THE ROLE OF EPITHELIAL SODIUM CHANNEL AND SYMPATHETIC NERVOUS POTENTIATION IN THE DEVELOPMENT OF SALT SENSITIVE HYPERTENSION AMONG NIGERIANS IN LAGOS

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BY

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DECLARATION

This work titled "The role of epithelial sodium channel and sympathetic nervous potentiation in the development of salt sensitive hypertension among Nigerians in Lagos" submitted to the School of Postgraduate Studies, University of Lagos, Lagos, Nigeria for the award of Doctor of Philosophy in Physiology is an original research carried out by ELIAS, Simiat Olanike in the Department of Physiology, Faculty of Basic Medical Sciences, College of Medicine, University of Lagos, under the supervision of Professor O.A. Sofola and Professor S.I. Jaja.

This work has not been submitted previously, in whole or in part, to qualify for any other academic award.

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"The role of epithelial sodium channel and sympathetic nervous potentiation in the development of salt sensitive hypertension among Nigerians in Lagos"

Submitted to the School of Postgraduate Studies, University of Lagos

for the award of

DOCTOR OF PHILOSOPHY (Ph.D)

is a record of original research work carried out

by

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DEDICATION

This work is dedicated

to

The loving memory of my parents

Dad - Alhaji Raimi Oduola ODETUNDE

who died on the 4th of June 1998

Mom - Alhaja Ajarah Boladale ODETUNDE

who died on the 4th of January 2007

For they put my feet on the straight path

May Almighty ALLAH (SWT) continue to grant them peaceful repose in Aljanat Firdaus

"(For them) Rest and Satisfaction

And a Garden of Delights"

- Holy Qur'an 56:89

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> **"Allah is sufficient for me (us), none has the right to be worshipped except Him, in Him I (we) put my (our) trust, and He is the Lord of the Mighty Throne"**
>
> Holy Qur'an Chapter 9:129

Simiat Olanike Elias

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ABSTRACT

The mechanisms by which salt causes hypertension are not conclusive. Increased sympathetic activity and systemic vascular dysfunction have both been implicated as well as mutations of the epithelial sodium channel (ENaC) that resulted in increased reabsorption of salt from the distal nephron. However, although an adrenergic overdrive has been observed in some hypertensive patients, it is not clear whether this adrenergic overdrive is the hallmark of hypertension. It has equally been a subject of debate whether exaggerated vascular reactivity is a cause or consequence of hypertension. Also, whereas ENaC mutations especially the β-T594M variant has been related to salt sensitive hypertension among Black individuals living in London, similar associations have not been recorded in African-Americans. On the other hand, among South Africans of black ancestry, some studies have shown no incidence of β-T594M ENaC mutation while a different type of ENaC mutation has been recorded in one study.

The present study was therefore designed to determine the role of salt sensitivity, autonomic potentiation and the epithelial sodium channel activity in the development of hypertension among Nigerians. Fifty-three otherwise healthy hypertensive (HT) adults and forty-seven age-matched normotensive (NT) subjects were studied. After baseline parameters had been obtained, the subjects were salt-loaded with 200 mmol/day of Na^+ as sodium chloride for 5 days. Thereafter salt sensitivity was determined in all the subjects as $\Delta MABP \geq +5mmHg$ following the salt-loading. As a test of mechanism for salt-sensitivity, plasma Na^+, urine Na^+ excretion ($U_{Na}V$), salt sensitivity index (SSI) and sodium clearance were determined. To assess the effect of the sympathetic nervous system on the cardiovascular system of the subjects, vascular reactivity and forearm vascular resistance (FVR) were determined before and after exposing the subjects to the cold pressor test for one minute. To assess the relationship between ENaC markers and salt sensitivity, the subjects were again salt-loaded with 200mmol/day of Na^+ with which 5mg Amiloride was co-administerd for the duration. Blood pressure,

plasma K^+, plasma renin activity (PRA) and serum aldosterone were determined thereafter. Blood was then collected for DNA isolation, amplification and sequencing.

Significant pressor responses to salt-loading alone were recorded in the NT ($p < 0.01$) and HT ($p < 0.001$) subjects. Salt sensitivity was significantly higher ($p<0.01$) among HT subjects when compared with NT subjects. At baseline and after salt-loading, plasma Na^+ concentration was similar among the NT and HT subjects though higher among the HT subjects. Urine Na^+ excretion was significantly lower ($p < 0.05$) in HT subjects compared with NT subjects. Salt sensitivity index (SSI) was higher ($p < 0.01$) among the HT subjects compared with in the NT subjects. Peak systolic and diastolic blood pressures were significantly higher ($p < 0.001$) among the two groups of subject compared to basal blood pressures before and after salt-loading; this was moreso in the hypertensive subjects. Hypertensive subjects showed higher systolic and diastolic vascular hyperreactivity compared to the NT subjects ($p < 0.05$) at baseline. However, following salt-loading, systolic vascular hyperreactivity increased significantly ($p < 0.05$) among the NT subjects and becoming higher than that recorded among the HT subjects at baseline. Systolic salt hyperreactivity was significantly higher ($p < 0.05$) among the NT subjects compared with the HT subjects. Similarly, diastolic salt hyperreactivity was higher in the NT subjects compared with HT subjects. Before salt-loading in NT subjects, there was a significant and positive correlation between salt sensitivity and systolic vascular reactivity ($p < 0.05$) while salt sensitivity showed a significant and negative correlation with diastolic vascular reactivity ($p < 0.05$). After salt-loading in NT subjects, there was a significant and negative correlation between salt sensitivity and systolic vascular reactivity ($p < 0.05$). Forearm vascular resistance (FVR) was significantly lower ($p < 0.05$) among the NT subjects compared with the HT subjects at baseline. Sympathetic nervous stimulation by means of the cold pressor test before and after salt-loading led to significant increases ($p < 0.001$) in FVR which were similar in both NT and HT groups. Co-administration of salt and amiloride led to significant decreases in systolic and diastolic blood pressure

compared with baseline in NT ($p < 0.05$) and HT ($p < 0.001$) subjects. Plasma K^+ was significantly lower ($p < 0.05$) among the HT subjects at baseline compared with the NT subjects. Following salt-loading, there was significant fall in plasma K^+ in the NT ($p < 0.05$) but not in the HT subjects. On co-administration of salt and amiloride however, plasma K^+ increased significantly ($p < 0.001$) in the HT subjects but not in the NT subjects. Plasma renin activity (PRA) was lower in the HT subjects ($p < 0.05$) compared with NT subjects before and after salt-loading. There were significant increases ($p < 0.05$) in PRA in both the NT and HT subjects after the salt load. The β-T594M mutation of the epithelial sodium channel was recorded among 5 percent of the study population while four previously undocumented non-synonymous mutations: β-E636V, β-E632V, β-D638Y and β-L628Q were observed in another 4 percent of the subjects.

The results of this study show that HT subjects were more salt sensitive than NT subjects and inabilty of the HT subjects to excrete a salt-load is contributory to this. Although the effect of sympathetic stimulation on FVR was similar in both the NT and HT subjects, NT subjects displayed enhanced vascular hyperreactivity after salt-loading, higher than that observed in hypertensive subjects at baseline. This is an indication that vascular hyperreactivity, a reflection of sympathetic potentiation is important in the development of hypertension among the subjects. Also there was significant correlation between systolic hyperreactivity and salt sensitivity among the NT subjects which is a strong indicator of development of hypertension in the future. Furthermore, amiloride studies indicated that enhanced epithelial sodium channel activity is important in the development of hypertension among Nigerians. The β-T594M mutation of the epithelial sodium channel was recorded among 5 percent of the study population confirming the presence of this mutation in Nigerian subjects. Also four new mutations of the epithelial sodium channel with the potential to affect blood pressure were recorded in 4 percent of the subjects.

CHAPTER ONE

1.0 INTRODUCTION

The role of salt in the pathogenesis of hypertension has engendered a lot of interest over the years. Hypertension is a leading non-communicable disease worldwide and it is the leading indication for prescriptions (Woodwell and Cherry, 2004). Approximately one in three adults in the United States suffers from hypertension (Soundararajan *et al.*, 2010). The figure in Nigeria is no less disturbing and has actually increased over the years. In 1997 the prevalence of hypertension in Nigeria was 16 percent (Cooper *et al.,* 1997) while Adedoyin *et al.,* (2008) reported a prevalence of 36.6 percent in 2008 and Ulasi *et al.,* (2010) report a current prevalence of 32.8 percent. Hypertension is important not just because of its prevalence, but especially because of its close relationship with cardiovascular morbidity and mortality (Ezzati *et al.,* 2002). Hypertension is the most prevalent cause of cardiovascular events (Kaplan, 2006) being responsible for about 50% of risk (Lawes *et al.,* 2008). Cardiovascular disease is a leading cause of death worldwide; in 2005 alone, about 17.5 million people died from cardiovascular diseases (CVD) representing 30% of deaths globally (World Health Organization, 2007). High dietary sodium intake is one of the most important environmental risk factors for hypertension (He and MacGregor, 2007). Extremes of salt intake have been documented in human populations ranging from about 20mmol/day (0.47g/day) sodium among the Yanomamo Indians of Brazil to 600mmol/day (13.8g/day) in Northern Japan (Panel on Dietary Reference intakes for Electrolytes and Water, 2004). In most industrialized societies, salt intake is about 150mmol/day (3.5g/day) an amount quite in excess of the daily requirement of 65mmol/day (1.52g/day) of sodium

recommended by the Panel on Dietary Reference Intakes for Electrolytes and Water (2004) as an adequate intake for most adults (Weinberger, 2004).

Based on this observation that salt intake varied as much as geographic locations, Dahl *et al.*, (1962a) reported that hypertension was common in societies with more than an average salt intake while it was rarely seen in populations that consumed diets with a low salt content. Communities with an average salt intake more than 3g/day experienced an increase in proportion of hypertensive subjects with age. This phenomenon became more pronounced as the salt intake increased even further while there is no incidence of hypertension whatsoever among populations that ingested less than 3g/day of sodium chloride (Meneton *et al.*, 2005). The relation of dietary salt intake with blood pressure among populations that ingest a salt level between 3 g/day and 20 g/day has been difficult to define (Meneton *et al.*, 2005).

The relationship between a high salt intake and the development of hypertension has been established in population studies although it has been observed that every population that ingests a high salt diet also has individuals who did not develop hypertension (Dahl, 1962b; Bashyam, 2007) which led to the suggestion that the development of hypertension is a consequence of both environmental (e.g. diet) and genetic background of the individual (Bashyam, 2007). In spite of the large number of studies suggesting a high salt intake is instrumental to the pathogenesis of hypertension in salt sensitive individuals (World Health Organization, 2002; Weinberger, 2004), most of these studies have revealed only a weak relationship between sodium intake/excretion and blood pressure in the general population (Hooper *et al.*, 2002; 2004). Some individuals

however do manifest large blood pressure changes in response to acute or chronic ingestion of salt or to salt restriction. These individuals are termed "salt sensitive" (Franco and Oparil, 2006). Salt-sensitivity is more evident in Blacks compared to Caucasians (Bragulat *et al.,* 2001) and it has been linked to increased cardiovascular events and reduced survival (Franco and Oparil, 2006).

A high incidence of salt sensitivity has been described among Africans living in Britain among whom incidence of hypertension may be as high as 50% (Cappuccio *et al.,* 1997a). There is no doubt that many of the cases of hypertension in Blacks are due to increased salt sensitivity (Sofola, 2004) although it has been difficult to establish a relationship between 24 hour urinary sodium excretion (an index of salt intake) and level of blood pressure among Nigerians (Azinge *et al.,* 1999) which may be attributable to the small number of subjects studied compared with the number in the INTERSALT study that involved thousands of subjects. Also, Sofola *et al.,* (1998) have been unable to demonstrate significant pressor responses to acute salt loading in some normal subjects probably due to the short duration of salt loading and the little number of subjects involved. Salt sensitivity might represent an independent cardiovascular risk factor for cardiovascular events due to the fact that a higher rate of cardiovascular events is recorded in salt sensitive individuals (Corruzi *et al.,* 2005). Salt sensitivity is also associated with increased mortality (Weinberger *et al.,* 2001) whether the salt sensitive individual is normotensive or hypertensive.

The pathogenic mechanisms responsible for salt sensitivity are far from being understood. Earlier attempts at explanation of the high incidence of hypertension among Africans and African Americans compared to Caucasians was attributed to the role of slavery in enhancing the survivability of individuals with the genetic ability to conserve salt in the slaves that survived transhipment (Wilson and Grim, 1991) but this has been questioned (Jackson, 1991). The demonstration of a linkage between the epithelial sodium channel (ENaC) and Liddle's syndrome, a severe and rare form of heritable human hypertension was a major breakthrough in the understanding of the genetics of hypertension (Shimkets *et al.*, 1994). This finding suggested the physiological involvement of ENaC in blood pressure regulation. The physiological significance of the β-T594M polymorphism of ENaC could partly explain the high incidence of salt sensitive hypertension in African Americans. Direct evidence that ENaC dysfunction is involved in the pathological processes leading to hypertension has come from the fact that all mutations identified in Mendelian forms of hypertension-related syndromes were found in genes involved in electrolyte transport functions including sodium channel, non-voltage-gated 1 beta subunit of the epithelial sodium channel (ENaC) gene encoding ENaC subunits A, B and G (SCNN1A, SCNN1B, SCNN1G) (Chang *et al.*, 1996; Tesson and Leenen, 2007).Variation in systolic blood pressure (SBP) is particularly thought to be explained by genes; a heritability of 41% was reported in the Victorian Family Heart Study (VFHS) (Harrap *et al.*, 2000) consistent with earlier studies (Mongeau *et al*, 1987; Hong *et al*, 1994). The VFHS reported suggestive linkage of systolic blood pressure to chromosome 16p, a finding consistent with earlier population studies (Atwood *et al.*, 2001; de Lange *et al.*, 2004; Hamet *et al.*, 2005). Further refined studies in the VFHS located the gene to 16p12 which is the location of the SCNN1B and SCNN1G genes that encode the β- and γ- subunits of the Epithelial Sodium Channel (ENaC)

respectively (Büsst *et al.*, 2007). Whereas the relationship between SCNN1B and systolic blood pressure has been investigated in different populations such as the Japanese (Iwai *et al.*, 2006), South Africans (Nkeh *et al.,* 2003) and Black Americans (Warnock, 1999) with variable results, such is not the case with regard to the SCNN1G (Büsst *et al.,* 2007).

It has been postulated that exaggerated vascular reactivity is a possible mechanism in the development of hypertension and cardiovascular disease (Stewart and France, 2001). According to the Reactivity Hypothesis, exaggerated responses to stress lead to a cascade of pathophysiological events that eventually result in sustained increases in blood pressure (Treiber *et al.,* 2003). Exaggerated vascular reactivity has also been linked to enhanced peripheral resistance, a hemodynamic alteration that is itself linked to hypertension. Increased and prolonged exposure to stress can cause serious strain on the arteries and myocardium. Jennings *et al.,* (2003) have reported that high reactors to acute stressors develop more extensive arterial plaques and more aggressive plaque growth which all contribute to the increased risk for cardiovascular events as well as increased mortality in these persons. Dietary salt intake promotes intrinsic changes in compliance and resistance vessels which effects are intensified by congenital and acquired sodium retentive states. This worsens the outcome of hyperreactivity.

1.1. STATEMENT OF PROBLEM

The prevalence of hypertension in Nigeria is increasing from 14.5% (Cooper *et al.,* 1997) to 32.8% (Ulasi *et al.,* 2010) but the exact mechanisms are still being investigated. Salt sensitivity has been linked to an increased risk for the development of cardiovascular events including heart failure and strokes as well as increased mortality irrespective of the blood pressure status of the individual (Weinberger *et al.,* 2001). Previously it had been thought that the sympathetic nervous system was important only in the short-term regulation of blood pressure but an adrenergic overdrive has been observed in some hypertensive patients (Grassi, 2009). It is however not clear whether the adrenergic overdrive is the hallmark of hypertension or if it is only present in certain conditions. It is also unclear whether the exaggerated vascular reactivity observed in hypertension is a cause or consequence of hypertension. The epithelial sodium channel (ENaC) is considered a major candidate in the development of hypertension more so that mutation in this channel has been shown to cause Liddle's syndrome, a rare form of heritable salt-sensitive hypertension (Bubien, 2010). Whereas ENaC mutations especially the β-T594M variant has been related to salt sensitive hypertension among African Americans and Blacks living in London, similar associations have not been recorded among South Africans of black ancestry. It therefore became important to carry out a study to determine whether salt sensitivity is present among Nigerians and the role played by autonomic potentiation in the development of hypertension. It was also important to demonstrate the role of the ENaC in the causation of salt sensitive hypertension in Nigerians (Africans) in their own native environment as well as genetic studies to identify the status of the ENaC among our population thereby providing a genetic basis for the modification of the treatment of hypertension in Nigerians.

1.2 OVERALL AIM OF THE STUDY

The overall aim of this study is to demonstrate the role of salt sensitivity and autonomic nervous potentiation in hypertension among adult Nigerians and the possible role of the epithelial sodium channel

1.3 SPECIFIC OBJECTIVES OF THE STUDY

The specifc aims of this study were to

1. Determine the prevalence of salt sensitivity among normotensive and hypertensive subjects
2. Investigate the effect of the sympathetic nervous system on the cardiovascular system of normotensive and hypertensive subjects by assessing vascular reactivity
3. Assess the role of sympathetic regulation on forearm vascular resistance among normotensive and hypertensive Nigerians
4. Assess the relationship between ENaC markers and salt sensitive hypertension among Nigerians
5. Identify genetic variants of ENaC and their association with salt sensitive hypertension among Nigerians

1.4 SIGNIFICANCE OF THE STUDY

This study demonstrates the role played by adrenergic potentiation in the development of hypertension among Nigerians. This is important because of the fact that hyperreactivity in

normotensive subjects is indicative of a high risk for developing hypertension in the future while systolic hyperreactivity in particular is predictive of future strokes in hypertensive subjects. This study also provides information on the status of the epithelial sodium channel (ENaC) among normotensive and hypertensive Nigerians. The outcome of this study is important in the development of amiloride as an anti-hypertensive drug in patients with epithelial sodium channel mutation. These results are therefore significant as they form a basis for the modification of the management of hypertension in Nigerians. The results of this study also add to the knowledge base on the determinants of hypertension in Nigerians.

1.5.LIMITATIONS

The renal sodium channel is inaccessible for clinical assessment therefore indirect measurements for the assessment of ENaC were carried out as described in the Methodology. Also short term effects of salt-loading were studied in the determination of salt sensitivity in Nigerians due to the fact that compliance is usually low in experimental studies of this nature when carried out over a long term in humans. It would have been interesting to study the effects of salt-loading over a longer period of time on the study parameters. The short duration of salt-loading is however acceptable as it has been used by many other workers in this field of study (Campese *et al.,* 1991; Coruzzi *et al.,* 2005; Tzemos *et al.,* 2008).

1.6. OPERATIONAL DEFINITION OF TERMS

Normotension: Repeated supine systolic blood pressure <140mmHg systolic and/ or <90mmHg diastolic

Hypertension: Repeated in supine blood pressure ≥140mmHg systolic and/ or ≥90mmHg diastolic; or being on pharmacological treatment for hypertension

Salt sensitivity: The phenomenon in which excess salt intake caused an increase of 5 mmHg or more in mean arterial blood pressure (MABP)

Salt resistance: The phenomenon in which excess salt intake caused an increase in MABP that is less than 5 mmHg or an outright decrease in MABP

Basal blood pressure (mmHg): The last of three almost identical blood pressure measurements taken at 10min intervals after a period of rest in the laboratory before exposure to the cold pressor test

Peak Blood Pressure (mmHg): The highest of three 15sec serial blood pressure readings taken from 1 minute after exposure to the cold pressor test (CPT)

Normoreactivity: Subjects were considered normoreactive if the difference between peak blood pressure after exposure to CPT and basal blood pressure was less than 15mmHg whether systolic or diastolic

Hyperreactivity: Subjects were considered hyperreactive if the difference between peak blood pressure after exposure to CPT and basal blood pressure was 15mmHg or more whether systolic or diastolic

Salt Reactivity: Subjects were considered salt reactive if they became hyperreactive only after a salt load

1.6.1. LIST OF ACRONYMS

SOE: Simiat Olanike Elias

OAS: Olusoga A Sofola

FASTA: Text-based format for representing nucleotides or peptides as single-letter codes in Bioinformatics

IUPAC-IUB: = Internationa Union of Pure and Applied Chemistry - International Union of Biochemistry

NT = normotensive

HT = hypertensive

MABP = mean arterial blood pressure

PCR = Polymerase chain reaction

SCNN1A = Sodium channel, non-voltage-gated 1 alpha subunit of the epithelial sodium channel (ENaC) gene

SCNN1B = Sodium channel, non-voltage-gated 1 beta subunit of the epithelial sodium channel (ENaC) gene

SCNN1G = Sodium channel, non-voltage-gated 1 gamma of the epithelial sodium channel (ENaC) gene

CHAPTER TWO

2.0 REVIEW OF LITERATURE

2.1 Salt and Blood Pressure

2.1.1. Influence of Diet and Environment

The role of salt in the pathogenesis of hypertension has generated a lot of research over the years. The earliest study that reported a positive correlation between salt intake and blood pressure in humans was published in 1904 (MacGregor and de Wardener, 1998) but was refuted in 1907 (Graudal, 2005). There ensued thereafter a lot of controversy but by the 1940s, the validity of this correlation was re-established following the successful treatment of hypertensive patients with a low-salt diet (Taubes, 1998). There seems to be some heterogeneity in the response of Man to salt intake. Dahl *et al.,* (1962b) reported that hypertension was common in societies with more than an average salt intake while it was rarely seen in populations that consumed diets with a low salt content. Communities with an average salt intake greater than 3g/day experienced an increase in proportion of hypertensive subjects with age; this phenomenon became more pronounced as the salt intake increased even further while there was no incidence of hypertension whatsoever among populations that ingested less than 3g/day of sodium chloride (Meneton *et al.,* 2005).

The relation of dietary salt intake with hypertension among populations that ingest a salt level between 3 g/day and less than 20 g/day has been difficult to define (Meneton *et al.,* 2005). The most well known communities with regard to low salt intake are the Yanomamo Indians on the border of Venezuela and Brazil who have been reported to ingest as little as 0.46g/day (20mmol/day) of sodium (Food and Nutrition Board, 2004). At the age of 50 years, the average blood pressure in this community is only 100/64 mmHg (Meneton *et al.,* 2005). The Eskimos and the Kalahari tribesmen of South Africa also record almost no incidence of high blood pressure at all (Dahl *et al.,* 1962a; Oliver *et al.,* 1975). Some researchers have attributed the low blood pressure in these communities to the fact that these are simple, peaceful communities ingesting simple traditional meals in contrast to the Western lifestyle and diet. However, considering the fact that the Yanomano Indians are reported to have a culture of aggression, warfare and violence, this reasoning has been debunked (Chagnon, 1968). Some other unacculturated communities that ingest a higher salt intake than the Yanomano Indians record a corresponding increase in blood pressure. Blood pressure has been shown to increase with age among the nomadic herdsmen of Quash'qai in Iran who consume the same amount of salt in their diet as most developed societies (Page *et al.,* 1981). Individuals in the Northern Kashmir region who were also unexposed to a Western lifestyle but who ingested a high salt level in their diet also recorded a high blood pressure with age; they had a culture of adding salt to their tea (Mir and Newcombe, 1988).

Some individuals manifest a rise in blood pressure upon changing their diet from low salt to high salt. From a carefully controlled study among farmers in Kenya who ingested low salt in their diet, migration to an urban community led to ingestion of high salt in their diet resulting in a rise

of 6.9 mmHg in their systolic blood pressure and 6.2 mmHg in their diastolic blood pressure after a few months whereas blood pressure remained unchanged among their non-immigrant colleagues (Poulter *et al.,* 1985). Indeed it has been reported that hypertension is less common among the nomadic Bushmen compared to their contemporaries who became prisoners and labourers (Kaminar and Lutz, 1960). Also among the Yi people in south western China, migration to an urban settlement with the associated change in diet to a higher salt-containing one led to an increase in blood pressure with aging. With age, an increase of 0.33 mmHg/y was recorded in both systolic and diastolic blood pressures; this increase was not recorded among those who remained in their rural farming environment (He *et al.,* 1991). On the other hand, communities that ingest a high salt diet like the Japanese community, north of the Japanese mainland that ingest as much as 27g/day of salt (with individual levels as high as 60g/day) had about 70% of the population aged 50 to 60 years being hypertensive with blood pressure greater than 150/90 mmHg (Meneton *et al.,* 2005) whereas the Japanese in the south who ingested about 14g/day of salt in their diet had only 10% of the population hypertensive between the ages of 50 to 60 years. (Meneton *et al.,* 2005). Dahl (1962a) observed that every population that ingests a high salt diet also has individuals who, in spite of the high salt intake did not develop hypertension. This led to the suggestion that the development of hypertension is a consequence of both environmental (e.g. salt) and genetic background of the individual (Bashyam, 2007).

2.1.2. Genetic Influence

Results from animal studies have so far backed up the hypothesis that the development of hypertension in individuals was influenced by both environmental and genetic factors. Dahl

(1960) observed that rats that were chronically fed a high salt diet developed varying degrees of hypertension. He went on to suggest that salt resistant animals might simply be statistical outliers within a genetically homogenous group; if salt sensitivity was a genetic trait, it should be possible to develop separate strains of salt-sensitive and salt resistant rats. This was done by genetic breeding and the different strains of rats responded distinctly to a diet of high salt content: blood pressure in Dahl salt-resistant rats (DR) remained the same while that in Dahl salt-sensitive (DS) rats increased dramatically (Dahl *et al.,* 1962b). Since then, salt-dependent hypertension has been studied extensively in animals. Different strains of rodents with genetically determined hypertension have been used to study the different aspects of the pathogenesis of essential hypertension arising as a result of complex interactions of different genetic determinants and the environment (Ferrari and Buachi, 1995). Experiments in Dahl Salt Sensitive (DS) rats (Nishida *et al*., 1998; Ambrosius *et al.,* 1999); Sprague-Dawley rats (Miyajima and Bunnang, 1985; Obiefuna *et al*., 1991a; Sofola *et al.,* 2002) and chimpanzees (Denton *et al*., 1995) have all been used to show that high salt diet results in elevated blood pressure.

Hypertension has been shown to be inherited in the Mendelian fashion. Genetic diseases with Mendelian transmission are often characterized by mutations with rare allelic frequency in human populations (<0.1%). On the other hand, allelic polymorphisms are more frequent and have an allelic frequency of >1% or 2% in human populations (Rossier and Schild, 2008). These allelic polymorphisms are usually single nucleotide polymorphisms (SNPs) but others such as copy number variations or short tandem repeats are also possibilities (Rossier and Schild, 2008). Genetic factors account for about 30% of the blood pressure variations in human populations

(Corvol *et al.,* 1999). It has been suggested that combined action of many genes as opposed to a single gene determines blood pressure response to salt ingestion. Each of the genes may affect one or more channel, transporter, or enzymes that may be associated with neural, hormonal, vascular and renal control mechanisms of blood pressure (Khalil, 2006), The Epithelial Sodium Channel (ENaC) is only one of these genes that have been associated with human essential hypertension. The critical role played by this channel in the control of sodium balance, blood volume and blood pressure (Verrey *et al.,* 2008b) will be discussed later.

2.2. SALT SENSITIVE HYPERTENSION

High salt has been shown to be instrumental to the pathogenesis of hypertension in salt sensitive individuals (INTERSALT, 1988; Stamler *et al*., 1991). Salt sensitivity describes the phenomenon in which excess salt intake causes high blood pressure in selective races and this is more evident in Blacks compared to Caucasians (Luft and Weinberger, 1997; Bragulat *et al.,* 2001). It refers to the propensity of individuals to show meaningful changes, increases or decreases, in mean arterial blood pressure (MABP) to sodium repletion or restriction respectively (Sanders, 2008). Salt sensitivity of blood pressure provides prognostic information with regard to cardiovascular events; it is important in predicting target organ damage like left ventricular hypertrophy, renal dysfunction and increased risk of death (Titze and Machnik, 2010). The outcome of salt sensitivity is irrespective of the individual's blood pressure measurement (Weinberger *et al.,* 2001).

A number of protocols have been used to define salt sensitivity, the most extensive being that of Weinberger *et al*., (1986) who defined salt sensitivity as a decrease of 10 mmHg or more in mean blood pressure from the value measured after a 4-h infusion of 2 L normal saline compared with the value measured the morning after one day of a 10-mmol Na/d diet during which 3 oral doses of furosemide were given at 1000, 1400, and 1800 hours. Using this criterion, these researchers observed that 51 percent of subjects with essential hypertension but only 26 percent of normotensive persons were salt sensitive (Weinberger, 1996). The study population was mixed, including both blacks and caucasians.

There have been many variations in the operational definition of salt-sensitivity. Salt sensitivity has been defined as an absolute change more than 5 mmHg to 10 mmHg in mean arterial blood pressure (MABP) or arbitrarily as an increase of at least 10 percent with a high sodium diet (250 mmol/day) compared with a low sodium diet (10 mmol/day) over a 7-day period (Kawasaki *et al.,* 1978). The different definitions result in different results. For example, using an absolute change of at least 5 mmHg in MABP in a study, 33.9% and 32.4% of the participants during low salt and high salt diets respectively were salt sensitive. On the other hand in the same study, if a proportional change of at least 5 percent in MABP had been used, 38.7 percent of the subjects during low salt intervention and 39.2 percent during high salt intervention will have been salt sensitive (He *et al.,* 2009). In the same vein, the diagnosis of salt sensitivity using an absolute difference in mean arterial blood pressure (MABP) of more than 10 percent between a low salt diet and a high salt diet period and diagnosis of salt-resistant as an absolute change in MABP lower than 5 percent after salt loading (Weinberger, 1996) does not allow the exploration of the

underlying causes for this change or non-change in MABP such as an impairment of the cardiovascular autonomic regulation (Coruzzi *et al.*, 2005).

The salt sensitivity index (SSI) which relates the changes in blood pressure induced by sodium loading with the concomitant changes in urinary sodium output without any arbitrary definition of threshold (Coruzzi *et al.,* 2005) has also been used to assess salt-sensitivity. Using SSI, Coruzzi *et al.,* (2005) observed a lack of correlation between the degree of salt-sensitivity and either baseline 24-hour blood pressure and heart rate, age or body mass index. This allowed these researchers to infer that increasing SSI levels observed in the study was the reason for the impaired autonomic control of the circulation displayed by the subjects.

Weinberger *et al*., (1986) have also noted that salt sensitivity has a typical bell-shaped distribution with a shift to the right in those who are hypertensive. Weinberger and Fineberg, (1991) later observed a further shift in the bell-shaped distribution with increasing age in normotensive persons and a greater shift in hypertensive persons. A high incidence of salt sensitivity has been demonstrated in Nigerians using salt taste threshold as an index of salt sensitivity (Obasohan *et al.,* 1992). In salt resistant persons on the other hand, ingestion of a high salt diet is associated with only a small increase in blood pressure in the absence of major endothelial cell dysfunction or alterations in vascular reactivity (Bragulat *et al.,* 2001).

2.2.1. Factors Defining Salt Sensitivity

Mechanisms defining salt sensitivity are complex and diverse. They include low birth weight with reduced nephron number, subtle renal injury and inflammation as well as changes in potassium intake (Ben-Dov and Bursztyn, 2011). Other possible factors include expression of ion channels and supporting cellular skeleton to non-modulation of the renin-angiotensin-aldosterone axis and ouabain-like activity (Na^+/K^+-ATPase inhibition) as well as diminished atrial and other natriuretic peptides (Ben-Dov and Bursztyn, 2011). Factors such as aging and diminished renal function have been associated with enhanced salt-sensitivity (Oparil *et al.,* 1988; He *et al.,* 2009) although genetic factors have also been implicated as evidenced by the familial aggregation and the higher prevalence of salt-sensitivity in certain ethnic groups such as the African Americans (Makaritsis *et al.,* 1999). Salt-sensitivity increases with age being higher in persons 45 years and older (Akita *et al.*, 2003; He *et al.*, 2009). Earlier attempts at the explanation for the difference in incidence of hypertension between African Americans, Africans and Caucasians was attributed to the role of slavery in enhancing the survivability of individuals with the genetic ability to conserve salt in the slaves that survived transhipment (Wilson and Grim, 1991) but this has been questioned (Jackson, 1991). The prevalence of hypertension in Nigeria is about 32.8% (Ulasi *et al.,* 2010) while it is as high as 50 percent among black Africans between the ages of 40 years and 59 years living in South London (Cappucio *et al.,* 1997a). Other explanations adduced for the high incidence of hypertension among blacks include an enhanced adrenergic vasoconstriction and reduced dilatory responses (Stein *et al.*, 2000) that will increase vascular tone and therefore blood pressure (Sofola, 2004).

Hypertensive subjects are generally more salt sensitive than normotensive subjects (He *et al.,* 2001; Akita *et al.,* 2003; Franco and Oparil, 2006; He *et al.,* 2009). Salt sensitivity also increased

with a higher baseline blood pressure especially with regard to a higher baseline systolic blood pressure (Volmer *et al*., 2001; He *et al.,* 2009). Although some researchers have opined that salt sensitivity may not be reproducible in the same individuals (Khalil, 2006); Weinberger *et al.*, have reported follow-up studies showing that salt sensitivity is persistent and reproducible over time (Weinberger and Fineberg, 1991). Variation in a person's daily intake of salt as well as urinary excretion of salt may affect this variation in the salt sensitivity status of the individual (Khalil, 2006).

It has also been reported that persons with low plasma renin activity tended to be salt sensitive (Weinberger, 1996). It is thought that blunting of the renin-angiotensin-aldosterone system may contribute to salt sensitive hypertension (Franco and Oparil, 2006). This system plays a pivotal role in the regulation of sodium excretion and balance is sensitive to changes in sodium intake (Rasmussen *et al.,* 2003). In a study in normotensive and hypertensive subjects, reductions in MABP was observed to correlate inversely with the increase in plasma renin activity and aldosterone concentration that occurred when the diet was changed from high to low sodium content; the renin aldosterone response was shown to be blunted in the hypertensive subjects. The findings suggested that the fall in blood pressure with acute salt restriction in hypertensive persons compared with normotensive individuals may have been due partly to a less responsive renin-angiotensin-aldosterone system in the hypertensive subjects (Franco and Oparil, 2006). Blunted activity of the renin-angiotensin-aldosterone system may therefore be contributory to salt-sensitivity of blood pressure in man.

Atrial Natriuretic Peptide may also play a role in the determination of salt-sensitivity (Feng *et al.,* 2003). When mice with homozygous deletion of the ANP gene (Nppa-/-) were fed a high salt diet, they developed hypertension but maintained a normal level of blood pressure when fed a low salt diet from weaning (Feng *et al.,* 2003). Also, a loss of function polymorphism of the ANP gene has been observed more in black salt sensitive hypertensive individuals compared with normotensive persons or white hypertensive individuals (Nakayama *et al.,* 2000).

The INTERSALT Study was a prospective study that began in 1981 designed to examine the relationship between salt and hypertension. This study was carried out in 52 centres across 32 countries. Twenty-four-hour urine was measured in 10,079 male and female subjects aged 20-59 years (INTERSALT, 1988; Elliott *et al.,* 1996; Meneton *et al.,* 2005). For all 52 centres, there was a positive correlation between sodium excretion and both systolic blood pressure (SBP) and diastolic blood pressure (DBP) and an even more significant association between sodium excretion and the changes in blood pressure with age among individuals; this association was not recorded across centres (Meneton *et al.,* 2005). In this study, after correcting for confounding factors such as age, sex, body mass index (BMI) and alcohol intake, subjects aged 25y to 59y who ingested a diet lower in salt content by about 5.7g/day recorded a 9 mmHg lower rise in blood pressure (Meneton *et al.,* 2005).

The INTERSALT study was important in that it reported a correlation between urinary sodium (Na) excretion and blood pressure (INTERSALT, 1988). Urinary sodium excretion has been shown to be an accurate index of total sodium intake because in the steady state, it represents about 93% of sodium intake (He and MacGregor, 2003). Few populations were found whose

sodium intakes were approximately 100 mmol/d. This (100mmol/d Na^+) is the likely threshold above which an effect of sodium on blood pressure is observed (Kaplan, 2000). In other words, hypertension was not observed until the sodium intake had exceeded 100mmol/d. This led to the suggestion of there being a sodium threshold below which hypertension is not observed.

It has also been suggested that salt sensitivity increases at menopause (Yamori *et al.*, 2001). The Cardiovascular Diseases and Alimentary Comparison (CARDIAC) study fashioned after the INTERTSALT study and overseen by the World Health Organization (WHO), examined the relationship between 24-h sodium excretion and blood pressure in at least 3,681 men and 3,653 women from 60 centres in 25 countries worldwide. It was reported from a cross-centre correlation analysis that systolic blood pressure and diastolic blood pressure were positively associated with 24-h sodium excretion in both men and women but the association was significant only in men (Yamori *et al.*, 1990). Cross centre analyses of results from 21 centres which had data on menopausal status however indicated that 24-h sodium excretion was positively associated with systolic and diastolic blood pressure in both pre- and post-menopausal women; the association was only significant in menopausal women (Yamori *et al.*, 2001).

Raising the salt intake of a normotensive individual acutely may raise the plasma sodium transiently without affecting the blood pressure (Khalil, 2006). On the other hand, effects of chronic increase in salt intake vary. Although it may be difficult to detect a small rise in plasma sodium, an increase less than 1% may be sufficient to stimulate the thirst centre in the hypothalamus (Khalil, 2006). Increased salt intake seems to result in a positive association between plasma sodium and systolic blood pressure of hypertensive individuals more than it does normotensive persons (Khalil, 2006).

Salt restriction has been shown to result in a modest though significant reduction in blood pressure (Sacks *et al.,* 2001). The Dietary Approach to Stop Hypertension (DASH) study was a prospective study used to demonstrate the fact that low salt diet resulted in reduction in both systolic and diastolic blood pressure in both normotensive and hypertensive subjects (Sacks *et al.*, 2001). In an earlier study, similar observations were made in older hypertensive individuals (Cappuccio *et al*., 1997b; He *et al*., 2000; Conlin, 2001). Grobee and Hoffman (1986) after reviewing 13 randomized trials, observed that the effect of sodium restriction was small and affected mainly systolic blood pressure which fell by an average of 3.6 mmHg (range -0.5 to -10.0 mmHg) while mean diastolic blood pressure fell by 1.98 mmHg (range +3.2 to -7.0 mmHg). The extent of the blood pressure reduction increased with age and with higher initial pressures (Robertson, 2003). Similar findings by Graudal *et al.,* (1998) from the analysis of 58 trials in hypertensive and 56 in normotensive individuals led to the conclusion that reduced sodium intake might be employed as supplementary therapy in hypertension although they stopped short of advising a reduced salt intake in the general population. A diet too low in salt content may lead to an increase in circulating plasma renin level as well as increased sympathetic nervous activity (Alderman, 2000). A low 24-hour sodium excretion has also been found to be associated with increased attacks of syncope which was corrected by ingestion of salt (El-Sayed and Hainsworth, 1996).

Guyton (1987) postulated that modification of the pressure-natriuresis relation always participates in the development of blood pressure regardless of the initiating factor. This hypothesis has been extended by Kimura and Brenner (1993) by proposing that there are three renal mechanisms that may lead to hypertension: an increased preglomerular vascular resistance,

a decrease in whole kidney ultrafiltration and an increase in tubular sodium reabsorption. Whereas the first mechanism will lead to a salt-insensitive or salt resistant hypertension, the other two will result in salt sensitive hypertension.

Blood pressure follows a circadian rhythm with 10 percent to 15 percent lower values recorded during the night than during the day (Bankir *et al*., 2008). This is thought to be due to the activity of the sympathetic nervous system although several other neurohormonal systems regulating blood pressure may contribute (Baumgart, 1991; Smolensky and Haus, 2001). This nocturnal dipping in blood pressure is however reduced or reversed in hypertension due to high sodium intake and salt sensitivity (Sachdeva and Weder, 2006; Uzu *et al*., 2006; Hoshide and Kario, 2008). It has been suggested that the nondipping pattern of blood pressure at night observed in the hypertensive patients is as a result of an impaired capacity to excrete sodium during the day time (Fukuda *et al.*, 2006; Kimura, 2008). This therefore suggests that to maintain 24-hour sodium balance, blood pressure increases at night to promote sodium excretion. The impaired capacity to excrete sodium may be as a result of reduced renal function as in persons with low glomerular filtration rate or to increased tubular sodium reabsorption as in persons with primary aldosteronism (Uzu *et al*., 1998). In a study of 325 subjects, hypertensive and non-hypertensive persons, Bankir *et al*., (2008) reported that the non-dipping blood pressure in some hypertensive subjects may be as a result of an inability to concentrate sodium in urine since urinary volume was similar in all categories of subjects studied.

Based on the hypothesis that the relative increase in blood pressure observed during the night is a pressure-natriuresis mechanism favouring a compensatory rise in sodium excretion and the

maintenance of sodium balance (Fukuda *et al.,* 2006; Sachdeva and Weder, 2006), salt sensitive patients have been treated with sodium restriction (Uzu *et al.,* 1997) leading to a recovery of a normal nocturnal dipping. A similar response has been obtained with treatment with thiazide diuretics (Uzu and Kimura, 1999). Salt insensitive persons however show a normal nocturnal dipping that is not modified by either a low salt diet or thiazide diuretic (Uzu *et al*., 1997).

2.2.1.2. Mechanism of Effect of Salt on Blood Pressure

Various mechanisms contribute to the observed effect of dietary salt intake on blood pressure as evidenced by the many epidemiological, experimental models (human and animal), physiological and biochemical studies as well as clinical trials, genetic and mortality studies (Meneton *et al.,* 2005). These mechanisms include enhanced responsiveness to constrictor agonists like noradrenaline (Obiefuna *et al.,* 1991a; Nishida *et al*., 1998; Sofola, *et al*., 2002) and reduced relaxation responses of the vascular smooth muscles to endothelium-dependent vasodilators like acetylcholine (Sofola *et al.,* 2004; Zhu *et al.,* 2007). Acute salt-loading impairs microvascular arterial functions in rats while it causes fibrosis in the long term (Boegehold, 1993; Frisbee and Lombard, 1999).

There is increasing evidence to show that salt-loading raises blood pressure via a neurogenic mechanism involving an early interaction between vasopressinergic and adrenergic neurons in the central nervous system (CNS) leading to a persistent hyperadrenergic state (Gavras and Gavras, 1989). High salt diet also acts via a mechanism that affects cardiac contractility. High salt diet causes cardiac hypertrophy; for instance, rats fed a high salt diet were shown to develop

left ventricular hypertrophy independent of pressure overload (Yan and Leenan, 1991). An increased left ventricular mass index has also been reported in spontaneous hypertensive rats as an independent pathogenic factor (Frohlich *et al*., 1993). Attenuated relaxation response to acetylcholine was observed in spontaneously hypertensive rats (Kagota *et al.*, 2001) or Dahl salt sensitive rats (Nishida *et al*., 1998) but not in non-genetically modified Sprague Dawley rats (Obiefuna *et al.,* 1991b; Adegunloye and Sofola, 1997; Giardina *et al.*, 2001). Some other workers have however documented the fact that high salt diet resulted in reduction of acetylcholine-induced vascular relaxation (Lenda *et al.,* 2000; Oloyo *et al.,* 2011). Salt has also been shown to increase the inotropic response of the heart to positive inotropic substances such as noradrenaline (NA) (Sofola *et al.,* 1999).

In some humans with salt sensitive essential hypertension, studies have shown reduced relaxation responsiveness to acetylcholine following high salt consumption (Bragulat *et al.*, 2001). Bragulat *et al.,* (2001) have also reported significantly lower forearm vasodilatory response to acetylcholine (endothelium-dependent vasodilation) in 26 salt sensitive persons compared to 16 salt resistant hypertensive patients following oral salt loading. It has however not been very easy to demonstrate the relationship between salt loading and blood pressure in humans presumably due to the short duration of such studies as well as the fact that very few healthy subjects are willing to participate (Kaplan 2000; Sofola, 2004). The development of high blood pressure in humans involves abnormal and persistent changes in the mechanisms that control blood pressure. In the model developed by Guyton *et al.,* there were multiple areas in which short-term, long-term and infinite gain systems controlling blood pressure interact (Guyton *et al.,* 1972; Luft, 2001). The Guyton model helped to explain the fact that all organisms

that rely on salt and water metabolism to regulate their internal environment exhibit a relationship between salt and water intake and excretion as well as blood pressure. This relationship is very steep in normotensive individuals (Luft, 2001). That the kidneys play a very key role in handling a salt load has been proven over and again by renal cross-transplantation experiments between hereditary strains of hypertensive rats and normotensive strains. Cross-transplanting the kidneys of a normotensive rat into a hypertensive rat in which nephrectomy had been performed does not allow the blood pressure in the latter to rise. On the other hand when the kidneys of a young hypertensive strain is harvested before it has developed hypertension and is transplanted into a bilaterally nephrectomised normotensive rat, blood pressure increases (Meneton *et al.,* 2005). In the same vein, blood pressure in patients with essential hypertension and terminal nephrosclerosis who received healthy kidney from young normotensive donors became normal (Meneton *et al.,* 2005). These confirm that although there may be functional abnormalities at other sites in the body leading to the observed hypertension, the fact still remains that the primary disturbance initiating the increase in blood pressure in hereditary form of essential hypertension is domiciled in the kidneys.

Skrabal *et al.,* (1989) observed an upregulation of α_2-receptors and downregulation of β_2-receptors during high salt intake. This led them to hypothesize that an increased ratio between α_2- and β_2-receptors during salt-loading can promote vasoconstriction and decrease vasodilation in resistance vessels and increase sodium reabsorption in the proximal tubules. They further reported reduced number of β_2-receptors and a correlation between the change in blood pressure with the alteration in salt intake and the number of β_2–receptors in salt sensitive subjects after measuring blood pressure and adrenoceptor activity in cultured fibroblasts from 20 normotensive

subjects fed a high salt diet (Kotanko *et al.*, 1992). However, the report of Mills *et al.*, (1995) suggests that hypertensive blacks have the most sensitive β-receptors as well as the highest receptor density compared with white hypertensive and normotensive black and white subjects.

2.3 Sympathetic Regulation of the Cardiovascular System

It was previously believed that the sympathetic nervous system was only involved in the short-term regulation of blood pressure. However, it is now believed that in selected persons with borderline elevation in blood pressure associated with a hyperkinetic circulation, there is sympathetic overdrive (Grassi, 2009). It has been difficult to confirm though whether the adrenergic overdrive is the hallmark of hypertension or if it is a specific feature of selected hypertensive states. It is also not clear if adrenergic overdrive affects all circulatory districts or is confined to some organs (Grassi, 2009). Some studies have shown that this sympathetic overdrive is observable in all stages of hypertension and that the magnitude of the overdrive goes in parallel with the magnitude of the blood pressure increase (Grassi *et al.*, 1998). It has been suggested that a derangement in the baroreceptor reflex function may be responsible for the adrenergic abnormalities observed in hypertension. It has been further suggested that baroreflex control of vagal and sympathetic influences to the heart and the peripheral circulation undergoes impairment in hypertension thereby favouring the increase in resting heart rate as well as the potentiation of the adrenergic drive to peripheral vessels (Grassi, 2009). Whereas it seems to be settled that there is impairment of the vagal-heart rate control exerted by the baroreflex in hypertension (Ogoh, *et al.*, 2003), there is still some controversy on the fact that there may be a similar impairment on the sympathetic component of the reflex which seems to be reset at a new normal in hypertension (Grassi, 2009; Holwerda *et al.*, 2011).

2.3.1. Vascular Reactivity

Cardiovascular reactivity refers to the blood pressure and heart rate responses to an externally applied stressor. It has been hypothesised that exaggerated cardiovascular reactivity is a possible mechanism in the development of hypertension and cardiovascular disease (CVD); hypertension is the single most important factor responsible for 62 percent of strokes and 49 percent of coronary heart disease worldwide (World Health Organization, 2002). Vascular hyperreactivity is an inherited trait and reflects underlying sympathetic system activation (Everson *et al.*, 2001). Cardiovascular reactivity varies with individual characteristics such as genetic predisposition to the disease including a family history of hypertension or other cardiovascular disease; level of stress that the individual is exposed to at work or from finances; personality factors and emotions (Everson *et al*, 2001). Race is equally a factor as Blacks have been shown to have a higher vascular reactivity to stress (Kelsey *et al*., 2000a; Stein *et al.*, 2000). The racial difference in vasoconstrictor reactivity to cold stress may be explained by the fact that β-adrenergic vasodilation partially masked racial differences in α-adrenergic vasoconstriction (Kelsey *et al.*, 2000a). According to the Reactivity Hypothesis, exaggerated physical or psychological responses to stress identify subgroups with increased cardiovascular risk (Lovallo, and Gerin, 2003). Conflicting results have been obtained from studies on the role of hyperreactivity and the development of hypertension but most follow-up studies carried out over a period of 5 years or longer have tended to support this hypothesis (Flaa *et al.*, 2008). Heightened reactivity or

delayed recovery is thought to be a process through which stress and other psychosocial factors promote cardiovascular disease (CVD) risk (Stewart *et al.,* 2006). By cardiovascular recovery is meant the time required for cardiovascular parameters to return to baseline after termination of stress; it has also been defined as the extent of elevation in cardiovascular activity during the post-stress period (Steptoe *et al,* 2006) or sustained cardiovascular activation above baseline levels during the post-task recovery period (Chida and Steptoe, 2010). Poor stress recovery has been associated with impaired cardiovascular risk status (Chida and Steptoe, 2010). It has been suggested that the intermittent blood pressure elevations caused by such stress could lead to structural vascular changes however Julius *et al.,* (1989) were not able to produce such sustained elevated blood pressure in a study on dogs. If the causal chain is from heightened reactivity and/or impaired recovery to CVD risk, different pathways might be involved. There could be a direct relationship between BP or haemodynamic responsivity and later CVD risk. Hyperreactive persons may experience repeated episodes of elevated BP or disturbed haemodynamics as they go about their everyday lives, so that over the course of time, their tonic BP will rise which will eventually lead to incident hypertension (Steptoe, 2007). Research has also focussed on catecholamines as an explanation for the link between hyperreactivity and the development of hypertension. It is known that sympathetic stimulation causes vascular hypertrophy therefore frequent surges of sympathetic activity in hyperreactive individuals may cause sustained increased total peripheral resistance followed by hypertension (Flaa *et al.*, 2008). Indeed the follow-up study of Flaa *et al* (2008) carried out over an 18-year period showed that arterial adrenaline and noradrenaline stress reactions predicted systolic blood pressure. Transient endothelial dysfunction may also be elicited by acute stressors along with the cardiovascular responses (Ghiadoni *et al.*, 2000); this may also contribute to hypertension later in the individual.

2.3.2. Cold Pressor Test

The Cold Pressor Test (CPT) is a standardised test used to characterize sympathetic nervous activity (Chen *et al.*, 2008). The test is known to cause global sympathetic activation and result in significant arteriolar constriction followed by an increase in blood pressure (Chen *et al.*, 2008). The CPT is a provocative test based on the premise that an excessive blood pressure response to an external stressful stimulus is indicative of future hypertension. It is a powerful stimulus of sympathetic activity that has been used to examine both peripheral and coronary circulation. Hyper-reactivity to the CPT is considered to be a useful indicator of future hypertension in normotensive individuals (Mathews *et al*., 2004; Siegrist *et al.*, 2006; Moriyama and Ifuku, 2010). High pressor and heart rate reactivity have been shown to predispose an individual to the development of hypertension, carotid atherosclerosis and coronary artery disease (Everson *et al*, 1996).

Several modifications of the original test have evolved over the decades but with the same general principle. These generally involve the immersion of a limb in ice water or the placement of a bag of ice water on the forehead (Kelsey *et al.*, 2000b). The traditional laboratory cold pressor test typically involves limited regional body surface exposure to very cold and often very painful ice water slurries held at approximately 4^{o}C. Vascular reactivity has also been tested by

exposing the entire human body to a cold environment. Subjects are tested in a walk-in refrigerated chamber held at a temperature of 8^{o}C to 10^{o}C and humidity between 85 percent and 95 percent. It is a relatively naturalistic form of environmental cold stress that offers a viable alternative to the traditional CPT for the assessment of cardiovascular reactivity especially when trying to circumvent the attendant pain. This method can be related to the observed effect of environmental temperature on blood pressure. There have been some reports that blood pressure is higher during winter than any of the other seasons (Alperovitch *et al.,* 2009). However most studies have reported that this seasonal variation in blood pressure affects to a larger extent morning blood pressure causing a BP surge in the mornings (Murakami *et al.,* 2011). The morning BP surge is associated with target organ damage and increased risk for cardiovascular disease. Using the whole body cold exposure (CE) method, Kelsey *et al.,* (2000a) have demonstrated that whole body cold exposure leads to increases in blood pressure and total peripheral resistance similar to those produced by the traditional CPT although the BP increases observed were higher with the CPT in spite of the greater vascular resistance during whole body cold exposure (CE). This was probably because convergent increases in total peripheral resistance (TPR) and cardiac output contributed to the increase in BP during CPT unlike in CE where an increase in TPR contributed to the increase in BP despite a drop in cardiac output (Kelsey *et al.*, 2000b). Although the cardiac output responses to CPT were opposite to those elicited by CE, these changes were relatively small (Kelsey *et al*, 2000b). Similar reports have suggested that β-adrenergic vasodilation partially masked α-adrenergic vasoconstriction during CPT (Kelsey *et al.*, 2000a).

The differences in the outcome of cardiovascular responses to the different types of cold stress tests remain unestablished. However, it has been suggested that the differences may emanate from variations in the intensity and duration of exposure (Kelsey *et al.*, 2000a), the amount and location of the exposed skin surface, painfulness of the cold stimulus, body posture during stimulation and the proximity of the experimenter to the subject during the test (Kelsey *et al.*, 2000b). These are factors that may influence various mechanisms that mediate increases in BP including β-adrenergic and α-adrenergic vasoactive mechanisms as well as noradrenergic vasoactive mechanisms involving angiotensin II, endothelin-I and so on (Treiber *et al*, 2000). Indeed stepdown tests by Kelsey *et al.,* (2000b) suggested that factors other than the changes in cardiac output and TPR are contributive to the BP reactivity to the CPT and CE. Some studies have suggested that not all individuals who show heightened reactivity to the cold stress or poor recovery experience accelerated progression to CVD risk. How much an individual is exposed to recurrent stress comes into play as well as a positive family history. Behaviours such as cigarette smoking, use of medications and excess adiposity also critically influence the individual's response to acute stress (Kajantie and Phillips, 2006) as well as play a role in the exacerbation of CVD. The cold pressor stress activates the sympathetic nervous system causing an increase in vascular tone of resistance arteries (Murakami *et al.,* (2011). Evidence is also accumulating that the dorsomedial hypothalamic nucleus plays a major role in integrating the cardiovascular response to acute stress (Dampney *et al.,* (2002). Experiments in rats have shown that this area is crucial to integrating the cardiovascular and autonomic responses to emotional stress (Flaa *et al.,* 2008).

2.3.3. Vascular Resistance

The most prevalent cause of vascular events is hypertension (Kaplan, 2006) being responsible for about 50% of risk (Lawes *et al.,* 2008). Patients with only episodic hypertension have a high risk for vascular events (Rothwell *et al.,* (2010). Resistance arteries are the primary determinants of blood flow and blood pressure within the capillary beds. Resistance to blood flow within a network of vessels depends mainly on the size of the individual vessels; changes in diameter especially in precapillary arteries maintain adequate blood flow and blood pressure within the capillary beds thereby promoting maximal gas and nutrient exchange. Optimal pressure and flow relationship is maintained primarily by small resistance artery pressure-dependent myogenic reactivity (Coats, 2010). Myogenic component of vascular tone refers to the correlation between intraluminal pressure and pressure-dependent tone; it is commonly exhibited by small arteries with lumen diameter less than 150μm. Myogenic reactivity is a very important determinant of vascular resistance and a regulator of regional blood flow (Coats, 2010).

2.3.3.1 Venous Occlusion Plethysmography

Venous Occlusion Plethysmography has been in use for about a hundred years (Joyner *et al.,* 2001) being first used to record organ blood flow in 1905 (Brodie and Russel, 1905). Plethysmography has been used to investigate the role of the vascular endothelium in health and disease (Frewin *et al.,* 1968; Linder *et al.,* 1990; Panza *et al.,* 1993); studying the role of the autonomic nervous system in regulating limb blood flow in humans (Jie *et al.,* 1987) and the vasodilator responses to different events such as exercise, ischaemia and mental stress (Joyner and Dietz, 1997). The principle behind venous occlusion plethysmography is that of a "collecting" cuff inflated around the upper arm or thigh to a pressure below the individual's diastolic blood pressure such that the arterial inflow to the limb continues while the venous

drainage is obstructed. Thus the limb "swells" from the increase in volume. If a vein of the limb is placed above the heart level, the rate of increase in limb volume is proportional to the arterial inflow (Joyner *et al.*, 2001).

2.4. Salt Sensitive Hypertension and the Epithelial Sodium Channel

Salt sensitive hypertension especially in Africans has been related to low renin activity (Osotimehin *et al.,* 1984; Ambrosius *et al.,* 1999) in subjects who are amiloride sensitive (Baker *et al.,* 2002). Salt sensitivity has been linked to mutation of the epithelial sodium channel (ENaC) gene in a T594M transmutation that occurs in about 5% of hypertensive Blacks in the United States of America as compared to about 1% of Caucasians in the same location (Baker *et al.,* 2002). Blood pressure was controlled in African American subjects with Amiloride, 20mg per day for 2 months implying increased activity of the epithelial sodium channel (ENaC). In another study in which Blacks and Caucasians participated however, the use of Amiloride 5mg per day for one week resulted in an increase in blood pressure among the black subjects while the white subjects recorded a fall in blood pressure (Pratt *et al.,* 2002) demonstrating a significant racial difference in the activity of ENaC. However, polymorphisms of the ENaC were not measured in the study.

Hypertension can be classified as either Mendelian hypertension (monogenic form of hypertension) or essential hypertension (polygenic form of hypertension) based on the mode of inheritance (Su and Menon, 2001). The Mendelian forms of hypertension are due to a single defective gene which is transmitted in a dominant or recessive manner. For example, mutations

within the SCNN1B and SCNN1G genes of the epithelial sodium channel are responsible for the Mendelian diseases characterized by high blood pressure (Hiltunen *et al.*, 2002; Furudashi *et al.*, 2005) and low blood pressure (Bonny and Rossier, 2002; Thomas *et al.*, 2004). On the other hand, essential hypertension does not follow Mendelian inheritance pattern. It occurs 3.8 times more in persons with a positive family history of hypertension therefore suggesting a genetic component. A role for environmental factors like diet and stress has also been implicated in the development of essential hypertension. Some individuals respond to an increase in dietary sodium intake with an increase in blood pressure and manifest a decrease in blood pressure with salt restriction (Weinberger, 2004) while there is no such change in others. This led to the suggestion that the development of hypertension is a consequence of both environmental (e.g. salt) and genetic background of the individual (Bashyam, 2007).

Sodium reabsorption is carried out by transporters present along the nephron. The transporters are localized to specific segments of the nephron and mediate the entry of sodium across the apical membrane (Figure 1). They include the Na^+/H^+ exchanger of the proximal tubule, the $Na^+/K^+/2Cl^-$ co-transporter of the thick ascending limbs of Henle, the Na^+:Cl^- co-transporter (NCC) expressed exclusively at the distal convoluted tubule especially in the early DCT (DCT1) becoming less along the late distal tubule (DCT2) and the α -, β- and γ- subunits of the epithelial sodium channel (ENaC) whose expression increases gradually along DCT2 and is robust in the connecting tubule (CNT) and the collecting duct (CD) (Loffing and Kaissling, 2003). This arrangement of the NCC and ENaC protein expression in DCT2 generates three distinctive segments: DCT1 where only NCC is expressed, DCT2 where NCC and ENaC are co-expressed, and CNT/CD where only ENaC is expressed (Loffing *et al.*, 2001). Although the bulk of

reabsorption of sodium is carried out in the proximal tubule, the fine control of sodium reabsorption is carried out in the distal nephron and collecting duct (Figure 1). The location of the epithelial sodium channel makes it an important candidate gene for its involvement in blood pressure control; increases in the activity of ENaC results in increased extracellular fluid volume and blood pressure (Drummond, 2009). Apart from their role in salt and water homeostasis, ENaC proteins and other related acid-sensing ion channels (ASICs) may contribute to the control of blood pressure through reflex regulation of the autonomic nervous system and local control of vascular tone (Drummond *et al.*, 2008b).

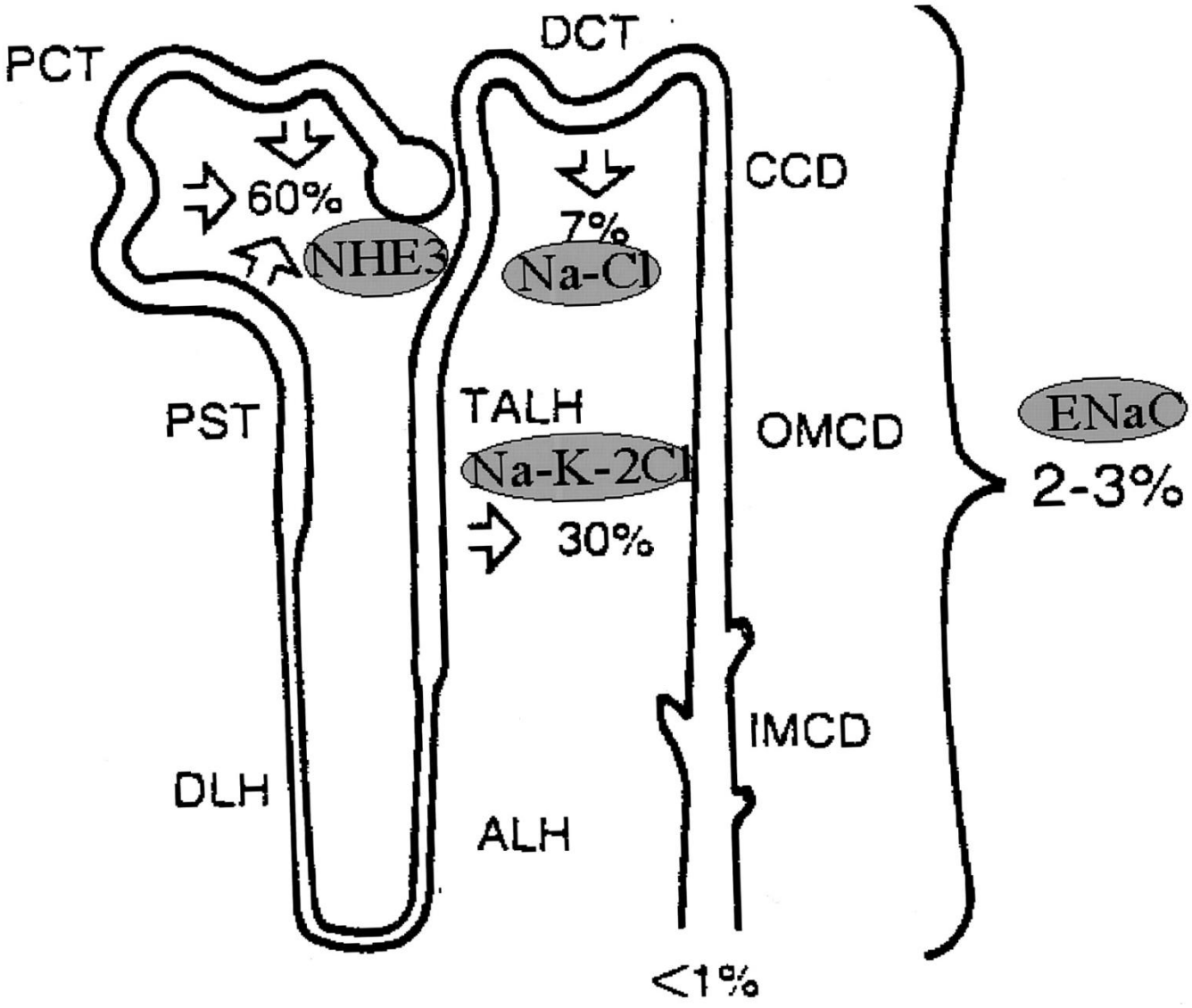

Figure 1: Localization of Na^+ transporters in the nephron

PCT = proximal convoluted tubule; PST, proximal straight tubule; DLH = descending limb of Henle's loop; DCT = distal convoluted tubule; TALH = thick ascending limb of Henle's loop; ALH = ascending limb of Henle's loop; CCD = cortical collecting ducts; OMCD = outer medullary collecting ducts; IMCD = inner medullary collecting ducts; NHE3 = epithelial sodium/proton exchanger (modified from Su and Menon, 2001)

2.4.1 Epithelial Sodium Channel in Salt Sensitive Hypertension

The epithelial sodium channel (ENaC) is responsible for the rate-limiting step of sodium reabsorption by the epithelial cells of the distal nephron. It is the final common pathway for the reabsorption of the final 2% of filtered sodium load in the distal nephron (Hollier *et al.,* 2006). ENaC is located in apical membrane of "tight" epithelia in the distal nephron, distal colon, and airways (Bize and Horisberger, 2007). It is also located in the salivary glands and sweat glands. ENaC therefore plays an important role in the maintenance of sodium balance, extracellular fluid volume and blood pressure by the kidney (Verrey *et al.,* 2008b) and the controlled fluid reabsorption in the airways (Chraibi and Horisberger, 1999). The activity of ENaC is regulated by several hormones such as aldosterone (Kellenberger and Schild, 2002) and vasopressin; intracellular and non-hormonal extracellular factors also modulate this activity; indeed the activity of ENaC is strongly regulated by sodium itself, via two distinct phenomena, feedback inhibition and sodium self-inhibition (Bize and Horisberger, 2007).

The epithelial sodium channel (ENaC) is composed of three structurally related subunits, α-, β-, and γ-ENaC (Figure 2). These subunits are each 85 to 95kD in size in the unmodified state (Bhalla and Hallows, 2008). The α-subunit was first identified by Canessa *et al.,* (1993) in expression cloning studies while the cloning of the β- and γ- subunits was accomplished by Canessa *et al.,* in 1994 (Benos and Stanton, 1999). Each subunit shares about 30% to 40% sequence identity with the others (Canessa *et al.,* 1994) and has two hydrophobic membrane-spanning domains – a large extracellular loop and intracellular N- and C-termini (Kellenberger and Schild, 2002). The channel has characteristic features that include high cation selectivity; the ratio of its permeability to sodium to its permeability to potassium is over 20 (Rossier, 2004).

Consequently, it allows Na^+ to enter the cell by moving down its concentration gradient but does not allow potassium to leak out of the cell (Snyder, 2002). This property is critical to the Na^+-absorptive activity of ENaC in epithelia. It has also been reported that the channel is more permeable to Lithium (Li^+) than to Na^+ (Snyder, 2002). The activity of a channel is determined by the amino acids that line the channel pore. In the case of ENaC, the pore is formed by residues from each of the three subunits (Snyder *et al.,* 1999). The selectivity filter that discriminates between different cations is formed by a sequence motif (G/S-X-S) at the extracellular end of the second membrane-spanning segment (Sheng *et al.,* 2000). Additional residues in this segment may also modulate conductance (Langloh *et al.,* 2000). ENaC is voltage-independent; changes in membrane potential have minimal effect on ENaC Na^+ currents (Snyder, 2002).

The channel also has a small single channel conductance of 4-6 picosiemens (pS) and is typically found in the apical membrane of sodium-transporting epithelial cells (Malik *et al.,* 2006). ENaC also exhibits gating kinetics characterised by long closing and opening times (Rossier, 2004) and the probability of the channel being open (P_o) varies widely between individuals (Palmer and Frindt, 1996). Although very little is known about this heterogeneity in gating or about the mechanisms that control channel gating, however, two segments of the channel appear to be important: mutation of specific residues within the cytoplasmic N-terminus results in channels that are nearly always closed (low P_o) (Grunder *et al.,* (1999). Interestingly, a mutation in this domain causes PHA (Chang *et al.,* 1996) consistent with an important function in vivo.

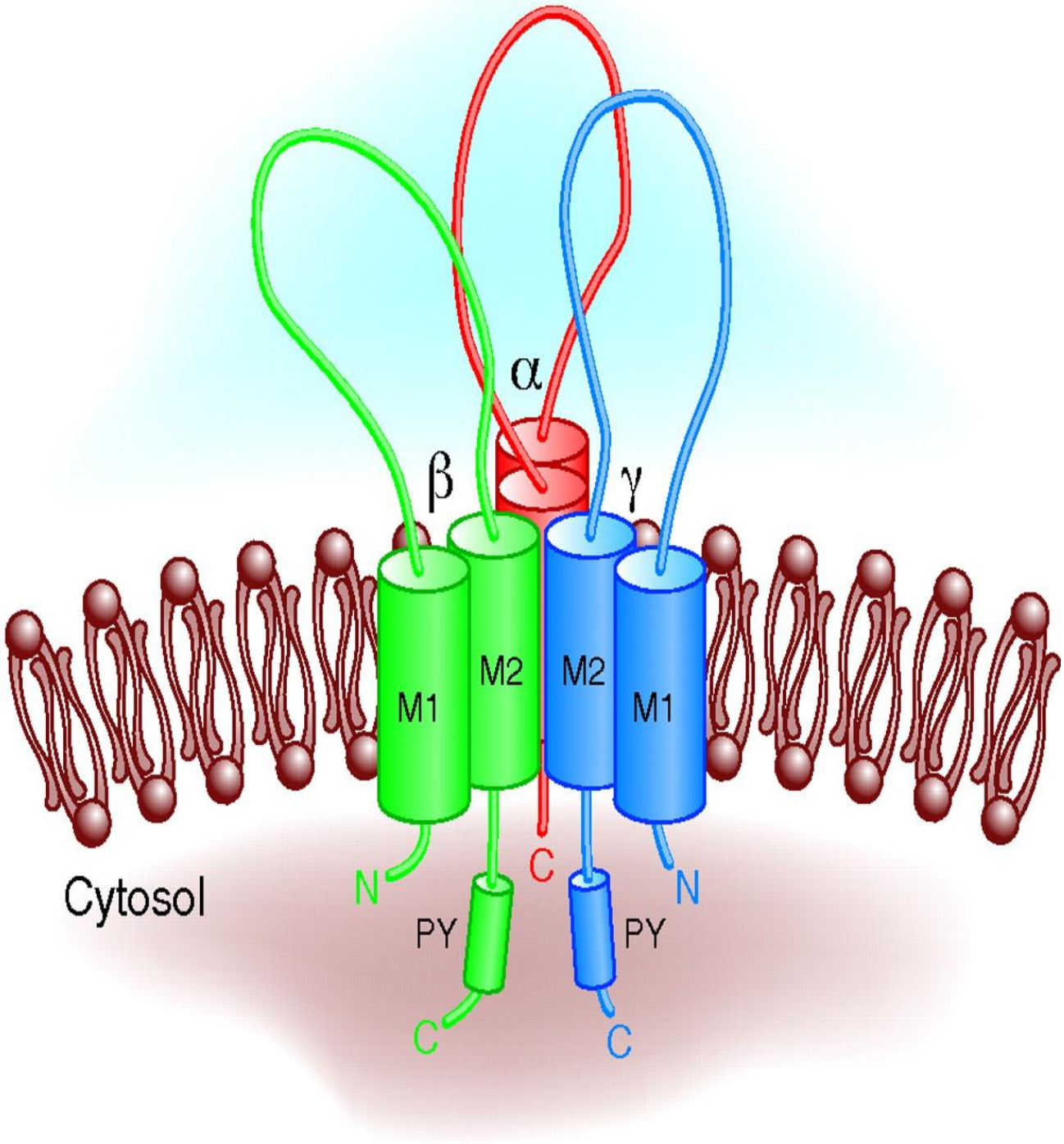

Figure 2: Structural features of the epithelial sodium channel (ENaC)

(Bhalla and Hallows, 2008)

Secondly, a domain just extracellular to the second membrane-spanning segment is also involved in channel gating. Mutation of residues in this 'DEG' domain increase Na^+ current by locking the channel in a high P_o state (Snyder *et al.,* 2000; Sheng *et al.,* 2001). These mutations introduce a bulky side chain suggesting that stearic hindrance might interfere with channel closing.

In the kidney, the three subunits combine in a 1-1-1 ratio to form the functional unit channel (Jasti *et al.,* 2007). Maximal ENaC activity observed by the amiloride-sensitive inward current is recorded only when all three subunits are present (Pochynyuk *et al.,* (2008): the α-subunit is absolutely necessary for channel activity while the β- and γ- subunits are required for maximal channel expression and activity at the cell surface (Schild, 2010). Neither the β- nor the γ-subunits expressed alone or together, produced any measurable Na^+ current (Schild *et al.,* 1995). When the α-subunit was injected into the Xenopus oocyte alone, (presumably α4 tetramers) only 1% or 2% of maximal activity was recorded; when αβ or αγ subunits were co-injected (α2β2 or α2γ2 respectively), 5% to 15% of maximal activity was recorded while injection of β- or γ-subunit alone did not lead to any significant channel activity and β2γ2 led to about 2% activity being recorded after as many as seven days of incubation (Bonny *et al.,* 1999). These results indicate that the α-subunit has a specific chaperone role to bring β- and γ- subunits to the cell surface but β- and γ- subunits are also required to bring the α-subunit to the plasma membrane.

Several gene inactivation experiments have been performed in vivo from which were observed that each subunit of the channel is required for survival as a lethal prototype is produced from these knockout experiments with death occurring soon after birth (McDonald *et al.,* 1999). When

the α-subunit was inactivated, the newborn mice died from failure of lung clearance and the inability to reabsorb fluid from the distal airways. Juxtaposing this with the data from in vitro studies in the Xenopus oocyte system, the α-knockout should leave the mice with only the possibility of making βγ channels (presumably β2γ2) with very low activity (Rossier, 2004). When the γ-subunit was inactivated the mice died from a severe salt-loosing syndrome and lethal hyperkalaemia, presumably the remaining αβ channels (α2β2) with 5% or 19% maximal activity were enough to prevent the severe failure in lung clearance observed in the α-knockout mice but not the lethal salt-loosing syndrome (Rossier, 2004). This was similar to the results obtained when the β-subunit was deleted (α2γ2 with 5% to 10% activity). This suggests that 5% or 10% ENaC activity is enough to establish a normal airway interphase but insufficient for normal kidney function. This is classically shown by one of the diseases resulting from a loss of function mutation of ENaC, Psuedohypoaldosteronism type I (PHA-I) in which there is early and severe salt wasting and dehydration, resulting in hypernatremia and hypotension; life threatening hyperkalaemia and metabolic acidosis as well as activation of the renin-angiotensin-aldosterone system in the presence of normal adrenal function (Kellenberger and Schild, 2002; Rossier, 2004). Both plasma aldosterone and renin are elevated indicating a peripheral resistance of target tissues to the hormones. Treatment with salt supplementation and the use of potassium chelators are sufficient to take care of the severe hypernatremia and hyperkalaemia respectively (Rossier, 2004). Whereas the renal symptoms of PHA-I mice models were similar to those of the PHA-I patients, (Kellenberger and Schild, 2002), the very severe lung phenotype is not observed in newborn PHA-I patients (Rossier, 2004). This led to further studies of the mucocillary clearance in the lungs of the patients consistent with an important inhibition of sodium and chloride reabsorption in the distal and proximal airways (Kerem *et al*, 1997). In a case of recessive

neonatal systemic PHA-I, the salt-loosing syndrome was accompanied by severe and recurrent episodes of respiratory problems at birth (Akcav *et al*., 2002) thereby suggesting that the difference between the human and mice lung phenotype appear to be quantitative and qualitative: the gene inactivation in the mouse demonstrates a lethal phenotype within four days of life, which is independent of genetic backgrounds while suggesting no redundancy between ENaC subunits mimicking human pathophysiology in lung and kidney (Rossier, 2004).

ENaC is a member of the gene superfamily DEG/ENaC/ASIC, a large family of proteins expressed in a diverse range of species including the nematode Caenorhabditis elegans, Drosophila and mammals and cell types including neurons, epithelia and muscle cells (Drummond *et al.*, 2008a). The genes were identified based on mutations that result in mechanosensation defects (mecs) or neurodegeneration (degs) (Corey and Garcia-Anoveros, 1996) acid sensing ion channels (ASIC) (Chen *et al.*, 1998) and mechanosensitive cation channels present in the skin and on cochlear hair cells (Benson *et al.*, 2002). Members of the ENaC/DEG gene family show a high degree of functional heterogeneity which is reflected by the wide variety of tissues within which they are located (Kellenberger and Schild, 2002).

The basic function of ENaC is to allow vectorial transcellular transport of Na^+ in a two-step process as shown in Figure 3:

There is a large electrochemical gradient for Na^+ across the apical membrane. This provides the driving force for the entry of Na^+ into the cell. Sodium ions are then transported actively across the basolateral membrane by the Na^+/K^+ ATPase in exchange for potassium (Bubien, 2010). Potassium then exits the apical membrane through potassium channels (Rossier and Stutts, 2009)

recycling across the basolateral through a different set of potassium channels (Bubien, 2010). This active transepithelial transport of Na^+ is important for maintaining the composition and volume of fluid on either side of the epithelium (Kellenberger and Schild, 2002) and is crucial for the maintenance of blood Na^+ and K^+ concentration and their homeostasis in the kidney and the colon (Kellenberger and Schild, 2002).

2.4.2 Regulation of ENaC

The epithelial sodium channel (ENaC) is regulated by a variety of intrinsic and extrinsic factors via several models of channel regulation. Changes in channel gating (P_o) or changes in the number of channels at the cell surface as well as changes in the unitary conductance of the channel (Rossier, 2004) are involved in the regulation of ENaC. Snyder (2002) reported that mechanisms that control the surface expression of ENaC are of critical importance in the regulation of epithelial Na^+ absorption based on two properties of ENaC. First is the fact that the channel does not require a stimulus to become active; it is constitutively active (Canessa *et al.*, 1994). This is contrary to what happens with voltage-gated and ligand-gated channels which require a stimulus for activity. The P_o of ENaC tends to increase at negative membrane potentials indicating that the gating properties are weakly voltage-dependent (Rossier, 2004).

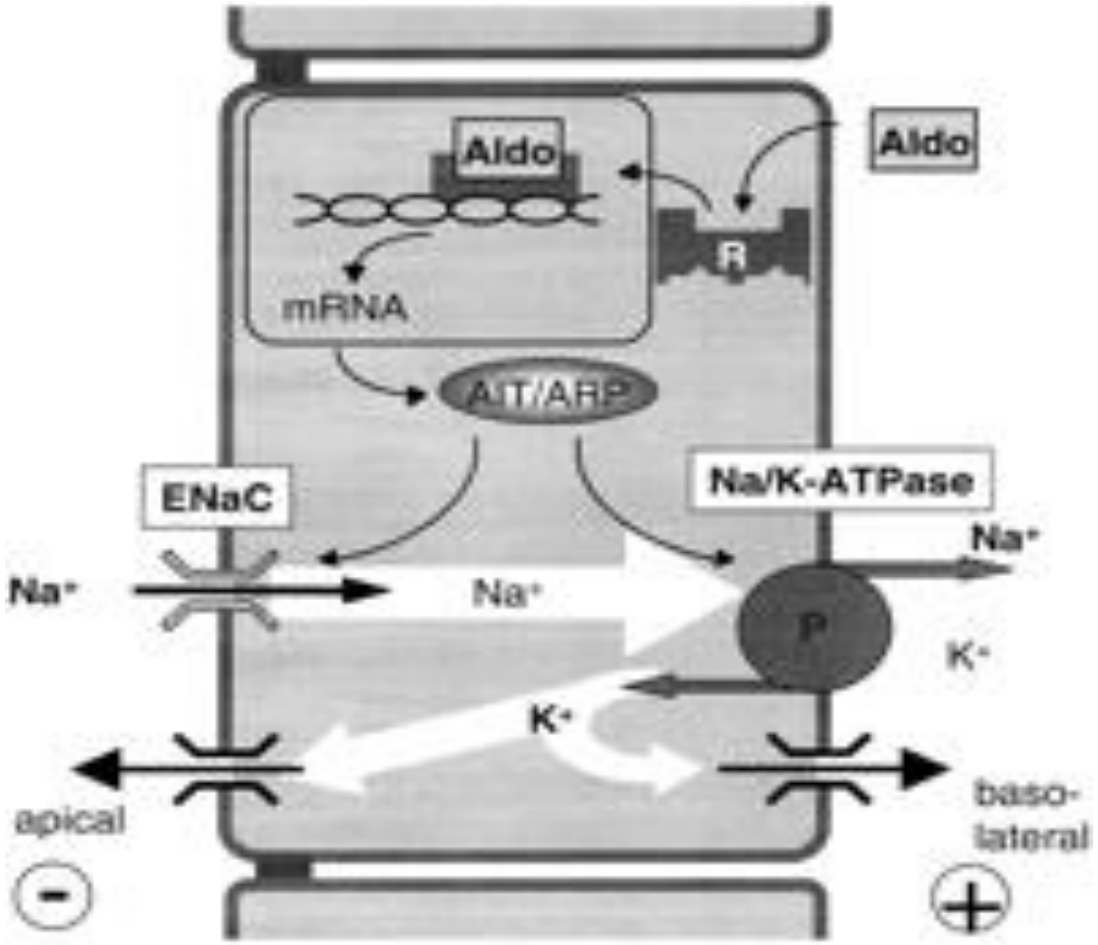

Figure 3: **Transepithelial ion transport in a principal cell of the cortical collecting duct (CCD)** ENaC mediates Na^+ entry from the tubule lumen at the apical membrane, and the Na^+-K^+-ATPase extrudes Na^+ at the basolateral side. K^+ channels are present on the basolateral and apical membranes. K^+ channels at the apical membrane mediate K^+ secretion into the tubular lumen. The diagram also illustrates the action of aldosterone (Aldo) which binds to intracellular receptors that are translocated to the nucleus and affect the expression and subcellular localization of ENaC and the Na^+-K^+-ATPase as well as other target proteins via aldosterone-induced transcripts (AITs) and aldosterone-repressed transcripts (ARTs). (Kellenberger and Schild, 2002).

The P_o varies greatly from channel to channel even within the same patch. Secondly, ENaC is regulated over a slow time frame, taking minutes to hours. This is compatible with mechanisms that alter the expression of a protein at the cell surface (Garty and Palmer, 1997) and differs significantly from those mechanisms that participate in neural transmission and contraction of myocytes that require milliseconds to take place (Snyder, 2000). The total number of ENaC subunits within the cell is relatively large compared with the number in the apical membrane (Malik *et al,* 2006). The exact point in the trafficking pathway at which ENaC subunits are assembled into a functional channel is unclear as is the unit stoichiometry of the assembled channel. Nonetheless, one of the mechanisms by which ENaC functional activity can be regulated is by altering the rate of delivery of assembled ENaC to the cell surface (synthesis, vesicle trafficking and exocytosis) and the removal of channels from the cell surface (endocytosis and degradation) (Snyder, 2002). It has been suggested that vasopressin and possibly aldosterone alter sodium transport via this mechanism (Malik *et al*, 2001). Malik *et al* (2006) suggest that these hormones not only affect the rate of insertion of ENaC but also appear to alter the number of functional channels at the apical membrane by reducing the rate of ENaC retrieval and degradation. The regulation of Na^+ absorption in the aldosterone-sensitive distal nephron (ASDN) thus relies more on the number of active ENaC at the apical membrane of principal cells than on the modulation of channel gating itself (Schild, 2010).

2.4.2.1 Ubiquitylation

This is the process upon which depends the stability of ENaC at the cell surface. Ubiquitylation is the process by which the stability of a variety of target proteins is regulated (Schild, 2010). Mono or multi-ubiquitylation can serve as signal for endocytosis of transmembrane proteins, a

process that usually results in their degradation by the lysosome and/or the proteasome (Rotin *et al.,* 2001). Nedd4 E3 protein-ubiquitin ligases that belong to the HECT family contain 3-4WW domains responsible for binding to canonical PY motifs of the target substrates; such PY motifs are found at the C-termini of the β- and γ- subunits of ENaC (Staub *et al.,* 1997). They are essential for efficient ubiquitylation of specific lysine residues in the N-termini of β- or γ- ENaC by the HECT domain of Nedd4-2. In *Xenopus* oocytes, Nedd4-2 was shown to efficiently suppress ENaC activity by ubiquitylation of the functional channel complex at the cell surface which led to clathrin-mediated endocytosis and degradation (Kamynina *et al.,* 2001). Enzymes such as UCH-L3 or Usp2-45 that are able to deubiquitylate ENaC, have been shown to increase ENaC-mediated Na^+ current *in vitro,* therefore supporting the role of ubiquitylation in regulating ENaC stability at the cell surface (Ruffieux-Daidie *et al.,* (2008).

2.4.2.2 Extrinsic Factors

Aldosterone (Shigaev *et al.,* 2000) and other volume-regulatory hormones like arginine vasopressin (AVP) (Ecelbarger *et al.,* 2001; Bens *et al.,* 2006) and atrial natriuretic peptide (ANP) (Zeidel *et al.,* 1988) as well as some other hormones like insulin and endothelin (Gilmore *et al.,* 2001) are involved in the regulation of ENaC (Bhalla and Hallows, 2008).

Hormonal regulation of ENaC is accomplished through receptor-mediated modulation of intracellular signalling pathways that involve several kinase cascades like stimulation of serum and glucocorticoid-regulated kinase 1 (SGK1) or inhibition of extracellular signal-regulated kinase (ERK) in the case of aldosterone (Shigaev *et al.,* 2000; Soundararajan *et al.,* 2005), protein kinase A (PKA) for AVP and ANP regulation (Snyder, 2000; Yamada *et al.,* 2007),

phosphatidyl inositol 3-kinase-dependent signalling for insulin (Blazer-Yost *et al.,* 2003) and SRC family kinases in the endothelin regulatory pathway (Gilmore *et al.,* 2001). Increased apical targeting of ENaC subunits is usually induced primarily by aldosterone-induced mineralocorticoid receptor (MR) action although it may also occur through MR-independent mechanisms (O'Neill *et al.,* 2008). Aldosterone binds intracellularly to the mineralocorticoid receptor (MR) causing genomic activation and increased expression of the Na^+/K^+ ATPase. This process causes ENaC-mediated sodium reabsorption in all species (Loffing and Korbmacher, 2009). The non-genomic effect of aldosterone on the channel may also be of great importance in the regulation of blood pressure (Bubien, 2010). It seems that the two mechanisms, genomic and non-genomic mechanisms, work together to increase sodium reabsorption and potassium excretion (Figure 4). Aldosterone regulation of ENaC at the cell surface is also partly mediated through inhibition of Nedd4-2-dependent ubiquitylation of ENaC that ultimately leads to an increase in the number of active channel at the cell surface (Fakitsas *et al.,* 2007; Verrey *et al.,* 2008a).

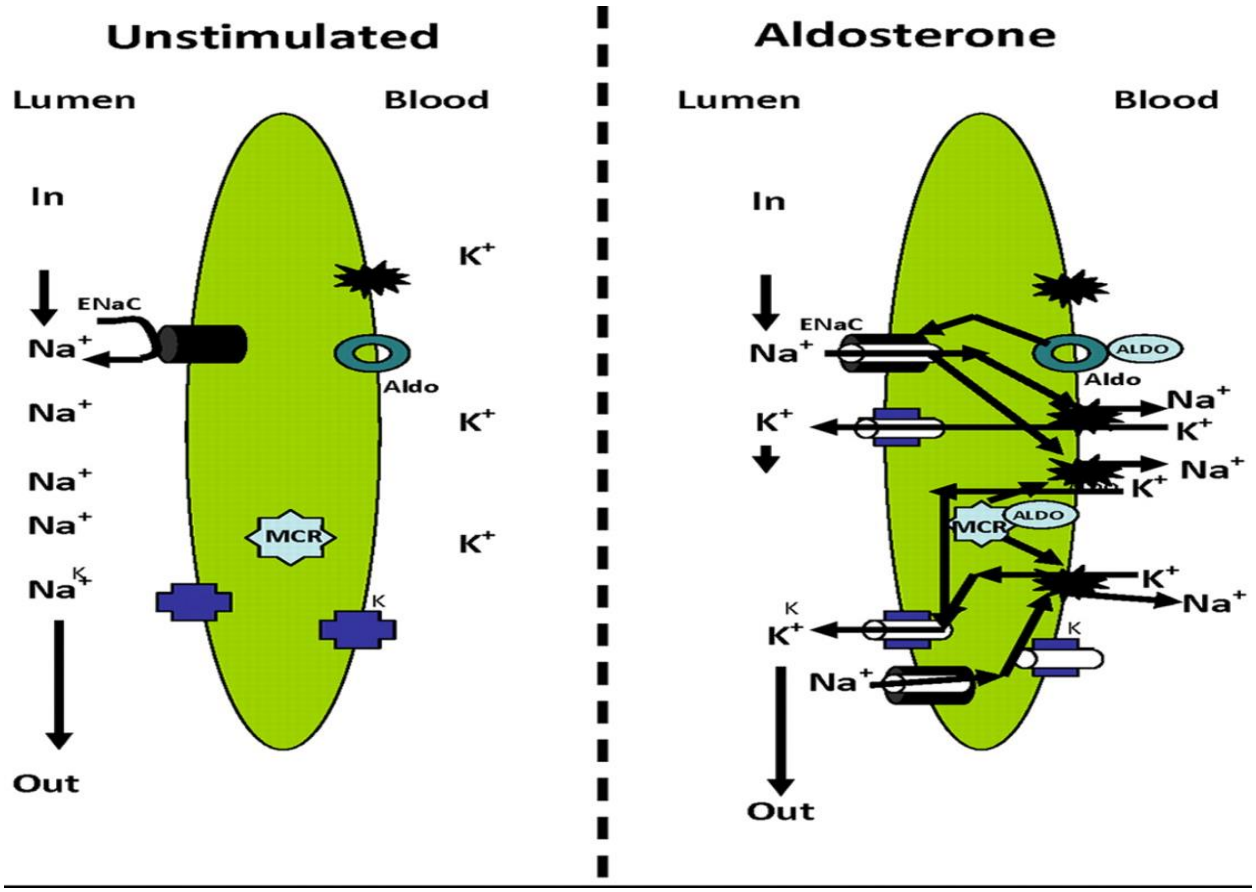

Figure 4: Model of the genomic and non-genomic effects of aldosterone in renal principal cells

KEY: Left panel: This illustration identifies the cellular components that mediate the mechanism of salt reabsorption by principal cells in the renal cortical collecting duct. Without stimulation, this mechanism is relatively inactive. Thus, there is little or no reabsorption of salt and water and very little loss of potassium.

Right panel: When aldosterone (Aldo) is secreted, there are two major effects on the principal cell reabsorptive mechanism. There is a non-genomic activation of ENaC, and after binding to the mineralocorticoid receptor (MCR), there is a genomic effect that results in the increased expression of the Na+/K+-ATPase in the basolateral membrane of the principal cells. Both of these mechanisms work concurrently to increase the reabsorption of sodium and the excretion of potassium. If this mechanism is overstimulated because of metabolic alterations, elevated blood pressure is the result. (Bubien, 2010).

Vasopressin also increases ENaC activity in the ASDN. The hormone binds the V2 receptors, stimulating the release of CAMP which in turn increases the density of ENaC at the cell surface (Auberson *et al.,* 2003). The cellular mechanism for ENaC regulation by vasopressin is provided by the action of Protein Kinase A (PKA). The enzyme is similar to SGK 1, and phosphorylates the same residues of Nedd4-2 as does SGK 1. However, additive effect of aldosterone and vasopressin on ENaC-mediated Na+ absorption in the ASDN cannot be wholly explained by the converging phosphorylation of Nedd4-2 by the two enzymes (Bugaj *et al.,* 2009) suggesting the participation of other mechanisms.

2.4.2.3 Proteolytic Cleavage

The extracellular domain is important in modulating ENaC activity. Proteolytic cleavage has been observed at two sites in the extracellular domains of α- and β- subunit of the channel. This converts an inactive ENaC to active ENaC (Hughey *et al.,* 2004a; Caldwell *et al.,* 2004). While one population of channels is cleaved by furin (Hughey *et al.,* 2004a), a second population reaches the cell surface uncleaved and is susceptible to cleavage and activation by proteases at the cell surface and in the extracellular fluid (Caldwell *et al.,* 2004; Hughey *et al.,* 2004b; Knight *et al.,* 2006).

2.4.2.4 Sodium Ion Self-Inhibition

Extracellular sodium ion (Na^+) inhibits ENaC activity by a process called Na^+ self-inhibition. This process serves as a negative feedback mechanism to control the Na^+ transport (Sheng *et al.,* 2006). The role of Na^+ in this regard has been predicated on the observation that mutation of conserved histidine residues in the extracellular domains of α- and γ- ENaC alter Na^+ self-

inhibition and also the observation that proteolytic cleavage of the extracellular domain prevents Na^+ self-inhibition (Sheng *et al.*, 2006).

2.4.3 Mutations of the Epithelial Sodium Channel

Improper functioning or regulation of the epithelial sodium channel is an independent cause of sustained severe hypertension. Mutations in the ENaC result in clearly defined syndromes involving dysregulation of ENaC activity with subsequent disorders of systemic blood pressure attributable to a primary renal mechanism (Warnock, 2001). Mutations of ENaC were originally described as truncations or frame shift in the β- or γ- subunit of ENaC (Meneton *et al.*, 2001). A missense mutation in the β-subunit of the human ENaC gene (T594M allele of SCNN1B or β-ENaC) has been implicated as a gain-of-function mutation as in Liddle syndrome causing impaired renal sodium excretion and salt sensitive hypertension (Swift and MacGregor, 2004).

The demonstration of genetic linkage between Liddle syndrome and ENaC suggests the physiological involvement of ENaC in blood pressure regulation. Liddle syndrome results from mutations in the cytoplasmic C terminus of either the β- or the γ-subunit of ENaC which leads to constitutively increased channel activity (Rossier and Schild, 2008). It represents a small minority of salt sensitive hypertension (Butterworth *et al.*, 2009). The "gain of function mutations" as in Liddle syndrome increase channel activity resulting in excess reabsorption of sodium leading to hypertension whereas the 'loss of function mutations' as in PHA-I decrease channel activity causing salt wasting, dehydration and hypotension (Su and Menon, 2001; Rossier and Schild, 2008). Liddle syndrome and PHA-I therefore serves as important 'proof of

principle' showing that altered function of the epithelial sodium channel can directly affect blood pressure. In addition to the Liddle and PHA-I mutations, several polymorphisms have been identified in the β- and γ- subunits of ENaC among which β-T594M (Swift and MacGregor, 2004) and β-G442V are only seen in individuals of African origin (Su *et al.*, 1996; Persu *et al.*, 1998). For example in the HYPERGENE data set in which sequence analysis of the β-ENaC subunit was carried out in 532 hypertensive probands including 101 probands with low renin hypertension, missense mutations were identified in seven unrelated individuals; three probands with low renin hypertension and three probands of African ancestry had mutations that changed threonine to methionine at position 594 (T594M) with an overall incidence of 6% of the hypertensive probands of African ancestry (Warnock, 2001). A glycine to valine (G442V) β-subunit variant was also identified in 19 probands all but one of whom were of African origin and eight were from the low renin hypertension subset. None of these missense mutations or polymorphisms has had any demonstrable effects on the in vitro expression systems used to examine ENaC activity (Warnock, 2001). The HYPERGENE reports go to confirm earlier reports by Su *et al* (1996) and Persu *et al* (1998) that β-T594M and β-G442V are seen in individuals of African origin.

Baker *et al.,* (1998) reported an association of β-T594M polymorphism with hypertension among Blacks in London; seventeen (8%) of the hypertensive Blacks and 2% of the normotensive Blacks studied had the T594M mutation. These individuals had a low plasma renin activity (PRA) suggesting an increased sodium reabsorption and therefore further suggesting that the T594M polymorphism may serve to explain, to some extent, the presence of salt-sensitivity in Blacks (Luft, 2001). It has been observed that about 9% of Finnish hypertensive individuals

carry genetic variants of β- and γ- subunits of ENaC; this is thrice the prevalence of these variants in non-hypertensive Finns as well as random controls from the same population (Hannila-Handelberg *et al.,* 2005). Although the functional importance of these variant alleles is still a subject of more research, however, patients with the variants showed an increase in urinary potassium excretion rate in relation to their renin levels (Hannila-Handelberg *et al.,* 2005). Different studies have provided evidence linking hypertension and chromosome 16p12, the genome that encodes β- and γ- subunits of ENaC (Atwood *et al,* 2001; de Lange *et al.,* 2004; Hamet *et al*, 2005). In the VHFS, more than 2900 persons were examined for genotypes and haplotypes related to 26 single nucleotide polymorphisms (SNPs) across SCNN1G and its promoter (Büsst *et al.,* 2007). The γ-subunit of ENaC has been implicated as a candidate gene in the determination of systolic blood pressure and it has been suggested that it may have specific role in ENaC activation by a novel mechanism, that is, proteolytic cleavage of its extracellular domain (Büsst *et al.,* 2007).

In spite of the fact that several studies have shown that the β- and γ- subunits of ENaC are linked to systolic blood pressure in Blacks, some researchers have not been able to record such relationship between the subunits of ENaC and blood pressure (Hollier *et al.,* 2006). As mentioned earlier, the β-T594M polymorphism has been associated with increased blood pressure in an English population of African ancestry (Baker *et al.,* 1998) whereas no similar association was found in a South African population (Nkeh *et al.,* 2003). Also a study of more than 300 affected sibling pairs collected in China failed to show linkage between the β- and γ-subunits of ENaC and hypertension indicating possible genetic heterogeneity in different ethnic groups for the involvement of ENaC in essential hypertension. Some workers have also not been

able to establish linkage between α-ENaC and hypertension (Su and Menon, 2001). This is however inconclusive as the power to detect linkage is affected by sample size and a large number of samples is required to either confirm or refute the linkage.

2.4.4 **AMILORIDE** (3,5- diamino-6- chloro-N-(diaminomethylene) pyrazine-2-carboxamide)

Amiloride is a guanidinium group-containing pyrazine derivative. It was first approved for use in 1967 as MK870. Its molecular formula is $C_6H_8ClN_7O$ (Figure 5) and has a molecular weight of 229.65.

Amiloride is a small molecule that inhibits channels formed by the epithelial sodium channel (ENaC)/degenerin (Deg) family of proteins (Qadri *et al.,* 2010). It blocks the epithelial sodium channel in the late distal convoluted tubules, connecting tubules and collecting ducts (Loffing and Kaissling, 2003) thereby causing a reduction in the reabsorption of sodium and water. This leads to loss of sodium and water sparing potassium. Epithelial sodium channel (ENaC) also exhibits a high sensitivity to amiloride–inhibition constant: 0.1µM (Rossier, 2004). At submicromolar and low micromolar doses, amiloride is a highly selective inhibitor of ENaC (Drummond *et al.,* 2008a); indeed the channel is blocked by amiloride and amiloride analogs as well as triamterene (Snyder, 2002). Although Amiloride is very selective for ENaC, it can inhibit sodium transport at sites other than ENaC. The Na^+/K^+ exchanger, the Na^+/Ca^{2+} exchanger as well as the Na^+/K^+ ATPase can all be inhibited by this molecule. The IC_{50} (µmol/L) of amiloride for ENaC has been reported to be 0.35 while those for the earlier mentioned channels are 84, 1100 and 1100 respectively (Pratt *et al.,* 2002). Amiloride is usually used in the treatment of hypertension in addition to thiazides or loop diuretics (Loffing and Kaissling, 2003).

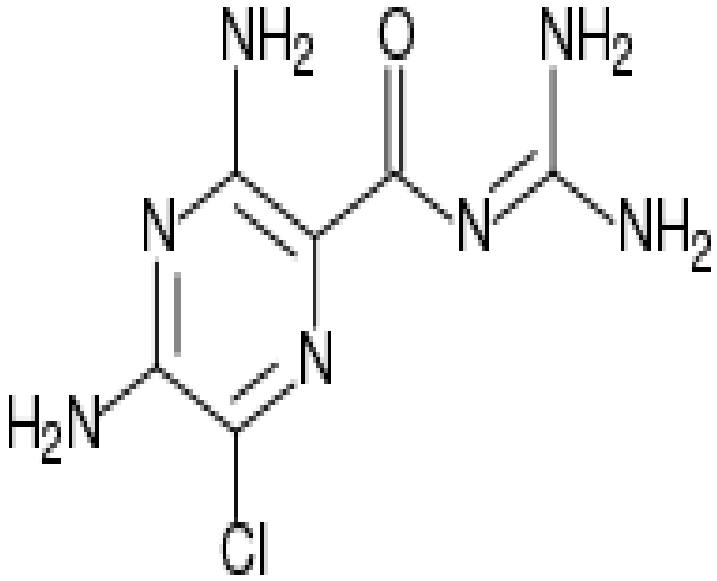

Figure 5: Structure of Amiloride
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2.4.5 Renin-Angiotensin-Aldosterone System

The renin-angiotensin-aldosterone-system (RAAS) is a major regulator of both the cardiovascular system and the renal system. It is important in the homeostasis of body fluids and electrolytes and therefore regulates blood pressure. The RAAS is an enzymatic cascade that begins with the cleavage of angiotensinogen to angiotensin I by renin, a hormone produced by the juxta-glomerular apparatus in response to an increased osmolarity of blood. Angiotensin I (Ang I) is then converted to angiotensin II (Ang II) by angiotensin converting enzyme (ACE) produced in the lungs. Angiotensin II exerts direct effects on the renal tubular sodium reabsorption. The hormone also acts via receptors in the adrenal glands to stimulate the secretion of aldosterone from the adrenal cortex.

2.4.5.1 Aldosterone

Aldosterone was characterised in 1953 but was initially termed electrocortin due to its mineralocorticoid action on transepithelial electrolyte transport. The name was later changed to aldosterone when it was observed to have a unique and highly reactive aldehyde (CHO) group instead of the expected methyl (CH_3) group at C_{18} of the steroid skeleton (Funder and Reincke, 2010). Aldosterone is a very important factor in the control of body fluids and blood pressure. The plasma concentration of aldosterone varies widely from about 0.05 nM to 0.5nM. It is secreted from the zona glomerulosa of the suprarenal glands within seconds in response to a decrease in extracellular fluid volume and/or a fall in blood pressure. The main targets for aldosterone action are the principal cells of the renal collecting duct in which the hormone binds to intracellular mineralocorticoid receptors which enhances NaCl reabsorption. Evidence is

beginning to accumulate that aldosterone acts on target cells of the cardiovascular system (Funder, 2006) especially the vascular endothelium (Schiffrin, 2006). Mineralocorticoid receptors are expressed by endothelial cells and they functionally respond to aldosterone (Oberleithner *et al.*, 2006: Rautureau *et al.,* 2011). Prolonged exposure to aldosterone *in vitro*, changes the morphology of endothelial cells considerably and the cells from different tissues grow in size and stiffen (Oberleithner *et al.*, 2006). The initial increase in cell size to aldosterone is probably due to an increased salt and water uptake mediated by a rise in apically expressed epithelial sodium channels (ENaC) (Golestaneh *et al.*,, 2001). This position is strongly supported by the inhibitory action of amiloride.

When aldosterone is applied to cells, it causes a fast non-genomic response that may comprise an influx of inorganic ions across the plasma membrane, a process that may involve classical intracellular receptors. There follows an endothelial cell swelling that is observed as early as 5 min after the application of the hormone which can be prevented by spironolactone, an aldosterone receptor blocker. However, this step can also be blocked by amiloride thereby suggesting the involvement of ENaC and the fact that sodium influx is closely related to the increase in cell volume (Fels *et al.*, 2010). The volume of the endothelial cells decrease to normal within 20 min. Reports from studies in oocytes suggest that within this first 20 min, the cell genome is already activated which leads to export extrusion of mRNA from the cell nucleus (Schäfer *et al.*, 2002) indicating that de novo protein synthesis has commenced and also emphasizing the proliferative nature of aldosterone.

Thus, aldosterone, on entering the cell, binds to cytosolic mineralocorticoid receptor (MR) and translocates into the nucleus. Here, it triggers a signal cascade and induces the transcription of a large repertoire of aldosterone-responsive genes and a *de novo* synthesis of proteins such as ROMK, Na^+/K^+ ATPase and ENaC (Kolla and Litwack, 2000). This leads to sodium retention and potassium excretion by the nephron. But aside from this slow (up to hours), genomic signalling pathway, there is also a fast non-genomic pathway which is characterised by an early onset (within seconds to minutes) and an insensitivity to transcription inhibitors (Fels *et al.,* 2010). The ENaC is also known to be expressed by vascular endothelial cells in addition to MR (Oberleithner *et al.,* 2006; Wang *et al.,* 2009) and its function here is also regulated by aldosterone.

Aldosterone causes some rapid effect in ENaC in the kidneys and the colon (Harvey *et al.,* 2008; Grossmann and Gekle, 2009) aside from the slow effects discussed above. In principal cells of the renal cortical collecting duct, aldosterone modifies the rapid surface expression and insertion of ENaC via a protein kinase D-dependent mechanism. These experiments detected a membrane receptor which could mediate the aldosterone effects and enable the rapid insertion of ENaC molecules into the plasma membrane (Wildling *et al.,* 2009). Therefore aldosterone inserts, as a non-genomic short-term response, preformed ENaC molecules into the plasma membrane of endothelial cells which activate sodium and water influx into the cell leading to a transient increase in the volume and surface of the cell.

On the other hand, it has been reported that long term aldosterone treatment of endothelial cells leads to an augmented synthesis and membrane abundance of ENaC molecules. The C-terminus of the α-ENaC subunit interacts with F-actin in the submembraneous cytoskeleton (Mazzochi *et al.,* 2006) which may be important for the proper function of endothelial cells (Golestaneh *et al.,* 2001). Once the number of ENaC molecules at the plasma membrane is so increased, the interaction with proteins of the cortical cytoskeleton could be strengthened leading to an increased mechanical stiffness of the cells. Mechanical stiffness of a cell is important for cell motility, division, tissue organisation and cellular responses to biochemical and biophysical signals. In terms of endothelial cell stiffness, aldosterone sensitizes the vascular endothelial cells to changes in plasma sodium and renders them rather insensitive to changes in plasma potassium (Oberleithner *et al.,* (2009).

Aldosterone also essentially increases the number of active ENaC at the cell surface. Aldosterone induces the phosphatidylinositide 3'-kinase (P13K)-dependent kinase SGK-1 (serum- and glucocorticoid-regulated kinase 1), a Ser/Thr kinase that elevates ENaC level and activity at the cell surface in heterologous expression systems. SGK-1 was found to phosphorylate Nedd4-2, leading to a decreased interaction and ubiquitylation of ENaC subunits, and finally resulting in an increased ENaC activity at the cell surface (Debonneville *et al.,* 2001).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1.0 Ethical Clearance

Ethical approval for this study was sought and obrained from the Medical Research Grants and Experimentation Ethics Committee, College of Medicine of the University of Lagos, Idiaraba (Appendix 1).

3.1.1 Consent for Experiments

The experimental procedure and requirements were explained to each subject before being screened for the study. Only subjects who gave verbal and written consent after the explanations were admitted into the study (Appendix II).

The study was thereafter carried out in the Department of Physiology, College of Medicine of The University of Lagos (CMUL), Idiaraba in accordance with the Helsinki Declaration (World Medical Association, 2008).

3.1.2 Ethical Consideration for Subjects

All subjects screened for this study received free counselling on prevention and management of salt sensitive hypertension. Subjects who were diagnosed as hypertensive during the screening were made aware of their diagnosis and referred to the Medical Out-patient Clinic of Lagos University Teaching Hospital (LUTH), Idiaraba or the General Hospital, Randle Road, Surulere. All the hypertensive subjects also had their prescriptions filled by the researcher (SOE) for one

month while the normotensive subjects received haematinics for the same period. This is an acceptable method of compensating the subjects for time spent participating in the study.

3.2. Subjects

The study was carried out in a multi-stage fashion:

Stage 1- Healthy human subjects numbering two hundred and twelve (212) were screened for the study. They included volunteers from among the staff and students of the College of Medicine of the University of Lagos, Idiaraba as well as from the communities surrounding the College

Stage 2- Out of the two hundred and twelve subjects, one hundred (100) subjects were selected based on the calculation below to participate in the second stage of the study:

a) Control Group – 47 Normotensive Nigerians

b) Test Group – 53 Hypertensive Nigerians

This number was calculated from the equation for determining sample size for a comparative research

$$N = \frac{4\sigma^2(Z_{crit} + Z_{pwr})^2}{D^2} \quad \text{..... Equation 1 (Eng } \textit{et al.}\text{, 2003)}$$

Where N = total sample size for the two groups, normotensive and hypertensive subjects

σ = the assumed standard deviation for each group (assumed to be equal for both groups)

Z_{crit} = standard normal deviate at the chosen level of significance (α) of 0.05

Z_{pwr} = desired statistical power for the study

D = minimum expected difference between the two means

Based on previous study (Pratt *et al.*, 2002), the assumed standard deviation for this study was 15.1.

The level of significance is denoted by α. In order to guard against Type 1 errors, α is usually set to small values; in this study, α = 0.05 at 95% confidence interval. The Z_{crit} is therefore 1.96. The desired statistical power for this study, (Type 2 Error), is 0.8. The Z_{pwr} is thus 0.842 (Eng *et al.*, 2003). The minimum acceptable difference between the two means, D, was taken as 10mmHg.

Thus,

$$N = \frac{4\sigma^2(Z_{crit} + Z_{pwr})^2}{D^2} \quad \text{..... Equation 1}$$

$$= \frac{4 \times (15.1)^2 (1.96 + 0.842)^2}{10^2}$$

$$= \frac{4 \times (228.01)(7.96)}{100}$$

$$= \frac{7259.84}{100}$$

= 72.6 or approximately 73 subjects required or 36.5 subjects per group

- This was made up to 37 subjects per group of subjects to be studied
- To take care of attrition including withdrawals, 20% extra was added to make a total of 90 subjects. Forty-five subjects were therefore initially recruited into each group However, based on the fear that subjects may have to be withdrawn from the study as it progressed, due to the Withdrawal Criteria (Section 3.2.4) set up to safeguard the health and well being of the subjects, an extra 10 subjects was added to each group. Therefore a total of fifty-five normotensive and fifty-five hypertensive subjects were selected to participate in the second stage of the study
- However, by the end of the study, following withdrawals and other reasons of attrition, 47 normotensive and 53 hypertensive subjects completed the study

3.2.1 Selection of subjects

Normotensive and hypertensive subjects were selected based on the definitions of the Joint National Committee Report given in Section 1.6 (JNC 7, 2003)

3.2.1.1 Normotensive Subjects

The normotensive subjects who participated in the study were selected by simple random sampling from among the pool of 124 healthy normotensive volunteers screened.

3.2.1.2 Hypertensive Subjects

Hypertensive subjects for the second stage of this study were selected from among the 88 otherwise healthy hypertensive subjects screened. Their selection was based on fulfilment of the Inclusion Criteria set up for this study. None of them ran foul of the Exclusion Criteria (Section 3.2.3).

3.2.2 Inclusion Criteria

The hypertensive subjects for this study were either

- Previously undiagnosed volunteers who were diagnosed during the screening
- Known hypertensive subjects who have never been on any orthodox treatment prior to being recruited for the study
- Known hypertensive subjects who had been on some form of antihypertensive medication
    - but who had not taken diuretics for a minimum of 4 weeks prior to the study (Baker *et al.*, 2001)

- and had not taken any other form of anti-hypertesive for a minimum of two weeks prior to the study (Baker *et al.,* 2001)

This was important in order to ensure that the results obtained were due to the intervention from this study and not from any of the previous medications.

In order to obey the terms of the Helsinski Declaration (World Medical Association, 2008), the subjects were monitored clinically and by means of laboratory investigations. Those who were at risk or whose condition may be worsening were withdrawn from the study as per the Withdrawal Criterion set up (Section 3.2.4).

3.2.3 Exclusion Criteria

Hypertensive patients with ischaemic heart disease, cerebrovascular disease, renal impairment, diabetes mellitus, secondary cause of hypertension or other concurrent illness were excluded from the study population (Baker *et al.,* 2001). Subjects were also excluded if plasma creatinine was greater than 150μmol/L or serum potassium concentration greater than 5mmol/L *ab initio.* Pregnant females were excluded for ethical reasons.

3.2.4 Withdrawal Criteria

Subjects were withdrawn from the study if they had

- Plasma creatinine that exceeded 200μmol/L
- Serum potassium greater than 5.5mmol/L

- Blood pressure consistently greater than 180mmHg systolic and 110mmHg diastolic at any time

All these factors indicated worsening of the individuals' condition.

- Systolic blood pressure less than 110mm Hg or diastolic blood pressure less than 65mmHg before the amiloride tests; this was to prevent the possibility of hypotension in these subjects (Pratt *et al.*, 2001)

3.3 Experimental Design

3.3.1 Study Protocol

The study was carried out in phases after the measurement of baseline clinical parameters of weight, height and body mass index (BMI):

Phase I – Identification of Salt Sensitivity in Normotensive and Hypertensive Nigerians

Phase II – Evaluation of Effect of Autonomic Nervous System on Cardiovascular System of Normotensive and Hypertensive Nigerians by the Determination of Vascular Reactivity and Effect of Sympathetic Stimulation in Normotensive and Hypertensive subjects

Phase III - Determination of Effect of Sympathetic Stimulation on Forearm Vascular Resistance among Normotensive and Hypertensive Subjects

Phase IV – Assessment of Relationship between ENaC Markers and Salt Sensitive Hypertension

Phase V – Identification of Genetic Variants of ENaC and their role in Salt Sensitive Hypertension among Nigerians

3.3.1.1 Clinical Measurements:

Clinical measurements were carried out before salt–loading, after salt-loading and after combined salt-loading plus amiloride; each subject acted as his/her own control. All clinical measurements were carried out by the same observer (SOE) to prevent inter-observer error. Randomly selected subjects were also evaluated, as a cross – check, by one of the supervisors (OAS). On the morning of the experiments, subjects were asked to avoid alcoholic drinks, cigarette smoking, coffee and teas as well as exercise (Chen *et al.*, 2008; He *et al.*, 2009) as these are all activities that can individually stimulate the sympathetic nervous system.

3.3.1.1.1 Measurement of Body Weight

Weight (kg) was measured to the nearest 0.01kg with the aid of a Mechanical Personal Scale Model BR9012. The scale was assessed for accuracy by the repeatability test. This involved the measurement of the weight of the same individual 10 times and the $x \pm$ S.E.M. calculated.

3.3.1.1.2 Measurement of Height

Height (m) was measured to the nearest 0.1cm using a stadiometer.

3.3.1.1.3 Determination of Body Mass Index

Body Mass Index (kg/m^2) was calculated from Equation 2

w/h^2 ..... Equation 2

where w = weight (kg)

h = height (m)

3.3.2 Phase I - Identification of Salt Sensitivity among Normotensive and Hypertensive Nigerians

Salt sensitivity was determined after the subjects had ingested a salt-load of 200 mmol Na^+ per day for 5 days. To start with, baseline clinical measurements were taken as described in the following sections.

3.3.2.1 Measurement of Arterial Blood Pressure

Arterial blood pressure measurements were carried out using a non-invasive indirect auscultatory method using the stethoscope and a mercury sphygmomanometer. The gold standard in the measurement of blood pressure is the use of the mercury sphygmomanometer and the Korotkoff sound technique using the auscultatory method (Pickering *et al.,* 2005). The auscultatory method is the mainstay of blood pressure methods but is gradually being supplanted by other techniques due to increasing use of automated blood pressure measuring devices (Pickering *et al.,* 2005). However, even where these newer non-mercury devices are used, the mercury sphygmomanometer is still used to validate the devices (Pickering *et al.,* 2005). The mercury sphygmomanometer consists of a mercury column inside a vertical column to which is attached the Riva-Rocci inflatable cuff and an inflator. This device has changed little since it was designed about six decades ago except for the fact that modern versions have very little risk of spilling mercury if dropped. The mercury sphygmomanometer is unique in its simplicity and there is negligible difference in the accuracy of different brands of the instrument (Pickering *et al.,* 2005).

Baseline blood pressure (mm Hg) was determined following the standardized protocol developed by the International Collaborative Study of Hypertension in Blacks (ICSHIB) (Ataman *et al.,*

1996) and the American Heart Association (AHA) Recommendations for Blood Pressure Measurement in Humans (Pickering *et al.,* 2005).

Procedure for Measuring Arterial Blood Pressure

All blood pressure measurements were carried out with subjects in the sitting position, their backs supported by the back of the chair and their feet flat on the floor. Subjects were allowed a 5-minute period of rest in this position before the commencement of blood pressure measurements. Subjects were not allowed to wear constrictive clothing on the upper arm in order to avoid tourniquet effect above the Riva-Rocci cuff. Subjects' arms were supported at heart level and the Riva-Rocci cuff of the sphygmomanometer placed in a way to encircle at least 80% of the arm circumference.

Arterial blood pressure measurements were taken from the brachial artery pulsation of the right arm with the arm in the supine position; systolic blood pressure (SBP) and diastolic blood pressure (DBP) were recorded as the first and fifth Korotkoff phases respectively (Ataman *et al.,* 1996; Pickering *et al.,* 2005). The cuff was deflated at the rate of 2mmHg/s (Ataman *et al.,* 1996). Measurements were taken to the nearest 2 mmHg to avoid digit preference. Digit bias or digit prejudice is a situation in which the observer records an inappropriate excess of "zero" as the last digit in blood pressure recording or depending on the subject's circumstance, the observer records the blood pressure just below or just above a cut-off point (Wingfield *et al.,* 2002). After initial measurement to avoid "White Coat" effect, three readings were taken with one minute interval in between for the subjects to relax; the average of the three measurements was recorded. The measurements were carried out by the same observer using the same

Accoson® sphygmomanometer to avoid inter-observation variations. Random cross-checks were carried out by OAS. Neither the subjects nor the observer talked during the procedure to ensure accuracy of the blood pressure measurement.

Pulse pressure (mmHg) was calculated as the difference between SBP and DBP. Mean Arterial Blood Pressure (MABP) (mm Hg) was calculated from Equation 3.

$$DBP + 1/3\ PP \quad \text{..... Equation 3 (Guyton and Hall, 1996)}$$

where DBP = Diastolic blood pressure (mm Hg)

PP = Pulse Pressure (mmHg)

3.3.2.2 Salt-Loading

After control parameters had been determined, subjects were given a salt-load at a dose of 200mmol/day Na^+ as NaCl Analar Grade (BDH, United Kingdom) for 5 days (Corruzi *et al.*, 2005; Tzemoz *et al.*, 2008). The NaCl had a 99.9% purity (BDH, United Kingdom). The salt was administered in two divided doses and given with 500ml of orange squash after subjects had eaten in the morning and in the evening. The treatment was well tolerated by all subjects.

3.3.2.2.1 Determination of Salt Sensitivity

Mean arterial blood pressure (MABP) of subjects was calculated before and after salt-loading. Subjects who showed a difference in MABP that was 5mmHg or more after salt-loading were considered salt sensitive (SS) while those who showed a difference in MABP that was less than 5mmHg after salt-loading were considered salt resistant (SR) (Cooper and Hainsworth, 2002;

Schmidlin *et al.*, 2007; McNeely *et al.*, 2008). Subjects were also considered salt resistant if they showed a fall in MABP following salt-loading (Weinberger, 1996).

3.3.2.2.2 Collection of 24-Hour Urine Sample

i. Before Salt-Loading

Subjects were given verbal and written instructions on how to collect a 24-hour urine sample:

At 7.00am on the first day of collection, subjects were asked to empty the bladder completely into a waste. Thereafter, 24-hour urine collection was commenced with the urine collected into a 5-Litre plastic container with toluene as preservative. The last collection was timed for 7.00am the next day (Day 2). The volume of the 24-hour urine sample was measured with the use of a measuring cylinder.

ii. After Salt-Loading

Salt-Loading was carried out over a period of 5 days. At 7.00am on Day 5, subjects were asked to empty the bladder completely into a waste. Thereafter, 24-hour urine collection was commenced with the urine collected into a 5-Litre plastic container with Toluene as preservative. The last collection was timed for 7.00am the next day (Day 6).

On day 6, subjects reported to the laboratory at about 9.00am. They were allowed a 10- minute period of rest following which their blood pressure was determined as earlier described (Section 3.3.2.1.).

The volume of the 24-hour urine sample was measured with the use of a measuring cylinder.

3.3.2.2.3 Determination of Urine Sodium Concentration

Stored urine samples were removed from the freezer and allowed to thaw at room temperature. The concentration of sodium [Na^+] in the 24-hour urine samples was analysed using a Coring Flame Photometer (Model 410C). The flame photometer was standardised before each measurement by setting the readout to zero using distilled water as blank and subsequently using appropriate Na^+ standard. The urine samples were diluted as appropriate and read against a "16.0" readout of 160 mmol/L Na^+ standard. The observed values for the samples were then multiplied by 10 and expressed as mmol/L Na^+.

3.3.2.2.4 Determination of Urinary Sodium Excretion

Urinary sodium excretion was also calculated before and after salt-loading (He *et al*, 2009; Castiglioni *et al.,* 2011). Urinary sodium excretion ($U_{Na}.V$) has been shown to provide an accurate index of total sodium intake as it represents approximately 93% of intake at steady-state condition (He and MacGregor, 2003).

$U_{Na}.V$(mmol/min) ..... Equation 4

where U_{Na} = Urine Na concentration (mmol/L)

V = Urine flow rate (ml/min)

3.3.2.2.5 Determination of Salt Sensitivity Index

The degree of salt sensitivity was determined among the normotensive and hypertensive subjects by means of the Salt Sensitivity Index (Coruzzi *et al.,* 2005; Castiglioni *et al.,* 2011). This index relates the changes in blood pressure induced by sodium loading with the concomitant changes in urinary sodium output without any arbitrary definition of thresholds (Coruzzi *et al.,* 2005).

Salt Sensitivity Index (SSI) was calculated as the ratio between change in mean arterial blood pressure (ΔMABP) (mmHg) and the difference between urinary sodium excretion rates (ΔUNaV) (mmol/day) before and after salt loading (Coruzzi *et al.,* 2005). The ratio was multiplied by a factor of 1000 to facilitate easy readability of the results.

$$SSI\ [mmHg/(mmol/d)] = \frac{\Delta MABP\ (mm\ Hg)}{\Delta U_{Na}V\ (mmol/d)} \times 1000 \quad \text{..... Equation 5}$$

(Coruzzi *et al.,* 2005; Castiglioni *et al.,* 2011)

where ΔMABP = difference in Mean Arterial Blood Pressure

$\Delta U_{Na}V$ = difference in Urinary Sodium Excretion

3.3.2.2.6 Determination of Sodium Clearance

Sodium clearance (ml/min) was calculated from Equation 6

$$C_{Na} = U_{Na}.Q/P_{Na} \quad \text{..... Equation 6}$$

where C_{Na} = Sodium clearance (ml/min)

U_{Na} = Urine sodium concentration (mmol/L)

Q = Urine Flow (ml/min)

P_{Na} = Plasma sodium concentration (mmol/L)

3.3.3 Phase II – Evaluation of Effect of Sympathetic Nervous System on Cardiovascular System of Normotensive and Hypertensive Nigerians

3.3.3.1 Determination of Effect of Sympathetic Activation on Blood Pressure in Normotensive and Hypertensive Subjects

The effect of sympathetic stimulation on blood pressure was examined by means of the cold pressor test (CPT).

Subjects were allowed 30 minutes rest in the laboratory in the sitting position before commencement of tests. Basal blood pressure (mmHg) (defined in Section 1.6) was determined before exposure to the CPT. With the sphygmomanometer left in place in readiness for determining blood pressure, the subject's foot was immersed for 1 minute up to the ankle, in ice slurry composed of equal parts water and crushed ice and maintained at 4^{o}C, (Rubenfire *et al.,* 2000; Stein *et al.,* 2000; Siegrist *et al.,* 2006; Kawano *et al.,* 2007; Flaa *et al.,* 2008). Subjects were asked to be still, breathe normally and to avoid muscle contractions as well all forms of Vasalva manoeuvre (Kawano *et al.,* 2007) as all these will modify their response to the cold pressor test. At the end of 1 minute and with the foot still immersed up to the ankle, peak blood pressure (mm Hg) was determined as the highest of three 15 sec serial blood pressure readings (Wood *et al.,* 1984; Stein *et al.,* 2000).

The foot was used in this study in order to obtain maximal haemodynamic and sympathetic responses to the CPT (Kawano *et al.,* 2007).

3.3.3.2 Determination of Effects of Sympathetic Activation on Blood Pressure in Normotensive and Hypertensive Subjects after Salt-Loading and after Co-Administration of Salt and Amiloride

After a 5-day period of salt-loading as described earlier (Section 3.3.2.2), the cold pressor test (CPT) was carried out to determine the effect of salt-loading on the subjects' blood pressure response to CPT.

3.3.3.3 Co-Administration of Salt and Amiloride

A wash-out period of one week was allowed during which no test was performed. This was to ensure that subjects returned to their baseline levels before further intervention was carried out. Blood pressure was measured to ascertain the subjects have returned to the baseline levels.

Subjects with systolic blood pressure less than 110mmHg or diastolic blood pressure less than 65mmHg were withdrawn from the study at this point to avoid potential for hypotension when amiloride is ingested (Pratt *et al.,* 2001).

Thereafter, subjects were again given a salt-load of 200 mmol/day of Na^+ for 5 days. In addition to this, 5mg of Amiloride was administered daily for 5 days (Pratt *et al.,* 2002).

Cold pressor test (CPT) was then carried out to determine the effect of combined salt-loading plus amiloride on the subjects' blood pressure response to CPT.

3.3.3.4` Determination of Vascular Reactivity and Effect of Salt-Loading in Normotensive and Hypertensive Subjects

To determine vascular reactivity, basal blood pressure was determined as defined in Section 1.6. This was followed by exposure of the subjects to the cold pressor test (Section 3.3.3.1.) At the end of the 1 minute of the cold pressor test but with the foot still immersed in the ice slurry, peak blood pressure (mm Hg) was determined as the highest of three 15 sec serial blood pressure readings taken after 1 minute with the foot still immersed (Wood *et al.*,1984; Stein *et al.,* 2000).

Subjects were considered hyperreactive if peak SBP or DBP minus basal SBP or DBP respectively was 15mmHg or higher, while they were considered normoreactive if the difference between peak SBP or DBP and basal SBP or DBP was less than 15mmHg SBP or DBP respectively following the CPT (Moriyama and Ifuku, 2010).

3.3.3.4.1 Effect of Salt-Loading on Vascular Reactivity

Following salt-loading with 200 mmol/day of Na^+ for 5 days (described in Section 3.3.2.2.), the effect of salt-loading on vascular reactivity was determined by re-exposing the subjects to the CPT as described above (Section 3.3.3.1). Basal and Peak Blood Pressures were determined as earlier described.

3.3.3.5 Determination of Heart Rate and Effect of Sympathetic Stimulation

Heart rate (beats/min) was determined from an electrocardiograph record which was obtained by using a portable electrocardiogram (Cardiosunny Electrocardiogram Model 5010, Fukuda Medical Electronics, Tokyo, Japan) at a paper speed of 25mm/sec. The machine was

standardized to show a deflection of 10mm/mV. Only the bipolar limb leads were recorded for 10 seconds (Jaja and Etemire, 2006).

Heart rate (beats/min) was calculated from R-R interval of Lead I or Lead III of the ECG using the formula 25/R-R x 60 (beats/min).

3.3.3.5.1 Determination of Effect of Sympathetic Stimulation on Heart Rate

This was carried out by means of the cold pressor test (CPT). The effect of the CPT on heart rate of subjects was determined by leaving the ECG Leads in place during the test. Heart rate was determined from the R-R interval of either Lead I or Lead III of the ECG as earlier described.

3.3.3.5.2 Determination of Effect of Salt-Loading on Heart Rate

Heart rate of the subjects was also determined after salt-loading as earlier described (Section 3.3.2.1).

3.3.3.5.3 Effect of Cold Pressor Test on Heart Rate after Salt-Loading and After Co- administration of Salt Plus Amiloride

The cold pressor test as described in Section 3.3.3.1 was repeated after a 5-day period of salt-loading described in Section 3.3.2.2. Thereafter, a washout period of one week was allowed followed by 5 days of salt-loading plus amiloride as described in Section 3.3.3.3.

3.3.4 Phase III - Determination of Vascular Resistance and Effect of Sympathetic Stimulation in Normotensive and Hypertensive Subjects

3.3.4.1 Test of Forearm Vascular Resistance

To determine vascular resistance, blood pressure of the subjects as well as rate of blood flow to the forearm were measured.

Blood pressure was measured using the auscultative method described above (Section 3.3.2.1.).

3.3.4.1.2 Measurement of Blood Flow

Measurement of blood flow was carried out by means of Venous Occlusion Plethysmography. The plethysmograph comprises a Perspex chamber sealed with a rubber cuff but with space through which subjects' forearm could be inserted. The plethysmograph was coupled to a Grass polygraph (Model 7D, Grass instruments, Mass, USA) via a volume transducer (Model PT5A) inserted in a side vent. The transducer was connected to the polygraph through a pre-amplifier and driver amplifier to an ink stylus. The transducer was calibrated every time before the plethysmograph was put to use. The polygraph and its writer amplifier were calibrated as well.

Calibration of Grass Polygraph

A polarity switch position for recording flow rate was selected. A convenient baseline and driver sensitivity were then selected. The driver amplifier was then switched to "use". The preamplifier was then calibrated by selecting an appropriate balance voltage. A sensitivity level of 1 mV/cm was selected; this was changed to 0.5mV/cm or 0.02mV/cm where necessary. Thereafter, the volume transducer was connected to the polygraph.

Calibration of Volume Transducer

This was carried out by taking consistent recordings of air displaced from the plethysmograph while infusing water in steps of 1ml from a burette connected to a Marriott bottle and connected to the transducer; water was then allowed to drop into the Marriott bottle in steps of 1 ml and the volume of air displaced from the plethysmograph was recorded on a tracing paper and measured.

Venous Occlussion Plethysmography

The subject's forearm was inserted into the plethysmograph and an airtight environment maintained (Figure 6). The cuff of the sphygmomanometer was then wrapped around the upper arm. With the polygraph set at a speed of 10mm/s and patient at rest, the sphygmomanometer was inflated to 60mmHg (venous occlusion) and kept there until the rate of rise of the stylus got to a plateau. Then the polygraph was stopped and the sphygmomanometer deflated rapidly.

Figure 6: Set up for Venous Occlusion Plethysmography

Rate of forearm blood flow (ml/s) was calculated from the initial rate of rise of the slope of the tracing obtained. A typical trace is shown in Figure 7.

3.3.4.2 Calculation of Forearm Vascular Resistance

Forearm Vascular Resistance (FVR) (mmHg/ml/s) was calculated as the ratio between mean arterial blood pressure (MABP) (mmHg) and the rate of forearm blood flow (ml/s) as shown in Equation 7.

$$\text{Forearm Vascular Resistance (FVR) (mmHg/ml/s)} = \frac{\text{MABP (mmHg)}}{\text{Rate of Blood Flow (ml/s)}} \quad \text{..... Equation 7}$$

(Arosio *et al.*, 2006)

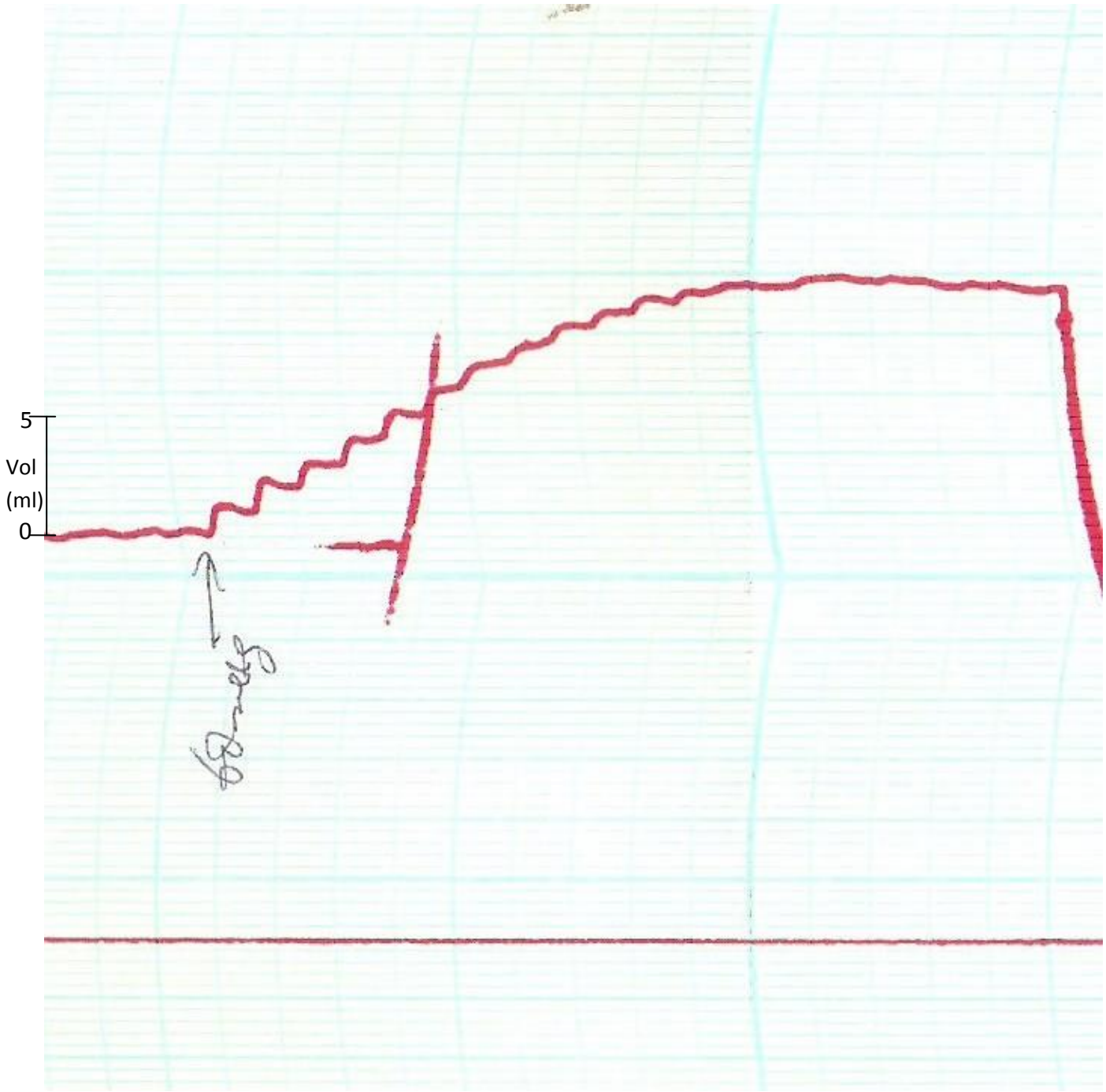


Figure 7: Typical blood flow tracing obtained from venous occlusion plethysmography (Waveform = Respiratory Excursions)

3.3.4.3 Determination of Effect of Salt-Loading on Forearm Vascular Resistance

The tests for vascular resistance were repeated after the subjects had been salt-loaded with 200 mmol/day Na^+ daily for 5 days as described above (Section 3.3.2.2.).

Briefly, blood pressure (mmHg) was measured with the aid of an Accosson (TM) mercury sphygmomanometer using the auscultation method earlier described (Section 3.3.2.1.). Thereafter, with the subject sitting in a comfortable position, the rate of blood flow (ml/s) was determined using Venous Occlusion Plethysmography described above (Section 3.3.5.1.) after salt-loading. Forearm vascular resistance was then calculated from Equation 7 as before.

3.3.4.3.1 Determination of Effect of Sympathetic Stimulation on Forearm Vascular Resistance

The effect of sympathetic stimulation on forearm vascular resistance (FVR) was carried out by means of the cold pressor test (Section 3.3.3.1). This was determined before salt-loading, after salt-loading and after salt-loading plus amiloride.

Briefly, subjects reported to the laboratory on the morning of experiments. They were allowed a 30-minute period of rest following which their blood pressure was measured as described earlier **(**Section 3.3.21.**)** using the Accosson sphygmomanometer and the auscultatory method of blood pressure determination. Thereafter, the subject's rate of blood flow to the fore-arm was determined using the Venous Occlusion Plethysmography as described earlier (Section 3.3.4.1.2). Forearm vascular resistance was calculated as above (Equation 7).

Subjects were then exposed to the cold pressor test by immersing their right foot for 1 minute in ice slurry composed of equal parts of water and crushed ice maintained at $4^{o}C$ (Section 3.3.3.1). Thereafter, blood pressure was measured as described earlier (Section 3.3.2.1) and the rate of blood flow to the fore-arm was also determined as earlier described (Section 3.3.4.1). Forearm vascular resistance was again calculated (Equation 7).

Following this baseline measurement, the subjects were then salt-loaded with 200 mmol/day Na^{+} dissolved in orange squash and taken in two divided doses for 5 days (Section 3.3.2.2). On the morning of day 6, subjects reported to the laboratory and the tests for forearm vascular resistance and effect of cold pressor test on forearm vascular resistance were repeated as described above.

Subjects were then allowed a wash-out period of one week following which salt-loading was repeated. In addition, 5mg of amiloride was taken per oral daily for 5 days (Section 3.3.3.2). The tests for forearm vascular resistance were then repeated as outlined above.

3.3.5 Phase IV - Assessment of Relationship Between ENaC Markers and Salt Sensitive Hypertension

3.3.5.1 Studies on the Epithelial Sodium Channel

Amiloride 5mg was used as blocker of the epithelial sodium channel (ENaC) for this phase of the study.

3.3.5.1.1 Co-administration of Salt plus Amiloride

After the initial salt-loading (Section 3.3.2.2), a wash-out period of one week was allowed during which no test was performed. As explained earlier (Section 3.3.3.3), subjects with systolic blood pressure less than 110mmHg or diastolic blood pressure less than 65mmHg were withdrawn from the study at this point. Thereafter, subjects were again given a salt-load of 200 mmol/day of Na^+ in addition to which 5mg of Amiloride was administered daily for 5 days (Pratt *et al.,* 2002).

The series of tests were repeated to determine the effect of blockade of the ENaC on the parameters.

3.3.5.1.2 Determination of Effect of Amiloride on Blood Pressure

Blood pressure response to Amiloride was used as an indirect assessment of ENaC activity. Blood pressure was determined as described in Section 3.3.2.1. This was carried out before salt-loading, after salt-loading and after salt-loading plus amiloride. A reduction in the blood pressure measured after amiloride ingestion gave an indication of the activity of the ENaC (Baker *et al.,* 2002).

3.3.5.1.3 Potassium Handling

Both plasma potassium and 24-hour urinary potassium excretion may be indicative of genetic variation in ENaC (Gaukrodger *et al.,* 2008).

i. Determination of Plasma Potassium

Plasma potassium was measured from stored plasma before salt-loading, after salt-loading and after salt plus amiloride-loading.

Protocol for Collection of Venous Blood

Subjects were counselled for venesection. With the subjects comfortably seated in the laboratory, blood was collected from a peripheral vein after cleaning the site with methylated spirit. Venous blood was collected into lithium heparinised bottle. The blood was centrifuged at 3000 revolutions per minute for 12 minutes. Thereafter plasma was pipetted into safe-lock Eppendorf bottles. These were stored at -20^{o}C until analyses.

Determination of Plasma Potassium Concentration

Stored plasma was removed from the freezer and allowed to thaw at room temperature. The concentration of potassium [K^+] in plasma samples was analysed using a Coring Flame Photometer (Model 410C). The flame photometer was standardised before each measurement by setting the readout to zero using distilled water as blank and subsequently using appropriate K^+ standards.

ii. Determination of 24-Hour Urine Potassium Excretion

This was estimated from stored aliquots of the 24-hour urine sample collected as described earlier (Section 3.3.2.2.3). 24-hour urine samples were collected at the end of every experiment before salt-loading, after salt-loading and after combined salt plus amiloride ingestion.

Determination of Urine Potassium Concentration

Stored urine samples were removed from the freezer and allowed to thaw at room temperature. The concentration of potassium [K^+] in the 24-hour urine samples were analysed using a Coring Flame Photometer (Model 410C). The flame photometer was standardised before each measurement by setting the readout to zero using distilled water as blank and subsequently using appropriate K^+ standards.

3.3.5.4 Hormonal Assays

Plasma Renin Activity (PRA) and Serum Aldosterone were analysed with the use of enzyme-linked immunosorbent assay (ELISA) kits after each experiment.

3.3.5.4.1 Determination of Plasma Renin Activity

Plasma renin activity (PRA) was analysed with the use of Human Renin ELISA kit (Biotech Co. Ltd., China).

Plasma samples were collected in cryo-vials and stored at -20^{o}C until ready for assay. These frozen samples were removed from the freezer and allowed to thaw at room temperature. The

thawed samples were then centrifuged at 1000 revolutions per minute (r.p.m.) for 15 minutes before the assay.

100µL each of standard (supplied in kit), blank (deionized water) and samples were pipetted into the labelled wells of the microplate reader taking care to ensure there was no contact between the inner wall of the wells and the pipette. The wells were then covered with adhesive strips and incubated for two hours at 37^{o}C. Following this, liquid of each well was removed without washing. Thereafter, 100uL of Biotin-antibody working solution (supplied in Kit) was added to each well and incubated for one hour at 37^{o}C. Each well was then aspirated and washed as follows:

Each well was filled with 200µL Wash Buffer (supplied with Kit) and allowed to stand for 2 minutes; then the liquid was removed by flicking the plate over a sink. In order to ensure good results, the remaining drops of were removed by patting the plate on a paper towel. This washing was repeated twice to make a total of three washes.

Following the third wash, 100µL of HRP-avidin working solution (supplied with Kit) was added to each well. The wells were then covered with a new adhesive strip and incubated for 1 hour at a temperature of 37^{o}C. Each well was then aspirated as before and washed 5 times with the Wash Buffer. Thereafter, 90µL of TMB Substrate (supplied with Kit) was added to each well and incubated for 30 minutes at 37^{o}C keeping the plates away from droughts and other temperature fluctuations. Then 50µL of Stop Solution (supplied with Kit) was added to each well after the first four wells containing the highest concentration of standards had developed obvious blue

colour. Finally, the optical density of each well was determined within 30 minutes using the microplate reader set to wavelength of 450nm.

The assays were carried out in duplicate for each standard, sample and blank. The average of the two results was calculated and the average zero standard optical density was subtracted from this. A standard curve was then drawn by reducing the data using computer software "Curve Exert 1.3" to generate a four parameter logistic (4-PL) curve-fit (Human Renin ELISA Kit User Manual, Cusabio Biotech Co., Ltd).

3.3.5.4.2 Determination of Serum Aldosterone

Aldosterone was assayed with the use of Aldosterone ELISA Kit (ALPCO Diagnostics, USA). Serum samples were collected in cryo-vials and stored at -20^{o}C until ready for assay. These frozen samples were removed from the freezer and allowed to thaw at room temperature. The thawed samples were then centrifuged at 1000 r.p.m. for 15 minutes before the assay.

50µL each of standard (supplied in kit), blank (deionized water) and serum samples was pipetted into the correspondingly labelled Rabbit Anti-Aldosterone Antibody-coated microwells taking care to ensure there was no contact between the inner wall of the wells and the pipette. The wells were then covered with adhesive strips and incubated for two hours at 37^{o}C. Following this, liquid of each well was removed without washing. Thereafter, 100uL of the Aldosterone Biotin-Avidin Horseradish-Peroxidase (HRP) conjugate working solution (supplied in Kit) was added to each well and incubated on a plate shaker working at approximately 200 r.p.m. for one hour at room temperature. Each well was then aspirated and washed three times as follows:

Each well was filled with 300µL of diluted Wash Buffer (supplied with Kit) and allowed to stand for 2 minutes; then the liquid was removed by flicking the plate over a sink. In order to ensure good results, the remaining drops were removed by patting the plate on a paper towel.

Thereafter, 150µL of TMB Substrate (supplied with Kit) was added to each well and incubated at room temperature for 10-15 minutes or until a dark blue colouration was observed. Then 50µL of Stop Solution (supplied with Kit) was added to each well. Then the optical density of each well was determined within 20 minutes using the microplate reader set to a wavelength of 450nm (Aldosterone ELISA Kit User Manual, ALPCO Immunoassays).

The assays were carried out in duplicate for each standard, sample and blank. The mean of the two results was calculated. A standard curve was then drawn plotting the mean optical densities of the standards on the y-axis and the concentrations of the standards on the x-axis. The optical density of each sample was then determined. The value of aldosterone (pg/ml) was then read off the standard curve.

3.3.6 Phase V - Genetic Studies

This phase of the study was carried out at the Department of Laboratory Medicine, College of Medicine, University of Capetown (UCT), South Africa and the Division of Women's Health, School of Medicine, King's College, London.

For this phase, whole blood samples were obtained from the cubital vein in potassium ethylenediaminetetraacetic acid (K-EDTA) anticoagulated sample bottles and stored on a filter paper which was then stored in the refrigerator until analysis.

3.3.6.1 Isolation of DNA

Isolation of DNA from the dried blood spots was carried out using MegaZorb$^{(R)}$ DNA Mini-Prep Kit (Promega, USA). The process was carried out in stages as indicated in the kit manual

i. Lysis

All reagents were brought to room temperature prior to commencing the reactions. The Proteinase K (PK) solution and the Lysis Buffer supplied in the kit were gently mixed by swirling by hand taking care not to produce bubbles. The blood spots were cut into smaller pieces and added to a clean microcentrifuge. Thereafter, 300µL of TE Buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0) was added to the tube. The mixture was incubated for 30 minutes at room temperature, mixing once every 5-10 minutes by manual swirling. Then 20µL of the well-mixed PK solution was added to the bottom centre of the tube while avoiding touching the side of the tube with the pipette to avoid contamination. The mixture was then mixed by gentle vortexing for about 15 seconds. Thereafter, 20µL of 20mg/ml RNAse A stock solution was added to the microcentrifuge tube. The cap of the tube was closed and the content mixed

gently by vortexing for 15 seconds followed by incubation at room temperature for 5 minutes. Care was taken to prevent the mixture from getting caught up in the cap of the microcentrifuge tube. Then 200µL of well-mixed, clear Lysis Buffer was added to the mixture and the tube placed on the vortex for 15 seconds. It was then incubated in a 56^{o}C water bath for 10 minutes using a foam float. After removing from the water bath, the liquid was pipetted from the tube into a clean 2ml microcentrifuge tube and the original tube was discarded.

ii. Binding

Before commencing the binding process, the buffer was mixed by pulse vortexing. The MegaZorb$^{(R)}$ Reagent was mixed by vortexing until all the particles were uniformly suspended. Then 500µL of the well-mixed Binding Buffer was added to the sample in the 2ml microcentrifuge tube. This was mixed thoroughly by pulse-vortexing until a homogenous mixture was obtained. The tube was then pressed against a magnetic rack for about 60-90 seconds in order to sediment the particles.

iii. Wash

The Wash Buffer was mixed by manual swirling before beginning this process. Then 1ml of the well-mixed Wash Buffer was added to the tube. The tube was removed from the magnet and mixed well by inverting several times to ensure the particles were completely dispersed. The tube was again returned to the magnetic rack to sediment the particles. The tube was inverted a few times while still being held against the magnet in order to "rinse" the tube cap with the supernatant. The supernatant was then removed by aspiration while holding the tube firmly against the magnet to ensure the magnetic particles in the tube are tightly attached to

the magnet. Thereafter, the Wash process was repeated to perform a second wash from which a clearer supernatant was obtained and discarded.

iv. Elution

The Elution Buffer was mixed by pulse-vortexing. Then 200µL of the well-mixed Elution Buffer was added to the tube containing the MegaZorb$^{(R)}$ particles. The magnet was removed and the mixture swirled gently to mix. The tube was then incubated for 10 minutes at room temperature by occasional manual mixing. The particles were again sedimented by placing the tube in the magnetic rack as above. The supernatant therefrom was then carefully transferred into a clean tube. This supernatant contained the purified DNA. This was stored at -20^{o}C until analysis.

3.3.6.2 Polymerase Chain Reacton Studies

All frozen reagents were thawed before use. All reagents were thoroughly mixed and centrifuged briefly before starting the polymerase chain reaction (PCR) tests.

The forward and reverse primers were designed from the SCNN 1B gene sequence on Exon 13 (Table 1 and Figure 8) following the method of Rayner *et al.*, (2003). The designed primers were tested in the Primer Test Suite (PCR Primer Stats; http://www.bioinformatics.org/sms2/pcr_primer_stats.html) to ensure they are of good quality with regard to the parameters examined.

Thereafter, the primers were checked in the Primer Basic Local Alignment Search Tool (BLAST) Suite http://www.ncbi.nlm.nih.gov/tools/primer-blast/).

Table 1: Primer Pairs for β-ENaC Polymerase Chain Reaction

TYPE OF PRIMER	SEQUENCE
FORWARD PRIMER*	5'-ATC GAG TTT GGG GAG ATC ATC–3'
REVERSE PRIMER#	5'-CAA CAG TCT TGG CTG CTC AGT-3' (Reverse Complement of actual sequence highlighted in green in Figure 8)

*= yellow highlight in Figure 8

#= green highlight in Figure 8

(Rayner *et al.*, 2003)

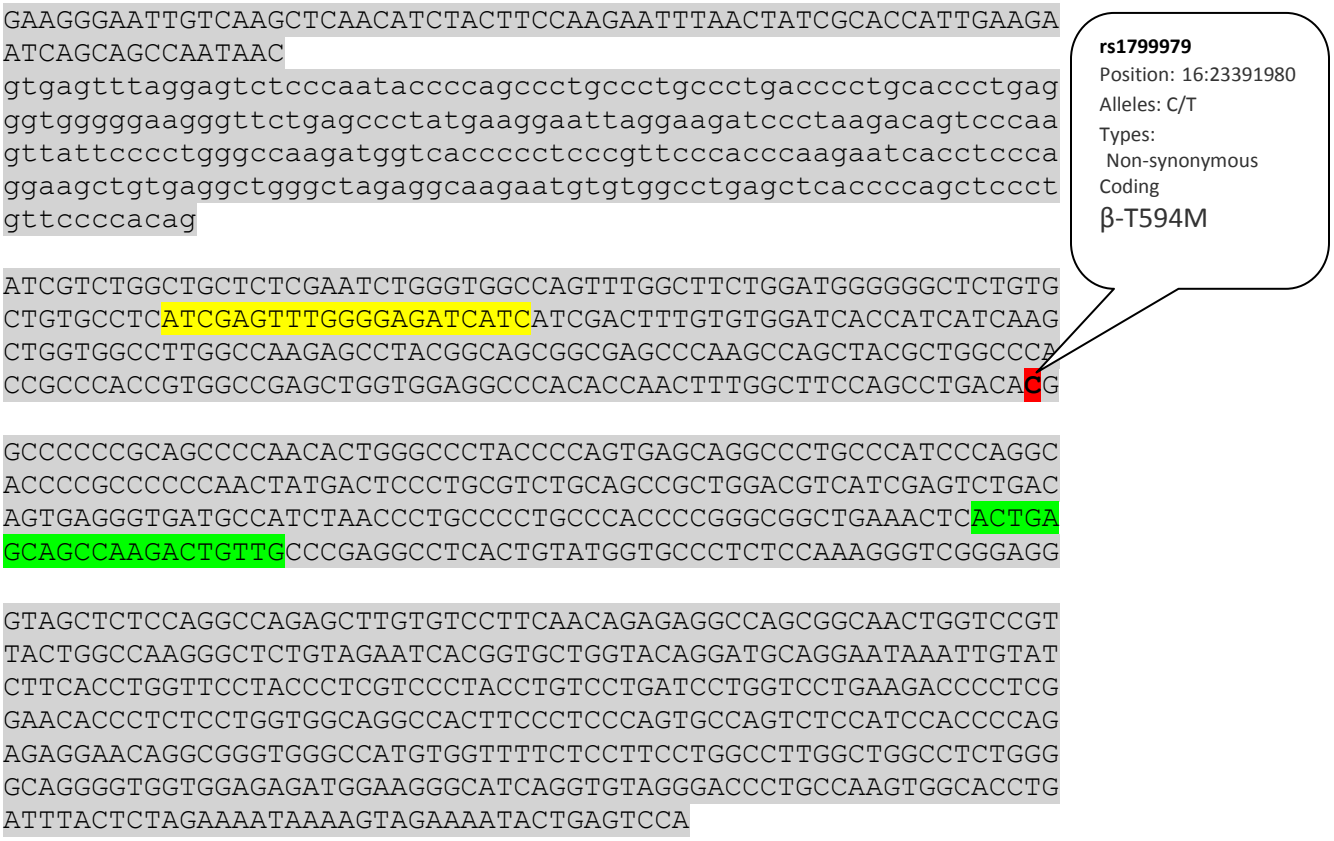


Figure 8: SCNN1 B EXON 13

(Ensembl www.ensenmbl.org/Homo_sapiens/Transcriot/Sequence)

KEY: Red = position of the β-T594M mutation

rs = Reference Sequence or Ascension number of the mutation in the Ensembl project

Yellow = Sequence of Forward Primer

Green = Sequence of Reverse Primer

PCR Primer Stats results

```
General properties:
-------------------
                    Primer name: Fwd
                Primer sequence: ATCGAGTTTGGGGAGATCATC
                Sequence length: 21
                    Base counts: G=7; A=5; T=6; C=3; Other=0;
                 GC content (%): 47.62
     Molecular weight (Daltons): 6501.30
                      nmol/A260: 4.76
                micrograms/A260: 30.94
           Basic Tm (degrees C): 52
   Salt adjusted Tm (degrees C): 47
Nearest neighbor Tm (degrees C): 62.46

PCR suitability tests (Pass / Warning):
-----------------------------------
               Single base runs: Pass
         Dinucleotide base runs: Pass
                        Length: Pass
                    Percent GC: Pass
          Tm (Nearest neighbor): Warning: Tm is greater than 58;
                     GC clamp: Pass
                Self-annealing: Pass
              Hairpin formation: Pass
General properties:
-------------------
                    Primer name: Rev
                Primer sequence: CAACAGTCTTGGCTGCTCAGT
                Sequence length: 21
                    Base counts: G=5; A=4; T=6; C=6; Other=0;
                 GC content (%): 52.38
     Molecular weight (Daltons): 6397.21
                      nmol/A260: 5.20
                micrograms/A260: 33.28
           Basic Tm (degrees C): 54
   Salt adjusted Tm (degrees C): 49
Nearest neighbor Tm (degrees C): 65.40

PCR suitability tests (Pass / Warning):
-----------------------------------
               Single base runs: Pass
         Dinucleotide base runs: Pass
                        Length: Pass
                    Percent GC: Pass
          Tm (Nearest neighbor): Warning: Tm is greater than 58;
                     GC clamp: Pass
                Self-annealing: Pass
              Hairpin formation: Pass
------------------------------------------------------------
```

Primer-BLAST Primer-Blast results

Specificity of primers

Target templates were found in selected database: Genome database (reference assembly only) for selected species (Organism limited to Homo sapiens)

Primer pair 1

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	ATCGAGTTTGGGGAGATCATC	21	51.38	47.62	5.00	5.00
Reverse primer	CAACAGTCTTGGCTGCTCAGT	21	54.98	52.38	5.00	2.00

Products on target templates

>NT_010393.16 Homo sapiens chromosome 16 genomic contig, GRCh37.p5 Primary Assembly

product length = 367
Features associated with this product:
amiloride-sensitive sodium channel subunit beta

```
Forward primer  1         ATCGAGTTTGGGGAGATCATC  21
Template        23331811  .....................  23331831

Reverse primer  1         CAACAGTCTTGGCTGCTCAGT  21
Template        23332177  .....................  23332157
```

>NT_007819.17 Homo sapiens chromosome 7 genomic contig, GRCh37.p5 Primary Assembly

product length = 3384
Features associated with this product:
BMP-binding endothelial regulator protein precursor

```
Forward primer  1         ATCGAGTTTGGGGAGATCATC  21
Template        34094916  TGA........CC........  34094936

Forward primer  1         ATCGAGTTTGGGGAGATCATC  21
Template        34098299  .GAA.A............T..  34098279
```

3.3.6.2.1 Preparation of Master Mix for PCR

The Master Mix was then prepared with content as follows:

		Volume	Final Concentration
a.	PCR Grade water	variable	50µL
b.	PCR reaction buffer with $MgCl_2$ 10x	1µ	2mM $MgCl_2$
c.	PCR Grade Nucleotide Mix 10mM	1µ	200µm (all nucleotides)
d.	Forward Primer	variable	200nM
e.	Reverse Primer	same as forward	200nM
f.	Fast Start Taq DNA Polymerase (GoTaq)	0.4µL	2µ

The contents of the Master Mix were mixed thoroughly and then transferred to a PCR tube. Equal volumes of this Master Mix were thereafter pipetted into separate 0.2ml thin PCR tubes. The mixture was mixed gently to produce a homogenous reaction mixture. Template DNA was added to the individual tubes cotaining Master Mix using the following guidelines:

	Volume	
DNA or cDNA Template	Variable	10pg-500ng complex DNA
FINAL VOLUME	50µL	1x Reaction Mix

The Master Mix was then transferred to a PCR tube. Equal volumes of this Master Mix were then pipetted into separate 0.2ml thin PCR tubes. The mixture was mixed gently to produce a homogenous reaction mixture. The PCR tubes were then placed immediately thereafter in the thermocycler (Perkin Elmer 2400, Wasrrington, UK).

Polymerase chain reaction was thereafter carried out following the method of Rayner *et al.,* (2003) after heating the lid of the thermocycler to 100^{o}C in order to keep the mixture in the tube:

I. DENATURATION/ACTIVATION

This is also known as "Hot Start". It was carried out at 95^{o}C for 5 minutes.

II. AMPLIFICATION

This was carried out for 35 cycles. Each cycle was made up of the following processes:

i. Denaturation which was carried out at 95^{o}C for 30seconds

ii. Annealing which was carried out at 56.5^{o}C for 30 seconds

iii. Elongation which was carried out at 72^{o}C for 45 seconds. At this stage, the reaction was looped to return to the Denaturation stage for 35 cycles

III. FINAL ELONGATION

After completing the Amplification stage, a final elongation stage was carried out at 72^{o}C for 7 minutes.

3.3.6.2.2 Sizing of PCR Products

Size and quality of PCR products were verified by electrophoresis on a 2% agarose gel with ethidium bromide staining (Baker *et al,* 1998). The gel casting trays and combs were selected. 1g of agarose was weighed into a conical flask and 100ml Tris/Acetate/EDTA (TAE) buffer added

to it. The mixture was mixed gently following which it was heated in a microwave while checking regularly to ensure the solution did not boil over. It was thereafter allowed to cool slightly to about 65^{o}C following which ethidium bromide (5mg/ml) was added to make the bands visible. The mixture was then poured into a gel casing with a comb in place. The agarose was allowed to set for 10 to 15 minutes. Thereafter, it was removed gently from the casing and the comb also removed leaving intact wells. The gel was then placed in a submarine (horizontal) electrophoresis tank filled with TAE buffer enough to cover the gel by 0.5mm. Then 10 to 15µL of PCR products was mixed with 6-8µL loading buffer/dye GoTaq flexibuffer in a mixing tray. This is to allow the amplicons to sink to the bottom of the wells. A marker-ladder was placed in the gel as well to confirm the correct fragment size. The samples were then loaded gently into the gel wells. The electrophoresis machine was switched on and run at 70 to 110 Volts for about 120 minutes to allow the DNA to migrate towards the anode. The machine was switched off and the gel tray removed. The gel was then observed under the ultra-violet (UV) light and a picture taken of the gel.

3.3.6.2.3 Purification of DNA Fragments

This was carried out using a QIAQUICK Purification Kit (QIAQUICK, Catalogue No. 28104 (50)). The PE Buffer supplied with kit was dissolved in 96-100% ethanol. This served as the Wash Buffer. Then 5 volumes of the PB Buffer (supplied with kit) was added to 1 volume of PCR sample. This was mixed gently. The sample was then aplied to a QIAQUICK spin column placed into a 2ml collection tube supplied with the kit. This was placed in the centrifuge for 30 to 60 seconds at 13000 rpm. The flow-through was discarded and the spin column placed back in the same collection tube. Thereater, 750µL of PE Buffer was added to the column to wash and

the tube returned to the centrifuge for 30 to 60 seconds at the same revolution as above. The flow-through was again discarded and the spin column returned to the collection tube. It was then centrifuged for 1 minute to completely remove the ethanol. The spin column was then placed in a clean 1.5ml microcentrifuge tube.

3.3.6.2.4 Elution of PCR Products for Sequencing

To elute the PCR products for sequencing, 30µL of distilled water (dH_2O) was added to the centre of the membrane. The sample was then allowed to stand for 1 minute. The concentration of the purified PCR product was then checked by means of the Nanodrop spectrophotometer. Purified PCR products were stored at -20^{o}C till sequenced.

3.3.6.2.5 Single Nucleotide Polymorphism (SNP) Genotyping Assay

This was carried out at the Division of Women's Health, School of Medicine, King's College, London made possible by an International Junior Research Grant of The Physiological Society of Great Britain. TaqMan$^{(R)}$ SNP Genotyping Assay Kit (Life Technologies, United Kingdom) for the β-T594M mutation using the ENSEMBL Reference Sequence number, rs1799979, for the single nucleotide polymoprhism (SNP) was procured for use in identifying the presence of the mutation. A master mix containing 5µL of Sybr Green for PCR and 0.5µL each of the TaqMan$^{(R)}$ SNP Genotyping Assay and molecular grade water was added to 4µL of the gDNA of each subject. Two samples of "No Template Control" (NTC) prepared by replacing gDNA with 4µL of tRNA were included in each SNP Genotyping PCR run as control. The samples were aliquoted by means of the Corbett Robotics$^{(R)}$ into PCR tubes. These were then placed in the RotorGene$^{(R)}$ for real time PCR with Two-Step PCR conditions as below:

1. Initial denaturation at 95^{o}C for 3 minutes
2. Cycling: Denaturation at 95^{o}C for 20 seconds
   Annealing/Extension at 60^{o}C for 15 seconds
   Cycling was carried out for 60 cycles for optimum results

3.3.6.2.6 Identification of β-ENaC Variants by DNA Sequencing

DNA sequencing was carried out at the DNA Sequencing core facility of the University of Stellenbosch, Cape Town, South Africa. The subjects' DNA samples were analysed for β-ENaC variants. Big Dye Terminator Cycle Sequencing kits (Applied Biosynthesis, Foster City, California, USA) were used according to the manufacturer's instructions. The sequencing reactions were carried out on double-stranded DNA with the same primers used for PCR in dye terminators cycle sequencing. Extension products were then purified by ethanol precipitation. The reactions were loaded onto an automated sequencer and run under standard conditions. DNA sequences were confirmed by sequencing both strands. A second set of sequencing to confirm the results of the TaqMan$^{(R)}$ SNP Genotyping Assay was carried out at the core facilities of Source Bioscience (United Kingdom).

3.4 Analysis of Data

Univariate and multivariate analysis of data were carried out as appropriate. The Graph Pad Statistical software was used. Data is presented as Mean±Standard Error of Mean (X±S.E.M.). Paired and unpaired Student t tests were used for within group and between group comparisons respectively. Mann-Whitney test was carried out for comparison of means when a Gaussian distribution could not be assumed. Analysis of variance (ANOVA) was also used with appropriate Post Hoc tests carried out to determine differences among means. Results are

presented as mean ± standard error of mean (X ± S.E.M.). Differences between means were accepted as significant at 95% confidence level; exact confidence intervals were calculated for each analysis.

CHAPTER FOUR

4.0 RESULTS

4.1 Biodata of Subjects

Two hundred and twelve (212) subjects were screened for this study after informed consent was obtained from them. One hundred and twenty-four (124) of this subject population were normotensive with a mean age of 35.17±1.0y and the remaining eighty-eight (88) subjects were hypertensive with a mean age of 44.88 ± 1.0y which was significantly higher ($p < 0.001$) than the age of the normotensive subjects (Table 2a). The normotensive subjects had a mean weight of 66.16±1.17 kg while the hypertensive subjects had a mean weight of 78.38±1.30 kg. This was significantly higher ($p<0.001$) than that of the normotensive subjects (Table 2a). Table 2a also shows that the body mass index (BMI) calculated in the normotensive subjects was significantly lower ($p < 0.001$) than that of the hypertensive subjects at 24.08±0.46 kg/m^2 and 29.14±0.50 kg/m^2 respectively.

Following the application of the selection criteria set out for the second stage of this study in which subjects were selected to participate in Phase I through V, a total of one hundred (100) age-matched subjects completed the study: forty-seven (47) normotensive subjects with mean age of 41.23±1.40y and fifty-three (53) age-matched hypertensive subjects with mean age 44.42±1.30y ($p > 0.05$). The normotensive subjects had a mean weight of 66.53±1.80 kg. This was significantly lower ($p < 0.001$) than the mean weight of 78.31±1.68 kg recorded in the hypertensive subjects (Table 2b). The mean body mass index (BMI) in normotensive subjects was 23.66±0.68 kg/m^2. This was significantly lower ($p < 0.001$) than the mean BMI of 28.69.1±0.70 kg/m^2 recorded in the hypertensive subjects (Table 2b).

Table 2: Biodata of subjects

Table 2a: Biodata of all subjects screened

	Normotensive (NT)	Hypertensive (HT)	p value
n	124	88	
Age (year)	35.17 + 1.10 (18-65)	44.88 ± 1.0 (25-70)	<0.001*
Weight (kg)	66.16 ± 1.17 (45 - 103)	78.38 ± 1.3 (56-109)	<0.001*
Height (m)	1.66 ± 0.01 (1.43 – 1.97)	1.64 ± 0.02 (1.47 – 2.1)	>0.05 N.S.
BMI (kg/m^2)	24.08 ± 0.46 (15.24 – 38.72)	29.14 ± 0.5 (17.1 - 41.3)	<0.001*

* = Significant; N.S. = Not significant

Table 2b: Biodata of selected normotensive and hypertensive subjects[#]

	Normotensive (NT)	Hypertensive (HT)	p value
n	47	53	
Age (year)	41.23 + 1.40 (27-65)	44.42 ± 1.30 (25-65)	>0.05 N.S.
Weight (kg)	66.53 ± 1.80 (45.00 – 103.00)	78.31 ± 1.68 (56.00-109.00)	<0.001*
Height (m)	1.68 ± 0.02 (1.43 – 1.97)	1.65 ± 0.01 (1.47 – 2.10)	>0.05 N.S.
BMI (kg/m^2)	23.66 ± 0.68 (16.50 – 38.72)	28.69 ± 0.70 (17.12 - 41.29)	<0.001*

* = Significant; N.S. = Not significant

These are subjects who took part in the second stage of the study

4.2 Salt Sensitivity in Normotensive and Hypertensive Subjects

4.2.1 Baseline Blood Pressure

Mean systolic blood pressure (SBP) was 117.1±1.49 mmHg among the normotensive subjects at baseline. This was significantly lower ($p < 0.001$) than baseline SBP of 145.50±2.60 mmHg recorded among the hypertensive subjects (Table 3).

Table 3 also shows the mean diastolic blood pressure (DBP) of 78.45±0.94 mmHg recorded among the normotensive subjects at baseline. This was significantly lower ($p < 0.001$) than the mean DBP of 96.07±1.54 mmHg recorded among the hypertensive subjects at baseline.

The mean arterial blood pressure (MABP) of 91.31±0.94 mmHg recorded among the normotensive subjects at baseline was also significantly lower ($p < 0.001$) than the 112.7±1.59 mmHg recorded among the hypertensive subjects at baseline (Table 3).

Table 3: Baseline blood pressure in normotensive and hypertensive subjects

	Normotensive (n = 47)	Hypertensive (n = 53)	p value
SBP (mmHg)	117.10 ± 1.49 (100.0 – 130.0)	145.4 ± 2.60 (110.0 – 190.0)	<0.001
DBP (mmHg)	78.45 ± 0.94 (70.0 – 89.0)	96.07 ± 1.54 (70.0 – 116.0)	<0.001
MABP (mmHg)	91.31 ± 0.94 (80.0 + 101.3)	112.7 ± 1.56 (90.0 – 136.7)	<0.001

KEY:

SBP = systolic blood pressure; DBP = diastolic blood pressure; MABP = mean arterial blood pressure

4.2.2 Salt Sensitivity

Among the normotensive subjects, 16 subjects responded to the salt-load with an increase in mean arterial blood pressure (MABP) that was greater than or equal to 5 mmHg. Thus salt sensitivity was observed in 34.0% (16/47) of the normotensive (NT) subjects while the remaining 66.0% (31/47) were salt resistant (Figure 9).

Salt sensitivity was higher in hypertensive subjects (HT) among whom 54.7% (29/53) responded to the salt-load with a change in MABP (ΔMABP) that was 5mmHg or more compared with the baseline value (Figure 9). The remaining 45.3% (24/53) were salt resistant.

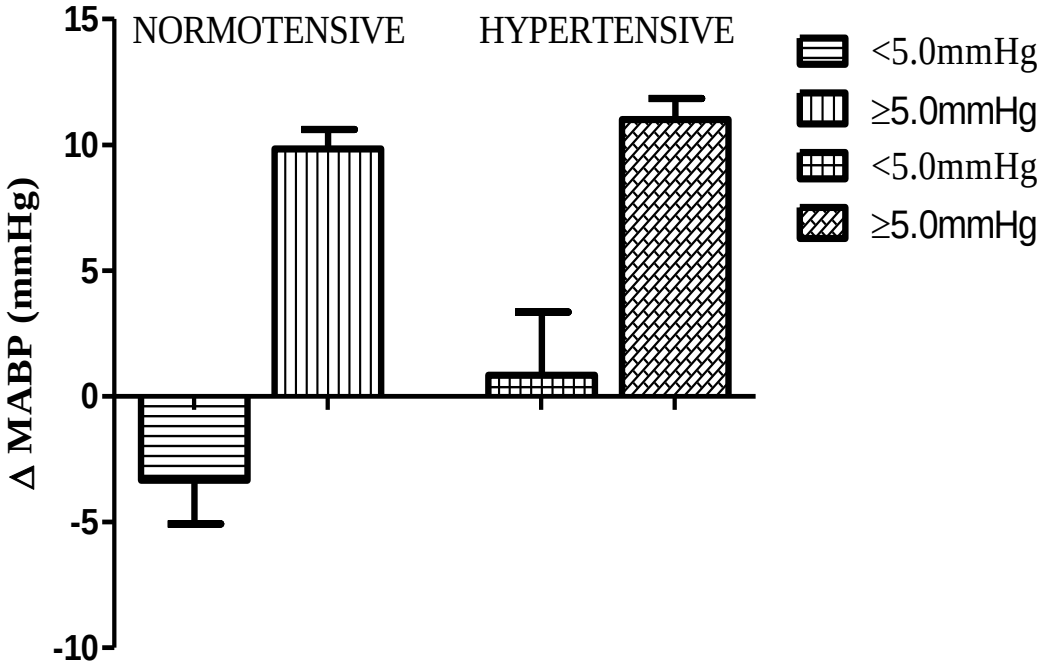


Figure 9a: Salt sensitivity among normotensive (NT) and hypertensive (HT) subjects

Salt sensitive = ΔMABP ≥ 5mmHg

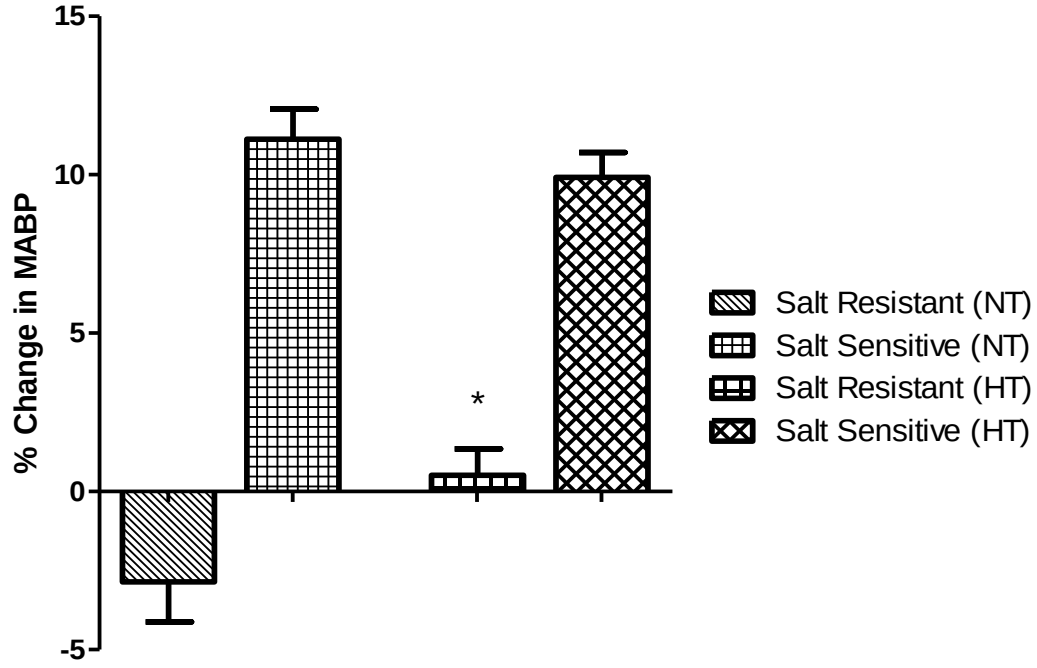


Figure 9b: Percentage change in mean arterial blood pressure (MABP) among normotensive and hypertensive subjects after salt-loading

*p < 0.05 compared with Salt Resistant (SR) Normotensive

4.2.4 Urine Sodium Excretion in Normotensive and Hypertensive Subjects

As shown on Table 4, urine excretion of sodium ($U_{Na}V$) was 114.6±10.1 mmol/min among the normotensive subjects before salt-loading. This was significantly higher ($p < 0.05$) than the baseline level of 84.4±8.7 mmol/min recorded among the hypertensive subjects.

Following salt-loading for 5 days, $U_{Na}V$ increased significantly ($p < 0.01$) to 163.0±16.7 mmol/min among the normotensive subjects. In the same vein, $U_{Na}V$ increased significantly ($p <0.01$) to 118.20±13.11 mmol/min among the hypertensive subjects following salt-loading (Table 4).

After combined ingestion of the salt-load and amiloride, $U_{Na}V$ reduced significantly ($p < 0.01$) in NT subjects to 123.1±19.6 mmol/min compared with the level after salt-loading alone (Table 4). This value after the combined ingestion of the salt-load and amiloride was slightly higher ($p > 0.05$) than the 114.3±10.1 mmol/min recorded among the NT subjects at baseline. Among the HT subjects on the other hand, salt plus amiloride caused a slight increase ($p > 0.05$) in $U_{Na}V$ to 122.9±18.6 mmol/min. However, this value was significantly higher ($p < 0.01$) than the 84.4±8.7 mmol/min recorded among the HT subjects at baseline (Table 4).

Groups / Intervention	Normotensive (NT) $U_{Na}V$ (mmol/min) n = 28	Hypertensive (HT) $U_{Na}V$ (mmol/min) n = 30
Before Salt	114.6±11.0	84.4±8.7*
After Salt	163.0±16.7**	118.2±13.1 ††
After Salt + Amil	123.1±19.6 ‡‡	122.9±18.6 ‡‡‡

Table 4: Urine sodium excretion in normotensive and hypertensive subjects

Data are expressed as X±S.E.M.

* p <0.05 NT Before Salt versus HT Before Salt

** p < 0.01 Before Salt NT versus After Salt NT

‡‡ p < 0.01 After Salt NT versus After Salt+ Amil NT

†† p < 0.01 Before Salt HT versus After Salt HT

‡‡‡ < 0.01 Before Salt HT versus After Salt+Amil HT

4.2.5 Salt Sensitivity Index

Mean salt sensitivity index was 0.09±0.02 mmHg/mol/day in the normotensive subjects. This was significantly lower ($p < 0.01$) than the 0.25±0.04 mmHg/mol/day recorded among the hypertensive subjects (Table 5).

Table 5: Salt Sensitivity Index in normotensive and hypertensive subjects

PARAMETER	NORMOTENSIVE	HYEPRTENSIVE
ΔUNaV (mmol/day)	180158 ± 27443	86870 ± 11932
ΔMABP (mmHg)	11.70 ± 1.70	12.38 ± 1.56
SSI (mmHg/mol/day)	0.09 ± 0.02	0.25 ± 0.04*

** $p < 0.01$ Significant difference in Salt Sensitivity Index (SSI) NT versus HT

KEY:

NT = normotensive; HT = hypertensive

4.2.6 Plasma Sodium Concentration in Normotensive and Hypertensive Subjects

Plasma sodium (Na^+) concentration was determined in the subjects before salt-loading, after salt-loading and after salt-loading plus amiloride.

Before salt-loading, the normotensive subjects had a mean plasma Na^+ concentration of 135.60±1.38 mmol/L. This was slightly lower ($p > 0.05$) than the plasma Na^+ level of 138.20±1.39 mmol/L in the hypertensive subjects.

Following salt-loading, plasma Na^+ concentration increased significantly among the normotensive subjects from 135.60±1.38 mmol/L to 142.80±1.39 mmol/L ($p < 0.001$). Similarly there was a significant increase in plasma Na^+ concentration among the hypertensive subjects from 138.20±1.39 mmol/L to 143.50±1.12 mmol/L ($p < 0.001$).

There was a significant fall in plasma Na^+ concentration in the normotensive subjects following salt plus amiloride-loading from the 142.80±1.39 mmol/L recorded after salt-loading alone to 132.40±3.59 mmol/L ($p < 0.01$). However, when compared with the plasma Na^+ concentration recorded in the normotensive subjects at baseline, there was only a slight difference ($p>0.05$).

Plasma Na^+ concentration in the hypertensive subjects also fell significantly ($p < 0.001$) from the 143.50±1.12 mmol/L after salt-loading alone to 135.10±1.61 mmol/L. This level of Na^+ following salt-loading plus amiloride was similar to the baseline concentration of 138.20±1.39 mmol/L ($p>0.05$).

Table 6: Plasma sodium concentration in normotensive and hypertensive subjects

Groups / Intervention	Normotensive (NT) (mmol/L) n = 30	Hypertensive (HT) (mmol/L) n = 33
Before salt	135.60±1.38	138.20±1.39
After salt	142.80±1.02***	143.50±1.12 ***
After salt+amiloride	132.40±3.59**	135.10±1.61‡‡‡

Data are expressed as Mean±S.E.M.

*** $p < 0.001$ Significant increase when compared with before salt NT and HT

**$p < 0.01$ Significant decrease when compared with after salt NT

‡‡‡ $p < 0.001$ Significant decrease when compared with after salt HT

4.2.7 Sodium Clearance in Normotensive and Hypertensive Subjects Before Salt-Loading, After Salt-Loading and After Co-administration of Salt Plus Amiloride

Sodium clearance (C_{Na}) was 0.65±0.1 mmol/min in the normotensive subjects before salt-loading. Following salt-loading, sodium clearance increased significantly ($p<0.05$) to 1.02±0.1 mmol/min. When the subjects were given the combined salt-loading and amiloride, C_{Na} fell to 0.86±0.2 mmol/min from the level after salt-loading alone (Figure 10).

Sodium clearance (C_{Na}) among the hypertensive subjects was 0.61±0.1 mmol/min before salt-loading. This was marginally lower ($p > 0.05$) than that recorded among the normotensive subjects at baseline. There was a significant increase ($p < 0.05$) in C_{Na} among the hypertensive subjects following salt-loading from 0.60±0.1 mmol/min before salt-loading to 0.92±0.1 mmol/min. Combined salt-loading plus amiloride led to a decrease in C_{Na} to 0.71±0.1 mmol/min which was lower ($p > 0.05$) than the C_{Na} after salt-loading alone (Figure 10).

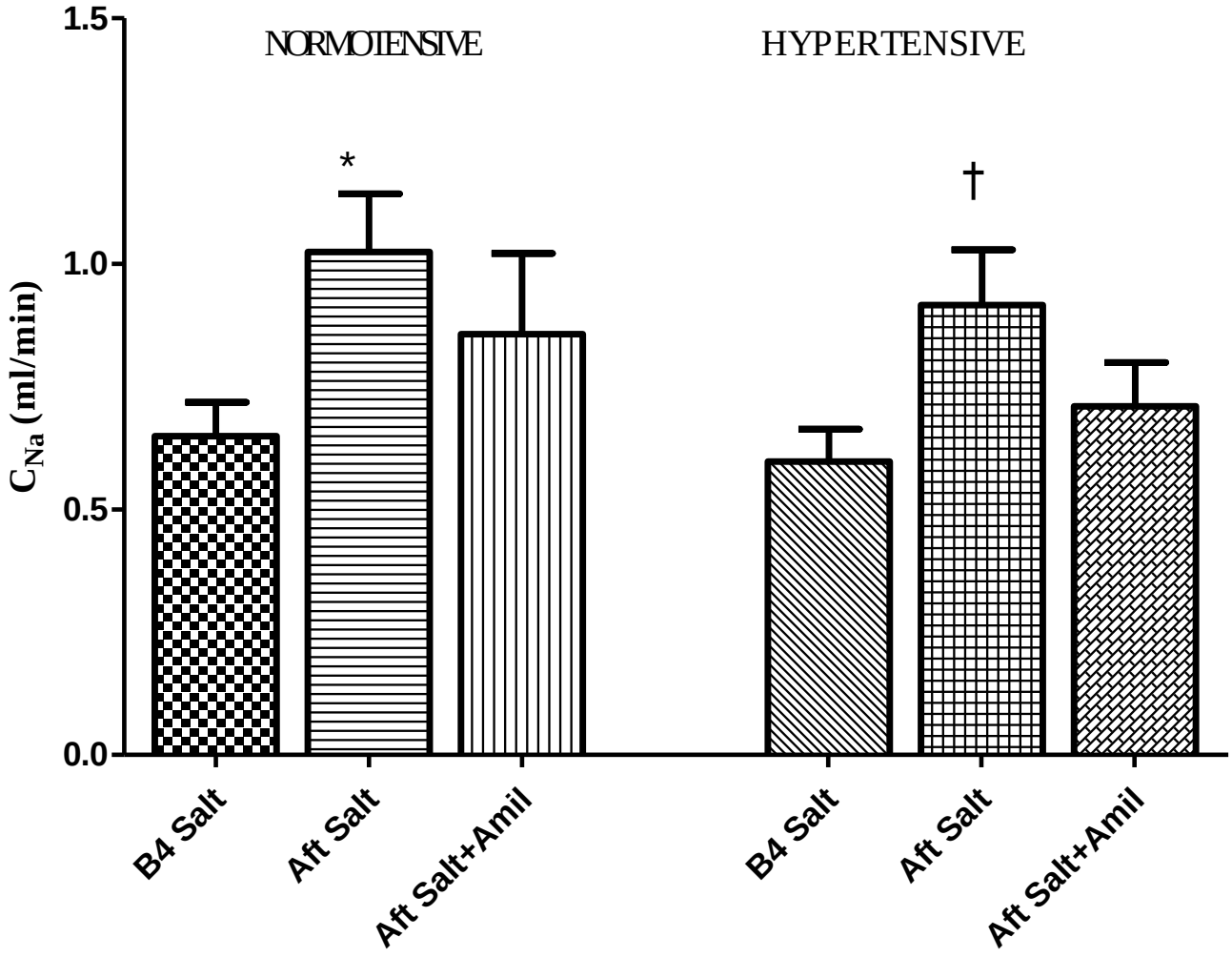


Figure 10: Sodium clearance in normotensive (NT) and hypertensive (HT) subjects before salt-loading, after salt-loading and after salt-loading plus amiloride

NT n = 28; HT n =30

* $p < 0.05$ Significant increase when compared with B4 Salt NT

† $p < 0.05$ Significant increase when compared with B4 Salt HT

KEY:

B4 = before; AFT = after; Amil = amiloride

4.3 Effect of Sympathetic Nervous System on Cardiovascular System of Normotensive and Hypertensive Subjects

4.3.1. Blood Pressure Response to the Cold Pressor Test in Normotensive and Hypertensive Subjects

4.3.1.1 Systolic Blood Pressure Response to Cold Pressor Test in Normotensive and Hypertensive Subjects

In normotensive subjects, exposure to the cold pressor test (CPT) before salt-loading led to an increase ($p < 0.001$) in systolic blood pressure (SBP) from 117.5±1.54 mmHg at baseline to 132.20±2.63 mmHg, (Figure 11a). This was a 12.28±1.6% increase in SBP following exposure to the CPT at baseline. Also as shown in Figure 11a, when hypertensive subjects were exposed to the CPT before salt-loading, there was a significant increase ($p < 0.001$) in SBP from the baseline level of 145.60±2.60 mmHg to 164±3.23 mmHg. This was a percentage difference of 13.57±1.6%.

Following salt-loading, exposure of the normotensive subjects to the CPT led to an increase ($p < 0.001$) in SBP from 121.10±2.05 mmHg before CPT to 137.9±2.69 mmHg. The percentage increase was 14.01±1.5% and this was not significantly higher ($p > 0.05$) than that recorded at before salt-loading. The figure also shows that when exposed to the CPT after salt-loading, there was a significant increase ($p < 0.001$) in SBP in hypertensive subjects from 152.40±3.03 mmHg to 171.20±3.28 mmHg. This was a percentage increase of 12.91%.

When normotensive subjects were exposed to the CPT after salt plus amiloride-loading for 5 days, SBP increased ($p < 0.001$) from 115.2±1.80 mmHg to 131.5±2.75 mmHg (Figure 11a); a percentage increase of 14.23±1.9%. This was not significantly higher ($p > 0.05$) than the percentage increases recorded before and after salt-loading respectively. On the other hand, there was a significant increase ($p < 0.001$) in SBP in hypertensive subjects following exposure to the CPT following ingestion of salt plus amiloride from 132.80±2.81 mmHg before CPT to 156.60±3.81 mmHg causing a percentage increase in SBP of 20.06±2.1%. This was significantly higher ($p < 0.01$) than the percentage increase recorded before salt-loading and significantly higher ($p < 0.01$) than that recorded after salt-loading.

4.3.1.2. Diastolic Blood Pressure Response to Cold Pressor Test in Normotensive and Hypertensive Subjects

In normotensive subjects before salt-loading, CPT caused an increase ($p < 0.001$) in diastolic blood pressure (DBP) from the baseline value of 79.74±0.86 mmHg to 93.49±1.75 mmHg (Figure 11b). This was an increase of 17.33±1.9%. When hypertensive subjects were exposed to the CPT before salt-loading, DBP increased significantly ($p < 0.001$) from 96.07±1.49 mmHg to 110.6±1.83 mmHg (Figure 11b). This was a percentage increase in DBP of 14.84±1.6%.

Following salt-loading, there was a significant increase ($p < 0.001$) in DBP among the normotensive subjects from 80.56±1.54 mmHg to 94.15±1.82 mmHg following exposure to the CPT (Figure 11b). This was a 17.49±2.5% increase. Figure 10b also shows that there was a significant increase ($p < 0.001$) in DBP among hypertensive subjects from 100.7±1.61 mmHg to

112.40±1.82 mmHg after salt-loading. The percentage increase of 11.35±1.3% was significantly less ($p < 0.05$) than that recorded before salt-loading.

The figure also shows the increase in DBP in normotensive subjects exposed to the CPT after salt plus amiloride-loading from 90.74±1.70 mmHg to 107.80±2.33 ($p < 0.001$). This was a 16.2±3.1% increase.

The percentage increases in DBP in normotensive subjects before salt-loading, after salt-loading and after salt plus amiloride-loading were similar ($p > 0.05$). Similarly, following salt plus amiloride-loading, exposure to the CPT led to a significant increase ($p < 0.001$) in DBP among the hypertensive subjects from 90.74±1.70 mmHg to 107.80±2.33 mmHg (Figure 11b). This was a percentage increase in DBP of 19.38±1.9% which was significantly higher ($p < 0.01$) than the percentage increase recorded after salt-loading.

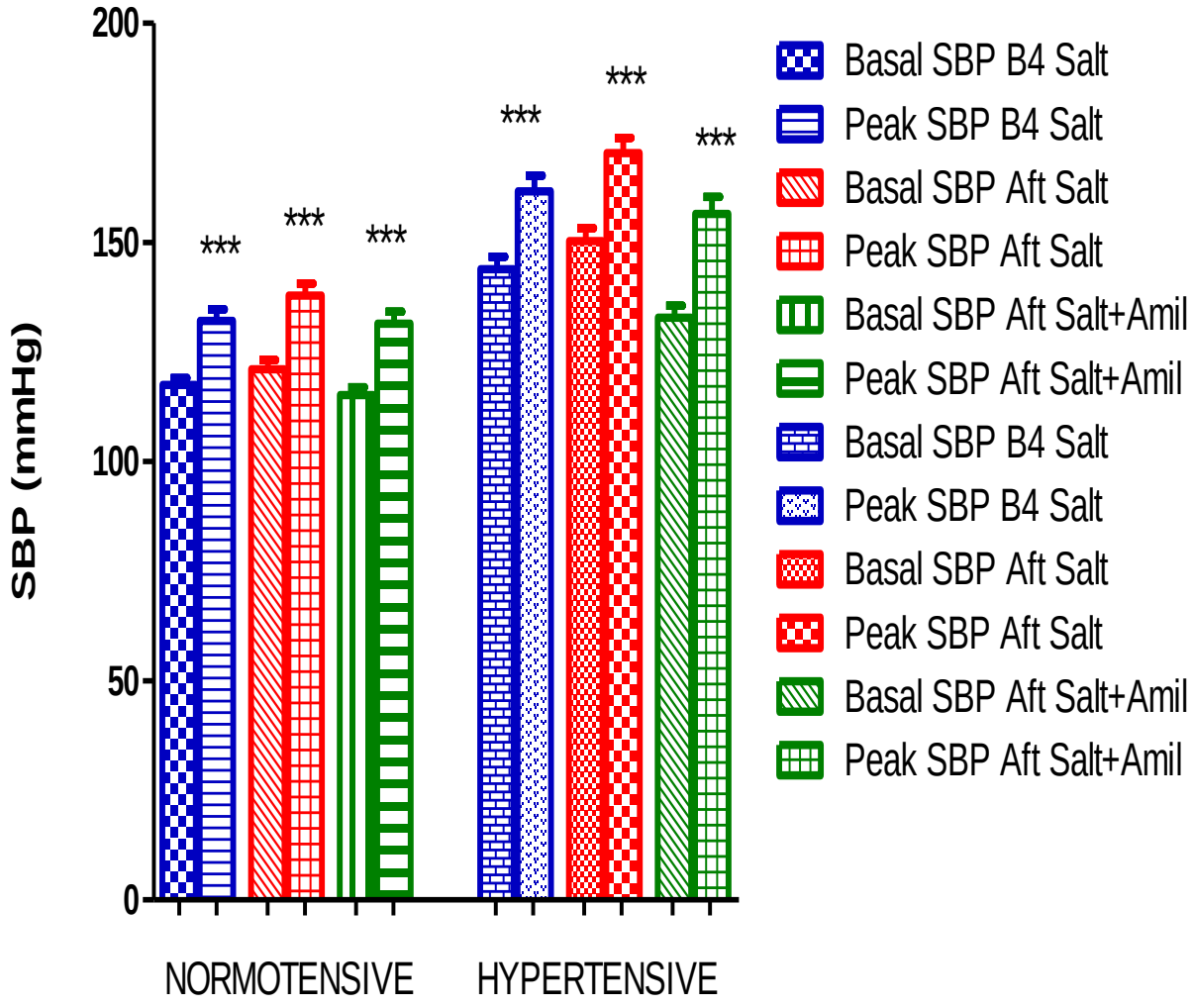


Figure 11a: Systolic blood pressure (SBP) response to the cold pressor test in normotensive and hypertensive subjects before salt, after salt-loading and after salt plus amiloride-loading

*** $p < 0.001$ between peak and basal SBP

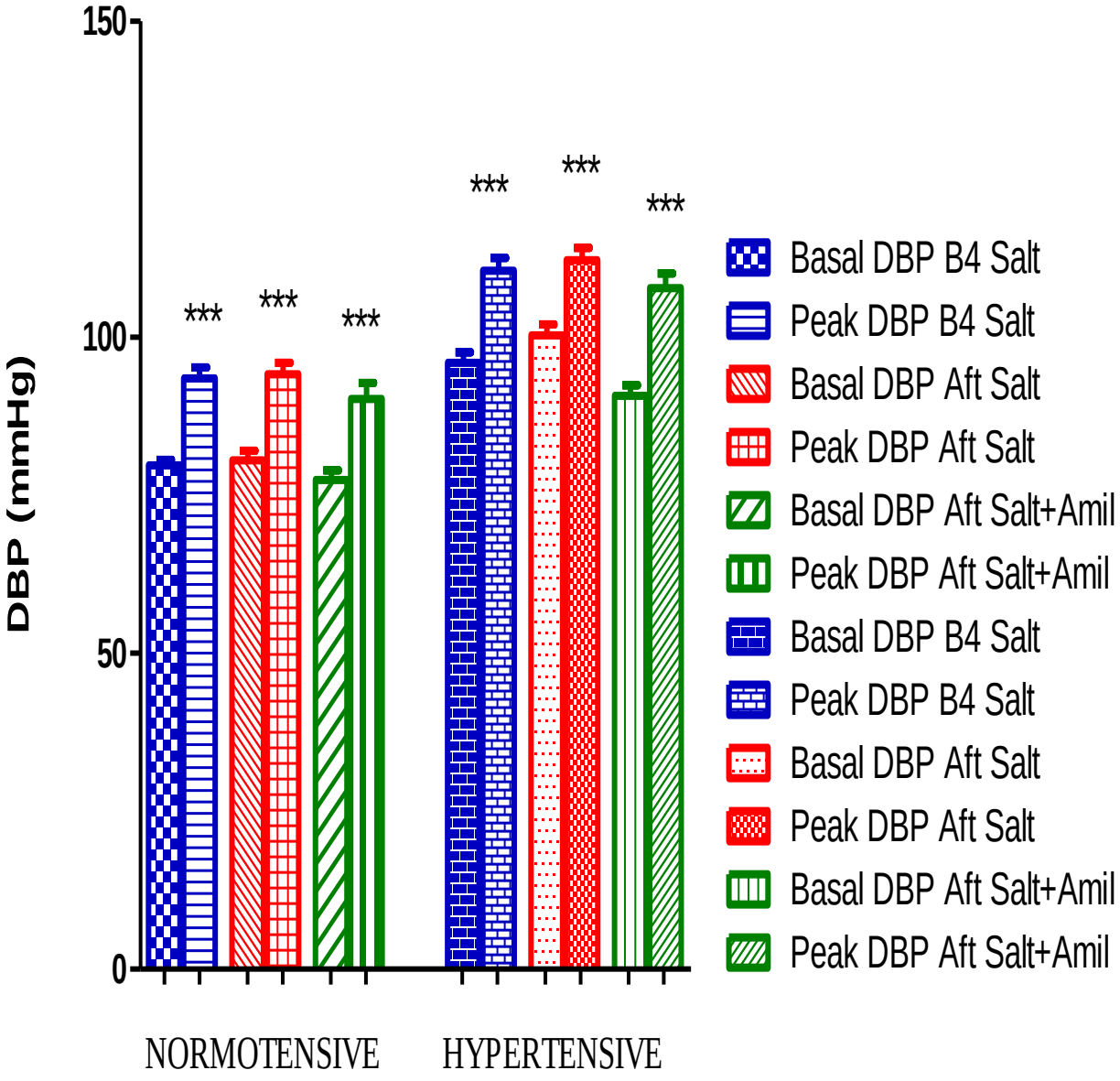


Figure 11b: Diastolic blood pressure (DBP) response to the cold pressor test in normotensive and hypertensive subjects before salt, after salt-loading and after salt-loading plus amiloride

*** $p < 0.001$ between peak and basal DBP

4.3.2 Vascular Reactivity among Normotensive and Hypertensive Subjects

4.3.2.1. Systolic Vascular Reactivity among Normotensive and Hypertensive Subjects

When exposed to the cold pressor test (CPT), 43.6% (17/39) of the normotensive subjects responded with systolic hyperreactivity before salt-loading. The mean difference in systolic blood pressure (ΔSBP) in the hyperreactive subjects was 24.5±1.9 mmHg and this was significantly higher ($p<0.001$) than the 7.4±1.6 mmHg recorded among the normoreactive subjects (Figure 12a).

Among the hypertensive subjects on the other hand, 49.0% (25/51) responded with systolic hyperreactivity to the cold pressor test. The mean difference in systolic blood pressure (ΔSBP) in the hyperreactive subjects was 31.0±3.1 mmHg and this was significantly higher ($p<0.001$) than the 8.2±1.0 mmHg recorded among the normoreactive hypertensive subjects (Figure 12a).

Also, when the normotensive subjects were exposed to the cold pressor test (CPT) after salt-loading, systolic hyperreactivity increased from the 43.6% before salt-loading to 64.1% (25/39). The mean difference in systolic blood pressure (ΔSBP) in the hyperreactive subjects after salt-loading was 22.6±1.7 mmHg and this was significantly higher ($p<0.001$) than the 6.6±1.7 mmHg recorded among the normoreactive normotensive subjects (Figure 12a).

Among the hypertensive subjects on the other hand, there was a decrease in systolic hyperreactivity from the 49.0% recorded before salt-loading to 33.3% (17/51) after salt-loading. The mean difference in systolic blood pressure (ΔSBP) in the hyperreactive subjects was

22.0±1.1 mmHg after salt-loading and this was significantly higher ($p<0.001$) than the 6.8±0.9 mmHg recorded among the normoreactive hypertensive subjects (Figure 12a).

Following the co-administration of the salt-load plus amiloride, systolic hyperreactivity reduced to 52.4% (11/21) among the normotensive subjects from the 64.1% recorded after salt-loading alone. The mean ΔSBP of 23.8±2.5 mmHg among these hyperreactive subjects was significantly higher ($p < 0.001$) than the 9.4±0.9 mmHg recorded among the normoreactive normoreactive subjects following salt plus amiloride-loading.

On the other hand, when hypertensive subjects were exposed to the cold pressor test after salt-loading plus amiloride, 67.5% (27/40) of these subjects responded with systolic hyperreactivity. This was a significant increase ($p < 0.001$) over the 33.3% recorded after salt-loading alone. It was also significantly higher ($p < 0.001$) than the number of normotensive subjects responding with systolic hyperreactivity following salt-loading plus amiloride. The mean ΔSBP of 36.2±2.9 mmHg was significantly higher ($p < 0.001$) than the 7.4±1.1 mmHg recorded among the normoreactive hypertensive subjects.

4.3.2.2 Diastolic Vascular Reactivity among Normotensive and Hypertensive Subjects

While 38.5% (15/39) of the normotensive subjects responded with diastolic hyperreactivity to the cold pressor test, 41.2% (21/51) of the hypertensive subjects responded with diastolic hyperreactivity before salt-loading. The mean difference in diastolic blood pressure (ΔDBP) among the hyperreactive normotensive subjects was 22.5±1.8 mmHg which was significantly ($p < 0.001$) higher than the mean ΔDBP of 7.8±1.5 mmHg recorded among the normoreactive normotensive subjects (Figure 12b). Also, the ΔDBP of 24.0±2.0 mmHg recorded among the hypertensive subjects that responded with diastolic hyperreactivity to the cold pressor test was significantly higher ($p < 0.001$) than the 7.3±0.9 mmHg recorded among the normoreactive hypertensive subjects (Figure 12b).

There was a reduction in the number of normotensive subjects that responded with diastolic hyperreactivity following salt-loading to 35.9% (14/39) compared with the 38.5% recorded before salt-loading. The mean difference in diastolic blood pressure (ΔDBP) among the hyperreactive normotensive subjects was 25.00±2.2 mmHg which was significantly ($p < 0.001$) higher than the mean ΔDBP of 6.80±1.1 mmHg recorded among the normoreactive normotensive subjects after salt-loading (Figure 12b).

Similarly, diastolic hyperreactivity reduced among the hypertensive subjects from the 41.2% before salt-loading to 31.4% (16/51) after salt-loading. The mean ΔDBP of 21.0±1.6 mmHg recorded among the hypertensive subjects that responded with diastolic hyperreactivity to the

cold pressor test was however significantly higher ($p < 0.001$) than the mean ΔDBP of 6.9±0.9 mmHg recorded among the normoreactive hypertensive subjects (Figure 12b).

Also, following exposure to the cold pressor test after salt plus amiloride-loading; daistolic hyperreactivity reduced to 33.3% (7/21) among the normotensive subjects from the 35.9% recorded following salt-loading alone. The mean ΔDBP of 24.6±3.3 mmHg among these hyperreactive normotensive subjects was higher ($p < 0.001$) than the 7.1±1.1 mmHg recorded in their normoreacitve counterparts (Figure 12b).

On the other hand, diastolic hyperreactivity increased among the hypertensive subjects following salt plus amiloride-loading to 57.5% (23/40) from the 31.4% recorded after salt-loading alone. The mean ΔDBP of 25.2±1.4 mmHg recorded among these hyperreactive subjects was higher ($p < 0.001$) than the 6.6±1.1 mmHg recorded among their normoreactive counterparts (Figure 12b).

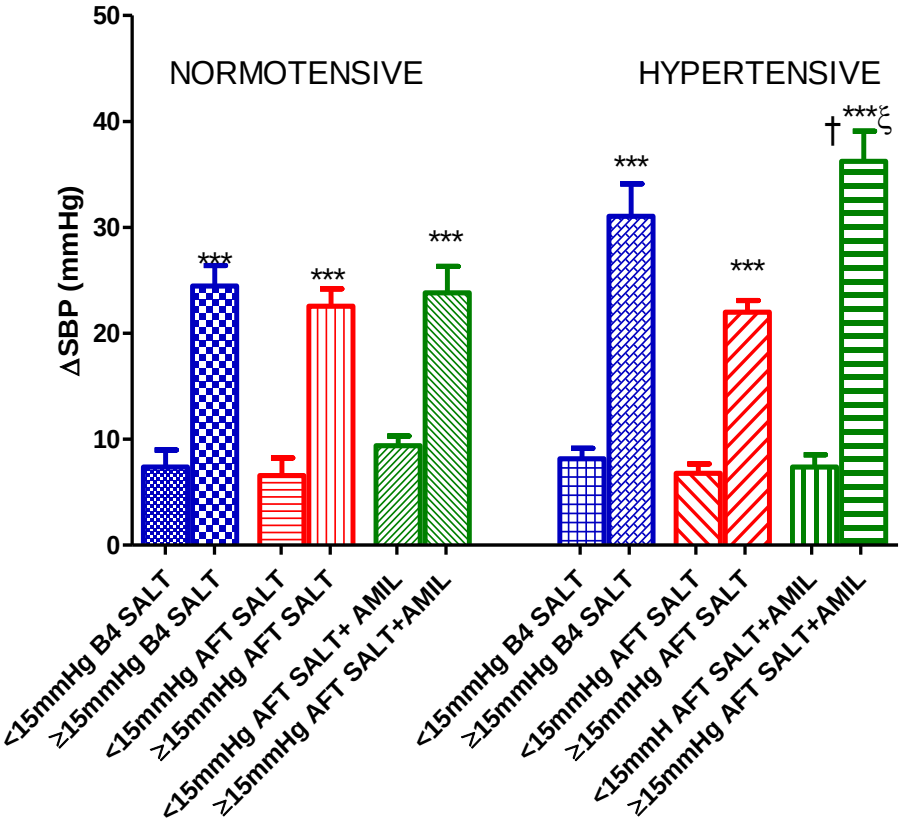


Figure 12a: Systolic vasular reactivity among normotensive (NT) and hypertensive (HT) subjects before salt, after salt-loading and after salt plus amiloride-loading

Hyperreactive = ΔSBP ≥ 15mmHg

NT: n = 17/39 (44%) B4 SALT; 25/39 (64%) AFT SALT; 11/21 (52%) AFT SALT+AMIL
HT: n = 25/51 (49%) B4 SALT; 17/51 (33%) AFT SALT; 27/40 (68%) AFT SALT+AMIL

***p < 0.001 hyperreactive versus normoreactive
† p < 0.05 NT aft salt+ amil versus HT; ξ = p < 0.001 (HT) aft salt versus aft salt+amil

KEY:
B4 SALT = before salt-loading; AFT SALT = after salt-loading; AMIL = amiloride

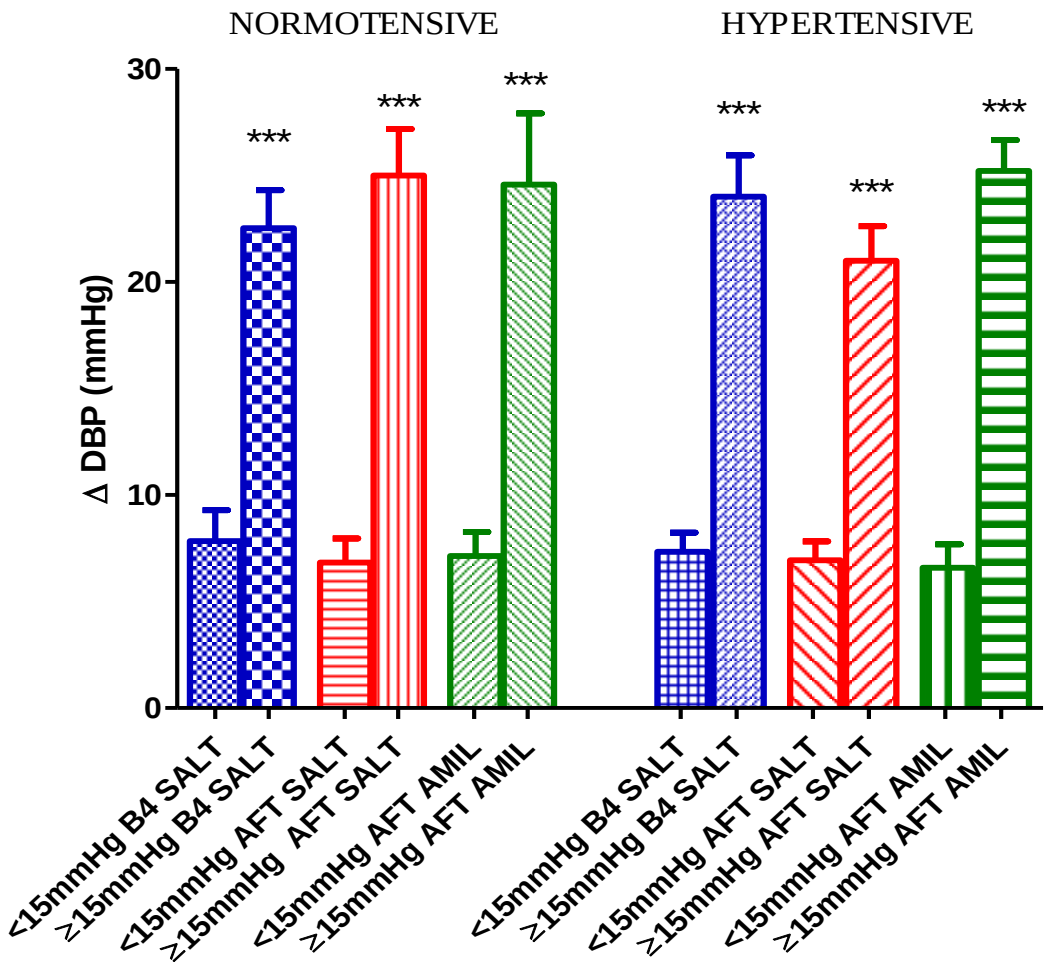


Figure 12b: Diastolic vascular reactivity in normotensive (NT) and hypertensive (HT) subjects before salt, after salt and after salt-loading plus amiloride

Hyperreactive DDBP ≥15mmHg:

NT: n = 15/39 (39%) B4 SALT; 14/39 (36%) AFT SALT; 7/21 (33%) AFT SALT+AMIL

HT: n = 21/51 (41%) B4 SALT; 16/51 (31%) AFT SALT; 23/40 (58%) AFT SALT+AMIL

***p < 0.001 normoreactive versus hyperreactive

KEY:
B4 SALT = before salt-loading; AFT SALT = after salt-loading; AMIL = amiloride

4.3.2.3 Salt Reactivity among Normotensive and Hypertensive Subjects

Salt reactivity, the phenomenon in which ingestion of a salt load leads to a hitherto normoreactive person becoming hyperreactive, was higher among the normotensive subjects compared with the hypertensive subjects.

As shown on Table 7, 33.3% of the normotensive subjects showed systolic salt reactivity while systolic salt reactivity was recorded among 17.7% of the hypertensive subjects.

Similarly, while 15.4% normotensive subjects demonstrated diastolic hyperreactivity, only 9.8% of the hypertensive subjects showed diastolic salt reactivity.

Table 7: Salt Reactivity in normotensive and hypertensive subjects

	Normotensive Subjects (n = 39)	Hypertensive Subjects (n = 51)
Systolic Salt Reactivity	13 (33.3%)	9 (17.7%)
Diastolic Salt Reactivity	6 (15.4%)	5 (9.8%)

4.3.2.4 Salt Sensitivity and Vascular Reactivity

Salt sensitivity was positively and significantly correlated with systolic vascular reactivity among normotensive subjects before salt-loading ($r = 0.47$; $p < 0.01$) (Figure 13a). In the same vein, Figure 13b shows that salt sensitivity in normotensive subjects was negatively and significantly correlated with diastolic vascular reactivity before salt-loading ($r = -0.38$; $p < 0.05$).

Among hypertensive subjects on the other hand, salt sensitivity was negatively correlated with both systolic vascular reactivity ($r = -0.008$; $p > 0.05$) (Figure 14a) and diastolic vascular reactivity ($r = -0.06$; $p > 0.05$) before salt-loading (Figure 14b).

As shown in Figure 15a, after salt-loading, salt sensitivity remained slightly positively correlated with systolic reactivity among normotensive subjects ($r = 0.25$; $p > 0.05$) while it was significantly negatively correlated ($r = -0.40$; $p < 0.05$) with diastolic reactivity (Figure 15b).

Among the hypertensive subjects on the other hand, salt sensitivity varied negatively with regard to systolic reactivity ($r = -0.07$; $p > 0.05$) (Figure 16a) and diastolic reactivity ($r = -0.22$; $p > 0.05$) (Figure 16b) after salt loading.

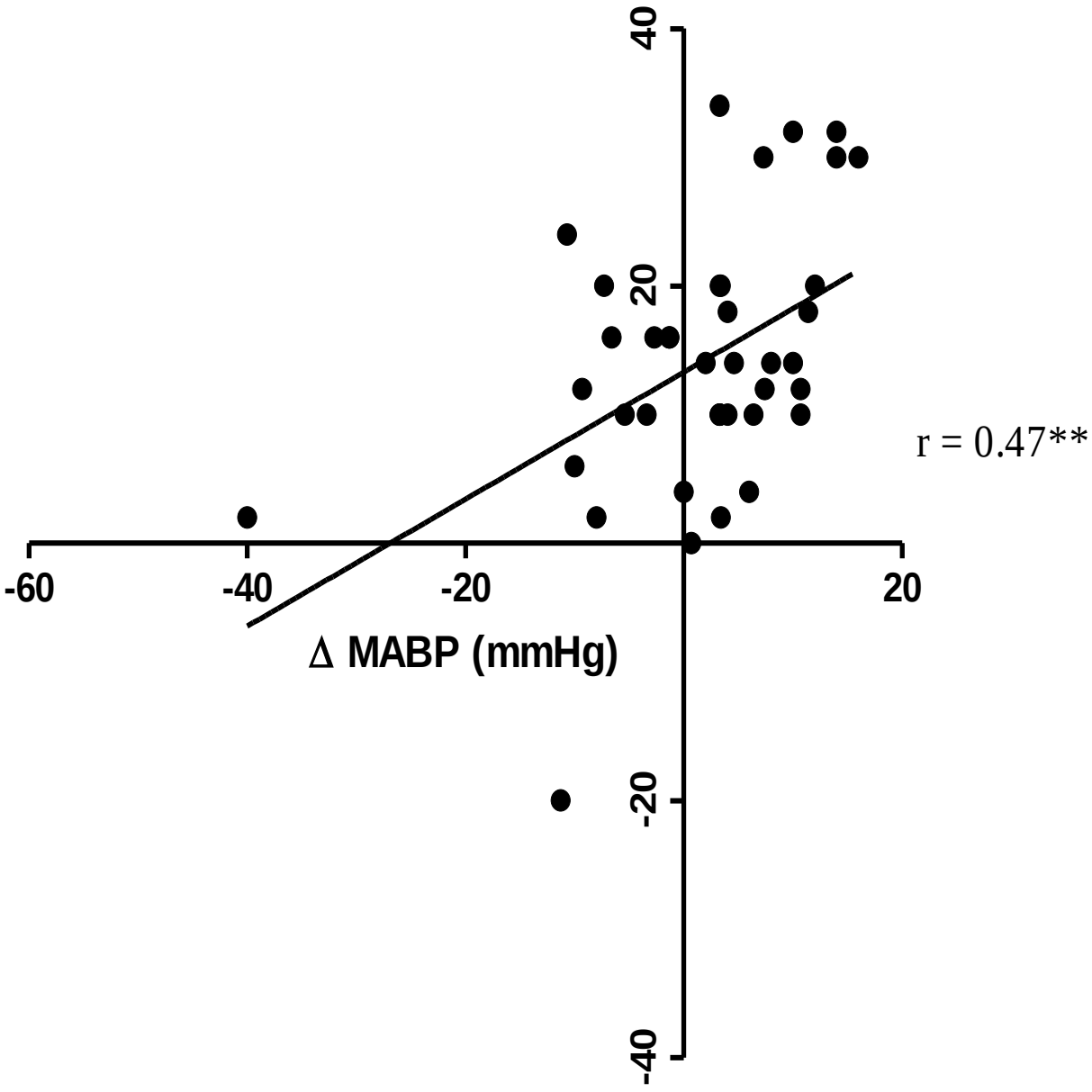


Figure 13a: Correlation between salt sensitivity (ΔMABP) and systolic reactivity in normotensive subjects before salt-loading

n = 40

*p < 0.01

KEY:

ΔSBP = change in systolic blood pressure; systolic reactivity = ΔSBP ≥15mmHg;
ΔMABP = change in mean arterial blood pressure

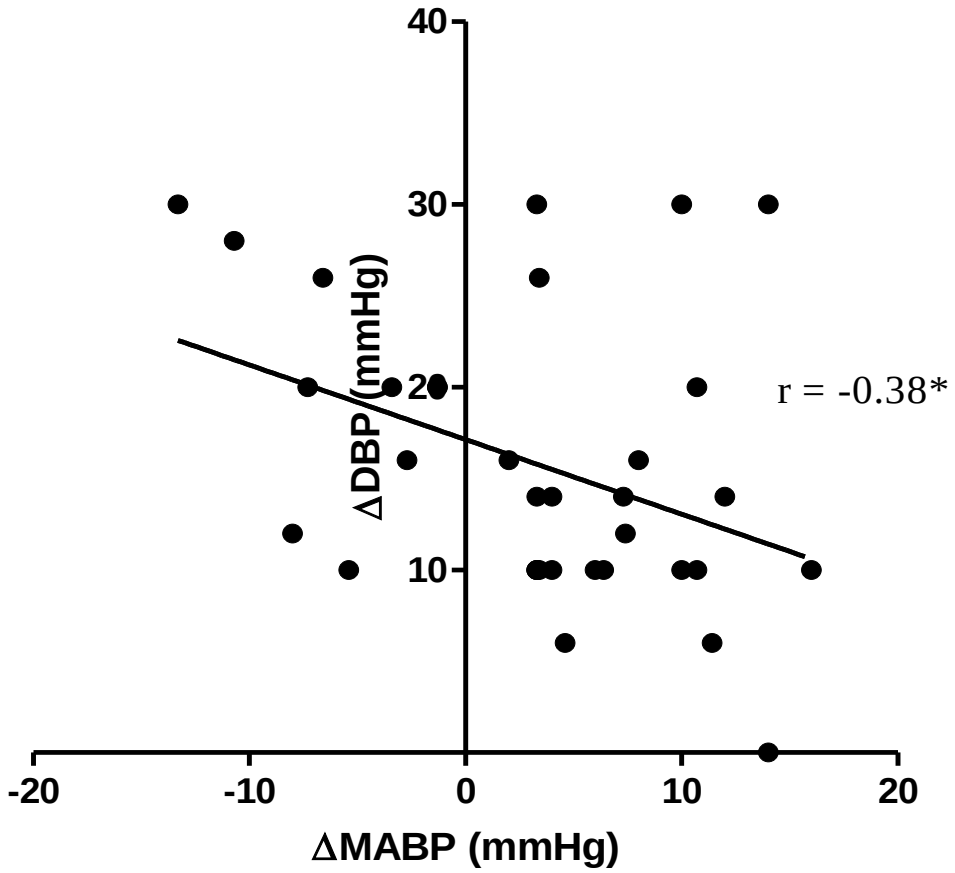


Figure 13b: Correlation between salt sensitivity and diastolic reactivity in normotensive subjects before salt-loading

n = 35

* $p < 0.05$

KEY:

ΔDBP = change in diastolic blood pressure; diastolic reactivity = ΔDBP ≥15mmHg;

ΔMABP = change in mean arterial blood pressure

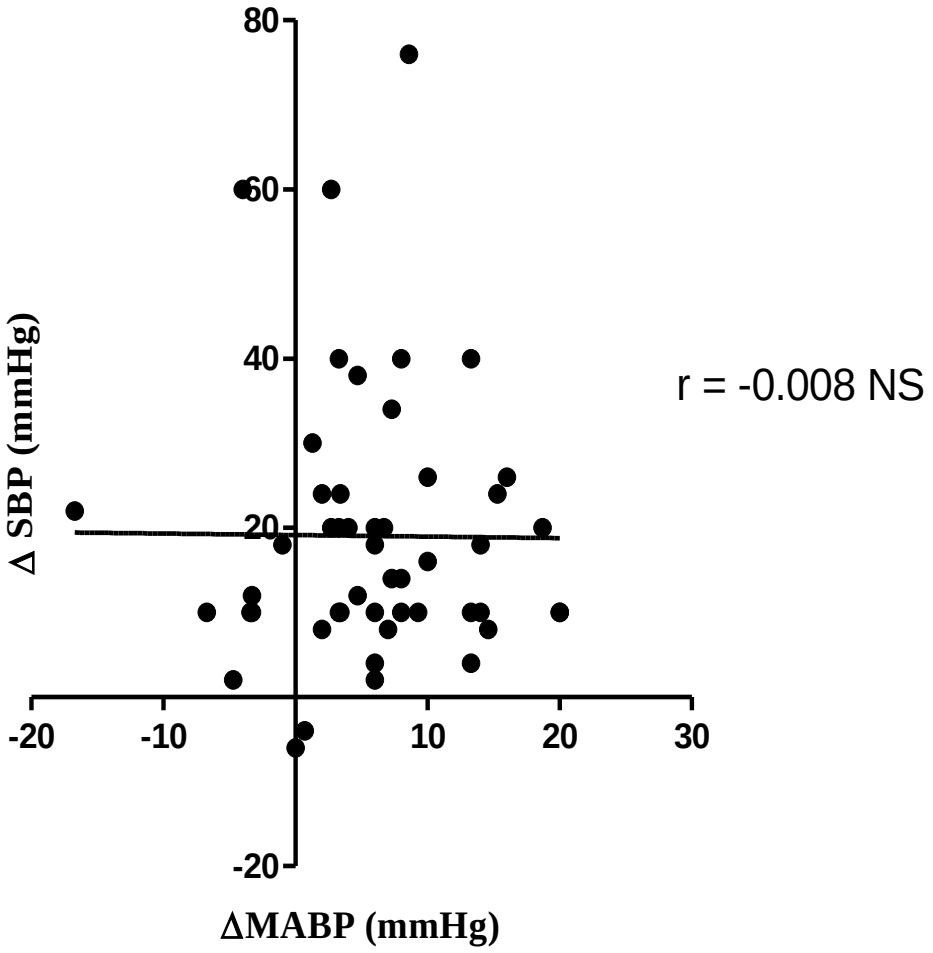


Figure 14a: Correlation between salt sensitivity and systolic reactivity in hypertensive (HT) subjects before salt loading

n = 50

NS = not significant

KEY:

ΔSBP = change in systolic blood pressure; systolic reactivity = ΔSBP ≥ 15mmHg;

ΔMABP = change in mean arterial blood pressure

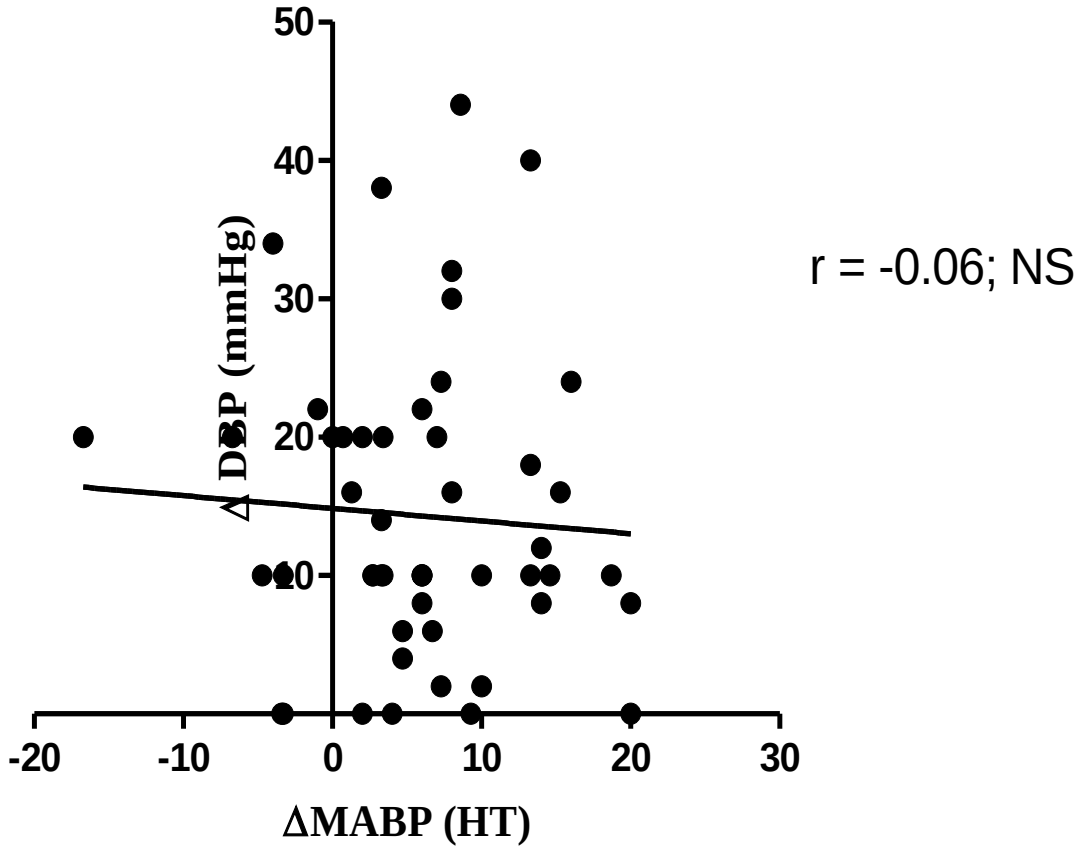


Figure 14b: Correlation between salt sensitivity and diastolic reactivity in hypertensive (HT) subjects before salt-loading

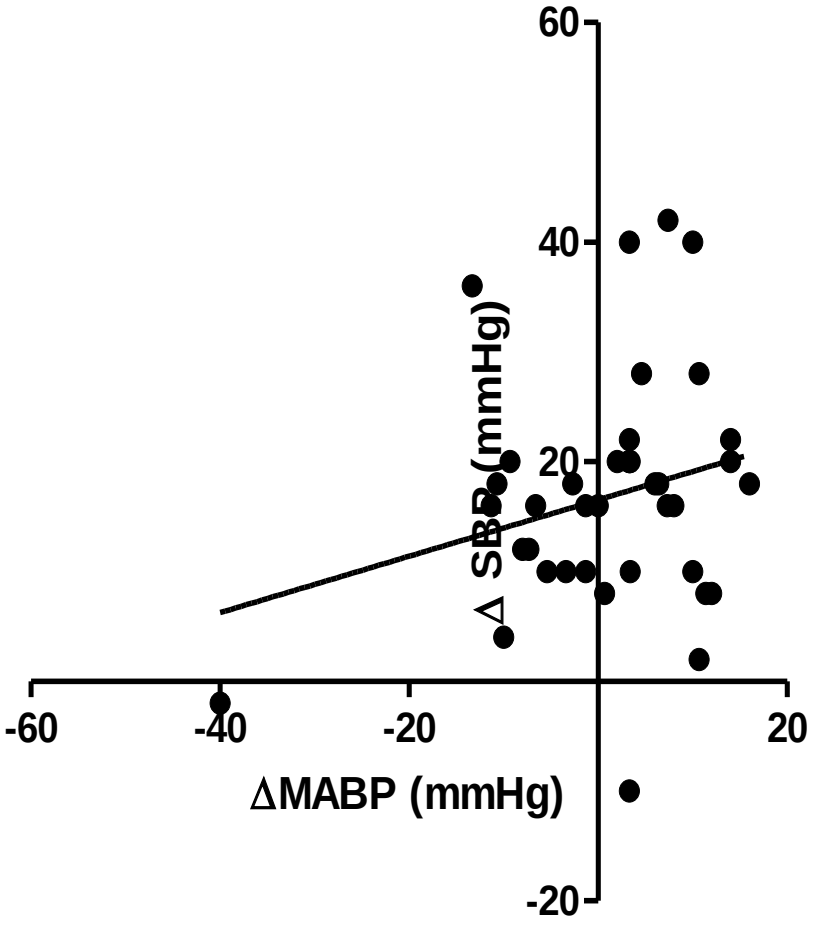
n = 50

KEY:

NS = not significant; ΔDBP = change in diastolic blood pressure;

diastolic reactivity = ΔDBP ≥15mmHg;

ΔMABP = change in mean arterial blood pressure



r = 0.25; NS

Figure 15a: Correlation between salt sensitivity (ΔMABP) and systolic reactivity in normotensive subjects after salt-loading

n = 39

KEY:

NS: not significant; ΔSBP = change in systolic blood pressure;

systolic reactivity = ΔSBP ≥15mmHg;

ΔMABP = change in mean arterial blood pressure

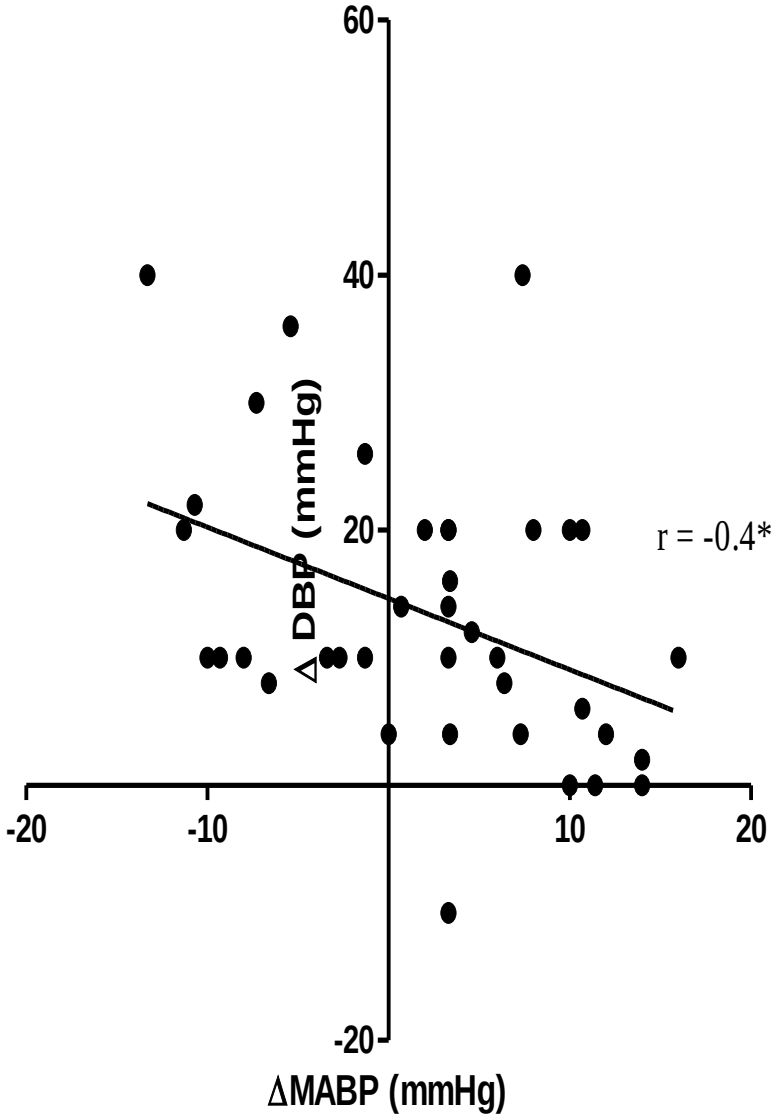


Figure 15b: Correlation between salt sensitivity and diastolic reactivity in normotensive (NT) subjects after salt-loading

n = 38

* $p < 0.05$

KEY:

ΔDBP = change in diastolic blood pressure;

diastolic reactivity = ΔDBP ≥15mmHg;

ΔMABP = change in mean arterial blood pressure

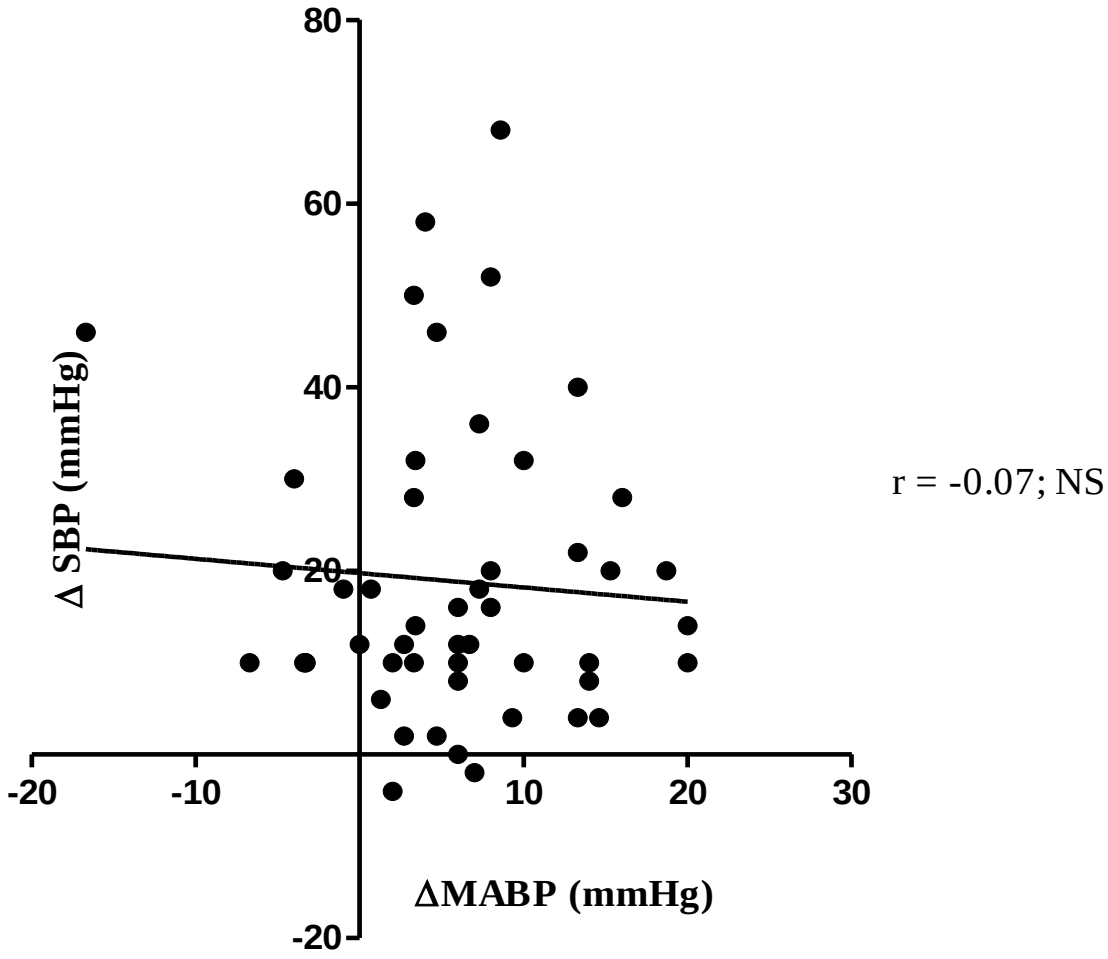


Figure 16a: Correlation between salt sensitivity and systolic reactivity in hypertensive subjects after salt-loading

n = 50

NS = not significant

KEY:

ΔSBP = change in systolic blood pressure;

systolic reactivity = ΔSBP ≥15mmHg;

ΔMABP = change in mean arterial blood pressure

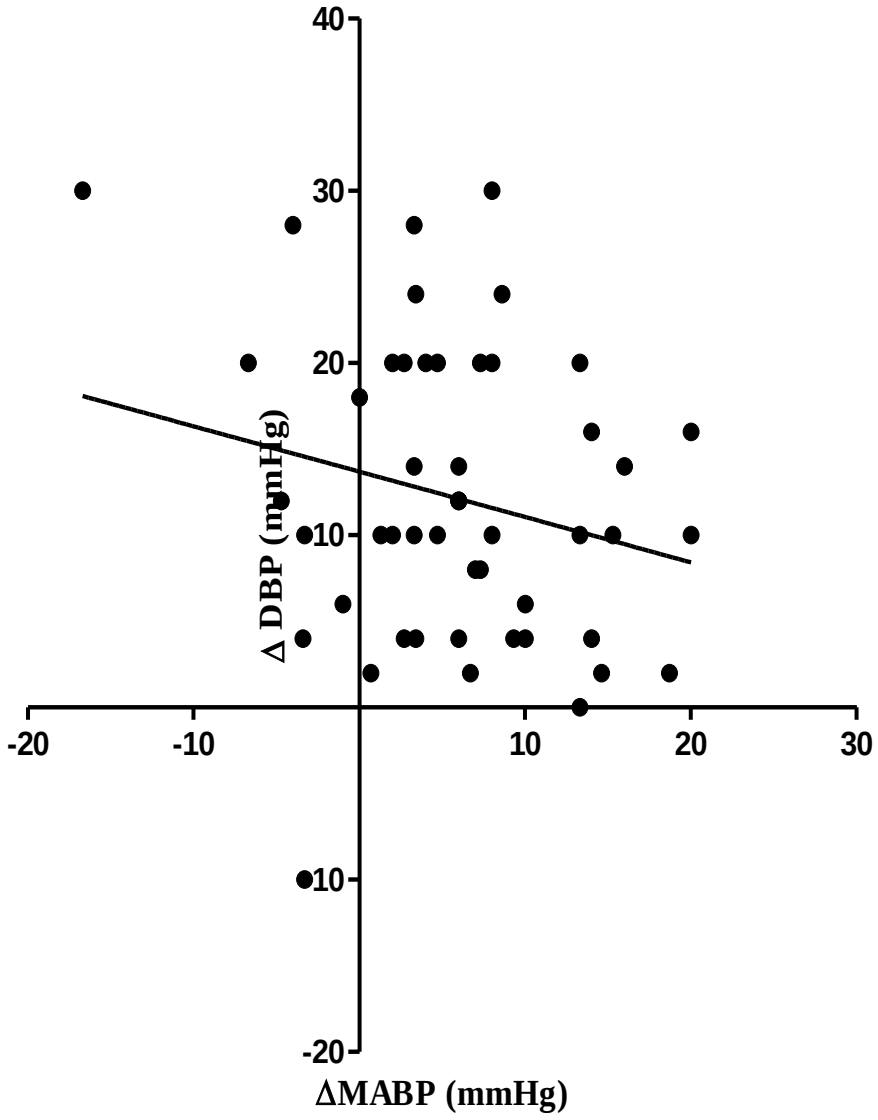


Figure 16b: Correlation between salt sensitivity and diastolic reactivity in hypertensive (HT) subjects after salt-loading

n = 50

NS = not significant

KEY:

ΔDBP = change in diastolic blood pressure; diastolic reactivity = ΔDBP ≥15mmHg;

ΔMABP = change in mean arterial blood pressure

4.3.3 Effect of Sympathetic Nervous System on Heart Rate of Normotensive and Hypertensive Subjects

4.3.3.1 Baseline Heart Rate in Normotensive and Hypertensive Subjects

Mean heart rate at baseline was 75.79±1.95 beats/min in the normotensive subjects which was significantly lower ($p < 0.05$) than the mean heart rate of 81.68 ± 1.77 beats/min recorded in the hypertensive subjects.

4.3.3.2 Heart Rate Response to Salt-Loading and Salt-Loading plus Amiloride in Normotensive and Hypertensive Subjects

Following salt-loading heart rate decreased slightly ($p > 0.05$) from 73.74±1.98 beats/min to 73.56±1.90 beats/min in normotensive subjects. Also heart rate reduced slightly ($p > 0.05$) among the hypertensive subjects from 81.17±1.73 beats/min to 79.37±1.72 beats/min following salt-loading (Figure 17).

Following ingestion of salt and amiloride for 5 days, heart rate in normotensive subjects fell to 71.28±2.49 beats/min (Figure 16). This was slightly lower than the heart rate when the subjects ingested salt alone (73.56±1.90 beats/min) for 5 days and also slightly lower ($p > 0.05$) than their heart rate at baseline (73.74±1.98 beats/min).

Ingestion of salt and amiloride caused a slight fall ($p > 0.05$) in heart rate from 79.37±1.72 beats/min in hypertensive subjects following salt-loading alone to 77.26±1.91 beats/min (Figure 17). When compared with baseline heart rate in these hypertensive subjects, the fall in heart rate caused by amiloride, from 81.17±1.73 beats/min to 76.89±1.91 beats/min was significant ($p <0.05$).

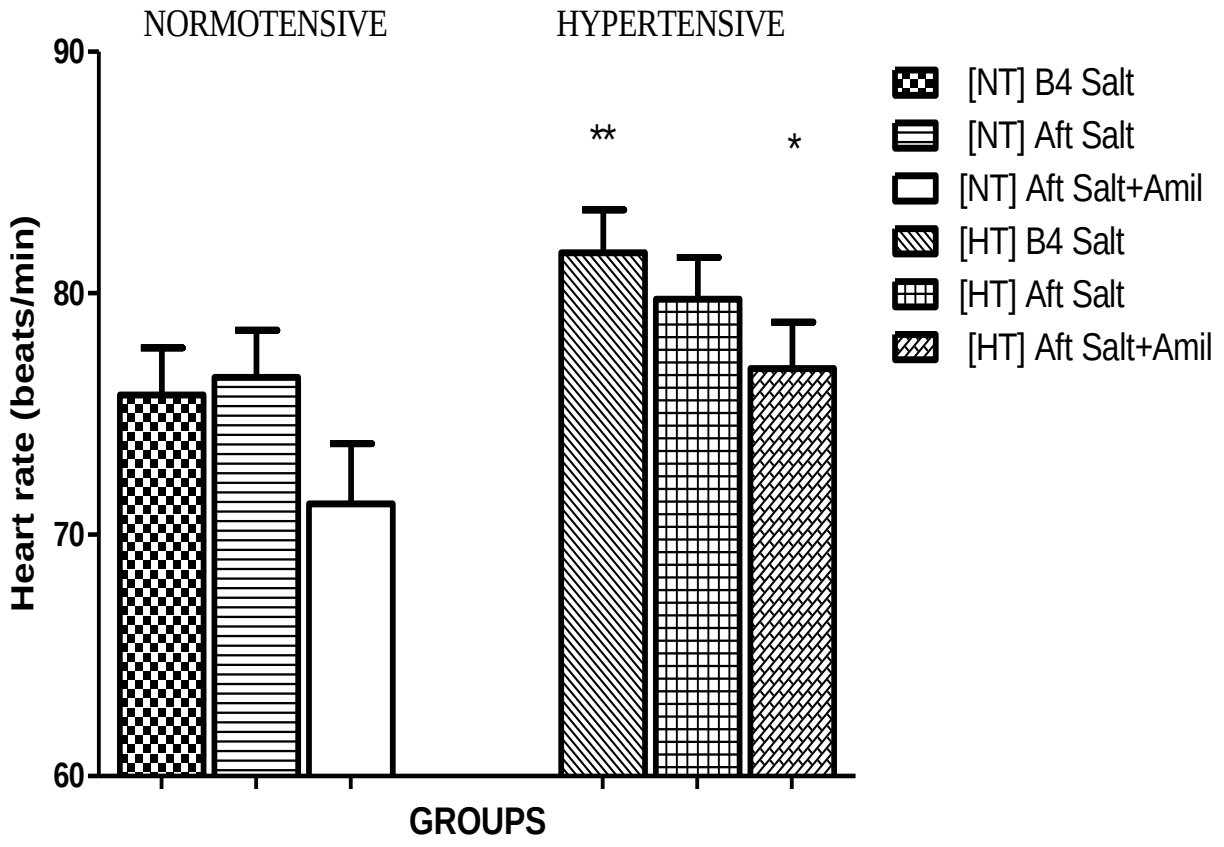


Figure 17: Heart rate response to salt-loading and salt-loading plus amiloride in normotensive (NT) and hypertensive (HT) subjects

NT n = 47; HT n = 53

** $p < 0.05$ - NT before salt versus HT before salt

* $p < 0.05$ HT B4 salt versus after salt + Amil in HT

KEY:

HR = heart rate; B4 = before salt-loading; Aft = after; Amil = amiloride

4.3.3.3 Heart Rate Response to the Cold Pressor Test in Normotensive and Hypertensive Subjects Before Salt-Loading, After Salt-Loading and After Salt-Loading Plus Amiloride

Before Salt-Loading

When exposed to the cold pressor test (CPT), heart rate increased slightly in normotensive subjects from 73.74± 1.98 beats/min to 74.24±2.19 beats/min before salt-loading. However, exposure to the CPT in hypertensive subjects before salt-loading led to a slight fall ($p > 0.05$) in heart rate from 81.17±1.73 beats/min to 80.28±1.85 beats/min (Figure 18).

After Salt-Loading and After Salt-Loading Plus Amiloride

After salt-loading, exposure of the normotensive subjects to the CPT caused a slight increase in heart rate from 73.56±1.90 beats/min to 75.50±1.84 beats/min (Figure 18).

In the same vein, following salt-loading for 5 days, exposure of the hypertensive subjects to the CPT led to a slight increase ($p > 0.05$) in heart rate from 79.37±1.72 beats/min to 80.71±1.73 beat/min.

Figure 18 also shows that following ingestion of the salt plus amiloride load, exposure of the normotensive subjects to the CPT led to a slight increase ($p > 0.05$) in heart rate from 71.28±2.49 beats/min to 72.06±2.59 beats/min. The figure also shows that following salt plus amiloride-loading, exposure to the CPT however caused a significant increase ($p < 0.01$) in heart rate from 77.26± 2.05 beats/min to 81.76±2.03 beats/min in the hypertensive subjects.

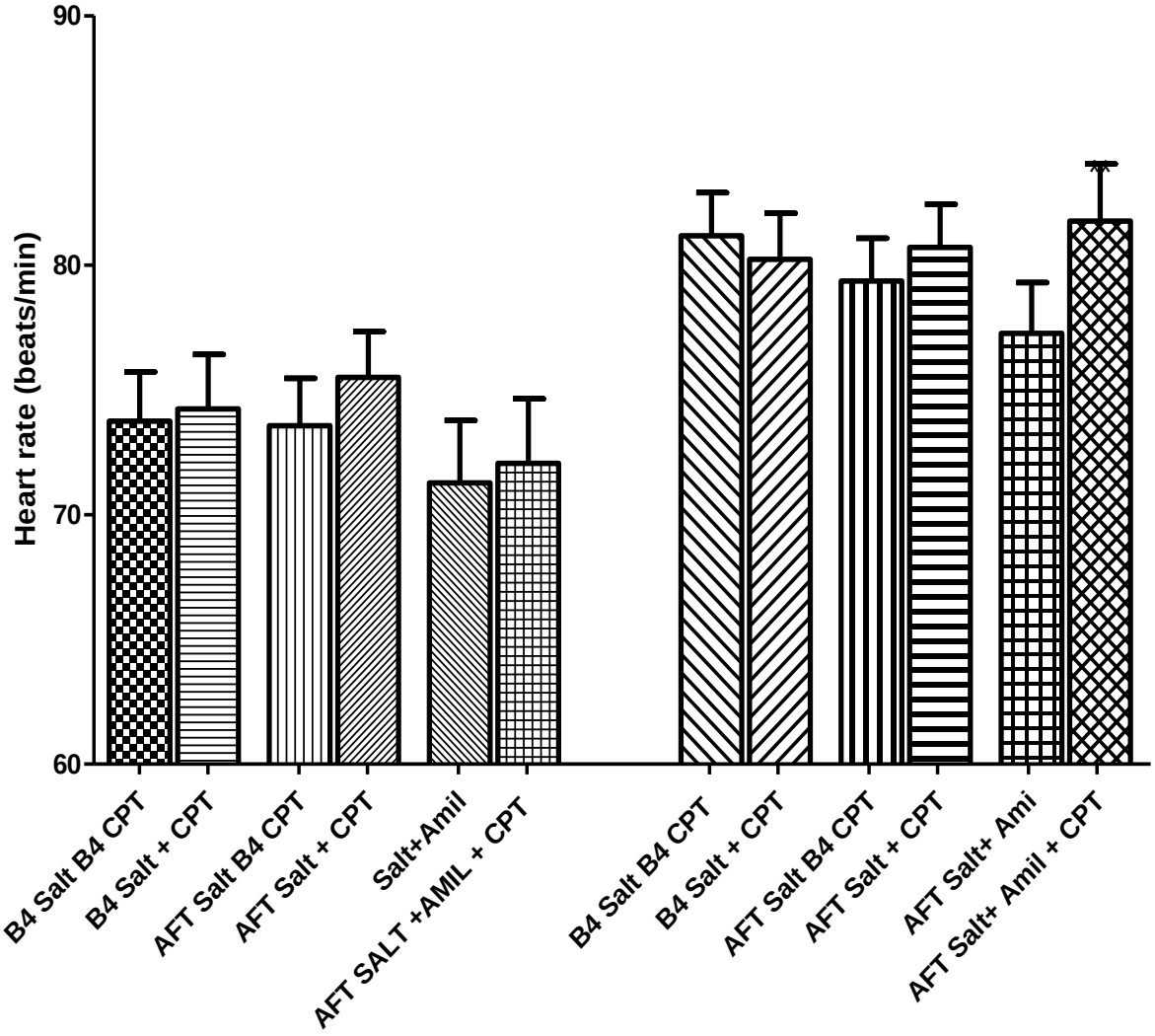


Figure 18: Heart rate response to the cold pressor test in normotensive (NT) and hypertensive (HT) subjects after salt-loading and after salt-loading plus amiloride

NT n = 41; HT n = 53

** = $p < 0.01$ - significant increase HT AFT Salt + Amil versus HT AFT Salt+Amil+CPT

KEY:

B4 = before; AFT = after; CPT = cold pressor test; AMIL = amiloride

4.4 Effect of Sympathetic Nervous System on Forearm Vascular Resistance of Normotensive and Hypertensive Subjects

Forearm vascular resistance is calculated as stated in Equation 7

$$\text{Forearm Vascular Resistance (mmHg/ml/s)} = \frac{\text{MABP (mmHg)}}{\text{Blood Flow (ml/s)}} \quad \text{..... Equation 7}$$

where MABP = mean arterial blood pressure

4.4.1 Forearm Blood Flow in Normotensive and Hypertensive Subjects Before Salt-Loading, After Salt-Loading and After Salt-Loading Plus Amiloride

Mean baseline forearm blood flow among the normotensive subjects was 1.14 ± 0.1 ml/s and this was slightly higher than the baseline forearm blood flow in the hypertensive subjects in whom mean baseline blood flow was 1.02 ± 0.09 ml/s.

Following salt-loading in normotensive subjects, forearm blood flow reduced slightly ($p > 0.05$) from mean baseline value of 1.14 ± 0.09 ml/s to 1.10 ± 0.12ml/s. There was also a slight lowering ($p > 0.05$) of blod flow among the hypertensive subjects from 1.014 ± 0.09 ml/s to 1.00 ± 0.08 ml/s after salt-loading (Figure 19).

Following ingestion of salt and amiloride, forearm blood flow in normotensive subjects reduced slightly ($p > 0.05$) from 1.10±0.12 ml/s to 1.03±0.09 ml/s. Similarly, forearm blood flow in hypertensive subjects reduced slightly ($p > 0.05$) after salt and amiloride ingestion from 1.00±0.08 ml/s after salt-loading only, to 0.94±0.07 ml/s (Figure 19).

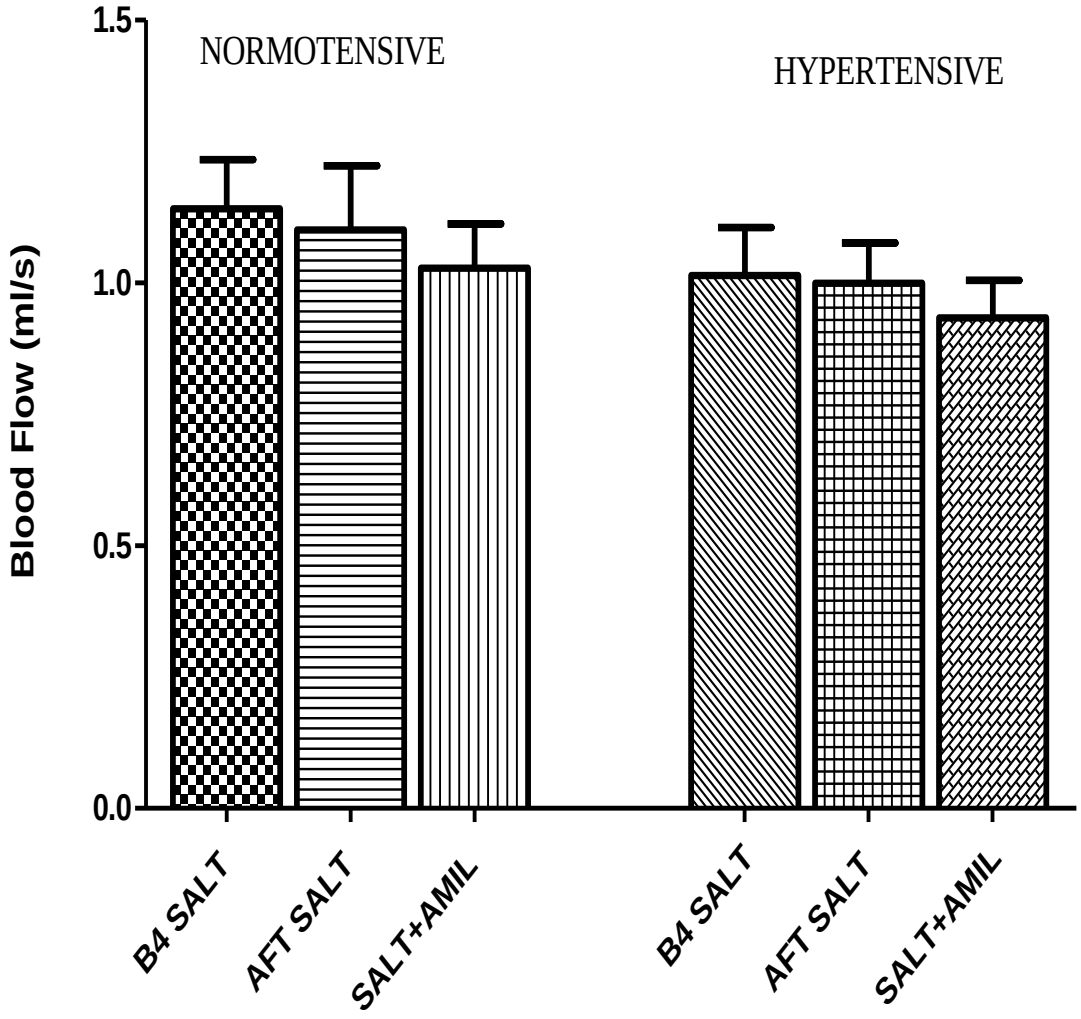


Figure 19: Forearm blood flow responses to salt-loading and salt-loading and amiloride in normotensive (NT) and hypertensive (HT) subjects

KEY:

B4 = before; AFT = after; AMIL = amiloride

4.4.1.2 Forearm Blood Flow Response to the Cold Pressor Test

Exposure to the cold pressor test (CPT) before salt-loading led to a significant reduction ($p < 0.001$) in forearm blood flow in normotensive subjects from 1.14±0.09 ml/s to 0.81±0.07 ml/s (Figure 20a). This was a percentage difference in blood flow of -25.48±3.9% (Figure 20b). Percentage diference in forearm blood flow was calculated as the percentage difference of the forearm blood flow after CPT and blood flow before CPT. It signifies the actual effect of the CPT on forearm blood flow.

Figure 20a also shows forearm blood flow reduced significantly ($p < 0.001$) in the hypertensive subjects when exposed to the CPT before salt-loading from 1.02±0.09 ml/s to 0.76±0.07 ml/s causing a percentage difference in forearm blood flow of -23.98±3.72% (Figure 20b). This was marginally lower ($p > 0.05$) than that in the normotensive subjects.

After salt-loading with 200mmol Na^+/day for 5 days, exposure to the cold pressor test (CPT) led to a significant reduction ($p < 0.001$) in forearm blood flow in normotensive subjects from 1.10±0.12 ml/s to 0.86±0.10 ml/s (Figure 20a). This led to a percentage difference in forearm blood flow of -21.42 ± 4.26% (Figure 20b).

Figure 20a also shows that forearm blood flow reduced significantly ($p < 0.001$) in the hypertensive subjects when exposed to the CPT after salt-loading from 1.02±0.08 ml/s to 0.74±0.05 ml/s. This was a percentage difference in forearm blood flow of -24.04±2.60% that was slightly higher ($p > 0.05$) than that recorded among the normotensive subjects (Figure 20b).

After salt-loading with 200mmol Na^+/day and 5mg amiloride daily for 5 days, exposure to the cold pressor test (CPT) led to a significant reduction ($p < 0.001$) in forearm blood flow in

normotensive subjects from 1.03±0.09 ml/s to 0.82±0.12 ml/s (Figure 20a). This led to an effect of -26.45±7.52% as percentage difference in blood flow (Figure 20b).

As shown in the same figure (Figure 20a), forearm blood flow also reduced significantly ($p < 0.001$) in the hypertensive subjects when exposed to the CPT after salt-loading plus amiloride from 0.95±0.07 ml/s to 0.71±0.06 ml/s. The percentage difference in forearm blood flow was -26.43±2.70% which was similar ($p > 0.05$) to that in the normotensive subjects (Figure 20b).

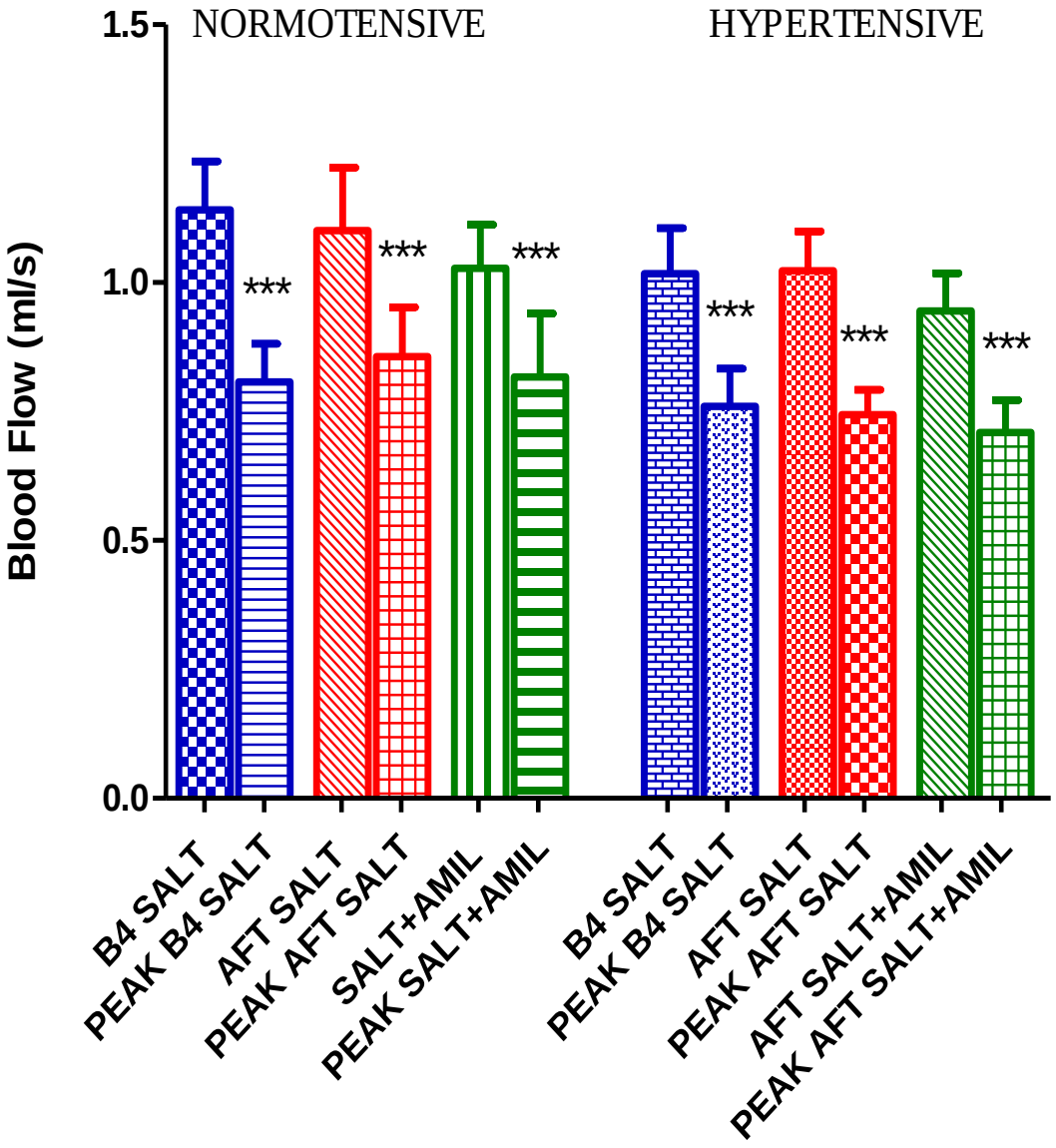


Figure 20a: Forearm blood flow responses to the cold pressor test (CPT) in normotensive (NT) and hypertensive (HT) subjects after salt-loading and salt-loading plus amiloride

***p <0.001 Significant difference before CPT versus after CPT

KEY:

B4 = Before salt; AFT = after salt; AMIL = amiloride; PEAK = after CPT

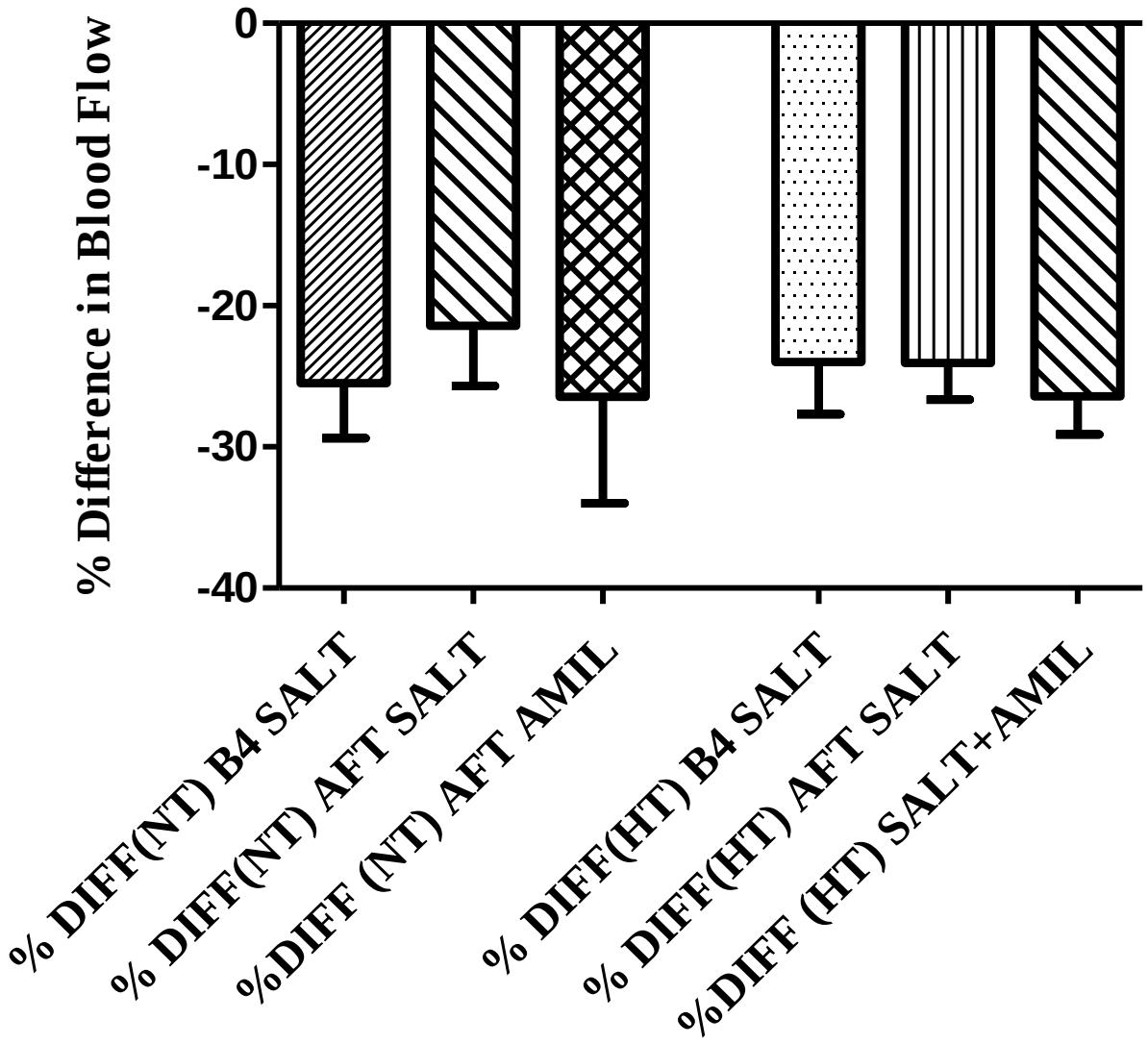


Figure 20b: Effect of the cold pressor test on rate of forearm blood flow in normotensive (NT) and hypertensive (HT) subjects

KEY:

DIFF = difference; B4 = before; AFT = after; AMIL = amiloride

4.4.2 Forearm Vascular Resistance in Normotensive and Hypertensive Subjects

The mean forearm vascular resistance in the normotensive subjects before intervention was 106.20±10.5 mmHg/ml/s (n = 38). This was significantly lower ($p < 0.05$) than the 149.30±13.4 mmHg/ml/s recorded among the hypertensive subjects at baseline (n = 51) (Figure 21).

As shown in the same figure, forearm vascular resistance increased slightly ($p > 0.05$) among normotensive subjects following salt-loading from the baseline value of 115.5±14.00 mmHg/ml/s to 117.70±10.0 mmHg/ml/s. On the other hand, there was a slight reduction ($p > 0.05$) in forearm vascular resistance among hypertensive subjects following salt-loading from 149.6±13.4 mmHg/ml/s to 136.30±9.1 mmHg/ml/s.

Results of this study show that in normotensive subjects, salt plus amiloride-loading caused a significant fall ($p < 0.01$) in forearm vascular resistance from 117.70±10.0 mmHg/ml/s recorded after salt-loading alone to 92.22±8.0 mmHg/ml/s. However, this value was only slightly less ($p > 0.05$) than the baseline value of 115.5±14.00 mmHg/ml/s (Figure 21).

Among the hypertensive subjects on the other hand, forearm vascular resistance increased slightly ($p > 0.05$) from 136.3±9.1 mmHg/ml/s recorded after salt-loading alone to 138.9±12.8 mmHg/ml/s. This forearm vascular resistance recorded after salt and amiloride ingestion was only slightly less ($p > 0.05$) than the baseline value of 149.6±13.4 mmHg/ml/s.

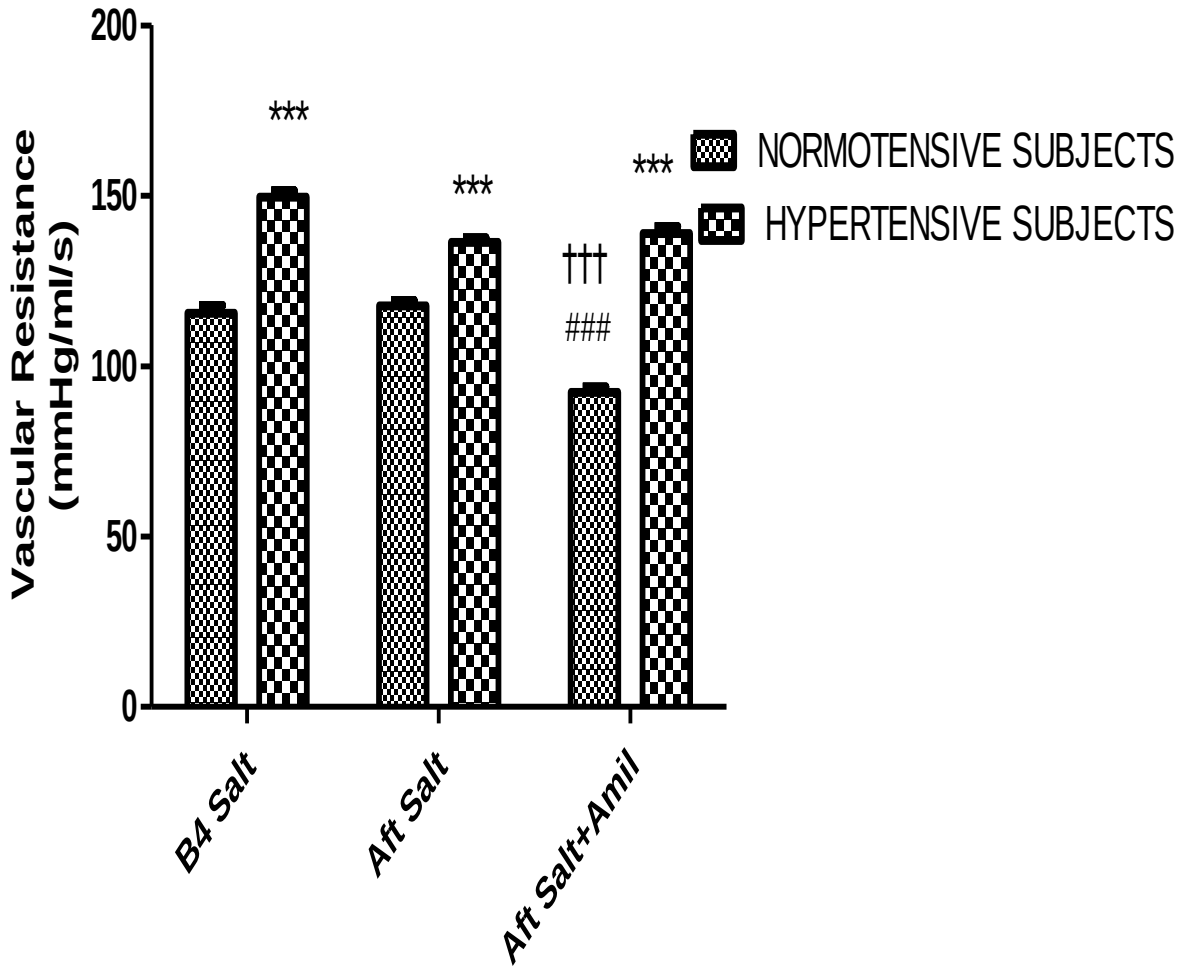


Figure 21: Effect of salt and amiloride on forearm vascular resistance in normotensive (NT) and hypertensive (HT) subjects

NT n = 39; HT n = 51

*** $p < 0.001$ NT versus HT all groups

$p < 0.001$ Baseline versus after salt+amiloride NT

††† $p < 0.001$ after salt-loading alone versus after salt+amiloride NT

KEY: B4 = before; Aft = after

4.4.3 Response of Forearm Vascular Resistance to the Cold Pressor Test Before Salt-Loading, After Salt-Loading and After Salt-Loading Plus Amiloride

Exposure to the cold stress resulted in significant increase ($p < 0.001$) in forearm vascular resistance among the normotensive subjects from 106.20±10.5 mmHg/ml/s before salt-loading to 176.20±18.0 mmHg/ml/s (Figure 22a) with a percentage increase (%Δ) of 74.93±11.3% (Figure 22c).

Similarly, forearm vascular resistance increased significantly ($p < 0.001$) in the hypertensive subjects from 149.30±13.4 mmHg/ml/s at baseline, to 264.20±29.0 mmHg/ml/s on exposure to the CPT before salt-loading (Figure 22b) giving a %Δ of 78.33±13.6%. These percentage increases were however similar ($p > 0.05$) in both groups of subjects (Figure 22c).

There was a significant increase in vascular resistance among the normotensive subjects from 118.00±9.75 mmHg/ml/s after salt-loading alone, to 202.90±26.97 mmHg/ml/s ($p < 0.001$) following exposure to the CPT after salt-loading (Figure 22a). This represented a percentage increase of 61.85±8.6% in vascular resistance and was less than ($p > 0.05$) the 74.93±11.3% observed before salt-loading among the normotensive subjects (Figure 22c).

Figure 22b also shows the response of the hypertensive subjects to the CPT after salt-loading. There was an increase ($p < 0.001$) in vascular resistance from 142.40±10.82 mmHg/ml/s to 227.70±18.75 mmHg/ml/s. This led to a percentage increase of 76.46±16.6% in vascular resistance following salt-loading which was slightly less ($p > 0.05$) than the 78.33±13.6% recorded before salt-loading (Figure 22c).

As shown also in Figure 22a, there was a significant increase in vascular resistance among the normotensive subjects from 89.92±7.91 mmHg/ml/s after salt plus amiloride-loading alone to

167.90±19.46 mmHg/ml/s ($p < 0.001$) on exposure to the CPT after salt plus amiloride-loading. This was a percentage increase of 91.78±18.4% in vascular resistance which was significantly higher ($p < 0.05$) than the 61.85±8.6% recorded after salt-loading alone but marginally different ($p > 0.05$) from the 74.93±11.3% recorded before salt-loading in these normotensive subjects (Figure 22c).

Figure 22b also shows that the hypertensive subjects also responded with an increase in vascular resistance from 144.60±13.70 mmHg/ml/s after salt plus amiloride-loading only to 270.20±32.10 mmHg/ml/s on exposure to the CPT after salt plus amiloride-loading ($p < 0.001$). The percentage increase in vascular resistance was 79.14±10.3% was not significantly different ($p > 0.05$) from the 76.46±16.6% and the 78.33±13.6% recorded in these subjects after salt-loading and before salt-loading respectively (Figure 22c).

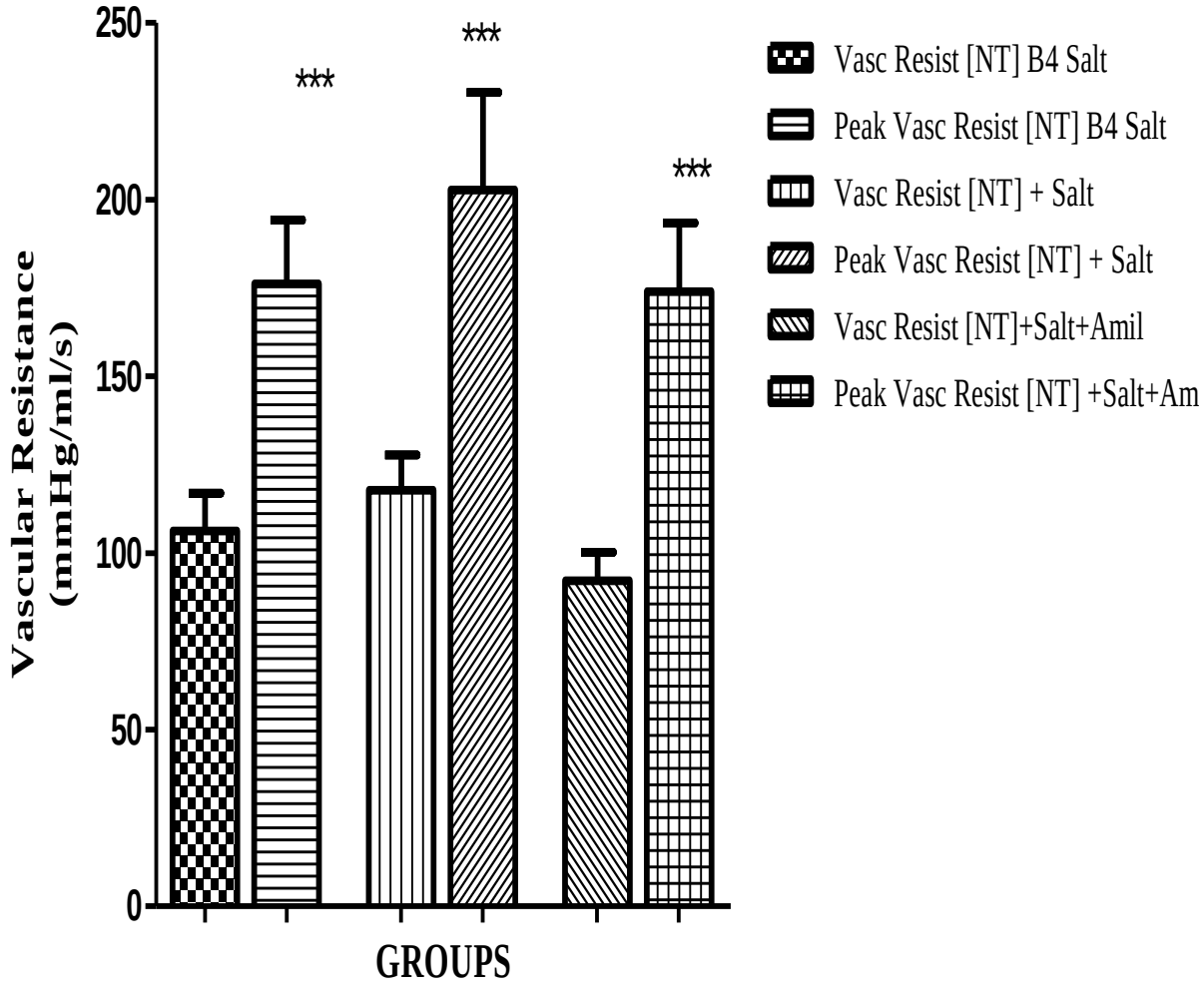


Figure 22a: Vascular resistance response to the cold pressor test (CPT) in normotensive subjects before salt-loading, after salt-loading and after salt-loading plus amiloride

n = 39

*** $p < 0.001$ = significant effect of CPT before salt, after salt-loading and after salt plus amiloride-loading

KEY:

Vasc Resist = vascular resistance; B4 = before; Amil = amiloride; PEAK = after CPT

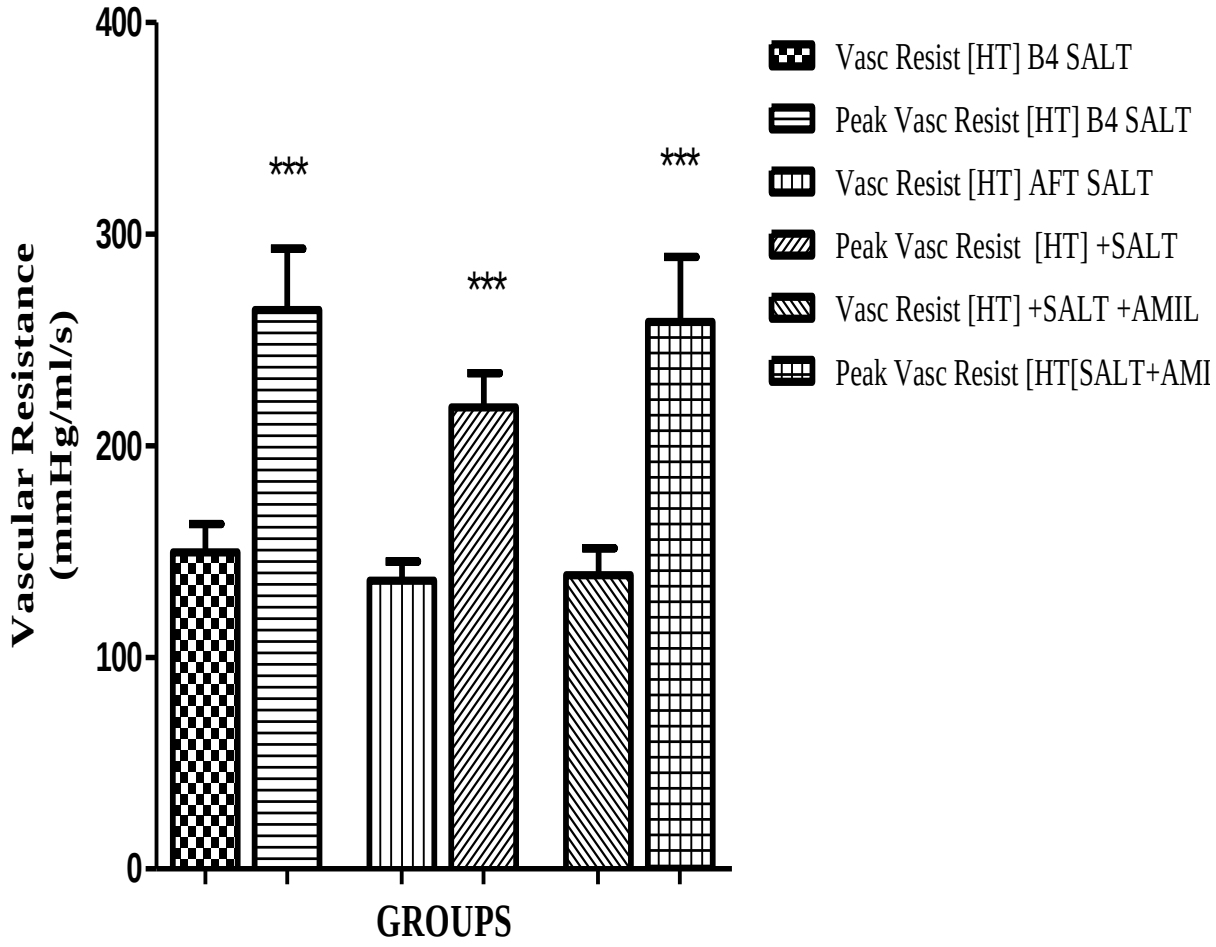


Figure 22b: Vascular resistance response to the cold pressor test (CPT) in hypertensive subjects before salt-loading, after salt-loading and after salt-loading plus amiloride

n = 52

*** $p < 0.001$ = significant effect of CPT before salt, after salt-loading and after salt plus amiloride-loading

KEY:

Vasc Resist = vascular resistance; B4 = before; Amil = amiloride; PEAK = after CPT

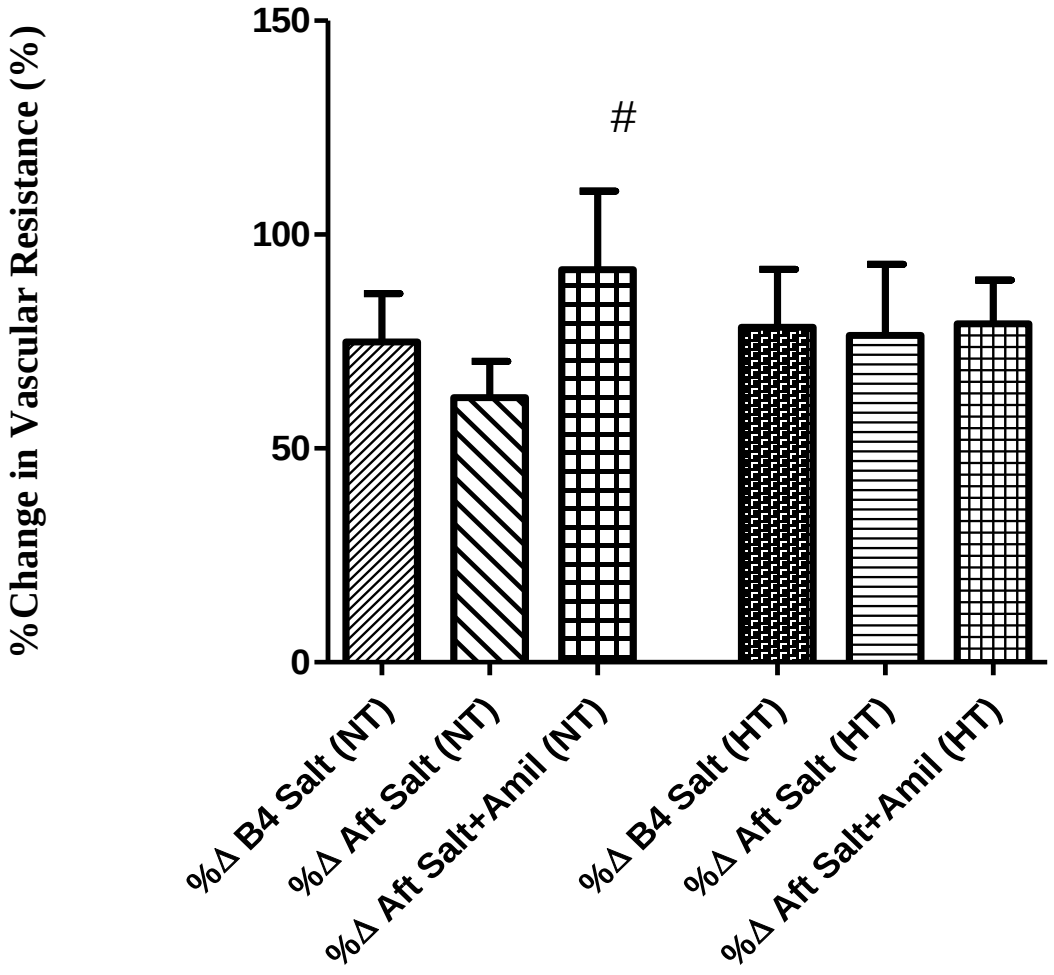


Figure 22c: Effect of the cold pressor test on vascular resistance in normotensive (NT) and hypertensive (HT) subjects before salt-loading, after salt-loading and after salt-loading plus amiloride

NT n =39; HT n = 52

# $p < 0.05$ %Δ Aft Salt+Amil compared with Aft Salt only (NT)

KEY: B4 = before; Aft = after; Amil = amiloride

4.5 Relationship between ENaC Markers and Salt Sensitive Hypertension

4.5.1 Blood Pressure Response to Salt-Loading and Salt-Loading Plus Amiloride

4.5.1.1 Blood Pressure Response to Salt-loading

After five days of salt-loading with 200mmol Na^+, systolic blood pressure (SBP) increased significantly in both groups of subjects. In normotensive subjects, systolic blood pressure (SBP) increased significantly ($p < 0.05$) from the baseline value of 117.50±1.54 mmHg to 121.10±2.05 mmHg (Figure 23a). The figure also shows that in hypertensive subjects, SBP increased significantly ($p < 0.001$) from 145.50±2.64 mmHg at baseline to 152.40±3.03 mmHg after salt-loading.

Following the same intervention, diastolic blood pressure (DBP) increased slightly among the normotensive subjects from 79.94±0.86 mmHg to 80.56±1.54 mmHg after salt-loading (Figure23b) while a more significant increase ($p < 0.001$) in DBP was recorded among the hypertensive subjects from 96.07±1.54 mmHg at baseline to 100.7±1.61 mmHg (Figure 23b).

In normotensive subjects, salt-loading led to a slight increase in mean arterial blood pressure (MABP) from 92.34±0.86 mmHg to 94.39±1.54 (Figure 23c). However in hypertensive subjects also shown in Figure 23c, MABP was significantly raised ($p < 0.001$) by salt-loading from 112.7±1.56 mmHg to 118.30±1.80 mmHg.

4.5.1.2. Blood Pressure Response to Salt-loading Plus Amiloride

When the subjects were given 5mg amiloride in addition to the 200 mmol/day Na^+, as shown in Figure 23a, SBP in normotensive subjects fell to 115.20±1.80 mmHg which was significantly less ($p < 0.05$) than the SBP at baseline and also lower ($p < 0.001$) than that after salt-loading alone. Amiloride also caused significant reductions in SBP in hypertensive subjects to 132.8±2.81 mmHg which was less than that at baseline ($p < 0.001$) and also less ($p < 0.001$) than that after salt-loading alone (Figure 23a).

As shown in Figure 23b, ingestion of salt and amiloride for 5 days resulted in a significant fall in DBP in normotensive subjects. Mean DBP fell to 77.45±1.46 mmHg which was lower ($p < 0.05$) than their DBP at baseline and also lower ($p < 0.01$) than their DBP after salt-loading alone. The figure also shows a significant reduction in DBP in hypertensive subjects following salt and amiloride ingestion to 90.74±1.70 mmHg. This new DBP was significantly lower ($p < 0.001$) than their DBP at baseline and lower ($p < 0.001$) than the DBP following salt-loading alone.

In normotensive subjects, mean arterial blood pressure (MABP) fell significantly ($p < 0.01$) from the baseline levels of 92.03±083 mmHg to 89.65±1.23 mmHg after salt plus amiloride ingestion (Figure 23c). This level of MABP after salt plus amiloride was significantly lower ($p < 0.001$)than the 94.39 mmHg recorded after salt-loading alone (Figure 23c). Also, in hypertensive subjects, salt plus amiloride led to a fall in MABP to 104.2±1.86 mmHg which was significantly lower ($p < 0.001$) than the 112.7±1.56 mmHg recorded at baseline and also significantly lower ($p < 0.001$) than the118.3±1.80 mmHg recorded after salt-loading alone (Figure 23c).

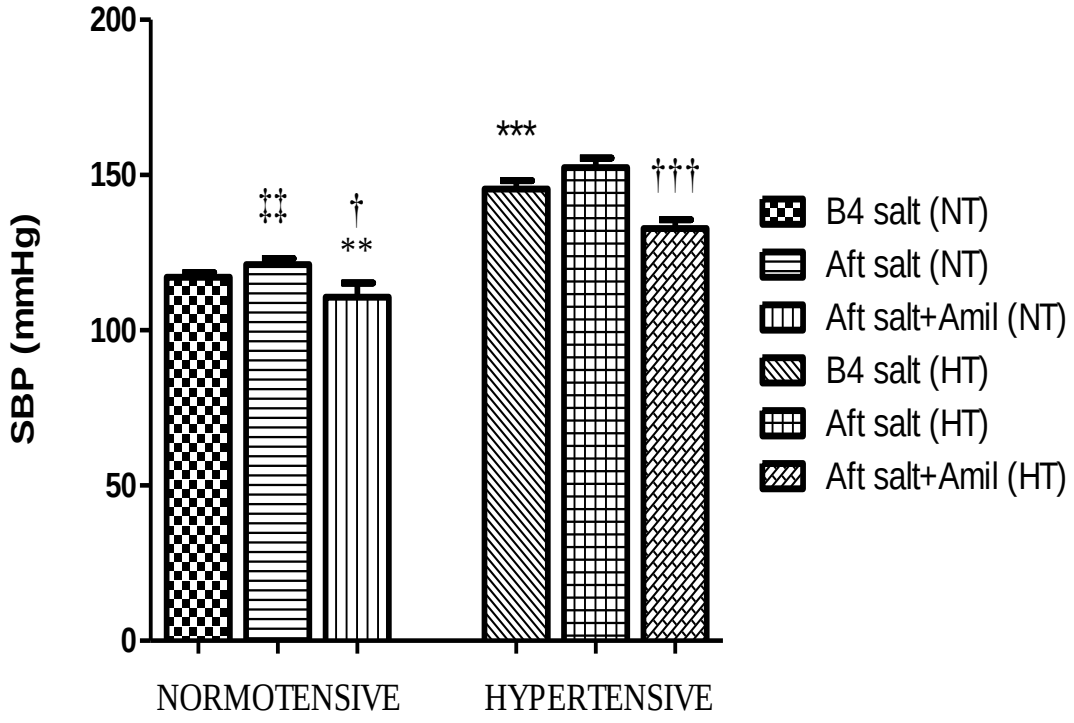


Figure 23a: Systolic blood pressure (SBP) response to salt-loading and salt-loading plus amiloride in normotensive (NT) and hypertensive (HT) subjects

NT n = 47; HT n = 53

*** p <0.001 NT B4 salt versus HT B4 salt

‡‡ p < 0.01 NT aft salt versus NT B4 salt

† p < 0.05 NT aft salt + Amil versus NT B4 salt

** p < 0.01 NT aft salt + Amil versus NT aft salt

††† p < 0.001 aft salt + Amil HT versus B4 salt HT

††† p < 0.001 aft salt + Amil HT versus aft salt HT

KEY:

B4 = before salt; aft = after salt; Amil = Amiloride

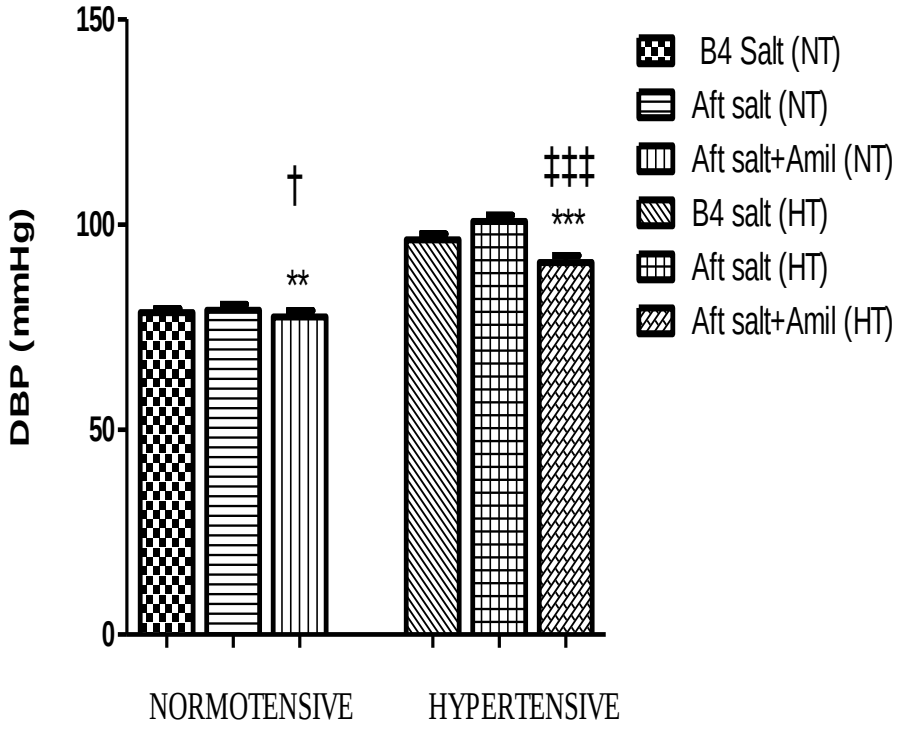


Figure 23b: Diastolic blood pressure response to salt-loading and salt-loading plus amiloride in normotensive (NT) and hypertensive (HT) subjects

NT n = 47; HT n = 53

*** p <0.001 NT B4 salt versus HT B4 salt

† p < 0.05 NT aft salt+Amil versus NT B4 salt

** p < 0.01 NT aft salt+Amil versus NT aft salt

††† p < 0.001 HT aft salt+Amil versus HT B4 salt

††† p < 0.001 HT aft salt+Amil versus HT aft salt

KEY:

B4 = before salt; aft = after salt; Amil = Amiloride

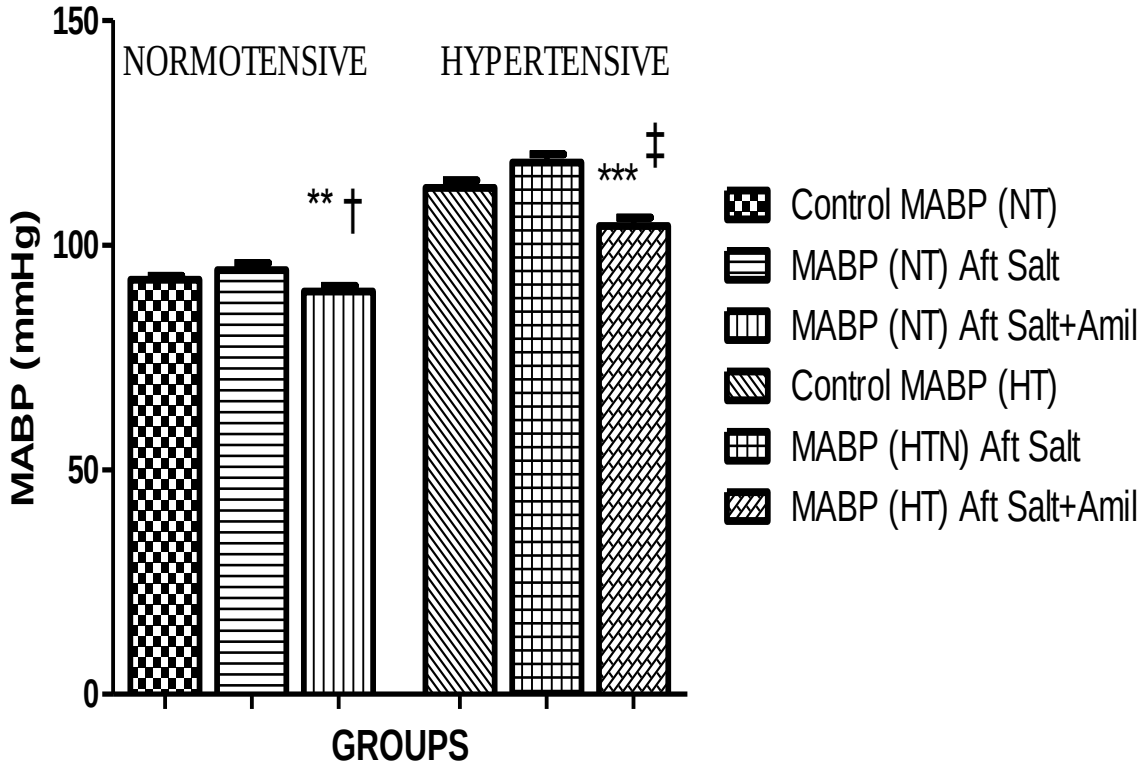


Figure 23c: Mean arterial blood pressure (MABP) response to salt-loading and salt-loading plus amiloride in normotensive (NT) and hypertensive (HT) subjects

NT n = 47; HT n = 53

** p <0.01 NT B4 salt versus NT Aft Salt+Amil

† p < 0.001 NT Aft Salt versus NT Aft Salt+Amil

** p < 0.01 NT Aft Salt+Amil versus NT Aft Salt

*** p < 0.001 HT B4 Salt versus HT Aft Salt+Amil

‡ p < 0.001 HT Aft Salt versus HT Aft Salt+Amil

KEY:

B4 = before salt; aft = after salt; Amil = Amiloride

4.5.2. Effect of Amiloride on Response to Salt-Loading

4.5.2.1. Effect of Amiloride on Mean Arterial Blood Pressure

The effect of amiloride on mean arterial blood pressure (MABP) was determined from the percentage difference in the MABP response to salt-loading alone and to salt-loading plus amiloride compared with baseline.

$$\%MABP = \frac{MABP_{Salt+amiloride} - MABP_{Salt}}{MABP_{Salt}} \times 100$$

As shown in Figure 24, whereas salt-loading alone caused an increase of 2.83±1.34% in MABP of normotensive subjects (% Diff salt minus B4 salt), amiloride reduced the MABP significantly ($p < 0.001$) by -8.17±1.96% from that recorded after the salt load alone [(% Diff salt minus B4 salt) – (%Diff Salt+Amil – Salt)]. When the effect of amiloride was compared with the baseline MABP (% Diff Salt+Amil – B4 Salt), there was a significant reduction ($p< 0.001$) in MABP of -3.64±1.31%.

As shown in the same figure, when amiloride was given with the salt load in hypertensive subjects, it caused a significant reduction ($p < 0.01$) of -11.08±1.06% in MABP compared with the increase of 4.79±1.01% caused by the ingestion of salt alone (% Diff salt minus salt + amiloride). Amiloride also had an effect of significantly reducing MABP by -6.91±1.21% ($p <0.001$) from that recorded at baseline in hypertensive subjects (% Diff Amil+salt – B4 Salt).

Figure 24 also shows however, that the magnitude of the effect of amiloride on MABP was similar among the normotensive and hypertensive subjects.

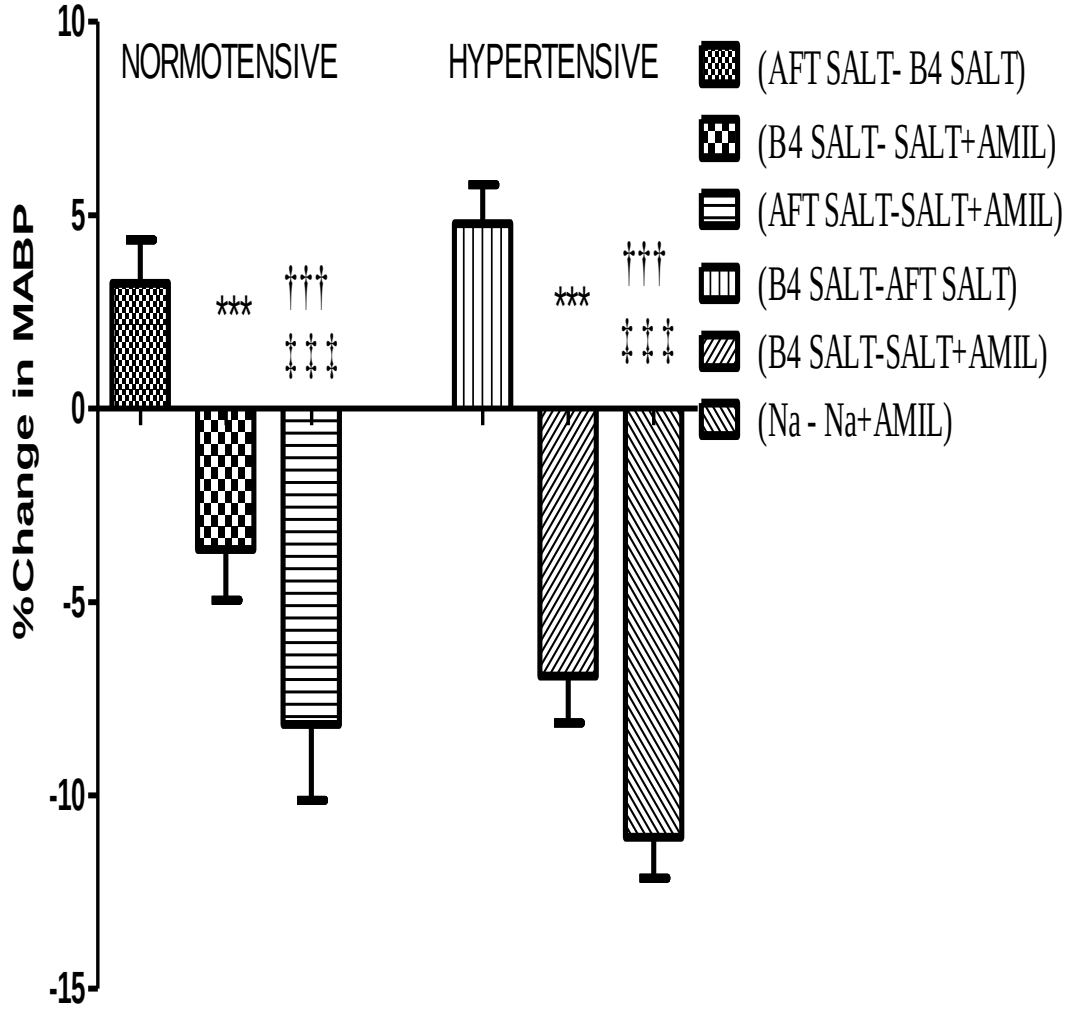


Figure 24: Effect of salt-loading plus amiloride on mean arterial blood pressure (MABP) in normotensive (NT) and hypertensive (HT) subjects

NT n = 22; HT n = 43

*** $p < 0.001$ effect of salt + amiloride versus effect of salt alone (NT) and (HT)

††† $p < 0.01$ effect of amiloride alone versus effect of salt + amiloride (NT) and (HT)

‡ ‡ ‡ $p < 0.01$ effect of amiloride alone versus effect of salt alone (NT) and (HT)

KEY:

B4 = before salt-loading; AFT = after salt-loading; Amil = amiloride; %Δ = percentage change

Effect of salt alone = %Δ (Aft salt - B4 Salt)

Effect of salt + amiloride = %Δ (Salt+Amil - B4 Salt)

Effect of Amiloride alone = %Δ (Salt+Amil - Aft Salt)

4.5.3. Plasma Potassium Concentration in Normotensive and Hypertensive Subjects Before Salt, After Salt-Loading and After Salt-Loading Plus Amiloride

Plasma potassium (K^+) concentration was 3.96±0.09 mmol/L in the normotensive subjects at baseline. In the hypertensive subjects, plasma K^+ concentration was 3.70±0.10 mmol/L before salt-loading. This was significantly lower ($p < 0.05$) than that in the normotensive subjects (Table 8).

Following salt-loading, plasma K^+ concentration reduced significantly from the baseline level to 3.73±0.10 mmol/L in the normotensive subjects, $p < 0.05$. Among the hypertensive subjects, there was a marginal reduction in plasma K^+ concentration to 3.40±0.08 mmol/L following salt-loading (Table 8).

When normotensive subjects ingested salt and amiloride, plasma K^+ concentration increased slightly ($p > 0.05$) from the 3.73±0.10 mmol/L recorded after salt ingestion alone to 3.79±0.14 mmol/L. This was slightly higher ($p > 0.05$) than the 3.96±0.09 mmol/L recorded at baseline (Table 8).

Among the hypertensive subjects on the other hand, plasma K^+ concentration increased significantly ($p < 0.001$) from the 3.40±0.08 mmol/L recorded following salt-loading alone to 3.92±0.08 mmol/L after salt+amiloride ingestion. The plasma K^+ concentration measured after salt+amiloride ingestion was also significantly higher than that at baseline in the hypertensive subjects ($p < 0.05$) (Table 8).

Table 8: Plasma potassium concentration in normotensive and hypertensive subjects

Groups / Intervention	Normotensive (NT) (mmol/L) n = 29	Hypertensive (HT) (mmol/L) n = 33
Before salt	3.96±0.09	3.70±0.08*
After salt	3.73±0.10#	3.40±0.08
After salt+amiloride	3.79±0.14	3.92±0.08 † ***

Data are expressed as Mean±S.E.M.

*p < 0.05 HT before salt compared with NT before salt

# p < 0.05 compared with before salt (NT)

† p < 0.05 compared with before salt (HT)

*** p < 0.001 compared with after salt (HT)

4.5.4 Urine Potassium Concentration in Normotensive and Hypertensive Subjects

Normotensive subjects had a mean urine potassium concentration [K^+] of 13.70±1.75 mmol/L before salt-loading. This reduced marginally to 13.25±1.74 mmol/L following salt-loading. However, upon salt+amiloride-loading, urine potassium excretion reduced significantly ($p < 0.01$) to 7.68±1.40 mmol/L when compared with the baseline level (Figure 25).

On the other hand, the hypertensive subjects had a mean urine [K^+] of 10.05±0.95 mmol/L before salt-loading. On ingestion of the sodium load, urine [K+] reduced slightly to 9.89±1.20 mmol/L. When given the salt plus amiloride for 5 days, mean urine [K+] fell further to 7.74±0.99 mmol/L (Figure 25). This was significantly lower ($p < 0.05$) than the baseline level.

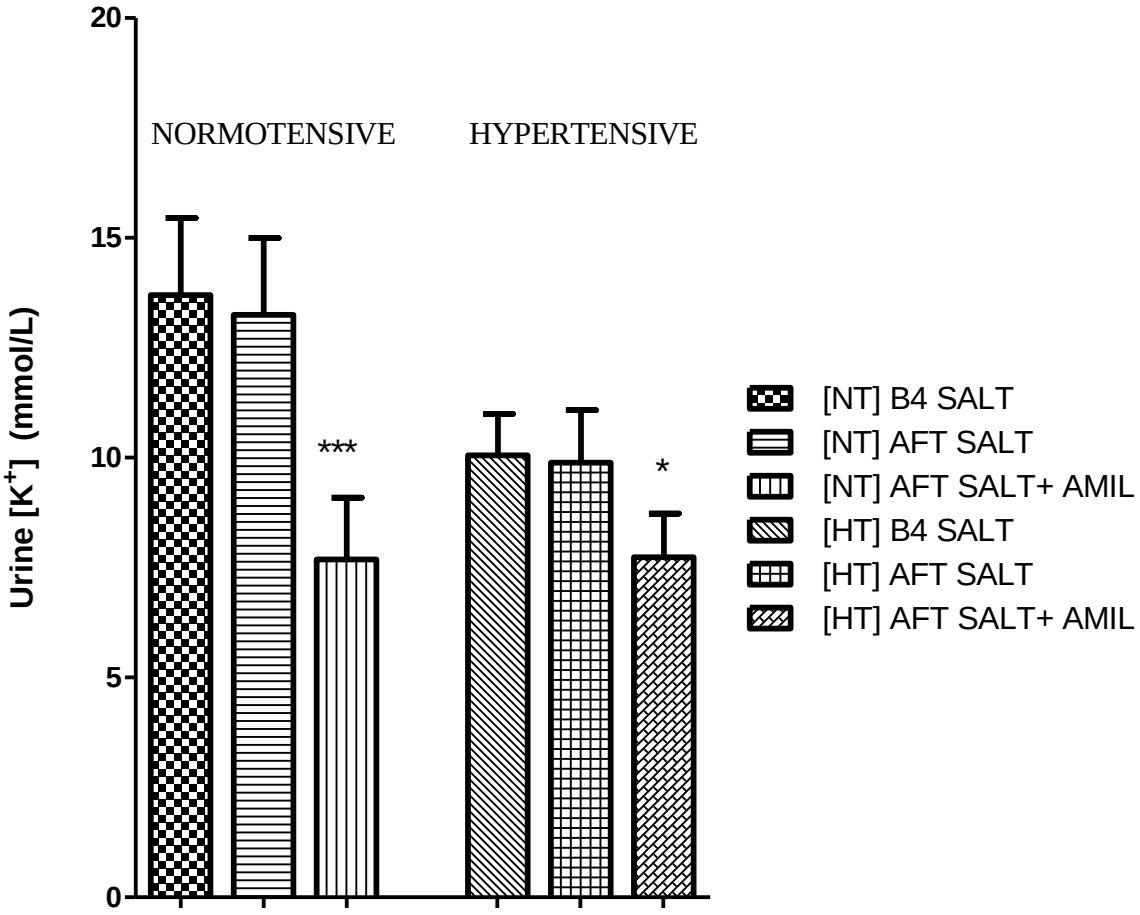


Figure 25: Urine potassium concentration in normotensive (NT) and hypertensive (HT) subjects before salt-loading, after salt-loading and after salt-loading plus amiloride

NT n = 24; HT n = 30

*** p <0.01 compared with B4 SALT

* p <0.05 compared with AFT SALT

KEY:

B4 = before; AFT = after

4.5.5 Effect of Salt-Loading and Salt-Loading Plus Amiloride on Plasma Renin Activity and Aldosterone

4.4.5.1 Effect of Salt-Loading and Salt-Loading Plus Amiloride on Plasma Renin Activity

At baseline, Plasma Renin Activity (PRA) was 22.67±5.13 mIU/L among normotensive subjects. This was significantly higher ($p < 0.05$) than the 11.68±2.58 mIU/L measured among the hypertensive subjects (Figure 26).

Following salt-loading, PRA of the normotensive subjects increased significantly ($p < 0.05$) from the baseline value to 37.24±5.92 mIU/L, (Figure 26). As shown in the same figure, salt-loading also caused a significant increase ($p< 0.05$) in PRA among the hypertensive subjects to 19.12±4.02mIU/L.

Following salt plus amiloride-loading, PRA decreased significantly ($p < 0.05$) to 26.07±3.37 mIU/L in the normotensive subjects from the level after salt-loading alone. Among hypertensive subjects, ingestion of salt plus amiloride resulted into a significant increase ($p < 0.05$) in PRA to 28.60±2.96 mIU/L from the level after salt-loading alone. This was also significantly higher ($p < 0.001$) than the baseline level in these hypertensive subjects (Figure 26).

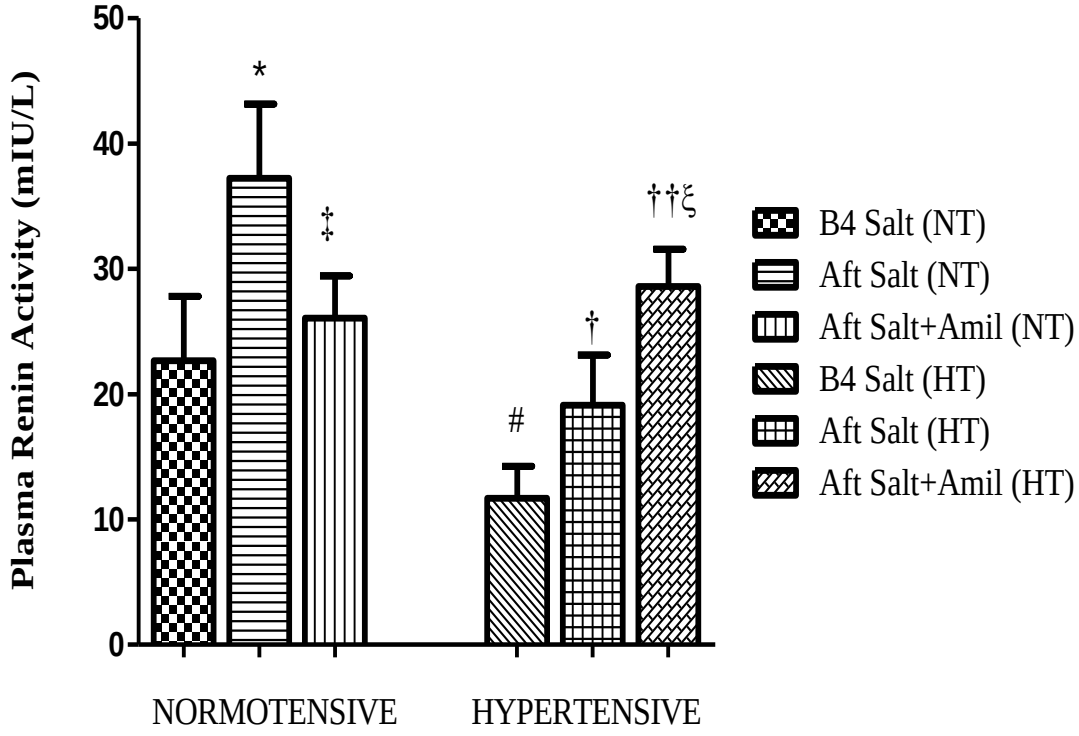


Figure 26: Effect of salt-loading and salt plus amiloride loading on Plasma Renin Activity (PRA) in normotensive (NT) and hypertensive (HT) subjects

NT n = 11; HT n = 22

* p <0.05 - significantly lower compared with NT B4 Salt

‡ p < 0.05 - compared with after salt-loading in NT

† p < 0.05 - compared with before salt-loading in NT and HT

†† p <0.001 - compared with B4 salt in HT

ξ p < 0.05 - compared with Aft salt-loading in HT

KEY:

B4 = before; Aft = after; amil = amiloride

4.5.5.2 Effect of Salt-Loading and Salt-Loading plus Amiloride on Serum Aldosterone

Before salt-loading, serum aldosterone level was 428.20±46.29 pg/ml among the normotensive subjects. Following salt-loading, serum aldosterone reduced slightly ($p > 0.05$) to 360.00±53.95 pg/ml. On ingestion of salt and amiloride for 5 days, serum aldosterone reduced significantly ($p < 0.001$) to 232.90±34.57 pg/ml from the level after salt-loading alone. The serum aldosterone concentration after salt plus amiloride ingestion was also significantly lower ($p < 0.01$) than that before salt-loading (Figure 27).

Among the hypertensive subjects, serum aldosterone level was 545.90±69.05 pg/ml before salt-loading. Following salt-loading, serum aldosterone reduced significantly to 419.50±48.95 pg/ml ($p < 0.05$). On loading with salt and amiloride for 5 days, serum aldosterone increased ($p > 0.05$) to 442.10±67.06 pg/ml. This was slightly less ($p > 0.05$) than the baseline value before salt-loading (Figure 27).

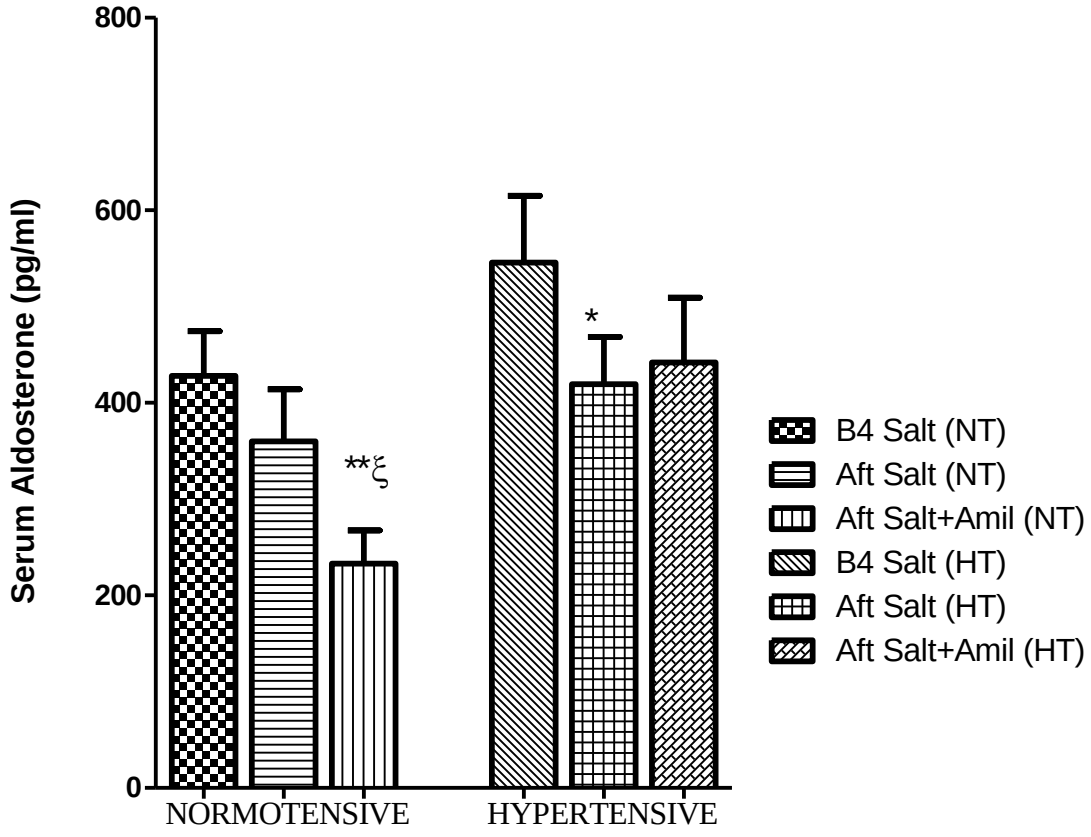


Figure 27: Serum aldosterone in normotensive (NT) and hypertensive (HT) subjects before salt-loading, after salt-loading and after salt-loading plus amiloride

NT n = 11; HT n = 15

** $p < 0.01$ - compared with before salt-loading NT

ξ $p < 0.001$ - compared with after salt-loading NT

* $p < 0.05$ - compared with before salt-loading HT

KEY:

B4 = before; Aft = after; Amil = amiloride

4.6.0 Genetic Variants of ENaC and Salt Sensitive Hypertension

4.6.1 Results of Polymerase Chain Reaction Tests

The agarose gel electrophoresis of the amplicons showed that the DNA fragments had between 350 base pairs (bp) and 400bp (Figure 28), averagely 375bp.

The concentration of the DNA in the PCR products as determined by the Nanodrop Spectrophotometer ranged between 98.9 ng/ul to 100.1 ng/Ul. However, the ratio of the absorbance at 260nm to 280nm varied typically as shown in Table 9. A typical tracing from the Nanodrop Spectrophotometer is shown in Figure 29.

Figure 30 shows the scatter plot obtained from data analysis of the TaqMan$^{(R)}$ SNP Genotyping assay. In this particular run, four (4) of the samples had the T594M polymorphism.

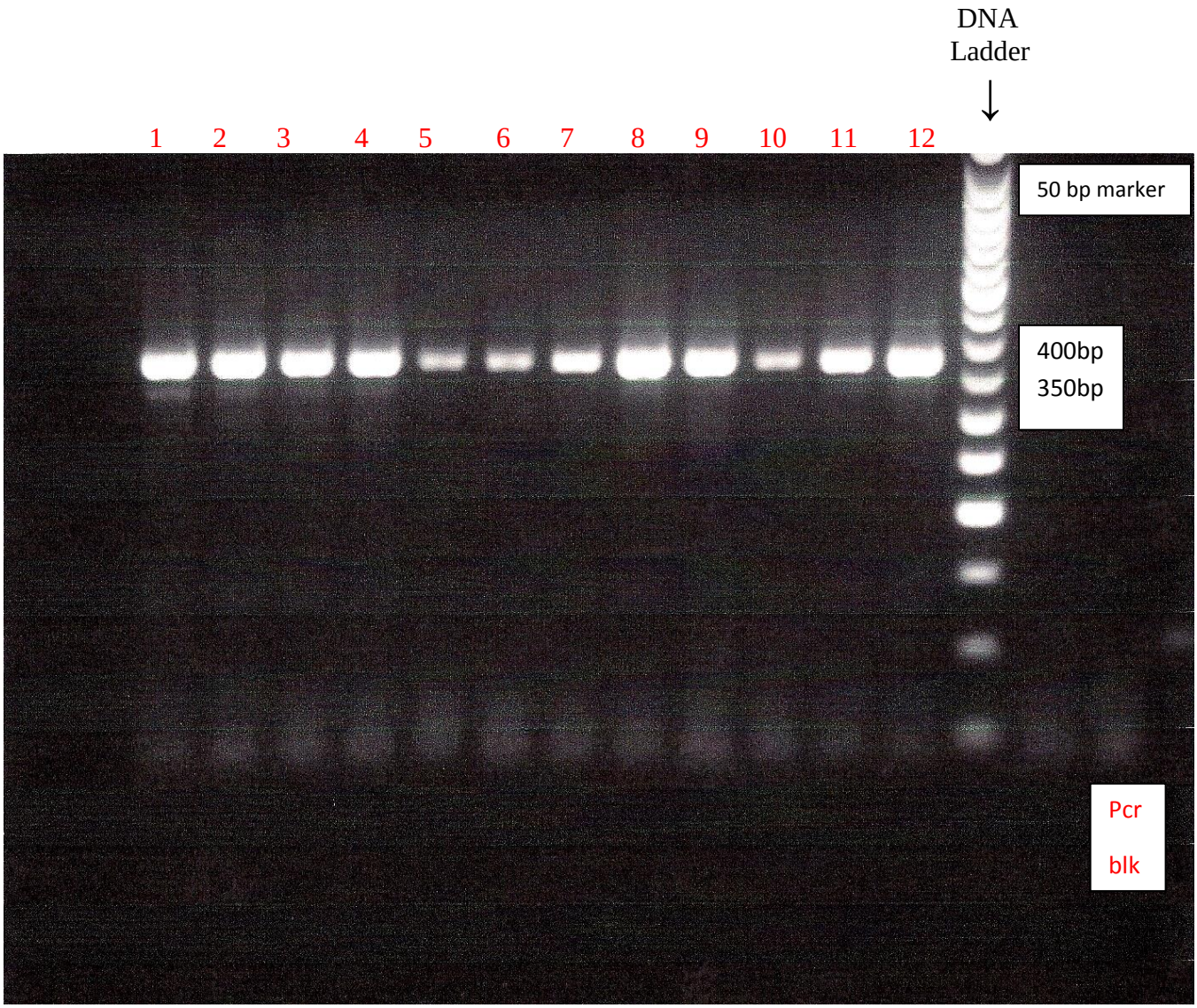


Figure 28: Picture of a typical gel of the polymerase chain reaction (PCR) products

KEY:

Blk = blank; bp = base pairs; PCR = Polymerase Chain Reaction

Table 9: DNA Concentration from some subjects

Plots | Report

Test type: Nucleic Acid | 9/29/2010 6:26 AM | Exit

Report Name | Report Full Mode: Ignore

Sample ID	User ID	Date	Time	ng/ul	A260	A280	260/280	260/230	Constant	Cursor Pos.	Cursor abs.	340 raw
48	Default	9/29/2010	4:57 AM	7.86	0.157	0.132	1.19	0.09	50.00	230	1.658	0.088
49	Default	9/29/2010	4:58 AM	10.90	0.218	0.156	1.40	0.57	50.00	230	0.382	0.258
50	Default	9/29/2010	4:58 AM	24.46	0.489	0.294	1.66	0.26	50.00	230	1.909	0.160
51	Default	9/29/2010	4:59 AM	-1.93	-0.039	0.004	-10.79	-0.14	50.00	230	0.274	0.088
52	Default	9/29/2010	5:03 AM	211.00	4.220	2.281	1.85	1.04	50.00	230	4.077	0.550
53	Default	9/29/2010	5:05 AM	13.68	0.274	0.237	1.15	0.14	50.00	230	1.887	0.085
54	Default	9/29/2010	5:06 AM	315.26	6.305	3.796	1.66	0.63	50.00	230	10.011	4.852
55	Default	9/29/2010	5:07 AM	25.70	0.514	0.293	1.75	0.65	50.00	230	0.789	0.127
56	Default	9/29/2010	5:08 AM	63.76	1.275	0.717	1.78	1.01	50.00	230	1.261	0.085
57	Default	9/29/2010	5:08 AM	57.77	1.155	0.656	1.76	0.53	50.00	230	2.161	0.301
58	Default	9/29/2010	5:09 AM	116.31	2.326	1.390	1.67	0.64	50.00	230	3.649	0.440
59	Default	9/29/2010	5:10 AM	48.90	0.978	0.621	1.58	0.59	50.00	230	1.665	0.378
60	Default	9/29/2010	5:11 AM	74.34	1.487	1.131	1.31	0.27	50.00	230	5.495	0.490
61	Default	9/29/2010	5:11 AM	22.29	0.446	0.269	1.66	0.45	50.00	230	0.985	0.165
63	Default	9/29/2010	5:12 AM	52.58	1.052	0.822	1.28	0.35	50.00	230	2.982	2.652
64	Default	9/29/2010	5:13 AM	40.85	0.817	0.449	1.82	0.63	50.00	230	1.305	0.240
65	Default	9/29/2010	5:14 AM	47.75	0.955	0.522	1.83	0.73	50.00	230	1.304	0.134
66	Default	9/29/2010	5:15 AM	71.75	1.435	0.841	1.71	0.59	50.00	230	2.422	0.539
67	Default	9/29/2010	5:15 AM	30.38	0.608	0.528	1.15	0.26	50.00	230	2.299	1.014
68	Default	9/29/2010	5:16 AM	5.39	0.108	0.082	1.31	0.18	50.00	230	0.598	0.117
69	Default	9/29/2010	5:17 AM	53.04	1.061	0.651	1.63	0.36	50.00	230	2.951	0.431
70	Default	9/29/2010	5:17 AM	36.02	0.720	0.430	1.68	0.85	50.00	230	0.846	0.230
71	Default	9/29/2010	5:18 AM	21.14	0.423	0.311	1.36	0.34	50.00	230	1.259	0.154
73	Default	9/29/2010	5:19 AM	24.06	0.481	0.372	1.29	0.26	50.00	230	1.858	0.167
74	Default	9/29/2010	5:20 AM	19.64	0.393	0.460	0.85	0.14	50.00	230	2.842	-0.055

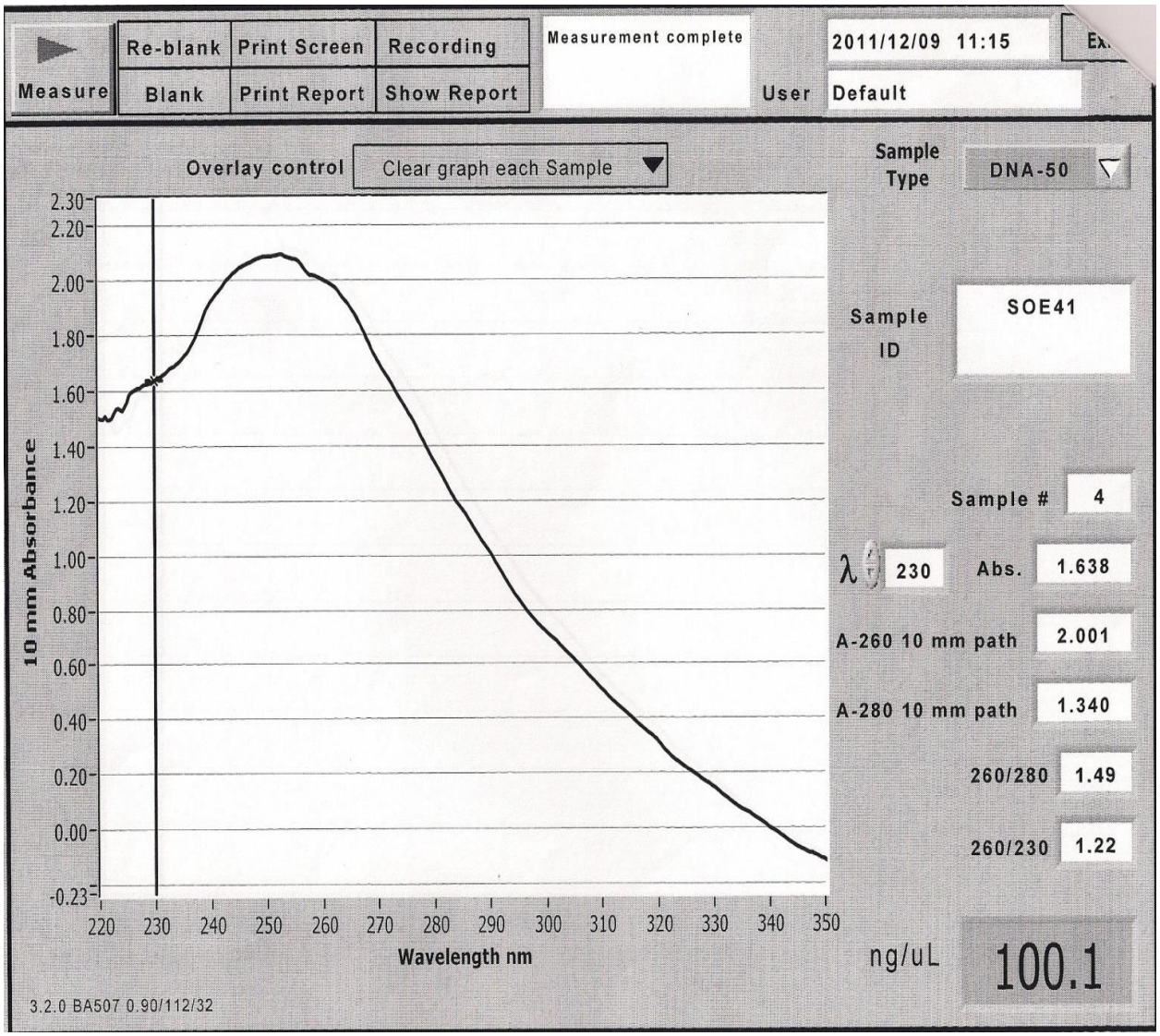
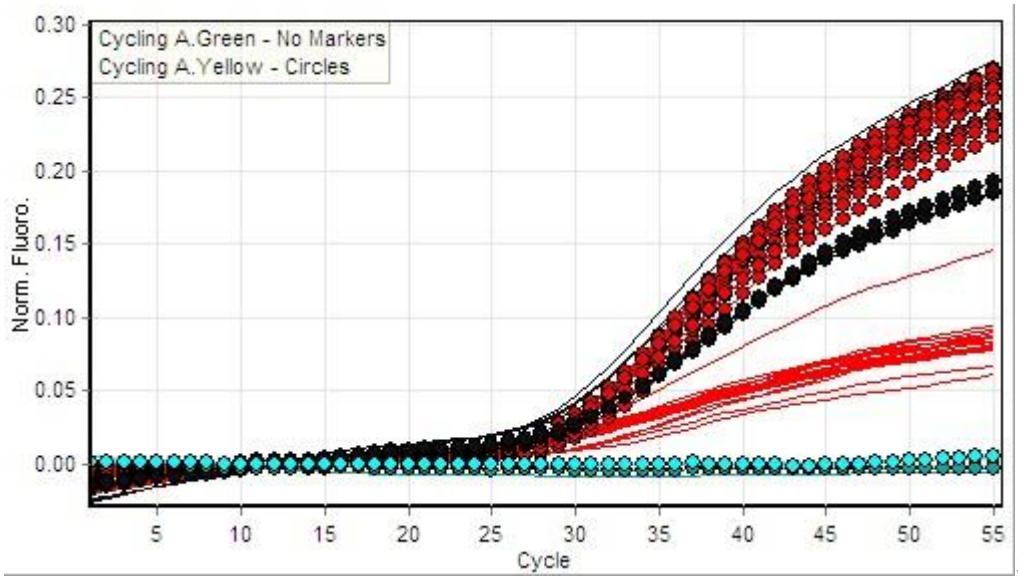


Figure 29: A typical tracing from the Nanodrop Spectrophotometer

Scatter Analysis data for Cycling A.Green, Cycling A.Yellow



Scatter Graph for Cycling A.Green, Cycling A.Yellow

Figure 30: Scatter analysis data and graph of TaqMan(R) SNP Genotyping Assay of subjects for the β-T594M Mutation of the epithelial sodium channel

4.6.2 Results of DNA Sequencing

Normotensive subjects as well as hypertensive subjects had the T594M mutation of β-ENaC subunit while other polymorphisms and new mutations were also observed in the two groups of subjects.

As shown in Table 10a, five (5.0%) of the subjects had the β-T594M polymorphism in which threonine was replaced by methionine due to the mutation of cytosine to threonine (ACG>ATG). The mutation is also written as T594M according to the IUPAC notation for amino acids (IUPAC-IUB Joint Commission on Biochemical Nomenclature, 1984). Out of these five, there were three hypertensive subjects constituting 5.7% (3/53) of the hypertensive subjects while the remaining two were normotensive constituting 4.2 percent (2/47) of the normotensive subjects. Typical chromatograms and FASTA sequences of the T594M polymorphism are shown in Figure 30 (normotensive) and Figure 31 (hypertensive).

As shown in Table10b, another polymorphism, Thr577thr polymorphism in which cytosine was replaced by thymidine, was observed in four subjects (4.0%). Three out of the four subjects were hypertensive (3/53) constituting 5.6% while one was normotensive (1/47) constituting 2.1% of the normotensive subjects. This polymorphism is also written as T577t (IUPAC-IUB Joint Commission on Biochemical Nomenclature, 1984). A typical chromatogram and FASTA sequence of this mutation is shown in Figure 33.

Table10c shows four previously unreported mutations. Of these, there were two glutamic acid to valine (Glu>Val) mutations at positions 632 and 636, (Glu632Val; E632V) and (Glu636Val; E636V) (IUPAC-IUB Joint Commission on Biochemical Nomenclature, 1984) respectively. These constituted 3.8% of the hypertensive subjects. Figure 34 shows the chromatogram of a typical Glu>Val mutation.

Another hypertensive subject (1/53; 1.9%) showed an aspartic acid to tyrosine (Asp>Tyr) mutation at position 638 (Asp638Tyr; D638Y), (IUPAC-IUB Joint Commission on Biochemical Nomenclature, 1984) . This is shown in Figure 35.

There was also a leucine to glutamine acid (Leu>Gln) mutation at position 628 (Leu628Gln; L628Q) (IUPAC-IUB Joint Commission on Biochemical Nomenclature, 1984). This was recorded in one normotensive patient (2.1%). The chromatogram is as shown in Figure 36.

TABLE 10: DNA SEQUENCING RESULTS

Table 10a: β-T594M mutations among normotensive and hypertensive subjects

SAMPLE NO	MUTATION	IUPAC-IUB CODE	BLOOD PRESSURE
34	ACG>ATG Thr594Met	T594M	Normotensive
45	ACG>ATG Thr594Met	T594M	Hypertensive
76	ACG>ATG Thr594Met	T594M	Hypertensive
107	ACG>ATG Thr594Met	T594M	Hypertensive
532	ACG>ATG Thr594Met	T594M	Normotensive

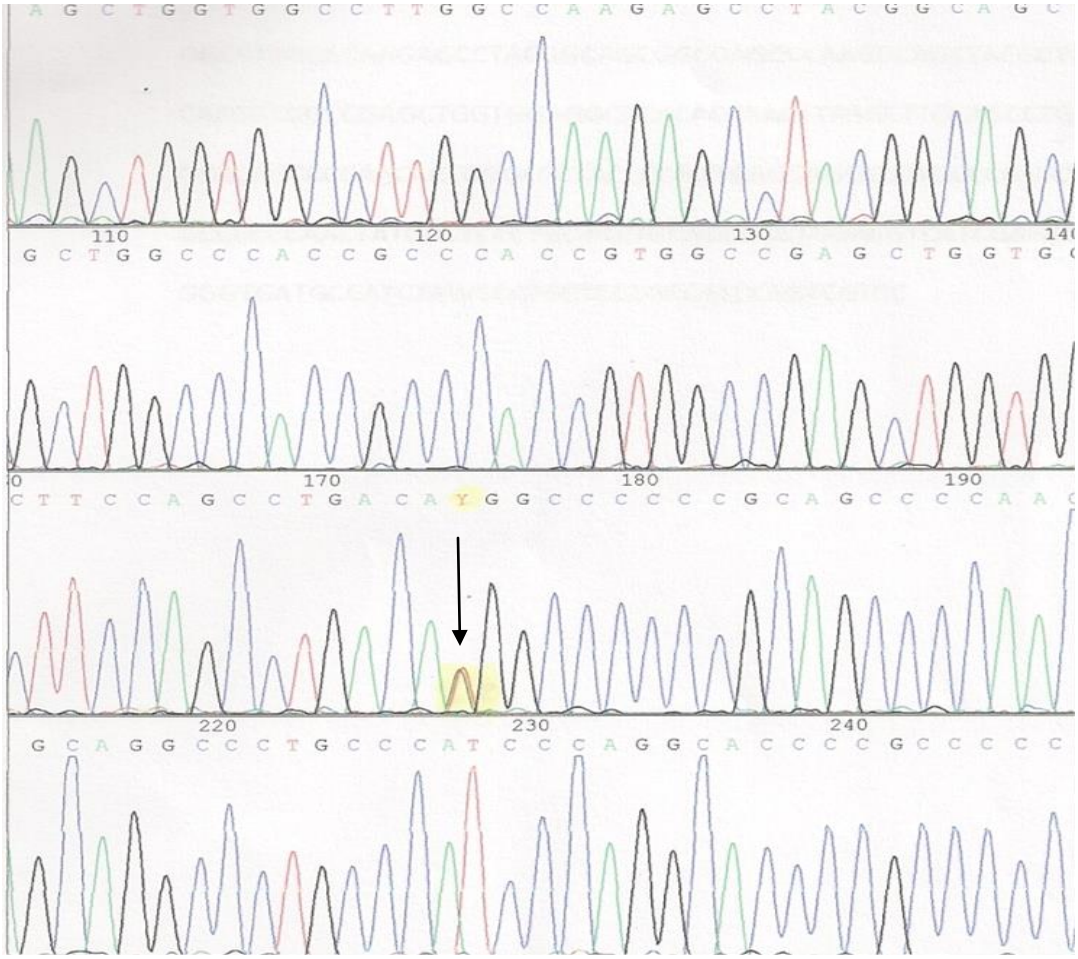
Table 10b: β-T577t polymorphisms among normotensive and hypertensive subjects

SAMPLE NO	POLYMORPHISM	IUPAC-IUB CODE	BLOOD PRESSURE
90	ACC>ACT Thr577thr	T577t	Normotensive
108	ACC>ACT Thr577thr	T577t	Hypertensive
309	ACC>ACT Thr577thr	T577t	Hypertensive
329	ACC>ACT Thr577thr	T577t	Hypertensive

KEY:
T577t is a synonymous or unmutated change

Table 10c: New mutations recorded among normotensive and hypertensive subjects

SAMPLE NO	MUTATION	IUPAC-IUB CODE	BLOOD PRESSURE
55	GAG>GTG Glu636Val	E636V	Hypertensive
52	GAG>GTG Glu632Val	E632V	Hypertenisve
33	GAT>TAT Asp638Tyr	D638Y	Hypertensive
50	CTG>CAG Leu628Gln	L628Q	Normotensive



>64_se_532_enac_4r sequence exported from B10_64_se_532_enac_4r_078.ab1

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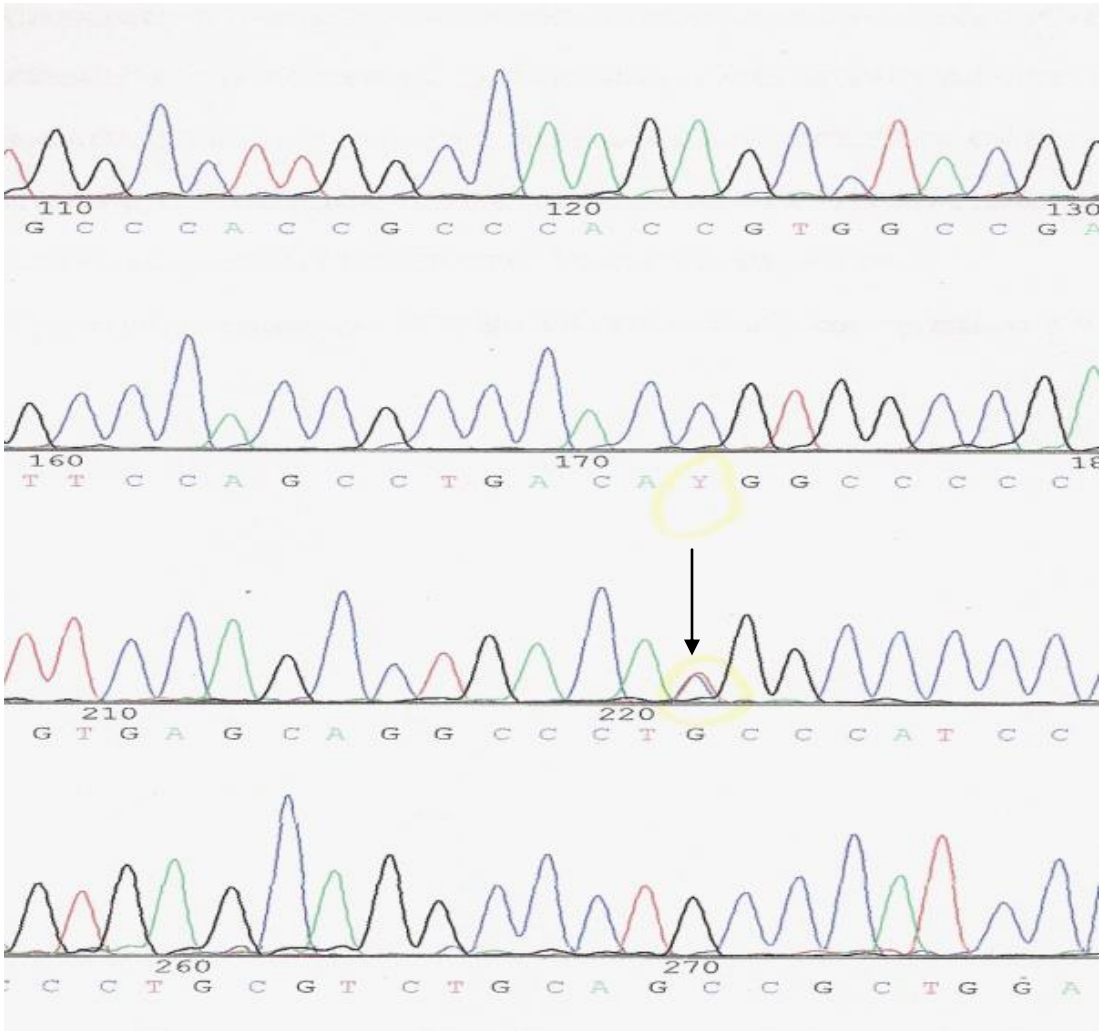
GGCCTTGGCCAAGAGCCTACGGCAGCGGCGAGCCCAAGCCAGCTACGCTGGCCCACCGCC
CACCGTGGCCGAGCTGGTGGAGGCCCACACCAACTTTGGCTTCCAGCCTGACAYGGCCCC
CCGCAGCCCCAACACTGGGCCCTACCCCAGTGAGCAGGCCCTGCCCATCCCAGGCACCCC
GCCCCCCAACTATGACTCCCTGCGTCTGCAGCCGCTGGACGTCATCGAGTCTGACAGTGA
GGGTGATGCCATCTAWCCCTGCTCCTGCCATCCAGTCGTCC
```

Figure 31: Chromatogram and DNA Sequence (FASTA Format) of a normotensive subject with β-T594M mutation

KEY:

Y = Point of missense or non-synonymous Mutation;
Y = Cytosine or Thymine (see IUPAC Codes, Appendix 3);
Thus mutation = ACG>ATG; ACG = Threonine, ATG = Methionine



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CCTTGGCCAAGAGCCTACGGCAGCGGCGAGCCCAAGCCAGCTACGCTGGCCCACCGCCCA
CCGTGGCCGAGCTGGTGGAGGCCCACACCAACTTTGGCTTCCAGCCTGACAYGGCCCCCC
GCAGCCCCAACACTGGGCCCTACCCCAGTGAGCAGGCCCTGCCCATCCCAGGCACCCCGC
CCCCCAACTATGACTCCCTGCGTCTGCAGCCGCTGGACGTCATCGAGTCTGACAGTGAGG
GTKATGCCATCTAACCCTGYTCCTRCTMTSKACTYWTCCTCG
```
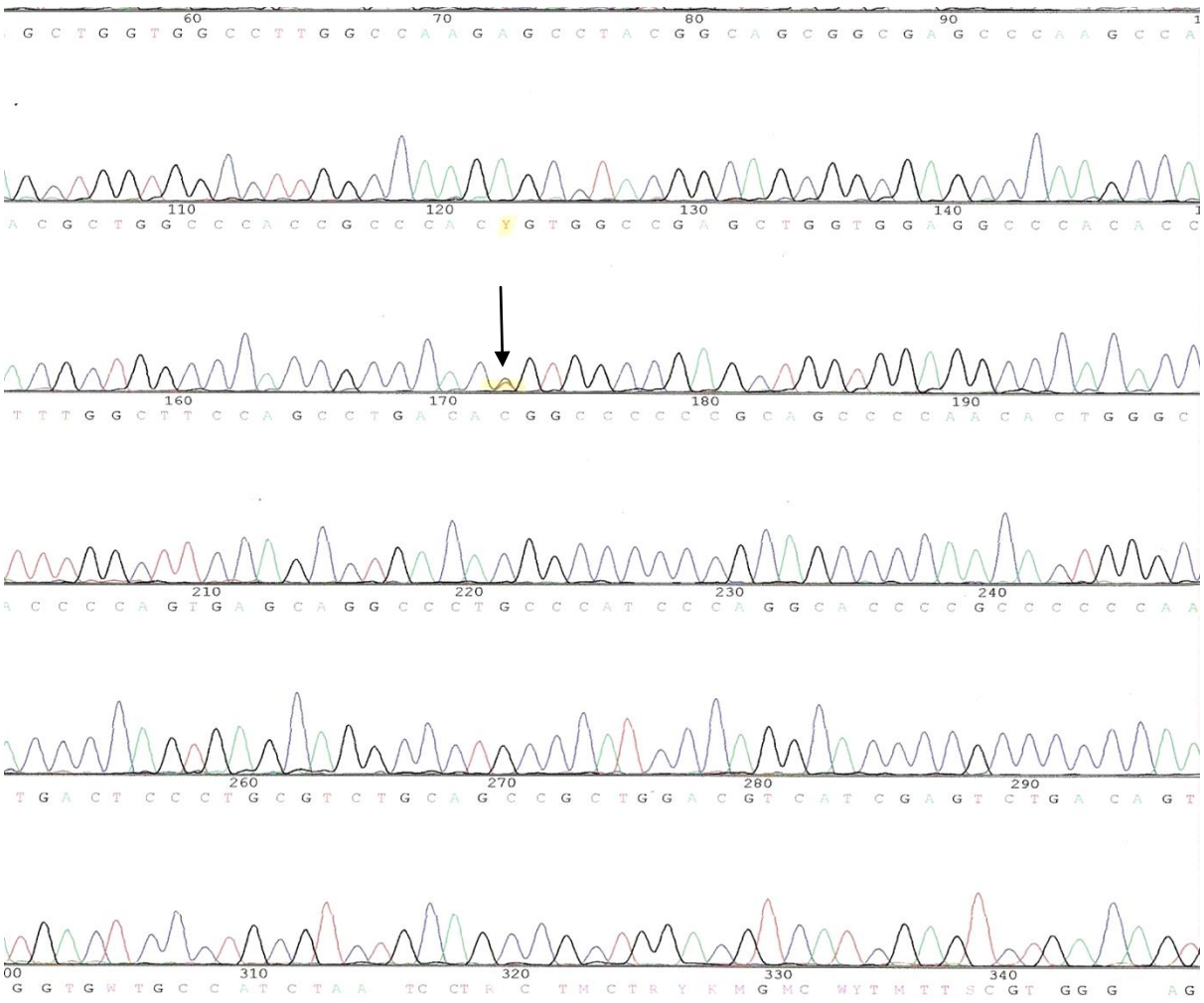

Figure 32: Chromatogram and DNA Sequence (FASTA Format) of a hypertensive subject with β-T594M mutation

KEY:

Y = Point of missense or non-synonymous Mutation;
Y = Cytosine or Thymine (see IUPAC Codes Appendix 3);
Thus mutation = ACG>ATG; ACG = Threonine, ATG = Methionine



>29_se_90_enac_4r sequence exported from G05_29_se_90_enac_4r_035.ab1

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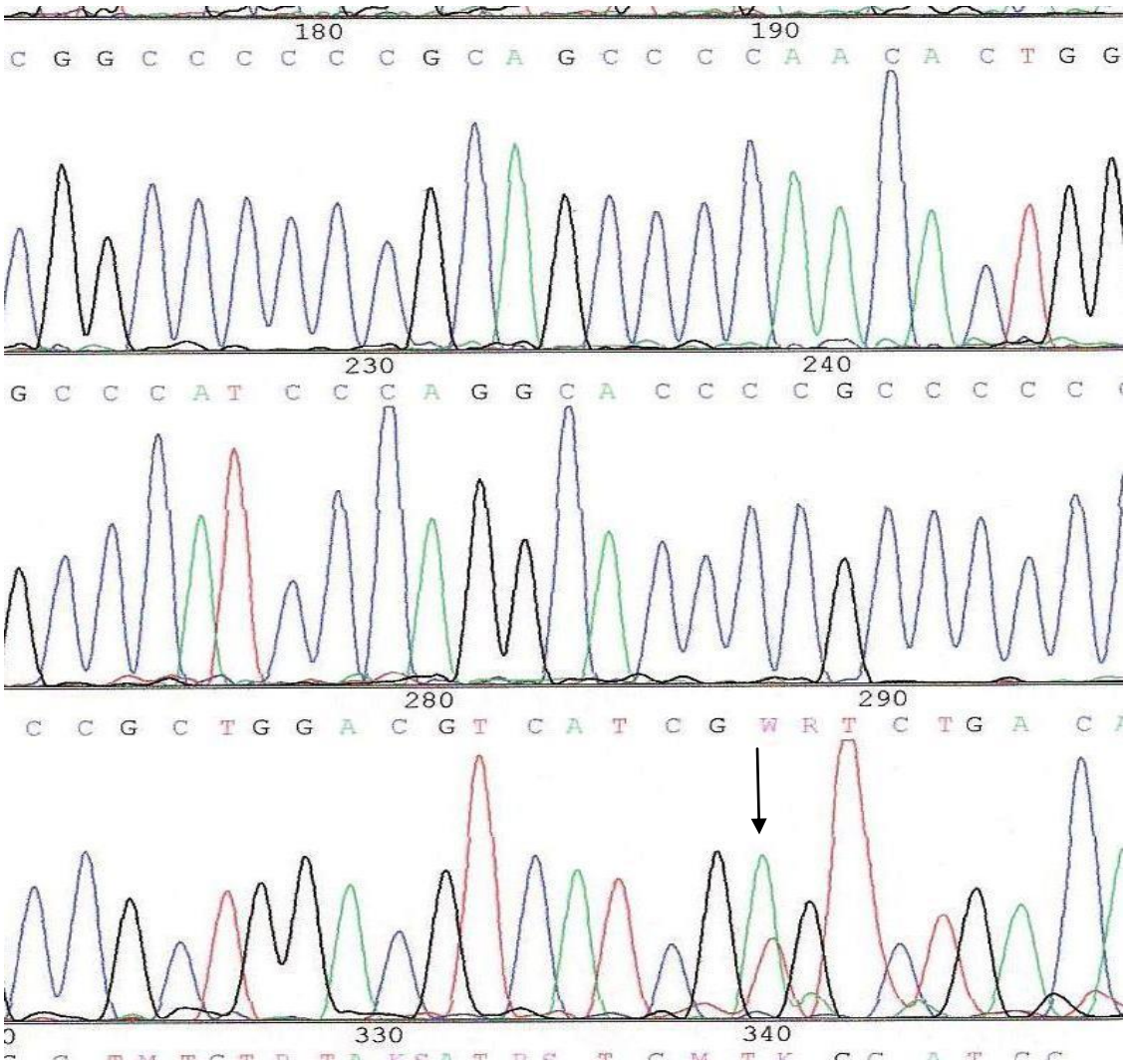
CYGTGGCCGAGCTGGTGGAGGCCCACACCAACTTTGGCTTCCAGCCTGACACGGCCCCCC
GCAGCCCCAACACTGGGCCCTACCCCAGTGAGCAGGCCCTGCCCATCCCAGGCACCCCGC
CCCCCAACTATGACTCCCTGCGTCTGCAGCCGCTGGACGTCATCGAGTCTGACAGTGAGG
GTGWTGCCATCTAATCCTRCTMCTRYKMGMCWYTMTTSCGTGGGAG

Figure 33: Typical Chromatogram and DNA sequence (FASTA Format) of Subject with β- T577t Polymorphism

KEY:

Y = Point of unmutated or synonymous change;
Y = Cytosine or Thymine ((see IUPAC Codes Appendix 3);
Thr>thr or ACC>ACT; ACC = Threonine, ATC = threonine



>23_se_52_enac_4r sequence exported from A05_23_se_52_enac_4r_047.ab1

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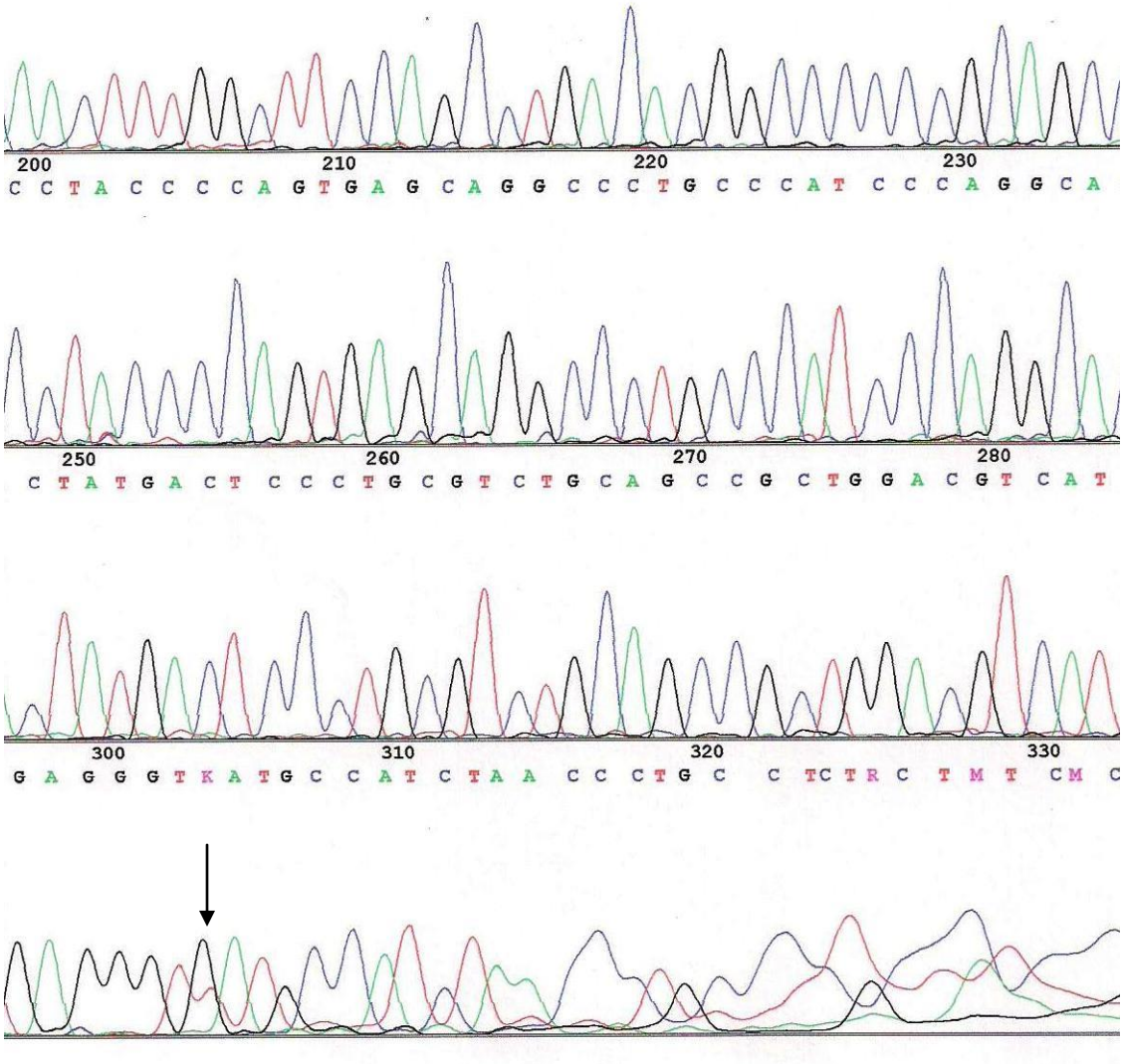
GCCTTGGCCAAGAGCCTACGGCAGCGGCGAGCCCAAGCCAGCTACGCTGGCCCACCGCCC
ACCGTGGCCGAGCTGGTGGAGGCCCACACCAACTTTGGCTTCCAGCCTGACACGGCCCCC
CGCAGCCCCAACACTGGGCCCTACCCCAGTGAGCAGGCCCTGCCCATCCCAGGCACCCCG
CCCCCCAACTATGACTCCCTGCGTCTGCAGCCGCTGGACGTCATCGWRTCTGACAGTGAG
GGTGATGCCATCTAACCCTGCTMTCTRTAKSATRSTCMTKGCATCC

Figure 34: Chromatogram and DNA sequence (FASTA Format) of Subject with GAG>GTG Glu632Val Mutation

KEY:
W = point of missense or non-synonymous change;
W = Adenine or Thymine (IUPAC code); R = Adenine or Guanine
Hence mutation = GAG>GTG = Glu>Val
Glu also represented as GAR (see IUPAC Codes Appendix 3)



>34_se_33_enac_4r sequence exported from
D06_34_se_33_enac_4r_042.ab1

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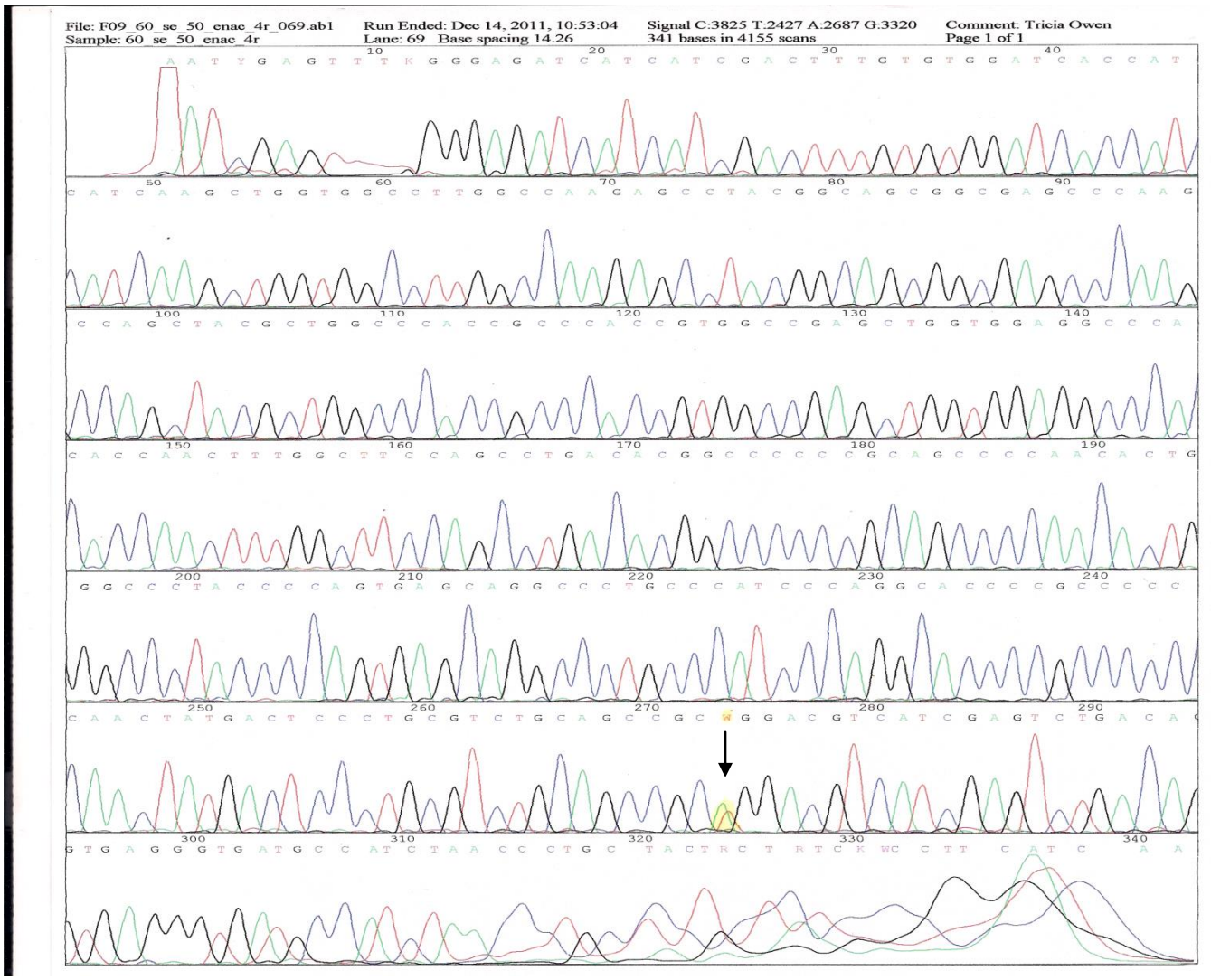
CCGTGGCCGAGCTGGTGGAGGCCCACACCAACTTTGGCTTCCAGCCTGACACGGCCCCCC
GCAGCCCCAACACTGGGCCCTACCCCAGTGAGCAGGCCCTGCCCATCCCAGGCACCCCGC
CCCCCAACTATGACTCCCTGCGTCTGCAGCCGCTGGACGTCATCGAGTCTGACAGTGAGG
GTKATGCCATCTAACCCTGCCTCTRCTMTCMCTAGTCSTCCGAT

Figure 35: Chromatogram and DNA sequence (FASTA Format) of Subject with GAT>TAT Asp638Tyr Mutation

KEY:
K = point of missense or non-synonymous change;
K = Thymine or Guanine ((IUPAC Codes Appendix 3);
Hence mutation = GAT>TAT = Asp>Tyr



>60_se_50_enac_4r sequence exported from F09_60_se_50_enac_4r_069.ab1

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GCAGGGCCTGCTCACTGGGGTAGGGCCCAGTGTTGGGGCTGCGGGGGGCCGTGTCAGGCT
GGAAGCCAAAGTTGGTGTGGGCCTCCACCAGCTCGGCCACGGTGGGCGGTGGGCCAGCGT
AGCTGGCTTGGGCTCGCCGCTGCCGTAGGCTCTTGGCCAAGGCCACCAGCTTGATGATGG
TGATCCACACAAAGTCGATGATGATCTCCCMAAACTCRATT

Figure 36: Chromatogram and DNA sequence (FASTA Format) of Subject with CTG>CAG Leu628Gln Mutation

KEY:
W = point of missense or non-synonymous change;
W = Adenine or Thymine ((IUPAC Codes Appendix 3);
Hence mutation = CTG>CAG = Leu>Gln

CHAPTER 5

5.0 DISCUSSION

5.1 Discussion of Methods

In determining blood pressure in this study, appropriate Riva-Rocci cuff sizes were used based on the middle upper arm circumference of each subject in order to prevent miscuffing (Pickering *et al.,* 2005; Doshi *et al.*, 2010). Blood pressure measurements are probably best done in subjects who are unaware of their blood pressure status because in most patients, just by being aware of their hypertensive status, the blood pressure, heart rate and responses to stress tests are heightened (Flaa *et al.,* 2008). However, in this study it was required that subjects be sorted into normotensive and hypertensive groups.

In this study, blood flow was determined by means of the venous occlusion plethysmography which is a very powerful tool for the measurement of limb blood flow in humans. Forearm vascular resistance (mmHg/ml/s) was calculated as the ratio between mean arterial blood pressure (MABP) (mmHg) and the rate of forearm blood flow (ml/s). This formula has also been used to determine local resistance to blood flow in carotid and humeral arteries (Arosio *et al.,* 2006).

The cold pressor test (CPT) is a provocative test designed by Hines (Hines and Brown, 1936) to predict future hypertension (Wood *et al.,* 1984). The test was used to determine vascular reactivity in this study. Usually the non-dominant hand is inserted up to the wrist in cold water and the blood pressure recording taken from the other hand (Weinberger, 2008; Moriyama and

Ifuku, 2010). In this study, the foot was immersed in the cold slurry in order to obtain maximal haemodynamic and sympathetic responses to the CPT (Kawano *et al.,* 2007); subjects' blood pressure and heart rate were determined simultaneously with the foot still immersed. This method differed from that used by Mei *et al.,* (2009) in which the hand was inserted into ice water for 2 min and blood pressure measured after the hand had been removed from the water. It was however similar to the method of Roy-Gagnon *et al.,* (2008) in which blood pressure was measured with the subjects' hand still immersed. The difference in methodology did not however affect the result of the studies.

Salt sensitivity was determined from the difference in mean arterial blood pressure before and after the ingestion of the salt load for 5 days. Unlike the diagnosis of hypertension, the determination of salt sensitivity status is usually a long and arduous task both for the patient and the physician. The protocols involved are usually time-consuming and depend on significant blood pressure changes at the end of a period of high-salt diet (five days to one week) compared with the baseline blood pressure or to the blood pressure at the end of an equal period of consumption of a low salt diet. A newer method which if validated will make the diagnosis of salt sensitivity easier and faster has been suggested by Castiglioni *et al.,* (2011). This involves the use of 24-hour ambulatory blood pressure monitoring without implementing controlled diets or monitoring salt intake. When in general use, this method is expected to be useful in determining the salt sensitivity status of persons with mild to moderate hypertension (Castiglioni *et al.,* 2011).

5.2 DISCUSSION OF RESULTS

5.2.1 Age of Subjects

The age of the subjects ranged from 25 years to 65 years. This was to avoid the effect of the extremes of life on renal function. This is because whereas renal function in the child is immature, there is a decline with age in the kidney's capacity to excrete sodium even in healthy humans and smaller increases in salt intake induce an increase in arterial blood pressure (Luft *et al.,* 1987; Meneton *et al.,* 2005). Glomerular filtration rate (GFR) also begins to decline from about the fourth decade of life there being only about 60 percent GFR remaining by the age of 80 years (Meneton *et al.,* 2005). There are of course individual variations as one-third of some individuals followed up for 8 years showed no decline in GFR (Meneton *et al.,* 2005) but the overall deterioration in GFR seems to be more marked in black persons (Mimran *et al.,* 1992).

5.2.2 Salt Sensitivity in Normotensive and Hypertensive Nigerians

Blood pressure was significantly lower among the normotensive subjects in this study; this is as expected based on the entry criteria for the study. However, on ingestion of 200mmol/day of salt daily for five days, systolic blood pressure increased significantly among both normotensive subjects and hypertensive subjects. Whereas both diastolic blood pressure and mean arterial blood pressure increased significantly among the hypertensive subjects in this study, normotensive subjects showed only a slight increase in these parameters of blood pressure after salt-loading. One of the consequences of ingesting a high salt diet is an increase in blood pressure. This has been clearly illustrated in animal models such as in chimpanzees (Denton *et al.,* 1995) and Sprague Dawley rats (Sofola *et al.,* 2002). However, while large-scale studies

have established a link between chronic salt intake and blood pressure, individual responses to a salt-load varies in man. The result of this study differs from that of Sofola *et al.,* (1998) who

reported no significant pressor response to a salt load in young normotensive Nigerians. The present results may be related to the longer duration of salt-loading over a period of 5 days compared with the 2 days of Sofola *et al.,* (1998). The present results tally with those of Farquhar *et al*., (2005) who reported an increase in blood pressure in a group of young normotensive subjects in a study using acute intravenous hypertonic saline infusion. On the other hand, like Sofola *et al.,* (1998), Andersen *et al.,* (2002) and Stachenfeld *et al.,* (2002) using hypertonic saline infusion did not record any appreciable increase in blood pressure.

Increasing the dietary salt in normotensive individuals on their normal salt diet has rarely led to significant pressor changes. In four of such five studies in which dietary salt was increased for a period of four weeks in young and middle-aged normotensive subjects, there was no increase in blood pressure (de Wardener and MacGregor, 2001). Interestingly, using this model in this study led to significant increases in blood pressure parameters in both the normotensive and hypertensive subjects; the only exception being that the increase in diastolic blood pressure and mean arterial blood pressure was marginal in normotensive subjects following the acute salt-load.

There have been suggestions that the initial blood pressure rise following a salt-load is as a result of volume-induced increase in cardiac output followed by a secondary increase in peripheral vascular resistance through an autoregulatory mechanism related to increased flow occurring at

the local tissues. Experimental studies in dogs have shown that high salt diet over a period of 6 weeks resulted in increased blood volume (Hainsworth *et al.,* 2003). According to this scheme, the eventual increase in blood pressure starts from a positive Na^+ balance, plasma volume expansion, a transient increase in cardiac output and a sustained increase in systemic vascular resistance (Schmidlin *et al.,* (2007). In the first three days following the increase in dietary salt intake, cardiac output alone causes the initial rise in blood pressure while systemic vascular resistance remains within normal limits initially to rise later as salt-loading is continued (Schmidlin *et al.,* (2007). Although cardiac output was not measured in the present study, forearm vascular resistance increased in normotensive and hypertensive subjects after 5 days of salt-loading which indicates that increased vascular resistance is causative in the development of hypertension in response to the salt load.

In the present study, salt sensitivity was higher among the hypertensive subjects (55%) compared with the normotensive subjects (34%). This report in normotensive subjects is lower than the original report by Weinberger *et al.,* (1986) in African Americans among whom 36% of the normotensive and 73% of the hypertensive subjects were salt sensitive (Weinberger *et al.,* 1986). What is evident however is that salt sensitivity occurs in both normotensive and hypertensive Nigerians. This is significant in that there have been reports that notwithstanding the blood pressure status of an individual, salt sensitivity confers greater risk for cardiovascular diseases and their sequelae on the person (Weinberger *et al.,* 2001). For instance, reports from a 10-year follow up study of mixed group of subjects showed a greater increase in blood pressure with age among those initially salt sensitive compared with their salt resistant counterparts (Weinberger and Fineberg, 1991). Weinberger *et al.,* (2001) have also demonstrated a similar cumulative risk of mortality in salt sensitive normotensive and hypertensive patients in a 30-year follow up study

of an initial cohort. Another study in 199 older normal and hypertensive white and black women involving salt loading and restriction demonstrated a similar prevalence of salt sensitivity in both races among the normotensive subjects but showed greater changes in blood pressure among the hypertensive black subjects (Wright *et al.,* 2003). It is also quite pertinent to note here that salt sensitivity status does not change over time (Weinberger and Fineberg, 1991) and that salt sensitive normotensive subjects develop hypertension more rapidly than their salt resistant counterparts. According to the "nephrogenic" formulation, dietary salt loading differs in effect between the salt sensitive and salt resistant in that the former would experience greater renal reclamation of salt and therefore experience greater sequential increases in sodium balance, plasma volume and cardiac output (Lifton *et al.,* 2001; Cruz *et al.,* 2001).

Malfunctioning of the neural and hormonal mechanisms that take part in blood pressure regulation contributes to this phenomenon with the kidneys playing a central role in long-term pressure homeostasis (Farquhar *et al.,* 2005). Salt sensitivity may occur with either hereditary or acquired defects in renal function. It may occur as a result of alterations in renal function that requires higher arterial pressure to maintain "steady state" homeostasis; this is reflected in a "resetting" or shifting to the right, of the pressure-natriuresis curve (Rodriguez-Iturbe and Vaziri, 2007). This means that a higher pressure is required to excrete a given amount of dietary sodium. Equally, a relationship between salt and impaired left ventricular dysfunction has been demonstrated in normotensive and hypertensive subjects (Tzemos *et al.,* 2008). A possible mechanism is that suggested by the Baustein hypothesis (Lim *et al.,* 2001) that excess salt intake leads to increased intracellular calcium which then impairs myocardial relaxation.

In the 30-year follow-up study mentioned earlier (Weinberger *et al.,* 2001), salt sensitivity was found to be associated with similar and higher mortality in normotensive as well as hypertensive subjects aged 25years or older when first studied. If being salt sensitive indeed confers on a normotensive subject a higher risk of developing hypertension in the future, and the results of the present study in which salt sensitive normotensive subjects responded with elevated blood pressure to an increased dietary salt intake confirm this suggestion, then of course, dietary intervention may be targeted at such an individual to slow down the progress to hypertension. On the contrary, increasing potassium in the diet has been shown to reduce salt sensitivity in normotensive and hypertensive subjects (Appel *et al.,* 1997); however, this was not studied in the present experiments.

Salt sensitivity index was higher among the hypertensive subjects when compared with the normotensive subjects. This index is important in that it supplies a measure of the sodium output without requiring information on the actual sodium intake so that the accuracy of the index is not dependent on the subjects' full compliance with the dietary salt-load (Coruzzi *et al.,* 2005). The advantage of determining a salt sensitivity index over plain salt sensitivity is that it relates the changes in blood pressure induced by salt-loading with the concomitant changes in urinary sodium output without any arbitrary definition of thresholds (Kimura and Brenner, 1997; Corruzi *et al.,* 2005). In this study, the index has been able to show that salt sensitivity is higher among the hypertensive than the normotensive subjects.

In this study, plasma sodium (Na^+) increased significantly in both the normotensive and hypertensive subjects after salt-loading. Plasma sodium level may play a role in the development

of hypertension since a small increase in plasma sodium (1-3mM) has been documented in hypertensive subjects (de Wardener *et al.,* 2004; He *et al.,* 2005). This triggered the suggestion that the vascular endothelium may participate in a sodium-mediated blood vessel dysfunction. The result of this study also suggests that there is impaired ability to excrete salt in the hypertensive subjects. At baseline and following the salt-load, plasma Na^+ was higher in the hypertensive subjects while Na^+ clearance and urine Na^+ excretion was lower among the hypertensive subjects compared with the normotensive subjects. In individuals such as those in this study who develop hypertension in response to a high dietary salt intake, the kidney has a limited ability to excrete the daily salt intake and may therefore retain the salt in skin and other extracellular compartments (Titze *et al.,* 2003). This internal sodium "escape" mechanism is probably insignificant in humans with high blood pressure indicating that external sodium balance plays a role in blood pressure regulation (Titze *et al.,* 2002).

5.2.3 Effect of Sympathetic Nervous Activation on Cardiovascular Functions

In order to examine the autonomic influences on the cardiovascular control in the subjects, the cold pressor test (CPT) was employed as a sympathetic stimulus in this study. Although very few reports have been concerned on the representativeness of this laboratory test of everyday stress undergone by human beings, yet laboratory stress responses have been observed to correspond very well with reactivity to challenges of daily life during ambulatory blood pressure monitoring (Kamarck *et al.,* 2003).

The blood pressure response to the CPT is primarily mediated through the activation of the sympathetic nervous system (Weinberger, 2008). The magnitude and duration of the blood pressure response is influenced by a number of factors including the basal blood pressure of the individual (Weinberger, 2008). The result of this study showed that all blood pressure responses to the CPT in the hypertensive subjects were higher than that recorded in the normotensive subjects at baseline. This was similar to a study among the Chinese in which a higher pressor response to the CPT was recorded in persons with higher baseline blood pressure and higher sodium ingestion (Chen *et al.,* 2008). However, after a second challenge was introduced in the form of salt-loading in this present study, vascular hyperreactivity was higher in the normotensive subjects probably indicating the higher effect of salt-loading on the vascular responsiveness of the normotensive subjects, or an uncovering of the greater endothelial dysfunction present in the hypertensive subjects *ab initio* since impaired endothelial function and arterial stiffening occur in the presence of cardiovascular disease (Najjar *et al.,* 2005). The ingestion of amiloride again restored the baseline response pattern observed in these subjects.

Vascular reactivity was higher in the hypertensive subjects at baseline compared with the normotensive subjects in this study. Heightened reactivity to the cold pressor test as shown by these hypertensive subjects indicates some vascular dysfunction in them. There have been some reports that hypertension is associated with increased cardiovascular and sympathoadrenal activity to physical stress such as the cold pressor test (Flaa *et al.,* 2008). Vascular hyperreactivity in normotensive subjects is said to indicate the risk of the development of hypertension in the future. It is also possible that heightened reactivity is a marker of other physiological processes that are more directly involved in cardiovascular pathology. However, there have been conflicting reports from studies attempting to show a strong association between

hyperreactivity and future hypertension. Thus there is a lot of controversy as to whether this relationship is causal or consequential, especially as there are so many mechanisms that contribute to the development of high blood pressure. However, results of the normotensive subjects in this present study in which normotensive subjects responded with greater hyperreactivity as well as salt hyperreactivity to the salt load indicate a causal role for heightened reactivity in the development of hypertension. This is similar to some earlier reports that have shown hyperreactivity to the cold pressor test is an indicator of future development of hypertension (Carroll *et al.,* 2003; Mathews *et al.,* 2004). Hasselund *et al.,* (2010) have demonstrated the stability of the cardiovascular and sympathetic responses to the cold pressor test; a condition required to consider hyperreactivity as being involved in the development of hypertension and cardiovascular disease. It is possible that intermittent blood pressure increases lead to structural changes in the vasculature. This intermittent blood pressure change in these individuals is accompanied by increased sympathetic activity and sympathetic tone is a trophic factor for vascular hypertrophy (Flaa *et al.,* 2008). However, attempts at producing irreversible, sustained blood pressure increases purely as a consequence of transient elevations in blood pressure in dogs have not been successful (Julius *et al.,* 1989). In established hypertension, there may be normal sympathetic activity but catecholamine receptor sensitivity and vascular hypertrophy may be increased (Flaa *et al.,* 2008). It is pertinent also to keep in focus the fact that underlying genetic and developmental processes may stimulate alterations in cardiovascular response as well as accelerate cardiovascular disease progression.

Systolic hyperreactivity was consistently higher than diastolic hyperreactivity in both the normotensive and hypertensive subjects before and after salt-loading. This finding is important

in that systolic hyperreactivity has been documented to confer greater risk for developing cardiovascular accidents compared with diastolic hyperreactivity (Everson *et al.,* 2001). In this study, systolic hyperreactivity increased among the normotensive subjects following salt-loading also suggesting a causal role for heightened hyperreactivity in the development of hypertension in Nigerians. There are suggestions that the absolute values of systolic blood pressure during the cold pressor test are better predictors of future hypertension compared with the changes in systolic blood pressure caused by the test (Flaa *et al.,* 2008; Hasselund *et al.,* 2010). Systolic hyperreactivity indicates an acute increase in cardiac force during systole whereas diastolic hyperreactivity represents increases in resistance during diastole. It is thus a possibility that systolic reactivity and the attendant increased rate and force of cardiac contractility exacerbates the risk for strokes by increasing the probability of an embolism (Everson *et al.,* 2001). In many black subjects, vascular dysfunction has been reported as a major pathogenic factor in initiating the pressor effect of dietary salt intake. In a study among a Chinese population, Chen *et al.* (2008) reported that systolic blood pressure response to the cold pressor test was a stronger predictor of salt sensitivity than diastolic reactivity which is similar to the report of this present study.

The results of the present study indicate significant correlation between vascular reactivity and salt sensitivity. Normotensive subjects in this study demonstrated positive and negative correlations between salt sensitivity and systolic and diastolic vascular reactivity respectively. On the other hand, hypertensive subjects displayed slight negative correlation with systolic and diastolic reactivity before and after salt-loading. The result among the hypertensive subjects in this study was similar to that obtained by Corruzi *et al.* (2005) among hypertensive subjects in

which salt sensitivity and baroreflex sensitivity showed a negative correlation during low and high salt intake. It is also similar to reports in a Chinese population involving normotensive and hypertensive subjects among whom it was demonstrated that individuals who were hyperreactive to the cold pressor test were also more sensitive to dietary salt and potassium interventions (Chen *et al.,* 2008). Alterations of the autonomic cardiovascular control are observed in salt sensitive individuals. Enhanced sympathetic drive has been described during high salt intake in salt sensitive subjects (Corruzi *et al.* 2005) as was observed in this study. This is thought to be as a result of deranged reflex cardiovascular control of the responsible mechanisms (Corruzi *et al.* (2005) and indicates further impairment in baroreceptor sensitivity caused by the cold pressor test in these salt sensitive hypertensive subjects who had underlying reduced baroreceptor sensitivity because of their hypertension. A similar outcome was also observed in the normotensive subjects with regard to diastolic reactivity before and after the ingestion of the salt load in this study.

Salt reactivity represents the phenomenon in which normoreactive subjects become hyperreactive following ingestion of a salt-load. This is a new terminology emanating from this study as it has not been previously described in any study before this. Salt reactivity was higher among the normotensive subjects which is not surprising since vascular dysfunction is expected to have occurred in the hypertensive subjects which will therefore impair the ability of their vascular smooth muscles to respond to the salt challenge. These results, salt reactivity in the normotensive subjects as well as the correlation between salt sensitivity and vascular hyperreactivity in these subjects, indicate a pathogenic role for both salt sensitivity and vascular hyperreactivity in the development of hypertension among Nigerians. Normotensive individuals

who are both salt sensitive and hyperreactive are therefore more likely to become hypertensive in future.

Amiloride ingestion led to an amelioration of heightened reactivity in the normotensive subjects in this study. Epithelial sodium channel (ENaC) proteins have been isolated in mechanotransduction sites that include the vascular smooth muscle cells (VSMCs) especially from myogenically active vascular beds (Jernigan and Drummond, 2005). Amiloride blocks ENaC at these sites as well (Drummond *et al.*, 2008b). It is therefore possible that amiloride had blocked ENaC at these sites in the normotensive subjects thereby causing an attenuation of heightened reactivity observed in the subjects following salt-loading alone. On the other hand, hypertensive subjects responded to amiloride by increased heightening of vascular reactivity. This may be due to the higher effect of amiloride in reducing blood pressure of hypertensive subjects in this study.

At baseline, heart rate was significantly higher among the hypertensive subjects. Following ingestion of the salt-load, heart rate reduced among both normotensive and hypertensive subjects; this is similar to earlier reports (Schmidlin *et al.,* 2007; McNeely *et al.,* 2008). The result in this study is quite different from that of Coruzzi *et al.,* (2005) in which heart rate increased proportionately with the degree of salt sensitivity in hypertensive subjects suggesting an altered cardiac autonomic regulation. The decrease in heart rate in the presence of high blood pressure following the salt-load may be due to a baroreceptor response. Baroreflex-mediated neural adjustments alter cardiac output mainly via changes in heart rate (Ogoh *et al.,* 2003) and

peripheral resistance in a bid to maintain arterial blood pressure around a perceived "normal" (Holwerda *et al.,* 2011).

Results from this study show a slight increase in heart rate following the cold pressor test (CPT) in the normotensive subjects before and after salt-loading. This is similar to earlier reports among healthy volunteers in whom CPT led to a consistent increase in heart rate (Siegrist *et al.,* 2006; Wirch *et al.,* 2006). However, hypertensive subjects showed a slight bradycardia and a slight increase in heart rate before and after salt-loading respectively. This may be viewed in the light of responses in young men with low, normal and high blood pressure among whom heart rate responses to the CPT were observed to be negatively correlated with resting heart rate (Flaa *et al.,* 2008). In the present study, heart rate at baseline was lower in the normotensive subjects compared with the hypertensive subjects. The cold pressor test stimulates the sympathetic system and the release of catecholamines, epinephrine and norepinephrine, from the adrenal medulla. These increase heart rate and arterial blood pressure and myocardial oxygen demand (Kiviniemi *et al.,* 2011). Few studies have reported severe bradycardia as part of vasovagal response to the CPT (Wirch *et al.,* 2006; Kiviniemi *et al.,* 2011). The vasovagal response is thought to be due to rapid reversal of sympathetic efferent neuron activation after the foot had been removed from the cold slurry leading to a fall in sympathetic tone below that required to maintain cardiac output and blood pressure in a state where cardiac output is already compromised (Wirch *et al.,* 2006). However, although the hypertensive subjects displayed a slight fall in heart rate on exposure to the CPT in this study, none of them experienced a full blown vasovagal response. The bradycardia observed among these subjects could be a reflex response to the increased blood pressure observed in the subjects. Ingestion of salt plus amiloride did not change the heart rate response to the CPT in both the normotensive and hypertensive subjects.

5.2.4 Sympathetic Regulation and Vascular Resistance

In this study, blood flow was slightly lower in the hypertensive subjects at baseline. The reduction in blood flow in both the normotensive and hypertensive subjects following salt-loading with 200 mmol/day of Na^+ suggests enhanced vasoconstrction in these subjects. There was further reduction in blood flow in the two groups following ingestion of salt plus amiloride which implies that amiloride augmented the enhanced vasoconstriction that occured in these subjects following salt-loading alone. Salt loading in humans has been shown to lead to a significantly lower forearm vasodilatory response to acetylcholine (endothelium-dependent vasodilatation) (Bragulat *et al.,* 2001).

An increased peripheral vascular resistance in response to the CPT as observed in this study, results in reduced regional blood flow. Both groups of subjects in the present study experienced a fall in their forearm blood flow on exposure to the CPT at baseline, after salt-loading and after ingestion of amiloride in addition to the salt-load. This was similar to an earlier report by Jaja *et al.,* (2003) in which reduced blood flow was obtained in normal Nigerians on immersion of the foot in cold water maintained at 7^oC. The regulation of peripheral vascular resistance is important in the control of regional blood flow. Also since the CPT causes increased sympathetic activity, this results in vasoconstriction leading to an increase in peripheral vascular resistance (Kelsey *et al.,* 2000b). At baseline, forearm vascular resistance was lower in the normotensive subjects compared with the hypertensive subjects in this study. Enhanced haemodynamic changes to physiological and psychological stressors have been recorded among Blacks. These are thought to be mediated largely through an increase in peripheral vascular resistance (Stein et al., 2000). Blacks have been shown to experience a greater increase in vascular tone in response

to stress probably due to increased sympathetic activity or altered vascular sensitivity to sympathetic stimulation. This may lead to increased peripheral vascular resistance which in addition to the high environmental stress to which blacks are exposed, predisposes them to sustained hypertension (Stein et al., 2000).

Following salt-loading in the present study, whereas forearm vascular resistance increased slightly among the normotensive subjects, there was a slight reduction among the hypertensive subjects. These results differ from that of Schmidlin *et al.,* (2007) in which a decrease in systemic vascular resistance was recorded in normotensive subjects over the first three days following a salt load. Such a fall in vascular resistance in their study does not tally with the nephrogenic explanation of salt-induced hypertension. By this formulation and as mentioned above, it is expected that during the initial days following a salt load, an increase in blood pressure is basically as a result of a change in cardiac output while vascular resistance remains normal. However, forearm vascular resistance was not measured on a daily basis in this present study, being measured only at the end of the 5-day salt-loading period. It is therefore possible that what was observed in the normotensive subjects in this study was at the point when autoregulation had occurred in response to the increased cardiac output that accompanies a salt load. On the other hand, the slight increase in forearm vascular resistance in the hypertensive subjects may be due to the fact that although peripheral vascular resistance is said to remain normal following a salt-load, this does not mean that it remains totally unchanged. On the contrary, this suggests a systemic vascular dysfunction that is expressed as impaired vasodilatory response to the increased salt intake (Schmidlin *et al.,* 2007).

In this study, stimualtion of the sympathetic nervous system by means of the cold pressor test resulted in significant increases in forearm vascular resistance among both the normotensive and hypertensive subjects. Peripheral vascular resistance and its regulation play a central role in the control of arterial blood pressure. Response of resistant arteries to cold may be as a result of a balance between adrenergic vasoconstriction and vasodilation; the latter is mediated by endothelial function. Endothelial function of resistant arteries is a vascular function that has been identified as a primary target of injury from hypertension (Moyna and Thompson, 2004). Increased arterial stiffness and reduced arterial compliance may both be associated with endothelial dysfunction which is again present in cardiovascular disease (Kawano *et al.*, 2007). Activation of the sympathetic nervous system by the cold pressor test increases vascular tone in resistant arteries. This simulates a natural occurrence in winter during which period it has been observed that blood pressure is higher compared to the other seasons (Modesti *et al.*, 2006; Murakami *et al.*, 2011).

5.2.5 ENaC Markers and Salt-Sensitivity

Subjects in the present study responded to blockade of the epithelial sodium channel (ENaC) with low-dose amiloride with a global reduction in blood pressure. This is more dramatic especially in the hypertensive subjects in whom amiloride caused blood pressure to reduce as low as the pre-intervention level observed in the normotensive subjects. The epithelial sodium channel (ENaC) is an amiloride-sensitive rate-limiting step of sodium reabsorption in the distal nephron. Mutations or polymorphisms have been found in the three genes encoding the α-, β- and γ-subunits of the gene. These are related to gain or loss of function of the channel resulting in increased or reduced reabsorption of sodium in the distal part of the nephron, and high or low

blood pressure. For example, Liddle syndrome, a rare clinical manifestation of gain of function mutation of the β-subunit of ENaC is characterised by low renin, low aldosterone form of hypertension. Psuedohypoaldosteronism (PHA-I), a loss of function of ENaC phenotype has equally been described. This is characterised by high renin, high aldosterone and unresponsiveness to mineralocorticoids. It has been reported that mutations in the β-subunit present especially in persons of the black race leads to hypertension through an increase in sodium reabsorption in the distal nephron. It has equally been suggested that ENaC could be intrinsically more active in the black race compared with the Caucasians based on the observation that plasma aldosterone levels as well as urinary excretion of aldosterone are consistently lower in blacks (Bloem *et al.,* 1996). This is possibly the consequence of enhanced sodium retention resulting from the inherent reduced abiltiy to excrete a salt load observed in blacks. This leads to the observed secondary suppression of the renin-angiotensin-aldosterone axis (Pratt *et al.,* 2002).

Results from the present study using low dose amiloride (5mg) is similar to those from an earlier study by Baker *et al.,* (2002) among black individuals living in London, in which blood pressure fell significantly in black individuals with the T594M mutation of the β-subunit of ENaC. However, Baker *et al.* had used a higher dose of amiloride (10mg) and for four weeks. The higher dose may have allowed the diuretic effect of amiloride to come into play while the long duration of the study may have given the opportunity for sodium reabsorptive mechanisms other than the ENaC to play a role in sodium reabsorption (Snyder, 2002). These will include mechanisms in the proximal convoluted tubule, thick ascending limb of Henle as well as the distal convoluted tubule. On the other hand, results from this study differ from that of Pratt *et al.* (2002) who reported no fall in blood pressure among a black population in Indianapolis (USA)

after treatment with 5mg amiloride daily for one week. Pratt *et al.* however recorded a fall in blood pressure among the white subjects in the same study leading them to suggest that black individuals had less ENaC activity to inhibit (Pratt *et al.,* 2002). Their results also imply that the increased sodium reabsorption in the black subjects studied did not occur in the distal nephron where ENaC is domiciled but rather in the proximal segments of the tubule whereas results from this present study indicate that increased sodium reabsorption occurs in the distal nephron hence the significant reduction in blood pressure in these subjects following the ingestion of 5mg amiloride for 5 days. The T594M mutation affects the last exon of the β-subunit of ENaC and results in a single amino acid change with substitution of methionine for threonine. This threonine residue is a potential target for phopshorylation by protein kinase C which inhibits sodium channel activity (Su *et al.,* 1996). It has been suggested that the β-T594M mutation could cause the channel to become resistant to the negative regulatory effects of protein kinase C (Baker *et al.,* 1998). Evidence from lymphocyte studies suggests that the β-T594M mutation may cause increased sodium-channel activity by causing affected cells to become insensitive to negative regulation (Baker *et al.,* 1998). If this mutation also has this effect on sodium-channels in the renal tubules, then it would contribute to the development of high blood pressure by reducing renal sodium excretion and causing sodium retention in the affected individuals.

Plasma sodium level was higher among the hypertensive subjects at baseline suggesting inherent sodium retention or less ability of the renal system to excrete sodium. This was corroborated by the urinary sodium excretion ($U_{Na}V$) which was also lower among the hypertensive subjects in this study. Plasma sodium increased significantly in both groups of subjects after ingestion of the salt load. However, although $U_{Na}V$ increased in both groups, it was still lower in the hypertensive subjects when compared with the normotensive subjects. Similarly sodium

clearance was lower among the hypertensive subjects before and after salt-loading. These observations indicate the fact that there is inherent reduction in the ability to excrete a salt load among the hypertensive subjects. This reduced ability to handle a salt load plays a significant role in the development of hypertension among Nigerians. When presented with a salt load, the kidney tends to retain salt since it has limited capacity to excrete even the daily uptake of salt (Oberleithner, 2007). There is therefore usually a concomitant increase in blood pressure which may be significant in salt sensitive individuals.

The effect of the salt load on plasma sodium in this study was quite different from that from an earlier study among normotensive Nigerians in whom salt-loading with 400 mmol/day of NaCl for two days failed to cause a significant increase in plasma sodium level or blood pressure (Sofola *et al.*, 1998). This could be because of the short duration of the salt-loading carried out in that study. There are reports that plasma sodium level is a determinant in the development of hypertension. There is accumulation of sodium in the extracellular space as a result either of the kidneys not being able to excrete a salt load efficiently or when the aldosterone level is raised (Oberleithner *et al.*, 2007). Although there was a concomitant increase in plasma potassium level in normotensive subjects following salt-loading, the hypertensive subjects showed a slight decrease in plasma potassium instead which is not surprising considering they were retaining sodium in exchange for the potassium.

Following combined ingestion of the salt load plus amiloride, there was a fall in plasma sodium level in the normotensive and hypertensive subjects in the present study implying a probable role for the ENaC in sodium reabsorption in these subjects. In addition, sodium clearance increased in

both groups though slightly higher among the hypertensive subjects. To complete the picture, there was also an associated fall in blood pressure in the two groups. Unlike the suggestion of Pratt *et al.,* (2002) that amiloride had less blood pressure-lowering effect in blacks indicating a centre for sodium reabsorption in black individuals other than the distal nephron, results from this study point to the involvement of the ENaC in sodium reabsorption and therefore blood pressure control in these Nigerian subjects. Other studies have shown a difference in the urinary excretion of vasodilatory and natriuretic substances like prostaglandins and dopamine between whites and black individuals which equally suggest that black individuals excrete sodium less efficiently than white subjects (Burnier, 2008). This renal dysfunction is thought to play a role in the prevalence of salt sensitive hypertension among black individuals (Bankir *et al.,* 2007).

Plasma potassium and its response to amiloride is a significant indicator of ENaC activity in the kidneys (Gaukrodger *et al.,* 2008). In this study, plasma potassium increased following ingestion of amiloride especially in the hypertensive subjects compared with the level at baseline and the level after ingesting the salt load alone. In the same vein, 24-hour urine potassium fell following the ingestion of amiloride in both normotensive and hypertensive subjects. Plasma potassium and 24-hour urine potassium level show significant heritability that is approximately 30 per cent to 60 per cent. This indicates that genetic influences on potassium handling may be identifiable (Gaukrodger *et al.,* 2008). No simple correlation has been drawn between potassium and blood pressure especially since total body potassium level in persons with essential hypertension is similar to that found in normotensive persons (Gaukrodger *et al.,* 2008). The observation in this study was not different as plasma potassium levels of normotensive and hypertensive subjects were similar. It is however possible that increased renal secretion of potassium leads to an associated decrease in renal sodium and chloride reabsorption (Gaukrodger *et al.,* 2008). These workers have also reported a small but significant effect of common genetic variation in the

SCNN1B gene on plasma potassium. Amiloride significantly reduced urine potassium concentration among the hypertensive subjects compared with that recorded following salt-loading alone. This is a further indication of a role for the ENaC as a determinant of hypertension among Nigerians.

Salt sensitivity in black individuals has been associated with a tendency towards expanded plasma volume, lower plasma renin activity (PRA) and increased renal vascular resistance (Burnier, 2008). Hypertensive subjects in this study were no different as they showed low plasma renin activity in comparison to the normotensive subjects. This suggests increased sodium reabsorption in these subjects and therefore indicates that an ENaC mutation, in particular β-T594M mutation, may be present in these subjects (Luft 2001). It also explains in part the high level of salt sensitivity recorded among these subjects. This report differs from that of an earlier study in South African blacks in which there was no association recorded between high blood pressure and ENaC mutation (Nkeh *et al.*, 2003). However results from this study are similar to another study among South African black pregnant women in whom an association was found between R563Q mutation of the β-subunit of ENaC and low-renin hypertension (Rayner *et al.*, 2003). Salt-loading led to an increase in plasma renin activity (PRA) in both groups of subjects in the present study. Blockade of the epithelial sodium channel with amiloride resulted in an increase in PRA as well as aldosterone in the hypertensive subjects in this study. The effect on aldosterone may be attributable to the increased serum potassium level caused at the same time by the ingestion of amiloride (Warnock and Bell, 2005; Bubien, 2010). Indeed elevated serum potassium has been reported to be the most powerful agonist for aldosterone secretion (Rossier and Stutts, 2009). Results of the present study also tally with earlier one by Pratt *et al.,* (2001) in which aldosterone concentration rose and fell as potassium concentration over time. The increase

in aldosterone level with amiloride may also be due to a feedback response to the blockade of ENaC by the drug. Similarly, the increase in PRA caused by amiloride administration will have contributed to the increased aldosterone observed in these hypertensive subjects via the renin-angiotensin-aldosterone system (RAAS).

Amiloride is not currently in use as an anti-hypertensive agent, being used as a mild diuretic agent in conjunction with other agents especially thiazide diuretics for its ability to reduce the loss of potassium (Baker *et al.,* 2002). Results of this study suggest that low dose amiloride will be useful as an anti-hypertensive agent especially in salt sensitive individuals. This has been demonstrated in an earlier study in which the effect of amiloride on blood pressure of hypertensive subjects with the T594M polymorphism of ENaC was similar to that obtained using the more potent thiazide diuretics (Baker *et al.,* 2002). However 10mg amiloride was used in that study in contrast to the present study in which 5mg has been used. Considering the attendant adverse effects of using these agents, it will be more propitious to use a single agent with little adverse effect. Although it may not be economically viable at present, knowing the status of the epithelial sodium channel among hypertensive Nigerians will also make it possible to select those patients who will benefit more from the use of amiloride as an anti-hypertensive agent. This is because the effect of amiloride in hypertensive persons with the T594M mutation is more than that expected from a general diuretic (Baker *et al.,* 2002) as amiloride counteracts the effect of the mutation on the epithelial sodium channel. The hyperkalaemia consequent upon the ingestion of amiloride may be a limitation to its use as an anti-hypertensive agent especially when used in conjunction with other agents that affect the RAAS (Page *et al.,* 2003). However, if amiloride is prescribed in lower doses as used in this study, there may be less incidence of hyperkalaemia (Saha *et al.,* 2005). This is important especially since no incidence of

hyperkalaemia was recorded in the present study. Another perspective is that the effect of amiloride in stimulating PRA will lead to increased aldosterone secretion which will enhance potassium secretion and therefore reduce the risk of an increase in potassium in the patients. This is quite different from the direct effect of the drug on ENaC which reduces potassium secretion.

5.2.6 Genetic Variants of the Epithelial Sodium Channel

The frequency of 5% (0.05) of the β-T594M mutation recorded in this study is comparable to that recorded previously in the EXOME project for African Americans (www.ncbi.nlm.nih.gov/SNP/snp-ref.cgi). It is also similar to that recorded among young Ghanaians living in Kumasi with average age 26.9±4.7y (Dong *et al.*, 2002) and the 6% recorded among a black population living in London (Baker *et al.*, 1998). The frequency is however much higher than the 0.022 recorded among older Ghanaians (age 49.1±5.5y) living in London (Dong *et al.*, 2002). All the subjects with the T594M mutation in this study were heterozygous for the mutation which was present on the reverse strand. It is interesting to note that two of the subjects were normotensive and salt sensitive. This could be an indication of high susceptibility to developing salt sensitive hypertension in future in these subjects. The β-T594M mutation of the channel may cause hypertension by causing affected channels to become insensitive to negative regulation by protein kinase C (PKC) (Su *et al.*, 1996; Dong *et al.*, 2002). This is because the threonine residue at this position which is the target site for phosphorylation by PKC has been changed in this mutation to methionine. The β-Thr577-thr polymorphism recorded in this study is not likely to be of any consequence with regard to the development of hypertension since this is a silent mutation also known as a synonymous change. This type of change specifies the same amino acid as the original. On the other hand, all the new mutations recorded in this study, β-

E632V, β-E636V, β-D638Y and β-L638Q, are all non-synonymous changes as a different type of amino acid resulted from the change. Three of the four subjects among whom these new mutattions were recorded were hypertensive with two of them also being salt sensitive. The fourth subject who was normotensive was also salt sensitive. It may well be that this latter subject will develop high blood pressure in future. This will require further investigation.

5.3 SUMMARY OF FINDINGS

i. Salt sensitivity was present among both groups of subjects being higher among the hypertensive subjects (55%) compared with the normotensive (34%) subjects. Reduced abilty to excrete sodium is a significant factor in the development of salt sensitive hypertension in Nigerians

ii. Potentiation of sympathetic nervous regulation of cardiovascular function plays a significant role in hypertension among Nigerians as evidenced by the fact that vascular hyperreactivity following salt-loading in the normotensive subjects tended towards that in hypertensive subjects at baseline. Systolic hyperreactivity was consistently higher than diastolic hyperreactivity in both groups of subjects. Salt reactivity was higher among the normotensive subjects especially with regard to systolic salt reactivity

iii. Forearm vascular resistance resistance increased significantly in response to sympathetic stimulation in normotensive subjects after salt-loading approaching the baseline level among the hypertensive subjects. This indicates that significant adrenergic nervous potentiation is important in the development of salt sensitive hypertension

iv. Blood pressure reduced significantly to below the baseline level in response to the blockade of the epithelial sodium channel with low dose amiloride. This indicates that enhaced ENaC activity is an important determinant of hypertension among Nigerian subjects

v. The β-T594M mutation was recorded in 5 percent of the subjects (frequency 0.05). Previously unrecorded mutations in this subunit of the epithelial sodium channel, E638V and E632V (3.8%) as well as D638Y (1.9%) have been observed among the hypertensive subjects. In the same vein, L628Q was recorded in 3.3 percent of the normotensive subjects

5.4 CONCLUSION

This study has established the fact that salt sensitivity is prevalent among normotensive as well as hypertensive Nigerians being more prevalent in the latter. Inherent reduced abilty to excrete salt by the kidneys played an important role in the development of salt sensitive hypertension among these subjects. The study has also shown that potentiation of sympathetic nervous activity is a significant cause of hypertension in Nigerains. That enhanced epithelial sodium channel activity plays a role in the development of salt sensitive hypertension has been established by the significant blockade of the channel resulting from administration of amiloride especially in hypertensive subjects. The result of this study is important for the fact that those persons who are salt sensitive and especially those who are also hyperreactive will benefit from adequate monitoring and dietary counselling. This is in order to prevent the development of hypertension among those who are as yet normotensive and modulate the course of hypertension in those who are already hypertensive. This is applicable to the salt reactive normotensive subjects as well.

This study also provides a basis for prospective studies to be carried out into the possibility of modifying present management of hypertension among our populace by using amiloride as an antihypertensive agent. This will be a more cost-effective public health intervention. The present study also provides a template upon which further work can be done which should ultimately lead to the development of a screening tool for hypertension especially in those considered to be at high risk, in particular with regard to the epithelial sodium channel (ENaC) mutation in Nigerians.

5.5 CONTRIBUTIONS TO KNOWLEDGE

1. Enhanced epithelial sodium channel activity is an important determinant of hypertension in Nigerians
2. β-T594M mutation of the epithelial sodium channel has been confirmed among five percent of the population studied; four previously unreported mutations of the β-subunit of the channel were also recorded among four of the subjects
3. Sympathetic autonomic potentiation is significant in the development of hypertension among Nigerians as evidenced by the enhanced vascular reactivity observed among the normotensive subjects
4. Salt sensitivity has been confirmed among normotensive and hypertensive Nigerians being higher among the latter; reduced renal ability to excrete salt plays a significant role in the development of salt sensitive hypertension among Nigerians
5. Significant positive correlation exists between salt sensitivity and systolic vascular hyperreactivity among normotensive subjects at baseline

6.0 REFERENCES

Adedoyin, RA, Mbada CE, Balogun MO, Martins T, Adebayo RA, Akintomide A and Akinwusi PO (2008). Prevalence and pattern of hypertension in a semiurban community in Nigeria. Eur J Cardiovasc Prev Rehab 15: 683-687

Adegunloye BJ and Sofola OA (1997). Effect of high dietary salt loading and high calcium diet on vascular smooth muscle responses and endothelial function in rats. Clin Exp Pharmacol Physiol 24: 814-818

Akcav A, Yavuz T, Semiz S, Bundak R and Demirdoven M (2002). Psuedohypoaldosteronism type I and respiratory distress syndrome. J Pediatr Endocrinol Metab 15: 1557-1561

Akita S, Sacks FM, Svetkey LP, Conlin PR, Kimura G. for the DASH-Sodium Trial Collaborative Research Group (2003). Effects of the Dietary Approach to Stop Hypertension (DASH) diet on the pressure-natriuresis relationship. Hypertension 42: 8-12

Alderman MH (2000). Salt, blood pressure and human health. Hypertension, 36: 890-893

Alperovitch A, Lacombe JM, Hanon O, Dartigues JF, Ritchie K, Ducimetiere P and Tzourio C (2009). Relationship between blood pressure and outdoor temperature in a large sample of elderly individuals: the Three-City Study. Arch Intern Med 169: 75-80

Ambrosius WT, Bloem U, Zhou L, Rebhun JF, Snyder PM, Wagner MA, Guo C and Pratt JH (1999). Genetic variants in the epithelial sodium channel in relation to aldosterone and potassium excretion and risk for hypertension. Hypertension, 34:631-637

Andersen LJ, Andersen JL, Pump B and Bie P (2002). Natriuresis induced by mild hypernatremia in humans. Am J Physiol Renal Integr Comp Physiol 282: R1754-1761

Appel LJ, Moore TJ, Obarzanek E, Vollmer WM, Svetkey LP, Sacks FM, Bray GA, Vogt TM, Cutler JA, Windhauser MM, Lin P-H, Karanja N, Simons-Morton D, McCullough M, Swain J, Steele P, Evans MA, Miller ER and Harsha DW for the DASH Collaborative Research Group (1997). A clinical trial of the effects of dietary patterns on blood pressure. N Engl J Med 336: 1117-1124

Arosio E, De Marchi S, Rigoni A, Prior M and Lechi A (2006). Effects of smoking on cardiopulmonary baroreceptor activation and peripheral vascular resistance. Eur J Clin Invest 36: 320-325

Ataman S, Cooper R, Rotimi C, Osotimehin B, Muna W, Kingue S, Muna W, Fraser H, Forrester T and Wilkis R (1996). Standardization of blood pressure in an international collaborative study. J Clin Epidemiol 49:869-877

Atwood LD, Samollow PB, Hixson JE, Stern Mp and MacCluer JW (2001). Genome-wide analysis of blood pressure in Mexicans. Genet Epidemiol 20: 373-382

Auberson M, Hoffmann-Pochon N, Vandewalle A, Kellenberger S and Schild L (2003). Epithelial Na+ channel mutants causing Liddle's syndrome retain ability to respond to aldosterone and vasopressin. Am J Physiol Ren Physiol 285: F459-F471

Azinge E, Mabayoje OM, Sofola OA and Oshibogun A (1999). 24-hour urinary sodium, potassium, and creatinine and their relationship to blood pressure in adult Nigerians. Nig Qt. J. Hosp. Med. 9: 17-20

Baker EH, Ireson NJ, Carney C, Markandu ND and MacGregor GA (2001). Transepithelial sodim absorption is increased in people of African origin. Hypertension 38: 76-80

Baker EH, Dong YB, Sagnella GA, Rothwell M, Onipinla AK, Markandu ND, Cappuccio FP, Cook DG, Persu A, Corvol P, Jeunemaitre X, Carter ND and McGregor GA (1998). Association of hypertension with T594M mutation in B subunit of epithelial sodium channels in black people resident in London. Lancet; 351: 1388-1392

Baker EH, Duggal A, Dong Y, Ireson NJ, Wood M, Markandu ND and McGregor GA (2002). Amiloride, a specific drug for hypertension in black people with T594M variant? Hypertension 40: 13-17

Bankir L, Bochud M, Maillard M, Bovet P, Gabriel A and Burnier M (2008). Night time blood pressure and nocturnal dipping are associated with daytime urinary sodium excretion in African subjects. Hypertension 51: 891-898

Bankir L, Perucca J and Weinberger MH (2007). Ethnic differences in urine concentration: Possible relationship to blood pressure. Clin J Am Soc Nephrol 2: 304-312

Bashyam H (2007). Lewis Dahl and the genetics of salt-induced hypertension. J Exp Med 204: 1507-1508

Baumgart P (1991). Circadian rhythm of blood pressure: internal and external triggers. Chronobiol Int 8: 444-450

Ben-Dov IZ and Bursztyn M (2011). Can salt sensitivity of blood pressure be assessed without changing salt diet? Hypertension 57: 156-157

Benos DJ and Stanton BA (1999). Functional domains within the degenerin/epithelial sodium channels (Deg?ENaC) superfamily of ion channels. J Physiol 520: 631-644

Bens M, Chassin C, Vandewalle A (2006). Regulation of NaCl transport in the renal collecting duct: Lessons from cultured cells. Pflugers Arch 453: 133-146

Benson CJ, Xie J, Wemmie JA, Price MP, Henss JM, Welsh MJ and Snyder P (2002). Heteromultimers of DEG/ENaC subunits form H^+-gated channels in mouse sensory neurons. Proc Natl Acd Sci USA 99: 2338-2343

Bhalla V and Hallows KR (2008). Mechanisms of ENaC regulation and clinical implication. J Am Soc Nephrol 19: 1845-1854

Bize V and Horisberger J (2007). Sodium self-inhibition of human epithelial sodium channel: selectivity and affinity of the epithelial sodium sensing site. Am J Physiol Renal Physiol 293: F1137 – F1146

Blazer-Yost BL, Esterman MA and Vlahos CJ (2003). Insulin-stimulated trafficking of ENaC in renal cells requires PI 3-kinase activity. Am J Physiol Cell Physiol 284: C1645-C1653

Bloem IJ, Manatunga AK and Pratt JH (1996). Racial difference in the relationship of an angiotensin 1-converting enzyme gene polymorphism to serum angiotensin 1-converting enzyme activity. Hypertension 27: 62-66

Boegehold MA (1993). Microvascular changes associated with high salt intake and hypertension in Dahl rats. Int J Microcirc Clin Exp 12: 143-156

Bonny O and Rossier BC (2002). Disturbances of Na/K balance: Psuedohypoaldosteronism revisited. J Amer Soc Nephrol 13: 2399-2414

Bonny O, Chraibi A, Loffing J, Jaeger NF, Grunder S, Horisberger JD and Rossier BC (1999). Functional expression of a Psuedohypoaldosteronism type 1 mutated epithelial Na^+ channel lacking the pore-forming region of its alpha subunit. J Clin Invest 104: 967-974

Bragulat E, de la Sierra A, Antonio MT and Coca A (2001). Endothelial dysfunction in salt-sensitive essential hypertension. Hypertension, 37: 444-448

Brodie TG and Russel AE (1905). On the determination of the rate of blood flow through an organ. J Physiol 32: 47-49

Bubien JK (2010). Epithelial Na^+ channel (ENaC), hormones and hypertension. J Biol Chem 285: 23527-23531

Bugaj V, Pochynyuk O and Stockand JD (2009). Activation of the epithelial Na+ channel in the collecting duct by vasopressin contributes to water reabsorption. Am J Physiol Ren Physiol 297; F1411-1418

Burnier M (2008). Ethnic differences in renal handling of water and solutes in hypertension. Hypertension 52: 203-204

Büsst CJ, Scurrah KJ, Ellis JA and Harrap SB (2007). Selective genotyping reveals association between the epithelial sodium channel γ-subunit and systolic blood pressure. Hypertension 50: 672-678

Butterworth MB, Edinger RS, Frizzell RA, Johnson JP (2009).Regulation of the epithelial sodium channel by membrane trafficking. Am J Physiol Renal Physiol 296: F10-F24

Caldwell RA, Boucher RC and Stutts MJ (2004). Serine protease activation of near-silent epithelium Na+ channels. Am J Physiol 286: C190-C194

Campese VM, Parise M, Karubian F and Bigazzi R (1991). Abnormal renal haemodynamics in black salt sensitive patients with hypertension. Hypertension 18: 805-812

Canessa CM, Horisberger JD and Rossier BC (1993). Epithelium sodium channel related to proteins involved in neurodegeneration. Nature 361: 47-470

Canessa CM, Schild L, Buell G, Thorens B, Gautschi I, Horrisberger JD and Rossier BC (1994). Amiloride-sensitive epithelial Na+ channel is made of three homologous subunits. Nature 367: 463-467

Cappuccio F, Cook DG, Atkinson RW and Strazullo P (1997a). Prevalence, detection and management of cardiovascular risk factors in different ethnic groups in South London. Heart 78: 555-562

Cappuccio FP, Markandu ND, Carney C Sagnella GA and MacGregor GA (1997b). Double-blind randomized trial of modest salt restriction in older people. Lancet, 350: 850-854

Carroll D, Ring C, Hunt K, Ford G and Macintyre S (2003). Blood pressure reactions to stress and the prediction of future blood pressure: effects of sex, age and socioeconomic position. Psychom Med 65: 1058-1064

Castiglioni P, Parati G, Brambilla L, Brambilla V, Gualerzi M, Di Rienzo M and Coruzzi P (2011). Detecting sodium-sensitivity in hypertensive patients. Information from 24-hour ambulatory blood pressure monitoring. Hypertension 57:180-185

Chagnon NA (1968). Yanomano. The fierce people. New York: Holt, Rinehart and Winston

Chang SS, Grunder S, Hanukoglu A, Rosler A, Mathew PM, Hanukoglu I, Schild L, Lu Y, Shimkets RA, Nelson-Williams C, Rossier BC and Lifton RP (1996). Mutation in subunits of the epithelial sodium channel cause salt wasting with hyperkalaemic acidosis, pseudohypoaldosteronism type I. Nat Genet 12: 248-253

Chen CC, England S, Akopian AN and Wood JN (1998). A sensory neuron-specific, proton-gated ion channel. Proc Natl Acad Sci USA 95: 10240-10245

Chen J, Gu D, Jaquish CE, Chen C-S, Liu D, Hixson JE, Hamm LL, Gu CC, Whelton PK and He J for the GenSalt Collaborative Research Group (2008). Association between blood pressure responses to the cold pressor test and dietary sodium intervention in a Chinese population. Arch Intern Med 168; 1740-1746

Chida Y and Steptoe A (2010). Greater cardiovascular responses to laboratory mental stress are associated with poor subsequent cardiovascular risk status. A meta-analysis of prospective evidence. Hypertension 55; 1026-1032)

Chraibi A and Horisberger J (1999). Stimulation of epithelial sodium channel by the sulfonylurea Glibenclamide. J Pharmac Exp Ther 290: 341

Coats P (2010). The effect of peripheral vascular disease on structure and function of resistance arteries isolated from human skeletal muscle. Clin Physiol Funct Imaging 30: 192-197

Conlin PR (2001). Dietary modification and changes in blood pressure. Curr Opinion Nephr Hypertens 10: 358-363

Cooper V, Rotimi C, Ataman S, McGee D, Osotimehin B, Kadiri S, Muna W, Kingue S, Fraser H, Forrester T, Bennett F and Wilks R (1997). The prevalence of hypertension in seven populations of West African origin. Am J Public Health 87: 160-168

Cooper VL and Hainsworth R (2002). Effects of dietary salt on orthostatic tolerance, blood pressure and baroreceptor sensitivity in patients with syncope. Clin Auto Res 12: 236-241

Corey DP and Garcia-Anoveros J (1996). Mechanosensation and the DEG/ENaC ion channels. Science 273: 323-324

Coruzzi P, Parati G, Brambilla L, Brambilla V, Gualerzi M, Novarini A, Castiglioni P and Jeunemaitre X (2005). Effects of salt sensitivity on neural cardiovascular regulation in essential hypertension. Hypertension 46: 1321 – 1326

Corvol P, Persu A, Gimenez-Roqueplo AP and Jeunemaitre X (1999). Seven lessons from two candidate genes in human essential hypertension: angiotensinogen and epithelial sodium channel. Hypertension 33: 1324-1331

Cruz DN, Simon DB, Nelson-Williams C, Farhi A, Finberg K, Burleson L, Gill JR and Lifton RP (2001). Mutations in the Na-Cl cotransporter reduce blood pressure in humans. Hypertension 37: 1458-1464

Dahl LK (1960). Effects of chronic excess salt feeding: elevation of cholesterol in rats and dogs. J Exp Med 112: 635 – 651

Dahl LK, Heine M and Tassinari L (1962a). Effects of chronic salt ingestion; evidence that genetic factors play an important role in susceptibility to experimental hypertension. J. Exp Med. 115: 1173-1180

Dahl LK, Heine M and Tassinari L (1962b). Role of genetic factors in susceptibility to experimental hypertension due to chronic excess salt ingestion. Nature 194: 480-482

Dampney RA, Coleman MJ, Fontes MA, Hirooka Y, Horiuchi J, Li YW, Polson JW, Potts PD and Tagawa T (2002). Central mechanisms underlying short and long-term regulation of the cardiovascular system. Clin Exp Pharmacol Physiol 29: 261-268

de Lange M, Spector TD and Andrew T (2004). Genome-wide scan for blood pressure suggests linkage to chromosome 11 and replication of loci on 16, 17 and 22. Hypertension 44: 872-877

de Wardener HE and MacGregor GA (2001). Blood pressure and the kidney. In: Diseases of the kidney and Urinary Tract. (7th Edn). New York, Lippincott Williams & Wilkins.

de Wardener HE, He FJ and MacGregor GA (2004). Plasma sodium and hypertension. Kidney Int 66: 2454-2466

Debonneville C, Flores SY, Kamynina E, Plant PJ, Tauxe C, Thomas MA, Münster C, Chraïbi A, Pratt JH, Horisberger J-D, Pearce D, Loffing J and Staub O (2001). Phosphorylation of Nedd4-2 by Sgk 1 regulates epithelial Na(+) channel cell surface expression. EMBO J 20: 7052-7059

Denton D, Weisinger R, Mundy MI, Wickings EJ, Dixson A, Moisson P, Pingard AM, Shade R, Carey D and Ardaillou R (1995). The effect of increased salt intake on blood pressure of chimpanzees. Nature Medicine, 1: 1009-1016

Dong YB, Plange-Rhule J, Owusu I, Micah F, Eastwood JB, Carter NB, Saggar-Malik Ak, Cappuccio FP and Jeffery S (2002). T594M mutation of the β-subunit of the epithelial sodium channel in Ghanaian populations from Kumasi and london and a possible association with hypertension. Genetic Testing 6: 63-65

Doshi H, Weder AB, Bard RL and Brook RD (2010). Does "hidden undercuffing"occur among obese patients? Effect of arm sizes and other predictors of the difference between wrist and upper arm blood pressures. J Clin Hyper 12: 82-87

Drummond HA (2009). The (F)low Down on the endothelial epithelial sodium channel. Hypertension 53: 903-904

Drummond HA, Grifoni SC and Jernigan NL (2008a). A new trick for an old dogma: ENaC proteins as mechanotransducers in vascular smooth muscle. Physiology 23: 23-31

Drummond HA, Jernigan NL and Grifoni SC (2008b). Sensing tension: epithelial sodium channel/acid sensing ion channel proteins in cardiovascular homeostasis. Hypertension 51: 1265-1271

Ecelbarger CA, Kim GH, Wade JB and Knepper MA (2001). Regulation of the abundance of renal sodium transporters and channels by vasopressin. Exp Neurol 171: 227-234

Elliott P, Stamler J, Nichols R, Dyer AR, Stamler R, Kestleloot H and Marmot M (1996). Intersalt revisited: further analyses of 24 hour sodium excretion and blood pressure within and across populations. INTERSALT Cooperative Research Group. BMJ 312: 1249–1253

El-Sayed H and Hainsworth R. (1996). Salt supplementation increases plasma volume and orthostatic tolerance in patients with unexplained syncope. Heart, 75: 134-140

Eng J (2003). Sample size estimation: How many individuals should be studied? Radiology 227: 309-313

Everson SA, Kaplan GA, Goldberg DE and Salonen JT (1996). Anticipatory blood pressure response to exercise predicts future high blood pressure in middle-aged men. Hypertension 27:1059-1064

Everson SA, Lynch JW, Kaplan GA, Lakka TA, Sivenius J and Salonen JT (2001). Stress-induced blood pressure reactivity and incident stroke in middle-aged men. Stroke 32: 1263-1270

Exome Project. www.ncbi.nlm.nih.gov/SNP/snp-ref.cgi Retrieved 05/05/2012

Ezzati M, Lopez AD, Rodgers A, Vander Hoorn S and Murray CJ (2002). Comparative Risk Assessment Collaborating Group. Selected major risk factors and global regional burden of disease. Lancet 360: 1347-1360

Fakitsas P, Adam G, Daidie D, van Bemmelen MX, Fouladkou F, Patrignani A, Wagner U, Warth R, Camargo SM, Staub O and Verrey F (2007). Early aldosterone-induced gene product regulates the epithelial sodium channel by deubiquitylation. J Am Soc Nephrol 18: 1084-1092

Farquhar WB, Paul EE, Prettyman AV and Stillabower ME (2005). Blood pressure and haemodynamic responses to an acute sodium load in humans. J Appl Physiol 99: 1545-1551

Fels J, Oberleithner H and Kusche-Vihrog K (2010). Menage a trois: Aldosterone, sodium, nitric oxide in vascular endothelium. Biochim Biopyhs Acta 1802; 1193-1202

Feng JA, Perry G, Mori T, Harashi T, Oparil S and Chen YF (2003). Pressure-independent enhancement of cardiac hypertrophy in atrial natriuretic peptide-deficient mice. Clin Exp Pharmacol Physiol 30: 343-349

Ferrari P and Buachi G. (1995). In Hypertension: Pathophysiology, Diagnosis and Management. Laragh JH, Brenner BM (Eds). NY Raven, p1261-1279

Flaa A, Eide IK, Kjeldsen SE and Rostrup M (2008). Sympathoadrenal stress reactivity is a predictor of future blood pressure. An 18 year follow-up study. Hypertension 52: 336-341

Food and Nutrition Board, Institute of Medicine (2004). Sodium and Chloride. In "Dietary Reference intakes, Water, Potassium, Sodium, Chloride, and Sulfate". Washington DC: Food and Nutrition Board, Institute of Medicine

Franco V and Oparil S (2006). Salt sensitivity, a determinant of blood pressure, cardiovascular disease and survival. J Am College of Diseases 25: 247S-255S

Frewin DB and Whelan RF (1968). The mechanism of action of tyramine on the blood vessels of the forearm in man. Br J Pharmacol Chemother 33: 105-116

Frisbee JC and Lombard JH (1999). Acute elevations in salt intake and reduced renal cell mass hypertension compromise arteriolar dilation in rat cremaster muscle. Microvasc Res 57: 273-283

Frohlich ED, Chen Y, Sosoko S and Pegram B (1993). Relationship between dietary salt intake, haemodynamics and cardiac mass in spontaneously hypertensive rats and Wistar Kyoto rats. Am J Physiol 264: R30-R34

Fukuda M, Goto N and Kimura G. (2006). Hypothesis on renal mechanism of non-dipper pattern of circadian blood pressure rhythm. Med. Hypotheses 67: 802-806

Funder JW (2006). Mineralocorticoid receptors and cardiovascular damage: it's not just aldosterone. Hypertension 47: 634-635

Funder JW and Reincke M (2010). Aldosterone: A cardiovascular risk factor? Biochem Biophys Acta 1802: 1188-1192

Furudashi M, Kitamura K, Adachi M, Miyoshi T, Wakida N, Ura N, Shikano Y, Shinshi Y, Sakamoto K, Hayashi M, Satoh N, Nishitani T, Tomita K and Shimamoto K (2005). Liddle's syndrome caused by a novel mutation in the praline-rich PY motif of the epithelial sodium channel beta-subunit. J Clin Endocrinol. Metab 90: 340-344

Garty H and Palmer LG (1997). Epithelial sodium channels: structure, function and regulation. Phys Rev 77: 359-396

Gaukrodger N, Avery PJ and Keavney B (2008). Plasma potassium level is associated with common genetic variation in the β-subunit of the epithelial sodium channel. Am J Physiol Regul Integr Comp Physiol 294: R1068-R1072

Gavras H and Gavras I (1989). Salt-induced hypertension: the interactive role of vasopressin and of the sympathetic nervous system. J. Hypertens; 7: 601-606

Ghiadoni L, Donald AE, Cropley M, Mullen MJ, Oakley G, Taylor M, O'Connor G, Betterridge J, Klein N, Steptoe A and Deanfield JE (2000). Mental stress induces transient endothelial dysfunction in humans. Circulation 120:2473-2478

Giardina JB, Green GM, Rinewalt AN, Granger JP and Khalil RA (2001). Role of endothelium-β receptors in enhancing endothelium-dependent nitric oxide-mediated vascular relaxation during high salt diet. Hypertension 37: 516-523

Gilmore ES, Stutts MJ and Milgram SL (2001). SRC family kinases mediate epithelial Na+ channel inhibition by endothelin. J Biol Chem 276: 42610-42617

Golestaneh N, Klein C, Valamanesh G, Suarez G, Agarwal MK and Mirshahi M (2001). Mineralocorticoid receptor-mediated signalling regulates the ion gated sodium channel in vascular endothelial cells and requires an intact cytoskeleton. Biochem Biophys Res Commun 280: 1300-1306

Grassi G (2009). Assessment of sympathetic cardiovascular drive in human hypertension. Achievements and perspectives. Hypertension 54: 690-697

Grassi G, Cattaneo BM, Seraville G, Lanfrachi A and Mancia G (1998). Baroreflex control of sympathetic nerve activity in essential and secondary hypertension. Hypertension 31: 68-72

Graudal N (2005). Possible Role of salt intake in the development of essential hypertension. Int. J. Epidemiol. 34: 972-974

Graudal NA, Galloe A, Garred P. (1998). Effects of sodium restriction on blood pressure, renin, aldosterone, catecholamines, cholesterols and triglyceride, a meta-analysis. JAMA 279: 1383-1391

Grobee DE and Hoffman A., (1986). Does sodium restriction lower blood pressure? BMJ 293: 27-29

Grossmann C and Gekle M (2009). New aspects of rapid aldosterone signalling. Mol Cell Endocrinol 308: 53-62

Grunder S, Jaeger NF, Gautschi I, Schild L and Rossier BC (1999). Identification of a highly conserved sequence at the N-terminus of the epithelial Na+ channel alpha subunit involved in gating. *Pflügers Arch* 438: 709-715

Guyton AC (1987). Renal function curve: a key to understanding the pathogenesis of hypertension. Hypertension 10: 1-16

Guyton AC and Hall JE (1996). Textbook of Medical Physiology. 9th Edition (WB Saunders Company) ISBN 0-7216-5944-6, P 1009-1012

Guyton AC, Coleman TG, Cowley AV Jr., Scheel KW, Manning RD Jr., and Norman RA Jr. (1972). Arterial pressure regulation (1972). Overriding dominance of the kidneys in long-term regulation and in hypertension. Am J Med 52: 584-594

Hainsworth R, Sofola OA, Knill AJP and Drinkhill MJ (2003). Influence of dietary salt intake on the response of isolated perfused mesenteric veins of the dog to vasoactive agents. Am J Hypertens 16: 6-10

Hamet P, Merlo E, Seda O, Broeckel U, Tremblay J, Kaldunski M, Gaudet D, Bouchard G, Deslauriers B, Gagnon F, Antonio G, Pausovà Z, Labuda M, Jomphe M, Gossard F, Tremblay G, Kirova R, Tonellato P, Orlov SN, Pintos J, Platko J, Hudson TJ, Rioux JD, Kotchen TA and Cowler AW Jr (2005). Quantitative founder-effect analysis of French Canadian families identifies specific loci contributing to metabolic phenotypes of hypertension. Am J Hum Genet 76: 815-832

Hannila-Handelberg T, Kontula K, Tikkanen T et al (2005). Common variants of the beta and gamma subunits of the epithelial sodium channel and their relation to plasma renin and Aldosterone levels in essential hypertension. BMC Med. Genet 6:4

Harrap SB, Stebbing M, Hopper JL, Hoang HN and Giles GG (2000). Familial patterns of covariation for cardiovascular risk factors in adults. Victorian Family Heart Study. Am J Epidemiol 152: 704-715

Harvey BJ, Alzamora R, Stubbs AK, Irnaten M, McEneaney V and Thomas W (2008). Rapid responses to aldosterone in the kidney and colon. J Steroid Biochem Mol Biol 108:310-317

Hasselund SS, Flaa A, Sandvik L, Kjeldsen SE and Rostrup M (2010). Long-term stability of cardiovascular and cathecholamine responses to stress tests. An 18-year follow up study. Hypertension 55: 131-136

He FJ and MacGregor GA (2003). How far should salt intake be reduced? Hypertension 42: 1093-1099

He FJ and MacGregor GA (2007). Salt, blood pressure and cardiovascular disease. Curr Opin Cardiol 22:298-305

He FJ, Markandu ND, Sagnella GA de Wardener HE and MacGregor GA (2005). Plasma sodium: Ignored and underestimated. Hypertension 45: 98-102

He FJ, Markandu ND and MacGregor GA (2001). Importance of the renin system to determining blood pressure fall with acute salt restriction in hypertensive and normotensive whites. Hypertension 38: 321-325

He J, Gu D, Chen J, Jaquish CE, Rao DC, Hixson JE, Chen JC, Duan X, Huang JF, Chen CS, Kelly TN, Bazzano LA and Whelton PK for the GenSalt Collaborative research Group (2009). Gender difference in blood pressure responses to dietary sodium intervention in the GenSalt study. J Hypertens 27:48-54

He J, Klag MJ, Whelton PK, Chen J-Y, Mo J-P, Qian M-C, Mo P-S and He G-Q (1991). Migration, blood pressure pattern, and hypertension: The Yi Migrant Study. Am J Epidemiol134: 1085-1101

He J, Whelton PK, Appel LJ, Charleston J and Klag MJ (2000). Long-term effects of weight loss and dietary sodium reduction on incidence of hypertension. Hypertension, 35: 544-549

Hiltunen TP, Hannila-Handelberg T, Petäjäniemi N, Kantola I, Tikkanen I, Virtamo J, Gautsci I, Schild L and Kontula K (2002). Liddle's syndrome associated with point mutation in the extracellular domain of the epithelial sodium channel gamma-subunit. J Hypertens 20: 2383-2390

Hines EA and Brown GE (1936). The cold pressor test for measuring the reactability of the blood pressure: data concerning 571 normal and hypertensive subjects. Am Heart J 11: 1-9

Hollier JM, Martin DF and Bell DM (2006). Epithelial sodium channel allele T594M is not associated with blood pressure or blood pressure response to amiloride. Hypertension 47: 428-433

Holwerda SW, Fulton D, Eubank WL and Keller DM (2011). Carotid baroreflex is impaired in normotensive African American men. Am J Physiol Heart Circ Physiol 301: H1639-H1645

Hong Y, de Faire U, Heller DA et al (1994). Genetic and environmental influences on blood pressure in elderly twins. Hypertension 24: 663-670

Hooper L, Bartlett C, Davey SG and Ebrahim S (2004). Advice to reduce dietary salt for prevention of cardiovascular disease. Cochrane Database Syst Rev 1: CD003656

Hooper L, Bartlett C, Smith GD and Ebrahim S (2002). Systemic review of long term effects of advice to reduce dietary salt in adults. BMJ 325: 628

Hoshide S and Kario K (2008). Determinants of nondipping in nocturnal blood pressure and specific nonpharmacological treatments for nocturnal hypertension. Am J Hypertens 21: 968

Hughey RP, Bruns JB, Kinlough CL, Harkleroad KL, Tong Q, Carattino MD, Johnson JP, Stockand JD and Kleyman TR (2004a). Epithelial sodium channels are activated by furin-dependent proteolysis. J Biol Chem 279: 18111-18114

Hughey RP, Bruns JB, Kinlough CL, Kleyman TR (2004b). Distinct pools of the epithelial sodium channels are expressed at the plasma membrane. J Biol Chem 279: 48491-48494

INTERSALT (1988). An international study of electrolyte excretion and blood pressure. Results of 24hr urinary sodium and potassium excretion. BMJ, 297: 311-328

IUPAC-IUB Joint Commission on Biochemical Nomenclature (1984). Nomenclature and symbolism for amino acids and peptides. Eur J Biochem 138: 9-37

Iwai N, Kajimoto K, Kokubo Y (2006). Extensive genetic analysis of 10 candidate genes for hypertension in Japanese. Hypertension 48: 901-907

Jackson FLC (1991). An evolutionary perspective on salt hypertension and human genetic variability. Hypertension 17: I-129 – I-132

Jaja SI and Etemire E. (2007). Blood pressure and electrocardiographic changes during face immersion in water in young men on regular exercise. Nig. Qt J Hosp Med. 2006; 16(1): 1-5

Jaja SI, Aisuodionwe SI, Gbenebitse S and Kehinde MO (2003). Effect of Vitamin C Supplementation on vascular responses induced by warmth or cold stimulation in normal Nigerians. Nig Qtly J Hosp Med 13: 61-64

Jasti J, Furukawa H, Gonzales EB and Gouaux E (2007). Structure of acid-sensing ion channel 1 at 1.9. A resolution and low pH. Nature 449: 316-323

Jennings JR, Kamarc TW, Everson-Rose SA, Kaplan GA, Manuck SB and Salonen JT (2003). Exaggerated blood pressure responses during mental stress are prospectively related to enhanced carotid atherosclerosis in middle-aged Finnish men. Circulation 110: 2198-2203

Jernigan NL and Drummond HA (2005). Vascular ENaC proteins are required for renal myogenic constriction. Am J Physiol Renal Physiol 289: F891-F901

Jie K, van Brummelen P, Vermey P, Timmermans PBMWM and van Zwieten PA (1987). Postsynaptic a1- and a2-adrenoceptors in human blood vessels: interactions with exogenous and endogenous catecholamines. Eur J Clin Invest 17: 174-181

Joint National Committee on Prevention, Detection, Evaluation and Treatment of High Blood Pressure, 7th Report (2003). National Institute of Health, United States

Joyner MJ and Dietz NM (1997). Nitric oxide and vasodilation in human limbs. J Appl Physiol 83: 1785-1796

Joyner MJ, Dietz NM and Sheperd JT. (2001). From Belfast to Mayo and beyond: the use and future of plethysmography to study blood flow in human limbs. J Appl Physiol 91: 2431-2441

Julius S, Li Y, Brant D, Krause L and Buda AJ (1989). Neurogenic pressor episodes fail to cause hypertension, but do induce cardiac hypertrophy. Hypertension 13:422-429

Kagota S, Tamashiro A, Yamaguchi Y, Sugiura R, Kuno T, Nakamura K and Kunimoto M (2001). Down regulation of vascular soluble guanylate cyclase by high salt intake in spontaneously hypertensive rats. Br. J. Pharmacol. 134: 737-744

Kajantie E and Phillips DIW (2006). The effects of sex and hormonal status on the physiological response to acute psychosocial stress. Psychonueroendocrinology 31: 151-178

Kamark TW, Schwartz JE, Janicki DL, Schiffman S and Raynor DA (2003). Correspondence between laboratory and ambulatory measures of cardiovascular reactivity: a multilevel modelling approach. Psychophysiology 40: 675-683

Kaminar B and Lutz WPW (1960). Blood pressure in Bushmen of the Kalahari Desert. Circulation, 22: 289-295

Kamynina E, Debonneville C, Bens M, Vandewalle A and Staub O (2001). A novel mouse Nedd4 protein suppresses the activity of the epithelial Na^+ channel. FASEB J 15: 204-214

Kaplan NM (2000). The dietary guideline for sodium: should we shake it up? No. American Journal of Clinical Nutrition, 71: 1020-1026

Kaplan NM (2006). Clinical Hypertension, 9th edn. Philadelphia, USA: Lippincott Williams and Wilkins

Kawano H, Tanimoto M, Yamamoto K, Sanada K, Gando Y, Tabata I, Higuchi M and Miyachi M (2007). Resistance training in men is associated with increased arterial stiffness and blood pressure but does not adversely affect endothelial function as measured by arterial reactivity to the cold pressor test. Exp Physiol 93: 296-302

Kawasaki T, Delea CS, Bartter FC and Smith H (1978). The effect of high-sodium and low-sodium intakes on blood pressure and other related variables in human subjects with idiopathic hypertension. Am J Med 64: 193-198

Kellenberger S and Schild L (2002). Epithelial sodium channel / Degenrin family of ion channels: a variety of functions for a shared structure. Physiol Rev 82: 735-767

Kelsey RM, Alpert BS, Patterson SM, Barnard M (2000a). Racial differences in haemodynamic responses to environmental thermal stress among adolescents. Circulation 101: 2284-2289

Kelsey RM, Patterson SM, Barnard M and Alpert BS (2000b). Consistency of haemodynamic responses to cold stress in adolescents. Hypertension 36: 1013-1017

Kerem E, Bistritzer T, Hanukoglu A, Hofmann T, Zhou Z, Bennet W, MacLaughlin E, Barker P, Nash M, Quittel L, Boucher R and Knowles MR (1997). Pulmonary epithelial sodium-channel dysfunction and excess airway liquid in pseudohypoaldosteronism. New Engl J Med 341: 156-162

Khalil RA (2006). Molecular mechanisms linking salt to hypertension. Dietary salt and hypertension: new molecular targets add more spice. Am J Physiol Regul Integr Comp Physiol 290: R509-R513

Kimura G (2008). Kidney and circadian blood pressure rhythm. Hypertension 51: 827-828

Kimura G and Brenner BM (1993). A method of distinguishing between salt-sensitive from non-salt-sensitive forms of human and experimental hypertension. Curr Opin Nephrol Hypertens 2: 341-349

Kimura G and Brenner BM (1997). Implications of the linear pressure-natriuresis relationship and importance of sodium sensitivity in hypertension. J Hypertens 15:1055-1061

Kiviniemi TO, Schindler T, Tulppo M, Knuuti J, Raitakari O and Koskenvuo JW (2011). Cold pressor test safety-the incidence of vasovagal reactions. Am J Cardiol 107: 492-493

Knight KK, Olson DR, Zhou R and Snyder PM (2006). Liddle's syndrome mutations increase Na^+ transport through dual effects on epithelial sodium channel surface expression and proteolytic cleavage. Proc Natl Acad Sci USA 103: 2805-2808

Kolla V and Litwack G (2000). Transcriptional regulation of the human Na/K ATPase via the human mineralocorticoid receptor. Mol Cell Biochem 204: 35-40

Kotanko P, Holinger O and Skrabal F (1992). Beta-2-adrenoceptor density in fibroblast culture correlates with human NaCl sensitivity. AM J Physiol 263: C623-C627

Langloh ALB, Berdiev B, Ji H-L, Keyser K, Stanton BA and Benos DJ (2000). Charged residues in the M2 region of α-hENaC play a role in channel conductance. Am J Cell Physiol 278: C277-C291

Lawes CMM, Hoorn SV and Rodgers A (2008) for The International Society of Hypertension. Global burden of blood-pressure-related disease, 2001. Lancet 371: 1513-1518

Lenda DM, Sauls BA and Boegehold MA (2000). Reactive oxygen species may contribute to reduced endothelium-dependent dilation in rats fed a high salt diet. Am. J. Physiol. 264: F44-F347

Lifton RP, Gharavi AG and Geller DS (2001). Molecular mechanisms of human hypertension. Cell 104: 545-556

Lim PO, Rana BS, Struthers AD and MacDonald TM (2001). Exercise blood pressure correlates with the maximum heart rate corrected QT interval in hypertension. J Hum Hypertens 15: 169-172

Linder L, Kiowski W, Bu"hler FR and Lu"scher TF (1990). Indirect evidence for release of endothelium-derived relaxing factor in human forearm circulation in vivo. Blunted response in essential hypertension. Circulation 81: 1762-1767

Loffing J and Korbmacher C (2009). Regulated sodium transport in the renal connecting tubule (CNT) via the epithelial sodium channel (ENaC). Pfflugers Arch 458: 111-135

Loffing J and Kaissling B (2003). Sodium and calcium transport pathways along the mammalian distal nephron: from rabbit to human. Am J Physiol Renal Physiol 284: F628 – F643

Loffing J, Loffing-Cueni D, Valderrabano V, Klausli L, Hebert SC, Rossier BC, Hoenderop JG, Bindels RJ and Kaissling B (2001). Distribution of transcellular calcium and sodium transport pathways along mouse distal nephron. Am J Physiol Renal Physiol 281: F1021-F1027

Lovallo WR and Gerin W (2003). Psychophysiological reactivity; mechanisms and pathways to cardiovascular disease. Psychosom Med. 65: 36-45

Luft FC (2001). Molecular genetics of salt-sensitivity and hypertension. Drug Metab Dispos 29: 500-504

Luft FC and Weinberger MH (1997). Heterogeneous responses to changes in dietary salt intake: the salt sensitivity paradigm. Am J Clin Nutr 65: 612S-617S

Luft FC, Weinberger MH, Fineberg NS, Miller JZ and Grim CE (1987). Effects of age on renal sodium homeostasis and its relevance to sodium sensitivity. Am J Med 82: 9-15

MacGregor GA and de Wardener HE. (1998). Salt, Diet and Health: Neptune's Poisoned Chalice. Cambridge University Press, Cambridge

Makaritsis KP, Handy DE, Johns C, Kobilka B, Gavras I and Gavras H (1999). Role of the $\alpha_{2\beta}$-adrenergic receptor in the development of salt-induced hypertension. Hypertension 33: 14-17

Malik B, Price SR, Mitch WE, Yue Q and Eaton DC (2006). Regulation of epithelial sodium channels by the ubiquitin-proteasome proteolytic pathway. Am J Physiol Renal Physiol 290: F1285-F1294

Malik B, Schlanger L, Al Khalil O, Bao H-F, Yue G, Price SR, Mitch WE and Eaton DC (2001). ENaC degradation in A6 cells by ubiquitin-proteosome proteolytic pathway. J Biol Chem 276: 12903-12910

Mathews KA, Katholi CR, McCreath H, Whooley MA, Williams DR, Zhu S and Markovitz JH (2004). Blood pressure reactivity to psychological stress predicts hypertension in the CARDIA Study. Circulation 110: 74-78

Mazzochi C, Bubien JK, Smith PR and Benos DJ (2006). The carboxyl terminus of the alpha-subunit of the amiloride-sensitive epithelial sodium channel binds to F-actin. J Biol Chem 281: 6528-6538

McDonald FJ, Yang B, Hrstka RF, Darr TE, McCray PB Jr, Stokes JB, Welsh MJ and Willaimson RA (1999). Disruption of the beta subunit of the epithelial Na+ channel in mice hyperkalaemia and neonatal death associated with Psuedohypoaldosteronism phenotype. Proc Natl Acad Sci USA 96: 1727-1731

McNeely, JD, Windham BG and Anderson DE (2008). Dietary sodium effects on heart rate variability in salt-sensitivity of blood pressure. Psychophysiology 45: 405-411

Mei H, Gu D, Rice TK, Hixson JE, Chen J, Jaquish GE, Zhao Q, Chen C-S, Chen J-C, Gu CC, Kelly TN and He J (2009). Heritability of blood pressure responses to cold pressor test in a Chinese population. Am J Hypertens 22: 1096-1100

Meneton P, Jeunemaitre X, De Wardener HE and MacGregor G (2005). Links between dietary salt intake, renal salt handling, blood pressure and cardiovascular diseases. Physiol Rev 85: 679-715

Meneton P, Oh YS and Warnock D (2001). Genetic renal disorders of renal ion channels and transporters. Semin Nephrol 21: 81-93

Mills PJ, Dimsdale JE, Ziegler MG and Nelesen RA (1995). Racial differences in epinephrine and β_2-adrenergic receptors. Hypertension 25: 88-91

Mimran A, Ribstein J and Jover B (1992). Aging and sodium homeostasis. Kidney Int Suppl 37: S107- S113

Mir MA and Newcombe R (1988). The relationship of dietary salt and blood pressure in three farming communities in Kashmir. J Hum Hypertens 2:241-246

Miyajima E and Bunnang RD (1985). Dietary salt loading produces baroreflex impairment and mild hypertension in rats. Am J. Physiol., 24: H27-H284

Modesti PA, Morabito M, Bertolozzi I, Massentti L, Panci G, Lumachi C, Giglio A, Bilo G, Caldara G, Lonati L, Orlandini S, Mancia G, Gensini GF and Prati G (2006). Weather related changes in 24-h blood pressure profile: effect of age and implication for hypertension management. Hypertension 47: 155-161

Mongeau JG (1987). Heredity and blood pressure in humans: an overview. Pediatr Nephrol 1: 69-75

Moriyama K and Ifuku H. (2010). Increased cardiovascular reactivity to the cold pressor test is not associated with increased reactivity to isometric handgrip exercise. Eur J Appl Physiol 108: 837-843

Moyna NM and Thompson PD (2004). The effect of physical activity on endothelial function in man. Acta Physiol Scand 180: 113-123

Murakami S, Otsuka K, Kono T, Soyama A, Umeda T, Yamamoto N, Morita H, Yamanaka G and Kitaura Y (2011). Impact of outdoor temperature on prewaking morning surge and nocturnal decline in blood pressure in a Japanese population. Hypertens Res 34: 70-73

Najjar SS, Scuteri A and Lakatta EG (2005). Arterial aging: is it an immutable cardiovascular risk factor? Hypertension 46: 454-462

Nakayama T, Soma M, Takahasi Y, Rehemudula D, Kanmatsuse K and Furuya K (2000). Functional deletion mutation of the 5′-flanking region of type A human natriuretic peptide and association with essential hypertension and left ventricular hypertrophy in the Japanese. Circ Res 86: 841-845

Nishida Y, Ding J, Zhou M, Chen Q, Murakami H, Wu X and Kosaka H (1998). Role of nitric acid in vascular hyper-responsiveness to norepinephrine in hypertensive Dahl rats. J Hypertens. 16: 1611-1618

Nkeh B, Samani NJ, Badenhorst D, Libhaber E, Sareli P, Norton GR and Woodiwiss AJ (2003). T594M Variant of the Epithelial Sodium Channel β-Subunit Gene and Hypertension in Individuals of African Ancestry in South Africa. Am J Hypertens 16: 847-852

O'Neill H, Lebeck J, Colins PB, Kwon TH, Frokiaer J and Nielsen S (2008). Aldosterone-mediated apical targeting of ENaC subunits is blunted in rats with streptozocin-induced diabetes mellitus. Nephrol Dial Transplantat 23: 1546-1555

Obasohan AO, Ukoh VA, Onyia KA and Isah AO (1992). Salt taste threshold in normotensive and hypertensive Nigerians. Tropical Cardiol. 18: 183-187

Oberleithner H, Callies C, Kusche-Virhog K, Schillers H, Shahin V, Riethmuller C, MacGregor GA and de Wardener HE (2009). Potassium softens vascular endothelium and reduces nitric oxide release. Proc Natl Acad Sci USA 106: 2829-2834

Oberleithner H, Riethmuller T, Ludwig V, Shahin V, Stock C, Schwab A, Hausberg M, Kusche K and Schillers H (2006). Differential action of steroid hormones on human endothelium. J Cell Sci 119: 1926-1932

Oberleithner H, Riethmuller T, Schillers H, MacGregor GA, de Wardener HE and Hausberg M (2007). Plasma sodium stiffens vascular endothelium and reduces nitric oxide. PNAS 104: 16281-16286

Obiefuna PCM, Ebeigbe AB, Sofola OA and Aloamaka PC (1991a). Altered responses from aortic smooth muscle from Sprague Dawley rats with salt-induced hypertension. Clin. Exp. Pharmacol. Physiol., 18: 813-818

Obiefuna PCM, Sofola OA and Ebeigbe AB (1991b). Dietary salt-loading attenuates endothelium-dependent relaxation to histamine but not to acetylcholine. Exp. Physiol. 76: 135-138

Ogoh S, Fadel PJ, Nissen P, Jans O, Selmer C, Secher NH and Raven PB (2003). Baroreflex-mediated changes in cardiac output and vascular conductance in response to alterations in carotid sinus pressure during exercise in humans. J Physiol 550: 317-324

Oliver WJ, Cohen EL and Neel JV (1975). Blood pressure, sodium intake and sodium-related hormones in Yamomono Indians, a "no salt" culture. Circulation, 52: 146-151

Oloyo AK, Sofola OA and Anigbogu CN **(**2011). Orchidectomy attenuates impaired endothelial effects of a high salt diet in Sprague-Dawley rats. Can J Physiol Pharmacol 89**:** 295–304

Oparil S, Meng QC, Chen YF Yang RH, Jin HK and Wyss JM (1988). Genetic basis of NaCl-sensitive hypertension. J Cardiovasc Pharmacol 12: S56-S69

Osotimehin B, Erasmus RT, Iyun AO et al. (1984). Plasma renin activity and plasma aldosterone concentrations in untreated Nigerians with essential hypertension. Afr. J. Med. Med. Sci. 13: 139-143

Page GP, George V, Go RC, Page PZ and Allison DB (2003). "Are we there yet?" Deciding when one has demonstrated specific genetic causation in complex diseases and quantitative traits. Am J Hum Genet 73: 711-719

Page LB, Vandevert DE, Nader K, Lubin NK and Page JR (1981). Blood pressure of Qash'qai pastoral nomads in Iran in relation to culture, diet and body form. Am J Clin Nutr 34:527-538

Palmer LG and Frindt G (1996). Gating of Na channels in the rat cortical collecting tubule: effects of voltage and membrane stretch. J Gen Physiol 107: 35-45

Panel on Dietary Reference Intakes for Electrolytes and Water (2004). Institute of Medicine, Food and Nutrition Board. Dietary Reference Intakes: Water, Potassium, Chloride and Sulfate. Washington, DC: National Academics Press

Panza JA, Cassino PR, Badar DM and Quyyumi AA (1993). Effect of increased availability of endothelium-derived nitric oxide precursor on endothelium-dependent vascular relaxation in normal subjects and in patients with essential hypertension. Circulation 87: 1475-1481

Persu A, Barbry P, Bassilana F, Bassilana F, Houot A, Mengual R, Lazdunski M, Corvol P and Jeunemaitre X (1998). Genetic analysis of the beta subunit of the epithelial Na^+ channel in essential hypertension. Hypertension 32: 129-137

Pickering TG, Hall JE, Appel LJ, Falkner BE, Graves J, Hill MN, Jones DW, Kurtz D, Sheps SG and Rocella EJ (2005). Recommendations for blood pressure measurement in humans and experimental animals. Part 1: Blood pressure measurement in humans. A statement for Professionals from the Subcommittee of Professional and Public Education of the American Heart Association Council on High Blood pressure Research. Hypertension 45: 142-161

Pochynyuk O, Bugaj V, Vandewalle A and Stockand JD (2008). Purinergic control of apical plasma membrane $PI(4,5)P_2$ levels set ENaC activity in principal cells. Am J Physiol Renal Physiol 294: F38-F46

Poulter NE, Khaw KT, Mugambi M, Peart WS and Sever PS (1985). Migration-induced changes in blood pressure: a controlled longitudinal study. Clin Exp Pharmacol Physiol 12: 211-216

Pratt JH, Ambrosius WT, Agarwal R, Eckert GJ and Newman S (2002). Racial difference in the activity of the amiloride-sensitive epithelial sodium channel. Hypertension 40: 903-908

Pratt JH, Eckert GJ, Newmann S and Ambrosius WT (2001). Blood pressure responses to small doses of amiloride and spironolactone in normotensive subjects. Hypertension 38; 1124-1129

Qadri YJ, Song Y, Fuller CM and Benos DJ (2010). Amiloride docking to acid-sensing ion channel-1. J Biol Chem 285: 9627-9635

Rasmussen MS, Simonsen JA, Sandgaard NCF, Hoilund-Carlsen PF and Bie P (2003). Mechanisms of acute natriuresis in normal humans on low sodium diet. J Physiol 546: 591-603

Rautureau Y, Paradis P and Schiffrin EL (2011). Cross-talk between aldosterone and angiotensin signalling in vascular smooth muscle cell. Steroids 76: 834-839

Rayner BL, Owen EP, King JA, Soule SG, Vreede H, Opie LH, Marais D and Davidson JS (2003). A new mutation, R563Q, of the beta subunit of the epithelial sodium channel associated with low renin, low-aldosterone hypertension. J Hypertens 21: 921-926

Robertson JIS (2003). Dietary salt and hypertension, a scientific issue or a matter of faith? Journal of Evaluation in Clinical Practice 9: 1-22

Rodriguez-Iturbe B and Vaziri ND (2007). Salt-sensitive hypertension – update on novel findings. Nephrol Dial Transplant 22: 992-995

Rossier BC (2004). The epithelial sodium channel. Activation by membrane-bound serine proteases. Proceedings of American Thoracic Society 1: 4-9

Rossier BC and Schild, L (2008). Epithelial sodium channel. Mendelian versus essential hypertension. Hypertension 52: 595-600

Rossier BC and Stutts MJ (2009). Activation of the epithelial sodium channel by serine proteases. Annu Rev Physiol 71: 361-379

Rothwell PM, Howard SC, Dolan E, O'Brien E, Dobson JE, Dahlof B, Sever PS and Poulter NR (2010). Prognostic significance of visit-to-visit variability, maximum systolic blood pressure and episodic hypertension. Lancet 375: 895-905

Rotin D, Kanelis V and Schild L (2001). Trafficking and cell surface stability of ENaC. Am J Physiol Ren Physiol 281: F391-F399

Roy-Gagnon MH, Weir MR, Sorkin JD, Ryan KA, Sack PA, Hines S, Bielak LF, Peyser PA, Post W, Mitchell BD, Shuldeiner AR and Douglas JA (2008). Genetic influences on blood pressure response to the cold pressor test: results from the Heredity and Phenotype Intervention Heart Study. J Hypertens 26: 729-736

Rubenfire M, Rajagopalan S and Mosca L (2000). Carotid artery vasoreactivity in response to sympathetic stress correlates with coronary disease risk and is independent of wall thickness. J Am Coll Cardiol 36P 2192-2197

Ruffieux-Daidie D, Poirot O, Boulkroun S, Verrey F, Kellenberger S and Staub O (2008). Deubiquitylation regulates activation and proteolytic cleavage of the ENaC. J Am Soc Nephrol 19; 2170-2180

Sachdeva A and Weder AB (2006). Nocturnal sodium excretion, blood pressure dipping and sodium sensitivity. Hypertension 48: 527-533

Sacks FM, Svetkey LP, Vollmer WM, Appel LJ, Bray GA, Harsha D, Obarzanek E, Conlin PR, Miller 3rd ER, Simons-Morton DG, Karanja N, Lin PH and DASH-Sodium Collaborative Research Group (2001). Effects on blood pressure of reduced dietary sodium and dietary approaches to stop hypertension (DASH) diet. N Engl. J. Med., 344: 3-10

Saha C, Eckert GJ, Ambrosius WT, Chun T-Y, Wagner MA, Zhao Q and Pratt JH (2005). Improvement in blood pressure with inhibition of the epithelial sodium channel in blacks with hypertension. Hypertension 46: 481-487

Sanders PW (2008). Salt sensitivity; It is not always in the genes. Hypertension 51: 823-824

Schäfer C, Shahin V, Albermann L, Hug MJ, Reinhardt J, Schillers H, Schneider SW and Oberleithner H (2002). Aldosterone signalling pathway across the nuclear envelope. PNAS 99: 7154-7159

Schiffrin EL (2006). Effects of aldosterone on the vasculature. Hypertension 47: 312-318

Schild L, Canessa CM, Shimkets RA, Gautschi I, Lifton RP and Rossier BC (1995). A mutation in the epithelial sodium channel causing Liddle disease increases channel activity in Xenopus Laevis oocyte expression system. Proc Natl Acad Sci 92: 5699-5703

Schild L. (2010). The epithelial sodium channel and the control of sodium balance. Biochim Biophys Acta 1802: 1159-1165

Schmidlin O, Forman A, Sebastian A and Morris RC Jr. (2007). What initiates the pressor effect of salt in salt-sensitive humans? Hypertension 49: 1032-1039

Sheng S, Carattino MD, Bruns JB, Hughey RP and Kleyman TR (2006). Furin cleavage activates the epithelial Na^+ channel by relieving Na^+ self inhibition. Am J Physiol 290: F1488-F1496

Sheng S, Li J, McNulty KA, Avery D and Kleyman TR (2000). Characterization of the selectivity filter of the epithelial sodium channel. J. Biol Chem 275: 8572-8581

Sheng S, Li J, McNulty KA, Kieber-Emmons T and Kleyman TR (2001). Epithelial sodium channel pore region. Structure and role in gating. J Biol Chem 276: 1326-1334

Shigaev A, Asher C, Latter H, Garty H and Reuveny E (2000). Regulation of SGK by aldosterone and its effects on the epithelial Na(+) channel. Am J Physiol 277: F319-F327

Shimkets RA, Warnock DG, Bositis CM, Nelson-Williams C, Hansson, JH, Schambelan M, Gill JR Jr, Ulick S, Milora RV and Findling JW (1994). Liddle's syndrome: Heritable human hypertension caused by mutations in the beta subunit of the epithelial sodium channel. Cell 79: 407-414

Siegrist PT, Gaemperli O, Koepfli P, Schepis T, Namdar M, Valenta I, Aiello F, Fleischmann S, Alkdhi H and Kaufmann PA (2006). Repeatability of cold pressor test-induced flow increase assessed with H2150 and PET. J Nucl Med. 47:1420-1426

Skrabal F, Kotanko P and Luft FC (1989). Inverse regulation of alpha-2 and beta-2 adrenoceptors in salt sensitive hypertension: an hypothesis. Life Sci 45: 2061-2076

Smolensky MH and Haus E (2001). Circadian rhythms and clinical medicine with applications to hypertension. Am. J. Hypertens 14: 280S-280S

Snyder PM (2000). Liddle's syndrome mutations disrupt cAMP-mediated translocation of the epithelial Na(+) channel to the cell surface. J Clin Inves 105: 45-53

Snyder PM (2002). The epithelial Na^+ channel: cell surface insertion and retrieval in Na^+ homeostasis and hypertension. Endocr Rev. 23: 258-275

Snyder PM, Bucher DB and Olson DR (2000). Gating induces a conformational change in the outer vestibule of ENaC. J Gen Physiol 116: 781-790

Snyder PM, Olson DR and Bucher DB (1999). A pore segment in the Degenerin/ENaC Na^+ Channels. J Biol Chem 274: 28484-28490

Sofola OA (2004). Salt, Blood pressure and cardiovascular changes in human and experimental studies – A review. Nig Med Pract 46: 22-26

Sofola OA, Adegunloye BJ and Iduwe G. (1999). Dietary salt loading effects on contractile response of atria of Sprague Dawley rats to noradrenaline and acetylcholine. J. Physiol. 518P: 43-44p (Proceedings).

Sofola OA, Azinge EC and Mabayoje O (1998). The cardiovascular and serum electrolyte responses to acute salt-loading in normotensive Nigerians. Nig Med Pract 35: 12-15

Sofola OA, Knill A, Hainsworth R and Drinkhill MJ (2002). Change in endothelial function in mesenteric arteries of Sprague Dawley rats fed a high salt diet. J. Physiol. 543: 255-260

Sofola OA, Knill A, Myers D, Hainsworth R and Drinkhill MJ (2004). High salt diet and responses of pressurized messenteric artery of the dog to noradrenaline and acetylcholine. Clin Exp Pharmacol Physiol 31: 696 –699

Soundararajan R, Pearce D, Hughey RP and Kleyman TR (2010). Role of epithelial sodium channels and their regulators in hypertension. J Biol Chem 285: 30363-30369

Soundararajan R, Zhang TT, Wang J, Vandewalle A and Pearce D (2005). A novel role for Glucocorticoid-induced leucine zipper protein in epithelial sodium channel-mediated sodium transport. J Biol Chem 280: 39970-39981

Stachenfeld NS and Keefe DL (2002). Estrogen effects on osmotic regulation of AVP and fluid balance. Am J Physiol Endocrinol Metab 283: E711-E721

Stamler J, Rose G, Elliot P, Dyer A, Marmot M, Kesteloot H and Stamler R (1991). Findings of the international co-operative INTERSALT study. Hypertension 17: I-9 - I-15

Staub O, Gautschi I, Ishikawa T, Breitschopf K, Clechanover A, Schild L and Rotin D (1997). Regulation of stability and function of the epithelial Na^+ channel (ENaC) by ubiquitynation. EMBO J 16: 6325-6336

Stein CM, Lang CC, Singh I, He HB and Wood, AJJ (2000). Increased vascular vasoconstriction and decreased vasodilation in blacks. Additive mechanisms leading to enhanced vascular reactivity. Hypertension; 36: 945-951

Steptoe A (2007). Psychological contributions to behavioural medicine and psychomatics. In: Cacioppo JT, Tassinary LG, Bernston G, eds. Handbook of Psychophysiology 3rd ed. Ney York, NY: Cambridge University Press. Pg 723-751

Steptoe A, Donald AE, O'Donnell K, Marmot M and Deanfield JE (2006). Delayed blood pressure recovery after psychological stress is associated with carotid intima-media thickness. Whitehall Psychobiology Study. Arterioscler Thromb Vasc Biol 26: 2547-2551

Stewart JC and France CR (2001). Cardiovascular recovery from stress predicts longitudinal changes in blood pressure. Biol Psychol 58: 105-120

Stewart JC, Janicki DL and Kamarck TW (2006). Cardiovascular reactivity to and recovery from challenges as predictors of 3-year change in blood pressure. Health Psychol 25: 111-118

Su YR and Menon AG (2001). Epithelial sodium channels and hypertension. Drug metabolism and Disposition 29: 553-556

Su YR, Rutkowski MP, Klanke CA, Wu X, Cui Y, Pun RY, Carter V, Reif M and Menon AG (1996). A novel variant of the beta-subunit of the amiloride-sensitive sodium channel in African Americans. J Am Soc Nephrol 7: 2543-2549

Swift PA and McGregor GA (2004). Genetic variation in the epithelial sodium channel: a risk factor for hypertension in people of African origin. Adv Renal Replace Ther 11: 76-86

Taubes G. (1998). The (political) science of salt. Science; 281: 898-907

Tesson F and Leenen FHH (2007). Still building on candidate-gene strategy in hypertension? Hypertension 50:607-608

Thomas CP, Zhou J, Liu KZ, Mick VE, MacLaughlin E and Knowles M (2004). Gene symbol SCNN1B. Disease: pseudohypoaldosteronism type 1. Hum Genet. 114: 402

Titze J and Machnik A (2010). Sodium sensing in the interstitium and relationship to hypertension. Curr Opin Nephrol Hypertens. 19: 385-392

Titze J, Krause H, Hccht H, Dictsch P, Rittweger J, Lang R, Kirsch KA and Hilgers KF (2002). Reduced osmotically inactive Na storage capacity and hypertension in the Dahl model. Am J Physiol 285: F134-F141

Titze J, Lang R, Ilics C, Schwind KH, Kirsch KA, Dictsch P, Luft FC and Hilgers KF (2003). Osmotically inactive skin Na^+ storage in rats. Am J Physiol 285: F1108-F1117

Treiber FA, Jackson RW, Davis H, Pollock JS, Kapuku G, Mensah GA and Pollock DM (2000). Racial differences in endothelin-I at rest and in response to acute stress in adolescent males. Hypertension 35: 722-725

Treiber FA, Kamarc T, Schneiderman N, Sheffield D, Kapuku G and Taylor T (2003). Cardiovascular reactivity and development of preclinical and clinical disease states. Psychosom Med 65: 46-62

Tzemos N, Lim PO, Wong S, Struthers AD and MacDonald TM (2008). Adverse cardiovascular effects of acute salt loading in young normotensive individuals. Hypertension 51: 1525 – 1530

Ulasi II, Ijoma CK and Onodugo OD (2010). A community-based study of hypertension and cardio-metabolic syndrome in a semi-urban and rural communities in Nigeria. BMC Health Serv Res 10: 71

Uzu T and Kimura G. (1999). Diuretics shift circadian rhythm of blood pressure from non-dipper to dipper in essential hypertension. Circulation 100: 1635-1638

Uzu T, Ishikawa K, Fujii T, Nakamura S, Inenaga T and Kimura G (1997). Sodium restriction shifts circadian rhythm of blood pressure from non-dipper to dipper in essential hypertension. Circulation 96: 1859-1862

Uzu T, Kimura G, Yamauchi A, Kanasaki M, Isshiki K, Araki S, Sugiomoto T, Nishio Y, Maegawa H, Koya D, Haneda M and Kashiwagi A (2006). Enhanced sodium sensitivity and disturbed circadian rhythm of blood pressure in essential hypertension. J Hypertens 24: 1627-1632

Uzu T, Nishimura M, Fujii T, Takeji M, Kuroda S, Nakamura S, Inenaga T and Kimura G (1998). Changes in the circadian rhythm of blood pressure in primary aldosteronism in response to dietary sodium restriction and adrenalectomy. J Hypertens 16: 1745-1748

Verrey F, Fakistas P, Adam G and Staub O (2008a). Early transcriptional control of ENaC (de)ubiquitylation by aldosterone. Kidney Int 73: 691-696

Verrey F, Hummler E, Schild L and Rossier BC (2008b). Mineralocorticoid action in the aldosterone-sensitive distal nephron. In: Alpern RJ, Hebert SC eds. The Kidney, Physiology and Pathophysiology, 4th ed. Burlington, VT: Academic Press, pg 889-924

Volmer WM, Sacks FM, Ard J, Appel LJ, Bray GA, Simons-Morton DG, Conlin PR, Svetkey LP, Erlinger TP, Moore TJ, Karanja N (2001) for the DASH-Sodium Trial Collaborative

Research Group. Effects of diet and sodium intake on blood pressure: subgroup analysis of the DASH-Sodium trial. Ann Intern Med 135: 1019-1028

Wang S, Meng F, Mohan S, Champaneri B and Gu Y (2009). Functional ENaC Channels expressed in endothelial cells: a new candidate for mediating shear force. Microcirculation 16: 276-287

Warnock DG (1999). T594M mutation in the ENaC beta subunit and low renin hypertension in Blacks. Am J Kidney Dis. 34: 579-587

Warnock DG (2001). Genetic forms of human hypertension. Curr Opin Nephrol Hypertens 10: 493-499

Warnock DG and Bell PD (2005). Improvement of blood pressure with inhibition of the epithelial sodium channel in blacks with hypertension. Hypertension 46: 469-470

Weinberger MH (1996). Salt sensitivity of blood pressure in humans. Hypertension 27: 481-490

Weinberger MH (2004). More on the sodium saga. Hypertension 44: 609-611

Weinberger MH (2008). The cold pressor test: A new predictor of future hypertension? Arch Intern Med 168: 1732

Weinberger MH and Fineberg NS (1991). Sodium and volume sensitivity of blood pressure. Age and pressure change over time. Hypertension 18:67–71

Weinberger MH, Fineberg NS, Fineberg SE and Weinberger M (2001). Salt sensitivity, pulse pressure and death in normal and hypertensive humans. Hypertension (2): 429-432

Weinberger MH, Miller JZ, Luft FC and Grim CE and Fineberg NS (1986). Definitions and characteristics of sodium sensitivity and blood pressure resistance. Hypertension 8: II-127 – II-134

Wildling L, Hinterdorfer P, Kusche-Vihrog K, Treffner Y and Oberleithner H (2009). Aldosterone receptor sites on plasma membrane of human vascular endothelium detected by a mechanical nanosensor. Pfluggers Arch 458: 223-230

Wilson TW and Grim CE (1991). Biohistory of slavery and blood pressure differences in blacks today. Hypertension, 17: I-122 - I-128

Wingfield D, Cooke J, Thijs L, Staessen JA, Fletcher AE, Fagard R and Bulpitt CJ (2002). Terminal digit preference and single-number preference in the Syst-Eur trial: influence of quality control. Blood Press Monit 7: 169-177

Wirch JL, Wolfe LA, Weissgerber TL and Davies GAL (2006). Cold pressor test protocol to evaluate cardiac autonomic function. Appl Physiol Nutr Metab 31: 235-243

Wood DL, Sheps SG and Elveback LR (1984). Cold pressor test as a predictor of hypertension. Hypertension 6: 301-306

Woodwell DA and Cherry DK (2004). National ambulatory medical care survey: 2002 summary. Adv Data 346: 1-44

World Health Organization (2002). Reducing risks, promoting healthy life. Geneva, Switzerland. World Health Report. http://www.who.int/whr/2002

World Health Organization (2007). World Health Organization 2007 fact sheet on cardiovascular disease. Available at http:llwww.who.int/mediacentre/factsheets/fs317/en/print.html.

World Medical Association (2008). World Medical Association Declaration of Helsinki - Ethical principles for medical research involving human subjects. 59th World Medical Assembly (WMA) General Assembly, Seoul

Wright JT Jr, Rahman M, Scarpa A, Fatholani M, Griffin V, Jean-Baptiste R, Islam M, Eissa M, White S and Douglas JG (2003). Determinants of salt sensitivity in black and white normotensive and hypertensive women. Hypertension 42: 1087-1092

Yamada T, Konno N, Matsuda K and Uchiyama M (2007). Frog atrial natriuretic peptide and cGMP activate amiloride-sensitive Na(+) channels in urinary bladder cells of Japanese tree frog. Hyla Japonica. J Comp Physiol [B] 177: 503-508

Yamori Y, Liu L, Ikeda K, Mizushima S, Nara Y and Simpson FO (2001). Different associations of blood pressure with 24-h urinary sodium excretion among pre- and post-menopausal women. WHO Cardiovascular Diseases and Alimentary Comparison (WHO-CARDIAC) Study. J Hypertens 19: 535-538

Yamori Y, Nara Y, Mizushima S, Mano M, Sawamura M Kihara M and Horie R (1990). International cooperative study on the relationship between dietary factors and blood pressure: a report from the Cardiovascular Diseases and Alimentary Comparison (CARDIAC) Study. J Cardiovasc Pharmacol 16: S43-S47

Yan R and Leenan FHH (1991). Dietary sodium intake and left ventricular hypertrophy in normotensive rats. Am. J. Physiol. 261: H1397-H1401

Zeidel ML, Kikeri D, Silva P, Burrows M and Brenner BM (1988). Atrial natriuretic peptides inhibit conductive sodium uptake by rabbit inner medullary collecting ducts. J Clin Invest 82: 1067-1074

Zhu J, Huang T and Lombard JH (2007) Effect of high-salt diet on vascular relaxation

and oxidative stress in mesenteric resistance arteries. J Vasc Res 44:382–390

7.0 APPENDIX

7.1: Ethical Approval

COLLEGE OF MEDICINE
UNIVERSITY OF LAGOS
P.M.B. 12003, LAGOS, NIGERIA

Provost: O. A. ATOYEBI, *MB, BS, (Hons), Lagos, FMCS, FWACS, FICS*

Deputy Provost: A. F. FAGBENRO-BEYIOKU, *B.Sc. (George Town), M.Sc. (Chicago), Ph.D. (Lagos)*

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CM/COM/8/VOL.XXI

May 7, 2009

Dr. Simiat O. Elias
(Ph.D Student)
Department of Physiology.
College of Medicine,
University of Lagos
Idi-Araba

Dear Dr. Elias,

RE: ETHICAL APPROVAL

The **Research Grants and Experimentation Ethics Committee** met on **Tuesday February 24, 2009** and considered your application for Ethical Clearance for a research proposal titled **"Role of Epithelial Sodium Channel (ENaC) in the Development and Management of Salt-Sensitive Hypertension".**

On behalf of the Committee, I hereby inform you that ethical approval has been given for you to conduct the research titled **"Role of Epithelial Sodium Channel (ENaC) in the Development and Management of Salt-Sensitive Hypertension".**

Thank you.

Yours sincerely,

Beyioku

Prof. A.F. Fagbenro-Beyioku
Chairman, Research Grants & Experimentation Ethics Committee

Appendix 2 Consent Forms

Appendix 2a Consent Form for Participation in the Study

TITLE OF STUDY: The Role of Epithelial Sodium Channel and Autonomic Nervous Potentiation in the Development of Salt-sensitive Hypertension among Nigerians in Lagos

I …… hereby consent to be included in the above-named study after having been fully informed about the details of the study.

I agree to have my blood and urine tested during the course of the study.

……. …….

Signature Date

Appendix 2b. Consent for Genotyping

TITLE OF STUDY: The Role of Epithelial Sodium Channel and Autonomic Nervous Potentiation in the Development of Salt-sensitive Hypertension among Nigerians in Lagos

I …… hereby agree that my blood should be used for Genotyping for the ENaC gene. I have been informed that the results will be used for research purposes only and will not be traced back to me.

……. …….

Signature Date

Appendix 3a: IUPAC-IUB Codes

Nucleotide ambiguity code

(as used in DNA Baser)

Code	Represents	Complement
A	Adenine	T
G	Guanine	C
C	Cytosine	G
T	Thymine	A
Y	Pyrimidine (C or T)	R
R	Purine (A or G)	Y
W	weak (A or T)	W
S	strong (G or C)	S
K	keto (T or G)	M
M	amino (C or A)	K
D	A, G, T (not C)	H
V	A, C, G (not T)	B
H	A, C, T (not G)	D
B	C, G, T (not A)	V
X/N	any base	X/N
-	Gap	-

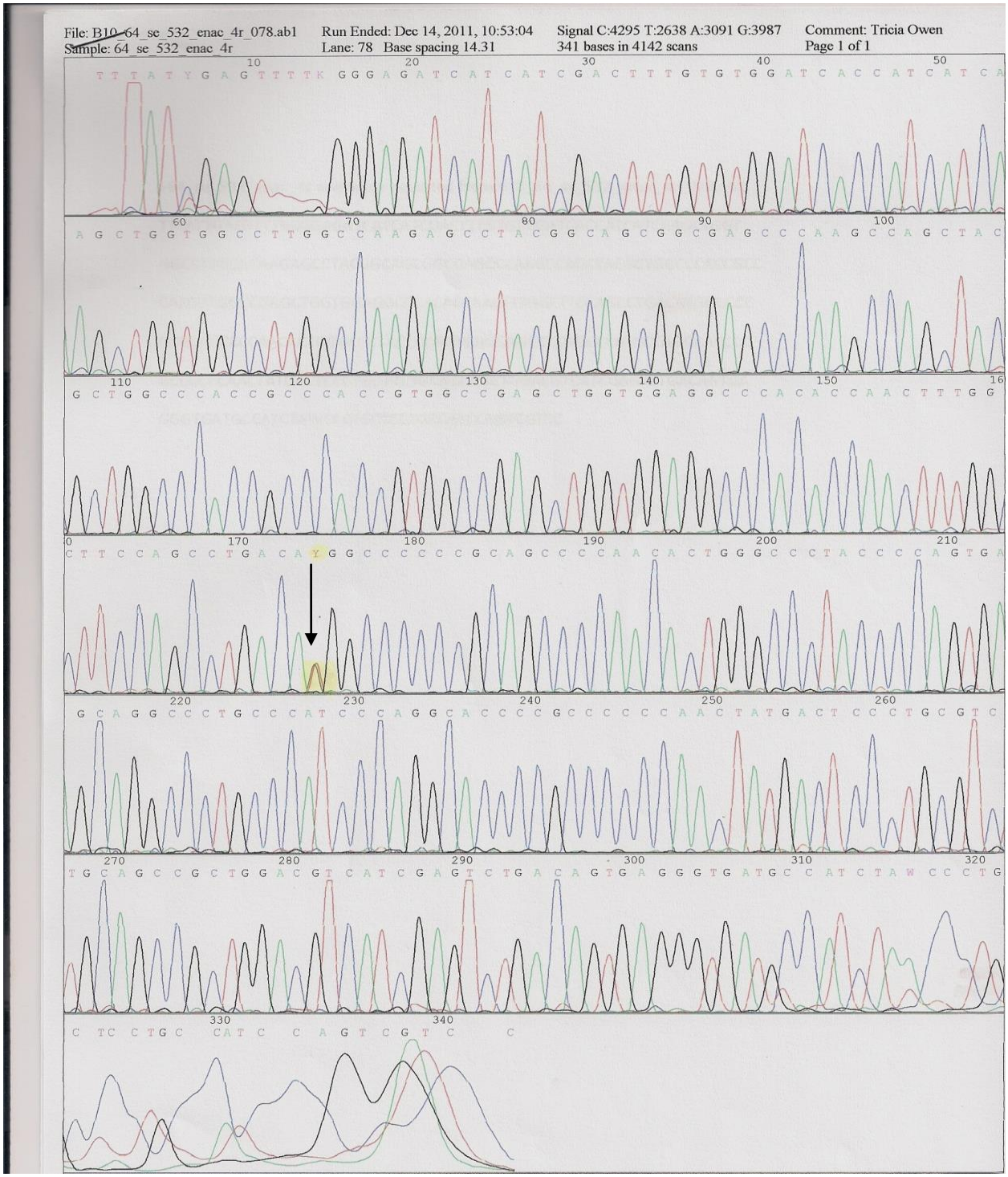
Appendix 3b: Standard Ambiguity Codes

The standard ambiguity codes for nucleotides and for the one-letter and three-letter designations of amino acids are given. The synonymous codons for the amino acids, and their depiction in IUB codes (Nomenclature Committee, 1985, Eur. J. Biochem. 150:1-5) are also shown.

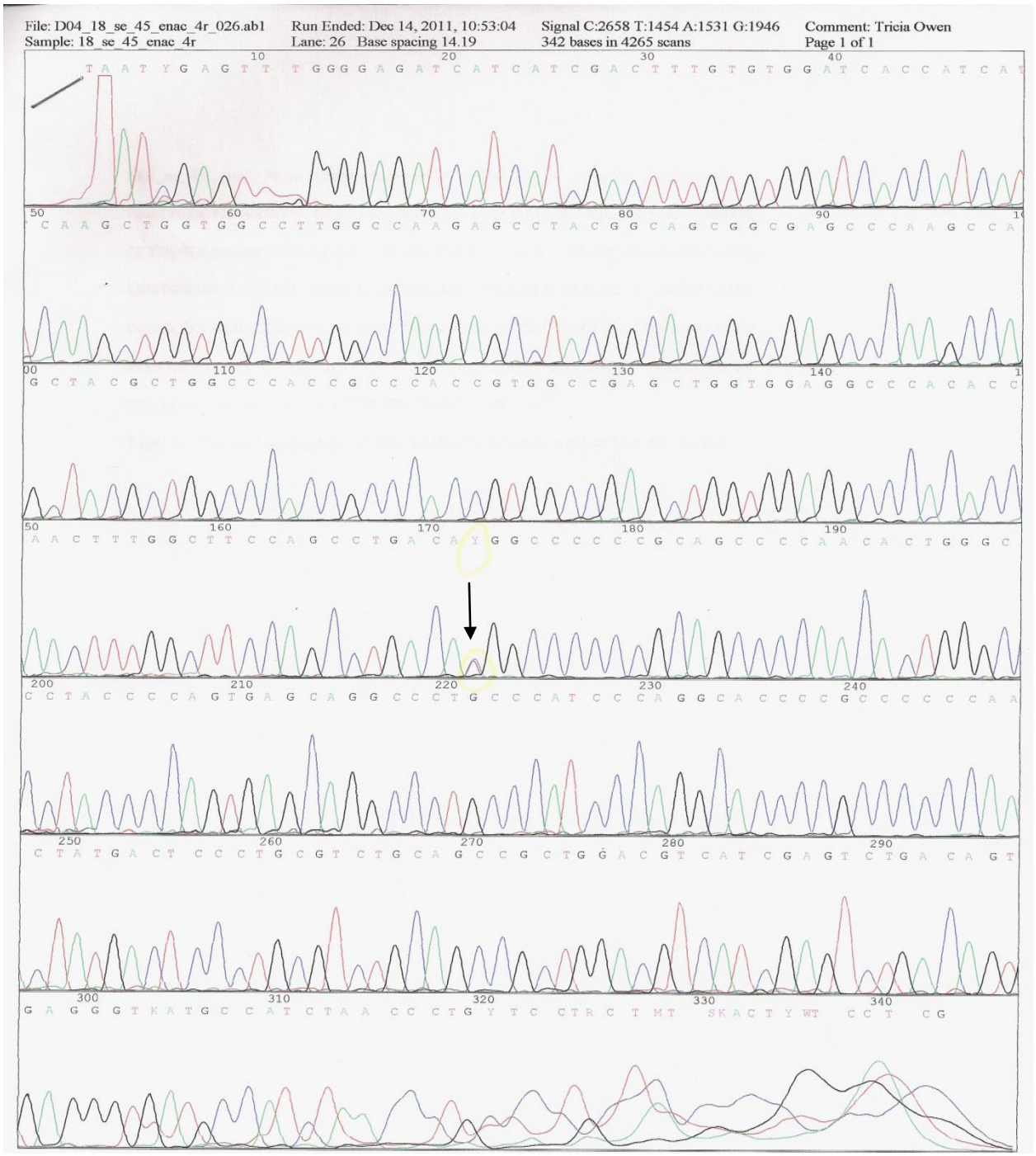
Nucleotide	Symbol	3-Let	Amino Acid	IUB
(Adenosine) A	A	Ala	Alanine	GCX
C or G or T/U	B	Asx	Aspartate or Asparagine	RAY
(Cytidine) C	C	Cys	Cysteine	UGY
A or G or T/U	D	Asp	Aspartate	GAY
-	E	Glu	Glutamate	GAR
-	F	Phe	Phenylalanine	UUY
(Guanosine) G	G	Gly	Glycine	GGX
A or C or T/U	H	His	Histidine	CAY
(Inosine) I	I	Ile	Isoleucine	AUH
-	J	-	-	-
G or T/U	K	Lys	Lysine	AAR
-	L	Leu	Leucine	UUR,CUX,YUR
A or C	M	Met	Methionine	AUG
unknown base	N	Asn	Asparagine	AAY
-	O	-	-	-
-	P	Pro	Proline	CCX
-	Q	Gln	Glutamine	CAR
(Purine) A or G	R	Arg	Arginine	CGX,AGR,MGR
C or G	S	Ser	Serine	UCX,AGY
(Thymidine) T	T	Thr	Threonine	ACX
(Uridine) U	U	-	-	-
A or C or G	V	Val	Valine	GUX
A or T/U	W	Trp	Tryptophan	UGG
unknown base	X	unknown amino acid		XXX
(Pyrimidine) C or T/U	Y	Tyr	Tyrosine	UAY
-	Z	Glx	Glutamate or Glutamine	SAR
no base (deletion/gap)	.	no amino acid (deletion/gap)	-	-
-	*	End	terminator	UAR,URA

Appendix 4: Full Chromatogram of Subjects

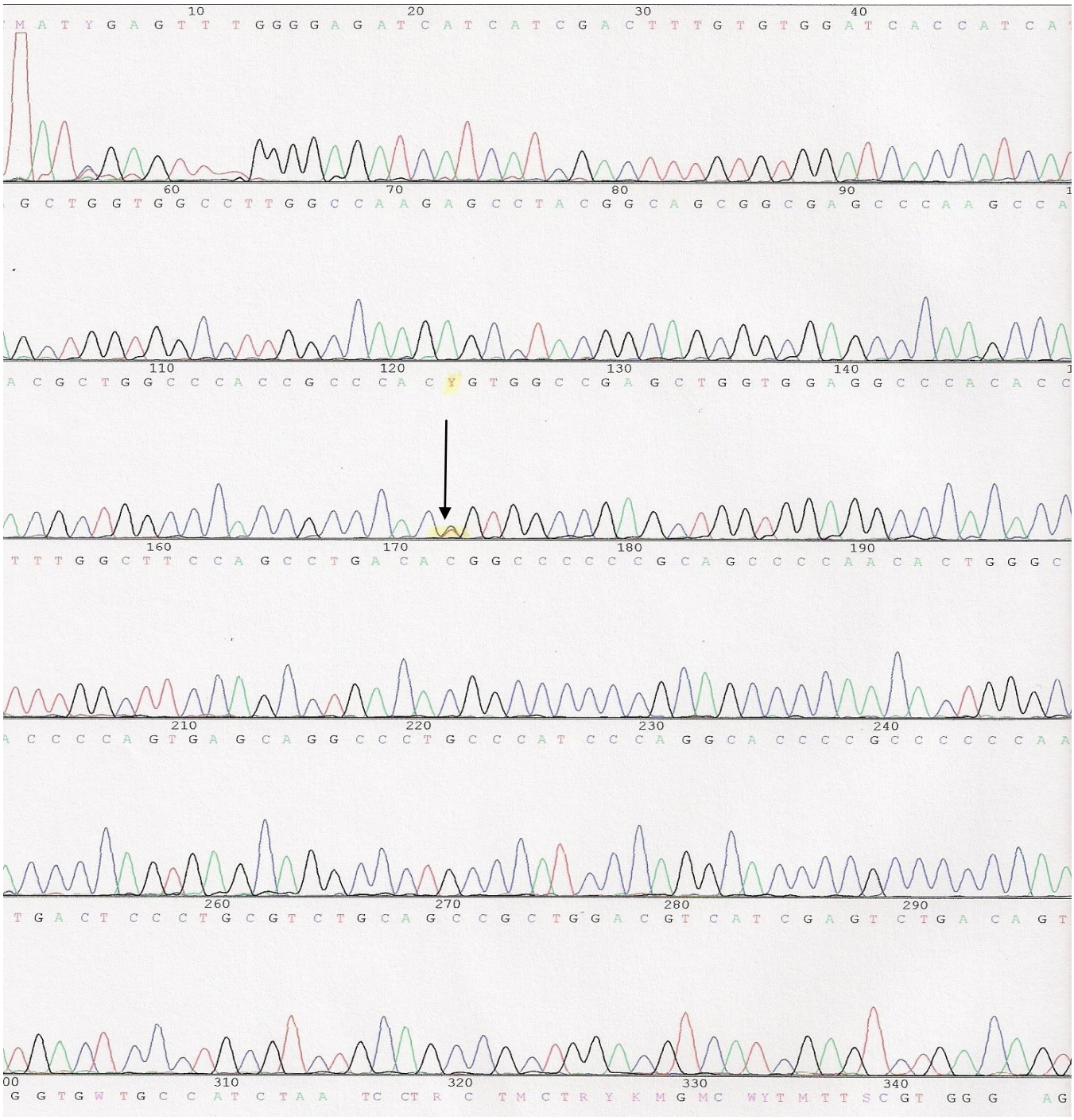
Appendix 4a: Full chromatogram of a normotensive subject with β-T594M Mutation
(see Figure 31, Page 179)



Appendix 4b: Full chromatogram of a hypertensive subject with β-T594M Mutation
(see Figure 32, Page 180)

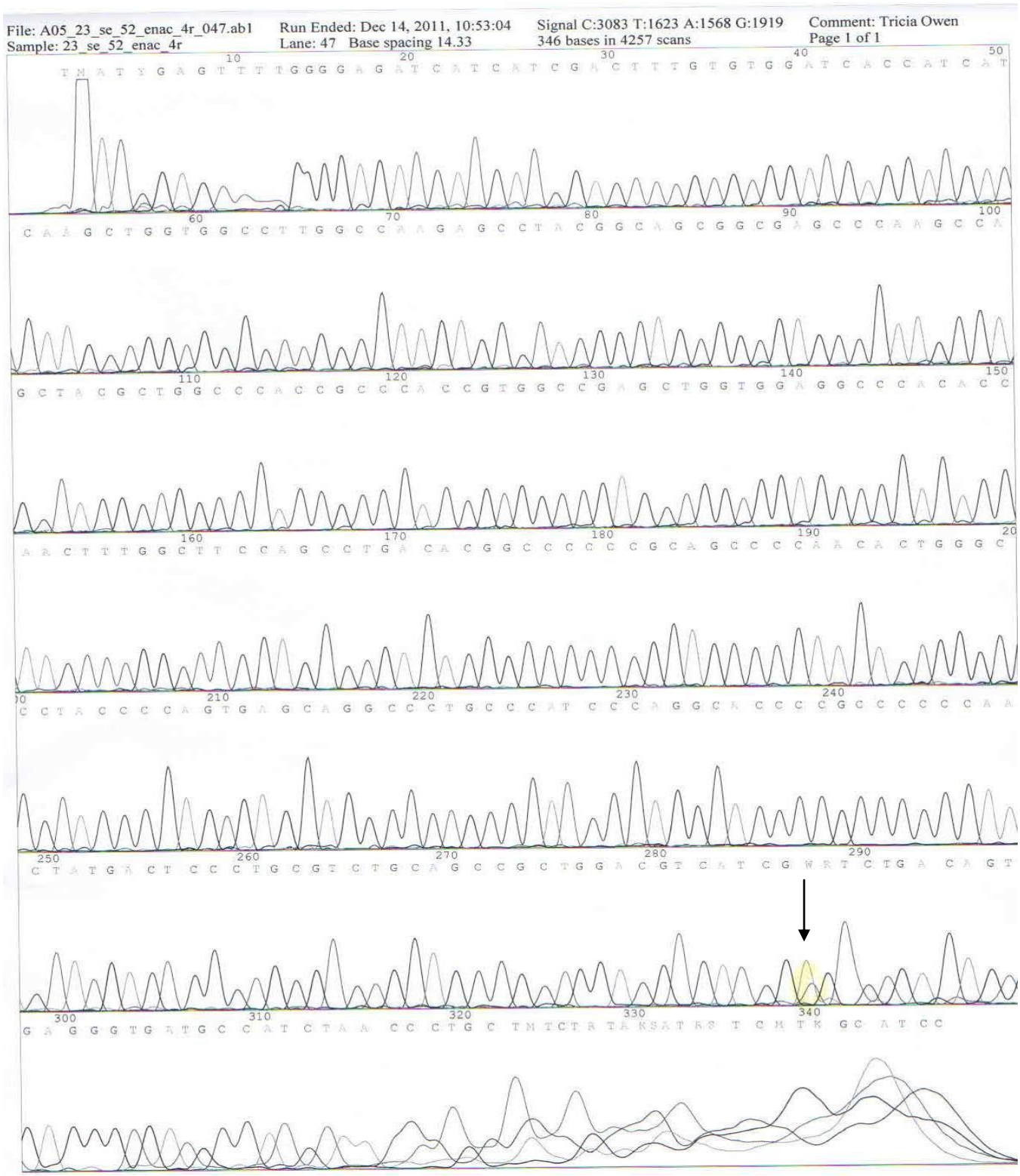


Appendix 4c: Full chromatogram of Subject with β- T577t Polymorphism
(see Figure 33, Page 181)

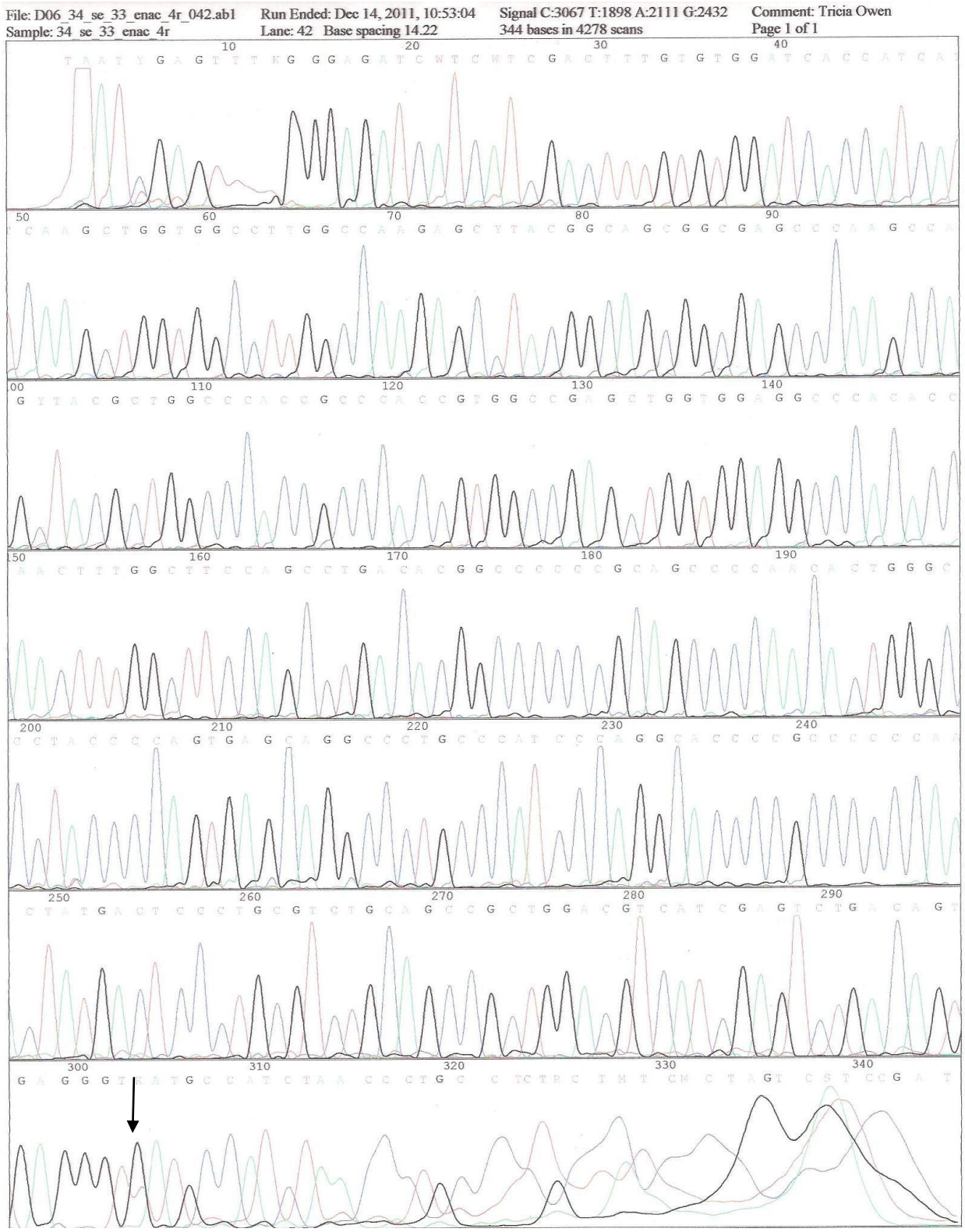


Appendix 4d: Full chromatogram of Subject with CTG>CAG Leu628Gln Mutation

(see Figure 34, Page 182)



Appendix 4e: Full chromatogram of Subject with GAT>TAT Asp638Tyr Mutation
(see Figure 35, Page 183)



Appendix 4f: Full chromatogram of Subject with CTG>CAG Leu628Gln Mutation
(see Figure 36, Page 184)