or occupation. These studies indicate that individuals with lower socioeconomic status were more likely to have cardiovascular diseases (Vargas, 2000). Socioeconomic differences play a significant role in the health status of a population and are likely to influence the pathogenesis of hypertension and access to preventive health services. It has been demonstrated that education may be the most judicious measure of socio economic status for epidemiological studies.

Education plays an important role in guarding against disease influenced by one's lifestyle (Liberatos *et al.*, 1988; Vargas, 2000). Hypertension, diabetes are common causes of cardiac, cerebrovascular and renal diseases, they are easily diagnosed and can be treated effectively to reduce death. Tedesco *et al.* (2001) reported that low knowledge of hypertension and its risk factor among the uneducated, made symptomless patients unwilling to alter their lifestyle, take

medication and visit health facilities when necessary to forestall some poorly perceived danger while the educated subjects are more likely to consider the health care need as a priority. In this study, the primary level of education had the highest number of hypertensives (40%), followed by the secondary level (27%). Those with no formal education were the least with 16.2%.This results suggest an association between low levels of education and the development of hypertension thus highlighting the need for increased awareness campaigns to enlighten the less educated to make them aware of the disease and the health facilities available to them. Adedoyin *et al.* (2005) reported low socio-economic status to have an inversely significant effect on systolic, heart rate and pulse rate thus implicating socio economic status in the development of hypertension among sedentary Nigerian adults.

Population geneticist study frequencies of genotypes and alleles within populations, by comparing these frequencies with those predicted by null models that assume no evolutionary mechanisms are acting within the population; they draw conclusions regarding the evolutionary forces in operation. The hardy-Weinberg Equilibrium law serves as the basis null model for population genetics. Populations will confirm to the Hardy-Weinberg law only if no evolutionary forces influence the loci under consideration. These evolutionary forces include large population size where there is no genetic drift. Chance can alter allele frequencies through mating processes and death within small populations. Random mating where choice of mates by individuals is by chance and not influence by the genotype of the individuals in question. In natural population matings are random with respect to certain characteristics such as blood group but for some other characters such as tribes, matings are not random. This will no doubt affect the distribution of allele frequencies. There are also differences in the mutation rates between alleles at the same locus with natural selection favouring some alleles in some population. Reproductive isolation from other populations which means that there is no gene flow or migration into the population. With our efficient systems of transportation, migration has introduced a lot of genes into populations where they were not found initially. There should be no differential survival or reproduction among phenotypes that is natural selection does not act on the individuals (Wigginton *et al.*, 2005). Due to selection, some phenotypes have been selectively favoured to suit different environments and of course reproduction will be more common among these phenotypes. The population under study does not conform to the hardy-Weinberg equilibrium theory which means some of these evolutionary forces are acting on the loci under consideration.

There was linkage disequilibrium between the C664G and M235T polymorphisms in the controls and between C664G and I/D alleles in the patients. LD implies correlation between loci. This could mean that the alleles of the C664G and I/D polymorphism belong to the same haplotype block. In otherwords they are inherited together more often than chance would dictate. LD throughout a particular genome reflects the population's history, breeding systems and the pattern of geographic subdivision. While LD in a genomic region reflects the history of natural selection, gene conversion, mutation and other forces that could cause gene-frequency evolution (Slatkin, 2008). Since the study population was not a completely homogenous population from a particular area, these associations need further investigations in a more homogenous population.

The results obtained from this study will form baseline information for these areas but more work still needs to be carried out to confirm these genotypes in a larger population.

Other genes like the Corin, fumin and genes that regulate other pathways outside the RAAS have also yielded results that have contributed to the molecular understanding of the molecular basis of hypertension but there still some polymorphism that act in linkage disequilibrium with the already identified gene to produce disease. These genes need to be identified to properly explain the molecular basis of hypertension.

Candidate gene analysis and Linkage studies are limited in its explanation for the molecular basis of hypertension due to conflicting results from various studies but with the advent of Genome wide Association Studies (GWAS), significant strides have been made in our understanding and knowledge of the genetic basis of human complex diseases compared with the pre GWAS approaches. Already Genome wide studies have identified over 300 genes associated with cardiovascular diseases in the last few years.

It is needful to investigate other genetic variations using Genome wide studies to discover additional disease-associated genes to explain the heritability of hypertension among the peoples of Calabar and Uyo, Nigeria.

Contributions to knowledge: In this study, the ethnic populations in Calabar and Uyo were screened for 3 polymorphisms associated with genes of the of the renin-angiotensin aldosterone system RAAS. Only one of the polymorphism, the Insertion/Deletion polymorphism of the angiotensin converting enzyme ACE gene was significantly associated with hypertension. Thus the ACE gene polymorphism is a molecular marker for hypertension in the study population.

Angiotensinogen levels in plasma samples of participants was also measured the mean O.D. values for angiotensinogen was significantly higher in the patients than in the control when the O.D. vales were compared between patients and controls using student T test.

The population was also screened for another polymorphism associated with hypertension, the atrial natriuretic peptide gene which plays an important role in a system that is in contrast to the RAAS. The mutant allele was not observed in the study population.

Enviromental factors that are predisposing factors to hypertension were studied. Except for age and gender, all other factors were not significant predisposing factors for hypertension in the study population when regression analysis was carried out.

This research will form baseline information for subsequent molecular studies in this population.

CONCLUSION

In this study, the frequency of the M235T variant of the AGT gene is high particularly the AGT M235T homozygous genotype. The M235T mutation, the angiotensin II type 1 receptor gene and atrial natriuretic peptide gene are not independent risk factors for hypertension in the sample population. However the I/D polymorphism of the ACE gene was associated with an increased risk for hypertension in the sample population collected from Calabar and the Uyo. The C664G variant of the atrial natriuretic peptide gene was not observed in this population. Linkage disequilibrium was observed among the patients, this is inconclusive due to the heterogeneity of the study population. Blood pressure is a complex phenotype with many physiologic pathways and compensatory system such that any gene or polymorphism within a gene will only explain a small part of the

variability when studied in isolation from other genes and polymorphism acting in concert to control blood pressure. Due to the fact that blood pressure is a physiologic series of checks and balances, it might be more meaningful to study the genetic effect of a group of genes acting on a particular physiologic pathway in a large more homogenous population to make definite conclusions.

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APPENDICES

Extraction buffer: To prepare 1000mls

100mM Tris ÷ 1000 = 0.1M

8.5mM EDTA ÷ 1000 = 0.0085M

500mM Nacl2 ÷ 0.5M

$$\text{Tris} = \frac{0.1 \times 121.14 \times 1000}{1000} = 12.114\text{g}$$

$$\text{EDTA} = \frac{0.0085 \times 372.24 \times 1000}{1000} = 3.164\text{g}$$

$$\text{Nacl} = \frac{0.5 \times 58.44 \times 1000}{1000} = 29.22\text{g}$$

The salts were weighed and added to about 500mls of distilled water, stirred until complete dissolution of the salts. Distilled water was added to make up to 1000 and the pH adjusted to 8.0. The solution was autoclaved.

20% Sodium dodecyl sulphate – SDS: 100ML 20G OF SDS salt was weighed and added to 60/80ml of distilled water. The solution was stirred for a while until complete dissolution of the salt. Distilled water was then added to make up to 100mls.

5M KOAC – Potassium Acetate

using the formular $Xg = \frac{\text{molarity} \times \text{molecular weight} \times \text{volume (ml)}}{1000}$

$$= \frac{5 \times 98.15 \times 10\text{ml}}{1000}$$

$$= 4.9075$$

Salt (4.90) was weighed and added to 5ml of sterile distilled water, stirred until the salt dissolved completely sterile distilled water was added to make up to 10mls.

Tris – Ethylene diamine tetra-acetate buffer (T.E.) buffer.

10mM Tris ÷ 1000 = 0.1M Tris

1mM EDTA ÷ 1000 = 0.001M EDTA

Tris Xg = $\frac{0.01 \times 121.14 \times 1000}{1000} = \frac{1.211}{2}$

5000ml = 0.605g of salt

EDTA Xg = $\frac{0.001 \times 372.24 \times 1000}{1000} = \frac{0.372}{2}$

500ml = 0.186

To prepare 500mls of T.E.: 0. 605g of Tris molecular grade and 0.186g of EDTA were added to 300mls of distilled water, stirred until complete dissolution of the salts and distilled water was then added to make 500mls and the solution was autoclaved.

Tris – EDTA buffer for filter paper extraction.

10mM ÷ 1000 = 0.1M Tris base

Xg = $\frac{0.1 \times 121.14 \times 500mls}{1000}$

= 0.6057g of Tris

0.1mM EDTA ÷ 1000 = 0.0001m

Xg = $\frac{0.0001 \times 372.24\ 50mls}{1000}$

= 0.0186 of EDTA.

Both salts were weighed and added to 350mls of sterile distilled water, stirred until complete dissolution of salts. Then sterile distilled water was added to make up500mls.

Reconstitution of Primer

R 31.4mol x 10 = 314ul
F 32.7nmol x 10 = 327ul

) This amount of T.E

was added to the lyophilised powder. For working dilution 90ul of T.E was added to 10ul of reconstituted lyophilized powder to make 100ul.

Gel preparation.

50 x TAE was diluted to 1 X by diluting 20mls of 50x in 1000ml of distilled water. The ends of the plastic tray supplied with the electrophoresis apparatus was sealed with adhesive tape. 100ml of 1× TAE was measured and put into a bottle; 1.5g of agarose powder was added and heated in the microwave oven for 5 min to dissolve the agarose.

The bottle was then allowed to cool to about 60^{o}C; 5µl of ethidium bromide was added.

When checking for DNA quality, 0.8g of agarose and 0.8ul of ethidium bromide was used. The agarose was poured into the tray and allowed to set at room temperature (30 – 45 mins).

Preparation of 10mM dNTP from 100mM stock (dNTP components comes as 100Mm stock concentration, the kit is made up of: dATP; dCTP; dGTP and dTTP. For a volume of 500µl

$$C1V1 = C2V2$$

$$100mM \times V1 = 10mM \times 500$$

$$V1 = \frac{10 \times 500}{100}$$

$$V1 = 50\mu l$$

50µl of dATP; dCTP; dGTP and dTTP were measured making a total of 200µl, 300µl of sterile water was added to make up 500µl.

Preparation of ELISA buffers.

4 Liters of Phosphate Buffer Saline – Tween (PBS-Tween) X10 pH 7.4

Sodium Chloride	320g
Potassium phosphate (monobasic)	8g
Sodium phosphate (diabasic)	44g
Potassium chloride	8g
Tween	20mls

All the salts were measured and dissolved in distilled water, put on a magnetic stirrer for proper dissolution of the salts, the pH was adjusted using a Ph meter and tween 20 was added.

To make 1 strength of PBS-Tween for washing, 200mls of the 10X stock was measured into measuring cylinder, distilled water was added to make 2000mls of wash solution.

1 liter of Conjugate Buffer

25mls of PBS without tween was added to 475 mls of distilled water containing

1g of PVP (Polyvinyl pyrrolidone)

0.1g of Albumin

250µl of Tween 20 was then added to make 500mls

1 Liter of Coating Buffer P^{H} 9.6

1.59g of Sodium Carbonate

2.93g of Sodium hydrogen Carbonate

Were measured and diluted with distilled water, the P^{H} was adjusted to 9.6

1 Liter of Substrate Buffer P^{H} 9.8

97 mls Diethanolamine

800 mls Distilled Water

The PH was adjusted using HCL and water added to make 1000mls.

Buffers were stored at 4^{o}C and fresh buffers were prepared if the already prepared buffers were not used within one month. Buffers were check every two weeks for contaminants.

INFORMED CONSENT FORM

My name is **Mary Esien Kooffreh,** I am a staff of the Dept of Genetics and Biotechnology, University of Calabar and currently a Ph.D student of the Dept of Zoology, University of Ibadan, Ibadan.

We are carrying out a survey to find how many people are hypertensive or not, have certain elements in their blood that could make them hypertensive, you will be given a questionnaire to fill to help us find out if hypertension runs in your family or not and other factors that could make you develop hypertension. Please note that your name will not be used anywhere. A code number will be used for your questionnaire, and blood sample. The information you give will help us study more on hypertension and how this disease can be controlled effectively.

Your weight, height and Blood pressure will be measured, about 3ml of blood will be collected from your arm, we will use this to look for certain elements in your blood that could make you develop hypertension and also check if you have HIV infection. You are free to turn down or refuse to participate in this survey. We will appreciate if you take part in all aspects of the survey as this will form baseline information for this area as not much work has been done in this region. The test is free and you do not have to pay any monies.

We thank you for your co-operation.

CONSENT: Now that the study has been explained to me and I understand the content of the study process I will be willing to take part in the programme.

{ } Hypertension { } HIV

Your wish will be strictly adhered to.

_____ _____

Signature/Thumb print of participant/Date Signature Interviewer/Date

_____

Signature/Thumb print of witness/Date

INFORMED CONSENT FORM (IBIBIO)

Ami nkere Mary Esien Kooffreh. Ndo kiet ke otu mmon enama utom ke department Genetics ye Biotechnology, Ufok nwed ntaifiok ke Calabar. Nko idaham nka nwed ke ufok ke Ibadan man otodo enno ekamba nwed itoro mmo ekoto Ph.D ke Zoology.

Nnyin idomo ukeme adi duno ndiono owo ifan edonoke nkonnkon iyip ye mmo mi idonoke. Iya uno fien nwed yak aboro nbime man otodo nnyin idiono mme nkonnkon iyip emi asanga ke ubon mfo ye mme nkpo afen ekemeke adi nam afo anie nkonnkon iyip. Ikpa ima adi neke nam afo adiono ke owo iyeme ayin nfo. Number ke idi dad inim idion ubana fien ye etok iyip nnyin isiogho fien ke ubok. Mme iboro afo noho aya wam nnyin adi neke mkpeep kpo mbana nkonnkon iyip, iyun iteme mmon edonoke enye nana ekpe kama idem ammo.

Iya udomo fien man idiono nana afo anion atre ye nana afo adopoke, inyun udomo fien ubok adi diono mme afomenie iden nkonnkon iyio ami. I ya inyun ise me afo me dono udono ado ekoto itiaita. Amekeme isin nkpo afo muyeme enam ye iyip mfo, ado, ikpa ukom fien tutu adieke afo yimeke adi wana ke efit se nyin inam. Nyim iyeme afo kpe akok nte kiet-O. Sosono nana anwam nyin adinam utom ami

UNYIME: Sia ema tan utom ami anwana mien, ami mme nyime adi wana ke esit nyun nno mbufo iyip mi eben ese mme menie
( ) nkonnkon iyip; ( ) udono itiaita
Se afo amek ke edi nam.

……. …….
Nsin ubok/ usen afion Nsin ubok andibip mbime /usen afion

…….
Nsin ubok ntiense/usen afion

INFORMED CONSENT FORM (EFIK)

Eyinn mi edi Mary Esien Kooffreh. Ndi kiet ke out mbon emi enamde uton ke department Genetics ye Biotechnology, ufok nwed ntaifiok ke Calabar. Ndien idaha emi, nka nwed ke ufok nwed ntaifiok ke Ibadan man eno mi akamba nwed itoro emi mmo ekotde Ph.D ke Zoology.

Nnyin iwana ndi dunnode ndiong owo ifang aemi edonnode nkonnkon iyip ye mmo emi mi donnoke. Iye inofi nwed oboro mbume man otodo nnyin idionno me nkonnkon iyip emi asnga ke ubon fo ye mme mkpo efen ekemede ndi nam fi eyene nkonkon iyip. Ikpi ma ndi neghede nnam fi ofiok ke owo iyomke eyinn fo. Number ke edi da inim idionno ibanga fi ye ekpri iyip nnyin isiode fi ke ubok. Mme iboro emi afo onode eye wam nnyin ndi neghede nkpep nkpo mbagha nkonnkon iyip, nyung mkpep mmo emi edonnode enye nte ekpe kamade idem mmo.

Iye domo fi man idionno nte afo onionde tre ye nte afo odobide, iyung idomo fi ubok man idionno me afo me yene idem nkonnkon iyip. Iye iyunn ise me afo mo donno udonno odo ekotde itiaita, eme keme ndi sin mkpo mi afo mu magha yak enam ye iyip fo, edi ikpi kom fi etieti edieke afo yimede ndi buana ke kpukpuru nkpo nyin inamde. Nyin iyomke fi ekpe okwuk ndomo kiet-O. Sosongo ke ndi wam nyin inam utom emi.

Mme yime: Sia ema ke tinn utom mmufo anwanga mi, me yime ndi buana ke esit nyung nno mbufo iyip mi eda ese me mmeyene:
( )nkonnkon iyip; ( ) udonno itiaita
Se afo mekde ke edi nam.

…… ……
Sin ubok nwed fo/ usen ofiong ubok nwed andibup mbume/usen ofiong

……...
Ubok nwed ntiense/usen ofiong

QUESTIONNAIRE
Please fill in the boxes and tick right where applicable.
CODE NO:

Age { }

Sex { }

Ethnic Group { }

Marital Status { }

Weight { }

Height {)

BP { }

Systolic { }

Diastolic { }

EDUCATIONAL QUALIFICATION

{ } Primary

{ } Secondary

{ } University

HIV STATUS:

Negative { }

Positive { }

Don't know { }

What is the level of your stress in your work place?

High { }

Low { }

Moderate { }

None { }

Occupation: ----------

Are you Hypertensive? { } Yes { } No

If yes when were you first diagnosed?

Is there anybody that has suffered from hypertension or stroke in your family before?

{ } Yes { } No

If yes, please indicate.

Brother/sister { }

Mother/father { }

Uncle/Aunties { }

Grandfather/mother { }

If you are hypertensive, what type of hypertensive drugs are you taking----------
--------- dosage ----------
Do you smoke? { } Yes { } No

How often do you take hot drinks.

	On a daily basis	at weekends
One glass	{ }	{ }
One bottle	{ }	{ }
Two or three bottles	{ }	{)
More than three bottles	{ }	{ }

Do you do any form of exercise daily like jogging { }
Playing games { } Swimming { }
Please mention any other form of exercise that you do ----------
Do you like plenty of salt in your food? { } Yes { } No
How often do you go to fast food joint? Daily { } Weekends { }
How often do you eat snacks for breakfast? { } for lunch
{ } dinner { } in between meals
What types of snack do you like? Meat pies { } Egg rolls { }
Cakes { } Sausage roll { } Bread { }

T test for popn

Group Statistics

	GROUPS	N	Mean	Std. Deviation	Std. Error Mean
HT	1	612	1.6196	.07947	.00321
	2	612	1.8754	6.76715	.27355
BMI	1	612	23.3201	5.83216	.23575
	2	612	27.4856	5.80769	.23476
WT	1	612	65.4199	12.75368	.51554
	2	612	70.6062	15.02097	.60719
SYS	1	612	1.1692E2	9.21780	.37261
	2	612	1.6111E2	23.26299	.94035
DIAST	1	612	72.4804	8.36550	.33816

	2	612	93.2516	13.76856	.55656
AGE	1	612	31.9216	10.40680	.42067
	2	612	51.2778	13.75807	.55614

T test for ELISAS

Group Statistics

	groups	N	Mean	Std. Deviation	Std. Error Mean
Onehr	0	199	.1753	.06879	.00488
	1	199	.1607	.04888	.00346
threehrs	0	199	.3806	.19552	.01386
	1	199	.2605	.12421	.00880
overnight	0	199	.6633	.33251	.02357
	1	199	.4949	.20958	.01486
Gene	0	199	2.8794	.34161	.02422
	1	199	2.8894	.33004	.02340
Age	0	199	34.5628	10.27426	.72832
	1	199	53.4523	14.14451	1.00268
Bmi	0	199	23.0556	6.51740	.46201
	1	199	26.3190	5.78990	.41043
systolic	0	199	1.1566E2	9.63119	.68274
	1	199	1.6307E2	23.87152	1.69221
diastolic	0	199	71.3970	8.50895	.60318
	1	199	94.8744	13.59067	.96342

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
HT	Equal variances assumed	3.156	.076	-.935	1222	.350	-.25587	.27356	-.79258	.28084
	Equal variances not assumed			-.935	611.169	.350	-.25587	.27356	-.79311	.28137
BMI	Equal variances assumed	1.555	.213	-12.520	1222	.000	-4.16552	.33270	-4.81826	-3.51279
	Equal variances not assumed			-12.520	1.222E3	.000	-4.16552	.33270	-4.81826	-3.51279
WT	Equal variances assumed	19.331	.000	-6.511	1222	.000	-5.18627	.79653	-6.74899	-3.62356
	Equal variances not assumed			-6.511	1.191E3	.000	-5.18627	.79653	-6.74903	-3.62352
SYS	Equal variances assumed	229.847	.000	-43.683	1222	.000	-44.18464	1.01148	-46.16907	-42.20021
	Equal variances not assumed			-43.683	798.249	.000	-44.18464	1.01148	-46.17012	-42.19916
DIAST	Equal variances assumed	70.250	.000	-31.895	1222	.000	-20.77124	.65124	-22.04891	-19.49358
	Equal variances not assumed			-31.895	1.008E3	.000	-20.77124	.65124	-22.04918	-19.49331

AGE	Equal variances assumed	29.229	.000	-27.758	1222	.000	-19.35621	.69732	-20.72428	-17.98814
	Equal variances not assumed			-27.758	1.138E3	.000	-19.35621	.69732	-20.72438	-17.98804

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
onehr	Equal variances assumed	.594	.441	2.445	396	.015	.01463	.00598	.00287	.02639
	Equal variances not assumed			2.445	357.305	.015	.01463	.00598	.00286	.02639
threehrs	Equal variances assumed	9.216	.003	7.314	396	.000	.12010	.01642	.08781	.15238
	Equal variances not assumed			7.314	335.433	.000	.12010	.01642	.08780	.15240
overnight	Equal variances assumed	19.229	.000	6.044	396	.000	.16840	.02786	.11363	.22318
	Equal variances not assumed			6.044	333.874	.000	.16840	.02786	.11359	.22321
gene	Equal variances assumed	.343	.559	-.298	396	.765	-.01005	.03367	-.07625	.05615
	Equal variances not assumed			-.298	395.531	.765	-.01005	.03367	-.07625	.05615
age	Equal variances assumed	11.527	.001	-15.242	396	.000	-18.88945	1.23928	-21.32584	-16.45306
	Equal variances not assumed			-15.242	361.440	.000	-18.88945	1.23928	-21.32655	-16.45234
bmi	Equal variances assumed	2.297	.130	-5.281	396	.000	-3.26337	.61799	-4.47831	-2.04842
	Equal variances not assumed			-5.281	390.579	.000	-3.26337	.61799	-4.47836	-2.04837
systolic	Equal variances assumed	95.678	.000	-25.977	396	.000	-47.40201	1.82474	-50.98941	-43.81461
	Equal variances not assumed			-25.977	260.797	.000	-47.40201	1.82474	-50.99512	-43.80890
diastolic	Equal variances assumed	24.392	.000	-20.655	396	.000	-23.47739	1.13666	-25.71204	-21.24274
	Equal variances not assumed			-20.655	332.552	.000	-23.47739	1.13666	-25.71334	-21.24143

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.	Correlations			Collinearity Statistics	
		B	Std. Error	Beta			Zero-order	Partial	Part	Tolerance	VIF
1	(Constant)	66.793	12.300		5.430	.000					
	AGT	-2.224	2.215	-.033	-1.004	.316	-.074	-.041	-.033	.978	1.023
	AGE	.192	.061	.113	3.150	.002	.105	.128	.103	.828	1.208
	SEX	.679	1.763	.014	.385	.700	-.015	.016	.013	.800	1.250
	ETHNIC	.246	.304	.028	.811	.418	.117	.033	.027	.874	1.145
	MARITAL	1.531	1.426	.037	1.074	.283	.086	.044	.035	.912	1.096
	BMI	-.192	.149	-.048	-1.290	.197	-.094	-.053	-.042	.778	1.286
	DIASTOLIC	.923	.057	.546	16.111	.000	.564	.552	.527	.932	1.073
	EDUCATION	-.858	.896	-.035	-.957	.339	-.139	-.039	-.031	.803	1.245
	STRESS	.146	.720	.007	.203	.839	-.106	.008	.007	.898	1.113
	OCCUPATION	-.190	.202	-.033	-.938	.349	-.103	-.038	-.031	.888	1.126
	KNOWLEDGE	2.255	1.757	.044	1.283	.200	.014	.053	.042	.899	1.112
	FAMHISTORY	1.602	.986	.054	1.625	.105	.054	.067	.053	.964	1.038
	SMOKING	7.361	6.492	.038	1.134	.257	.070	.047	.037	.947	1.056
	ALCOHOL	-1.324	.993	-.045	-1.333	.183	-.077	-.055	-.044	.927	1.079
	EXERCTYPE	-.393	.607	-.022	-.647	.518	-.062	-.027	-.021	.943	1.060
	SALT	1.906	2.094	.031	.911	.363	.056	.037	.030	.943	1.061
	FASTFOOD	-5.591	2.565	-.075	-2.179	.030	-.150	-.089	-.071	.913	1.095
	SNACKUSE	.220	.602	.012	.366	.714	.076	.015	.012	.949	1.053

a. Dependent Variable: SYSTOLIC

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.	Correlations			Collinearity Statistics	
		B	Std. Error	Beta			Zero-order	Partial	Part	Tolerance	VIF
1	(Constant)	48.074	7.274		6.609	.000					
	AGT	-.076	1.326	-.002	-.057	.954	-.047	-.002	-.002	.976	1.025
	AGE	-.144	.036	-.144	-3.989	.000	-.050	-.162	-.132	.836	1.196
	SEX	-1.533	1.053	-.054	-1.456	.146	-.054	-.060	-.048	.803	1.246
	ETHNIC	.191	.182	.037	1.051	.293	.121	.043	.035	.874	1.144
	MARITAL	.430	.854	.017	.504	.614	.036	.021	.017	.911	1.098
	BMI	.091	.089	.039	1.028	.304	-.040	.042	.034	.777	1.287
	EDUCATION	-.178	.536	-.012	-.332	.740	-.060	-.014	-.011	.802	1.247
	STRESS	-1.199	.428	-.097	-2.802	.005	-.144	-.114	-.093	.910	1.099
	OCCUPATION	-.171	.121	-.050	-1.417	.157	-.084	-.058	-.047	.890	1.124
	KNOWLEDGE	-1.495	1.051	-.050	-1.423	.155	-.023	-.058	-.047	.900	1.111
	FAMHISTORY	-.670	.591	-.038	-1.135	.257	-.001	-.047	-.038	.962	1.040
	SMOKING	1.576	3.887	.014	.406	.685	.039	.017	.013	.945	1.058
	ALCOHOL	.397	.595	.023	.668	.504	-.027	.027	.022	.925	1.081
	EXERCTYPE	.141	.363	.013	.387	.699	-.010	.016	.013	.943	1.061
	SALT	.365	1.253	.010	.291	.771	.040	.012	.010	.941	1.062
	FASTFOOD	-.906	1.540	-.020	-.588	.557	-.109	-.024	-.019	.907	1.103
	SNACKUSE	.222	.360	.021	.617	.538	.061	.025	.020	.950	1.053
	SYSTOLIC	.330	.020	.558	16.111	.000	.564	.552	.533	.913	1.096

a. Dependent Variable: DIASTOLIC

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.	Correlations			Collinearity Statistics	
		B	Std. Error	Beta			Zero-order	Partial	Part	Tolerance	VIF
1	(Constant)	82.217	6.324		13.000	.000					
	ATIR	-2.110	3.231	-.023	-.653	.514	.006	-.027	-.022	.972	1.029
	ACE	.115	.478	.008	.242	.809	-.007	.010	.008	.989	1.011
	AGT	-.048	1.125	-.001	-.042	.966	.006	-.002	-.001	.971	1.030
	AGE	.076	.042	.086	1.790	.074	.169	.073	.061	.507	1.974
	SEX	-3.195	.694	-.172	-4.606	.000	-.206	-.186	-.157	.829	1.206
	ETHNIC	.162	.149	.038	1.089	.276	.002	.045	.037	.976	1.024
	MARITAL	-.871	.857	-.049	-1.017	.310	.059	-.042	-.035	.498	2.010
	BMI	.064	.056	.040	1.132	.258	.069	.046	.039	.917	1.091
	DIASTOLIC	.550	.039	.499	13.962	.000	.521	.498	.476	.909	1.100
	EDU	-.163	.415	-.015	-.392	.695	-.008	-.016	-.013	.790	1.266
	STRESS	-.028	.244	-.004	-.113	.910	.029	-.005	-.004	.867	1.153
	OCCUPATION	-.117	.082	-.053	-1.424	.155	-.012	-.058	-.049	.852	1.174
	FAMHISTORY	-.388	.384	-.036	-1.009	.313	-.032	-.041	-.034	.937	1.067
	SMOKING	-.193	1.930	-.004	-.100	.920	-.033	-.004	-.003	.926	1.079
	ALCOHOL	.240	.358	.024	.671	.503	.055	.028	.023	.922	1.085
	EXERCISE	-.086	.145	-.022	-.596	.552	.014	-.024	-.020	.866	1.155
	SALT	.470	.749	.022	.628	.530	.005	.026	.021	.972	1.029
	FASTFOOD	-.391	.727	-.020	-.538	.591	-.004	-.022	-.018	.876	1.142
	SNACKUSE	-.172	.251	-.024	-.683	.495	-.006	-.028	-.023	.964	1.037

a. Dependent Variable: SYSTOLIC

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.	Correlations			Collinearity Statistics	
		B	Std. Error	Beta			Zero-order	Partial	Part	Tolerance	VIF
1	(Constant)	7.013	6.487		1.081	.280					
	ATIR	4.120	2.922	.049	1.410	.159	.050	.058	.048	.974	1.026
	ACE	-.262	.433	-.021	-.606	.545	-.029	-.025	-.021	.990	1.010
	AGT	.592	1.018	.020	.582	.561	.015	.024	.020	.971	1.030
	AGE	.100	.038	.125	2.623	.009	.222	.107	.089	.510	1.962
	SEX	.473	.639	.028	.740	.460	-.076	.030	.025	.801	1.248
	ETHNIC	-.264	.135	-.067	-1.960	.051	-.066	-.080	-.067	.981	1.020
	MARITAL	.708	.776	.044	.913	.362	.149	.037	.031	.497	2.010
	BMI	.085	.051	.060	1.680	.093	.114	.069	.057	.919	1.088
	EDU	.266	.375	.027	.709	.479	-.017	.029	.024	.791	1.265
	STRESS	.476	.220	.079	2.164	.031	.063	.089	.074	.874	1.144
	OCCUPATION	.021	.075	.010	.281	.779	.040	.012	.010	.849	1.178
	FAMHISTORY	.211	.348	.021	.606	.545	.008	.025	.021	.936	1.068
	SMOKING	-.407	1.747	-.008	-.233	.816	-.006	-.010	-.008	.926	1.079
	ALCOHOL	.154	.325	.017	.475	.635	.026	.020	.016	.921	1.085
	EXERCISE	.009	.131	.003	.071	.943	-.021	.003	.002	.865	1.156
	SALT	-.731	.678	-.037	-1.079	.281	-.050	-.044	-.037	.973	1.028
	FASTFOOD	.235	.658	.013	.357	.721	-.008	.015	.012	.875	1.142
	SNACKUSE	.408	.227	.062	1.795	.073	.050	.074	.061	.969	1.032
	SYSTOLIC	.451	.032	.497	13.962	.000	.521	.498	.475	.914	1.095

a. Dependent Variable: DIASTOLIC

PTSYSTOLIC

Model Summary[b]

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Durbin-Watson
1	.604[a]	.365	.346	18.81339	1.938

a. Predictors: (Constant), SNACKUSE, SEX, FAMHISTORY, AGT, OCCUPATION, STRESS, KNOWLEDGE, SMOKING, EXERCTYPE, MARITAL, DIASTOLIC, FASTFOOD, SALT, ALCOHOL, ETHNIC, EDUCATION, AGE, BMI

b. Dependent Variable: SYSTOLIC

PTDIASTOLIC

Model Summary[b]

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Durbin-Watson
1	.593[a]	.352	.332	11.25225	1.875

a. Predictors: (Constant), SYSTOLIC, KNOWLEDGE, SEX, FAMHISTORY, AGT, EXERCTYPE, OCCUPATION, SNACKUSE, STRESS, SMOKING, MARITAL, SALT, ALCOHOL, FASTFOOD, ETHNIC, EDUCATION, AGE, BMI

b. Dependent Variable: DIASTOLIC

CTSYSTOLIC

Model Summary[b]

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Durbin-Watson
1	.559[a]	.313	.291	7.76312	1.868

a. Predictors: (Constant), SNACKUSE, EXERCISE, ACE, BMI, ETHNIC, SALT, AGT, SMOKING, ATIR, OCCUPATION, ALCOHOL, DIASTOLIC, FASTFOOD, FAMHISTORY, STRESS, SEX, EDU, AGE, MARITAL

b. Dependent Variable: SYSTOLIC

CTDIATOLIC

Model Summary[b]

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Durbin-Watson
1	.559[a]	.313	.291	7.76312	1.868

a. Predictors: (Constant), SNACKUSE, EXERCISE, ACE, BMI, ETHNIC, SALT, AGT, SMOKING, ATIR, OCCUPATION, ALCOHOL, DIASTOLIC, FASTFOOD, FAMHISTORY, STRESS, SEX, EDU, AGE, MARITAL

b. Dependent Variable: SYSTOLIC

UNIVERSITY OF UYO TEACHING HOSPITAL

P. M. B. 1136
UYO
AKWA IBOM STATE
085 - 204313

Our Ref: UUTH/AD/S/96/VOL.III/170 Date: 4th December, 2007

Your Ref:.....

UUTH, UYO INSTITUTIONAL REVIEW COMMITTEE

APPROVAL CERTIFICATE LETTER

Principal Investigator: **Mrs. Mary Essien Kooffreh**

Protocol Title: **THE PREVALENCE OF HYPERTENSION IN SOUTH EAST NIGERIA: RELATIONSHIP WITH M235T ALLELE OF ANGIOTENSINOGEN GENE AND INTERACTION WITH HIV.**

STATUS

The UUTH, Uyo Institutional Review Committee has reviewed your Protocol Titled: "The Prevalence of Hypertension in South East Nigeria; Relationship with M235T Allele of Angiotensinogen Gene and Interaction with HIV"

The aim of the study is to ascertain the genetic epidemiology of hypertension in South East Nigeria (Calabar and Uyo) and examine the effect of HIV infection (if present) on hypertension in the study population.

Findings from the study will educate the patients and control subjects to know if they have the genes for hypertension and this will help change their lifestyles. Patients who are HIV positive will be advised to see a doctor for a proper follow up.

THE RESEARCH PROTOCOL DESCRIBED ABOVE HAS BEEN REVIEWED BY THE UUTH, IRC AND APPROVAL GIVEN AS INDICATED.

J. E. Inyang
Secretary
University of Uyo Teaching Hospital, Uyo. IRC

ETHICAL COMMITTEE

UNIVERSITY OF CALABAR TEACHING HOSPITAL

P. M. B. 1278, CALABAR, NIGERIA

CHIEF MEDICAL DIRECTOR:
Prof. E. E. J. Asuquo
MB, BS, FRCOG, FWACS, FMCOG, FICS

SECRETARY:
Barr. B. F. I. Anyatang
B.A. HONS (English & Litt. Studies) LL.B. (HONS), B.L, ANIM, IHSAN

CHAIRMAN:
Professor A. A. Asindi
MBBS (Ibadan), DCH (Glasg), FWAC (Paed.), FRCP (Glasg).

Our Ref:..... Date:.....

Your Ref:.....

5th March, 2007

Mary Esien Kooffreh (Mrs.),
Dept. of Genetics & Biotechnology,
University of Ibadan,
Ibadan.

YOUR REQUEST FOR ETHICAL CLEARANCE: PREVALENCE OF HYPERTENSION IN SOUTH EAST NIGERIA: RELATIONSHIP WITH M235T ALLELE OF ANGIOTENSINOGEN GENE & INTERACTION WITH HIV

Your protocol on this subject has been thoroughly examined.

The methodology and information to participants have met our ethical conditions.

Approval is hereby granted for you to commence the project.

It is expected that you will be resident in Calabar during the period of the research.

The Ethical Committee will from time to time monitor this project.

PROF. A. A. ASINDI
CHAIRMAN, ETHICAL COMMITTEE

INSTITUTE FOR ADVANCED MEDICAL RESEARCH AND TRAINING (IMRAT)
COLLEGE OF MEDICINE, UNIVERSITY OF IBADAN. IBADAN, NIGERIA.
Telefax: 234-2-2412170; 234-2-2410088 /3310, 3120, 3114, 3594 Fax: 234-2-2413545
Ag. Director : Prof F. A. A. Adeniyi

UI/UCH IRC Registration Number: **Pending**
: **Date: 01/11/2007**

Notice of Full Approval after full Committee Review

Re: The Prevalence of Hypertension in South East Nigeria: Its Relationship with M235T Allele of the Angiotensinogen Gene alongside Its Interaction with HIV.

UI/UCH Institutional Review Committee assigned number: UI/IRC/06/0116

Name of Principal Investigator: Mrs. Mary E. Kooffreh

Address of Principal Investigator: Department of Zoology,
University of Ibadan, Ibadan

Date of receipt of valid application: 04/12/2006

Date of meeting when final determination of research was made: 01/11/2007

This is to inform you that the research described in the submitted protocol, the consent forms, and other participant information materials have been reviewed and *given full approval by the UI/UCH Institutional Review Committee.*

This approval dates from 01/11/2007 to 31/10/2008. If there is delay in starting the research, please inform the UI/UCH IRC so that the dates of approval can be adjusted accordingly. Note that no participant accrual or activity related to this research may be conducted outside of these dates. *All informed consent forms used in this study must carry the* UI/UCH IRC *assigned number and duration of* UI/UCH IRC *approval of the study.* In multiyear research, endeavor to submit your annual report to the UI/UCH IRC early in order to obtain renewal of your approval and avoid disruption of your research.

The National Code for Health Research Ethics requires you to comply with all institutional guidelines, rules and regulations and with the teneis of the Code including ensuring that all adverse events are reported promptly to the UI/UCH IRC. *No changes are permitted in the research without prior approval by the* UI/UCH IRC *except in circumstances outlined in the l Code. The* UI/UCH IRC *reserves the right to conduct compliance visit to your research site without previous notification.*

UI/UCH IRC APPROVED 01 NOV 2007 COLLEGE OF MEDICINE U. I.

Prof. C. A. Adebamowo
Chairman, UI/UCH IRC
E-mail:uiuchirc@yahoo.com

GOVERNMENT OF CROSS RIVER STATE

CENTRE FOR CLINICAL GOVERNANCE RESEARCH AND TRAINING
MINISTRY OF HEALTH, CALABAR
CROSS RIVER STATE. TEL: 087-239403
08056333512, 08032758315

OFFICE:
1ST FLOOR ADMIN. BLOCK
GENERAL HOSPITAL COMPLEX,
MARY SLESSOR AVENUE,
CALABAR.

Our Ref: **CRS/MOH/CCGRT/**

October 3, 2007

Mrs. Mary Koofreh
Department of Genetics & Biotechnology
University of Calabar
Calabar, Cross River State

Dear Madam,

Re: Prevalence of Hypertension in Southeast Nigeria and its relationship with M235T allele of the angiotensin gene and interaction with HIV

I am directed to advise you that, upon review of your application, the State Research Ethics Committee has granted **INTERIM ETHICAL APPROVAL** for your research.

This interim approval will enable you proceed with your work subject to the following considerations:

- **Scope of the research**: It is recommended that you strengthen your protocol by defining the scope to focus on one or two key variables. This will significantly improve its potential for producing an outcome that may beneficially influence scientific knowledge and the change clinical management of enuresis.

- **Sample size determination**: It is recommended that you define your sample size with the help of a statistician, who is also able to provide advise on the appropriate statistical software to use when you are ready to analyse your data. In addition, clarifying the sample size will also help you account for adjusted response rates in the two phases of your research.

- **Informed consent**: The REC strongly recommends that you inform your participants that there will be two phases to the research and they should be aware that you will need to come back for post treatment review which will also include a second round of sample collection: urine and anal swabs; and responding to a questionnaire.

While you proceed, please be aware that the research ethics committee reserves the right to call for an audit of your research at any point during or after the study.

With full assurance of our highest regards,

Yours sincerely,

Dr. Joseph Ana
Chairman, REC