ANGIOTENSIN CONVERTING ENZYME AND ENDOTHELIAL NITRIC OXIDE SYNTHASE GENES POLYMORPHISM IN MALARIA-INFECTED PATIENTS ATTENDING SELECTED HOSPITALS WITHIN KANO METROPOLIS, KANO STATE, NIGERIA

BY

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Declaration

I hereby declare that this work is the product of my research efforts undertaken under the supervision of Dr. Mansura Abdulazeez and has not been presented anywhere for the award of a degree or certificate. All sources have been duly acknowledged.

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CERTIFICATION

This is to certify that the research work for this dissertation and the subsequent write-up (Kabiru Abubakar Musa SPS/13/MBC/00048) were carried out under my supervision.

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This dissertation is dedicated to my beloved wife and children Hajiya Zainab Aliyu Muhammad Gidan Kanawa and Abubakar Kabiru Abubakar (Maheer) and Aliyu Kabiru Abubakar (Sabir).

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ABSTRACT

The present study was aimed at exploring the hypothesis that susceptibility to malaria is influenced by genetic variations of Angiotensin converting enzyme (ACE) and Endothelial nitric oxide synthase (eNOS) polymorphisms. Also, the association of these polymorphisms with hypertension in malaria patients was examined. Two hundred subjects (one hundred and twenty malaria-infected and eighty control subjects) attending Sheikh Muhammad Jidda General Hospital and Murtala Muhammad Specialist Hospital, Kano were recruited for the studies between the months of August to November 2015. DNA was extracted from blood samples and ACE and eNOS (Glu^{298}→Asp substitution) genotypes analyzed by polymerase chain reaction (PCR). The PCR products were separated on 1% agarose gel and some samples randomly selected for sequencing. From the results, 41.70% of the infected patients had severe malaria, while 58.30% had mild malaria, with 26.70% of malaria patients having hypertension. There was a significance association of D/D (P=0.03, odd's ratio (OR)=0.50, 95%CI=0.20-1.40) and I/D (P=0.01, OR=0.30, 95%CI=0.1-0.8) genotypes of ACE and G/T (P=0.04, OR=4, 95%CI=0.2-1.1) and T/T (P=0.03, OR=0.60, 95%CI=0.20-1.80) genotypes of eNOS with mild malaria. Also, a significant association of D/D (P=0.004, OR=0.20, 95%CI=0.1-0.6) and G/G (P=0.01, OR=1.011, 95%CI=0.42-2.42) genotypes with hypertension in malaria patients was observed. In conclusion, this study has demonstrated that patients with D/D, I/D, G/T and T/T genotypes are less susceptible to severe malaria, suggesting the dominant effect of 'D' allele of ACE and 'T' allele of eNOS polymorphisms against the susceptibility to malaria. The study also showed that malaria-infected patients with D/D and G/G genotypes might be prone to hypertension. However, additional genetic studies are necessary to investigate the genetic contributions of these polymorphisms to the pathology of malaria.

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CHAPTER ONE

INTRODUCTION

Malaria remains one of the world's most deadly diseases, causing approximately 600,000 deaths, mostly among African children (WHO, 2012). The malaria parasite, *Plasmodium spp,* has an intricate and complex life cycle in vertebrate (intermediate) and invertebrate (definitive) hosts and the multiple developmental forms of Plasmodium species are potential targets of distinct anti-parasite molecules. The parasites are among the major human pathogens annually associated with 300 to 500 million clinical cases worldwide and 1 million to 3 million deaths (WHO, 2012). Despite high infection rates, only 1% to 2% of malaria patients develop life-threatening complications, such as cerebral malaria and profound anemia, hence the involvement of natural selection, to a large extent, on severity (Kwiatkowski, 2005). Cerebral Malaria (CM), a syndrome characterized by impaired consciousness, generalized convulsions, coma and neurological sequelae (Idro *et al*., 2005), causes a high proportion of these deaths. However, efforts to control this disease are hampered by drug resistance in parasites, insecticide resistance in mosquitoes and the lack of effective vaccine (O'Neil & Posner, 2004).

Studies have demonstrated that Angiotensin II (Ang II) has beneficial effects on malaria and particularly on CM (Callera *et al*., 2007). Different lines of evidence suggest a possible 'protective' effect that could affect parasite development and host susceptibility to Plasmodium-induced pathology. Angiotensin II is a peptide within the renin angiotensin aldosterone system (RAAS). It is one of the most important regulatory systems of blood volume, arterial pressure and cardiovascular homeostasis. It is the

principal effect or hormone of the RAAS in vascular biology, mediating effects via two main receptors: Angiotensin receptor type 1 (AT1) and type 2 (AT2) (Callera *et al*., 2007). When Ang II binds to AT1 on vascular smooth muscle cells, it mobilizes intercellular Ca^{2+}, leading to cellular contraction which when sustained increases peripheral vascular resistance, resulting in high blood pressure (Touyz & Schiffain, 2000).

High blood pressure is an important public health challenge that results in morbidity and mortality (Kamel & Wilson, 1999). It is responsible for about 45% of deaths due to heart disease and 51% of deaths due to stroke, adding to the tremendous number of 9.4 million deaths every year (Lim *et al.*, 2012). In contrast, recent studies have shown that the actions of Ang II via the type 2 receptor are counter regulatory to those mediated via the AT1 receptor as Ang II acts via AT2 to produce a vasodilator effect through bradykinin-dependent activation of endothelial nitric oxide synthase. This has been attributed to the *in vivo* and *in vitro* antiplasmodial activity of Ang II (Dhangadamajhi *et al*., 2010). Other reports have shown that angiotensin peptides can cause impairment of the erythrocyte cycle of Plasmodium, reducing the parasite growth *in vitro* (Maciel *et al*., 2008; Saraiva *et al*., 2011), since the development of malaria depends on the initial levels of parasite in mice and possibly in humans (Bejon *et al*., 2005). This could be a potential explanation for angiotensin II protection from malaria. It is possible that Ang II could modulate malaria severity through additional mechanisms, especially since the inhibitory effect observed in parasite growth is modest (Maciel *et al*., 2008; Saraiva *et al*., 2011).

It is a known fact that genetic and environmental factors are important determinants of the pathogenesis and progression of almost all diseases (Yan *et al.,* 2004). A number of

genetic variations have been shown to have clinical or physiological impacts, for example, different human polymorphisms, such as those causing thalassemia or sickle cell anaemia have been associated with a natural selection process, though not well established. Sampson *et al*. (2014) reported that the association of polymorphisms of ACE and protection from CM might also be due to the natural selection process. The ACE I/D polymorphism known to have a major influence on plasma and tissue ACE levels also affects generation of Ang II. Studies by Dhangadamajhi *et al*. (2010) demonstrated that differences in ACE polymorphisms are associated with the levels of Ang II and consequently the susceptibility to malaria. They reported that the "D" allele of ACE I/D polymorphism, responsible for increased Ang II production is significantly associated with mild malaria. However, the available data are scarce and genetic studies are necessary, especially in malaria endemic regions, such as sub-Saharan Africa to determine whether malaria could have a driving evolutionary force for RAAS polymorphisms, and therefore for hypertension.

The severity of malaria depends largely upon the capacity of *Plasmodium falciparum*-infected erythrocytes (RBCs) to adhere to the endothelia of micro vessels (cytoadherence) and to form rosettes with uninfected RBCs resulting in high parasite burden and severe proinflammatory responses in localized areas, causing endothelial damage and organ dysfunction (Craig& Scherf,2011). The endothelium plays a critical role in the regulation of vascular tone, inhibits leukocyte adhesion, platelet aggregation, endothelial cell activation, and modulates expression of adhesion molecules such as nitric oxide (NO) and prostacyclin (Moncada & Higgs, 2003). Nitric Oxide is derived from L-

arginine by the action of endothelial nitric oxide synthase (eNOS) and has been shown to be beneficial because of its anti-parasitic and anti-disease effects (Anstey *et al.,* 2009).

Polymorphisms in eNOS gene have been found to be associated with hypertension and severity of malaria (Dhangadamajhi *et al*., 2009; 2010) in different human populations. The eNOS gene is assigned to chromosome 7 and contains 26 exons spanning approximately 21kb of genomic DNA. Three genetic variant of the eNOS gene have been identified: a variant with a T/C substitution in 5' flanking region near the promoter at position -786 (T-786C); a variant with a 27-bp variable number of tandem repeat (VNTR) region in intron 4, and a variant with a G/T substitution at position 894in exon 7 that codes for replacement of glutamic acid by aspartic acid at residue 298 in the mature eNOS protein (G894T). All three variants have been determined to be clinically important for several diseases, but the mechanisms by which they affect eNOS activity have not been demonstrated (Dhangadamajhi *et al*., 2009). In a study to investigate the association between the three eNOS polymorphisms and clinical manifestations of malaria, Dhangadamajhi *et al*. (2009) demonstrated a positive association between Glu^{298}/Asp (G894T) and mild malaria, and concluded that this variant had protective effects against cerebral malaria.

Since malaria and human have been co-evolving for millions of years and there exist a complex nature of host-pathogen interaction, several genetic variants have been ascribed to malaria than any other disease (Dhangadamajhi *et al*., 2009). Also, it is possible that polymorphisms acquired because of their protective effect against malaria have turned out to contribute together with life-style to the worldwide epidemic of hypertension. Due to scarce data necessary to establish these facts, the present study aims to investigate the

ACE and eNOS polymorphisms and their association with malaria and hypertension in malaria-infected patients attending two Government hospitals within Kano metropolis: Sheikh Muhammad Jidda General Hospital and Murtala Muhammad Specialist Hospital, Kano.

1.1 STATEMENT OF THE PROBLEM

Malaria and hypertension are among the world's deadly diseases and major causes of mortality and morbidity, particularly in endemic areas of sub-Saharan Africa. In Nigeria, malaria accounts for an estimated 100 million cases and over 300,000 deaths per year, while hypertension affects 11.2 % Nigerians (i.e. about 4.33 million)(Snow *et al*., 2005). The etiology of malaria is variable and attributable to environmental factors, host genetics and virulence (Mackinnon *et al*., 2005). Host genetic factor contributes to the variability of malaria phenotypes (Weatherall & Clegg, 2002) and thus, help to determine some of the mechanisms involved in susceptibility to P. *falciparum* infection. The knowledge gained since 1980s using molecular genetics approaches has produced undisputed evidence about polymorphisms associated with malaria resistance and their complex interactions. Indeed, several gene mutations and polymorphisms in the human hosts confer survival advantage and have increased in frequency through natural selection over generations. These include sickle cell trait (Hb.AS) and hemoglobinopathies such as thalassaemias and glucose-6-phosphate detydrogenase (G6PD) deficiency (Verra *et al;* 2009).

There are evidences suggesting that polymorphisms of ACE and eNOS influence the severity of malaria and hypertension, and also contribute to their pathology, though the

mechanism underlying the association of these polymorphisms to hypertension and malaria remains to be clarified.

1.2 JUSTIFICATION

Although malaria and hypertension do not have an obviously direct cross influence, there are evidences suggesting that the Renin angiotensin aldosterone system (RAAS), and most specifically Ang II, may influence the severity of Malaria. Though available data are still very limited, it is has been suggested that Ang II may be protecting the integrity of the Blood Brain Barrier (BBB) in the setting of CM, giving a survival advantage to those that carry polymorphisms resulting in higher level of systemic Ang II. However, the role of Ang II in inflammation, endothelial cell barriers formation, sodium conservation and parasite growth with respect to malaria are needed before any solid conclusion can be reached. It is possible that polymorphisms acquired because of their protective effect against malaria have turned out to contribute together with the western society's lifestyle to the worldwide epidemic proportions of hypertensions (Gallego-Delgado and Rodriguez, 2014).

Furthermore, polymorphisms of eNOS have been associated with severity of malaria. Genetic variants of eNOS have been shown to have protective effect on malaria due to their association to high NO production known to be beneficial due to its antiparasitic and antidisease effect. According to Yayama and Okamoto (2008), activation of AT2 receptor results in increased production of nitric oxide by endothelial Nitric Oxide Synthase (eNOS) in endothelial cells, which could be protective against cerebral malaria since low

nitric oxide bioavailability may exacerbate endothelial dysfunction and contribute to the pathogenesis of severe malaria.

However, available data in this sense are limited and additional genetic studies are necessary in other malaria endemic regions (specifically sub-Saharan Africa) to determine whether malaria could have been a driving evolutionary force for these polymorphisms and therefore for hypertension. In line with above, the ACE and eNOS gene polymorphisms and its association with malaria and hypertension in malaria patients in our region are of paramount important.

1.3 AIM AND OBJECTIVES

The general aim of this work is to examine ACE and eNOS polymorphisms and their association with malaria and hypertension in malaria-infected patients attending some government hospitals within Kano metropolis.

The specific objectives are:

1. To determine the severity of infection in malaria patients and to determine the prevalence of hypertension in the malaria patients.
2. To determine the ACE gene polymorphisms and its relationship with severity of malaria and hypertension in the malaria patients.
3. To determine the eNOS gene polymorphisms and its relationship with severity of malaria and hypertension in the malaria patients.

CHAPTER TWO

LITERATURE REVIEW

2.1 POLYMORPHISM

Polymorphism is said to occur when two or more clearly different phenotypes exist in the same population of a species. In other words, it is the occurrence of more than one *form* or *morph*. In order to be classified as such, morphs must occupy the same habitat at the same time and belong to a panmictic population (one with random mating). Polymorphism as described here involves morphs of the phenotype. The term is also used somewhat differently by molecular biologists to describe certain point mutations in the genotype, such as single nucleotide polymorphisms (SNPs) and restriction fragment length polymorphism (RFLPs). Polymorphism is common in nature; it is related to biodiversity, genetic variation and adaptation; it usually functions to retain variety of form in a population living in a varied environment. The most common example is sexual dimorphism, which occurs in many organisms. Other examples are mimetic forms of butterflies, and human hemoglobin and blood types. According to the theory of evolution, polymorphism results from evolutionary processes, as does any aspect of a species. It is heritable and is modified by natural selection. In polymorphism, an individual's genetic make-up allows for different morphs, and the switch mechanism that determines which morph is shown is environmental. In genetic polymorphism, the genetic make-up determines the morph (Clark, 2006).

2.1.1Genetic Polymorphism

According to Cavalli-Sforza and Bodmer (2011), genetic polymorphism is the occurrence in the same population of two or more alleles at one locus, each with appreciable frequency. In simple words, the term 'polymorphism' was originally used to describe variations in shape and form that distinguish normal individuals within a species from each other. These days, geneticists use the term genetic polymorphisms to describe the inter-individual, functionally differences in DNA sequence that make each human genome unique.

All the common blood types, such as the ABO blood group system, are genetic polymorphisms. Here we see a system where there are more than two morphs: the phenotypes A, B, AB and O are present in all human populations, but vary in proportion in different parts of the world. The phenotypes are controlled by multiple alleles at one locus. A study of disease statistics by Cavalli-Sforza and Bodmer (2011) demonstrated that these polymorphisms are seemingly never eliminated by natural selection.

2.2 ANGIOTENSIN CONVERTING ENZYME (ACE)

Angiotensin converting-enzyme (ACE) is known as peptidyl-dipeptidase A. It is also known as kininase II, CD143, or EC 3.5.15.1. Although the cleavage of angiotensin I to the eight–amino acid peptide angiotensin II is central to its action, ACE, its peptide substrates and products affect many physiologic processes in addition to blood pressure control. These include hematopoiesis, reproduction, renal development, renal function, and the immune response (Acharya *et al*., 2003).Two enzymes, renin and ACE act sequentially to produce angiotensin II. Renin an aspartyl protease cleaves only the single

substrate angiotensinogen at only a single position, releasing the amino-terminal peptide angiotensin I. Renin specialization is further evidenced by its extremely limited tissue distribution: active renin is made in the kidney by granular cells in the wall of the afferent arteriole at the base of the glomerulus in the juxtaglomerular apparatus (Kurtz, 2011).

ACE was discovered in the mid-1950s, and was the last of the traditional components of the RAAS to be identified. In fact, ACE was found more than 50 years after the identification of renin by physiologist Robert Tigerstedt working in Stockholm with a medical student, Per Gustav Bergman. Understanding how these proteins were discovered gives insight into why ACE and the renin angiotensin system (RAS) are associated with blood pressure control. In 1896, Tigerstedt began a series of experiments in which a cold-water extract of rabbit kidney was injected into the jugular vein of a recipient rabbit. Within little more than a minute, the blood pressure of the recipient increased more than 40 mm Hg. Over the next year, Tigerstedt showed that the active agent in the renal extract was both water- and alcohol-soluble, non dialyzable, and heat sensitive. It was found in the renal cortex and renal venous blood, but not in the renal arterial blood. The pressor effect was not mediated by a change in heart rate and was not prevented by destruction of the spinal cord or other nerves. Tigerstedt named his discovery renin (Hall, 2003).

When ACE was initially identified, the bioassays used to measure its activity were poorly suited for extensive biochemical purification and analysis of the enzyme. This constitutes a major impediment to detailed characterization, and was finally solved when model tripeptide substrates such as hippuryl-His-Leu, hippuryl-Gly-Gly, and benzyloxycarbonyl (Z)-Phe-His-Leu were developed. ACE has been purified from the lungs of pig, rabbit,

dog, and cow, and from the sera of rabbit and humans (Soffer, 2011). ACE was identified on the luminal surface of endothelial cells and in organs such as the lungs and retina that have heavy vascularization. The enzyme was found bound to cell membranes by its C-terminal amino acid sequence. ACE expression is reported to be higher in the cultured endothelium from arterial sources than from cultured venous endothelial cells (Johnson & Erdös, 2014). According to Skidgel and Erdös (2013), ACE is present in high concentrations in epithelial cells with brush borders, including proximal tubules of the kidney, gut, choroid plexus, and placenta. Although most of the enzyme is associated with tissues, soluble ACE is also found in virtually all body fluids, including serum, urine, cerebrospinal fluid, seminal fluid, and amniotic fluid.

Initially, ACE was thought to be a metal dependent peptidase because enzymatic activity was virtually totally inhibited by EDTA. The purified enzyme was shown to contain zinc and it was estimated that each molecule of ACE contained only one metal atom; but it is now known that each molecule of ACE contains two zinc atoms (Ehlers & Riordan, 2011).The enzyme acts as a C-terminal dipeptidase for angiotensin I, bradykinin, and other small peptide hormones, including neurotensin, substance P, enkephalins, N-formyl-Met-Leu-Phe, acetyl Ser-Asp-Lys-Pro (AcSDKP) and angiotensin 1-7. It cleaves a C-terminal tripeptide from des-Arg9- bradykinin, and it can cleave substrates in which the carboxyl terminus is amidated, including the release of either a dipeptide or a tripeptide from the C-terminus of substance P (a peptide that affects bone marrow development by stimulating production of several myeloid growth factors, such as IL-1, IL-3, stem-cell factor, and granulocyte/monocyte CSF by bone marrow stromal cells) and a tripeptide from GnRH (previously referred to as LH-RH). *In vitro*, ACE cleaves a C-

terminal dipeptide from the amidated peptide cholecystokinin-8. Although ACE is best known for cleaving small peptides, there have been reports that it cleaves the insulin b chain. Also, several studies have now identified ACE as capable of cleaving the amyloid peptides (Ab1-40 and Ab1-42) implicated in the pathogenesis of Alzheimer's disease. The enzyme was found to have both carboxypeptidic and endopeptidic activity against Ab peptides, as determined by mass spectrometry (Sun *et al.,* 2008; Zou *et al*., 2009). Further, analysis of the brains of ACE knockout mice found that the lack of ACE does not significantly raise steady-state Ab levels (Eckman *et al.,* 2006). Although all of these findings indicate that ACE is not the major peptidase responsible for Ab cleavage *in vivo*, there is some genetic evidence, suggesting that ACE levels may influence the risk of Alzheimer's disease (Sun *et al*., 2008).

ACE was first cloned by Corvol and colleagues in 1988 from human endothelial cells and by Bernstein and colleagues in 1989 from mouse kidneys. Both group isolated cDNA based on probes derived from a partial amino acid sequence. Human ACE is synthesized as a 1306–amino acid polypeptide; the mature enzyme contains 1277 residues after cleavage of a hydrophobic amino acid leader sequence.ACE has a predicted molecular mass of 146.6 kDa. Mouse ACE is synthesized as a 1312–amino acid peptide and then processed to contain 1278 amino acids (predicted molecular mass of 147.4 kDa). Human and mouse ACE are highly homologous, with 1088 of the 1312 amino acids being identical (83%), whereas another 60 are conserved substitutions. A surprise derived from cloning and from a subsequent study was that ACE has two internal areas of amino acid homology, each about 612 amino acids. These are now termed the ACE N- and C-domains. For human ACE, the two domains are about 60% homologous in both DNA and

amino acid sequences. Higher homology (89%) is observed in a 40–amino acid "core" region containing residues comprising part of the catalytic site of the enzyme.

For mouse ACE, the structure is similar, with predicted amino acids 47–610 (the N-domain) aligning with amino acids 650–1208 (the C domain). In each domain, 300 amino acids are identical and an additional 47 are conserved substitutions. Each of the two catalytic domains contains the zinc-coordinating amino acid motif His-Glu-XX-His, which is a structural feature of many zinc peptidases. The exact motif in ACE is His-Glu-Met-Gly-His (HEMGH), with the two histidines coordinating zinc. A third zinc coordinating amino acid residue is glutamate, which is present on the C-terminal side of the HEMGH motif, and is found in its own characteristic motif EXIXD.

Originally, the importance of these residues for binding zinc was deduced by comparison with the sequence of thermolysin, but their functional roles have been substantiated by site-directed mutagenesis and ultimately by X-ray crystallography (Anthony *et al.*, 2012).

As a function of its amino acid sequence, ACE is classified as a member of the gluzincin family (i.e., thermolysin-like peptidases). The MEROPS database classification is clan MA, subclan MA(E), family M2, and peptidase XM02-001. Expression of each ACE domain in Chinese hamster ovary cells and the creation of mice with ACE genetic mutations have shown that each domain binds zinc and is independently catalytic. Our understanding of ACE structure is further enhanced by 1) the availability of ACE sequence from several species; 2) the three-dimensional crystal structure of the N-domain of ACE, the C-domain, and the enzyme ACE2, which is a C-domain homolog of ACE; and 3) the three-dimensional electron microscopic reconstruction of porcine ACE followed by the fitting of these data to the human atomic models (Chen *et al.,* 2010).

According to Danilov *et al*., (2011), The N-domain of the molecule begins with Leu1 and extends to Pro601. Within this region, H361EMGH365 and Glu389 coordinate zinc binding. The interdomain linker is about 11 amino acids from Pro602 to Asp612, followed by the C-terminal domain, which comprises Leu613 to about Pro1193 (the last amino acid seen in the crystal structure of the C domain). In this domain, H959EMGH963 and Glu987 coordinate zinc. The C-domain is followed by a stalk from approximately Gln1194 to Arg1227, a hydrophobic transmembrane domain from Val1228 to Ser1248, and an intracellular C-terminal tail from Gln1249 to Ser1277 at the end of the molecule. Limited proteolysis of ACE with endoproteinase Asp-N will cleave between the Thr615–Asp616 and the Leu1219–Asp1220 peptide bonds to generate the two catalytic domains in active form that can be separated by a lisinopril affinity column. Several groups have suggested that in somatic ACE, the catalytic activity of the C-domain is negatively regulated by the presence of the N-domain. X-ray and electron microscopic studies provide a detailed picture of the deep substrate-binding clefts suspected by earlier biochemical studies (Chen *et al.,* 2010).

In this model of ACE, the interdomain linker keeps the two domains separate by about 2.0–2.5 nm and is somewhat different from other models positing contact between the N- and C-domains. Recent analyses, using both X-ray crystallographic data and monoclonal antibody–-mediated epitope mapping and blocking studies, have indicated that membrane-bound ACE may form dimmers by association of two N-terminal domains (Danilov *et al*., 2011).

2.2.1 Functions of Angiotensin-Converting Enzyme

Production of angiotensin II and blood pressure regulation

Whereas the pioneering studies of ACE were oriented toward understanding blood pressure, a more modern view recognizes many other physiologic roles for this enzyme. In part, this is due to the diversity of ACE substrates and products. However, even if we only consider a single product—angiotensin II, the physiologic effects of ACE are extraordinarily diverse. Angiotensin II has effects on the kidney, the vasculature, the heart, the nervous system, metabolism, cell proliferation, and many other processes (Xiao *et al.,* 2003).

In considering ACE, it is often important to discriminate between the effects of angiotensin II and other ACE substrates and products. There are two major approaches. First, mice having null mutations in ACE can be compared with mice lacking other components of the renin-angiotensin system, such as angiotensinogen or the AT1 receptor. A second method, usable in mice and humans, is to compare the results of an ACE inhibitor to those of an angiotensin II receptor antagonist. Both of these approaches indicate that ACE-mediated production of angiotensin II is critical in blood pressure regulation. In knockout mice with a genetic mutation that eliminates angiotensinogen, renin, ACE, or all AT1 receptors (i.e., AT1A and AT1B), the blood pressure is very markedly reduced. For example, as measured using a tail cuff manometer, a wild-type mouse averages a systolic blood pressure of about 110 mmHg. This is essentially unchanged in mice with only a single functional copy of the ACE gene. In contrast, an ACE-null animal has an average systolic blood pressure of approximately 73 mm Hg. An equivalent reduction in blood pressure in animals lacking angiotensinogen, renin, or ACE

indicates that, at least in mice, the effect of eliminating ACE activity is a marked inability to produce angiotensin II, and that this is the key feature resulting in low blood pressure. The blood pressure in a mouse lacking both ACE and the bradykinin B2 receptor did not differ from a mouse lacking just ACE (Xiao *et al.,* 2003). Although equivalent genetic studies in humans are not possible, a recent comparison of the benefits of ACE inhibitors versus angiotensin II receptor antagonists for treating essential hypertension showed that both classes of inhibitors had similar long-term effects on blood pressure. This meta-analysis was not able to identify consistent differential effects on death, cardiovascular events, quality of life, or other outcomes (except cough) between ACE inhibitors and angiotensin II receptor antagonists in clinical trials). Thus, these human studies are grossly analogous to the animal data in suggesting that the major role of ACE affecting blood pressure is the production of angiotensin II (Maciel*et al*., 2008).

Another important substrate of ACE is bradykinin, whose levels are elevated in the absence of ACE activity. The kinin system, similar to the RAS, is composed of multiple peptides and receptors (Leeb-Lundberg *et al.,* 2005). The two major active kinins are bradykinin and kallidin. The latter is a decapeptide and is very rapidly converted to bradykinin by the action of aminopeptidases. Bradykinin has a short half-life of approximately 15 seconds in plasma due to the action of multiple metalloproteases (Moreau *et al*., 2005). Neutral endopeptidase and ACE are the two major bradykin in degrading enzymes (Duncan *et al*., 2000). Pharmacologic inhibition of ACE increases plasma bradykinin levels in patients, but has very little or no effect on the concentration of kallidin. Kinins bind two types of receptors called B1 and B2. The B2 receptor is the predominant receptor and is constitutively expressed, whereas the B1 receptor is induced

by tissue injury such as ischemia and inflammation (Madeddu *et al*., 2007; Maurer *et al*., 2011).Leeb- Lundberg *et al.* (2005) reviewed the pharmacology and physiology of these receptors. In blood vessels, bradykinin binds the B2 receptor and induces the production of nitric oxide (NO) and the release of prostacyclin, resulting in vasodilation and increased vascular permeability. In the kidney, bradykinin has natriuretic effects. Inducing bradykinin formation in hypertensive models reduces blood pressure (Wang *et al.,* 2012). Thus, besidesreducing angiotensin II production, ACE inhibitors maycontribute to the control of blood pressure by increasingthe concentration of bradykinin (Trabold *et al*., 2012; Maestri *et al.,* 2013).

Role of angiotensin II in renal development

Angiotensin II plays an important role in renal development, as shown by the phenotype of mice null for angiotensinogen, ACE, or all AT1 receptors. Such mice cannot effectively concentrate urine due to a marked expansion of the renal pelvis with a resulting underdevelopment of the renal medulla and papilla. In extreme cases, the renal medulla is virtually absent. The mice also show juxtaglomerular cell hypertrophy, medial thickening of small arteries and arterioles, interstitial fibrosis, and tubular dilatation. These renal lesions are not present in a newborn ACE knockout mouse but begin to be seen by 16 days after birth. Renal pathology was also observed in rats treated with either an ACE inhibitor or an AT1 receptor antagonist during the first two weeks of life. Despite limited exposure to the drug, the adult animals demonstrated widening of the renal papillary space and a reduction in the ability to concentrate urine (Ertoy and Bernstein, 2000). Great insight into the role of angiotensin II in renal development showed that the expansion of the renal pelvis results from a functional hydronephrosis

and elevation of intrapelvic urinary pressure (Matsusaka *et al.,* 2012). In the absence of the angiotensin II AT1 receptor, hypoplastic development of smooth muscle along the renal pelvis and ureter occurs. Further, wild-type mice exhibit rhythmic pulsatile pressure elevations in the renal pelvis which, roughly every 2 seconds, cycle the renal pelvic pressure from about 5 to 15 mm Hg and then back down. This rhythmic contraction and the resulting peristaltic movement from the renal hilum toward the bladder was absent in mice lacking AT1 receptors. These mice showed a constant elevation of intrapelvic pressure to just less than 20 mm Hg. Although an equivalent experiment has not been performed in ACE-null mice, the fact that these animals have a virtually identical phenotype strongly suggests that similar pathophysiologic mechanisms are at play. There are several other abnormalities in the kidneys of ACE-null mice. These animals have a significantly reduced glomerular filtration rate (GFR) and single nephron GFR due, undoubtedly, to their low blood pressure. Although proximal tubular fractional re-absorption was normal in the ACE-null mice, tubuloglomerular feedback was essentially absent. Interestingly, a different line of mice with one ACE-null allele and a second ACE allele targeting ACE expression to the liver also showed a marked reduction in tubuloglomerular feedback, despite a normal blood pressure, GFR, and single-nephron GFR. The authors concluded that the expression of ACE in renal tissues was an important component of tubuloglomerular feedback.

Additional work has shown that ACE-mediated intrarenal generation of angiotensin II works in conjunction with adenosine to induce afferent arteriolar contraction and regulate GFR (Schnermann & Briggs, 2008). The studies in animals have correlations in humans, where reports have shown that ACE inhibitors taken during the second and third

trimesters of pregnancyare associated with intrauterine growth retardation, neonatal hypotension, renal failure, oligohydramnios, and patent ductus arteriosus (Quan, 2006). The kidneys of newborns exposed to ACE inhibitors show juxtaglomerular hyperplasia, dilatation of Bowman's space, renal tubular dilatation, and increased cortical and medullary fibrosis. Similar effects on newborns have also been observed with AT1 receptor antagonists (Quan, 2006).

Role of angiotensin-converting enzyme in testis

Early work identified testis as having abundant ACE. Although the enzyme was catalytically similar to somatic ACE (the isozyme of ACE made by somatic tissues), the molecular mass of testis ACE at about 95 kDa was substantially different from that of the somatic isozyme. Also, different mRNAs encoded the two isozymes, and hormones regulated them differently. Using immunologic approaches, testis ACE was identified in male germ cells, whereas somatic ACE was found in the epididymal epithelium, cells of the vas deferens, and within seminal fluid. Part of the mystery surrounding testis ACE was solved with the cloning of this isozyme. This showed a protein of 732 amino acids. In the human, the amino-terminal 67 amino acids (66 amino acids in the mouse) are not found in somatic ACE. The remainder of the protein (665 amino acids in the human, 666 amino acids in the mouse) is completely identical to the C-terminal sequence of somatic ACE. In other words, whereas somatic ACE is composed of two catalytic domains, testis ACE, after beginning with a unique sequence, comprises only a single catalytic domain, as well as the stalk, C-terminal transmembrane domain, and C-terminal intracytoplasmic tail, which are identical to the C-terminal portion of somatic ACE (Schnermann &Briggs, 2008).

The mystery of this unique structure was solved when it was shown, using RNase protection and primer extension techniques that mouse testis ACE transcription begins at the 13th exon of the ACE gene. This is 7.2 kilobases 39 of the translation start site of somatic ACE in mice. Somatic tissues treat exon 13 as intronic and splice from exon12 to exon 14. It is the male germ cell specific initiation of transcription at exon 13 that endows testis ACE with 66 amino acids of unique N-terminal sequence. After that, exon 14 and the remainder of testis ACE correspond exactly to the carboxyl half of somatic ACE. That male germ cells begin transcription at a different location from somatic tissues is due to a tissue-specific promoter located immediately 59 of the transcription start site. This was demonstrated in two studies of transgenic mice in which Escherichia coli lacZ geneexpression was placed under the control of the putative testis ACE promoter region, comprising either 682 or91 base pairs of DNA immediately upstream from the start of testis ACE transcription (Langford *et al.,* 2011). According to Howard *et al.,*(2013) mice transgenic for these constructs expressed B-galactosidase only within elongating spermatozoa within seminiferous tubules, a histologic pattern identical to that of testis ACE. Thus, these experiments establish that a testis-specific promoter is positioned between the somatic ACE N- and C-domains.

In terms of function, male mice lacking ACE are severely compromised in their ability to reproduce. In contrast, reproduction in female mice lacking ACE is normal. This defect in males was shown to be directly attributable to the lack of testis ACE when mice expressing only testis ACE were studied (Hagaman *et al*., 2008). Male mice with this phenotype have low blood pressure, since they lack somatic ACE, but they reproduce normally. The role of testis ACE is unique, as male fertility was not restored in ACE

knockoutmice made transgenic for the expression of somatic ACE in sperm. In contrast, an equivalent experiment performed with testis ACE did restore normal fertility. This nonequivalence of the two isozymes of ACE, despite the correspondence of the testis isozyme to the C-terminal domain of somatic ACE, suggests a unique functional role for the testis isozyme. In addition, analysis of several lines of transgenic mice suggests that angiotensin II is not the crucial ACE product necessary for fertility. For example, the fertility of angiotensinogen null mice (mice unable to produce angiotensin II) was reported as 8.3 pups per litter, which is quite normal for laboratory mice (Hagaman *et al.,* 2008). Further, mice lacking both isoforms of the angiotensin II AT1 receptor have not been reported as having male reproductive defects. The light microscopic histology of testes from male mice null for ACE is not different from wild-type mice. Such mice have normal numbers of sperm and normal in vitro sperm motility. When mated with female mice, ACE-null animals inseminate females with normal frequency, as indicated by the presence of vaginal plugs. Further, when sperm from male ACE-null mice were collected. From the uteruses of normal females 1 hour after mating, there were no significant differences in the mean number of sperm or the viability, mobility, capacitation, or acrosome reaction as compared with a similar analysis using wild-type males. A major question in understanding the role of testis ACE is whether its enzymatic activity is critical for biologic function or whether the reproductive effect is only due to the presence of the testis ACE protein, perhaps in some structural capacity within sperm. This was examined in a mouse model in which testis ACE was expressed in its normal location and quantity but in an enzymatically inactive form, due to a genetic mutation eliminating zinc-binding and catalytic activity (Fuchs *et al*., 2005). Despite the presence

of testis ACE protein, the lack of catalytic activity severely reduced male fertility, a phenotype identical to mice lacking testis ACE protein. In terms of magnitude of effect, consider that when six wild-type males were mated with wild-type females, 19 vaginal plugs were observed that resulted in 15 litters and 153 offspring. In contrast, seven male mice lacking testis ACE catalytic activity produced 22 vaginal plugs, but only one litter and one offspring. A major defect in the *invitro* binding of sperm to ovocytes was also observed in that the binding of sperm from mice lacking testis ACE activity was only 4% the number of sperm from wild type controls. Thus, male mice lacking testis ACE activity do mate, but this is so ineffective as to be highly noncompetitive in an evolutionary sense.

Role of ACE in erythropoiesis

An early study of the ACE inhibitor enalapril in both hypertensive patients and normal volunteers found a small reduction in hematocrit levels. Others have reported anemia as a side effect of treatment with ACE inhibitors. ACE-null mice have a mild normocytic anemia, with the hematocrit consistently lowered by approximately 20% as compared with control mice. However, ACE knockout mice also have other abnormalities, including reduced renal function, which could induce a secondary anemia. To understand the anemia, Cole *et al*. (2010) studied a mouse line expressing a truncated form of ACE in which the enzyme was secreted from tissues. In these mice, plasma ACE activity was 35% the normal level, and there was no evidence of renal insufficiency, but the mice did have anemia similar to ACE-null mice. The anemia was due to a reduction of red blood cell mass, and not volume expansion. In this model, a 2- week infusion of angiotensin II corrected the hematocrit to near wild type levels, strongly implicating a direct role of

angiotensin II in erythropoiesis. Consistent with this finding, angiotensin II receptor antagonists reduce hemoglobin, hematocrit, and erythrocyte counts in rats. The role of ACE and angiotensin II in erythropoiesis is complex. First, in vitro culture suggests that angiotensin II is associated with the production of endogenous erythropoietin from peritubular fibroblasts of the kidney. However, there is still uncertainty about the exact relationship of ACE inhibition and erythropoietin levels in vivo. Some studies suggest that the administration of ACE inhibitors reduces plasma erythropoietin levels or induces resistance to this hormone. Angiotensin II modulation of erythropoietin production is hypothesized to occur via activation of AT1 receptors and/or altered renal hemodynamics (Gossmann *et al*., 2011; Benohr *et al*., 2014). However, other studies found no causative link between erythropoietin and anemia induced with ACE inhibitors or AT1 receptor antagonists. Second, angiotensin II is a mitogen for erythroid progenitors. *In vitro* culture of either human peripheral blood mononuclear cells or murine bone marrow cells showed that burst-forming unit erythroid and CFU erythroid were elevated by the addition of angiotensin II in the culture medium (Lin *et al.,* 2011). This effect was inhibited by losartan. Further, losartan alone retarded the formation of CFU erythroid. It should be noted that a variety of clinical reports have observed an association between activation of the renin-angiotensin system and increased erythropoiesis (Cole *et al*., 2010). These studies come from analyses of patients with a variety of chronic diseases, including chronic obstructive pulmonary disease, heart failure, and renal transplantation.

Myelopoiesis

Besides erythropoiesis, ACE appears to affect myelopoiesis. This is best appreciated in ACE knockout mice that have increased numbers of immature myelocytic cells

(myeloblasts and myelocytes) in theirbone marrow (Lin *et al*., 2011). These mice also have diminished numbers of mature segmented neutrophilsin the bone marrow. Extensive extramedullary hematopoiesis is present in ACE knockout mice. Moreover, ACE knockout mice have an increased susceptibility to Staphylococcus aureus infection (Okwan-Duodu *et al*., 2010). *In vitro* analysis of bone marrow proliferation and differentiation showed that ACE inhibition expanded the number of colony-forming units (i.e., increased proliferation) but slowed the formation of mature myeloid cells (decreased maturation), which is consistent with the bone marrow phenotype of ACE knockout mice (Lin *et al*., 2011). Substance P (SP) is a peptide that affects bone marrow development by stimulating production of several myeloid growth factors, such as IL-1, IL-3, stem-cell factor, and granulocyte/monocyte CSF by bone marrow stromal cells. ACE is a major peptidase responsible for hydrolyzing (and inactivating) SP, and elevated SP is found in the bone marrow of ACE knockout mice (Lin *et al.,* 2011). In fact, ACE appears to regulate myeloid proliferation through SP. ACE inhibitor treatment increases the number of colonies seen in an in vitro myeloid colony-forming assay. However, when the ACE inhibitor–treated cultures were co-incubated with either an anti–SPneutralizing antibody or an SP receptor antagonist, the increase in colony numbers was completely eliminated. ACE inhibitor mediated myeloproliferation was not observed using purified hematopoietic stem cells, but was found when these cells were co-cultured with a stromal cell line, implying an important role for bone marrow stromal cells in modulating myeloproliferation, possibly by SP production. In addition to destroying SP, ACE also produces angiotensin II, and it is the lack of angiotensin II in the ACE knockout bone marrow that retards myeloid maturation.

Role of ACE in immune response

Major Histocompatibility Complex Class I: The surface presentation of peptides by proteins comprising the major histocompatibility complex (MHC) class I molecules is a critical part of almost all CD8+ T cell-adaptive immune responses (Jensen, 2007). MHC class I molecules are on the surface of all nucleated cells, and thus all cells bear the biochemical machinery necessary to process proteins into peptides and load these peptides onto MHC class I molecules for cell-surface display. A major function of MHC class I molecules is to display viral proteins on a virally infected cell, leading to activation of T cells and destruction of the cell. That ACE may play a role in the processing of such peptides was first suggested in 1992, when serum ACE activity was found capable of trimming a synthetic influenza antigen into a form capable of being bound and presented by MHC class I molecules. Using a cell-free system, a second group described serum ACE activity as necessary to trim the C terminus of a peptide epitope of human immunodeficiency virus 1 into the final presented 10-mer peptide (Nakagawa *et al.,* 2010). Although these reports of extracellular processing are interesting, MHC class I peptides are typically generated within antigen presenting cells. The first evidence that ACE is capable of affecting intracellular class I peptide processing was presented by Yewdell and colleagues (1992). This group used a vaccinia virus expression system to over express ACE in a fibrosarcoma cell line and convincingly demonstrate that ACE was capable of processing the influenza nucleoprotein peptide M147-158/R2 into the final MHC class I epitope M147-155via C-terminal dipeptide release. Further analysis showed that the M147-158/R2 peptide was transported to the endoplasmic reticulum, where it was cleaved by ACE. The authors speculated that ACE acted on the peptide

prior to antigen binding to MHC class I molecules. This study used ACE over expression as an artificial tool to investigate the biochemistry of MHC class I peptide transport and trimming. The authors concluded that peptidases do play a role in final antigen processing. However, the authors indicated that they were not suggesting that ACE normally plays a role in antigen processing. Little follow-up work was performed on the role of ACE and MHC class I. In fact, the detailed study of antigen processing led to the belief that carboxypeptidases do not play a natural role in MHC class I peptide trimming (Kunisawa and Shastri,2003). In 2008, this question was re-examined using genetically modified macrophages and other cells designed to over express ACE (Shen *et al*., 2008). Several different antigens were studied, and antigens were presented to cells in a variety of fashions. All studies suggested that the over expression of ACE was able to cleave MHC class I peptide precursors within the endoplasmic reticulum. Additional *in vivo* experiments using an adoptive transfer strategy with wild type macrophages or macrophages over expressing ACE demonstrated enhanced generation of antigen-specific CD8+ T cells in mice receiving the macrophages over expressing ACE. Thus, this work was similar to that of Eisenlohr *et al.* (2002) in that it showed that ACE over expression had significant effects on antigen presentation.

Understanding the physiologic role of ACE in MHC class I antigen processing began with experiments showing that macrophages and dendritic cells (antigen-presenting cells) increase their expression of ACE following immune activation, either *in vitro* by interferon g (IFNg) or *in vivo* by infection of a mouse with Listeria monocytogenes (Shen *et al.,* 2011). Such data suggested a role of ACE in response to immune challenge. An analysis of the role of ACE in MHC class I peptide processing demonstrates that

carboxypeptidases play an important role in the natural processing of the MHC class I repertoire. Although further work is necessary to investigate the role of ACE in immunogenic peptide processing, several conclusions are appropriate. The finding that ACE affects presentation of immunologic peptides, and that ACE is up regulatedby macrophages and dendritic cells after infection with listeria, raises the question of whether this ACE up regulation diversifies the presented MHC class Ipeptides and enhances the immune response. It is also important to recognize that, in humans, the natural immune response consists of many different overlapping responses, and we know of no credible evidence that medical treatment with ACE inhibitors significantly reduces immune function.

2.3 ANGIOTENSIN-CONVERTING ENZYME POLYMORPHISMS

The insertion/deletion (alu-repeat) polymorphism in ACE was used as a marker genotype in a study involving healthy subjects (n = 80), where it was found that the allele frequency was 0.4 for the insertion (I) and 0.6 for the deletion (D). The I and D genotypes correlated with serum ACE concentration, where individuals with the DD genotype had the highest average serum ACE levels, those with the II genotype averaged the lowest, and heterozygous individuals (ID) had an intermediate value. It was concluded that the I/D polymorphism accounted for 47% of the total phenotypic variance of serum ACE. The effect of the I/D gene polymorphism was found not only in people of European ancestry, but also among Japanese and Pima Indians, where it was associated with serum ACE activity (Foy *et al*., 2006). In contrast, there was no such association in African Americans (Bloem *et al*., 2006). The I/D polymorphism is not the only polymorphism in the human ACE gene; ACE gene sequencing in 11 individuals (a total of 22 copies of the

ACE gene) identified 78 different polymorphisms, of which 17 were in absolute linkage disequilibrium with the intron 16 I/D Alu repeat (Rieder *et al.,* 2009). Thirteen distinct haplotypes were identified. Early studies examined the association of the ACE I/D polymorphism with coronary artery disease. Cambien *et al.* (2012) reported that the D/D genotype, associated with high levels of circulating ACE, was found more frequently in patients with myocardial infarction than in control subjects. Leatham *et al*. (2012) corroborated this finding in Caucasian patients with myocardial infarction, but not in patients with unstable angina. Nakai *et al.* (2012) also found an association of the D/D genotype with coronary artery disease in Japanese patients. However, clinical studies eventually began to show conflicting results. For example, whereas Schunkert *et al*. (2012) reported that, among subjects with left ventricular hypertrophy (n = 141 females, 149 males), there was an excess of the D/D genotype, and that this association was especially strong in males 45— 59 years of age, an analysis of echo cardiographic data from the Framingham Heart Study (n = 2439) found no association of left ventricular mass with the ACE I/D polymorphism, nor any increased risk of left ventricular hypertrophy. Kupari *et al*. (2012) also concluded that, in the absence of heart disease, the ACE I/D polymorphism had no major influence on left ventricular mass or function that was detectable with echo cardiography.

Although the early studies implicated an association of the ACE D/D genotype with myocardial infarction, no convincing evidence was initially found associating this polymorphism with hypertension. For example, Schmidt *et al.* (2013) reported findings from the Dutch Hypertension and Offspring Study that the allele frequencies for both the I and D allele were similar in parents and offspring with high and low blood pressure(n =

111 parents and 75 offspring). Gu *et al.* (2013) also reported a lack of association between the I/D polymorphism in a hypertensive Belgian population (n = 119) compared with controls, regardless of age or sex. In Japanese patients with essential hypertension, Ishigami *et al*. (2012) reported that there was no association with ACE I/D polymorphism. Barley *et al.* (2012) suggested that there may be racial differences in the association of ACE I/D polymorphism with hypertension in that subjects of European descent showed no association between ACE genotype and blood pressure, whereas subjects of African Caribbean descent did. However, the same group also did not find any association between I/D polymorphism and plasma renin or aldosterone levels in either Caucasians or African Caribbeans. Eventually, a very large study was conducted as part of the Framingham Heart Study to examine the association between the I/D ACE polymorphism and hypertension. This study showed that there was a statistically significant association of the ACE locus with small effects on hypertension and with diastolic blood pressure, but these findings were only present in men (O'Donnell *et al*., 2012). Recently, the Cohorts for Heart and Aging Research in Genomic Epidemiology Consortium studied 29,136 subjects and compared their findings with those in the Global Blood Pressure Genetics Consortium (n = 34,433) and the Women's Genome Health Study (n = 23,019) (Johnson *et al*., 2013). A single-base polymorphism in ACE termed rs4305, which is different from the traditional I/D polymorphism referred to in the study by Johnson *et al*. (2011) as rs4350-I/D, showed modest replication of an association with increased hypertension. However, the effect was very modest (b = 0.06, where b is the log odds of hypertension per allele dose). Thus, the available evidence suggests that any

effect of ACE on blood pressure in humans is very small. This conclusion is consistent with the results of a recent meta-analysis (Takeuchi *et al.,* 2013).

2.3.1 Angiotensin-Converting Enzyme I/D Polymorphism and Malaria

Recent reports have shown that angiotensin peptides can induce impairment of the erythrocytic cycle of *Plasmodium*, reducing the parasite growth *in vitro* (Maciel *et al.,*2008; Saraiva *et al*., 2011). Since the development of CM depends on the initial levels of parasitemia in mice and possibly in humans (Bejon *et al*., 2005), this could be a potential explanation for Ang II protection from CM. It is possible that Ang II could modulate malaria severity through additional mechanisms, since the inhibitory effect observed in parasite growth is modest (Maciel *et al.,*2008; Saraiva *et al.,*2011).

A protective effect of Ang II against CM may also be mediated by effects on the host vascular endothelial cells. Ang II exerts its vasoconstriction effect through the AT1 receptor on vascular smooth muscle cells. However, since the BBB is impermeable to Ang II and other RAAS peptides, circulating Ang II does not reach underlying vascular muscular cells and vascular pressure in the brain is locally regulated by Ang II produced within the brain parenchyma (Bader, 2010). In the context of CM, higher levels of circulating Ang II would not increase vasoconstriction in the brain, but could affect endothelial cells that form the BBB and express both, AT1 and AT2 receptors. Using monolayers of human brain microvascular endothelial cells, we have observed that incubation with erythrocytes infected with *Plasmodium falciparum* promotes the disruption of interendothelial cell junctions between these cells. Activation of AT2 or inhibition of AT1 receptor preserves the integrity of interendothelial cell junctions after incubation with *P. falciparum in vitro* and protect against experimental CM in mice

(Gallego-Delgado *et al*., 2013). This could be key in the association of Ang II with lower development of CM since AT2 receptor stimulation has protective effects on brain injury (McCarthy *et al*., 2009; Habashi *et al.,*2011) and inhibits vascular endothelial cells migration (Falcon *et al*., 2005). Gene expression analysis comparing CM-susceptible mice vs. CM-resistant mice also showed higher expression levels of the AT2 receptor gene in CM-resistant mice compared to susceptible ones (Delahaye *et al.,*2007).

Finally, it has also been proposed that the effects of Ang II in malaria severity could be mediated by immune mechanisms, since activation of AT1 by Ang II is a pro-inflammatory stimulus (Benigni *et al.,*2010). In this case, elevated levels of Ang II would result in higher inflammation that would contribute to the activation of endothelial cells and would be detrimental for the outcome of severe malaria. Treatment of *Plasmodium*-infected mice with losartan, an inhibitor of AT1, and captopril, an inhibitor of ACE that reduces the levels of Ang II, inhibit T cell activation induced by infection (Silva-Filho *et al.,*2013). Treatment with losartan, which inhibits the signaling of Ang II through AT1, while potentiating the effects mediated by AT2, would result in decreased endothelial cell migration and lower disruption of interendothelial cell junctions, in addition to anti-inflammatory systemic effects (Marchesi *et al*., 2008) and would align with the hypothesis of a beneficial effect of Ang II in malaria severity. However, the effects of captopril, reducing Ang II, decreasing parasitemia and protecting against experimental CM (Silva-Filho *et al.,*2013), apparently do not agree with the hypothesis that increased Ang II provides protection against severe malaria. Further experiments *in vivo* are necessary to elucidate the signaling pathways, AT1 vs. AT2, mediating the observed effects on malaria severity of different concentrations of systemic Ang II in parasite

development and generation of CM.Humans have been infected by *P. falciparum* for over 50,000 years (the estimated date for the out-of-Africa migration) and have co-evolved during this time (Tanabe *et al.,*2010). Different human polymorphisms, such as those causing thalassemia or sickle cell anemia, have been associated to this natural selection process. Although there is not sufficient evidence available to demonstrate it, the association of polymorphisms of the ACE and protection from severe malaria may also be the result of a natural selection process. This apparent contradiction would be explained if higher levels of Ang II at early ages protect from CM. In this case, those polymorphisms would have a higher transmission ratio, despite their deleterious effects during adulthood, as observed in people with African American genetic background (Sampson *et al*., 2014). While hypertension has been traditionally considered a disease of western countries, in the latest years it has become obvious that hypertension is coming to epidemic levels in Africa, even presenting higher prevalence than in wealthier countries, possibly because of the higher levels of circulating Ang II found in people with African genetic background (Lloyd-Sherlock *et al.,*2014). As a consequence, a significant number of African natives are currently taking anti-hypertensive medication, and this number is expected to increase exponentially in the coming years (Lloyd-Sherlock *et al.,*2014).

A protective role for Ang II against cerebral malaria was proposed based on a gene polymorphism analysis of angiotensin-related enzymes in patients with severe or mild malaria, suggesting that elevated levels of Ang II would reduce the incidence of severe disease (Dhangadamajhi *et al.,* 2010). Additionally, Ang II was found to inhibit the growth of *P. falciparum in vitro* (Saraiva *et al.,* 2011). To study the effect of Ang II in

malaria *in vivo*, an experimental model in mice exposed to increased levels of circulating Ang II was developed. Since Ang II has a rapid turnover, continuous delivery is required to maintain elevated levels in plasma. Using subcutaneous mini-pumps for constant delivery of Ang II in a malaria rodent model, it was observed that increased levels of Ang II result in a moderate decrease of levels of blood parasitemia and incidence of experimental cerebral malaria (Saraiva *et al.,* 2011).

According to Mulder *et al.,* (2003), the ACE genotypes include the presence (I allele) or absence (D allele) of a 287 bp Alu repeat sequence in intron 16, resulting in 3 genotypes (D/D and I/I homozygote, and I/D heterozygote). An I/D polymorphism of ACE gene at this region correlates with circulating ACE plasma activity. An I/D (region in intron 16) polymorphism of ACE gene correlates with circulating ACE plasma activity. Higher plasma ACE activity is observed in subjects with ACE-D/D genotype. A raised plasma ACE activity may elevate blood pressure through increased production of angiotensin II. Studies have demonstrated that ACE Insertion (I)/Deletion (D) polymorphism are associated with common diseases like malaria, hypertension, diabetic nephropathy, coronary heart disease and tuberculosis.

2.4 ENDOTHELIAL NITRIC OXIDE SYNTHASE (eNOS)

Endothelial NOS (eNOS), also known as nitric oxide synthase 3 (NOS3) or constitutive NOS (cNOS), is an enzyme encoded by the *NOS3*genein humans and located in the 7q35-7q36 region of chromosome 7. This enzyme is one of three isoforms that synthesize nitric oxide (NO), a small gaseous and lipophilic molecule that participates in several biological processes (Marsden *et al*., 2002). The other isoforms include neuronal nitric

oxide synthase (nNOS), which is constitutively expressed in specific neurons of the brain and inducible nitric oxide synthase (iNOS), whose expression is typically induced in inflammatory diseases (Oliveira-Paula *et al*., 2014). eNOS is primarily responsible for the generation of NO in the vascular endothelium, a monolayer of flat cells lining the interior surface of blood vessels, at the interface between circulating blood in the lumen and the remainder of the vessel wall (Fish and Marsden, 2006).

The endothelial nitric oxide synthase (eNOS) is a constitutively expressed enzyme that oxidizes L-arginine to generate L-citrulline and nitric oxide (NO). The catalysis of this reaction requires a number of essential cofactors such as calmodulin (CaM), tetrahydrobiopterin (H4B), flavin mononucleotide, FAD, and NADPH. The NO thus generated exerts a number of functions on the cardiovascular system. Acute activation of eNOS in blood vessels in response to the application of an agonist such as acetylcholine or bradykinin results in the activation of the soluble guanylyl cyclase in smooth muscle cells and the production of cGMP. An increase in intracellular cGMP levels may affect vascular tone by a number of mechanisms, for example by decreasing the intracellular concentration of free Ca^{2+} as well as by activating protein kinase G and phosphorylating heat shock protein (Hsp) 20, which is reported to regulate force by binding to thin filaments and inhibiting cross-bridge cycling (10, 104, 105). A basal NO production or "vasodilator tone" can also be said to exist *in vivo* as the fluid shear stress generated by the flowing blood and pulsatile stretch of the vascular wall, as a consequence of the cardiac cycle, continually stimulate endothelial NO production. Although important for the regulation of blood flow, the continuous production of NO also helps to maintain the endothelium in an anti-atherogenic state, in part by preventing the activation of

transcription factors that determine the expression of proatherogenic gene products such as the adhesion molecules required for the attachment and sequestration of monocytes through the endothelial cell monolayer.

NO is produced by nitric oxide synthase (NOS) enzymes, of which there are three main isoforms: neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS) (Alderton *et al*., 2001).The three NOS isoforms are encoded on separate chromosomes by separate genes. They share homology in regions involved in cofactor binding (for example, FAD, FMN, and NADPH ribose and adenine binding sites), and have similar enzymatic mechanisms that involve electron transfer for oxidation of the terminal guanidino nitrogen of L-arginine. However, their expression patterns differ, as do the detailed regulations of their activity. nNOS is predominantly expressed in certain neurons and in skeletal muscle, whereas eNOS is predominantly expressed in endothelial cells. iNOS is expressed by macrophages and cells of macrophage/monocyte lineage. Despite their names, a variety of cell types express these isoforms, with many tissues expressing more than one isoform. Furthermore, the innervations and vasculature in all tissues have the potential to express NOS and eNOS, while circulating blood elements may express iNOS. Both nNOS and eNOS are generally constitutively expressed; their activities are primarily regulated by intracellularCa2/calmodulin levels. In contrast, iNOS expression is induced in activated macrophages as an immune response. For enzymatic activity, NOS proteins must bind cofactors anddimerize. NOS proteins first bind to the cofactors FAD and FMN. The additions of L-arginine, BH4 and heme allow the NOS protein to form dimers. eNOS and nNOS dimmers formed this way are inactive, and depend on calmodulin binding stimulated by increases in intracellular calcium. In contrast, the iNOS

dimers bind calcium/calmodulin and are active even at low (resting intracellular) concentrations of calcium. Thus, the main switch for activity for nNOS and eNOS is a transient increase in intracellular calcium concentration, whereas the main switch for iNOS is at the level of transcription. The DNA and protein sequences of the NOS isoforms are conserved between species, with nNOS, eNOS and iNOS sharing up to 96%, 93% and 80% amino acid sequence identity between mice and humans. Within each species, the NOS isoforms share about 51-59% amino acid sequence identity (Alderton *et al*., 2001).

Each isoform of nitric oxide synthase has notable structural features. The nNOS gene encodes a PDZ domain in exon 2 that is required for membrane association. Several nNOS splice variants lack exon 2, resulting in expression of cytoplasmic nNOS that lacks subcellular localization sequences (Brenman *et al.,* 2006). In endothelial cells, eNOS is localized to caveolae by N-terminal fatty acid modifications-myristoylation and palmitoylation, as well as interactions with heat shock protein hsp90 and caveolins. Caveolins (caveolin-1 in endothelial cellsand caveolin-3 in cardiac muscle) bind to eNOS and inhibit its activity. eNOS is also regulated by phosphorylation at multiple sites, including serine 1179 and threonine 497.In addition to nNOS, eNOS, and iNOS, there is a constitutive lyactive NOS isoform present in mitochondria, referred to as mtNOS.23,24mtNOS is located in the inner mitochondrial membrane, and likely plays key roles in modulating mitochondrial respiration and mitochondrial transmembrane potential. However, whether mtNOS corresponds to one of the three known isoforms is not known (Lamas *et al.,* 2012).

2.4.1 Physiological Functions of Endothelial Nitric Oxide Synthase

Vasodilation and inhibition of platelet aggregation and adhesion

Endothelial NOS appears to be a homeostatic regulator of numerous essential cardiovascular functions. Endothelial NOS-derived NO dilates all types of blood vessels by stimulating soluble guanylyl cyclase and increasing cyclic GMP in smooth muscle cells (Rapoport *et al*., 2003). Deletion of the eNOS gene leads to elevated blood pressure. Nitric oxide released towards the vascular lumen is a potent inhibitor of platelet aggregation and adhesion to the vascular wall. Besides protection from thrombosis, this also prevents the release of platelet-derived growth factors that stimulate smooth muscle proliferation and its production of matrix molecules. Endothelial NOS is also critical for adaptive vascular remodeling to chronic changes in flow (Lin*et al.,*2011).

Inhibition of leucocyte adhesion and vascular inflammation

According to Zeiher *et al.,* (2005), Endothelial NO controls the expression of genes involved in atherogenesis. Nitric oxide decreases the expression of chemo attractant protein MCP-1.Nitric oxide can also inhibit leucocyte adhesion to the vessel wall by either interfering with the ability of the leucocyte adhesion molecule CD11/CD18 to bind to the endothelial cell surface or by suppressing CD11/CD18 expression on leucocytes. Leucocyte adherence is an early event in the development of atherosclerosis, and therefore, NO may protect against the onset of atherogenesis. A disturbed integrity of the endothelial monolayer barrier can initiate proinflammatory events. Endothelium-derived NO prevents endothelial cell apoptosis induced by proinflammatory cytokines and proatherosclerotic factors including reactive oxygen species (ROS) and angiotensin II

(AT). The suppression of apoptosis may also contribute to the anti inflammatory and anti-atherosclerotic effects of endothelium-derived NO (Garg and Hassid, 2009).

Control of vascular smooth muscle proliferation

Furthermore, NO has been shown to inhibit DNA synthesis, mitogenesis, and proliferation of vascular smooth muscle cells. These anti proliferative effects are likely to be mediated by cyclic GMP. The inhibition of platelet aggregation and adhesion protects smooth muscle from exposure to platelet-derived growth factor(s). Therefore, NO also prevents a later step in atherogenesis, fibrous plaque formation. Based on the combination of those effects, NO produced in endothelial cells can be considered an anti-atherosclerotic principle (Murohara *et al.,* 2008).

Stimulation of angiogenesis by endothellial nitric oxide synthase-derived NO

Endothelial NOS-derived NO plays a critical role in post-natal angiogenesis, mediating signals downstream of angiogenic factors. Recent findings in eNOS-deficient mice point to a novel and previously unrecognized role of NO in foetal lung development and lung morphogenesis. The lung phenotype of eNOS-deficient mice closely resembles alveolar capillary dysplasia in humans, a form of malignant pulmonary hypertension of the newborn that presents with defective lung vascular development and respiratory distress. Similarly, eNOS had been found to be critical for collateral formation and angiogenesis post-ischaemia. Furthermore, the positive effects of NO on endothelial cell survival are likely to also contribute to the pro-angiogenic effects of NO (Murohara *et al.,* 2008).

2.5ENDOTHELIAL NITRIC OXIDE SYNTHASE (eNOS) POLYMORPHISM AND MALARIA

The eNOS is constitutively expressed by vascular endothelium, and its gene is assigned to chromosome 7. This gene contains 26 exons spanning ~21 kb of genomic DNA and encodes an mRNA with 4,052 nucleotides, and a single copy is present in the haploid genome. Several allelic variants of the eNOS gene have been identified, and a variant with a T/C substitution in the 5′ flanking region near the promoter at position −786, a variant with a 27-bp variable number of tandem repeat (VNTR) region in intron 4, and a variant with a G/T substitution at position 894 in exon 7 that codes for replacement of glutamic acid by aspartic acid at residue 298 in the mature eNOS protein, which together span ~6.2 kb in the human genome, have been determined to be clinically important for several diseases (Fairchild *et al*., 2001).

According to Yayama and Okamoto, (2008), activation of AT2 receptor results in increased production of nitric oxide by endothelial Nitric Oxide Synthase (eNOS) in endothelial cells, which could be protective against cerebral malaria since low nitric oxide bioavailability may exacerbate endothelial dysfunction and contributes to the pathogenesis of severe malaria (Gramaglia *et al*., 2006; Miller *et al.*, 2013). Interestingly, poly- morphisms in eNOS that are responsible for increased expression and nitric oxide production have been associated with mild malaria (Dhangadamajhi *et al.,* 2010).In addition, in recent studies, increased NO production has been shown to be beneficial because of its anti parasitic and anti disease effect, although this is controversial. This effect is due to inhibition of the cytoadherence process through down regulation of the expression of ICAM1, VCAM1, and E-selectin, which is involved in cytoadherence and

microvascular sequestration of parasitized RBCs and decreased production of tumor necrosis factor by macrophages (Serirom, *et al*., 2003). NO is produced during the enzymatic conversion of l-arginine to l-citrulline by three isoforms of nitric oxide synthase (NOS), namely inducible NOS, endothelial NOS (eNOS), and neuronal NOS. The eNOS-derived NO mediates vasodilation, inhibits platelet aggregation and endothelial cell activation, and modulates expression of cell adhesion molecules. Further, endothelial dysfunction in malaria is nearly universal when the disease is severe, is reversible with l-arginine, and likely contributes to pathogenesis (Yeo *et al.,* 2007). Therefore, we hypothesized that functionally important variants of eNOS could influence individual susceptibility to malaria by altering the amount of NO generated by the endothelium. The eNOS is constitutively expressed by vascular endothelium, and its gene is assigned to chromosome 7. This gene contains 26 exons spanning ~21 kb of genomic DNA and encodes an mRNA with 4,052 nucleotides, and a single copy is present in the haploid genome (Marsden *et al*., 2003). Several allelic variants of the eNOS gene have been identified, and a variant with a T/C substitution in the 5′ flanking region near the promoter at position −786, a variant with a 27-bp variable number of tandem repeat (VNTR) region in intron 4, and a variant with a G/T substitution at position 894 in exon 7 that codes for replacement of glutamic acid by aspartic acid at residue 298 in the mature eNOS protein, which together span ~6.2 kb in the human genome, have been determined to be clinically important for several diseases. However, the mechanisms by which these genetic variants can affect eNOS enzyme activity have not been demonstrated. Impaired NO production as a result of the polymorphism in exon 7 and lower serum nitrite-nitrate (NO_x) levels due to the −786C variant have been observed (Miyamoto *et al*., 2003). The

VNTR polymorphism in intron 4 of eNOS (eNOS4b/a polymorphism) has been reported to be significantly associated with the plasma NO_x concentration and affects the transcription efficiency in a haplotype-specific fashion in linkage disequilibrium with the T-786C polymorphism in the promoter region (Wang*et al*., 2002). To the best of our knowledge, no other coding or functional variants in this region which impair endothelial NO production have been reported so far. Moreover, because of different gene pools, lifestyles, and gene-environment interactions among populations, there may be ethnic differences in the allelic frequencies of eNOS polymorphisms, as well as the genetic associations between disease and the plasma NO_x concentration (Wang *et al.,* 2002).

CHAPTER THREE

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Subjects

A total number of two hundred (200) samples were collected. One hundred and twenty (120) malaria-infected patients (males and females) and eighty (80) control subjects (males and females) samples were obtained from patients attending Sheikh Muhammad Jidda General Hospital and Murtala Muhammad Specialist Hospitals, Kano State, Nigeria.

3.1.2 Ethical Approval

Ethical approval was obtained from the ethics committee of the Hospital Management Board, Kano state, with an ethical clearance number HMB/GEN/488/VOL.I. Individual patients were duly informed about the research work and consent forms were signed by the patients. All information was kept confidential.

3.1.3 Inclusion and Exclusion Criteria

Patients studied were those that had been diagnosed as having malaria using the WHO criteria (2012), of parasites per microlitre of blood assuming a leukocyte count of 8,000/µlitre and within the age of 20-70years. Only those who consented were included in the study. Individuals below the age of 20 years and those who refused to give their consent were excluded.

3.1.4 Administration of Questionnaires

A standard questionnaire was made available to the patients. This reflected their background information, socioeconomic status, awareness about hypertension and malaria and essentially, the drugs prescribed to the patients for the treatment of these conditions.

3.1.5 Apparatus and Equipment

Weighing balance, beaker, measuring cylinder, syringe and needle, cotton wool, sample containers, test-tubes, tube racks, centrifuge, Micropipettes, Pipette tips, refrigerator, cuvettes, High Speed Refrigarated Centrifuge (PM-180R), Gel Documentation Machine (BIORAD ET-997061AA), Votex mixer (CAT-S8220), Gel tank, Gel combs, Casting tray, Triacetate buffer, Agarose gel powder, Eppendorf centrifuge, Eppendorf tubes, Incubator (Multi block heater-Lab line), Programmable thermal cycler (PTC-100^{TM}), Power pack (Biorad).

3.1.6 Reagents

Lysis buffer, Proteinase-K, Phenol chloroform, Chloroform, 100% ethanol, 70% ethanol, 3M sodium acetate, Trisacetate buffer, Ethidium bromide, Buffer GB, Washing buffer, Purification buffer (all were obtained from Bioneer Corporation, United State and were of analytical grade).

3.2 METHODS

3.2.1 Specimen Collection

Venous blood (5ml) was aseptically collected from the subjects and controls, with the use of a sterile 22G needle and syringe after sterilizing the site with methylated spirit. The samples were properly labeled and 3mls reserved for genomic DNA isolation and parasitaemia determination. The remaining was allowed to clot to obtain the serum which was stored at 4^0C. Blood collected for genomic DNA isolation was stored at -20^0C until required.

3.2.2 Determination of Blood Pressure

The blood pressure of the individual patients was determined using the digital blood pressure monitor (Boots Upper Arm Blood Pressure Monitor, Omron HEM-742-UK). Hypertension was defined as Systolic Blood Pressure greater than or equal to 140 mmHg and Diastolic Blood Pressure greater than or equal to 90 mmHg.

3.2.3 Determination of Parasitemia

Microscopic diagnosis for malaria was carried out by examination of thick and thin blood smears stained with Giemsa. Blood was collected from a single finger prick. Thick smears were considered negative if no parasite was seen after examination of approximately 100 oil immersion fields. Blood parasitemia was calculated on the basis of positive smears collected from patients. The malaria parasite density was graded as follows: 1 parasite/field: low density (+), moderate: 2-9 parasites/field: medium density, moderate (++); and >20 parasites/field: high density, severe (+++) (Cheesbrough, 1998).

Patients were considered having severe P. *falciparum* malaria if they met the predefined, modified WHO criteria for severe malaria (hyperparasitaemia >10% parasitaemia).

3.2.4 DNA Extraction

DNA was extracted from whole blood containing EDTA by a standard salting out procedure according to Miller *et al, (*1998). Briefly, 200µl of the blood sample was mixed with 400µl of lysis buffer which breaks down the cell, after which 10µl of proteinase-K was added to the mixture and vortex. The mixture was incubated for 1hr and 400µl of phenol chloroform was added to the lysate and vortex briefly in order to separate the DNA. The sample was centrifuged at 10000rpm for 10minutes and the upper layer carefully removed using a pipette and transferred to a new 1.5ml tube. Ethanol (100%) and 20µl of 3M sodium acetate were added and mixed by inverting the tube several times. This was then incubated at -20^0C over night, centrifuged at maximum speed for 10-30 minutes at 4^0C and the 100% ethanol decanted. Another 400µl of 70% ethanol was added and centrifuged at maximum speed for 5 minutes at 4^0C, after which ethanol was removed as much as possible until all traces were absent, after several rounds of centrifugation. The DNA was dried out by leaving the tube open for 3-10 minutes and DNA pellet was re-suspended in 50µl sterile water.

3.2.5 Polymerase Chain Reaction (PCR)

Primer design

The DNA sequences of the genes of interest (ACE and eNOS) were obtained from the NCBI website to design appropriate primers. Primer sequences were as follows:

ACE

Forward Primer: 5'CTG GAG ACC ACT CCC ATC CTT TCT 3'

Reverse Primer: 5' GAT GTG GCC ATC ACA TTC GTC AGA T 3'

eNOS

Forward primer: 5'CAT GAG GCT CAG CCC CAG AAC 3'

Reverse primer: 5'AGT CAA TCC CTT TGG TGC TCA A 3'

To reconstitute the primers, 43µl of distilled water was added to the lipolized primer and 5µl of primer was diluted using 45µl of water. The concentration of the primer after addition of water was 430nmol.

Conditions for PCR amplification

To determine the ACE and eNOS genotypes of test and control groups, the genomic DNA fragments on the intron 16 of ACE gene and exon 7 of eNOS gene were amplified by PCR. The conditions for amplification were optimized.

For ACE, the following conditions were used:

1. Initial denaturation: 94^{o}C for 5mins
2. Denatuation: 94 ^{o}C for 30s
3. Annealing: 58 ^{o}C for 30s
4. Extension: 72^{o}C for 30s
5. Cycling condition: 35 cycles
6. Final extension: 72 ^{o}C for 10min

For eNOS, the following conditions were used:

1. Initial denaturation: 94^{o}C for 5min
2. Denatuation: 94 ^{o}C for 30s
3. Annealing: 56 ^{o}C for 30s
4. Extension: 72^{o}C for 30s
5. Cycling condition: 35 cycles
6. Final extension: 72 ^{o}Cfor10min

For the reaction set-up, 2µl of templates, 1µl for each reverse and forward primer and 16µl of water were added to the premix making up a 20µl.

3.2.6 Agarose Gel Electrophoresis

PCR products were separated by electrophoresis on a 1.0 % agarose gel using TAE buffer and ethidium bromide and were visualized by ultraviolet-induced fluorescence. The agarose gel was prepared by adding 100 ml of the buffer to 1.0 g of agarose in a 250 ml conical flask. The suspension was heated in the microwave oven for 2-3 minutes to melt the agarose, and then poured into the agarose gel rack with the combs well placed. Thereafter, the gel was loaded with samples after solidification, was placed in the electrophoresis compartment and the buffer added. The set-up was then connected to a power pack and run for 40 minutes at 12 V/cm, after which it was visualized under gel documentation machine.

Purification of PCR product

After gel electrophoresis, DNA was extracted and purified from the gel using PCR purification kit. Briefly, to 100mg of the gel, 300mg of buffer was added and incubated for 10minutes at 50^{0}C to dissolve the gel. The sample was then transferred to DNA

binding column inside collection tube and centrifuged 50^0Cfor 30seconds to precipitate the DNA. About 500µl of wash buffer was added and allowed to stand for 5minutes, before centrifuging for one minute, and the buffer discarded while the DNA binds the column. Wash buffer (750µl) was then added and incubated for one minute and the buffer was discarded again. The tube containing the DNA pellet was again centrifuged for two minutes at 50^0C and allowed to stand for 5minutes. This was centrifuged for one minute at 50^0C in order to collect the pure DNA (Zhang *et al.,* 2005).

Restriction Enzyme digest

Genotyping of the Glu^{298}Asp polymorphism was done by PCR amplifying exon 7 using sense primer 5'-CATGAGGCTCAGCCCCAGAAC-3' and antisense primer 5'-AGTCAATCCCTTTGGTGCTCAC-3', followed by digestion with the MboI restriction enzyme overnight at 37^0C using the method by Colombo *et al.* 2003. In the presence of a T at position 894, which corresponds to Asp298, the 206-bp PCR product was cleaved into two fragments (119 and 87 bp).

3.2.6 DNA Sequencing

Sequencing reaction was prepared in a 2ml tube as described by Smith *et al*., (2006) method. All reagents were kept on ice while preparing the sequencing reactions. 2µl of primer (antisense), 4µl of the DNA template and 14µl of distilled water were mixed together and the sequencing PCR was ran using the following conditions:

For both ACE and eNOS:

Denaturation-96^0C for 20seconds

Annealing-50^0C for 20seconds

Elongation-60^0C for 4minutes

Number of cycles-30 cycles.

DNA clean up and elution

About 100µl of Purification buffer was added to the DNA template and centrifuged for one minute and the buffer was discarded while the DNA binds to the column. Also 400µl of DNA wash buffer was then added and allowed to stay for 5minutes and centrifuged for 1minute and the buffer discarded again. However, 15µl of distilled water was used to elute the DNA and centrifuged for 1minute at 50^0C and the same distilled water was used to double elute so that more DNA will be washed from the DNA binding column and collected in the collection tube and the tube finally was centrifuged for the DNA to settle down. The DNA was then transferred to 96-wild plate and the DNA sequence was identified using CEQ^{TM}2000XL DNA analysis system version 8.0 (Smith *et al*., 2006).

3.3 STATISTICAL ANALYSIS

Pearson chi-square test, confidential interval and odd ratio were applied to check the association between the selected variables. Allele and genotype frequencies were compared using Hardy-Weinberg equation. The statistical softwares used were MedCal version 16.8 and SPSS version 20.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 RESULTS

Table 4.1 shows the percentage male and female subjects with malaria attending Sheikh Muhammad Jidda and Murtala Muhammad Specialist Hospitals, Kano. There were more male patients in both experimental and control groups as clearly presented in Table 4.1.

Table 4.1: Percentage Male and Female with Malaria Attending Sheikh Muhammad Jidda and Murtala Muhammad Specialist Hospitals.

Gender	**Malaria Subject**		**Control**	
	Frequency	Percentage	Frequency	Percentage
Male	70	58.3%	48	60%
Female	50	41.8%	32	40%
Total	120	100%	80	100%

However, with respect to age group distribution 40-44 years has the highest percentage with subject ≥65 having the lowest for both (Table 4.2).

Table 4.2: Age Group Distribution of Malaria Patients Attending Sheikh Muhammad Jidda and Murtala Muhammad Specialist Hospitals, Kano.

Age(years)	Malaria-infected		Control	
	Frequency	Percentage	Frequency	Percentage
40-44	40	33.3%	23	28.8%
45-49	28	23.3%	19	23.8%
50-54	20	16.7%	14	17.5%
55-59	15	12.5%	11	13.8%
60-64	12	10%	9	11.3%
≥65	5	4.2%	4	5%
Total	120	100%	80	100%

Result of severity of malaria based on age group of malaria-infected patients attending Sheikh Muhammad Jidda and Murtala Muhammad Specialist Hospitals, Kano is presented in Table 4.3. With respect to age group, 20 (16.67%) patients between 40-44 years had mild (+) malaria and 11 (9.17%) had severe (++) malaria. For those between 45-49 years, 15 (12.50%) had mild and 7 (5.83%) had severe (++) malaria. Furthermore, there were 13 (10.83%) with mild (+), and 8 (6.67%) severe (++) malaria patients between 50-54 years. However, those between 55-59 years the trend was 10 (8.33%) had mild and 9 (7.5%) severe. For those between 60-64 years patients 8 (6.67%) had mild, and 8 (6.67%) severe. Finally, 65years and above, 4 (3.33%) had mild malaria and 7 (5.83%) severe.

Table 4.3: Severity of Malaria Based on Age Groups in Patients with Malaria Attending Sheikh Muhammad Jidda And Murtala Muhammad Specialist Hospitals, Kano.

Age Group	Frequency of Malaria Severity	
	Mild	**Severe**
40-44	20 (16.67%)	11 (9.17%)
45-49	15 (12.50%)	7 (5.83%)
50-54	13 (10.83%)	8(6.67%)
55-59	10 (8.33%)	9 (7.5%)
60-64	8 (6.67%)	8 (6.67%)
65 and above	4 (3.33%)	7 (5.83%)
Sub-total	70 (58.33%)	50 (41.67%)
Total	120 (100%)	

Figure 4.1 shows the result of prevalence of hypertension in malaria patients attending Sheikh Muhammad Jidda and Murtala Muhammad Specialist Hospitals, Kano. From the results, (22) 18.3% of malaria-infected male patients were hypertensive, while (10) 8.3% of the females had hypertension.

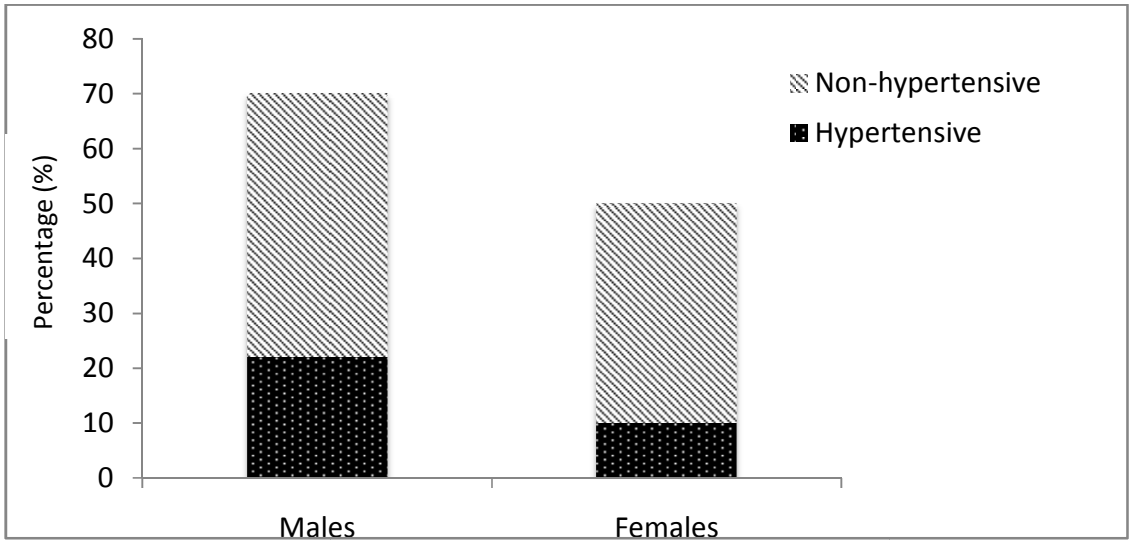

Figure 4.1: Prevalence of Hypertension in Malaria-Infected Patients Attending Sheikh Jidda and Murtala Muhammad Specialist Hospitals, Kano (Subject is hypertensive when the ratio of systolic to diastolic is greater than 140 to 90mmHg).

4.1.1 Polymorphisms and Malaria Infection

Tables 4.4 and 4.5 shows the genotype and allele frequencies of ACE and $Glu^{298}Asp$ polymorphisms in control and malaria-infected patients attending Sheikh Jidda and Murtala Muhammad Specialist Hospitals, Kano. Evaluation of the genotype frequencies of these two polymorphisms showed significant deviation of $Glu^{298}Asp$ polymorphism (χ^2 = 4.14, P = 0.03) in malaria-infected patients from the Hardy-Weinberg equilibrium, while ACE (I/D) polymorphism (χ^2 = 0.012, P = 0.91) of malaria infected patients showed no deviation.

Distribution of ACE polymorphisms in malaria-infected patients

The distribution of different ACE genotypes and alleles of malaria-infected patients attending Sheikh Jidda and Murtala Muhammad Specialist Hospitals, Kano is presented on table 4.4. From the results, 60 (50%) of the patients had the I/I genotype, 36 (30%) had I/D, 96 (80%) had ID+DD and 24(20%) had D/D genotypes. As for the control subjects, I/I were 20 (25%), I/D were 30 (37.5%), D/D were 30(37.5%) and ID+DD were 60 (75%). For the alleles, 108 (45%) malaria-infected patients had I allele and 132 (55%) had D allele, while 70 (43.75%) control subjects had I allele and 90 (56.25%) had D allele. Statistical comparison of the frequencies of all genotypes using Chi-square test showed there were no significant (P>0.05) differences between the control subjects and malaria-infected patients.

Table 4.4: Distribution of ACE (I/D) Genotypes and Alleles in Control and Malaria-Infected Patients Attending Sheikh Jidda and Murtala Muhammad Specialist Hospitals, Kano.

Genotypes and alleles	Malaria-infected Patients (N=120)	Control Group (N=80)
I/I	24(20%)	20(25%)
I/D	60(50%)	30(37.5%)
D/D	36(30%)	30(37.5%)
ID+DD	96(80%)	60(75%)
I	108(45%)	70(43.75%)
D	132(55%)	90(56.25%)

Chi-square test (χ^2)was used to determine the differences between genotype frequencies.

Hardy-Weinberg (HW) equilibrium ($\chi^2 = 0.012$, P = 0.91)

Table 4.5: Genotype and Allele Frequencies of ACE (I/D) Polymorphism in Patients with Mild and Severe Malaria attending Sheikh Jidda and Murtala Muhammad Specialist Hospitals, Kano.

Genotypes and alleles	Mild (N=70)	Severe (N=50)
I/I	9 (12.8%)	15 (25%)
I/D	31(44.3%)[a]	19 (37.5%)[b]
D/D	30 (42.9%)[c]	16 (37.5%)[d]
ID+DD	61 (87%)[e]	35 (75%)[f]
I	49(35%)	49 (49%)
D	91(65%)	51(51%)

The Chi-square test (χ^2)was used to determine the differences between genotype frequencies.

Values with different superscripts in the same row are significantly different (P<0.05)

A total of seventy (70) malaria-infected patients had mild malaria, out of which 9 (20%) had I/I genotype, 31 (44.3%) and 30 (42.9%) had I/D and D/D genotypes, respectively, while of the fifty (50) with severe malaria, 15 (25%) were I/I, 19 (37.5%) were I/D and 16 (37.5%) had D/D genotypes. When the genotype and allele frequency of ACE I/D polymorphism were compared between mild and severe group of malaria patients, there was a significant (P<0.05) difference, indicating an association of I/D (P=0.01, OR=0.3, 95%CI=0.1-0.8) and D/D (P=0.03, OR=0.5, 95% CI=0.2-1.4) genotype with mild malaria (Table 4.5).

Table 4.6 shows the genotypes and allele frequency of malaria-infected patients with or without hypertension attending the hospitals. There were thirty-two (32) malaria-infected patients with hypertension and eighty-eight (88) non-hypertensive patients. There was a significant ($P<0.05$) difference in D/D genotype between hypertensive and non-hypertensive patients (P=0.004, OR=9.07, 95%CI=0.1-0.6) signifying an association of D/D genotype with hypertension. Also, the results showed a significant ($P<0.05$) in D allele when compared to I allele of hypertensive and non-hypertensive malaria-infected patients. However, the difference in I/I and I/D genotypes were not significant ($P>0.05$).

Table 4.6: Comparison of Different ACE (I/D) Genotypic and Allelic States of Malaria-Infected Patients with or without Hypertension attending Sheikh Muhammad Jidda and Murtala Muhammad Specialist Hospitals, Kano.

Genotypes and alleles	Malaria-Infected (Hypertensive) (N=32)	Malaria-Infected (Non-hypertensive) (N=88)
I/I	4(12.5%)	20(22.7%)
I/D	8(25%)	52(59.1%)
D/D	20(62.5%)[a]	16(18.2%)[b]
ID+DD	28(87.5%)	68(77.3%)
I	16 (25%)	92 (52.3%)
D	48 (75%)[c]	84 (47.7%)[d]

The Chi-square test (χ^2)was used to determine the differences between genotype frequencies.

Values with different superscripts in the same row are significantly different ($P<0.05$)

Distribution of Glu^{298}Asp polymorphisms in malaria-infected patients

In Glu^{298}Asp polymorphism, the genotype distributions were GG 28 (23.3%), G/T 48 (40%), TT 44(36.7%) and GT+TT 92(76.7%) in malaria-infected patients, while for control subjects, G/G were 20 (25%), G/T were 33 (41.3%), T/T were 30(37.5%) and GT+TT were 60 (75%). The frequency of G allele was 104 (43%) and T allele 136 (57%) in malaria-infected patients, while in control subjects, 73(45.6%) had G alleles and 87 (54.4%) had T allele. There were no significant (P>0.05) differences in frequencies of genotypes and alleles between control subjects and malaria-infected patients (Table 4.7).

Table 4.7: Distribution of Glu^{298}Asp genotypes and Alleles in Malaria-Infected Patients and Control Subjects attending Sheikh Jidda and Murtala Muhammad Specialist Hospitals, Kano State, Nigeria

Genotypes and alleles	Malaria-infected Patients (N=120)	Control Group (N=80)
G/G	28(23.3%)	20(25%)
G/T	48(40%)	33(41.3%)
T/T	44(36.7%)	27(33.8%)
GT+TT	92(76.7%)	60(75%)
G	104(43%)	73(45.6%)
T	136(57%)	87(54.4%)

The Chi-square test (χ^2)was used to determine the differences between genotype frequencies.

Hardy-Weinberg (HW) disequilibrium ($\chi^2 = 4.14$, P = 0.03)

Table 4.8: Genotype and Allele Frequencies of Glu^{298}Asp Polymorphism in Patients with Mild and Severe Malaria attending Sheikh Jidda and Murtala Muhammad Specialist Hospitals, Kano.

Genotypes and alleles	Mild (N=70)	Severe (N=50)
G/G	11 (15.7%)	14 (28%)
G/T	39(55.7%)[a]	20 (40%)[b]
T/T	20 (28.6%)[c]	16 (32%)[d]
GT+TT	59 (84.3%)	36 (72%)
G	61(43.6%)	48(48.5%)
T	79(56.4%)	51(51.5)

The Chi-square test (χ^2)was used to determine the differences between genotype frequencies.

Values with different superscripts in the same row are significantly different (P<0.05)

Results of the genotype and allele frequencies of Glu^{298}Asp polymorphism in subjects with mild and severe malaria attending the hospitals are presented on Table 4.8. From the results, 11 (15.7%), 39 (55.7%) and 20 (28.6%) of patients with mild malaria, while 14 (28%), 20 (40%) and 16 (32%) of those with severe malaria had G/G, G/T and T/T genotypes, respectively. Comparison of the genotype frequency between mild and severe group of malaria patients, showed a significant (P<0.05) difference in G/T (P=0.04, OR=0.4, 95% CI=0.2-1.1) and T/T (P=0.03, OR=0.6, 95% CI=0.2-1.8) genotypes indicating a protective association of the genotypes (Table 4.8).

Table 4.9 shows that 16 (50%), 7 (21.9%) and 9 (28.1%) malaria-infected patients with hypertension, and 16 (18.2%), 43 (48.9%) and 29 (33%) of those without hypertension had G/G, G/T and T/T genotypes, respectively. A significant ($P<0.05$) difference was obtained when G/T ($P=0.001$, OR=6.1, 95%CI=2.1-17.7) and T/T ($P=0.03$, OR=3.2, 95%CI=5.31) genotypes were compared with G/G between hypertensive and non-hypertensive malaria patients.

Table 4.9: Comparison of different Glu^{298}Asp Genotypic and Allelic States of Malaria-Infected Patients with or without Hypertension Attending Sheikh Muhammad Jidda and Murtala Muhammad Specialist Hospitals, Kano.

Genotypes and alleles	Malaria-Infected (Hypertensive) (N=32)	Malaria-Infected (Non-hypertensive) (N=88)
G/G	16(50%)	16(18.2%)
G/T	7(21.9%)[a]	43(48.9%)[b]
T/T	9(28.1%)[c]	29(33%)[d]
GT+T	16(50%)[e]	72(81.9%)[f]
G	39	75
T	25[g]	101[h]

The Chi-square test (χ^2)was used to determine the differences between genotype frequencies.

Values with different superscripts in the same row are significantly different ($P<0.05$)

Figures 4.2 and 4.3 below show the agarose gel electrophoresis map of the two types of polymorphisms studied. Once the amplicons were obtained, they were subjected to 1% agarose gel electrophoresis with ethidium bromide and the bands visualized under UV light. With the help of DNA ladder, Deletion (D allele) and insertion (I allele) were identified. The ACE gene insertion/deletion (I/D) polymorphism was characterized by the presence (insertion) or absence (deletion) of a 287 bp Alu-repeat sequence in Intron 16. The homozygous individuals for insertion allele (II genotype) were identified by the presence of a single 490 bp product, the homozygous individuals for deletion allele (DD genotype) were identified by the presence of a single 190 bp product and the heterozygous individuals` insertion, deletion (ID genotype) was identified by the presence of both 190 and 490 bp products (Figure 4.2).

The eNOS $Glu^{298}Asp$ polymorphism is characterized by the presence of a T at position 894, which corresponds to Asp298. The homozygous G/G was identified by presence of bands at 87 and 119 bp, T/T was identified by a band at 206 bp, while G/T was identified by the presence of three bands at 87, 119 and 206 bp (Figure 4.3).

Figures 4.4 and 4.5 show the Basic Local Alignment Search Tool (BLAST) analysis of the ACE and eNOS genes, respectively, when compared to the human genome (reference). From the results, the sequence of the ACE gene had an alignment score of 99%, but was found to be 96%, while eNOS sequence had a score of 96%, but was found to be 95%.

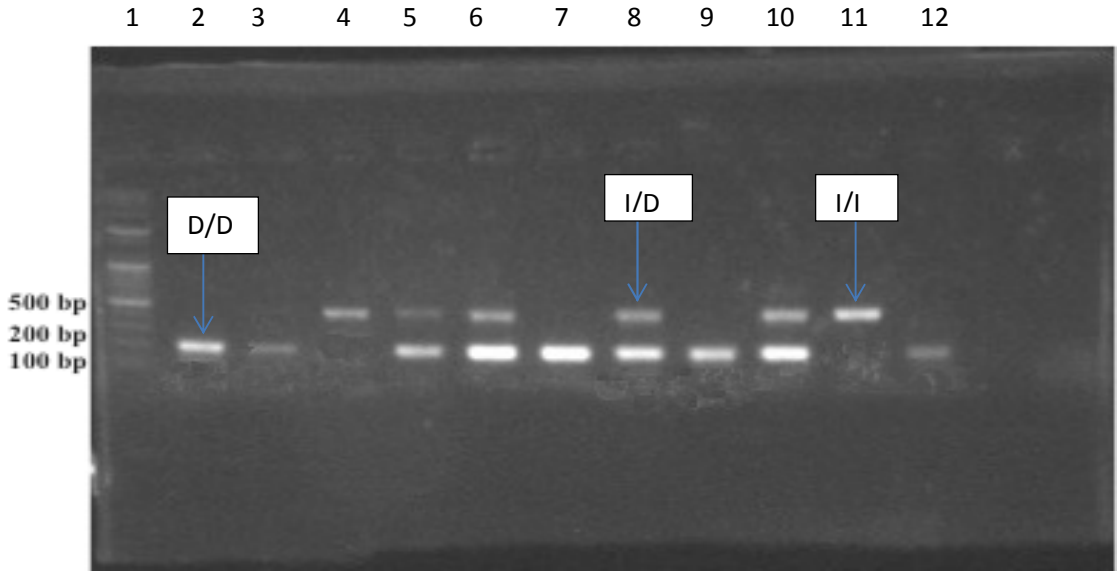

Figure 4.2: Angiotensin Converting Enzyme Gene I/D Polymorphism agarose gel electrophoresis map visualized under UV light. Lane 1: DNA Ladder; Lanes 2, 3, 7, 9 and 12: D/D =190 Bp; Lanes 4 and 11: I/I = 490 Bp; Lanes 4, 5, 7 and 9: I/D = 190 and 490 Bp.

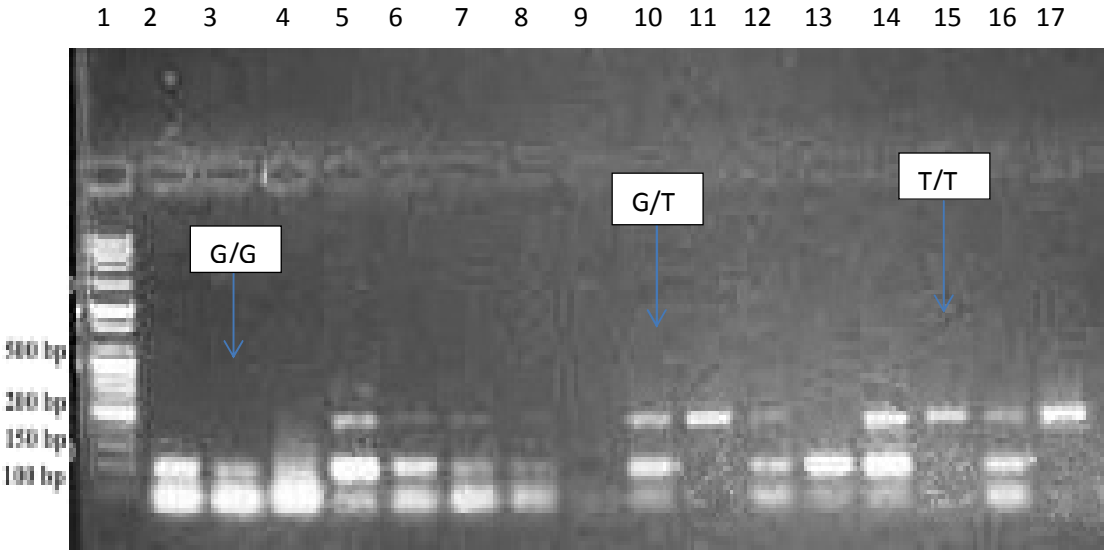

Figure 4.3: Endothelial Nitric Oxide Synthase Gene Polymorphism Agarose Gel Electrophoresis Map Visualized under UV Light. Lane 1: DNA Ladder; Lanes 2, 3, 4, 6, 7, 8 and 12: G/G = 87 & 119 Bp; Lanes 5, 10, 14 and 16: G/T = 87, 119 & 206 Bp; Lanes 11, 15 and 17: T/T = 206 Bp.

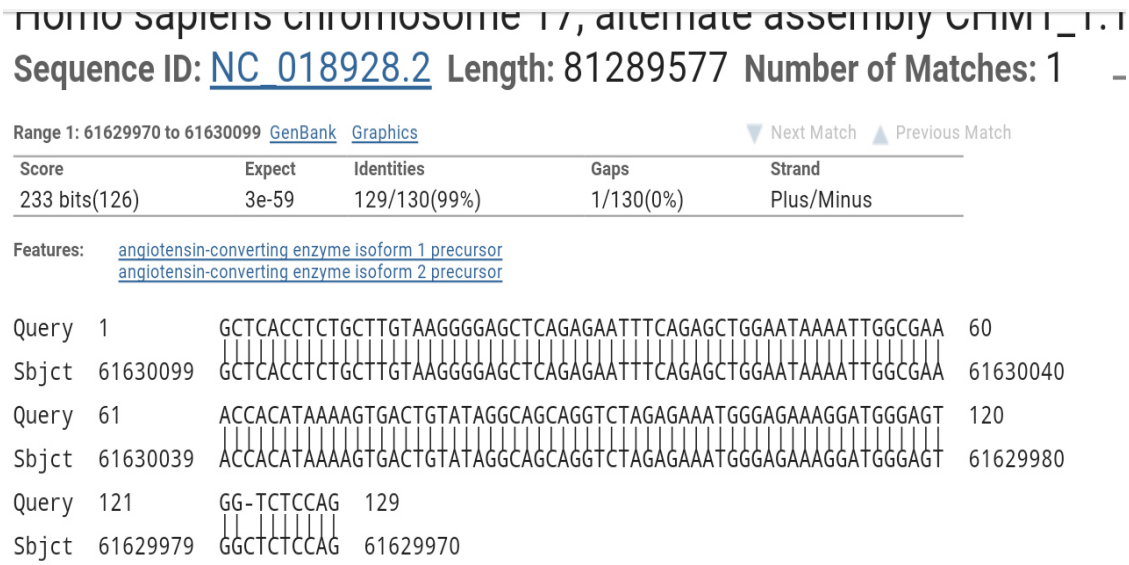

Figure 4.4: A Basic Local Alignment Search Tool (BLAST) analysis of the sequenced angiotensin converting enzyme gene.

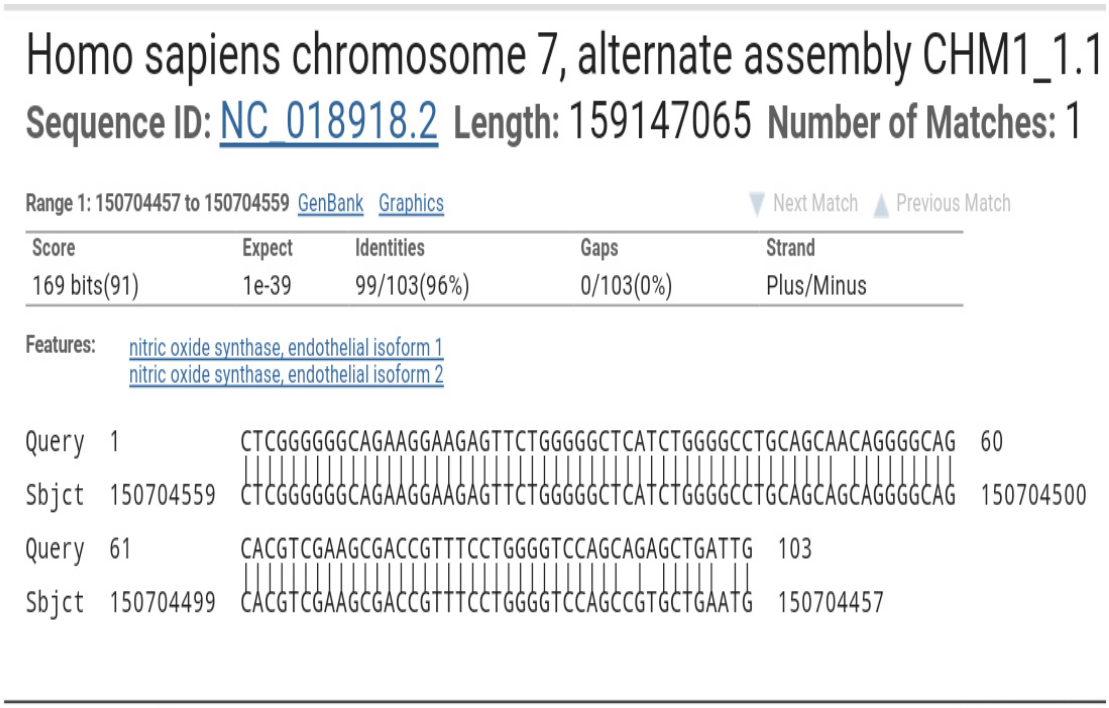

Figure 4.5: A Basic Local Alignment Search Tool (BLAST) analysis of the sequenced endothelial nitric oxide synthase gene.

4.2 DISCUSSION

The present study was carried out to explore the hypothesis that susceptibility to malaria is influenced by genetic variations in eNOS and ACE, as genetic and environmental factors have been shown to be important determinants of the pathogens and progression of all diseases (Yan *et al*., 2004). Initial results from the study show that 41.8% of the malaria patients were female while 58.3% were males. With respect to age groups (Table 4.2), 33.3% of patients were between ages of 40-44 years, while only 4.2% were above 65 years. This shows that male patients had higher prevalence of malaria compared to the females and with respect to age group; younger patients had higher prevalence than older patients. This is in line with a study conducted by Akanbi *et al.*(2010) who reported that males had higher prevalence of malaria than females while it contradicts reports by Azfar *et al*. (2009) that prevalence was higher in females (56%) than male patients (44%). Also, males were found to have high prevalence of hypertension than females (Figure 1). This contradicts reports by Akanbi *et al.* (2010) who showed high prevalence in females than males. It is possible that the high prevalence of hypertension in males represents the increasing prevalence in hypertension noted across the globe, and may be explained in part by their sedentary lifestyle, as most were market people who sit in one place and conduct their businesses. Apart from their sedentary life style, most of them do not exercise regularly and also depend on food vendors who use a lot of salt and seasoning to improve the taste of food (Sanusi *et. al.,* 2005).

With respect to severity of malaria (Table 4.3), the result shows that 58.30% patients had mild malaria and 41.70% had severe malaria. The distribution of severity among age groups was not consistent, with 5.83% of patients between 45-49, and 60 years and

above having severe malaria, while 9.17% between 40-44 years had severe malaria. This study contradicts Akanbi *et al*. (2010) who demonstrated that older people are more prone to malaria than younger ones. However, despite high infection rates, only 1-2% of malaria patients usually have the severe form of the disease that results into CM and anaemia. They concluded that natural selection might have played a role in this. The result of the present study may be attributed to the fact that there were fewer patients above 60 years at the time of sample collection compared to those below 55 years. Also, studies have shown that older patients are usually less willing to go to hospitals when they are sick (Dhangadamajhi *et al.* 2009).

Several polymorphisms have been associated with higher prevalence of arterial hypertension. There are 2 polymorphisms in the Angiotensin Converting Enzyme (ACE) and Angiotensin Converting Enzyme 2 (ACE2) that leads to elevated Ang II levels (Giner *et al.,*2000; Di Pasquale *et al*., 2004; Fan *et al*., 2007). Interestingly, these same genetic variations (the "D" allele of ACE I/D polymorphism and the ACE2 C→T substitution) have been associated with a lower incidence of cerebral malaria (CM) in Indian adults, although the later one only in women. A protective role for Ang II against human cerebral malaria has been proposed based on a gene polymorphism analysis of angiotensin-related enzymes in patients with severe or mild malaria. It was observed that an insertion/deletion polymorphism of ACE, that is associated with increased levels of Ang II, was also strongly associated with mild malaria (Dhangadamajhi *et al*., 2010).

In the present study, most of the patients with mild malaria had D/D and I/D genotypes, of ACE I/D, indicating that these genotypes are associated with mild malaria (Table 4.5).

This observation is in line with study by Dhangadamajhi *et al*. (2010) who reported the high frequency of these genotypes in patients with mild malaria in an Indian population, and suggests the dominant effect of 'D' allele against susceptibility to malaria. Angiotensin II (Ang II) is a peptide hormone with well-characterized effects on circulatory homeostasis that induces vasoconstriction and results in high blood pressure. It is derived from angiotensinogen through sequential enzymatic cleavages: first renin cleaves angiotensinogen, forming Ang I that is then converted to Ang II by angiotensin converting enzyme (ACE). Circulating Ang II not only contributes to increase blood pressure, but it is also involved in key inflammatory events, including the activation of endothelial cells to express higher levels of leukocyte adhesive molecules and the increase in vascular permeability (Benigni *et al*., 2010). Studies have demonstrated that the 'D' allele increases Ang II production, and results in conversion of Ang II to Ang (1-7) by ACE2. Ang (1-7) have been shown to decrease the invasion of human erythrocytes by P. *falciparum*, by binding to its specific receptor; Mas receptor (expressed in the human erythrocyte membrane) thus, inhibiting protein kinase A activity and consequently impairs merozoite invasion in the erythrocytes (Saraiva *et. al.,* 2011).Gallego-Delgado *et. al.* (2015) demonstrated a significant reduction in blood parasitemia, delayed establishment of CM, decreased incidence of brain hemorrhage and modest increase in survival of P. *berghei*-infected mice treated with Ang II. They attributed this to the conversion of Ang II to Ang (1-7) and its consequent effects on parasite invasion.

Additionally, Ang II has been shown to inhibit the growth of *P. falciparum in vitro* (Saraiva *et al*., 2011). It is possible that Ang II modulates malaria severity through other

mechanisms, since the inhibitory effect observed in parasite growth is modest (Maciel *et al.,*2008; Saraiva *et al*., 2011).

In order to explore the hypothesis that susceptibility to malaria is influenced by genetic variation in eNOS, the commonly defined polymorphic loci of eNOS, Glu^{298}→Asp was genotyped in this study. From the results, a protective association of eNOS Glu^{298}→Asp substitution G/T (P=0.04, OR=0.4 95% CI=0.2-1.1) and T/T (P=0.03, OR=0.6, 95% CI=0.2-1.8) against severe malaria was observed (Table 4.5). This is in line with reports of Dhangadamajhi *et al*. (2009) and Dhangadamajhi *et al*. (2010) who evaluated and compared the distributions of eNOS genotypes in patients with mild and severe malaria. They suggested that the Glu^{298}→Asp substitution may enhance the expression of eNOS leading to increased NO production that impairs the parasite erythrocytes' cycle, preventing it from invading red blood cells, hence protection against malaria. It is important to note that very few studies have reported the specific association between the genetic polymorphisms of eNOS and plasma NO concentrations in different diseases, and hence, there is no clear evidence for impaired NO production as a result of the G/T polymorphism in exon 7 that codes for replacement of glutamic acid by aspartic acid at residue 298 in the mature eNOS protein (Fairchild *et al*., 2001). However, recent studies have shown that increased NO production is beneficial because of its anti-parasitic and anti-disease effect. This effect has been attributed to the inhibition of the cytoadherence process resulting in down regulation of ICAM1, VCAM1, and E-selectin, involved in cytoadherence and microvascular sequestration of parasitized RBCs and decreased production of tumor necrosis factor by macrophages (Serirom *et al.,* 2003). NO is produced during the enzymatic conversion of L-arginine to L-citrulline by three isoforms

of nitric oxide synthase (NOS), namely inducible NOS, endothelial NOS, and neuronal NOS. The eNOS-derived NO mediates vasodilation, inhibits platelet aggregation and endothelial cell activation, and modulates expression of cell adhesion molecules (Laroux, *et al.,* 2000).

Endothelial dysfunction, known to contribute to the pathogenesis of malaria has been shown to be reversible with l-arginine (Yeo *et al.,*2007), therefore, functionally important variants of eNOS could influence individual susceptibility to malaria by altering the amount of NO generated by the endothelium. According to Yayama and Okamoto (2008), activation of AT2 receptor results in increased production of nitric oxide by eNOS and protects against cerebral malaria since low nitric oxide bioavailability may exacerbate endothelial dysfunction and contributes to the pathogenesis of severe malaria (Gramaglia *et al*., 2006; Miller *et al*., 2013). Therefore, the association of Glu^{298}→Asp genotypes with mild malaria in the present study may be due to elevated levels of NO, and conforms to reports by Yoon *et al.*(2000) who demonstrated a relationship between increased plasma NO levels and polymorphisms in the eNOS gene. However, earlier reports by Tesauro *et al.*(2000) demonstrated that the Glu^{298}→Asp change increases the susceptibility to cleavage of the eNOS enzyme and results in low levels of NO production. Recent reports have shown that the cleavage is due to *in vitro* acidic hydrolysis during sample preparation and does not appear to influence the stability, half-life, or biologic activity of the enzyme (Fairchild *et al*., 2001; Dhangadamajhi *et al*., 2010). Thus, the protective association of 'D' allele responsible for enhanced expression of Ang II can be explained on the basis of its antiplasmodial activity (Maciel *et al*., 2008) together with NO (Dhangadamajhi *et al.,* 2009) and the improvement of Ang II mediated

endothelial dysfunction, if occurs by NO (Yeo *et al*., 2007). Further, the actions of Ang II via the type 2 (AT2) receptor leading to the NO/cGMP pathway cannot be ruled out and the AT2 receptor has been shown to play a protective role via the NO/cGMP pathway in ischemic diseases of the cardiovascular and renal tissues (Tanabe *et al.,* 2010).

Comparison of the individual frequencies of the three genotypes of ACE polymorphism in hypertensive and non-hypertensive malaria patients showed that the D/D (P=0.04, OR=0.2, 95% CI=0.1-0.6) genotype was associated with hypertension (Table 4.6). The results also showed that malaria patients with D/D genotypes were more prone to develop hypertension with an odd's ratio (OR) of 0.5, while eNOS Glu^{298}→Asp substitution, G/T (P=0.001, OR=6.1 95% CI=2.1-17.7) and TT (P=0.03, OR=3.2, 1.2-8.9) genotypes, were significantly associated with normal blood pressure (Table 4.9). However, patients with G/G genotype may be more prone to develop hypertension as 50% of malaria patients with hypertension had this genotype (Table 4.9). Although, malaria and hypertension do not have an obviously direct cross influence, certain genotypes of ACE polymorphism have been associated to high Ang II and NO levels, both of which play a significant role in the development of hypertension. The D/D genotype of ACE (I/D) polymorphism has been associated with high ACE levels. ACE is responsible for the conversion of Angiotensin I to Angiotensin II. Angiotensin II is a potent vasoconstrictor, and a stimulator of aldosterone synthesis, which causes increase in blood pressure. Results of the present study agrees with studies by Ipsa *et al.,* (2002) who reported that D/D genotypes were more prone to develop hypertension in South Indian population. Also, Yan *et al.* (2004) demonstrated that the ACE (I/D) polymorphism has a major influence on plasma and tissue levels of ACE, and so described this polymorphism as genetic factor

for cardiovascular diseases. Also, eNOS has been shown to be homeostatic regulator of numerous essential cardiovascular functions. NO derived from eNOS dilates all types of blood vessels by stimulating soluble guanylyl cyclase and increasing cyclic GMP in smooth cells (Rapoport *et al.,*2003). Nitric oxide released towards the vascular lumen is a potent inhibitor of platelet aggregation and adhesion to the vascular wall (Lin *et al.,* 2013). The association of G/T and TT genotypes with hypertension conforms to studies by Li *et al.* (2011) who demonstrated the association of eNOS polymorphism with hypertension in Northern and Southern Chinese Han population. Though the mechanism underlying the association of G/G genotype with hypertension in malaria patients remains to be clarified, the possibility that eNOS Glu^{298}→Asp polymorphism may be under natural selection cannot be ruled out, as results of the present study demonstrates that the polymorphism deviated from Hardy-Weinberg equilibrium (Dhangadamajhi *et al*., 2009). The Hardy-Weinberg model requires seven assumptions: an unlimited population size, mutation and natural selection not occurring, all members of the population breed and produce the same number of offspring, mating is random and there is no migration in or out of the population (Masel, 2012). Nevertheless, further studies involving larger sample size is required to better understand and come to a logical conclusion.

CHAPTER FIVE

SUMMARY, CONCLUSION AND RECOMMENDATION

5.1 SUMMARY

Malaria and hypertension are among the world's deadly diseases. Malaria is caused by the Plasmodium species which have two major life-cycles. Genetic polymorphism is the occurrence in the same population of two or more alleles at one locus each with appreciable frequency. In other word it is inter-individual, functionally silent differences in DNA sequence that make each human genome unique. Angiotensin converting enzyme (ACE) is an enzyme together with rennin which convert Angiotensin I to Angiotensin II whereas, endothelial nitric oxide synthase (eNOS) is an enzyme which oxidizes L-arginine to generate L-citrulline and nitric oxide (NO) in the vascular endothelium. The type of genetic polymorphism in ACE we focused in the present research is an insertion/insertion (I/I), deletion/deletion (D/D) and insertion/deletion (I/D), however, with respect to eNOS we focused on $Glu^{298} \rightarrow$Asp substitution.

Studies have shown that the renin angiotensin aldosterone system (RAAS), and most specifically Ang II may influence the severity of the malaria. Genetic variants of ACE and eNOS have been shown to have protective effect on malaria due to their association to high NO and Ang II production, both of which play a significant role in the development of hypertension. Evidences suggest that polymorphisms of ACE and eNOS influence the severity of both malaria and hypertension, and contribute to their pathogenicity, though the mechanism underlying the association of these polymorphisms

to hypertension and malaria remains to be clarified. Hence, in the present study the ACE and eNOS polymorphisms of malaria-infected patients were investigated.

Results of this study demonstrated the following:

- There were more male than female patients with malaria.
- Age group played a significant role in malaria prevalence as most malaria-infected patients were below 60 years.
- The prevalence of hypertension in malaria patients was 26.6% with males having a higher prevalence of 18.3%, about twice that of females (10%).
- There was no association in the genotypes and allele frequencies of ACE (I/D) and eNOS polymorphisms between malaria-infected patients and control subjects.
- There was a significant association of I/D and D/D genotypes of ACE(I/D) polymorphism with mild malaria.
- Also, there was a protective association of T/T and G/T genotype of eNOS Glu298→Asp against severe malaria
- The I/D and D/D genotype of ACE(I/D) and G/G genotype of eNOS Glu298→Asp may be associated with hypertension in malaria patients.
- BLAST sequence analysis of the ACE and eNOS genes showed alignment scores of 99 and 95%, respectively.

5.2 CONCLUSION

This study has shown that genetic polymorphisms in ACE and eNOS responsible for increased Ang II and NO production are significantly associated with mild malaria in people within Kano metropolis. The findings also suggest that certain genetic variants of

ACE and eNOS Glu^{298}→Asp substitution may be associated with hypertension in malaria-infected patients within this population.

5.3 RECOMMENDATIONS

1. Additional genetic studies involving larger sample size are necessary in this region and other geopolitical zones within Nigeria to determine whether malaria could have been a driving evolutionary force for RAAS and eNOS polymorphisms and therefore hypertension.

2. These genetic findings warrant further investigation of genetic contribution of these polymorphisms to the pathogenesis of malaria.

3. More research on protective polymorphisms against malaria will lead to better understanding of the mechanisms underlying malaria severity, which can be used in developing therapeutic solutions.

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APPENDIX I

APPENDIX II

QUESTIONNAIRE

ANGIOTENSIN CONVERTING ENZYME GENE POLYMORPHISM AND ITS ASSOCIATION WITH HYPERTENSION IN MALARIA PATIENTS ATTENDING SHEIK MUHAMMED JIDDA GENERAL HOSPITAL, KANO STATE, NIGERIA

SECTION 1: BACKGROUND INFORMATION

Questionnaire No……

1. Date:____/____/_____
2. State:……. L.G.A: ……. Community…….
3. Sex: [ ], Age: [ ], years [ ].
4. Weight: [ ]kg, Height [ ]m, BMI [ ]kg/m2
5. Ethnic group [ ]:

   1 = Hausa, 2 = Yoruba, 3. Igbo, 4 = Others (Specify)……

SECTION 2: SOCIO-ECONOMIC STATUS

6. What is your highest level of education [ ].
   1 = Primary education, or First stage of basic education, or less. 2 = Junior secondary education, or second stage of basic education. 3 = Senior secondary school. 4 = Post-secondary non-tertiary education. 5 = Tertiary Education-First Degree. 6 = Tertiary Education- Post graduate Degree

7. What is your current employment status? [ ]. 1 = employed. 2 = doing house work at home. 3 = student. 4 = retired/long-term disabled. 5 = unemployed.

8. What is your net monthly income? [ ]

1 = <₦10, 000. 2 = ₦10, 000- ₦20, 000. 3 = ₦20, 000 – ₦29, 999. 4 = ₦30, 000 – ₦39, 999. 5 = ₦40, 000 – ₦49, 999. 6 = ₦50, 000 – ₦99, 999. 7 = ₦100, 000 and above

SECTION 3: AWARENESS OF MALARIA INFECTION

9. Please indicate below the chronic condition(s) you have?

Chronic malaria [ ] High Blood pressure [ ] Heart disease, type of heart disease: ……[ ] Lung disease, type of lung disease …….. [ ]

Others, Specify……..

10. How often do you visit the hospital for fever symptoms? [ ] 1 = once a month. 2 = once in 3 months. 3 = once a year

11. When last did you have malaria? ----------

12. Which drug is often prescribed to you when you are positive for malaria infection?

.....

13. How quickly do you respond to treatment using the above medication?

.....

14. Do you experience any side effects when you treat malaria with the medication

A = Yes. B = No

SECTION 4: AWARENESS OF HYPERTENSION

15. When was your blood pressure last measured by a health professional [ ]. 1= within the past 1 month. 2 = within the past 2-12 months. 3. = 1-5 years 4 = Others Specify ……..

16. Have you been told by a health professional in the past year (12 months) that you have elevated blood pressure or hypertension. [ ] 1 = Yes. 2 = No 3 = I did not see a health professional within the period.

17. If Yes in the above question, do you have any prescribed medication that you often use?

A = Yes. B = No; If Yes, State the Drug name .....

SECTION 5: PHYSICAL ACTIVITIES

During the past week, even if it was not a typical week for you, how much total times did you spend on each of the following?

18. Stretching or strengthening exercise (ranging of motion, using weight e.t.c.) [ ]. 1 = None. 2 = less than 30 min/wk. 3 = 30 – 60min/wk. 4 = 1-3hrs per wk. 5 = more than 3hrs/wk

19. Walk for exercise? [ ] 1 = None. 2 = less than 30min/wk. 3 = 30-60 min/wk. 4 = 1-3 hrs per week. 5 = more than 3hrs/wk.

20. Swimming or aquatic exercise [ ]. 1 = None. 2 = less than 30min/wk. 3 = 30-60min/wk. 4 = 1-3hrs per week. 5 = more than 3hrs/wk

20. Bicycling (including stationary exercise bikes) [ ]. 1= None. 2 = less than 30 min/wk. 3 = 30-60min/wk. 4 = 1-3hrs per week. 5 = more than 3hrs/wk.

21. Other aerobic exercise equipment (stair master, rowing, skiing machine) [ ]. 1= None. 2 = less than 30min/wk. 3 = 30-60min/wk. 4 = 1-3hrs per week. 5 = more than 3hrs/wk.

22. Other aerobic exercise [ ]. 1 = None. 2 = less than 30min/wk. 3 = 30-60min/wk. 4 = 1-3hrs per week. 5 = more than 3hrs/wk. specify……..

APPENDIX III

INFORMED CONSENT FORM

I am …… of Department of Biochemistry, Bayero University Kano, Kano State.

I am conducting a research work on ANGIOTENSIN CONVERTING ENZYME (ACE) GENE POLYMORPHISM AND ITS ASSOCIATION WITH HYPERTENSION IN MALARIA PATIENTS.

I will be grateful if you participate in this research.

Your participation will entail some questioning and collection of blood sample for analysis.

Please note that this participation is voluntary and you have the right to refuse or withdraw from this research work without any ill feelings towards you from me.

I understand the aim of the research work and have agreed to participate in the research.

Signature of subject Signature of investigator

……. …….

Signature of witness

……

APPENDIX IV

QUESTIONNAIRE

ANGIOTENSIN CONVERTING ENZYME GENE POLYMORPHISM AND ITS ASSOCIATION WITH HYPERTENSION IN MALARIA PATIENTS ATTENDING MURTALA MUHAMMAD SPECIALIST HOSPITAL, KANO STATE, NIGERIA

SECTION 1: BACKGROUND INFORMATION

Questionnaire No……

6. Date:____/____/_____

7. State:……. L.G.A: ……. Community…….

8. Sex: [ ], Age: [ ], years [ ].

9. Weight: [ ]kg, Height [ ]m, BMI [ ]kg/m2

10. Ethnic group [ ]:

1 = Hausa, 2 = Yoruba, 3. Igbo, 4 = Others (Specify)…….

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6. What is your highest level of education [ ].
1 = Primary education, or First stage of basic education, or less. 2 = Junior secondary education, or second stage of basic education. 3 = Senior secondary school. 4 = Post-secondary non-tertiary education. 5 = Tertiary Education-First Degree. 6 = Tertiary Education- Post graduate Degree

7. What is your current employment status? [ ]. 1 = employed. 2 = doing house work at home. 3 = student. 4 = retired/long-term disabled. 5 = unemployed.

8. What is your net monthly income? [ ]

1 = <₦10, 000. 2 = ₦10, 000- ₦20, 000. 3 = ₦20, 000 – ₦29, 999. 4 = ₦30, 000 – ₦39, 999. 5 = ₦40, 000 – ₦49, 999. 6 = ₦50, 000 – ₦99, 999. 7 = ₦100, 000 and above

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Chronic malaria [ ] High Blood pressure [ ] Heart disease, type of heart disease: ……[ ] Lung disease, type of lung disease …….. [ ]

Others, Specify……..

10. How often do you visit the hospital for fever symptoms? [ ] 1 = once a month. 2 = once in 3 months. 3 = once a year

11. When last did you have malaria? ----------

12. Which drug is often prescribed to you when you are positive for malaria infection?

.....

13. How quickly do you respond to treatment using the above medication?

.....

14. Do you experience any side effects when you treat malaria with the medication

A = Yes. B = No

SECTION 4: AWARENESS OF HYPERTENSION

15. When was your blood pressure last measured by a health professional [ ]. 1= within the past 1 month. 2 = within the past 2-12 months. 3. = 1-5 years 4 = Others Specify ……..

16. Have you been told by a health professional in the past year (12 months) that you have elevated blood pressure or hypertension. [ ] 1 = Yes. 2 = No 3 = I did not see a health professional within the period.

17. If Yes in the above question, do you have any prescribed medication that you often use?

A = Yes. B = No; If Yes, State the Drug name .....

SECTION 5: PHYSICAL ACTIVITIES

During the past week, even if it was not a typical week for you, how much total times did you spend on each of the following?

18. Stretching or strengthening exercise (ranging of motion, using weight e.t.c.) [ ]. 1 = None. 2 = less than 30 min/wk. 3 = 30 – 60min/wk. 4 = 1-3hrs per wk. 5 = more than 3hrs/wk

19. Walk for exercise? [ ] 1 = None. 2 = less than 30min/wk. 3 = 30-60 min/wk. 4 = 1-3 hrs per week. 5 = more than 3hrs/wk.

20. Swimming or aquatic exercise [ ]. 1 = None. 2 = less than 30min/wk. 3 = 30-60min/wk. 4 = 1-3hrs per week. 5 = more than 3hrs/wk

20. Bicycling (including stationary exercise bikes) [ ]. 1= None. 2 = less than 30 min/wk. 3 = 30-60min/wk. 4 = 1-3hrs per week. 5 = more than 3hrs/wk.

21. Other aerobic exercise equipment (stair master, rowing, skiing machine) [ ]. 1= None. 2 = less than 30min/wk. 3 = 30-60min/wk. 4 = 1-3hrs per week. 5 = more than 3hrs/wk.

22. Other aerobic exercise [ ]. 1 = None. 2 = less than 30min/wk. 3 = 30-60min/wk. 4 = 1-3hrs per week. 5 = more than 3hrs/wk. specify……..

APPENDIX V

(STATISTICAL ANALYSIS)

TABLE 1: ACE GENOTYPES AND ALLELES FREQUENCY IN MALARIA-INFECTED PATIENTS AND CONTROL SUBJECTS.

Malaria-infected Patients Hardy-Weinberg Equilibrium (HWE)

	Observed Frequency N(%)	Expected Frequency (N%)	χ^2 p-value
Genotypes			
I/I	**24(20%)**	**24.3(20.3%)**	
I/D	**60(50%)**	**59.4(49.5%)**	**0.012 0.91**
D/D	**36(30%)**	**36.3(30.3%)**	
Alleles			
I	**108(45%)**		
D	**132(55%)**		

Control Subjects Hardy-Weinberg Equilibrium (HWE)

	Observed Frequency N(%)	Expected Frequency N(%)	χ^2 p-value
Genotypes			
I/I	**20(25%)**	**15.3(19.1%)**	
I/D	**30(37.5%)**	**39.4(49.3%)**	**4.55 0.031**
D/D	**30(37.5%)**	**25.3(31.6%)**	
Alleles			
I	**70(43.75%)**		
D	**90(56.25%)**		

From above the

TABLE 2: COMPARISON OF DIFFERENT GENOTYPIC AND ALLELIC STATE IN MALARIA-INFECTED PATIENTS AND CONTROL SUBJECTS.

	Malaria-infected	Control	OR (95%CI) p-value
ACE I/D	**N = 120**	**N = 80**	
I/I	**24(20%)**	**20(25%)**	**Ref**
I/D	**60(50%)**	**30(37.5%)**	**0.6(0.2-1.3) 0.17**
D/D	**36(30%)**	**30(37.5%)**	**1.0(0.5-2.2) 1.00**
ID+DD	**96(80%)**	**60(75%)**	**0.7(0.4-1.5) 0.40**
D	**0.55**	**0.563**	**1.1(0.7-1.6) 0.81**

TABLE 3: COMPARISON OF DIFFERENT GENOTYPIC AND ALLELIC STATE IN MALARIA-INFECTED PATIENTS AND CONTROL SUBJECTS BASED ON GENDER.

	Malaria-infected	Control	OR (95%CI) p-value
ACE I/D : Male	**N = 66**	**N = 40**	
I/I	**13(19.7%)**	**8(20%)**	**Ref**
I/D	**33(50%)**	**14(35%)**	**0.7(0.2-2.0) 0.50**
D/D	**20(30.3%)**	**18(45%)**	**1.5(0.5-4.3) 0.49**
ID+DD	**53(80.3%)**	**32(80%)**	**0.9(0.4-2.6) 0.97**
D	**0.553**	**0.625**	**1.4(0.8-2.4) 0.30**

	Malaria-infected	Control	OR (95%CI) p-value
ACE I/D : Female	N = 54	N = 40	
I/I	11(19.7%)	12(20%)	Ref
I/D	27(50%)	16(35%)	0.5(0.2-1.5) 0.24
D/D	16(30.3%)	12(45%)	0.7(0.2-2.1) 0.51
ID+DD	43(80.3%)	28(80%)	0.6(0.2-1.5) 0.29
D	0.546	0.50	0.8(0.5-1.5) 0.53

TABLE 4: COMPARISON OF DIFFERENT GENOTYPIC AND ALLELIC STATE OF MALARIA-INFECTED PATIENTS AND CONTROL SUBJECTS WITH MILD AND SEVERE

	Mild	Severe	OR (95%CI) p-value
ACE I/D	N = 70	N = 50	
I/I	9(20%)	15(25%)	Ref
I/D	31(50%)	19(37.5%)	0.3(0.1-0.8) 0.01
D/D	30(30%)	16(37.5%)	0.5(0.2-1.4) 0.03
ID+DD	61(80%)	35(75%)	0.3(0.1-0.9) 0.02
D	0.579	0.51	0.8(0.5-1.3) 0.29
ACE I/D : Male	N = 40	N = 22	
I/I	5(12.5%)	7(31.8%)	Ref
I/D	24(60%)	9(40.1%)	0.3(0.1-1.1) 0.06

D/D	**11(27.5%)**	**6(27.3%)**	**0.4(0.1-1.8)**
0.22			
ID+DD	**35(87.5%)**	**15(80%)**	**0.3(0.1-1.1)**
0.07			
D	**0.575**	**0.625**	**0.7(0.3-1.4)**
0.30			
ACE I/D	**: Female**	**N = 30**	**N = 28**
I/I	**4(13.3%)**	**8(28.5%)**	**Ref**
I/D	**17(56.7%)**	**10(35.7%)**	**0.3(0.1-1.2)**
0.09			
D/D	**9(30%)**	**10(35.7%)**	**0.6(0.1-2.5)**
0.44			
ID+DD	**26(86.7%)**	**20(71.4%)**	**0.4(0.1-1.5)**
0.16			
D	**0.583**	**0.536**	**0.8(0.4-1.7)**
0.61			

TABLE 3: COMPARISON OF DIFFERENT GENOTYPIC AND ALLELIC STATE OF MALARIA-INFECTED PATIENTS AND CONTROL WIH OR WITOUT HYPERTENSION

	Malaria-infected	**Control**	**OR (95%CI)**
p-value			
ACE I/D	**: Hypertension N = 32**	**N = 0**	
I/I	**4(12.5%)**	**0**	**Ref**
I/D	**8(25%)**	**0**	**0.5(0.01-31.4)**
0.76			
D/D	**20(62.5%)**	**0**	**0.2(0.01-12.6)**
0.46			
ID+DD	**28(87.5%)**	**0**	**0.2(0.003-9.0)**
0.37			

D	**0.75**	**0**	**0.3(0.01-17.8)**
0.59			
ACE : non-hypertensive N = 88		**N = 80**	
I/I	**20(22.7%)**	**20(25%)**	**Ref**
I/D	**52(59.1%)**	**30(37.5%)**	**0.6(0.3-1.2)**
0.16			
D/D	**16(18.2%)**	**30(37.5%)**	**1.9(0.8-4.5)**
0.16			
ID+DD	**68(77.3%)**	**60(75%)**	**0.9(0.4-1.8)**
0.73			
D	**0.477**	**0.563**	**1.4(0.9-2.2)**
0.12			

APPENDIX VI

(STATISTICAL ANALYSIS II)

TABLE 1: Glu^{298} Asp GENOTYPES AND ALLELES FREQUENCY IN MALARIA-INFECTED PATIENTS AND CONTROL SUBJECTS.

Malaria-infected Patients Hardy-Weinberg Equilibrium (HWE)

	Observed Frequency N(%)	Expected Frequency(N%)	χ^2 p-value
Genotypes	**N=120**	**N=120**	
G/G	**28(23.3%)**	**22.2(18.5%)**	
G/T	**48(40%)**	**58.8(49%)**	**4.14 0.035**
T/T	**44(36.7%)**	**39(32.5%)**	
Alleles			
G	**104(43%)**		
T	**136(57%)**		

Control Subjects Hardy-Weinberg Equilibrium (HWE)

	Observed Frequency N(%)	Expected Frequency N(%)	χ^2 p-value
Genotypes	**N=80**	**N=80**	
G/G	**20(25%)**	**16.6(20.7%)**	
G/T	**33(41.3%)**	**39.7(49.6%)**	**2.29 0.34**
T/T	**27(33.8%)**	**23.7(29.7%)**	
Alleles			
G	**73(45.6%)**		
T	**87(54.4%)**		

From above the

TABLE 2: COMPARISON OF DIFFERENT GENOTYPIC AND ALLELIC STATE IN MALARIA-INFECTED PATIENTS AND CONTROL SUBJECTS.

	Malaria-infected	Control	OR (95%CI) p-value
Glu298 Asp	**N = 120**	**N = 80**	
G/G	**28(23.3%)**	**20(25%)**	**Ref**
G/T	**48(40%)**	**33(41.3%)**	**1.0(0.5-2.0) 0.92**
T/T	**44(36.7%)**	**27(33.7%)**	**0.9(0.4-1.8) 0.69**
GT+TT	**92(76.7%)**	**60(75%)**	**0.9(0.5-1.8) 0.79**
T	**0.567**	**0.544**	**0.9(0.6-1.4) 0.65**

TABLE 3: COMPARISON OF DIFFERENT GENOTYPIC AND ALLELIC STATE IN MALARIA-INFECTED PATIENTS AND CONTROL SUBJECTS BASED ON GENDER.

	Malaria-infected	Control	OR (95%CI) p-value
Glu298Asp: Male	**N = 66**	**N = 40**	
G/G	**16(24.2%)**	**9(22.5%)**	**Ref**
G/T	**30(45.5%)**	**16(40%)**	**1.0(0.3-2.6) 0.92**
T/T	**20(30.3%)**	**15(37.5%)**	**1.3(0.5-3.8) 0.59**
GT+TT	**50(75.8%)**	**31(77.5%)**	**1.1(0.4-2.8) 0.84**
T	**0.530**	**0.575**	**1.2(0.7-2.1) 0.53**

	Malaria-infected	Control	OR (95%CI) p-value
Glu298Asp: Female	**N = 54**	**N = 40**	

G/G	**12(22.2%)**	**11(27.5%)**	**Ref**	
G/T	**18(33.3%)**	**17(42.5%)**	**1.0(0.4-3.0)**	**0.96**
T/T	**24(44.5%)**	**12(30%)**	**0.6(0.2-1.6)**	**0.27**
GT+TT	**42(77.8%)**	**29(72.5%)**	**0.8(0.3-1.9)**	**0.56**
T	**0.611**	**0.513**	**0.7(0.4-1.2)**	**0.18**

TABLE 4: COMPARISON OF DIFFERENT GENOTYPIC AND ALLELIC STATE OF MALARIA-INFECTED PATIENTS AND CONTROL SUBJECTS WITH MILD AND SEVERE

	Mild	**Severe**	**OR (95%CI)**	**p-value**
Glu298Asp	**N = 70**	**N = 50**		
G/G	**11(15.7%)**	**14(28%)**	**Ref**	
G/T	**39(55.7%)**	**20(40%)**	**0.4(0.2-1.1)**	**0.02**
T/T	**20(28.6%)**	**16(32%)**	**0.6(0.2-1.8)**	**0.03**
GT+TT	**59(84.3%)**	**36(72%)**	**0.5(0.2-1.2)**	**0.11**
T	**0.564**	**0.52**	**0.8(0.5-1.4)**	**0.50**
Glu298Asp: Male	**N = 40**	**N = 22**		
G/G	**7(17.5%)**	**6(27.3%)**	**Ref**	
G/T	**21(52.5%)**	**9(40.9%)**	**0.5(0.1-1.9)**	**0.31**
T/T	**12(30%)**	**7(31.8%)**	**0.7(0.2-2.9)**	**0.60**

GT+TT	33(82.5%)	16(72.7%)	0.6(0.2-2.0)	0.37
T	0.563	0.523	0.9(0.4-1.8)	0.67
Glu298Asp: Female	N = 30	N = 28		
G/G	4(13.3%)	8(28.6%)	Ref	
T/G	18(60%)	11(39.3%)	0.3(0.1-1.3)	0.10
T/T	8(26.7%)	9(32.1%)	0.6(0.1-2.6)	0.46
GT+TT	26(86.7%)	20(71.4%)	0.4(0.1-1.5)	0.16
T	0.567	0.518	0.8(0.4-1.7)	0.61

TABLE 5: COMPARISON OF DIFFERENT GENOTYPIC AND ALLELIC STATE OF MALARIA-INFECTED PATIENTS AND CONTROL WIH OR WITHOUT HYPERTENSION

	Malaria-infected	Control	OR (95%CI)	p-value
Glu298Asp: Hyperten	N = 32	N = 0		
G/G	16(50%)	0	Ref	
G/T	7(21.9%)	0	2.2(0.04-121.8)	0.70
T/T	9(28.1%)	0	1.7(0.03-94.8)	0.79
GT+TT	16(50%)	0	1.0(0.02-53.5)	1.00
T	0.391	0	1.5(0.03-80.6)	0.82

Glu : non-hypertensive	**N = 88**	**N = 80**	
I/I	**16(18.2%)**	**32(40%)**	**Ref**
I/D	**43(48.8%)**	**22(27.5%)**	**0.3(0.1-0.6)**
0.001			
D/D	**29(33%)**	**26(32.5%)**	**0.5(0.2-1.0)**
0.049			
ID+DD	**72(81.8%)**	**48(60%)**	**0.4(0.2-0.7)**
0.002			
D	**0.574**	**0.463**	**0.6(0.4-1.0)**
0.04			

TABLE 5: COMPARISON OF DIFFERENT GENOTYPIC AND ALLELIC STATE OF MALARIA-INFECTED PATIENTS WITH AND WITOUT HYPERTENSION

	Hypertensive	**Non Hypertensive**	**OR (95%CI)**
p-value			
Glu298Asp:	**N = 32**	**N = 88**	
G/G	**16(50%)**	**16(18.2%)**	**Ref**
G/T	**7(21.9%)**	**43(48.9%)**	**6.1(2.1-17.7)**
0.001			
T/T	**9(28.1%)**	**29(33%)**	**3.2(1.2-8.9)**
0.03			
GT+TT	**16(50%)**	**72(81.9%)**	**4.5(1.9-10.8)**
0.001			
T	**0.391**	**0.574**	**2.1(1.2-3.8)**
0.01			
ACE I/D	**N = 32**	**N = 88**	
I/I	**4(12.5%)**	**20(22.7%)**	**Ref**
I/D	**8(25%)**	**52(59.1%)**	**1.3(0.4-4.8)**
0.69			

D/D	**20(62.5%)**	**16(18.2%)**	**0.2(0.1-0.6)**
0.004			
ID+DD	**28(87.5%)**	**68(77.3%)**	**0.5(0.2-1.6)**
0.22			
D	**0.75**	**0.477**	**0.3(0.2-0.6)**
0.04			