Mode of Impact of Genetic Determinants of Hypertension in People of African Descent

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ABSTRACT

Blood pressure (BP) is a heritable trait. However, the loci responsible and the mechanisms by which these genes determine BP are uncertain. Based on widely published data regarding frequent phenotypic characteristics that exemplify essential hypertension (EHT) in persons of African ancestry, in the present thesis I explored the role of gene candidates most likely to contribute to BP in this group. In this regard a high frequency of persons of African descent experience increases in BP in response to an enhanced salt intake (salt-sensitive hypertension). In addition, many patients of African origin with EHT fail to respond to inhibition of angiotensin-converting enzyme (ACE) with an appropriate decrease in BP, a factor that cannot be explained entirely on the basis of reduced plasma renin levels in this group. Thus, I evaluated the role of several gene variants that could influence either renal salt handling or the activity and effects of the renin-angiotensin system on BP in subjects of African ancestry.

Although the angiotensinogen (AGT) gene has at least 3 variants in the promoter region that influence angiotensinogen expression and which occur with a remarkably high frequency in populations of African ancestry, their role in this group is still controversial. To-date, interactions between these variants have not been considered. Using a case-control study design in a sample of 1325 subjects, as well as association analysis with 24 hour ambulatory BP (ABP) values in 626 hypertensives, I confirmed that an independent effect of functional AGT gene variants on the risk for EHT or 24 hour ABP was weak at best. Importantly, however, interactions between the -20A→C and -217G→A variants were noted to strongly impact on the risk for EHT as well as ABP. Furthermore, interactions between the -20A→C and -217G→A variants played a major role in

contributing toward the variability of ABP responses to ACE inhibitors, but not calcium channel blockers in this population group, with genotype determining whether or not ACE inhibitor responses occurred.

Although the 825C→T polymorphism of the guanosine triphosphate (G) protein β3 subunit (GNB3) gene influences the activity of a substance that modifies renal salt handling, namely the Na^+/H^+ exchanger, its impact in hypertensives of African descent is controversial. In the present thesis I confirmed in a large sample that the GNB3 variant was not associated with the risk for EHT or ABP values in subjects of African ancestry. However, because the activity of the exchanger is enhanced in obesity I hypothesised that the GNB3 gene variant could mediate a clinically relevant BP effect by modifying the impact of body size on BP (type I or II genetic effect). Indeed, GNB3 genotype proved to be a strong determinant of the impact of body size on systolic BP values, with genotype determining whether or not the effect occurred.

The epithelial sodium channel (ENaC) and atrial natriuretic peptide (ANP) have an important influence on renal salt handling. The T594M polymorphism of the β-subunit of the ENaC gene only exists with a relatively high frequency in subjects of African ancestry. Previous studies conducted in this population group in relatively small samples have indicated that the ENaC and ANP gene variants determine BP in subjects of African descent. In a larger sample of subjects of African descent I demonstrated that the T594M polymorphism of the ENaC gene has no impact on BP in this population group. However, my results suggest that the ANP gene may be a candidate worthy of further study.

In conclusion, the results described in this thesis provide evidence that lends some clarity to the role of likely gene candidates for BP control in people of African descent.

Importantly, data from this thesis suggest that interactions between functional variants of specific loci (e.g the AGT gene), and clinically relevant type I or II genetic effects (no independent actions, but modifier gene effects, e.g, GNB3) should be considered before excluding loci as playing an important role in BP control. Moreover, this thesis provides the first substantial data to indicate that gene variants determine the variability of BP responses to pharmacological agents in hypertension in this population group.

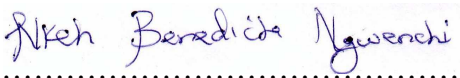
DECLARATION

I declare that this thesis is my own unaided work. It is being submitted for the degree of Doctor of Philosophy in the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg. The work contained in this thesis has not been submitted for any degree or examination in this University, or any other University.

Nkeh, Benedicta Ngwenchi……..

……*9th*……day of ……*November*……..2005

I certify that the studies contained in this thesis have the approval of the Human Research Ethics Committee (Medical) of the University of the Witwatersrand, Johannesburg. The ethics approval numbers are M951122, M980811 and M010111



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STATEMENT OF MY CONTRIBUTION TO DATA COLLECTION AND ANALYSIS

The studies described in this thesis were designed by myself in consultation with my supervisors. I genotyped all subjects for most variants studied, although approximately one third of the genotyping for the angiotensinogen -20A→C and M235T

polymorphisms was performed by colleagues in the laboratory. Though clinical data was collected by clinical personnel registered to practice in South Africa, I participated in collecting these data under their supervision. I performed all of the data analysis for this thesis and obviously interpreted these data.

LIST OF ABBREVIATIONS.

-6G→A	Guanine-to-adenine substitution at nucleotide position -6 of the angiotensinogen gene
-20A→C	Adenine-to-cytosine substitution at nucleotide position -20 of the angiotensinogen gene
-217G→A	Guanine-to-adenine substitution at nucleotide position -217 of the angiotensinogen gene
-532C→T	Cytosine-to-thymidine substitution at nucleotide position -532 of the angiotensinogen gene
704T→C	Thymidine-to-cytosine substitution at nucleotide 704 of the angiotensinogen gene and is synonymous with M235T
M235T	Methionine/threonine polymorphism at codon 235 of the angiotensinogen gene
825C→T	Cytosine-to-thymidine substitution at nucleotide 825 of the inhibitory G protein beta 3 subunit gene
T594M	Threonine-to-methionine polymorphism at codon 594 of the epithelial sodium channel β-subunit gene
1364C→A	Cytosine-to-adenine substitution at nucleotide 1364 of the ANP gene
1766T→C	Thymidine-to-cytosine substitution at nucleotide 1766 of the ANP gene
ABP	ambulatory blood pressure

ACE	angiotensin-converting enzyme
ACEI	ACE inhibitor
AGT	angiotensinogen
ANCOVA	analysis of covariance
Ang II	angiotensin II
ANOVA	analysis of variance
ANP	atrial natriuretic peptide
BMI	body mass index
BP	blood pressure
cAMP	cyclic adenosine monophosphate
CCB	calcium channel blocker
C/EBP	CAAT/enhancer binding protein
CI	confidence interval
DAG	diacylglycerol
DBP	diastolic blood pressure
DNA	deoxyribonucleic acid
EHT	essential hypertension
ENaC	epithelial sodium channel
G protein	guanosine trisphospate protein
GDP	guanosine diphosphate
Gi	inhibitory G protein
GNB3	guanosine triphosphate protein beta 3 subunit
Gs	stimulatory G protein

GTP	guanosine triphosphate
HbA_{1C}	glycated haemoglobin
IP_3	inositol triphosphate
$kg.m^{-2}$	kilogram per metres squared
MANCOVA	multivariate analysis of covariance
mm Hg	millimeters of mercury
mRNA	messenger ribonucleic acid
Na^+	sodium
Na^+/H^+	sodium ion/hydrogen ion
Na^+-K^+-$2Cl^-$	sodium-potassium-2chloride
OR	odds ratio
p value	probability value
PCR	polymerase chain reaction
PRA	plasma renin activity
r	correlation coefficient
RAS	renin angiotensin system
RFLP	restriction fragment length polymorphism
SBP	systolic blood pressure
SEM	standard error of the mean
SHR	spontaneously hypertensive rats
UV	ultra violet

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PREFACE

Although blood pressure (BP) is a heritable trait, the genes responsible for essential hypertension (EHT) and the mode of impact of these genes on BP have only recently begun to be identified. People of African ancestry appear to have relatively unique phenotypic characteristics that may be genetically predetermined. In this regard, the work performed in this thesis was prompted largely by the relative lack of data attempting to identify genes that are more likely to contribute to EHT in subjects of African ancestry. Moreover, despite the poor relationship between gene variants and either BP or the risk for EHT in subjects of African descent, no studies have been designed to account for the spectrum of genetic effects that can occur in polygenic traits. Indeed, highly polymorphic loci, with an abundance of functional variants all of which influence gene expression, could theoretically produce effects through interactions between variants as opposed to just producing independent variant effects. Also, independent gene effects only occur if a type III or IV genetic effect prevails. Little regard has been given to the possibility that type I or II gene effects where genetic variants produce no independent actions, but rather modify the impact of an alternative risk factor for hypertension on BP, could occur. Therefore, the studies described in this thesis were not only designed to assess independent gene effects, but also modifier gene effects.

In chapter I, I describe the relevant “unique” characteristics of BP control that prevail in subjects of African ancestry, with particular emphasis on the predominant phenotype, “salt-sensitive” EHT and the very limited antihypertensive responses to angiotensin-converting enzyme inhibitors in this group. This chapter therefore provides

background to the reasons for selecting the gene candidates explored in this thesis. Chapter I also provides a review of the present state of knowledge regarding the role of the gene variants evaluated in this thesis, with a particular emphasis on data obtained in subjects of African descent. This chapter is therefore meant to provide context to the thesis.

Chapters II-to-VI describe the studies performed in this thesis and are designed to provide a brief background to each aspect of the thesis, a methodology section, a results section and a general discussion. This approach has been employed as in each section separate hypotheses have been tested and the methodological aspects, although overlapping to some degree, require descriptions of different genotyping techniques. In chapter II, I provide the first evidence to indicate that despite unremarkable independent gene effects, interactions between genetic variants within the same locus, with similar effects on the function of the protein product, indeed produce a profound impact on the risk for EHT and ambulatory BP. These data were obtained for the angiotensinogen gene and provide the first strong evidence in favor of a role for this locus in contributing to EHT in subjects of African descent. In chapter III, I provide the third set of data to my knowledge to indicate that despite the lack of independent effects of gene variants on BP, these same variants have a profound influence on the impact of body size on ambulatory BP. These data were obtained for the guanosine triphosphate protein β3 subunit gene and provide the first strong evidence in favor of a role for this locus in contributing to BP through modifier gene effects (type I or II effects). In chapters IV and V as compared to previous studies in the literature, I have more thoroughly assessed the role of two functional gene variants with protein products that could influence salt handling

(epithelial sodium channel β-subunit and atrial natriuretic peptide) in subjects of African descent. Lastly, in chapter VI, I provide the first evidence to indicate that a genetic variant can account for a large degree of the variable BP changes that occur in response to angiotensin-converting enzyme inhibitors in subjects of African ancestry. In this regard the angiotensinogen gene was investigated as it has been the only variant demonstrated by our group to mediate important effects on the risk for EHT and ambulatory BP.

The work presented in my thesis has culminated in two manuscripts which have already been published (see below) and in three further manuscripts which are either currently under review or under preparation for submission.

Publications

1. Nkeh B, Tiago A, Candy GP, Woodiwiss AJ, Badenhorst D, Luker F, Netjhardt M, Brooksbank R, Libhaber C, Sareli P, Norton GR. Association between an atrial natriuretic peptide gene polymorphism and normal blood pressure in subjects of African ancestry. *Cardiovasc J S Afr*. 2002;13:97-101.

(data presented in chapter V)

2. Nkeh B, Samani NJ, Badenhorst D, Libhaber E, Sareli P, Norton GR, Woodiwiss AJ. T594M variant of the epithelial sodium channel β-subunit gene and hypertension in individuals of African ancestry in South Africa. *Am J Hypertens*. 2003;16:847-852.

(data presented in chapter IV)

Manuscripts under review/in preparation

3. Woodiwiss AJ, Nkeh B, Samani NJ, Badenhorst D, Tiago AD, Candy GP, Libhaber E, Sareli P, Brooksbank R, Norton GR. Functional variants of the angiotensinogen gene determine anti-hypertensive responses to angiotensin-converting enzyme inhibitors in subjects of African origin. Under review for publication in the journal *Journal of Hypertension.*

(data presented in chapter VI)

4. Interactions between functional angiotensinogen gene variants determine ambulatory blood pressure and hypertension risk in subjects of African ancestry. (in preparation)

(data presented in chapter II)

5. 825C→T variant of the G protein β3 subunit gene modifies the impact of body size on ambulatory blood pressure. (in preparation)

(data presented in chapter III)

Chapter I

Introduction

The epidemiology and pathophysiology of hypertension with an emphasis on subjects of African ancestry: A guide to exploring gene candidates in this ethnic group.

1.0 **The epidemiology of hypertension and its impact on the risk of cardiovascular disease**.

Hypertension is a condition often reported to affect 15-to-20% of adults in industrialized countries and hence is estimated to involve over 600 million people world-wide. More than 25% of North Americans become hypertensive in adult life (National Centre for Health Statistics, 1997). Hypertension tends to target people in nearly all geographic regions with the frequency varying widely from 14% in underdeveloped countries such as West-Africa to 33% in some regions of developed countries such as the United States of America (Cooper et al 1997, Cooper and Rotimi 1997). With urbanization in underdeveloped countries, the frequency of hypertension may increase to 30% (Cooper et al 2003). Prior to 1990, hypertension accounted for most cardiovascular deaths (Kannel 1989). By far the most common form of hypertension and indeed the form that accounts for the burden of cardiovascular disease is primary or essential hypertension (EHT) as opposed to secondary forms of hypertension (Dustan 2000).

1.1 **Epidemiological aspects of hypertension in people of African ancestry**

It is presently well recognized that the characteristics of hypertension and blood pressure (BP) control in people of African as opposed to European ancestry are distinct in many respects. Indeed, differences in the prevalence and frequency of essential hypertension (EHT) between urban ethnic groups had been noted some time ago with higher prevalence rates and frequencies reported in groups of African ancestry (Epstein et

al 1965, Sever et al 1980) as compared to other groups (Lin et al 1959, Epstein et al 1965, Ueshima et al 1987). More recent data indicates that the higher frequency of EHT amongst people of African origins living in urban communities as compared to Caucasoid groups occurs in a variety of geographic regions including in people living in the United States (Anderson et al 1989), Brazil (Ribeiro and Ribeiro 1986) and in Nigeria (Akingkube 1985). In African-Americans there is currently a frequency rate for EHT of 35% accounting for 20% of all deaths in this group (Cooper at al 1997, Cooper and Rotimi 1997). Importantly, hypertension in subjects of African ancestry is now well recognized as being characterized by an earlier onset, more rapid progression and less appropriate BP control in comparison to other ethnic groups (Flack and Staffileno 2000). Although pre-adolescent children of African ancestry have similar BPs as compared to Caucasian groups, the increase in BP accompanying post-pubertal years is greater than that in Caucasians (Rabinowitz et al 1993). This augmented rise in BP is apparently maintained into adult life (Webber et al 1986) when subjects of African ancestry more frequently develop clinically relevant EHT (Wattigney et al 1995).

Possibly as a consequence of the greater prevalence and incidence of essential hypertension in urban communities of African ancestry, or less appropriate BP control, there is a higher prevalence of and mortality from strokes, renal failure and congestive heart failure in subjects of African as opposed to European descent (Walter et al 1992, Klag et al 1997). Furthermore, subjects of African ancestry with untreated hypertension have higher left ventricular mass indices, relative wall thickness values and a greater frequency of impaired diastolic ventricular function in comparison to other ethnic groups (Mayet et al 1998, Stanton et al 2002). Left ventricular mass and relative wall thickness

are presently considered intermediate phenotypes for the development of cardiovascular disease (Savage et al 1987, Koren et al 1991, Yu et al 1996) and diastolic ventricular dysfunction is an important cause of heart failure in hypertension (Vasan 2003).

The greater incidence, frequency and prevalence of essential hypertension in urban communities, less appropriate BP control, and the higher incidence of target organ damage in subjects of African ancestry prompted me to focus my studies on this "high risk" population group.

1.2 The pathophysiology of essential hypertension with special reference to patients of African ancestry

Although important steps have been taken toward this end, the causes of EHT have yet to be fully elucidated. Factors thought to contribute to the development of EHT are too numerous to summarize and go beyond the scope of this thesis. Only those factors that provide some insights into the reasons for conducting the studies performed in this thesis will be discussed. Central to this thesis is the concept that salt intake and renal salt handling are important determinants of BP control and hypertension, especially in groups of African ancestry. Consequently, I will focus the initial part of this chapter on describing the current knowledge on the control of salt balance in the context of its impact on BP and the development of hypertension. As ethnic differences in the pathophysiology of hypertension may account for the earlier onset, greater severity, and the poor control of EHT observed in people of African ancestry, I will also highlight key

features which may partly account for the unique epidemiological characteristics of peoples of African descent.

1.2.1 **Sodium and hypertension.**

Importantly, BP may decrease to normal values in patients with EHT after kidney transplantation (Curtis et al 1983) and transplantation of kidneys from hypertensive into normotensive animals increases BP in the latter (Rettig et al 1990). These data provide compelling evidence to indicate that the kidney is the organ of long-term BP control. An impaired ability to excrete salt and volume loads through renal changes is likely to increase plasma and thus blood volume, and subsequently enhance cardiac output and BP. Vascular autoregulatory changes tend to maintain cardiac output in the face of raised blood volumes, but at the expense of an enhanced total peripheral resistance and consequently BP. It is therefore not surprising that salt intake and a decreased capacity of the kidney to excrete excess salt loads is considered an important cause of EHT.

With respect to salt intake, EHT is almost non-existent in populations of sub-Saharan Africa who consume less than 50 mmol of salt per day (Trowell 1980, Weinberger 1996). In addition, the rise in BP that normally accompanies aging is absent in these populations (Mbanya et al 1998). In contrast, a sharp rise in BP occurs with age in African-Americans and in descendants of groups originating from sub-Saharan Africa residing in developed countries (Stamler 1997) who generally consume more than 100 mmol of salt per day.

In the “Inter-salt Co-operative Research Group” (INTERSALT) study, which drew participants from over 32 nations and backgrounds, a salt consumption of 100 mmol/day greater than the recommended daily allowance was associated with increases in systolic (SBP) and diastolic (DBP) BP in both normotensive males and females irrespective of age (Stamler 1997). However, a convincing relationship between BP and salt excretion (an index of intake) was not evident in this study. Moreover, subsequent studies could not confirm a relationship between 24 hour urinary salt excretion and BP. Notwithstanding the considerable criticism drawn by the INTERSALT study, this and other studies nevertheless provided some evidence to indicate that an average salt consumption of more than 100 mmol/day over a period of 30 years would result in a SBP/DBP increase of 10/6 mm Hg (Miller et al 1983, Miller et al 1987, Stamler 1997).

Despite the relatively accepted evidence that salt intake contributes to BP control and the development of hypertension, it is apparent that not all individuals respond in a quantitatively similar manner to variations in salt intake. On high salt diets (>220 mmol/day of Na^+), BP may either increase by at least 5 mm Hg, remain unchanged, or even decrease by up to 5 mm Hg (Gerdts et al 1999). On low salt diets (20 mmol/day) those whose BP increases in response to a high salt intake will subsequently react with a decline in BP. These individuals are described as “salt-sensitive” (Ruppert et al 1993). Those whose BP does not change by 5 mm Hg or more and may even decrease on a high salt diet and increase on a low salt diet are said to be “salt-resistant” (Ruppert et al 1993, Gerdts et al 1999).

“Salt-sensitivity” is an indicator of alterations in kidney function, which necessitates a higher BP to excrete increased amounts of salt (Weinberger et al 1986,

Weinberger 1996). “Salt-sensitivity” is a familial trait (Overlack et al 1993) that is more common in older subjects and those with a family history of EHT, whilst “salt-resistant” are young with no family history of EHT. The former group exhibits lower plasma renin and aldosterone concentrations than the latter group as fluid retention in “salt-sensitive” individuals suppresses renin release (Sharma et al 1989, Ruppert et al 1993). Importantly, “salt-sensitive” BP responses to a salt load occur in 68% of normotensive individuals with a family history of EHT, whereas it occurs in only 20% of normotensive individuals with no family history of EHT (Cowley Jr 1997).

The “renal” hypothesis of EHT is even more compelling when considering data obtained in subjects of African ancestry. Indeed, the ability of renal transplantation from normotensive donors to hypertensive patients with renal failure to normalize the BP of the transplant recipients, was originally demonstrated in patients who were largely African-Americans (Curtis et al 1983). That a greater degree of “salt-sensitivity” exists in subjects of African ancestry was recognized some years ago (Luft et al 1979, Campese et al 1991). Indeed, about 50% of EHTs of African descent are “salt-sensitive” as compared to less than 20% of their Caucasian counterparts (Peters and Flack 2000). Consistent with more subjects of African ancestry being “salt-sensitive”, generally they have lower than normal levels of plasma renin activity (PRA)(Fisher et al 1999). Moreover, normotensive offspring of hypertensive parents of African origins have aberrations in urinary sodium (Na^+) and potassium excretion, a lower PRA and higher post-exercise BP, in comparison to Caucasian subjects (Hohn et al 1983, Liberman et al 1986, Treiber et al 1989, Anderson et al 1989). A potential consequence of the difficulties that subjects of African ancestry experience with renal Na^+ handling is an enhanced capacity to retain fluid and

hence increase blood volume (Arthur et al 1999) and hence BP. They may also develop higher intracellular Na^+ concentrations in circulating cells (M'Buyamba-Kabangu et al 1984), a change which if reproduced in vascular smooth muscle is likely to lead to a greater systemic vascular resistance and hence even higher BP values.

Although there is substantial evidence to indicate that in people of African ancestry an increased Na^+ intake contributes to BP, data obtained in South African groups of African ancestry do not necessarily support this contention (Hoosen et al 1985). Despite data to indicate that South Africans of African ancestry have lower PRA than white South Africans (Touyz et al 1995), Hoosen et al (1985) report that there is no relation between PRA and BP in South African blacks and also no relation between plasma Na^+ and BP. In other words the importance of the impact of plasma Na^+ on BP in South Africans of African ancestry is still controversial.

Aviv at al (2004) have indicated that a major cause of "salt sensitivity" in persons of African ancestry is through an augmentation of renal epithelial cell Na^+-K^+-$2Cl^-$ cotransporter activity. An enhanced activity of the Na^+-K^+-$2Cl^-$ cotransporter favors salt and hence water reabsorption in the ascending limb of the loop of Henle. However, there are a number of other potential alterations in a variety of cellular systems that could contribute toward abnormalities in renal Na^+ handling in subjects of African ancestry. These will be discussed in the following sections of this thesis.

1.2.1.1 **Salt sensitivity and blood pressure profiles**.

As compared to daytime BP, nocturnal BP is considerably lower under normal

circumstances. An important predictor of target organ damage in hypertension is an inability to decrease BP to appropriate levels during the night (Cuspidi et al 2001). “Salt sensitivity” is a predisposing factor to an inability to appropriately decrease BP at night in both normotensives (Wilson et al 1999, Arthur et al 1999) and hypertensives (Cicconetti et al 2003). In this regard the decrease in nocturnal BP in subjects of African ancestry is far less compared to Caucasian groups with similar daytime BP values (Flack and Staffelino 2000). Thus it is thought that a potential deleterious effect of the high incidence of “salt-sensitivity” in patients of African ancestry is excessive target organ damage mediated through abnormal 24 hour BP profiles.

1.2.2 **The renin-angiotensin system in blood pressure control and hypertension**

It is now well accepted that the renin-angiotensin system (RAS) is a major determinant of BP control. The RAS is activated by pro-renin secreted from the juxta-glomerular cells of the kidney in response to changes in the pressure within the afferent renal arterioles, Na^+ concentration in the macula densa, and sympathetic tone (β_1-adrenoreceptor mediated). The conversion of pro-renin to renin may take place in any tissue, even in the heart (Skinner et al 1986). Renin catalyses the conversion of angiotensinogen to angiotensin I which is converted to angiotensin II (Ang II) by angiotensin-converting enzyme (ACE) found in the plasma membrane of endothelial cells and in the lungs. Ang II is one of nature’s strongest pressor substances. Ang II increases total peripheral resistance through vasoconstrictor effects and even at very low concentrations it enhances the rate of aldosterone secretion which is a potent determinant

of renal Na^+ and water retention. Ang II also stimulates the sympathetic nervous system to further promote vascular smooth muscle contraction and Ang II acts directly on the kidney to augment Na^+ and water retention (Guyton 1980). The importance of the RAS in BP control is exemplified by the fact that activation of renin release is well established as being responsible for renovascular hypertension (Laragh, 1986) whilst transgenic mice overexpressing the gene for renin clearly have higher BPs than their normal littermates (Stec et al 2002, Morimoto et al 2002).

Although renin is a significant determinant of Ang II production it is now well accepted that angiotensinogen is also a major regulator of Ang II production independent of renin concentrations (Duggan and Ye 1998). The importance of angiotensinogen in BP control was recently underscored when hypotension was induced in angiotensinogen "knockout" mice (Tanimoto et al 1994) and an increased BP was produced in mice with an increased number of copies of the angiotensinogen gene (Kim et al 1995).

Angiotensinogen is now recognized as playing a major role in the pathogenesis of hypertension (Catanzaro 2000). In early studies in patients with EHT and their offspring, higher circulating Ang II concentrations were reported as compared to normotensive controls (Fasola et al 1968). More recently, Harrap et al (1996) demonstrated that Ang II concentrations are associated with an increased risk of developing hypertension. The mechanism responsible for potential changes in Ang II in EHT was suggested as early as 1979, when Walker et al (1979) showed an association between plasma angiotensinogen concentrations and diastolic blood pressure (DBP) in hypertensives, a finding to some extent reproduced by Ito et al (1980) one year later. More recently, Watt et al (1992) reported that the hypertensive offspring of hypertensive

parents had higher plasma angiotensinogen levels than normotensive offspring of hypertensive parents. Thus, angiotensinogen appears to be an important determinant of the development of human EHT. However, the mechanisms responsible for changes in angiotensinogen concentrations in EHT have only recently been elucidated. These mechanisms appear in-part to be related to angiotensinogen expression in adipose tissue and the impact of gene variants. These mechanisms will be underscored in subsequent discussion.

In subjects of African ancestry it may be argued that the RAS is unimportant as "salt-sensitive" hypertension suppresses PRA. Supporting the notion that the RAS is relatively inactive in patients of African ancestry is the well-documented evidence that hypertensive patients of African ancestry do not respond to inhibitors of ACE (Norton et al 1999, Sareli et al 2001) when used as monotherapy. However, although a lower PRA may occur in subjects of African ancestry with EHT as compared to age and gender-matched normotensive controls, circulating Ang II and aldosterone concentrations are usually unchanged (Meade et al 1983). Moreover, as PRA tends to decrease with age in hypertensive patients of African origin, plasma aldosterone concentrations may remain normal (Sagnella 2001). These data obviously reflect a "relatively" overactive RAS downstream from renin. Thus, although "salt-sensitivity" apparently suppresses renin release, factors downstream from renin may maintain the activity of the RAS at normal levels. One such factor could include abnormalities in angiotensinogen production. Indeed, a relationship exists between BP and angiotensinogen concentrations in subjects of African ancestry (Bloem et al 1997, Forrester et al 1996). Whether alterations in circulating angiotensinogen concentrations in subjects of African descent are the

consequence of specific phenotypic characteristics associated with excess angiotensinogen production, such as obesity; environmental effects; or because of distinct genotypic characteristics, has yet to be determined. In support of the notion that factors downstream from renin are equally as important in patients of African ancestry as compared to other groups are data that show that ACE inhibitors are equivalent in efficacy as in other population groups when used in conjunction with diuretic agents (Ajayi et al 1989, Ajayi et al 1996). In this regard, diuretic agents activate renin and hence potentiate the RAS.

1.2.3 **Atrial natriuretic peptide in blood pressure control and hypertension**

It is well recognized that an increase in blood volume increases BP. An increase in blood volume also stimulates stretch receptors within the atria, triggering the production of atrial natriuretic peptide (ANP) which causes vasodilatation and induces an increased renal excretion of water and Na^+ (Lang et al 1985, Hollister and Inagami 1991, Charles et al 1993). The outcome is both a reduction in systemic peripheral resistance and plasma volume, thus attenuating BP. ANP promotes a natriuresis and diuresis by inhibiting the reabsorption of Na^+ in proximal renal tubules (Seymour et al 1985) as well as in the inner macula densa collecting tubules (Gunning et al 1990) and as such inhibits the release of renin. Importantly, ANP also modulates the RAS at an adrenal level in that the response of plasma aldosterone to angiotensin II is inhibited by ANP in humans (Hunt et al 1996). ANP also promotes renal fluid excretion by increasing glomerular filtration through direct effects on mesangial cells (Weidmann et al 1986) and by constricting

efferent whilst dilating afferent glomerular arterioles (Camargo et al 1984). The impact of ANP on the control of renal fluid handling is probably best underscored by data showing that ANP has the capacity to counteract aldosterone-induced salt retention following Ang II stimulation (Anand Srivastava et al 1986). As indicated in the preceding section, it is well recognized that aldosterone is a potent stimulator of Na^+ and water retention. ANP also regulates blood volume through central nervous system effects in that it is found in the hypothalamus where it regulates Na^+ "hunger" and thirst (Blackburn et al 1995). If renal control of Na^+ excretion is abnormal, such as in "salt-sensitive" EHT, blood volume may increase. As a consequence, atrial stretch should stimulate mechano-sensitive receptors in the atria, thus triggering the release of ANP (Campese and Widerhorn 2000). Abnormalities of ANP production could thus prevent sufficient compensatory changes to fluid loads in "salt-sensitive" EHT.

A number of lines of evidence obtained from pre-clinical studies suggest that abnormalities in ANP may predispose to hypertension. Mice with disruption of the pro-ANP gene do indeed develop "salt-sensitive" hypertension (John et al 1995), whilst transgenic mice over-expressing the gene for ANP have lower BPs than their normal litter-mates (Ogawa et al 1994). Normotensive Wistar Kyoto rats challenged with a high "Na^+-load" respond by increasing plasma ANP concentrations, whereas "salt-sensitive" spontaneously hypertensive rats (SHR) do not increase the secretion of ANP in the presence of a similar challenge (Jin et al 1988). Importantly, when SHR receive physiological amounts of ANP they respond by eliminating a "Na^+-load" (Jin et al 1990). Pre-hypertensive SHRs exhibit an impaired response of ANP secretion to stretch stimuli

whereas in hypertensive SHRs, ANP is appropriately secreted in response to stretch (Onwochei and Rapp 1989).

Results obtained from human studies have also provided evidence to support the notion that abnormalities in ANP play a role in EHT. Following administration of Na^+, the quantity of ANP secreted in normotensive offspring of hypertensive parents is reduced in comparison to that noted in offspring of non-hypertensive parents (Ferrari et al 1990, Weidmann et al 1991, Allemann and Weidmann 1995). Similarly, in "salt-sensitive" EHT, ANP release is attenuated in response to a "Na^+-load" (Niimura 1991, Ferri et al 1994). Moreover, in hypertension *per se* a reduced natriuretic response to ANP release occurs (Ishimitsu et al 1998, Agaike et al 1994). With regards to the role of ANP in determining BP control in persons of African ancestry, ANP secretion in response to a high Na^+ diet in hypertensive African-Americans is also lower than it is in "salt-resistant" individuals (Campese and Widerhorn 2000). Hence, abnormal ANP responses to "Na^+-loads" in patients of African ancestry could contribute to the development of EHT in this population group.

1.2.4 **The epithelial sodium ion channel in blood pressure control and hypertension.**

Aldosterone-induced renal salt reabsorption is mediated via epithelial Na^+ channels (ENaC), which account for close to 5% of the total amount of Na^+ reabsorbed by the kidney tubules and is therefore fundamental to BP control (Rossier 1997, Fuller 1996, Gormley et al 2003).The ENaC is the rate-limiting step in Na^+ reabsorption in the kidney.

In the distal nephron, the functional ENaC is made up of three subunits: α-, β- and γ-subunits (Canessa et al 1994; Firsov et al 1998). The number of each of these subunits per channel is still a matter of debate (Canessa et al 1994, Snyder et al 1998, Kosari et al 1998, Eskandri et al 1999). The α-subunit induces channel activity (Snyder et al 1998) and is crucial for the assembly and targeting of the channel. The β- and γ-subunits support very low Na^+ conductance when expressed alone in *Xenopus* oocytes, but when co-expressed with the α-subunit they show a greatly augmented Na^+ conductance (Canessa et al 1994). The epithelial Na^+ channel is well recognized as being a target for amiloride diuretic agents. The amiloride sensitivity of the ENaC is attributed to the α-subunit (Canessa et al 1994).

Defects in Na^+ transport have been reported in leukocytes and erythrocyte membranes of hypertensives as compared to their normotensive relatives and patients with secondary hypertension (Edmondson et al 1975, Hilton 1986). Immortalized erythrocyte membranes from patients with EHT indeed have structural alterations compared to normotensives (Postnov and Orlov 1985). Abnormalities of the ENaC may in-part explain defects in cellular membrane Na^+ transport in hypertensives. Importantly, the ENaC is more active in hypertensives than in normotensives and in hypertensive subjects there is an increased expression of the α-subunit (Fuller 1996). An increased expression of the α -subunit in the tubular epithelium of hypertensives is thought to be one reason for an increased renal tubular Na^+ reabsorption in these patients.

1.2.5 The Na^+/H^+ exchanger in blood pressure control and hypertension

The Na^+/H^+ counter-transport mechanism is present in all eukaryotic cell membranes and exchanges external Na^+ for internal H^+ to raise the pH of the intracellular milieu (Seifter and Aronson 1986). At least four isoforms (1-3 and 5) have been cloned in mammals with type 1 being the most abundant (Siffert and Rainer 1995). The functions of the Na^+/H^+ exchanger are the regulation of intracellular pH, entry of Na^+ into cells and transfer of solutes across epithelial cells, and cell growth and proliferation (Grinstain et al 1989). Whereas the function of the ENaC is dependent on the concentration gradient for Na^+ across the epithelial cell membrane, the Na^+/H^+ exchanger controls cell pH by electroneutral cotransportation of Na^+ and H^+ and consequently determines water movement across membranes, including renal tubular epithelial cells, through osmotic means (Weder 1991, Orlov et al 1999). The role of the Na^+/H^+ exchanger in renal salt handling is supported by data obtained in transgenic mice overexpressing the Na^+/H^+ exchanger type 1 protein. These genetically engineered mice exhibit a decreased urinary Na^+ excretion and an elevated SBP after excessive salt intake as compared to “wild-type mice” (Kuro-o et al 1995).

Modulation of renal salt handling is not the only mechanism by which the Na^+/H^+ exchanger influences BP. Ang II increases the activity of the Na^+/H^+ anti-port in vascular tissue, thus rendering the intracellular milieu more alkaline, consequently stimulating cell growth and contributing toward vascular hypertrophy (Peiro et al 1997). Vascular hypertrophy mediates an increase in vascular resistance and hence contributes to long-term increments in BP. A rise in intracellular pH also releases cytosolic Ca^{2+} (Siskind et

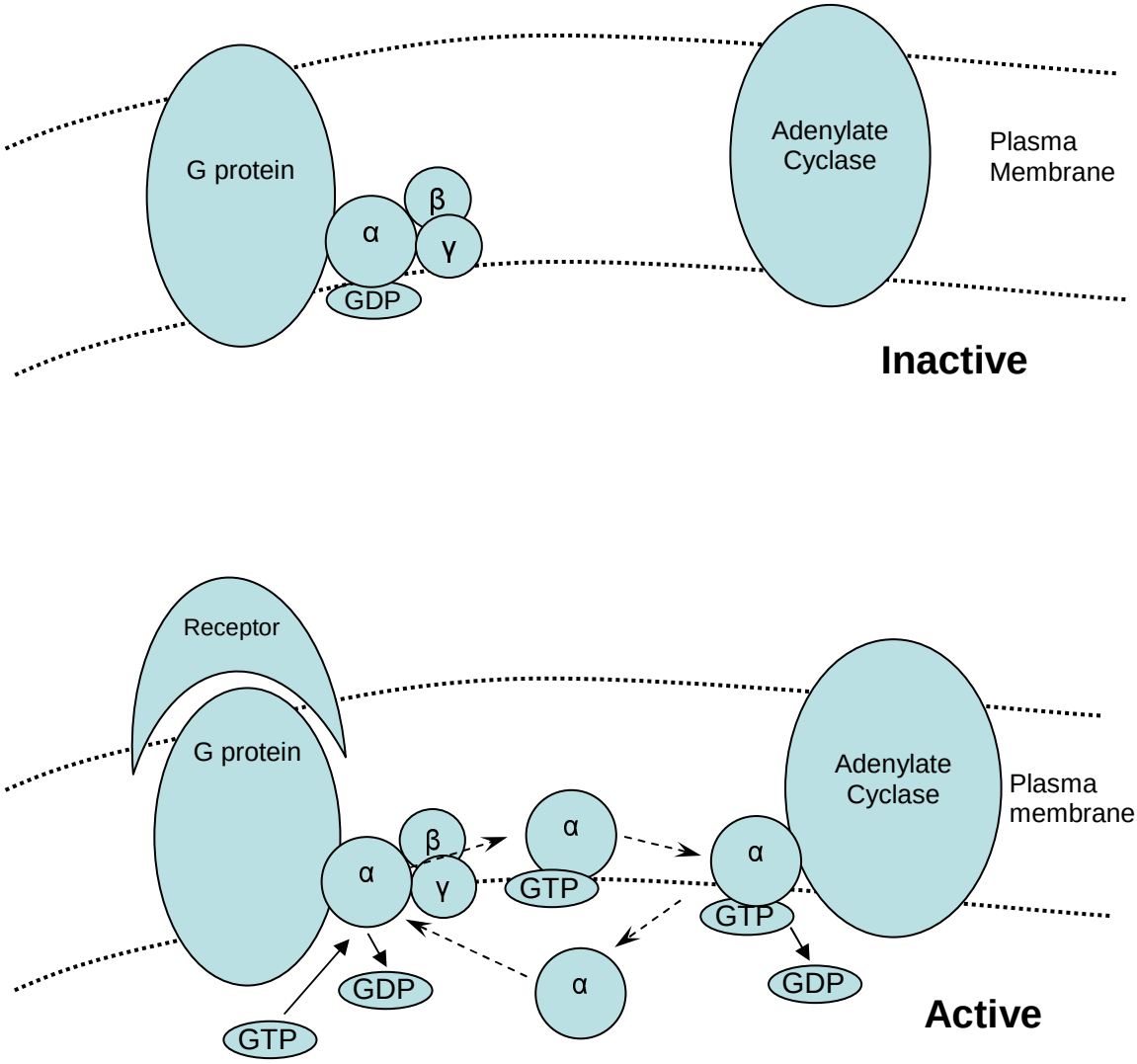
al 1989, Heming et al 2003) and enhances myosin affinity for Ca^{2+}, thus increasing the sensitivity of vascular smooth muscle to vasoconstrictors. Importantly, the activity of the Na^+/H^+ exchanger is determined in-part by the activity of inhibitory guanosine trisphospate (G_i) protein intracellular signaling pathways (Bouaboula et al 1999, van Willigen et al 2000) as will be discussed in the subsequent section of this chapter.

Abnormalities in the Na^+/H^+ exchanger may also in-part explain those defects in cellular membrane Na^+ transport noted in hypertensives as discussed above (see preceding section). Indeed, as compared to leucocytes obtained from age and sex-matched normotensive controls, hypertensives exhibit an increased activity of the Na^+/H^+ anti-port (Siffert and Dusing 1995, Siffert and Rainer 1995, Rosskopf et al 1993, Gruska et al 1997). At least 50% of hypertensive patients are reported to have enhanced Na^+/H^+ exchanger activity and this enhanced activity may be due to G_i protein changes rather than to alterations in the structure of the Na^+/H^+ exchanger itself (Pietruck et al 1996).

1.2.6 **Inhibitory guanosine triphosphate protein effects on blood pressure and hypertension**

Guanosine triphosphate (G) proteins are heterotrimeric membrane bound proteins with three distinct dissociable subunits, including α- β- and γ- subunits (Neer 1995). The α-subunit has a guanine nucleotide-binding site that accommodates either GTP or GDP. The β- and γ-subunits are tightly bound to each other and function as a single unit whereas the association between the βγ complex and the α-subunit is loose. In the inactive or resting state, the complete G-protein complex is associated with a GDP

molecule bound to a guanine nucleotide-binding site in the α -subunit (see 'Inactive' component of schematic diagram below). The binding of a hormone to its receptor results in a change in the conformation of the G-protein. The α-subunit loses its affinity for GDP which is replaced by GTP. The latter activates the α-subunit causing it to dissociate from the receptor. This is followed by a breakup of the G protein into independent α-GTP and βγ complexes. Both complexes bind to separate membrane bound effectors, that is, enzymes such as adenylate cyclase. As soon as the message is transmitted, the α -subunits' intrinsic GTPase activity breaks down the GTP to GDP and causes the subunit to dissociate from the enzyme and to re-associate with the βγ complex and the membrane bound receptor (see 'Active' component of schematic diagram below). In humans the G-protein subunits are not identical. There are about 20 different types of α-subunits (Kaziro et al 1991), 5 different β-subunits (Simon et al 1991, Watson et al 1994) and 6 different γ-subunits (Cali et al 1992). These different subunits are expressed in various combinations in different cell types. G-proteins are classified into families according to their α-subunit function where G_s (stimulatory G-protein) stimulates adenylate cyclase resulting in the synthesis of the second messenger, cAMP; G_i (inhibitory G protein) inhibits adenylate cyclase and decreases intracellular cAMP; G_q stimulates phospholipase C to form the inositol 1,4,5-triphosphate (IP_3) and 1,2, diacylglycerol (DAG) second messengers; and G_{12} regulates ion channels.



In the presence of a hormone bound to a receptor coupled to G_i, activation of the G_i complex results in fixation of a GTP molecule to the guanine nucleotide-binding site of the α_i-subunit. This is followed by the dissociation of the α_i-subunit from the $\beta\gamma_i$ complex. Subsequent inhibition of adenylate cyclase activity is either by interaction of the α_i with the stimulatory α-subunit or an interaction of the former with adenylate cyclase.

What is the potential role of G_i proteins in the regulation of BP? Pertussis toxin inhibits G_i proteins. Importantly, a single injection of pertussis toxin produces a decrease in BP in SHR, an effect that is maintained for over two weeks (Kost et al 1999). The toxin also increases renal blood flow and reduces renal vascular resistance (Kost et al 1999) suggesting that pertussis toxin-sensitive G_i proteins contribute to the maintenance of hypertension through an elevated renal vascular tone. Whether inhibition of G_i proteins results in an antihypertensive effect through increments in vascular smooth muscle cAMP concentrations (which relax smooth muscle) or by modifications in Na^+/H^+ exchanger activity (see previous section) were not explored (Kost et al 1999), but are obvious potential mechanisms.

Some hypertensive patients show a loss of 41 amino acids from the β-subunit of the G_i protein (Siffert at al 1998). The loss of this protein fragment is associated with an increased stimulation and GTP-binding in the α_i-subunit, a change which is thought to account for increased activation of the Na^+/H^+ exchanger noted in hypertensives (Siffert at al 1998) and discussed in the preceding section.

1.2.7 **Obesity and hypertension**

Obesity and weight gain are associated with EHT (Kannel et al 1967, Cassono et al 1990, Hall 2000, Montani et al 2002), whereas weight loss results in a decline in BP in both normotensives and hypertensives (McCowen et al 2000, Masuo et al 2000, Masuo et al 2001, Stevens et al 2001). Even in children, increasing body weight is associated with

greater BP levels later on in life (Burke et al 2004). The mechanisms responsible for increases in BP following obesity have gradually emerged over the past two decades.

Up to 50% of obese people have concomitant hypertension (Reisner and Hutchinson 2000) associated with both a high cardiac output (not accompanied by increased heart rates) and an increased vascular tone (de Simone et al 1994) thus supporting a volume retention and vascular mechanism. An enhanced sympathetic nervous system activity is thought to contribute toward obesity-induced hypertension. Indeed, an increased muscle sympathetic activation has been described in both normotensive and hypertensive obese individuals (Gudbjornsdottir et al 1996). The relationship between obesity and sympathetic nerve activation in hypertension was substantiated by Kassab et al (1995) who reported that complete bilateral renal nerve denervation abolished the development of hypertension in obese dogs. Importantly, obese subjects tend to be “salt-sensitive” possibly in-part as a result of increased sympathetic activity (Hall et al 2000, Redon 2001). Renal nerve sympathetic activation is known to be capable of decreasing renal excretory function by vasoconstriction, a decreased renal blood flow, a reduced glomerular filtration rate, and an increased proximal tubular Na^+ reabsorption (DiBona 2004).

The pathogenesis of the enhanced sympathetic nervous system activity in obesity is not entirely clear. Animal studies previously suggested that food intake increases sympathetic nervous system activity (Sechi et al 1997). More recent data indicate that leptin released from fat cells increase sympathetic neuronal traffic specifically to the kidney the consequence being an enhanced salt and fluid retention (Ciriello and de Oliveira 2002). Indeed, in human subjects, plasma leptin concentrations correlate with

renal norepinephrine spillover (Eikelis et al 2003). Importantly, higher plasma norepinephrine, insulin and leptin concentrations have been found in obese as compared to lean subjects regardless of BP status (Masuo et al 2000).

Activation of the RAS is most likely equally as important as that of the sympathetic nervous system in the pathobiology of obesity-induced hypertension. Indeed, obesity is associated with increased circulating angiotensinogen and ACE activity (Forrester et al 1996). These changes would further enhance renal sodium reabsorption through all of those changes discussed above (see 1.2.2). The mechanisms of RAS activation in obesity have recently been clarified. Importantly, a significant portion of circulating angiotensinogen is derived from its expression in adipose tissue (Giacchetti et al 2000). Adipose tissue is second only to the liver in the amount of angiotensinogen produced (Sharma et al 2001). The importance of the contribution of adipose tissue to plasma angiotensinogen is underscored by the strong relationship between leptin levels (an index of the degree of adiposity) and angiotensinogen concentrations (Schorr et al 1998). The relevance of adipose tissue-derived angiotensinogen as a determinant of obesity-induced hypertension was recently underscored in an animal-based study (Boustany et al 2004). In this study, the plasma concentration of angiotensinogen was shown to increase by as much as three fold in the obesity rats compared to the controls.

Activation of the Na^+/H^+ exchanger by adrenaline is greatly enhanced in obese as opposed to lean persons (Bourikas et al 2003). In addition, the increased activity of the Na^+/H^+ exchanger observed in hypertension appears to be largely in obese patients (Delva et al 1993). In animal models, angiotensin II stimulation of the Na^+/H^+ exchanger in the proximal tubule is greater in obese than in lean rats (Becker et al 2003). Consequently, a

potential downstream target from the RAS and sympathetic nervous system that appears to mediate hypertension in obesity is the Na^+/H^+ exchanger.

1.3.0 **Evidence for a genetic basis to hypertension**

There is now clear evidence that BP is a polygenic trait (resulting in the complex interplay of various genes). Several population and family studies have shown that EHT tends to aggregate in families (Pausova et al 1999). Many patients with EHT can cite another member of their family either with the disease or who has died from its complications (Kaplan 1990). The offspring of two hypertensive parents have a 3.8 fold greater risk of developing EHT in comparison to those who have one hypertensive parent who in turn have a 2.5 fold greater risk of developing EHT than the offspring of two normotensive parents (Williams et al 1988). Studies with twins indicate that the rate of developing EHT is higher in monozygotic than in dizygotic twins (Berg 1987, Romanov et al 1990). The correlation coefficient for BP between monozygotic twins is approximately 0.75 (Carmelli et al 1994) whereas for non-twin siblings it is about 0.25 (Longini et al 1984). Blood pressure in both groups of European (Perusse et al 1991, Lifton 1995) and African (Adeyemo et al 2002, Sneider et al 2003) descent is a heritable trait. Family and twin studies indicate that 30% of BP variance may be attributed to genetic factors (Corvol and Jeunemaitre 1997). Despite the strong genetic background to BP, the causal genes and the mode of impact of genes contributing to the disease have only recently begun to emerge. The potential that this emerging field offers and the relatively unique phenotypic characteristics of those of African descent, effects that could

be inherited, prompted me to contribute toward the knowledge base on the genetic determinants of EHT in this ethnic group. The following summarises the potential impact that gene candidates may have on BP, the prior work that has been performed on the gene candidates that I further explored in the present thesis, and the reasons for conducting the studies reported on in the present thesis.

1.3.1 **Mode of impact of gene variants on polygenic traits**

There are essentially four mechanisms by which genes may influence a polygenic trait of interest, such as BP. These include a type I effect, where neither the gene nor a given environmental factor can independently produce the disease, however when both coexist they cause the disease (Khoury et al 1988). A type II effect occurs when an environmental or alternative phenotypic factor independently contributes to a disease, but a genetic effect only occurs through an interaction with environmental or phenotypic factors (Luft 1997). A type III effect occurs when a gene is capable of independently producing the disease in the absence of the environmental influence. However, environmental or alternative phenotypes potentiate the genetic effect. Lastly, in the type IV effect, genetic and environmental/phenotypic effects are both independent and interactive, with the synergism potentiating the risk for the disease (Pausova et al 1999). To my knowledge all studies published to-date, with the exception of three, two recently published by our group (Tiago et al 2001, Tiago et al 2002), and one by an alternative group (Turner et al 1999), have reported on data obtained from studies designed to test only type III or IV gene effects (the gene of interest has independent effects on blood

pressure). In the present thesis, whilst exploring the impact of gene variants on BP, I largely designed my studies assuming that either type I and II or type III or IV effects could occur.

1.3.2 **Candidate genes explored in the present thesis**.

The following summarises the present state of knowledge regarding the gene candidates studied in the present thesis. The main reason for focusing on the gene candidates examined is that they are responsible for the synthesis of protein products that ultimately influence renal salt handling or are derived from adipose tissue. These issues have been highlighted in preceding sections. As indicated in the above discussion this is particularly pertinent for the population studied which are reported to be largely "salt-sensitive", and as noted during data collection (see chapters II-to-V) were largely overweight or obese.

1.3.2.1 **The angiotensinogen gene**

Of all the cellular systems that ultimately influence BP, by far the most extensively investigated system with respect to attempting to elucidate whether genetic factors contribute to EHT, is the RAS. Of the RAS genes investigated, without question the most comprehensively studied is the angiotensinogen (AGT) gene (Jeunemaitre et al 1992, Jeunemaitre et al 1993, Jeunemaitre et al 1997, Kunz et al 1997, Staessen et al 1999, Sethi et al 2001).

The AGT gene is highly polymorphic with polymorphisms occurring in the promoter region (C-3889T, A-1178G, G-1074T, G-792A, T-775C, C-532T, A-217G, A-20C, A-6G) and in exons (C172T, G384A, G400A, G507A, C670T, A676G, A698G, T704C, A1035G, A1164G, C2079T, G2624A, A3189G, C3889T, C4070T, C4072T, A5093C, C5343T, G5556A, G5593A, A5878C, A6066C, G6152A, C6233T, C6309A, C6420T, C6428G, G6442A, G7369A, C8357T, T9597C, G9669T, A9770G, C11535A, C11608T, G12058A, A12194A, C12429T, T12822C). Several variants identified as being functional. One such AGT gene variant is the 704T→C polymorphism in exon 2 of the AGT gene which gives rise to a methionine for threonine substitution at position 235 of the amino acid sequence (M235T) (Jeunemaitre et al 1992). The 235T allele of the M235T polymorphism is in linkage disequilibrium with a functional polymorphism at position -6 (-6G→A in the promoter region) (Corvol and Jeunemaitre 1997, Inoue et al 1997). The first study performed on the AGT gene demonstrated a relationship between the 235T variant and both plasma angiotensinogen concentrations and the risk of developing EHT in Caucasians (Jeunemaitre et al 1992). This same variant was subsequently reported to be associated with EHT in a number of studies that culminated in meta-analyses on data obtained in both Japanese and European groups, confirming a strong relationship between the 235T variant and the risk of developing EHT (Kunz et al 1997, Staessen et al 1999). Nevertheless, the meta-analyses underscored the fact that many of the studies were likely to be subject to selection bias. As a consequence, large cross-sectional studies were conducted in Caucasian populations and provided stronger evidence to indicate that the AGT gene M235T polymorphism was indeed associated with EHT in females (Sethi et al 2001) and with a higher BP (Ortlepp et al 2003).

Despite the relatively convincing relationship between the AGT gene and the risk for EHT and BP in Caucasians, the same level of confidence cannot be expressed regarding a role for the M235T polymorphism and EHT in subjects of African descent. Although the AGT locus is linked to EHT in Jamaicans of African descent (Caulfield et al 1995), the M235T polymorphism is not associated with EHT in Nigerians (Rotimi et al 1994, Rotimi et al 1996, Rotimi et al 1997)), in subjects of African descent living in London (Barley et al 1994), African-Americans (Rotimi et al 1996), or in South Africans of African origin (Tiago et al 2002). In addition, not all studies conducted in Caucasian hypertensives have provided data supporting the notion that the AGT gene is important in BP control and the development of EHT (Kunz et al 1997, Staessen et al 1999). It is possible that discrepant data regarding the impact of the M235T polymorphism on BP could be explained by its relative lack of functionality. Indeed, there is a polymorphism in linkage disequilibrium with the M235T polymorphism, namely the -6G→A polymorphism, which clearly influences angiotensinogen transcription rates (Inoue et al 1997) and is also associated with BP changes in response to weight loss and Na^+ restriction (Hunt et al 1998). Hence, linkage disequilibrium between the M235T polymorphism and the -6G→A is more likely to explain any relationship between the M235T polymorphism and BP. However, again, in large studies conducted in African-Americans, no relationship between the M235T polymorphism and EHT was noted (Larson et al 2000). Yet, what has not been adequately addressed is the fact that the high prevalence of the 235T and -6A alleles of the M235T and -6G→A polymorphisms respectively in subjects of African ancestry (Tiago et al 2002) may reduce the chances of showing an association between these variants and EHT in this population group.

Further upstream from the -6G→A polymorphism, a -20A→C polymorphism has also been demonstrated to alter transcriptional efficiency of the AGT gene (Zhao et al 1999). This core promoter region polymorphism is located within a zone that mediates the responsiveness of the AGT gene to various regulatory signals (Yanai et al 1997). Ishigami et al (1999) showed that this variant increased gene transcription levels *in vitro* and was associated with EHT in Japanese subjects. Zhao et al (1999) found that this variant was coupled with higher plasma angiotensinogen concentrations, though Hilgers et al (2001) failed to demonstrate an association between the -20A→C polymorphism and plasma Ang II concentrations. Recent data obtained from our laboratory indicate that although the -20A→C polymorphism is not associated with EHT or ambulatory BP in subjects of African ancestry, it does have type I or II genetic effects in that it modulates the impact of body size on BP (Tiago et al 2002). In this regard, the variant determines whether body size influences SBP and hence could predict BP responses to weight gain or loss (Tiago et al 2002). This is obviously of clinical relevance as obesity is an ever increasing problem in urban South Africans of African ancestry (Punyadeera et al 2001).

Further upstream from either the -6G→A, or -20A→C polymorphisms of the AGT gene, is the -217G→A polymorphism which is partially homologous to the CAAT/enhancer-binding protein (C/EBP) site. Jain et al (2002) recently demonstrated that as compared with the G nucleotide, the A nucleotide of this polymorphism increases promoter region activity when transfected into hepatic cells. A second study confirmed the impact of the -217G→A polymorphism on angiotensinogen transcription rates (Wu et al 2003). Using a small sample of patients and controls, Jain et al (2002) also demonstrated that the -217G→A polymorphism was strongly associated with EHT in

African-Americans but not in Caucasians. Only two other studies have assessed the relationship between the -217G→A polymorphism and EHT. One study confirmed an impact on the risk for developing EHT (Wu et al 2003). However, Zhu et al (2003) failed to show a relationship between the -217G→A polymorphism of the AGT gene and EHT in African-Americans. It is important to note that in contrast to the innumerable studies performed, assessing the relationship between the other variants of the AGT gene and BP, to-date only the three studies mentioned above, have reported on the potential role of the -217G→A polymorphism on the development of EHT (Jain et al 2002, Wu et al 2003, Zhu et al 2003). All of these studies were conducted in small samples of subjects with office BP assessments, which often result in spuriously high or low BP values being utilized (Myers 2001).

Although there are at least three functional polymorphisms of the promoter region of the AGT gene (-217G→A, -6G→A, and -20A→C) (Jain et al 2002, Wu et al 2003, Inoue et al 1997, Zhao et al 1999) no study has been conducted to assess potential interactions of these polymorphisms on the risk for EHT. It is entirely logical that combined effects of genetic variants within the same locus, all of which modify protein expression, would produce more robust effects on a phenotype of interest. This hypothesis has not previously been tested as most populations do not have a sufficiently high frequency of the variants to evaluate interactive effects.

As a consequence of the limited data produced on the clinical and epidemiological relevance of the -217G→A polymorphism of the AGT gene, and the lack of data on interactions between AGT gene variants, in my studies I further explored the role of the -217G→A polymorphism in hypertension. In this thesis I assessed whether the -217G→A

polymorphism could independently, or through interactions with alternative functional AGT polymorphisms (-6G→A, and -20A→C), determine the risk of EHT and ambulatory BP (off medication) in subjects of African ancestry. These data are described and discussed in chapter II and provide strong evidence in support of a role for interactive effects of the -217G→A and -20A→C polymorphisms as risk factors for EHT and ambulatory BP in subjects of African descent. Moreover, having identified a potentially important role for these polymorphisms in EHT in subjects of African ancestry, I subsequently explored their impact on antihypertensive responses to ACE inhibitor agents in this population group. These data are presented in chapter VI.

1.3.2.2 **The inhibitory guanosine triphosphate gene**

The loss of 41 amino acids from the β-subunit of the G_i protein in patients with EHT is now recognized as being the consequence of gene variation in exon 9 which results in the deletion of 23 nucleotide base pairs (Siffert at al 1998). The splice variant in exon 9 is in linkage disequilibrium with a 825C→T polymorphism in exon 10. As previously indicated the loss of 41 amino acids in the protein product is associated with an increased stimulation and GTP-binding in the α_i-subunit, a change which is thought to account for an increased activation of the Na^+/H^+ exchanger in hypertensives (Siffert at al 1998).

The 825C→T polymorphism of the β-subunit of the G_i protein (GNB3) is associated with EHT in Caucasians (Siffert et al 1998, Benjafield et al, 1998, Beige et al, 1999), aboriginal Canadians (Hegele et al 1998), Carribbeans and West Africans living in

London (Dong et al 1999) and elevated diastolic blood pressure (DBP) in Caucasian EHTs (Hengstenberg et al 2001, Schunkert et al 1998). Nevertheless, several studies have failed to demonstrate an association between the 825C→T polymorphism of GNB3 and EHT in either Caucasians (Brand et al 2000), Japanese (Kato et al 1998, Ishikawa et al 2000), or African-Americans (Larson et al 2000). In the causation of hypertension the impact of the GNB3 polymorphism has also been proposed to mediate affects through its ability to induce obesity (Siffert et al 1999).

The T allele of the GNB3 gene 825C→T polymorphism appears to be more prevalent amongst people of African ancestry, although it is not always associated with EHT. Without exception all studies assessing the impact of the 825C→T polymorphism of the GNB3 gene have designed studies that test for type III or IV genetic effects. To-date, no study has been conducted evaluating whether type I and II effects may occur. As the GNB3 gene variant is associated with an altered Na^+/H^+ exchanger, and obesity enhances the activity of the Na^+/H^+ exchanger (Bourikas et al 2003), I tested the hypothesis that the GNB3 gene variant modifies the impact of body size on BP in a typically “salt-sensitive” population of subjects of African origins. This study is described and discussed in chapter III of this thesis. Importantly, a strength of this study was the large sample sizes utilized and the use of ambulatory monitoring to assess BP as a continuous trait.

1.3.2.3 **The sodium epithelial channel β-subunit gene**

Several functional mutations that result in truncation of encoded products have

been described in genes for the subunits of the ENaC, including those in the carboxy terminal region of the β- and γ- subunits. “Salt-sensitive” hypertension in Liddle`s syndrome, a monogenic form of hypertension, has now been established as occurring as a consequence of a point mutation in the β-subunit of the ENaC gene (Shimkert et al 1994). This mutation results in increased renal tubular Na^+ reabsorption by inhibiting feedback regulation of the eNaC by intracellular Na^+ in apical cells (Shimkert et al 1994).

The discovery of the genetic cause of Liddle`s syndrome led investigators to speculate that relatively common and functional polymorphisms of the ENaC genes could also be involved in the pathogenesis of EHT. In this regard, a polymorphism in the β-subunit gene in the last exon of the ENaC gene, which results in the substitution of threonine (T) by methionine (M) at amino acid 594 (T594M) was considered as a potential cause of EHT (Melander et al 1998). This polymorphism of the ENaC β-subunit gene modifies the electrophysiological properties of and second messenger effects in B-lymphocytes (Su et al 1996, Cui et al 1997), but not the elctrophysiological properties of *Xenopus* oocytes (Persu et al 1998) (the experimental model used to assess the functional role of genetic mutations in Liddle’s syndrome). The changes noted in response to the T594M polymorphism are thought to mediate an increased Na^+ reabsorption in the kidneys (Rossier 1997).

The ENaC β-subunit gene T594M polymorphism has been reported to be absent in a population group (other than those of African ancestry) with a high prevalence of “salt-sensitive” EHT, namely the Japanese, irrespective of hypertension status (Matsubara et al 2000, Chang and Fujita 1994). The T594M polymorphism is also rare in Caucasians and importantly is generally present only in subjects of African descent (Su et al 1996,

Persu et al 1998). As about 50% of patients of African ancestry with EHT are “salt-sensitive” (Peters and Flack 2000), the T594M polymorphism was considered as a gene candidate in elucidating the possible etiology of EHT in patients of African ancestry.

The role of the ENaC β-subunit T594M polymorphism as a determinant of BP is generally inconclusive. The first studies evaluating the role of the ENaC β-subunit gene T594M polymorphism in the pathogenesis of EHT were conducted in African-Americans and demonstrated an absence of association with EHT (Su et al 1996). However, in contrast, Baker et al (1998) in a study population of 206 subjects of African ancestry living in London, demonstrated an association with EHT. In the study by Baker et al (1998) only 17 subjects had the risk allele. Further work by Persu et al (1998) demonstrated a lack of association between the T594M mutation and EHT in a very small sample of 50 subjects of African descent.

With respect to the role of the ENaC β-subunit gene T594M polymorphism as a determinant of BP and hypertension in subjects of African ancestry, the present data are inconclusive for a number of reasons. The relative lack of data (study sizes were by-and-large very small); and the realization that clinic BP measurements are now generally considered inappropriate (Myers 2001), prompted me to further pursue this issue in a large sample of appropriately phenotyped patients (ambulatory BP was determined off medication) of African descent. These data are presented in chapter IV.

1.3.2.4 **The atrial natriuretic peptide gene**

The potential role of dysregulation of ANP secretion in "salt sensitive" patients with EHT (Niimura 1991, Campese and Widerhorn 2000) and impaired natriuretic responses (Agaike et al 1994) recently prompted studies exploring the possibility that alterations in the ANP gene may contribute toward the pathogenesis of EHT in groups with a high prevalence of "salt-sensitivity". Several polymorphisms have been described in the ANP gene, both in intronic and coding regions (Kato et al 2000).

An *HpaII (SmaI)*-sensitive polymorphism of intron 2 of the ANP gene which is in complete linkage disequilibrium with an exon 1 polymorphism (Kato et al 2000) that alters the amino acid sequence of the preprohormone of ANP (Seidman et al 1984), is associated with EHT in African-Americans (Rutledge et al 1995). However, this same polymorphism is not associated with "salt-sensitivity" in a Caucasian group (Schorr et al 1997) or EHT in Chinese (Chung et al 1999) or Japanese (Rahmutula et al 2001) subjects.

A *ScaI*-sensitive polymorphism (1766T→C) in exon 3 of the ANP gene, which leads to an extension of ANP by two additional arginine residues (Ramasawmy et al 1992), is associated with a greater degree of renal target organ damage (microalbuminuria) in patients with EHT (Nannipieri et al 2001). This same polymorphism is however not associated with EHT in Jamaicans of African ancestry (Daniel et al 1997) or Japanese (Rahmutula et al 2001). Moreover, Nannipieri et al. (2001) provided evidence to indicate that the frequency of this gene polymorphism is possibly lower in hypertensives as compared to controls.

Again the inconclusive nature, and relative lack of data (study sizes were by-and-large very small and BP was determined using office as opposed to ambulatory measures) on the role of the ANP gene polymorphisms as determinants of BP and hypertension in

subjects of African ancestry, prompted me to further pursue this issue in a relatively large sample of appropriately phenotyped patients (ambulatory BP was determined off medication). These data are presented in chapter V.

Chapter II

Interaction Between Functional Polymorphisms of the Angiotensinogen Gene Determines the Risk for Hypertension and Ambulatory Blood Pressure in Subjects of African Origin.

Abstract

Background. As multiple functional, but relatively discordant polymorphisms of the angiotensinogen (AGT) gene occur with a high prevalence in subjects of African ancestry, I examined whether AGT gene polymorphisms interact with each other to influence hypertension risk and ambulatory blood pressure (ABP) in this ethnic group.

Methods and results. 1325 subjects of African ancestry (684 hypertensives phenotyped with ABP measurements determined off therapy and 641 controls) were genotyped for functional AGT gene polymorphisms. Although the -217G→A polymorphism was only modestly associated with hypertension risk, interactions between the -217G→A and -20A→C polymorphisms influenced both hypertension risk ($p<0.02$) and ABP ($p<0.05$). In those subjects homozygous for the -20A allele of the functional -20A→C polymorphism, the -217G→A polymorphism predicted hypertension risk (odds ratio=1.65, confidence interval=1.20-2.27, $p<0.005$; stepwise regression analysis), 24 hour ($p<0.002$), day ($p<0.005$) and night ($p<0.01$) diastolic ABP, and 24 hour systolic ABP ($p<0.01$). In contrast, the impact of the -217G→A polymorphism on hypertension risk and ABP was abolished in those subjects with at least one copy of the -20C allele.

Conclusions. Although the AGT gene -217G→A polymorphism is only modestly associated with hypertension, an interaction between the -217G→A and -20A→C polymorphisms of the AGT gene determines hypertension risk and ambulatory BP in subjects of African ancestry. These data support the notion that the AGT gene is important in BP control in this ethnic group, but through interactions between functional promoter region polymorphisms.

INTRODUCTION

The angiotensinogen (AGT) gene is highly polymorphic (Jeunemaitre et al 1992) and three polymorphisms within the promoter region (-6G→A, -20A→C, and -217G→A) have been shown to enhance angiotensinogen expression (Inoue et al 1997, Zhao et al 1999, Jain et al 2002, Wu et al 2003). Two meta-analyses (Kunz et al 1997, Staessen et al 1999) and a recent large population-based study (Sethi et al 2001) support an association between the 704T→C polymorphism (M235T polymorphism) and hypertension in Caucasian groups, a relationship that is likely to occur because the 704T→C polymorphism is in linkage disequilibrium with the -6G→A polymorphism (Inoue et al 1997). Despite relatively convincing data to support a role for the AGT gene in hypertension in Caucasian groups, the role in groups of African descent is unclear. Indeed, association studies conducted in relatively large samples indicate that the AGT gene 704T→C polymorphism, and its associated functional polymorphism (-6G→A) are unimportant as risk factors for hypertension in subjects of African ancestry (Larson et al 2000, Tiago et al 2002).

Although no association between the AGT gene and hypertension has been noted in subjects of African origins, there is still good reason to believe that the locus is important in BP control in this ethnic group. Subjects of African descent have higher plasma angiotensinogen concentrations than other population groups (Bloem et al 1995), an effect partly attributed to the AGT gene (Bloem et al 1997). Affected sibling-pair analysis indicates that the AGT locus is indeed important in contributing to hypertension in subjects of African ancestry (Caulfield et al 1995). Moreover, there is a high

prevalence of risk alleles of polymorphisms within the AGT gene in populations of African ancestry (Tiago et al 2002, Larson et al 2000, Bloem et al 1997, Rotimi et al 1997). The controversy regarding the role of the AGT gene in BP control in this ethnic group, could be explained by the high frequency of multiple functional AGT gene polymorphisms that occur in these populations (Tiago et al 2002, Jain et al 2002). As these polymorphisms share the same end-effect in that they largely influence angiotensinogen expression (Inoue et al 1997, Zhao et al 1999, Jain et al 2002, Wu et al 2003), the possibility that interactions influence BP in subjects of African descent requires consideration. In the present study, I therefore evaluated whether interactions between multiple functional but relatively discordant AGT gene polymorphisms could determine hypertension risk and ambulatory blood pressure (ABP) in subjects of African origins.

METHODS

Subjects and BP measurements. A total of 1325 subjects of African ancestry were studied. 684 hypertensive patients of African ancestry initially randomly screened from a variety of district clinics in suburban areas of Johannesburg were recruited if they had office and mean daytime ambulatory diastolic BPs (DBP) >90 mm Hg (Spacelabs model 90207) off medication. Patient's with auscultatory BPs <200/115 mm Hg had ambulatory measurements performed after at least two weeks off medication. A minority of patients (4%) with either first visit auscultatory BPs ≥200/115 mm Hg and with either target organ damage or two or more additional risk factors for cardiovascular disease had 24 hour BP monitoring performed within a shorter period off medication. To avoid

population stratification, only patients of the Nguni, Sotho and Venda chiefdoms of South Africa were selected. Also, patients with either type I diabetes mellitus, uncontrolled type II diabetes mellitus (defined as an HbA_{1C} of >10%), renal and endocrine disease and clinically important cardiac pathology (clinically significant arrhythmias; heart failure; valvular disease; ischemic heart disease – previous myocardial infarction or unstable angina) were excluded. Ambulatory BP (ABP) measurements were performed at least half hourly during the day (06:00–22:00 hours) and hourly during the night (22:00-06:00 hours), and ambulatory monitors were calibrated using standard techniques. Repeat ABP measurements were made if <85% of intended readings were obtained. All patients were advised not to smoke, imbibe alcohol or ingest caffeine during this period. Control subjects of African origin (Nguni, Sotho and Venda)(n=641) without a family history of hypertension were randomly recruited from community centers in the same suburban areas of Johannesburg and were considered normotensive if auscultatory and oscillometric (Spacelabs model 90207) DBPs were <90 mm Hg after 5 minutes of rest in the seated position, and the subjects had been residents of an urban area for at least 2 years. Subjects with isolated systolic hypertension were excluded from both case and control groups.

DNA preparation and genotyping. Deoxyribonucleic acid (DNA) was extracted from whole blood by lysing red blood cells and digesting the remaining white cell pellet with proteinase K (Lahiri et al 1992). In initial genotyping using direct sequencing techniques in 300 subjects (150 cases and 150 controls) we have previously established that the -6G and -532T alleles only occurred with a frequency of 3-to-4% and 11% respectively (Tiago et al 2002). Nevertheless, the 235T allele occurred with the -6A allele

in 94.3% of subjects and with the –532C allele in 100 % of subjects, but with the –20C and -20A alleles of the -20A→C polymorphism in only 12.0% and 88.0% of subjects respectively (Tiago et al 2002), and with the -217A allele of the -217G→A polymorphism in only 40.4% of subjects (present study). Consequently, the 704T→C, -20A→C, and -217G→A polymorphisms were studied, with the 704T→C used as an index of -6G→A, -532C→T effects. With respect to linkage disequilibrium between the -20A→C, and -217G→A polymorphisms, although the -217A allele occurred with the -20A allele in 95.7% of subjects; the -20A allele occurred with the -217G allele in 54.3% of subjects. Subsequent genotyping of the 704T→C, -20A→C, and -217G→A polymorphisms was undertaken using polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP)-based techniques employing the appropriate primer pairs and restriction enzymes as follows:

-20A→C; Sense primer: 5`-AGA GGT CCC AGC GTG AGT GTC-3` (Roche)

Antisense primer: 5`-AGC CCA CAG CTC AGT TAC ATC-3` (Roche) (Ishigami et al 1999)

Restriction enzyme – *Eco*OR 109I (Life Sciences)

-217G→A; Sense primer: 5`-CTCBAGG CTG TCA CAC ACC TAG-3` (Roche)

Antisense primer: 5`-GTT ACA TCA CTT GGC CAG AG-3` (Roche) (Jain et al 2002)

Restriction enzyme – *Msp* I (Roche)

704T→C; Sense primer: 5`-CAG GGT GCT GTC CAC ACT GGA CCC C-3` (Roche)

Antisense primer: 5`-CCG TTT GTG CAG GGC CTG GCT CTC T-3` (Roche) (Russ et al, 1992)

Restriction enzyme: *Asp* I (Boehringer Mannheim)

To genotype for the -20A→C gene polymorphism, PCR was performed utilizing ~50 ng DNA, 10 x PCR buffer (Takara), 1.5 mM $MgCl_2$, 0.2 mM dNTP, 2.5 mM forward and reverse primers and 1 unit Taq polymerase (Takara) in a total volume of 20 µl. DNA amplication was performed using the following PCR cycles: one 94^{o}C cycle for 5 minutes, followed by 30 cycles of denaturing (94^{o}C for 30 seconds), annealing (64^{o}C for 1 minute), and extension (72^{o}C for 1 minute) with a final extension step at 72^{o}C for 5 minutes. The PCR yielded a 342 base pair (bp) product. Restriction enzyme digestion was performed by incubating 8 µl of the amplicon with 1U of the *Eco*OR 109I restriction endonuclease overnight at 37^{o}C. Restriction enzyme digestion yielded 205 bp and 137 bp (-20C allele) products. These products and the 342bp (-20A allele) product were visualized on a 2% agarose gel under ultraviolet light (UV) light (Figure 1).

To genotype for the -217G→A polymorphism, PCR was performed utilizing ~50 ng DNA, 1 x PCR buffer (Takara), 2 mM $MgCl_2$, 0.2 mM dNTP, 2.5 mM forward and reverse primers, 3 % dimethylsulfoxide, 1 $\mu g.ml^{-1}$ bovine serum albumin, and 1 unit Taq polymerase (Takara) in a total volume of 20 µl. DNA amplication was conducted using the following PCR cycles: 94^{o}C for 5 minutes once, followed by 30 cycles of denaturation (94^{o}C for 1 minute), annealing (60^{o}C for 1 minute), and extension (72^{o}C for 1 minute) with a final extension at 72^{o}C for 5 minutes. The PCR yielded a 595 bp product. Incubation at 37^{o}C for at least 16 hours of aliquots of 10 µl PCR product with 1 unit of the restriction enzyme, *Msp*I, resulted in 336, 182, 40 and 37 bp fragments in subjects with the -217A allele. In subjects with a -217G allele an additional *Msp*I restriction site is recognized and 206, 182, 130, 40 and 37 bp fragments are produced. Restriction enzyme digestion products were visualized on a 3% agarose gel under UV light (Figure 2).

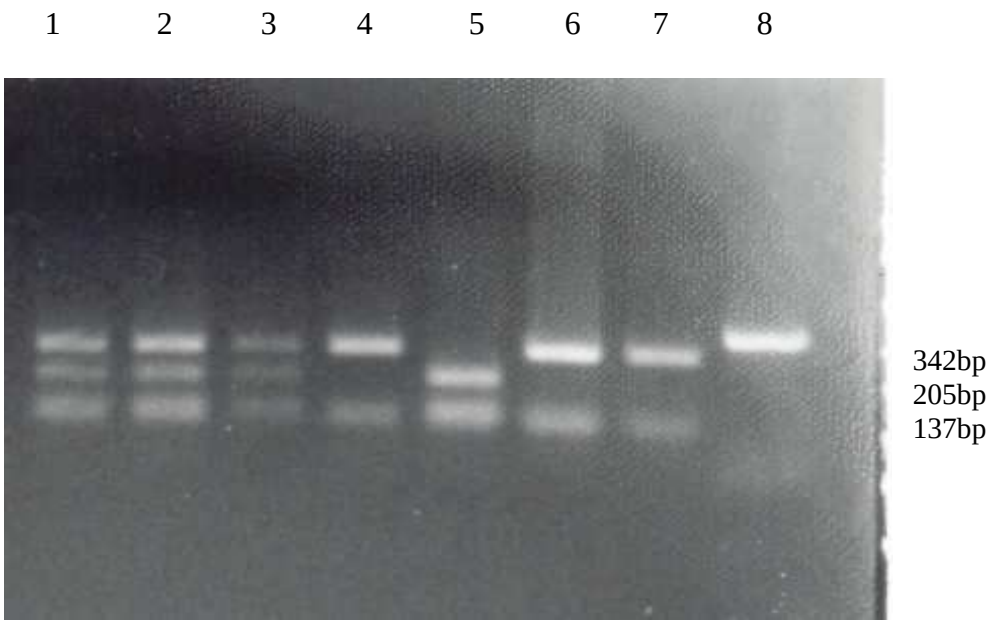


Figure 1. Typical example of an agarose gel image with electrophoretic patterns obtained when genotyping for the angiotensinogen gene -20A→C polymorphism. Samples from subjects homozygous for the risk allele (-20AA) are in lanes 4, 6 and 7. Samples from subjects heterozygous (-20AC) are in lanes 1, 2 and 3. Lane 5 represents patterns from a sample of a subject homozygous for the C allele (-20CC) whilst lane 8 shows an undigested -20A→C amplicon.

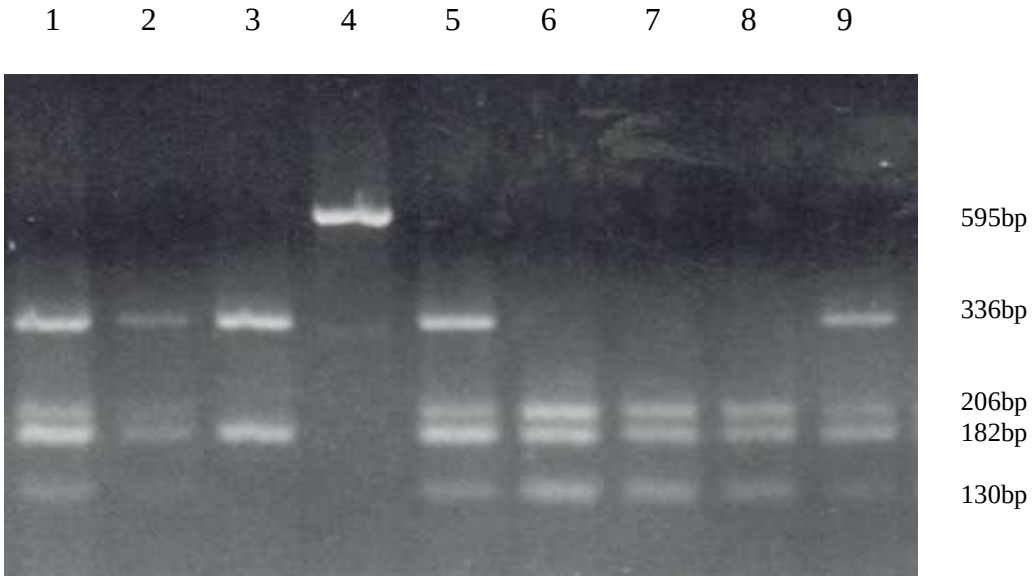


Figure 2. Typical example of an agarose gel image with electrophoretic patterns obtained when genotyping for the angiotensinogen gene -217G→A polymorphism. Lane 2 represents patterns obtained from a sample of a subject with the -217AA genotype, whilst lanes 1, 2, 5 and 9 are patterns from samples of subjects with the -217GA genotype. Lanes 6 , 7 and 8 are patterns from samples of subjects with the -217GG genotype, whilst lane 4 is a sample of undigested -217G→A amplicon.

The accuracy of genotyping of promoter region polymorphisms was confirmed with direct sequencing techniques in 300 samples.

Sequencing Protocol

The DNA fragment containing the angiotensinogen gene -217 polymorphism was amplified using standard PCR techniques as described above. PCR products were cleaned using a Shrimp Alkaline Phosphatase/Exonuclease mix (1:1), and a High Pure PCR Product Purification Kit (Roche). Purified PCR products were cycle sequenced according to the ABI PRISM Big-Dye Terminator Cycle Sequencing Ready Reaction Kit protocol with AmpliTaq DNA Polymerase, FS (Applied Biosystems). Briefly, samples were prepared by adding 8 µl Terminator Ready Reaction Mix (containing A-Dye Terminator labeled with dichloro[R6G], C-Dye Terminator labeled with dichloro[ROX], G-Dye Terminator labeled with dichloro[R110], T-Dye Terminator labeled with dichloro[TAMRA], deoxynucleoside triphosphates (dATP, dCTP, dITP, dUTP), AmpliTaq DNA polymerase, FS, with thermally stable pyrophosphatase, $MgCl_2$ and Tris-HCl buffer, pH=9.0) and 3.2 pmol sequencing primer to 100 ng PCR product. After cycle sequencing, extension products were purified using the Spin Column method. The dried sample pellets were resuspended in Template Suppression reagent, denatured and evaluated on an ABI PRISM 310 and 377 Genetic Analyzer. A typical example of a spectrophoretogram obtained is illustrated in figure 3.

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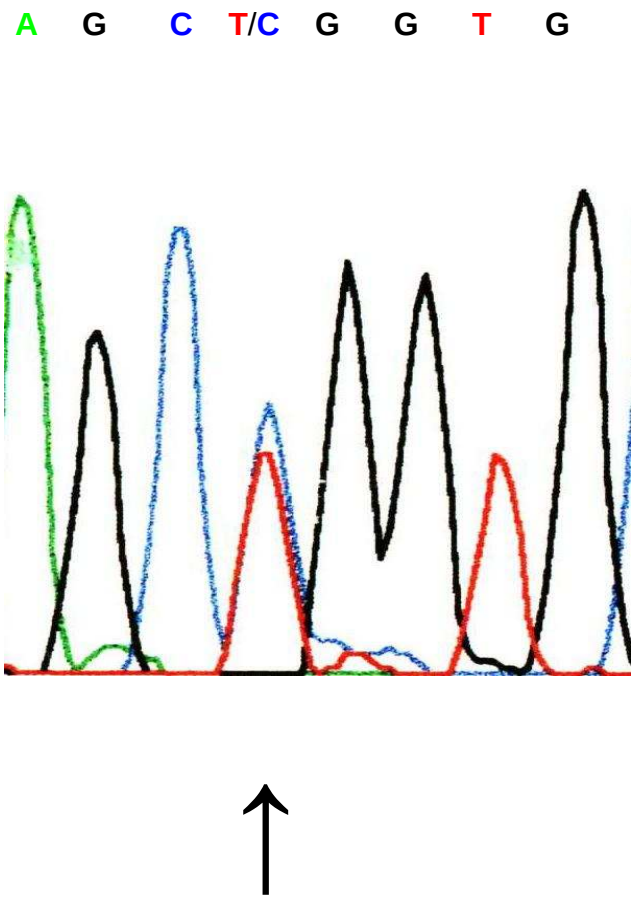

Figure 3. Typical example of spectrophoretograms obtained when genotyping for the AGT -217G→A polymorphism. This spectrophoretogram is from a sample of a subject with the -217GA genotype. It should be noted that sequencing occurred in the reverse direction and therefore the T/C corresponds to the GA genotype.

To genotype for the 704T→C polymorphism, PCR was performed with ~50 ng DNA, 10 x PCR buffer (Takara), 1.5 mM $MgCl_2$, 0.2 mM dNTP, 2.5 mM forward and reverse primers, 3 % dimethylsulfoxide, 1 $\mu g.ml^{-1}$ bovine serum albumin, and 1 unit Taq polymerase (Takara) in a total volume of 20 µl. DNA amplication was conducted using the following PCR cycles: 90°C for 3 minutes once, followed by 30 cycles of denaturation (91°C for 45 seconds), annealing (68°C for 1 minute) and extension (72°C for 1 minute), with a final extension cycle of 72°C for 7 minutes. Restriction enzyme digestion was performed by incubating 10 µl of the final PCR product with *AspI*, the restriction endonuclease, overnight at 37°C. Digested products were separated on a 17.5% polyacrylamide gel. PCR-RFLP yielded 165 bp products in subjects with the 704C allele, whilst in subjects with the 704T allele 141+24 bp products were produced (Figure 4). Heterozygotes for the gene polymorphism had 165 bp and 141+24 bp products (Figure 4). All samples had repeat genotyping for the 704T→C (M235T) polymorphism, which showed 100% reproducibility.

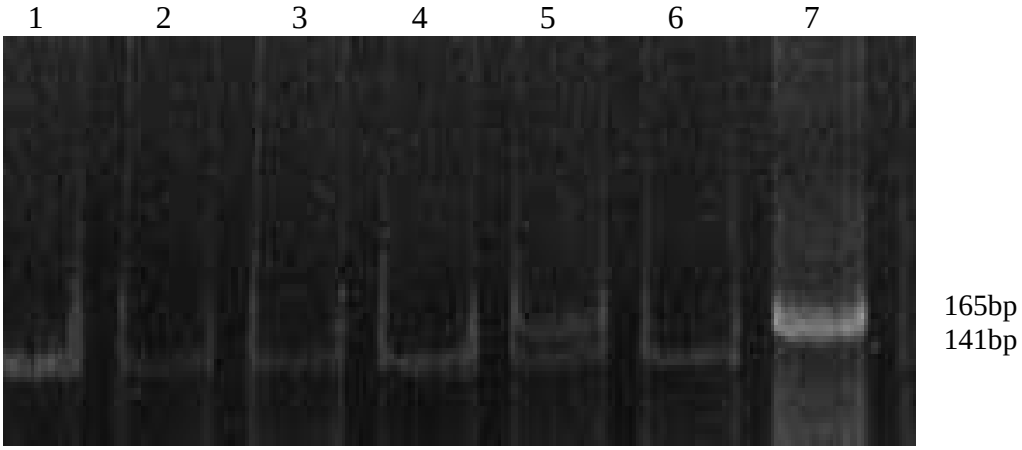

Figure 4: Typical example of a polyacrylamide gel image with electrophoretic patterns obtained when genotyping for the angiotensinogen 704T→C polymorphism. Lanes 1, 2, 3, 4 and 6 are from samples obtained from subjects homozygous for the *Asp* I restriction site (704TT or 235TT genotype), whilst lane 5 shows a sample from a subject heterozygous (704CT or 235MT genotype) and lane 7 the undigested 704T→C amplicon.

Statistical analysis. The estimated sample size required for the -217G→A polymorphism in this study to provide 80% power with a p value of 0.05 was based on an odds ratio from previous data in African Americans (Jain et al 2002) and the allele frequency in the first 100 control subjects genotyped. An estimated 183 subjects were required in each group. Sample size calculations based upon previous data obtained in this group (Tiago et al 2002) revealed that over 1500000 and over 9800 subjects would be required in each group for the -20A→C and M235T polymorphsims respectively. Case and control group mean values were compared with the use of a two-sample Student's t test or a Mann-Whitney test depending on whether variables were nominal or ordinal (Bartlett's test). To test for Hardy-Weinberg equilibrium the expected genotype numbers were calculated from the allele frequencies and deviation from the observed genotype numbers determined using a χ^2 test. Effects of alleles on the presence of hypertension were evaluated using a χ^2 test. Genotype effects on the presence of hypertension were assessed with a general MANCOVA with age, gender, body mass index (BMI), chiefdom, and alternative AGT genotype included as covariates and also by general stepwise regression analysis with age, gender, BMI, chiefdom, and AGT genotypes included in a multivariate regression model. To assess the effect of AGT genotype on either office BP or ABP, general stepwise regression analysis was also performed with age, gender, BMI, chiefdom, duration of hypertension, and AGT genotypes included in the regression model. Only patients with >90% of ABP measurements successfully recorded (n=626) were used to assess the effects of genotype on ABP. Risk genotypes were identified from a knowledge of alleles that mediate increases in angiotensinogen expression (Inoue et al 1997, Zhao et al 1999, Jain et al 2002, Wu et al 2003). Genotype

interactions that are likely to increase angiotensinogen expression were evaluated. As the -20A→C polymorphism modifies the response of the AGT promoter region to transcription factors, with the -20A allele being estrogen responsive and the -20C allele responsive to adenoviral major late transcription factor (Zhao et al 1999), both alleles were assumed to be of importance. Because of the distribution of alleles in the population sampled, genotype analysis was statistically powered assuming only recessive effects of the risk -217A, -20A, and 704C (235T) alleles. As the 704T→C (M235T) polymorphism of the AGT gene is associated with hypertension in females but not males (Sethi et al 2001), gender-specific analysis for all AGT gene polymorphisms was also performed. Formal interaction analysis between the polymorphisms was performed using ANCOVA followed by Tukey *post hoc* test, with age, gender, BMI and alternative genotype included as covariates. Continuous data are expressed as mean ± SEM.

RESULTS

Demographic and clinical data. Both the case and the control groups had a preponderance of females and individuals with an increased BMI (Table 1). The higher percentage of females reflects the gender distribution of patients visiting district clinics and community centres, rather than a profoundly greater incidence of hypertension in African women compared to men. Except for a higher mean BMI in the case group, the case and the control groups were matched according to other demographic features (Table 1) including the frequency of patients and control subjects from Nguni, Sotho and Venda chiefdoms. The low proportion of Venda subjects included in both the case and the control groups reflects the low prevalence of Venda subjects living in urban

Johannesburg regions. There were no differences in demographic and clinical data between subjects grouped according to genotype (data not shown).

Genotype and allele frequencies of AGT gene polymorphisms. The genotype frequencies of the -20A→C, -217G→A, and 704T→C (M235T) polymorphisms were in Hardy-Weinberg equilibrium (Table 2). With regards to the 704T→C (M235T) and the -20A→C polymorphisms of the AGT gene, neither polymorphism was associated with the risk for hypertension (Figure 5, Tables 2 and 3). In addition, there was no gender-specific effect of the -20A→C polymorphism on the risk for hypertension (Figure 5, Table 4). The T allele of the 704T→C (M235T) was associated with the risk for hypertension in males (Figure 5, Table 4). In contrast, the -217A allele and -217AA genotype were independent risk factors for the development of hypertension (Figure 5, Tables 3 and 4).

Genotype and allele frequencies of combined AGT gene polymorphisms. Neither the 704T→C (M235T), nor the -20A→C polymorphisms effect on the risk for hypertension was altered by alternative AGT genotype (data not shown). However, a significant interaction between the -217G→A and -20A→C was noted ($p<0.02$). Consequently, the -217G→A polymorphism was strongly associated with hypertension in subjects homozygous for the -20A allele of the -20A→C polymorphism (Tables 3 and 5, Figure 6). However, in subjects with at least one copy of the -20C allele of the -20A→C polymorphism, the impact of the -217G→A polymorphism on the risk for hypertension was abolished (Tables 3 and 5, Figure 6). In contrast to the -20A→C polymorphisms modifier effect on the -217G→A polymorphisms association with hypertension, genotype of the 704T→C (M235T) polymorphism did not influence the impact of the -217G→A polymorphism on the risk for hypertension (Tables 5 and Figure 6).

Table 1. Demographic and clinical characteristics of the hypertensive and control subjects of African ancestry recruited for the angiotensinogen (AGT) gene study.

	Hypertensives	Controls
n =	684	641
Age (years)	52.3±0.4	52.5±0.4
Nguni/Sotho/Venda (%)	64/34/2	67/31/2
Gender (% female)	67	63
Body mass index ($kg.m^{-2}$)	31.1±0.3*	28.9±0.3
Type II diabetes mellitus (%)	4	3
Office SBP/DBP (mm Hg)	166±1*/102±1*	132±1/ 79±1
24 hour SBP/DBP (mm Hg)	151±1/96±1	-
Day SBP/DBP (mm Hg)	155±1/102±1	-
Night SBP/DBP (mm Hg)	146±1/91±1	-
Duration of hypertension (years)	3.0±0.2*	0

SBP/DBP, systolic blood pressure, diastolic blood pressure. *$p<0.001$.

Table 2. Genotype and allele frequencies of AGT gene polymorphisms in hypertensive and control subjects of African ancestry.

	Genotype			Allele	
		704T→C (M235T)			
	MM	MT	TT	M	T
Controls	2 (0.3)	119 (18.6)	520 (81.1)	123 (9.6)	1159 (90.4)
Hypertensives	1 (0.1)	143 (20.9)	540 (79.0)	145 (10.6)	1223 (89.4)
		-20A→C			
	AA	AC	CC	A	C
Controls	494 (77.1)	136 (21.2)	11 (1.7)	1124 (87.7)	158 (12.3)
Hypertensives	523 (76.5)	149 (21.8)	12 (1.7)	1195 (87.4)	173 (12.6)
		-217G→A			
	AA	AG	GG	A	G
Controls	86 (13.4)	296 (46.2)	259 (40.4)	468 (36.5)	814 (63.5)
Hypertensives	131 (19.2)	314 (45.9)	239 (34.9)	576 (42.1)	792 (57.9)

Numbers are sample numbers (%). Odds ratios and 95% confidence intervals that genotypes or alleles are associated with hypertension are given in Figure 5.

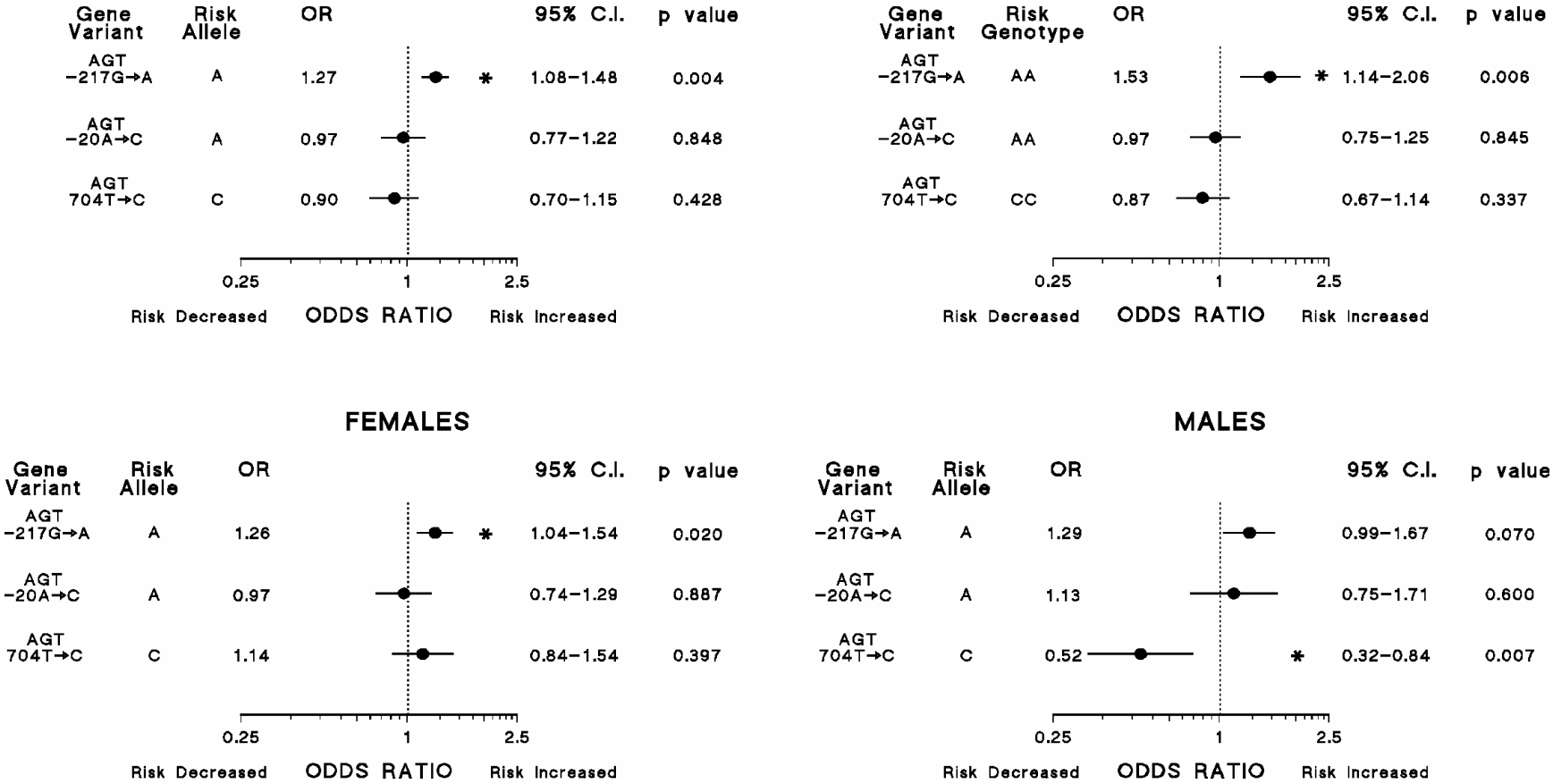

Figure 5. Impact of polymorphisms of the angiotensinogen gene on the risk of developing hypertension in subjects of African ancestry irrespective of gender (top panels) and grouped according to gender (lower panels). The -217G→A polymorphism was associated with the risk for hypertension. Probability values are for chi-squared analysis (alleles) and MANCOVA (genotype). * $p<0.05$. Outcomes of stepwise regression analysis are indicated in table 3.

Table 3. Impact of AGT genotype and other phenotypic factors on the risk for hypertension in subjects of African ancestry.

Factors associated with hypertension	β values for general stepwise regression analysis*	p value
	Total group	
Body mass index	0.17±0.03	<0.00001
-217AA genotype	0.07±0.3	<0.01
	Females only	
Age	0.09±0.03	<0.02
Body mass index	0.16±0.03	<0.00001
-217AA genotype	0.08±0.03	<0.03
	Males only	
Age	0.09±0.03	<0.02
Body mass index	0.16±0.03	<0.00001
	-20AA genotype only	
Body mass index	0.16±0.03	<0.0001
-217AA genotype	0.09±0.03	<0.005
	-20AC + CC genotype only	
Body mass index	0.16±0.03	<0.0001

* standardized regression coefficient.

Table 4. Allele frequencies of AGT gene polymorphisms in hypertensive and control subjects grouped by gender.

	Females		Males	
		704T→C (M235T)		
	M	T	M	T
Controls	94 (11.1)	716 (88.9)	29 (7.0)	443 (93.0)
Hypertensives	95 (10.3)	825 (89.7)	50 (11.2)	398 (88.8)
		-20A→C		
	A	C	A	C
Controls	701 (86.5)	109 (13.5)	423 (89.6)	49 (10.4)
Hypertensives	799 (86.8)	121 (13.2)	396 (88.4)	52 (11.6)
		-217G→A		
	A	G	A	G
Controls	288 (35.6)	522 (64.4)	180 (38.1)	292 (61.9)
Hypertensives	378 (41.1)	542 (58.9)	198 (44.2)	250 (55.8)

Numbers are sample numbers (%).Odds ratios and 95% confidence intervals that genotypes or alleles are associated with hypertension are given in Figure 5.

Table 5. Genotype distribution of different combinations of polymorphisms of the AGT gene in subjects of African ancestry.

	AA	AG	GG	A	G
-217G→A in -20A→C genotype-specific groups					
Homozygous for the -20A allele					
Controls	75 (15.2)	232 (47.0)	187 (37.8)	382 (38.7)	606(61.3)
Hypertensives	119 (22.8)	237 (45.3)	167 (31.9)	475 (45.4)	571 (54.6)
At least one copy of the -20C allele					
	AA	AG	GG	A	G
Controls	11 (7.5)	64 (43.5)	72 (49.0)	86 (29.3)	208 (70.7)
Hypertensives	12 (7.5)	77 (47.8)	72 (44.7)	101 (31.4)	221 (68.6)
-217 G→A in 704T→C (M235T) genotype -specific groups					
Homozygous for the 235T allele					
	AA	AG	GG	A	G
Controls	84 (16.2)	247 (47.5)	189 (36.3)	415 (39.9)	625 (60.1)
Hypertensives	117 (21.7)	252 (46.7)	171 (31.7)	486 (45.0)	594 (55.0)
At least one copy of the 235M allele					
	AA	AG	GG	A	G
Controls	2 (1.6)	49 (40.5)	70 (57.9)	53 (31.2)	189 (78.1)
Hypertensives	14 (9.7)	62 (43.1)	68 (47.2)	90 (31.2)	198 (68.8)

Numbers are sample numbers (%). Odds ratios and 95% confidence intervals that genotypes or alleles are associated with hypertension are given in Figure 6.

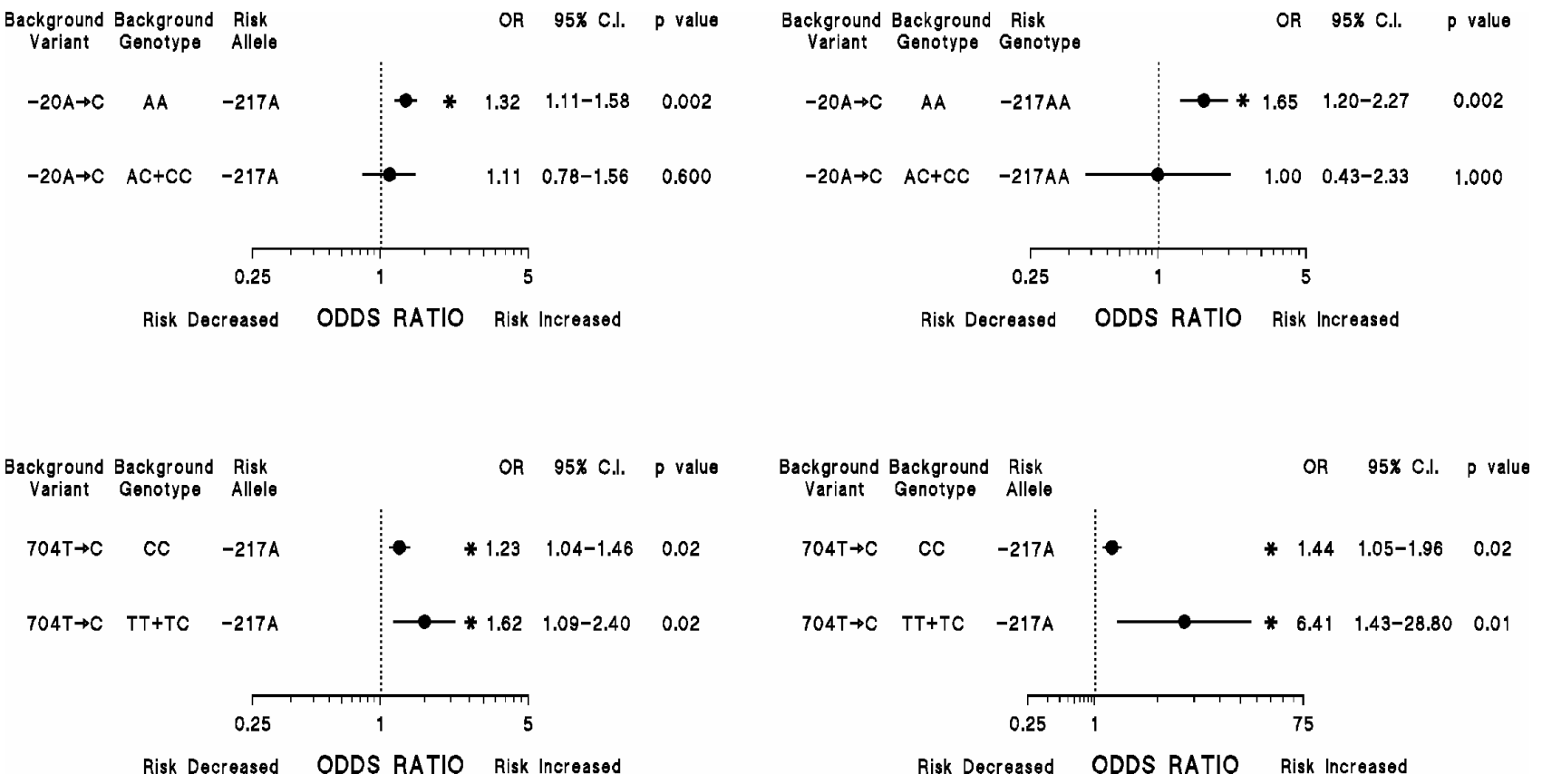

Figure 6. Impact of combinations of polymorphisms of the angiotensinogen gene on the risk of developing hypertension in subjects of African ancestry. See text for explanation. Probability values are for chi-squared analysis (allele) and MANCOVA (genotype). *$p<0.05$. Outcomes of stepwise regression analysis are indicated in Table 3.

Genotype effects on blood pressure. Angiotensinogen genotypes of individual polymorphisms were not independently associated with BP (Figure 7,. Table 6). However, an interaction between the -20A→C and -217G→A polymorphisms was noted for ambulatory 24 hour ($p=0.002$) day ($p=0.002$) and night ($p= 0.006$) diastolic BP and 24 hour ambulatory systolic BP ($p<0.05$). Consequently, patients with the -217AA genotype had a higher 24 hour, day and night ambulatory diastolic BP and 24 hour ambulatory systolic BP, but only if they were -20AA genotype (Figure 8; adjusted regression coefficients are indicated in Table 6). The only other factor that determined diastolic ABP values was a longer duration of hypertension (Table 6). Ambulatory systolic BP was also determined by a greater age, BMI and duration of hypertension (Table 6). No gene effects on office BP were noted in either case or control groups (data not shown). In contrast to the -20A→C polymorphisms modifier effect on the -217G→A polymorphisms association with ABP, genotype of the 704T→C (M235T) polymorphism did not influence the impact of the -217G→A polymorphism on ABP (data not shown).

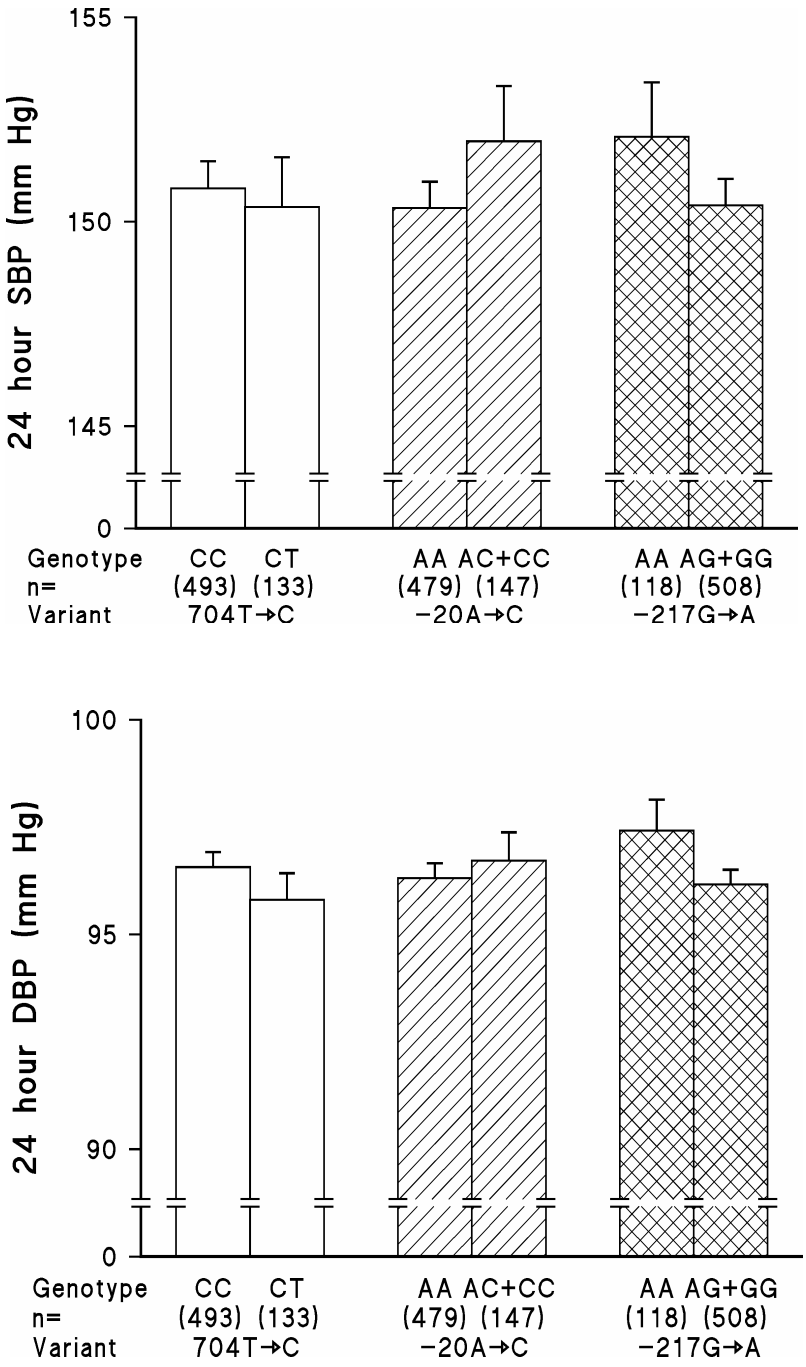

Figure 7. Impact of angiotensinogen genotype on ambulatory blood pressures (BP) in hypertensive patients of African ancestry. No differences in BP were noted between genotype-specific groups.

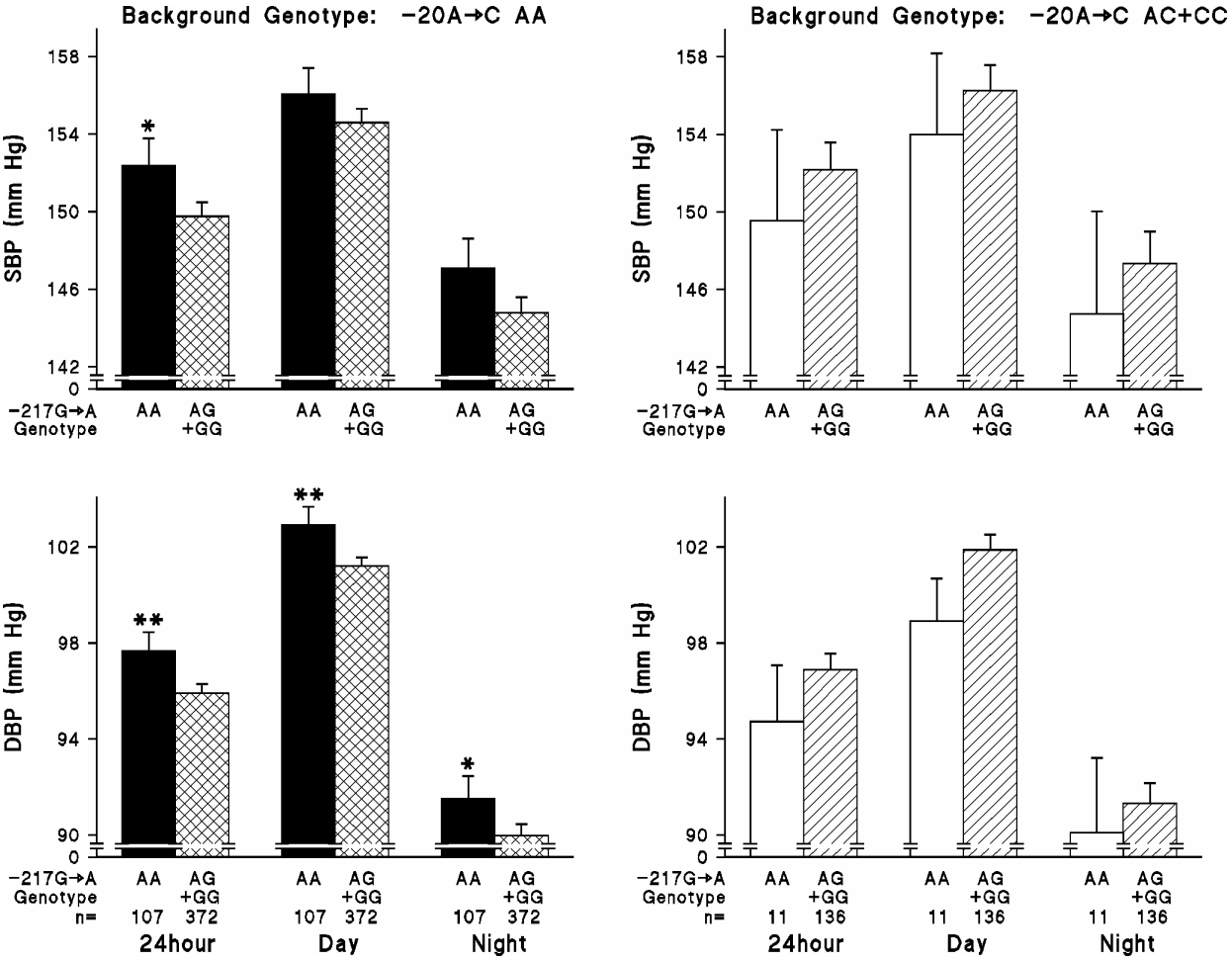

Figure 8. Impact of combinations of polymorphisms of the angiotensinogen gene on ambulatory blood pressures (BP) in hypertensive patients of African ancestry. See text for explanation. * $p<0.01$, ** $p\leq0.002$ for stepwise regression analysis as summarised in Table 6.

Table 6. Impact of AGT genotype and other phenotypic factors on ambulatory blood pressure in hypertensives of African ancestry.

Time of day	Factors associated with BP	Adjusted regression coefficients*	p values
	Diastolic ambulatory BP		
Mean 24 hour	Duration of hypertension	0.15±0.04	<0.001
	-20AA + -217AA genotype	0.13±0.04	<0.002
Mean Day	Duration of hypertension	0.17±0.04	<0.001
	-20AA + -217AA genotype	0.13±0.04	=0.002
Mean Night	Duration of hypertension	0.14±0.04	<0.001
	-20AA + -217AA genotype	0.12±0.04	=0.006
	Systolic ambulatory BP		
Mean 24 hour	Age	0.22±0.04	<0.0001
	Body mass index	0.10±0.04	<0.01
	Duration of hypertension	0.16±0.04	<0.0001
	-20AA + -217AA genotype	0.11±0.04	=0.005
Mean Day	Age	0.22±0.04	<0.0001
	Body mass index	0.10±0.04	<0.01
	Duration of hypertension	0.18±0.04	<0.0001
Mean Night	Age	0.22±0.04	<0.0001
	Body mass index	0.14±0.04	<0.001
	Duration of hypertension	0.14±0.04	<0.001

* standardized regression coefficient for general stepwise regression analysis. Blood pressure values for genotype-specific groups are indicated in Figures 7 and 8.

DISCUSSION

The main finding of the present study is that an interaction between the functional -217G→A and -20A→C polymorphisms of the AGT gene (Jain et al 2002, Wu et al 2003, Zhao et al 1999) determines hypertension risk and ABP in subjects of African ancestry. The -217A allele and the -217AA genotype were associated with hypertension in subjects homozygous for the -20A allele, but not in those subjects with at least one copy of the -20C allele. Moreover, -217AA genotype determined ambulatory systolic and diastolic BP in hypertensive subjects homozygous for the -20A allele, but not in those subjects with at least one copy of the -20C allele. In contrast, the 704T→C (M235T) polymorphism, and hence the -6G→A and -532C→T polymorphisms which are in strong linkage disequilibrium with the M235T polymorphism, was associated with neither hypertension risk, nor ABP when considered either alone or in an interactive manner with either the -20A→C or the -217G→A polymorphisms.

Although there is substantial evidence to support a role for the AGT gene in hypertension (Jeunemaitre et al 1992, Kunz et al 1997, Staessen et al 1999, Sethi et al 2001), there are still a number of outstanding issues in this regard. In particular, studies assessing the impact of the AGT gene in hypertension in groups of African ancestry have repeatedly demonstrated a lack of association with hypertension even in relatively large samples (Tiago et al 2002, Larson et al 2000). This is despite a number of lines of evidence indicating that this population group is the most likely to be affected by functional AGT polymorphisms. Indeed, subjects of African origins having higher plasma angiotensinogen concentrations than other population groups and the AGT gene is associated with serum angiotensinogen in this group (Bloem et al 1997). Second, sibling-

pair analysis indicates that the AGT locus is indeed important in contributing to hypertension in this group (Caulfield et al 1995). Lastly, in groups of African ancestry the prevalence of risk alleles of polymorphisms within the AGT gene is much higher than in other population groups (Tiago et al 2002, Larson et al 2000, Bloem et al 1997, Rotimi et al 1997).

A number of factors may explain the lack of impact in subjects of African origin, of AGT gene polymorphisms previously assessed. First, the high prevalence of the polymorphisms assessed (704T→C and -6G→A) may have limited the outcome of these studies (unequal distribution of alleles limits statistical power). The apparent association of the non-risk allele (T) of the 704T→C polymorphism in males is likely to be spurious due to the high prevalence of the risk allele (C) and the small sample size of males in our study. In this regard, the present study shows an important impact of functional polymorphisms of the AGT gene (-217G→A and -20A→C) in subjects of African origins with a distribution of alleles that provides appropriate statistical power. Second, most studies assessing the role of AGT gene polymorphisms have assumed independent gene polymorphism effects rather than accounting for the highly polymorphic nature of the AGT gene (Jeunemaitre et al 1992), many polymorphisms of which are functional (Inoue et al 997, Zhao et al 1999, Jain et al 2002, Wu et al 2003). It is reasonable to assume that if three functional promoter region polymorphisms exist, all of which influence angiotensinogen expression, that interactions between them would occur to determine BP. In this regard, in the present study I provide clear evidence to indicate that the impact of the -217G→A polymorphism on both hypertension risk and ambulatory BP in hypertensives is evident only in one -20A→C polymorphism genotype group (subjects

homozygous for the -20A allele). In subjects homozygous for the -20A allele, the -217G→A polymorphism was a strong risk factor for hypertension and was associated with ABP, effects that were abolished in those subjects with at least one copy of the -20C allele.

The results of the present study provide clarity on the role of the -217G→A polymorphism of the AGT gene in hypertension where data showing a strong association (Jain et al 2002) are contrasted with data showing no association (Zhu et al 2003) of the -217A allele with hypertension in African-Americans. Previous findings (Jain et al 2002, Zhu et al 2003) were obtained in small samples of hypertensives selected without ABP monitoring utilized to confirm the presence of hypertension. These studies also did not assess BP as a continuous trait, account for interactions between the -217G→A and the-20A→C polymorphisms, or necessarily employ analysis that accounts for confounding phenotypic effects (Jain et al 2002, Zhu et al 2003).

A generic problem in studies attempting to associate gene polymorphisms with risk of hypertension is that these studies generally produce highly variable results. Sample sizes inadequate to limit the risk of false positive or negative results, poor phenotypic characterization, and population admixture may limit the outcome of association studies (Gambaro et al 2000). In this regard the present study is one of the largest case-control studies conducted in the field. In addition, the present study is the only case-control study to my knowledge where the presence of hypertension was confirmed in all patients using 24 hour ABP monitoring, with patients off medical therapy. This approach excludes recruitment of subjects with isolated office hypertension (Myers 2001) and utilizes a technique that is more closely associated with end-organ

damage and complications of hypertension (Mallion et al 1999). Importantly, although I detected an association between genotype and ABP, I was unable to show a similar relationship between genotype and office BP. A further strength of the present study is that patient and control groups were not only matched for their usual demographic characteristics, including racial background, but also for more precise ethnic backgrounds (chiefdoms that are historically derived from the same gene pool). With regards to population stratification, the chance of spurious findings occurring through non-random ascertainment of subjects was limited by random recruitment from community clinics and community centers. Moreover, similar genotype effects were noted for the risk of hypertension and ABP considered as a continuous trait. Lastly, statistical procedures that account for alternative AGT gene polymorphisms and phenotypic confounders were employed.

The mechanism responsible for the impact of the -217G→A polymorphism on BP, could be explained by the ability of the polymorphism to increase binding of the CAAT/enhancer-binding protein, and subsequently both basal and interleukin 6-stimulated AGT gene transcription (Jain et al 2002). The mechanisms responsible for the modifying effect of the -20A→C polymorphism on the impact of the -217G→A polymorphism on BP were not evaluated in the present study. Interestingly, the -20A allele determines estrogen responsive angiotensinogen expression (Zhao et al 1999) and estrogen synthesis occurs in adipose tissue (via aromatase enzymes) (Ackerman et al 1981). Consequently, a potential hypothesis is that the -217G→A polymorphism influences angiotensinogen synthesis by adipose tissue, but only in response to intracrine estrogen synthesis in estrogen responsive genotype individuals (homozygous for the -20A

allele). This hypothesis is supported by the high mean body mass index of the hypertensive group assessed in the present study.

Patients with clinically important cardiac pathologies were excluded in the present study because, for their safety, they could not be taken off medical therapy and therefore ABP measurements could not be performed. Although a selection bias may have been introduced as a result of the exclusion of such patients, this bias would have been against the outcomes of the present study, and therefore, if anything, underscores the present data. As renin–angiotensin system (RAS) polymorphisms have been reported as possible risk factors for cardiovascular mortality, one would be more likely to observe a significant genetic finding in this population, than in one which is limited to patients with only essential hypertension. Thus, the present data cannot be attributed to or confounded by the associations previously demonstrated between RAS gene polymorphisms and the risk of cardiovascular mortality.

In conclusion, taken together with data showing linkage of the AGT locus with hypertension in subjects of African ancestry (Caulfield et al 1995), the functional relevance of the -20A→C (Zhao et al 1999) and -217G→A (Jain et al 2002, Wu et al 2003) polymorphisms, and modifier effects of the -20A→C polymorphism on body size-BP relations in patients of African descent (Tiago et al 2002), the present data suggest that the AGT gene, and hence the RAS is likely to play an important role in hypertension in subjects of African ancestry. Nevertheless, in keeping with the complex nature of polygenic traits, AGT gene effects in subjects of African origins are likely to be expressed through complex interactions between functional AGT gene polymorphisms (present data) and between these polymorphisms and body size (Tiago et al 2002).

Chapter III

825C→T Polymorphism of the G Protein β3 Subunit Gene Modifies the Impact of Body Size on Blood Pressure.

ABSTRACT

Background. As the 825C→T polymorphism of the guanosine triphosphate (G) protein β3 subunit (GNB3) gene influences the activity of the Na^+/H^+ exchanger and the activity of the exchanger is enhanced in obesity, I evaluated the role of the 825C→T polymorphism as a potential modifier of the impact of body size on BP.

Methods. 661 hypertensives sampled from a group with a high mean body mass index (BMI) and who had 24 hour ABP measurements determined whilst off therapy and 629 control subjects of African descent were genotyped for the GNB3 gene polymorphism.

Results. No independent effects of the GNB3 gene polymorphism on either the risk for hypertension, ABP values in hypertensives or office BP values in controls were noted. However, the polymorphism had a marked influence on the impact of body size on systolic BP (SBP) with a higher SBP noted in overweight and obese subjects (BMI>25 kg/m^2) in both case (ambulatory SBP, $p<0.01$) and control (office SBP, $p<0.01$) groups homozygous for the 825T allele. In addition, the GNB3 gene polymorphism produced a marked effect on the BMI-ambulatory SBP relation in the hypertensive group. A relationship between BMI and ambulatory SBP was noted in patients homozygous for the 825T allele ($n= 458$, $r= 0.20$, $p=0.00002$), but was absent in those with at least one copy of the C825 allele ($n=205$, $r=0.02$, $p=0.78$) ($p<0.04$ for comparison of the slopes of the BMI-SBP relationship between genotype-specific groups). A relationship between BMI and BP was not evident in the control group.

Conclusions. These results suggest that the functional GNB3 gene polymorphism is an important modifier of the impact of body size on BP in subjects of African ancestry. This

study provides further evidence to support the notion that gene modifiers can produce a profound impact on BP-phenotypic relations.

INTRODUCTION

Although body size is an important determinant of blood pressure (BP) (Kannel et al 1967, Masuo et al 2000, Stevens et al 2001, Montani et al 2002), the relationship between body size and BP is heterogenous (Johnson et al 1975, Hseuh and Buchanan 1994). Factors that influence body size-BP relations in hypertension require elucidation as they may predict the impact of weight reduction on BP. The BP response to obesity may be influenced by genetic factors that in turn depend on modifying alleles in the genetic background (Mark et al 1999). Hence, modifier genes have been suggested to have a substantial effect on the impact of body size on BP (Mark et al 1999). Indeed, recent data indicate important gene modifier effects on the impact of body size on BP in human hypertension (Turner et al 1999, Tiago et al 2002). However, a number of gene modifiers are likely to contribute to the impact of body size on BP.

The activity of the Na^+/H^+ exchanger, an intermediate phenotype linked to renal Na^+ balance (Weder et al 1991) and which is enhanced in hypertension (Siffert and Dusing 1995) but only in obese subjects (Delva et al 1993), is increased in subjects with a C→T substitution in position 825 of the guanosine trishosphate (G) protein β3 subunit (GNB3) gene (Siffert et al 1998). The 825C→T polymorphism has been variably linked to hypertension and BP (Siffert et al 1998, Schunkert et al 1998, Benjafield et al 1998, Hegele et al 1998, Kato et al 1998, Beige et al 1999, Dong et al 1999, Larson et al 2000, Ishikawa et al 2000, Brand et al 2000). As obesity may induce effects on BP in-part through an influence on the activity of the Na^+/H^+ exchanger (Delva et al 1993), and the 825C→T polymorphism determines a higher activity of the exchanger (Siffert et al

1998), I hypothesized that the 825C→T polymorphism of the GNB3 gene could influence the impact of obesity on BP through type I or II effects. To determine whether the GNB3 gene polymorphism substantially influences the impact of body size on BP in human hypertension, subjects of African ancestry were recruited. This approach was adopted as the GNB3 gene 825T allele is not associated with hypertension in large case-control studies (Larson et al 2000), and our group have previously noted a high mean body mass index (BMI) in this ethnic group (Tiago et al 2002).

METHODS

Study groups and BP measurements. In total 1300 (661 consecutive hypertensive patients and 629 controls) subjects of African ancestry were recruited utilizing selection criteria described in chapter 2 in the "methods" section. Office and ambulatory BP measurements were performed also as described in chapter 2. The estimated sample size employed to avoid false negative results was based on the reported 825T allele frequency in black subjects of African descent living in London (Dong et al 1999) and an odds ratio of 1.10 for the 825T allele being associated with hypertension.

Genotyping. Genomic DNA was extracted from whole blood as described in chapter 2. Genotyping of the 825C→T polymorphism of the GNB3 gene was undertaken using PCR-RFLP-based techniques employing the appropriate primer pairs and restriction enzymes (Siffert et al 1998). The sequence of the primers utilized were:

Sense primer: 5’ TGA CCC ACT TGC CAC CCG TGC 3’

Antisense primer: 5’ GCA GCA GCC AGG GCT GGC 3’ (Roche).

To genotype for the GNB3 gene polymorphism, PCR was performed with ~50 ng DNA, 10 x PCR buffer (Takara), 2 mM $MgCl_2$, 0.2 mM dNTP, 2.5 mM sense and antisense primers and 1unit Taq polymerase (Takara) in atotal volume of 20 µl. DNA amplification was achieved using the following PCR cycles: one 94^{o}C cycle for 5 minutes, followed by 30 cycles of: denaturing (94^{o}C for 1 minute), annealing (60^{o}C for 45seconds), and extension (72^{o}C for 1 minute), with a final extension step at 72^{o}C for 5 minutes. PCR resulted in a 268 bp product. Restriction enzyme digestion was performed by incubating10 µl of the amplicon with 0.8 U restriction enzyme (*Bsa JI*) for 3 hours at 60^{o}C. To ensure that no misgenotypig occurred, samples genotyped as TC were selected at random and subjected to a repeat PCR, restriction enzyme digestion and genotyping. 100% reproducibility was noted. Restriction enzyme products were visualized on a 3% agarose gel under UV light (Figure 9). Digestion yielded 268 bp (825T allele) and 152 and 116 bp (825C allele) fragments (Figure 9).

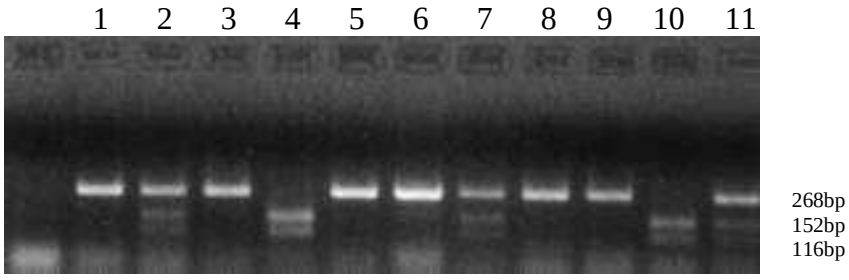

Figure 9. Typical example of an agarose gel image with electrophoretic patterns obtained when genotyping for the GNB3 gene 825C→T polymorphism. The 825TT genotype was characterized by a single band of 226 bp as seen in lanes 1, 3, 5, 6, 8 and 9. The 825CT genotype was characterized by three bands of 226, 152 and 116 bp (lanes 2, 7, 11) whilst the 825CC genotype was characterized by two bands of 152 and 116 bp (lanes 4 and 10). Lane 6 is an undigested 825C→T amplicon.

Data analysis. Hardy-Weinberg equilibrium, independent effects of either alleles or genotypes on the presence of hypertension and BP, and differences between case and control groups in clinical and demographic characteristics were determined as described in chapter II. As our group have previously provided data on and assessed three other genes in the group reported on in this study (Tiago et al 2002, Nkeh et al 2003, chapters II, IV and V) I adjusted probability values for multiple genotyping using Bonferroni's method (Bland and Altmann 1996). As the AGT gene is associated with hypertension in the group studied (see chapter II), when performing MANCOVA, AGT genotype was included as a covariate. To determine whether the GNB3 gene polymorphism influences the impact of an increased body size on BP, ANCOVA was performed in genotype-specific groups assigned to groups consisting of individuals with either a BMI >25 $kg.m^{-2}$ or ≤25 $kg.m^{-2}$. These BMI values were employed to subdivide case and control groups as BMI values >25 $kg.m^{-2}$ include both overweight and obese individuals (Morris et al 2001, WHO 1998). To determine GNB3 gene polymorphism effects on body size-BP relations, regression relations between BP and other phenotypic parameters in GNB3 genotype-specific groups were constructed. Comparisons between the slopes of these relations were determined using a Student's t test. Continuous data are expressed as mean ± SEM.

RESULTS

Demographic and clinical data. As the case and control subjects were largely the same as described in chapter 2, similar characteristics were noted (Table 7).

Genotype effects on the risk of hypertension, office BP, ambulatory BP, and body size. The genotype frequencies of the 825C→T polymorphism were in Hardy-Weinberg

Table 7. Demographic and clinical characteristics of the hypertensive and control subjects of African ancestry recruited for the guanoine triphosphate protein β3 subunit (GNB3) gene study.

	Hypertensives	Controls
n =	661	629
Age (years)	52.1±0.4	51.3±0.5
Nguni/Sotho/Venda (%)	59/39/2	68/30/2
Gender (% female)	74	70
Body mass index ($kg.m^{-2}$)	30.7±0.3*	29.2±0.3
Type II diabetes mellitus (%)	5.0	2.7
Clinic SBP/DBP (mm Hg)	168±1*/102±1*	128±1/78±1
24 hour SBP/DBP (mm Hg)	151±1/97±1	-
Day SBP/DBP (mm Hg)	155±1/102±1	-
Night SBP/DBP (mm Hg)	145±1/91±1	-
Duration of hypertension (years)	2.66±0.09*	0

SBP/DBP, systolic blood pressure, diastolic blood pressure. *$p<0.01$.

equilibrium. Neither the 825T allele, nor the 825TT genotype polymorphism were independently associated with the presence of hypertension in the total group or in the two major chiefdoms (Nguni and Sotho) (Table 8). Further, the polymorphism did not show a quantitative association with 24 hour, day, or night ABP in cases (see Figure 10 for systolic BP), office BP in cases and controls (Table 9), or with body size in either cases or controls (Table 9).

Genotype effects on the impact of an increased BMI on BP. Although GNB3 genotype failed to influence BP in either the hypertensive or the control groups (Figure 10 and Table 9), GNB3 genotype determined whether a BMI >25 $kg.m^{-2}$ produced a higher SBP in both the case and control groups (Figure 11). Indeed, in comparison to subjects with a BMI ≤25 $kg.m^{-2}$, those with a BMI >25 $kg.m^{-2}$ had a higher ambulatory SBP and office SBP ($p<0.02$) in the hypertensives, and office BP in the control group only if subjects were homozygous for the 825T allele (Figure 11). In subjects with at least one copy of the 825C allele, a BMI >25 $kg.m^{-2}$ failed to influence either ambulatory SBP (Figure 11) or office SBP ($p>0.05$) in the cases or office BP in the controls (Figure 11).

Genotype effects on BP-phenotypic relations. BMI showed a significant correlation with ambulatory SBP in the case ($r= 0.144767$, $p<0.0002$), but not office SBP in the control group. BMI was not correlated with diastolic BP in either the hypertensive or the control group. In the case group, the 825C→T gene polymorphism markedly modified the relation between BMI and ambulatory SBP. In patients with the 825TT genotype, the relationship was more pronounced, while in the 825TC + 825CC genotype group a relationship was absent (Figure 12). The β –coefficient for the BMI-ambulatory SBP relations was different between the two genotype groups (Figure 12).

Table 8. Genotype and allele frequencies of the 825C→T polymorphism of the GNB3 gene in hypertensive and control subjects of African ancestry.

	Genotype			Allele	
	TT	CT	CC	T	C
Total group					
Hypertensives	458 (69.3)	191 (28.9)	12 (1.8)	1107 (83.7)	215 (16.3)
Controls	469 (74.6)	156(24.8)	4 (0.6)	1094 (87.0)	164 (13.0)
Nguni chiefdom					
Hypertensives	272 (69.2)	116 (29.5)	5 (1.3)	660 (84.0)	126 (16.0)
Controls	325 (75.4)	103 (23.9)	3 (0.7)	753 (87.4)	109 (12.6)
Sotho chiefdom					
Hypertensives	178 (69.8)	70 (27.5)	7 (2.7)	426 (83.5)	84 (16.5)
Controls	135 (73.0)	49 (26.5)	1 (0.5)	319 (86.2)	51 (13.8)

Numbers are sample number (%). The odds ratios for the risk of hypertension for the 825T allele are 0.77, p= 0.096 (total group), 0.76, p=0.23 (Nguni chiefdom), 0.81, p=1.19 (Sotho chiefdom) and for the 825TT genotype are 0.77, p= 0.36 (total group), 0.65, p=0.10 (Nguni chiefdom), 1.05, p=0.83 (Sotho chiefdom). Probability values for genotype are for MANCOVA.

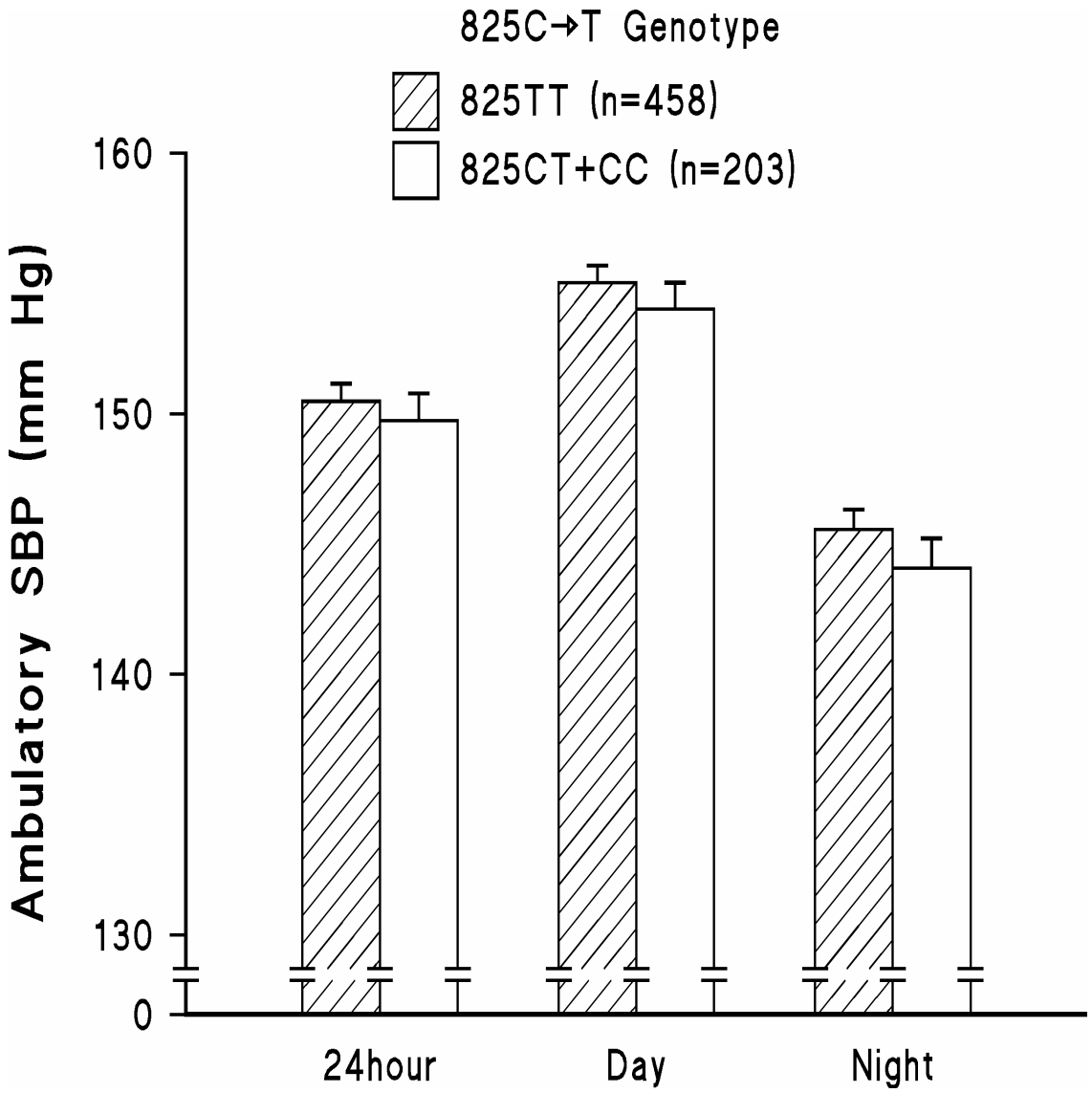

Figure 10. Effects of GNB3 gene 825C→T polymorphism on mean 24 hour ambulatory systolic blood pressure (SBP) in hypertensives. No differences were noted in BP between genotype-specific groups.

Table 9. Effect of the 825C→T polymorphism of the GNB3 gene on office blood pressures and body mass index in hypertensive and control subjects of African ancestry.

	Genotype					
	Hypertensive			Controls		
	TT	CT	CC	TT	CT	CC
Office SBP (mm Hg)	161±1	160±2	167±4	133±1	137±2	127±1
Office DBP (mm Hg)	98±1	99±1	99±3	80±1	81±1	64±1
BMI ($kg.m^{-2}$)	30.7±0.3	30.0±0.5	31.9±1.7	28.9±0.3	28.9±0.5	22.5±1.1

SBP, systolic BP; DBP, diastolic BP; BMI, body mass index. See table 8 for sample sizes. No differences were noted between genotype-specific groups.

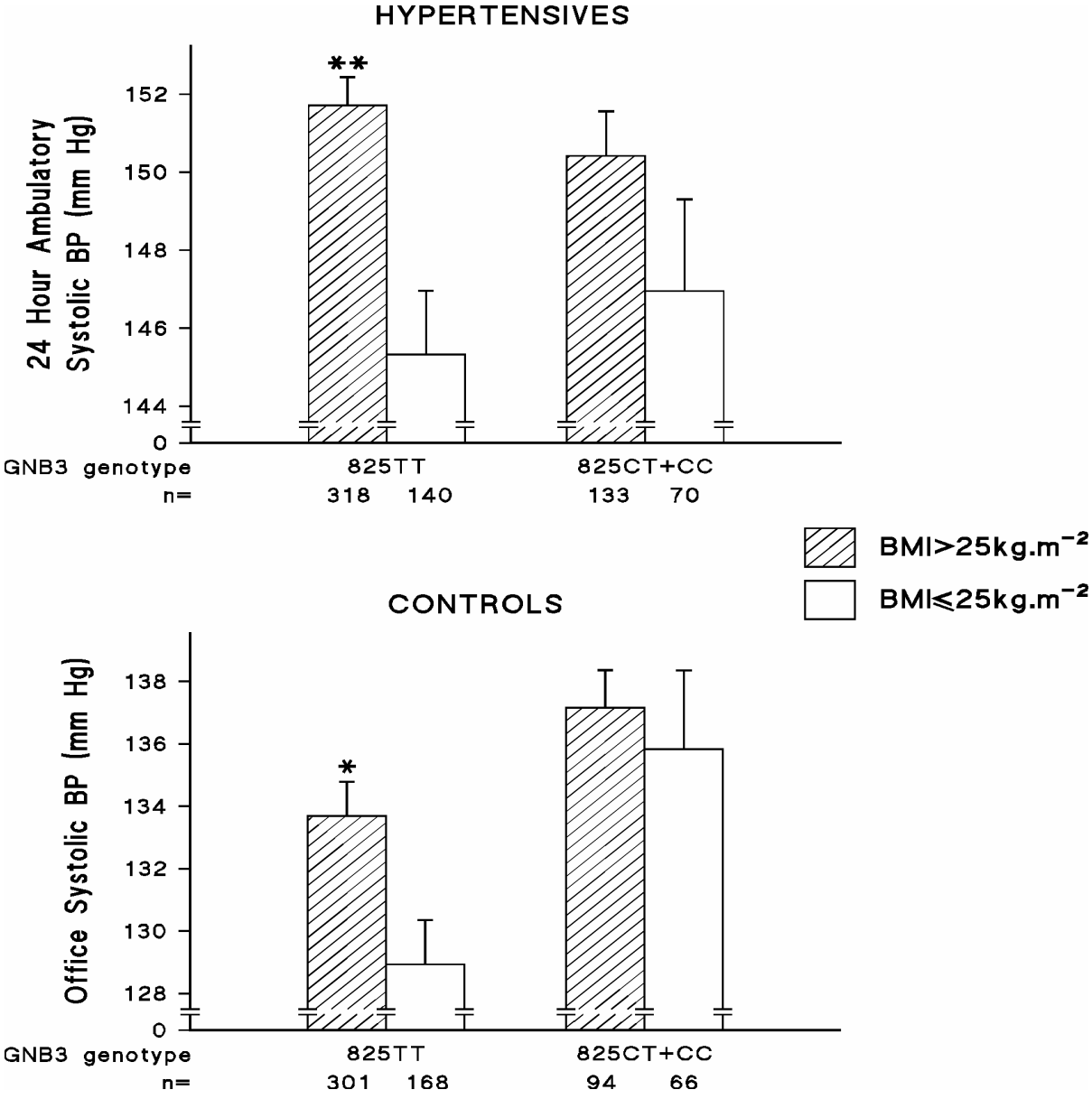

Figure 11. Effect of the 825C→T polymorphism of the GNB3 gene on the impact of a body mass index > 25 $kg.m^{-2}$ on either ambulatory systolic blood pressure (SBP) in hypertensives or office SBP in normotensive controls of African descent. * $p<0.05$, **$p<0.002$ compared to BMI ≤ 25 $kg.m^{-2}$.

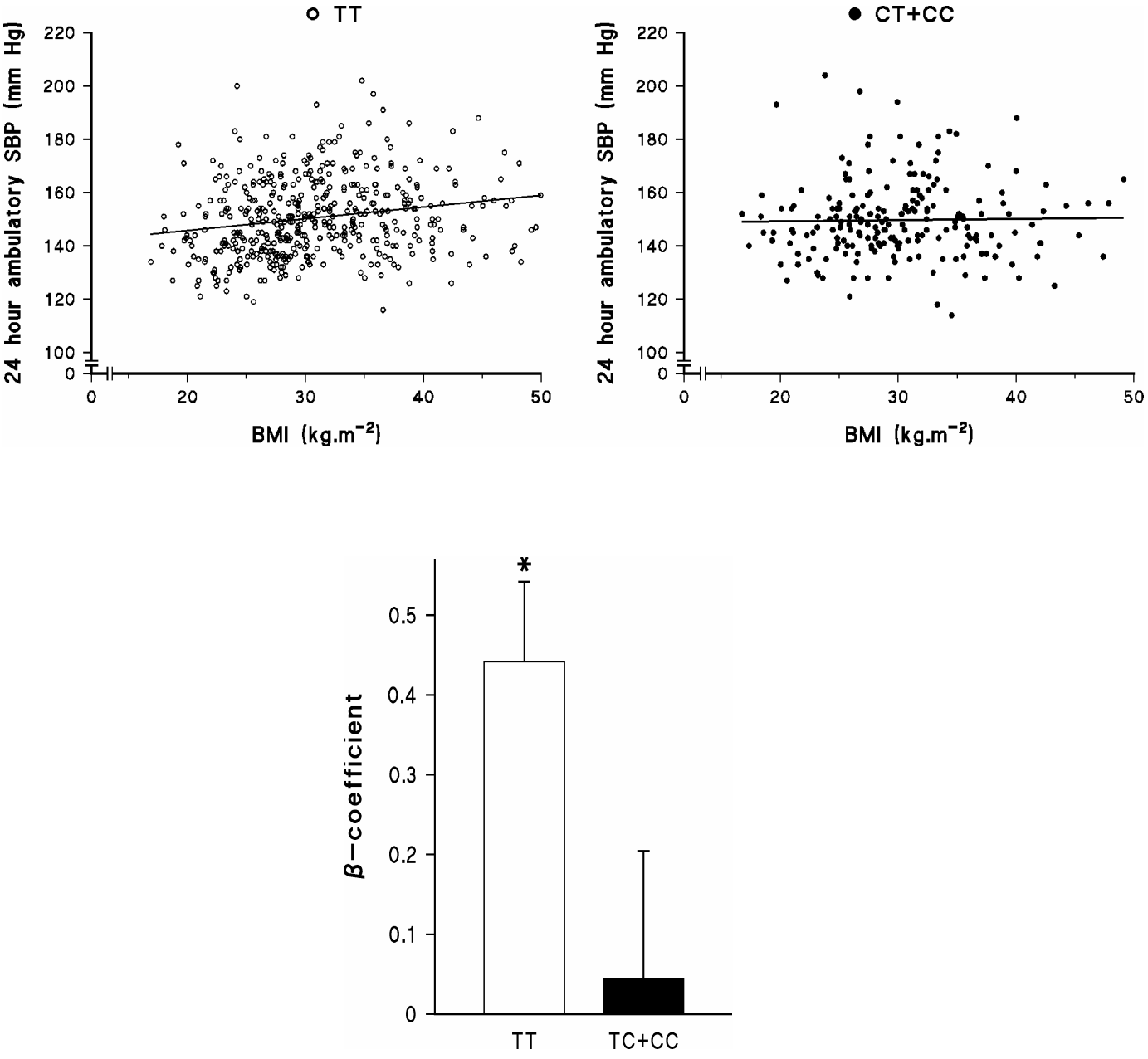

Figure 12. Effect of GNB3 gene 825C→T polymorphism on ambulatory systolic blood pressure (SBP)-body mass index (BMI) relations in hypertensives (upper panels). The β-coefficients for the individual SBP-BMI relations are provided in the lower panel. *p<0.04

DISCUSSION

The main finding of the present study is that a functional polymorphism of the GNB3 gene (Siffert et al 1998) has a marked influence on the impact of body size on BP. In both case and control groups an increased body size (BMI >25 $kg.m^{-2}$) determined a greater SBP (office SBP in controls and ambulatory SBP in cases) in subjects homozygous for the risk allele (825T), but not in those with at least one copy of the 825C allele. Moreover, the relation between body size and SBP in hypertensives was noted only in those patients homozygous for the risk allele (825T), but not in those patients with at least one copy of the 825C allele. Since the 825C→T polymorphism did not produce an independent effect on either BP in hypertensives, or the risk for hypertension when accounting for body size differences between subjects, the effect of the polymorphism reflects a moderating influence of genotype on the BMI effects on BP, rather than an effect of body size on the relationship between genotype and BP. This distinction is important as it implies that the presence of the risk genotype is insufficient to account for a BP effect alone.

The results of the present study support the notion that type I or II genetic effects can have a profound impact on the relationship between body size and BP. In this regard, both the ACE gene insertion/deletion polymorphism (Turner et al 1999) and a functional AGT gene promoter region variant (Tiago et al 2002) have previously been shown to influence the relationship between body size and BP in humans. These profound context-dependent effects of genetic polymorphisms is in keeping with the complex nature of polygenic traits and could assist in predicting the effect of body size on BP in humans.

A number of studies attempting to associate the GNB3 gene 825C→T polymorphism with risk of hypertension have produced variable results (Siffert et al 1998, Schunkert et al 1998, Benjafield et al 1998, Hegele et al 1998, Kato et al 1998, Beige et al 1999, Dong et al 1999, Larson et al 2000). Importantly, with respect to the ethnic group studied in this thesis (African ancestry), the 825C→T polymorphism has been associated with hypertension in a small sample (n=428) of subjects of Caribbean and West African origins (Dong et al 1999), but in a much larger sample of African Americans (n=904), no association with hypertension was noted (Larson et al 2000). As indicated in chapter two sample sizes inadequate to limit the risk of false positive or negative results, poor phenotypic characterization, and population admixture may limit the outcome of case-control studies (Gambaro et al 2000). With respect to these limitations, the present study fulfills many of the criteria required to be considered a relatively thorough case-control study. All of these strengths have already been highlighted in chapter two. The potential limitations of the present study design have also been discussed in chapter two.

In the present study I evaluated genetic effects on BP considered as a continuous trait, in a hypertensive group, rather than in a cross-section of the population. Reasons for the choice of study sample were two-fold. First, to improve on the statistical power of detecting genotype-phenotype interactions using ABP measurements, a group sampled with a relatively wide distribution of BPs as opposed to a group sampled where BPs cluster around the median (general population) was necessary. Second, effects of body size on BP are more relevant to hypertensives, as it is to this group that advice regarding weight reduction would be given to assist with BP control. Nevertheless, the modifying

influence of GNB3 genotype on BMI-BP relations was noted in both a hypertensive (ABP) and a normotensive control (office BP) group and hence it is reasonable to assume that the effect is a general effect rather than specific to the hypertensive group.

Recently, the molecular defect explaining the enhanced Na^+-H^+ exchange phenotype preserved in immortalized cell lines from hypertensive patients (Rosskopf et al 1995) was localized to the 825C→T polymorphism (Siffert et al 1998). Importantly, Na^+-H^+ exchanger activity is increased in obese hypertensives (Delva et al 1993, Bourikas et al 2003). Therefore, a potential explanation for the impact of the GNB3 gene variant on body size-BP relations is that obesity influences BP in-part through Na^+-H^+ exchanger activity, an effect that is determined by the GNB3 825C→T polymorphism. However, enhanced sensitivity to agonists that stimulate intracellular signaling via pertussis toxin-sensitive G proteins is tightly associated with the 825C→T polymorphism (Siffert et al 1998). Therefore, the impact of the GNB3 gene variant on body size-BP relations could also be through a modifier effect of obesity-induced increases in sympathetic activity (Mark et al 1999), on α-adrenoreceptors (Meirhaeghe et al 2001) or other receptor targets of the sympathetic nervous system that are coupled to G proteins.

If the results from the present study are confirmed in other studies and populations, then it is conceivable that the effect of the GNB3 gene could, for example, influence therapeutic choices. If the GNB3 gene 825C→T polymorphism modifies the effect of BMI on BP, then pharmacological agents targeting Na^+ excretion or the sympathetic nervous system may be particularly efficacious in obese hypertensive subjects with the deleterious genotype. Moreover, if body size effects on BP are genotype-dependent, then one would expect that weight reduction would be beneficial

with respect to BP effects in patients with the risk, but not the non-risk genotype. These hypotheses have yet to be tested.

In conclusion, the results of the present study indicate that in the absence of an independent effect on BP, a functional GNB3 gene polymorphism markedly influences the effect of body size on BP in both hypertensives and normotensives as well as the body size-BP relationship, at least in hypertensives. These data support the notion that genetic factors, through type I or II effects, modify the relation between body size and BP, an effect previously purported to significantly influence BP in human hypertension (Mark et al 1999).

Chapter IV

T594M Polymorphism of the Epithelial Sodium Channel β-Subunit Gene and Hypertension in Individuals of African Ancestry in South Africa.

ABSTRACT

Background. The T594M polymorphism of the β-subunit of the sodium epithelial channel (ENaC) β-subunit gene may contribute to hypertension in subjects of African origin.

Methods. A case-control study was performed to assess the role of the ENaC β-subunit gene polymorphism as an independent risk factor for hypertension in subjects of African ancestry. Moreover, the effect of the ENaC β-subunit gene polymorphism on ambulatory blood pressure (BP) in hypertensives, and office BP in hypertensives and controls was assessed. 519 hypertensive patients with 24 hour ambulatory BP (ABP) values determined whilst off medication, and 514 normotensive South Africans of African descent were genotyped for the T594M polymorphism of the ENaC β-subunit gene.

Results. 22 (4.2%) hypertensive participants compared with 23 (4.5%) normotensive participants possessed the T594M polymorphism (odds ratio = 1.06, confidence interval = 0.58-1.92, not significant). A similar genotype frequency distribution was noted in subjects representing the two predominant chiefdoms (Nguni and Sotho) in both case and control groups. Furthermore, no differences in frequency distribution of the T594M polymorphism were noted with respect to either body mass index or gender. There were no differences in clinic or ambulatory mean, day or night BP between hypertensive patients with or without the variant. Similarly, no differences were noted in clinic BP between control subjects with or without the variant. In addition, other phenotypic parameters (including age, duration and severity of hypertension) were similar between hypertensive patients with or without the variant.

Conclusion. These results do not support an important role for the T594M polymorphism of the ENaC β-subunit gene in contributing to either the development or severity of hypertension in subjects of African descent.

INTRODUCTION

The amiloride-sensitive epithelial sodium channel (ENaC) is well established as being important in the control of sodium balance and thus blood pressure (BP) (Rossier 1997, Fuller 1996). Discovery that Liddle's syndrome, a rare monogenic form of hypertension associated with pseudoaldosteronism, is mediated by mutations of the β-subunit of the ENaC gene (Shimkert et al 1994), led to the recent interest in the potential role of genetic mutations of the ENaC. Based on these findings further work was conducted to evaluate whether additional, more frequent polymorphisms in the β-subunit of the ENaC gene, could contribute to more common and hence epidemiologically more relevant forms of hypertension. A recently described polymorphism of the β-subunit of the ENaC gene, which is in last exon of the β-subunit gene, results in a change of threonine to methionine at amino acid 594 (T594M) has been shown to alter the electrophysiological properties of and second messenger effects in B-lymphocytes (Su et al 1996, Cui et al 1997), but not the electrophysiological properties of *Xenopus* oocytes (Persu et al 1998)(the experimental model utilized to assess the functional role of genetic mutations in Liddle's syndrome). With the exception of data obtained in subjects of African ancestry (Baker et al 1998), studies designed to assess the role of the T594M polymorphism in hypertension have been unrevealing (Persu et al 1998, Matsubara et al 2000). The T594M polymorphism occurs with a higher frequency in subjects of African ancestry (Su et al, 1996, Baker et al 1998) than in those of Caucasian (Su et al 1996, Persu et al 1998) or Japanese ancestry (Matsubara et al 2000). However, even in subjects of African ancestry the prevalence of the polymorphism is very low. Nevertheless, in

subjects of African ancestry the variant is associated with both the presence of hypertension and a low renin status in subjects living in London (Baker et al 1998). However, these data are inconsistent with data reported on in African-Americans where no relationship between the variant and the presence of hypertension was demonstrated (Su et al 1996). In neither of these case-control studies (Su et al 1996, Baker et al 1998) were the sample sizes employed particularly large, a criticism of many genetic epidemiology studies (Gambaro et al 2000). The aim of our study was therefore to assess the role of the T594M polymorphism of the β-subunit of the ENaC gene in hypertension in a larger group of subjects of African ancestry. As inadequate phenotyping of subjects recruited in case-control studies is also considered a reason for discrepancies between studies (Gambaro et al 2000) we confirmed the presence of hypertension in the patient group through ambulatory BP monitoring performed with patients off medical therapy.

METHODS

Study groups, BP measurements and hypertension grading. 1031 (519 consecutive hypertensive patients and 514 controls) subjects of African ancestry were recruited utilizing selection criteria described in chapter II in the methods section. Office and ambulatory BP measurements were performed as described in chapter II. A smaller sample size was utilized as compared to that described in chapters II and III, as this study was terminated prior to that described in chapters II and III at which stage less subjects had been recruited. The estimated sample size required for this study to provide 80% power with an α value of 0.05 (after adjusting for multiple genotyping performed in the

group) was based on an odds ratio calculated from data provided by Baker et al (1998) and the allele frequency in the first 100 cases and controls genotyped in our study.

Genotyping. Genomic DNA was extracted from whole blood as described in chapter II. The single nucleotide substitution of T for C in the T594M polymorphism was identified using a standard PCR-RFLP-based technique employing primer pairs and the *Nla III* restriction enzyme as previously described (Persu et al 1998). The sequence of the primers utilized was:

Sense: 5' ACC GTG GCC GAG CTG GTG GAG 3'

Antisense: 5' CAG TCT TGG CTG TCT AGT GAG 3'

To genotype for the GNB3 gene polymorphism, PCR was performed with ~50 ng DNA, 10 x PCR buffer (Takara), 2 mM $MgCl_2$, 0.2 mM dNTP, 2.5 mM forward and reverse primers and 2.5 unit Taq polymerase (Takara) in a total volume of 20 μl. DNA amplication was performed using the following PCR cycles: one 94^{o}C cycle for 5 minutes, followed by 30 cycles of denaturing (94^{o}C for 30 seconds), annealing (65^{o}C for 90 seconds), and extension (72^{o}C for 30 seconds) with a final extension step at 72^{o}C for 5 minutes. PCR yielded a 343 bp product. Restriction enzyme digestion was performed by incubating 10 μl of the amplicon with 3.3 U of the *Nla III* restriction endonuclease (Biolabs) for 2 hours at 37^{o}C. Restriction enzyme fragments were visualized on a 2% agarose gel under UV light (Figure 13). In the absence of the restriction site PCR-RFLP yielded a 226 bp product (594CC genotype) and in both the presence and absence of the restriction site 226 and 117+109 bp products were produced (594CT genotype). To avoid misgenotyping, all samples genotyped as 594CC were subjected to a repeat PCR and restriction digestion and genotyped a second time. 100% reproducibility was noted.

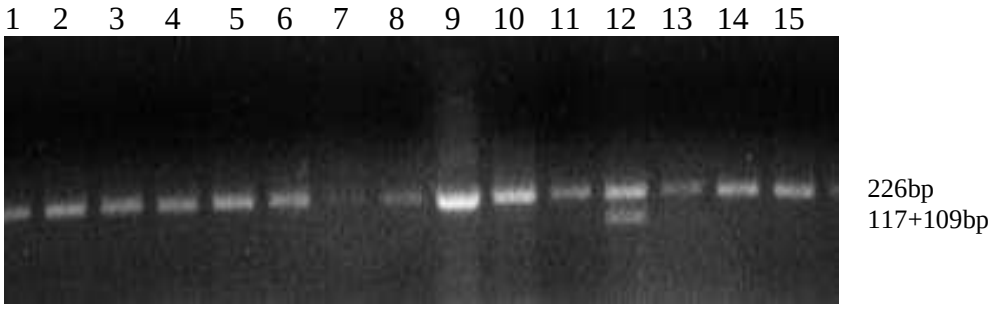

Figure 13. Typical example of an agarose gel image with electrophoretic patterns obtained when genotyping for the T594M polymorphism of the ENaC β-subunit gene. Lanes 1-8, 10, 11, 13-15 are samples lacking the restriction site (594CC genotype) while lane 12 is a sample from a subject with a heterozygote genotype (594CT genotype) and lane 9 is an undigested amplicon.

Data analysis. Hardy-Weinberg equilibrium, independent effects of either alleles or genotypes on the presence of hypertension and BP and differences in clinical and demographic characteristics between case and control groups were determined as described in chapter II. The effects of the variant on body size were determined by assessing the allele and genotype frequencies in overweight/obese individuals as defined by a BMI>25 $kg.m^{-2}$ (Morris et al 2001, WHO 1998). As our group has previously described data on other gene variants in the group reported on in this study (Tiago et al, 2002, Chapters II and III), I adjusted probablility values for multiple genotyping using Bonferroni's method (Bland and Altmann 1996). Continuous data are expressed as mean±SEM.

RESULTS

Demographic and clinical data. As the case and control subjects were largely the same as described in chapter II, similar characteristics were noted (Table 10). No differences were noted in demographic and clinical characteristics in genotype-specific groups (Table 11).

Table 10: Demographic and clinical characteristics of the hypertensive and control subjects of African ancestry recruited for the epithelial sodium channel (ENaC) β-subunit gene study.

	Hypertensives	Controls
n =	519	514
Age (years)	52.1±0.5	51.7±0.4
Nguni/Sotho/Venda (%)	337/177/5(65/34/1)	344/160/10(67/31/2)
Gender (female/male) (% female)	386/133(74)	359/155(70)
Body mass index ($kg.m^{-2}$)	30.6±0.3*	28.3±0.3
Type 2 diabetes mellitus (%)	5.0	2.0
Office SBP/DBP (mm Hg)	168±1/102±1**	127±1/77±1
24 hour SBP/DBP (mm Hg)	151±1/ 96±1	-
Day SBP/DBP (mm Hg)	155±1/102±1	-
Night SBP/DBP (mm Hg)	145±1/90±1	-
Duration of hypertension (years)	2.5±0.08	0

SBP/DBP, systolic blood pressure, diastolic blood pressure. *$p<0.01$. **$p<0.0001$.

Table 11. Demographic and clinical data for subjects grouped according to ENaC β-subunit genotype in hypertensive and control groups

	Hypertensives		Controls	
Genotype	CC	CT	CC	CT
n =	497	22	491	23
Age (years)	52.3±0.5	48.5±2.0	51.9±0.5	49.4±2.0
Gender (female/male)	368/129	18/4	344/147	15/8
(% female)	(74)	(82)	(70)	(65)
Body mass index ($kg.m^{-2}$)	30.6±0.3	31.7±1.3	28.4±0.3	26.6±1.4
Duration of hypertension (years)	2.7±0.2	2.2±0.8	-	-
Newly diagnosed (new/previous)	218/279	11/11	-	-
(% new)	(44)	(50)		
Hypertension grades I/II/III	183/220/94	10/9/3	-	-
(%)	(37/44/19)	(45/41/14)		

T594M polymorphism and hypertension. The genotype and allele distributions for the T594M polymorphism between cases and controls are shown in Table 12. The T594M polymorphism was not associated with the presence of hypertension in either the whole group or in the two predominant chiefdoms represented in the sample (Table 12, Figure 14). Furthermore the T594M polymorphism was not associated with the presence of hypertension in either females or males (Table 12, Figure 14). The gender-specific genotype and allele frequency distributions for the T594M polymorphism in both cases and controls are shown in Table 12. The T594M polymorphism was not associated with gender in the total group, the cases or the controls.

T594M polymorphism and body size. No associations between the T594M polymorphism and overweight/obesity (BMI>25 $kg.m^{-2}$) were noted in the total group, the cases or the controls (Table 13).

T594M polymorphism and blood pressure. There were no differences between genotype groups in clinic BP in either cases or controls (Table 14). Moreover, there was no relationship between genotype and ambulatory BP in the hypertensive group (Figure 15).

Table 12. Association between a T594M polymorphism of the ENaC β-subunit gene and hypertension in subjects of African ancestry.

Group	Genotype		Allele	
		Total group		
Controls	CC=491 (95.5)	CT=23 (4.5)	C=1005 (97.8)	T=23 (2.2)
Hypertensives	CC=497 (95.7)	CT=22 (4.2)	C=1016 (97.9)	T=22 (2.1)
		Nguni chiefdom		
Controls	CC=328 (95.3)	CT=16 (4.7)	C=672 (97.7)	T=16 (2.3)
Hypertensives	CC=323 (95.8)	CT=14 (4.2)	C=660 (97.9)	T=14 (2.1)
		Sotho chiefdom		
Controls	CC=153 (95.6)	CT=7 (4.4)	C=313 (97.8)	T=7 (2.2)
Hypertensives	CC=169 (95.5)	CT=8 (4.5)	C=346 (97.7)	T=8 (2.3)
		Females		
Controls	CC=344 (95.8)	CT=15 (4.2)	C=703 (97.9)	T=15 (2.1)
Hypertensives	CC=368 (95.3)	CT=18 (4.7)	C=754 (97.7)	T=18 (2.3)
		Males		
Controls	CC=147 (94.8)	CT=8 (5.2)	C=302 (97.4)	T=8 (2.6)
Hypertensives	CC=129 (97.0)	CT=4 (3.0)	C=262 (98.5)	T=4 (1.5)

Numbers are sample numbers (%); Odds ratios for genotype being associated with the risk of hypertension are indicated in figure 14.

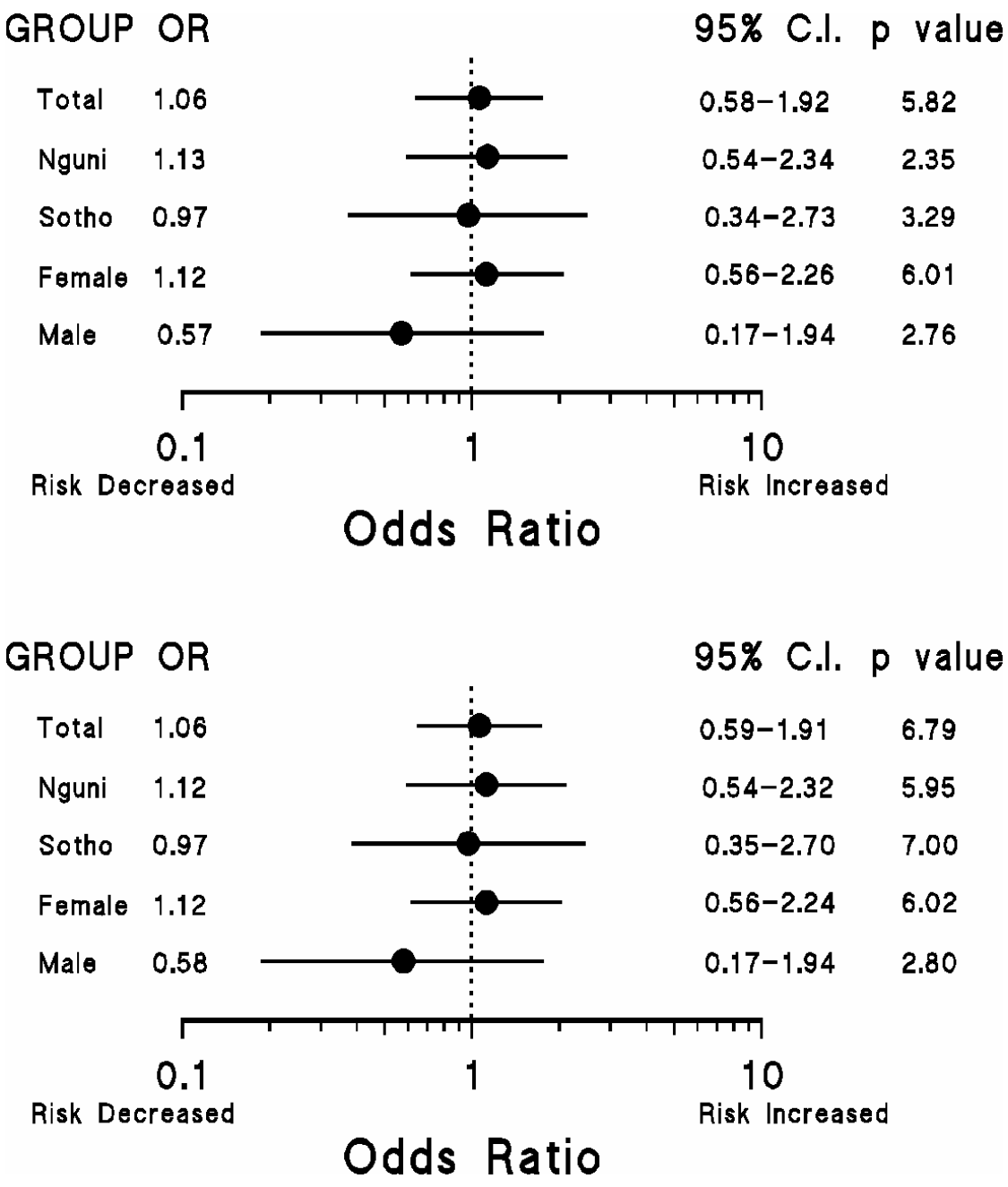

Figure 14. Impact of the ENaC β-subunit gene T594M polymorphism on the risk for hypertension in subjects of African descent. Allele-specific effects are indicated in the upper panel and genotype-specific effects in the lower panel.

Table 13. Association between a T594M polymorphism of the ENaC β-subunit gene and body size in hypertensive and control subjects of African origin

Group	Genotype	Allele
Total		
Overweight/obese	CC 688(95.4) CT 33(4.6)	C 1409(97.7) T 33(2.3)
Normal weight	CC 300(96.2) CT 12(3.8)	C 612(98.1) T 12(1.9)
Hypertensives		
Overweight/obese	CC 389(95.3) CT 19(4.7)	C 797(97.7) T 19(2.3)
Normal weight	CC 108(97.3) CT 3(2.7)	C 219(98.6) T 3(1.4)
Controls		
Overweight/obese	CC 299(95.5) CT 14(4.5)	C 612(97.8) T 14(2.2)
Normal	CC 192(95.5) CT 9(4.5)	C 393(97.8) T 9 (2.2)

Overwight/obese, BMI>25 $kg.m^{-2}$; Normal weight, BMI≤25 $kg.m^{-2}$

Table 14. Clinic blood pressures in hypertensive and control subjects of African ancestry grouped according to ENaC β-subunit genotype**.**

	SBP (mm Hg)		DBP (mm Hg)	
	Genotype			
	CC	CT	CC	CT
Hypertensives (n=519)	168±0.7	167±0.9	102±0.3	101±0.3
Controls (n=514)	128±0.7	127±3.0	78±0.3	79±2.0

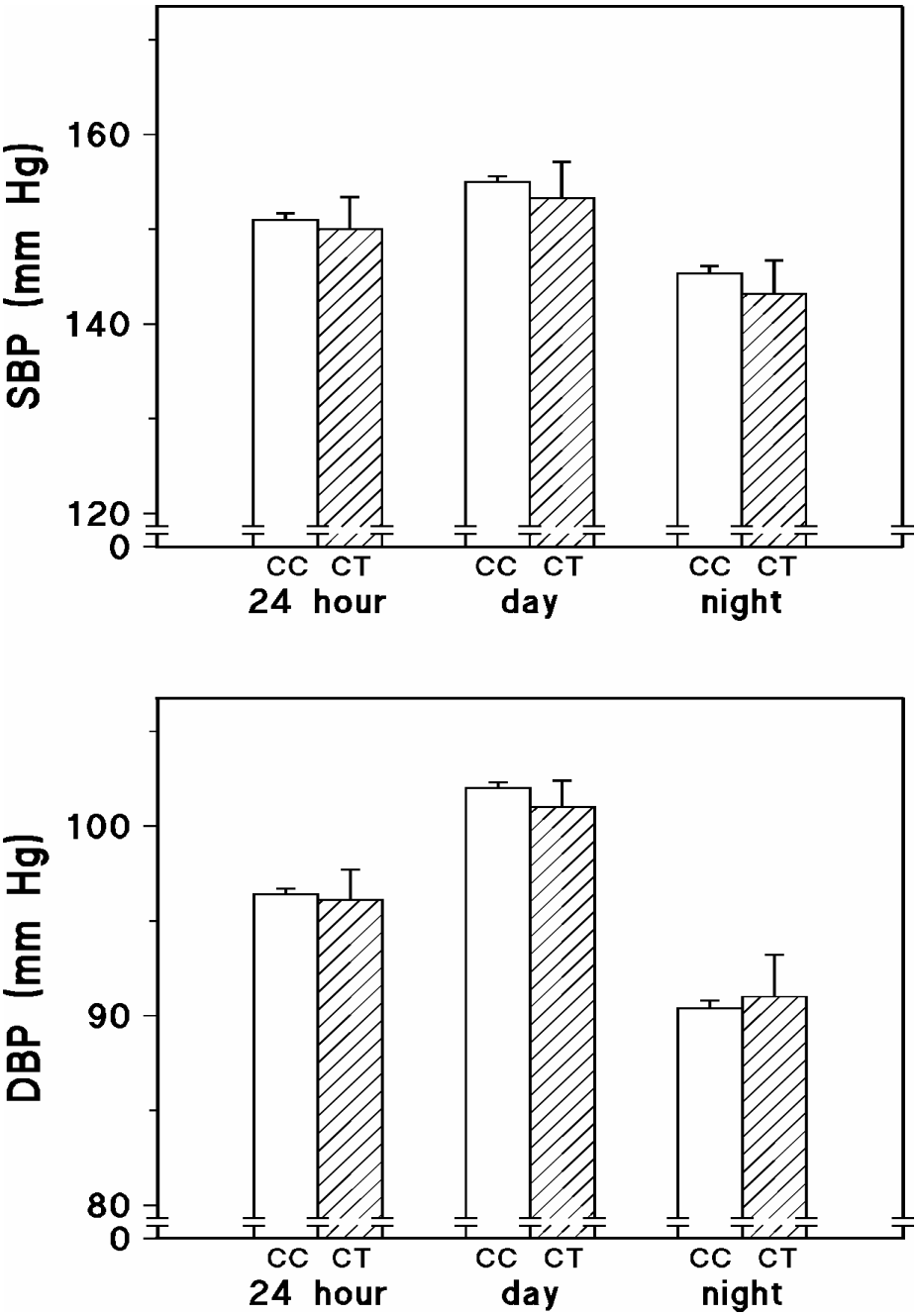

Figure 15. Ambulatory blood pressure measurements in hypertensive subjects grouped according to ENaC β-subunit genotype. SBP, systolic blood pressure; DBP, diastolic blood pressure. No differences were noted between the groups. Sample sizes for genotype groups as given in Table 11.

DISCUSSION

The main finding of the present study is that in a sample of subjects from a population of African origin, the T594M polymorphism of the β-subunit of the ENaC gene is neither associated with the presence of hypertension, nor with BP in hypertensive or normotensive subjects.

A number of strengths of the present study design have been underscored in chapter II. These include the fact that ambulatory BP monitoring was used to assess the hypertensive phenotype. Also, as indicated in chapter II, the issues of limited sample size, poor phenotypic characterisation and population admixture that are thought to contribute to producing spurious results (Gambaro et al 2000) have been addressed. In this regard, in the present study the relatively large sample size employed (1031 subjects evenly distributed between cases and controls) produced an equal distribution of the T594M polymorphism (between case and control groups (4.2 versus 4.5%). In addition, the lack of association between genotype and hypertension was clearly demonstrated in cohorts of subjects from specific chiefdoms, namely the Nguni and Sotho chiefdoms.

The frequency of the T594M polymorphism of the ENaC β-subunit gene in both the hypertensive and the normotensive groups reported on in the present study is similar to that reported on in hypertensive black subjects living in London (Baker et al 1998) as well as hypertensive and normotensive African-Americans (Su et al 996). In contrast, the frequency of the T594M polymorphism is lower in control subjects living in London (Baker et al 1998). Thus, the current evidence linking this polymorphism to the risk of

hypertension in subjects of African ancestry is based on an apparent lower prevalence of the T594M polymorphism in normotensive black subjects living in London.

In accordance with a previous report that the majority (90%) of the subjects with the T594M polymorphism were women (Baker et al 1998), in the present study 73% of subjects with the T594M polymorphism were women. However, no differences in the frequencies of the variant were noted between females and males in the total group, in the hypertensives or in the controls. The greater prevalence of females noted in subjects with the T594M gene polymorphism is likely to be based upon the greater percentage of females in both the case and control groups in our study. The gender distribution of the variants for the group of African descent is not reported in the study conducted in London (Baker et al 1998).

Recently, a genetic variant in the promoter region of the ENaC gamma subunit gene was shown to be associated with an increased body mass index in patients with hypertension; but not with the presence of hypertension (Morris et al 2001). With respect to the T594M polymorphism of the ENaC β-subunit gene, the present study shows no relationship between genetic variation and overweight/obesity in either cases or controls.

It has been argued that the outcome of case-control studies could be further improved on if subgroups characterized by specific phenotypes are selected (Gavras et al 1999). In this regard, the T594M polymorphism of the ENaC β-subunit gene is associated with lower plasma renin activity in black hypertensives in London (Baker et al 1998) and hence could predispose to low renin hypertension in subjects of African ancestry. However, we have previously shown that this variant does not account for a substantial proportion of variability of plasma renin activity, or renin-to-aldosterone ratios in South

Africans of African ancestry with typical low mean values for plasma renin activity (Tiago et al 2001).

In summary, the present results do not support a significant role of the T594M polymorphism of the ENaC β-subunit gene as an independent risk factor for hypertension or BP in subjects of African ancestry. However, these data do not preclude a minor role for the T594M polymorphism in contributing to essential hypertension in subjects of African ancestry.

Chapter V

Association Between an Atrial Natriuretic Peptide Gene Polymorphism and Normal Blood Pressure in Subjects of African Ancestry.

ABSTRACT

Background. The roles of the atrial natriuretic peptide (ANP) gene in hypertensive groups of African ancestry are unclear. The aim of the present study was to assess the relationship between ANP gene polymorphisms and hypertension in Black South Africans.

Methods. 289 patients diagnosed as having essential hypertension according to 24 hour ambulatory BP measurements (mean daytime diastolic BP >90 mm Hg) whilst off medication, and 278 normotensive control subjects of a similar African ancestry, were genotyped for polymorphic markers in intron 2 (which is in complete linkage disequilibrium with a potentially functional exon 1 polymorphism) and exon 3 (which leads to the extension of ANP by two additional arginines) of the ANP gene.

Results. No relationship between the exon 3 polymorphism and either the presence (odds ratio = 1.075) or the severity (24-hour BP) of hypertension was noted. The intron 2 polymorphism occurred at a low frequency in the control group (frequency of subjects heterozygous for the variant = 6.1%), but was almost absent in the hypertensive group (frequency of heterozygotes = 1.7%). Consequently, a relationship between a normal BP and the intron 2 polymorphism was noted (odds ratio = 0.28, confidence interval=0.10-0.76, $p<0.01$, <1% chance of false positive results).

Conclusions. The results of the present study suggest that the ANP locus contributes to BP in subjects of African ancestry.

INTRODUCTION

The diuretic, natriuretic and vasodilator effects of atrial natriuretic peptide (ANP) are well established (Lang et al 1985, Hollister et al 1991) and consequently there is considerable interest in the role of ANP in the development of hypertension. Previous studies have indicated that a reduced release of and responsiveness to plasma ANP may mediate salt-sensitive hypertension in humans (Agaike et al 1994, Ferrari et al 1990, Niimura 1991, Allemann and Weidmann 1995). Importantly, genetically engineered mice with a decreased production of ANP develop salt-sensitive hypertension (John et al 1995).

Recent data indicate a potential relationship between a polymorphic marker of the ANP gene in intron 2 and the presence of hypertension in a typical salt-sensitive hypertensive group of African-Americans (Rutledge et al 1995). The ANP gene intron 2 polymorphism has been shown to be in complete linkage disequilibrium with an exon 1 polymorphism (Kato et al 2000) that alters the amino acid sequence of the preprohormone of ANP (Seidman et al 1984) and consequently has potential functional relevance. Moreover, an ANP gene exon 3 polymorphism, which leads to an extension of ANP by two additional arginine residues (Ramasawmy et al 1992) has also been shown to be associated with microalbuminuria, an index of target organ damage, in hypertensives (Nannipieri et al 2001). However, other investigators have failed to show relations between ANP intron 2 genotypes and both salt-sensitivity in Caucasians (Schorr et al 1997) as well as hypertension in Chinese (Chung et al 1999). As the question of whether the ANP gene is a candidate for essential hypertension in subjects of African

ancestry is unclear, I assessed the relationship between ANP gene polymorphisms and essential hypertension in Black South Africans.

METHODS

Study groups and phenotypic measurements**.** 567 (289 consecutive hypertensive patients and 278 controls) subjects of African ancestry were recruited utilizing selection criteria described in chapter II in the "methods" section. Office and ambulatory BP measurements were performed as described in chapter 2. A smaller sample size was utilized as compared to that described in previous chapters as this study was the initial study that I conducted. The estimated sample size required for this study to provide 80% power with an α value of 0.05 (after adjusting for multiple genotyping performed in the group) was based on an odds ratio calculated from data provided by Rutledge et al (1995) and the allele frequency in the first 100 cases and controls that I genotyped.

Genotyping. Genomic DNA was extracted from whole blood using techniques described in chapter 2. Genotyping for the 1766T→C polymorphism of exon 3 of the ANP gene was accomplished using a PCR-RFLP-based technique employing oligonucleotide primers and a *Sca*I restriction endonuclease as previously described (Ramasawmy et al 1992, Masharani et al 1988). The sequences of the sense primer utilized were:

Sense primer: 5' GGC ACA CTC ATA CAT GAA GCT TGA CTT TT 3'

Antisense primer: 5' GCA GTC TGT CCC TAG GGC CCA 3'

To genotype for the exon 3 polymorphism of the ANP gene, PCR was performed with ~30 ng DNA, 10 x PCR buffer (Takara), 10 mM $MgCl_2$, 0.1 mM dNTP, 2.5 mM forward and reverse primers and 5 units Taq polymerase (Takara). DNA amplification was performed using the following PCR cycles: one 94^{o}C cycle for 2minutes, followed by 30cycles of denaturing (94^{o}C for 1minute), annealing (63^{o}C for 1minute), and extension (73.^{o}C for 45seconds) with a final extension step at 72^{o}C for 5minutes. PCR yielded a 133 bp product. Restriction enzyme digestion was performed by incubating 8 µl of the amplicon with 3 U *Sca*I restriction endonuclease overnight at 37^{o}C. Restriction enzyme digestion yielded 77 and 56 bp fragments in the presence of the restriction site (1766TT genotype). PCR-RFLP products were visualised using polyacrylamide gel electrophoresis (Figure 16).

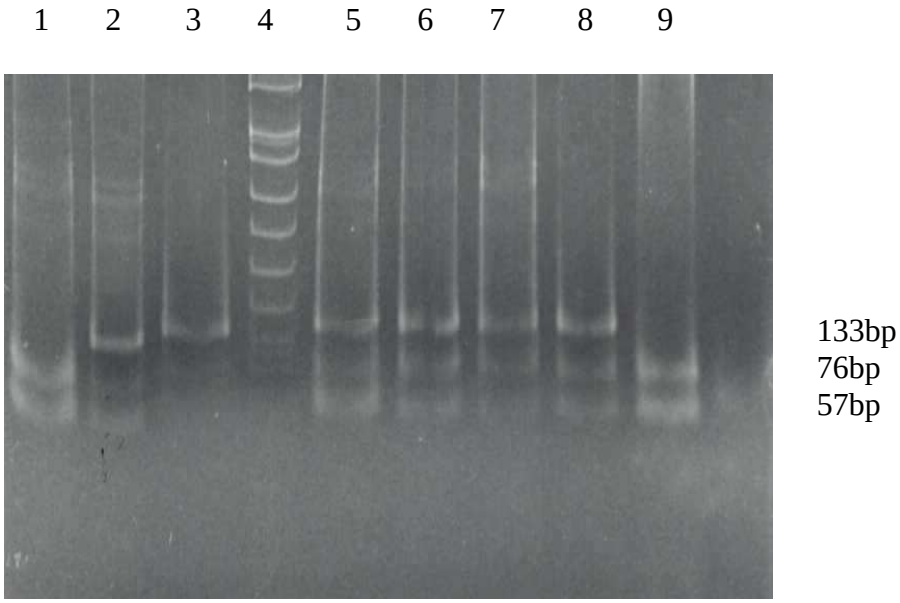

Figure 16. Typical example of a polyacrylamide gel image with electrophoretic patterns obtained when genotyping for the ANP gene *Sca*I polymorphism. Lanes 1 and 9 show samples from subjects positive for the presence of the restriction site, genotyped as 1766TT. Lane 3 depicts a sample from a subject homozygous for the absence of the *Sca*I restriction site (1766CC genotype). Samples from subjects heterozygous for the *Sca*I restriction site are in lanes 2, 5, 6, 7 and 8 (1766TC genotype). A molecular weight marker is in lane 4.

Genotyping for the 1364C→A polymorphism of intron 2 of the ANP gene was also achieved using PCR-RFLP-based techniques. A 1620 bp fragment from intron 2 of the ANP gene was amplified using the following oligonucleotide primers modified from Ramasawmy et al (1993):

sense: 5’ GGA AGT CAG CCC AGC CCA GAG AGA T 3’

antisense: 5’ GCA GTC TGT CCC TAG GCC CA 3’

To genotype for the intron 2 polymorphism of the ANP gene, PCR was performed with ~60 ng DNA, 10 x PCR buffer (Takara), 10 mM $MgCl_2$, 0.1 mM dNTP, 1.75 mM forward and reverse primers and 5 units Taq polymerase (Takara) in a total volume of 20 µl. DNA amplication was conducted using the following PCR cycles: one 95^{o}C cycle for 2 minutes, followed by 30 cycles of denaturing (95^{o}C for 1minute), annealing (65^{o}C for 1minute), and extension (74^{o}C for 1minute) with a final extension step at 72^{o}C for 5 minutes. PCR yielded a 1620 bp product. Restriction enzyme digestion was performed by incubating 8 µl of the amplicon with 5 U of *Sma*I restriction endonuclease overnight at 27^{o}C. Restriction enzyme digestion yielded 891 and 729 bp fragments in subjects with the 1364A allele. PCR-RFLP products were visualized on a 2% agarose gel under UV light (Figure 17). To avoid misgenotyping through failure of restriction enzyme digestion, a known heterozygous sample was included in each PCR digestion procedure and gel, and all samples that were genotyped as homozygous for the undigested product were re-analysed.

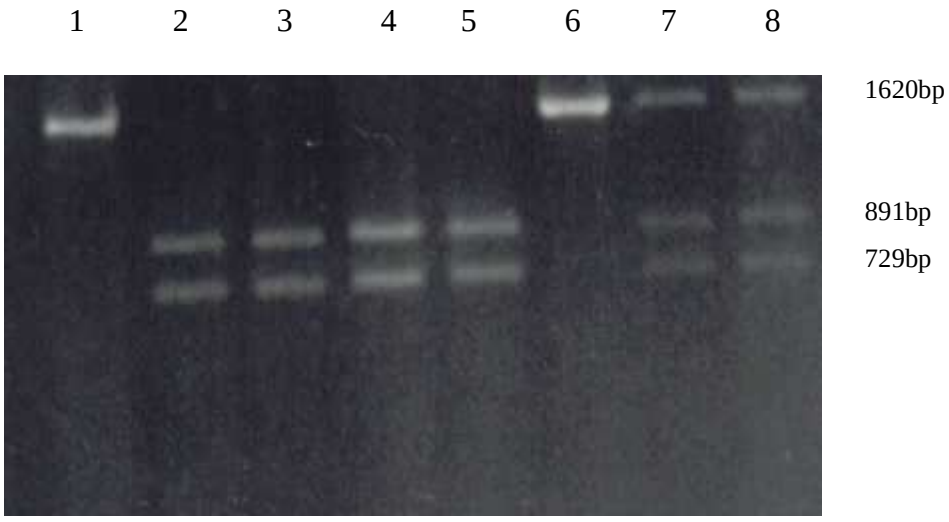

Figure 17. Typical example of an agarose gel with electrophoretic patterns obtained when genotyping for the ANP gene *Sma*I polymorphism. Samples from subjects with the 1364AA genotype are in lanes 2-to-5 while samples from subjects with the 1364CC genotype are in lanes 1 and 6. Samples from heterozygous subjects are shown in lanes 7 and 8.

Data analysis**.** Hardy-Weinberg equilibrium, independent effects of either alleles or genotypes on the presence of hypertension and BP and differences in clinical and demographic characteristics between case and control groups were determined as described in chapter II. A Bonferroni's correction was not applied in the present study as this was the first study done in the sample described in this thesis. Continuous data are expressed as mean±SEM.

RESULTS

Patient demographics and clinical characteristics**.** As the case and control subjects were largely the same as described in chapter II, similar characteristics were noted (Table 16). Importantly, however, unlike in other studies where the only differences between the groups were in BMI and BP, in the present study we also noted differences in gender distribution and age as well (Table 16). This occurred as the initial recruitment process for all of the studies discussed in the present thesis resulted in gender and age inequalities that were only rectified once further recruitment occurred.

Impact of ANP genotype on the risk for hypertension**.** Both case and control groups were in Hardy-Weinberg equilibrium for the gene polymorphisms examined. No difference in either genotype or allele frequencies for the exon 3 polymorphism was noted between hypertensive and control subjects (Table 16, Figure 18). In contrast, despite the low frequency of the ANP gene intron 2 polymorphism in both the case and control groups, a far lower frequency was noted in the hypertensive group (Table 16) and

Table 15. Demographic and clinical characteristics of hypertensive and control subjects of African ancestry recruited for the atrial natriuretic peptide (ANP) gene study.

	Hypertensives	Normotensives
N	289	278
Age (years)	52.8±0.6*	49.1±0.6
Gender (% males)	27*	45
Body mass index ($kg.m^{-2}$)	30.9±0.4*	28.6±0.5
Nguni/Sotho/Venda *n* (%)	170/114/5 (59/39/2)	181/89/8 (65/32/3)
Duration of hypertension (years)	3.13±0.28**	0
Clinic BP (SBP/DBP) (mm Hg)	169±1**/102±1**	126±1/78±1
ABP (SBP/DBP)		
Mean 24 hour ABP (mm Hg)	151±1/97±1	-
Mean day ABP (mm Hg)	155±1/102±1	-
Mean night ABP (mm Hg)	146±1/91±1	-

ABP, ambulatory BP; SBP, systolic blood pressure; DBP, diastolic BP.*$p<0.01$, **$p<0.001$ versus the control group.

Table 16. Genotype and allele frequencies of polymorphisms of the ANP gene in South Africans of African origins.

	Genotype			Allele	
	ANP gene 1766T→C polymorphism in exon 3				
	TT	TC	CC	T	C
Hypertensives	87(30.1)	159(55.0)	43(14.9)	333(57.6)	245(42.4)
Controls	85(30.6)	141(50.7)	52(18.7)	311(55.9)	245(44.1)
	ANP gene 1364C→A polymorphism in intron 2				
	CC	CA	AA	C	A
Hypertensives	284(98.3)	5(1.7)	0(0)	573(99.1)	5(0.9)
Controls	261(93.9)	17(6.1)	0(0)	539(96.9)	17(3.1)

Numbers are sample numbers (%). Odds ratios and the risk of developing hypertension are shown in figure 18.

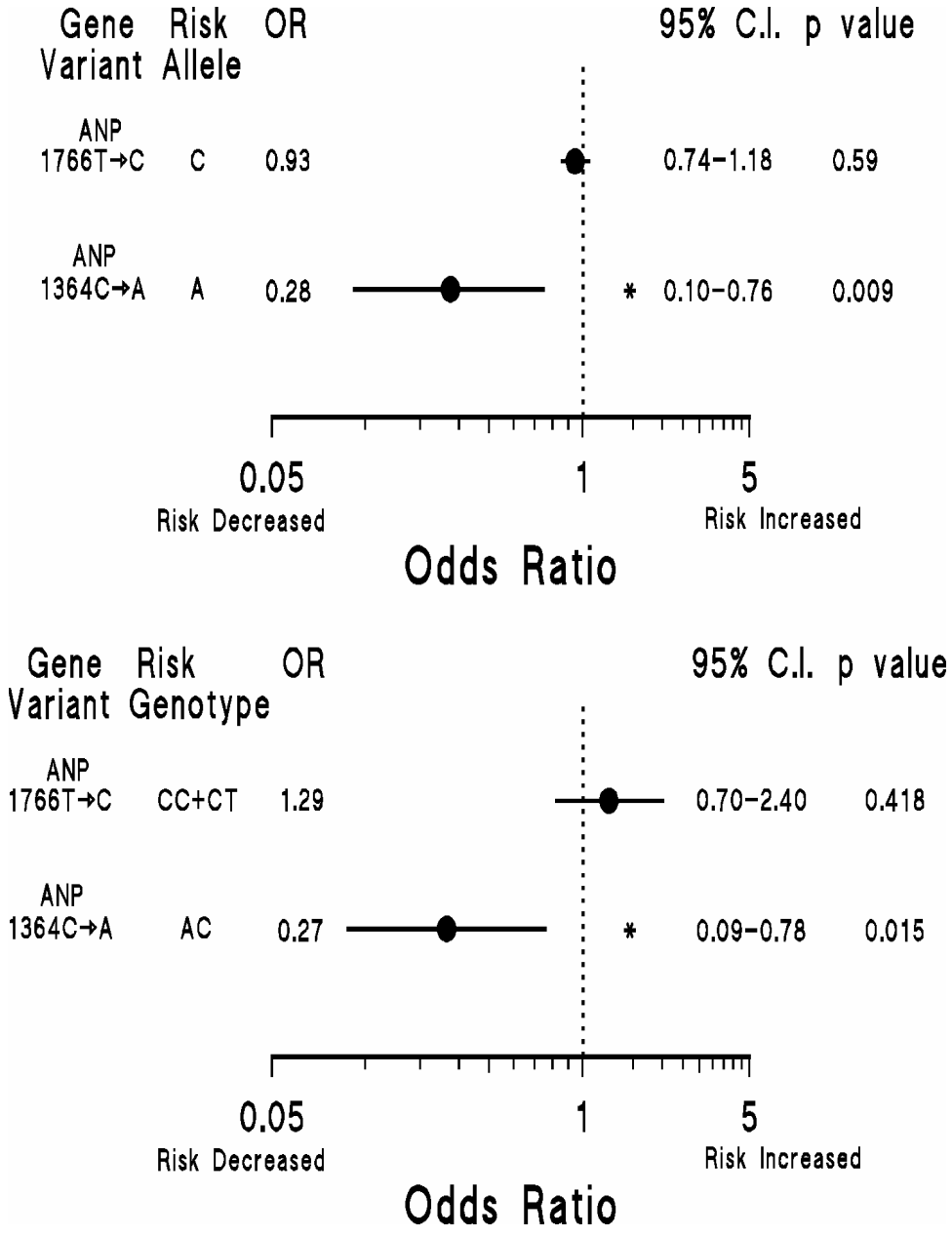

Figure 18. Impact of atrial natriuretic peptide gene polymorphisms on the risk for hypertension in subjects of African ancestry. The upper panel shows the impact of alleles and the lower panel the impact of genotypes. Probability values are for chi-squared analysis (alleles) and MANCOVA (genotype). *$p<0.05$.

consequently a strong relationship between the ANP 1364A allele and the absence of hypertension was noted (Figure 18). There was a less than 1% chance of false positive or negative data for each of the polymorphisms assessed.

Impact of ANP genotype on ambulatory blood pressure in hypertensives. Age ($r = 0.22$, $p<0.002$) and BMI ($r=0.18$, $p<0.01$) were associated with SBP in the hypertensive group. However, the exon 3 polymorphism of the ANP gene was not associated with BP in the hypertensives (Table 17). The intron 2 polymorphism occurred at too low a frequency to assess their impact on BP when considered as a continuous trait.

Table 17. Effect of an exon 3 polymorphism of the atrial natriuretic peptide (ANP) gene on ambulatory blood pressures (ABP) in hypertensives of African descent.

	ANP gene 1766T→C polymorphism in exon 3 genotype		
	TC (n=87) SBP/DBP	TC (n=159) SBP/DBP	CC (n=43) SBP/DBP
24 hour ABP (mm Hg)	153±2/96±1	152±1/98±1	152±3/98±1
Daytime ABP (mm Hg)	157±2/101±1	156±1/103±1	157±2/103±1
Nighttime ABP (mm Hg)	147±2/90±1	147±1/92±1	146±3/92±2

SBP, systolic BP; DBP, diastolic BP. No differences were noted between genotype groups.

DISCUSSION

The main finding of the present study is that an ANP gene intron 2 polymorphism, previously shown to be in complete linkage disequilibrium (Kato et al 2000) with a potentially functional exon 1 polymorphism (Seidman et al 1984) and related to the presence of hypertension in African-Americans (Rutledge et al 1995) is protective against mild-to-moderate essential hypertension in Black South Africans. In contrast, we found no relationship between hypertension and a potentially functional polymorphism in exon 3 of the ANP gene (Ramasawmy et al 1992) in the same population.

The strengths of the present study design have already been addressed in chapter II. Some potential study design limitations have also largely been discussed in chapter II. Importantly, unlike in studies described in chapters II-to-IV, the case and the control groups were not matched for age and gender in the present study. This occurred as the first study conducted was the one discussed in the present chapter when recruitment had only progressed to the sample sizes described. However, when assessing the impact of genotype on the presence of hypertension, multiple logistic regression analysis (performed with age, gender and body size as covariates) showed a marked association between the intron 2 polymorphism of the ANP gene and the absence of hypertension.

The frequency of the alleles of the 1364C→A and 1766T→C polymorphisms of the ANP gene in the control subjects studied are similar to those reported on in normotensive African-Americans (Rutledge et al 1995). However, the allele frequencies of the 1364C→A polymorphism obtained in the hypertensives in the present study were considerably different from those obtained in hypertensive African-Americans (Rutledge

et al 1995). An explanation for the differences between the present study and that performed in African-Americans is not readily apparent. One potential explanation is that population admixture in African-Americans might have obscured the results. In our study the groups were matched not only for racial characteristics, but also for more precise ethnic origins (that is, for chiefdoms). Alternatively, subjects of West African origin may have a different form of hypertension compared to Bantu tribes.

Although no significant relationship between plasma ANP concentrations and the ANP gene exon 1/intron 2 polymorphisms have been demonstrated, a trend for higher ANP concentrations in the genotype group shown to be protective against hypertension in our study, has been reported on (Kato et al 2000). Moreover, it is also interesting that although no significant association between the ANP gene intron 2 polymorphism and "salt-sensitivity" has been shown (Schorr et al 1997) the group with the allele of the ANP gene intron 2 polymorphism (which represented only a small portion of the study sample) shown to be protective against hypertension in our study, tended to have a reduced BP with "salt-loading" (Schorr et al 1997).

In summary, the present study shows an association between an infrequent polymorphic variant in intron 2 of the ANP locus, previously shown to be in complete linkage disequilibrium with a potentially functional exon 1 polymorphism (Kato et al 2000, Seidman et al 984), and hypertension in subjects of African descent. These results support a role for the ANP gene in hypertension and underscore a need for further work to be performed to evaluate the importance of exon 1 of the ANP gene.

Chapter VI

Functional Polymorphisms of the Angiotensinogen Gene Determine Anti-hypertensive Responses to Angiotensin-Converting Enzyme Inhibitors in Subjects of African Origin.

ABSTRACT

Background. The antihypertensive efficacy of angiotensin-converting enzyme inhibitors (ACEI) in subjects of African origin is variable. The aim of this study was to determine whether the response to ACEI therapy in subjects of African origin is genetically determined.

Methods. 194 hypertensive patients of African ancestry were recruited from district clinics in Johannesburg, South Africa. 80 patients received open-label ACEI (enalapril or lisinopril) monotherapy, and 114 received open-label calcium channel blockade (nifedipine) monotherapy as a class comparator. Twenty-four hour ambulatory BP (ABP) monitoring was performed at baseline (off medication) and after 2 months of therapy. DNA was analysed for functional polymorphisms (-217G→A and -20A→C) of the angiotensinogen (AGT) gene and the impact of genotype on outcome measures assessed. Plasma aldosterone and renin levels were determined after ACEI therapy.

Results. Adjusting for baseline ABP and type of ACEI in the ACEI-treated group, the -217G→A polymorphism predicted ABP responses to ACEI (n=77) ($p<0.01$), but not to nifedipine (n=108) monotherapy as determined after 2 months of therapy. ACEIs in patients with the AA genotype of the -217G→A polymorphism failed to elicit an antihypertensive response (change in ABP: systolic BP [SBP]= +0.84±2.89 mm Hg p=0.78, diastolic BP [DBP]= -0.47±1.74 mm Hg p=0.79). In contrast, those patients with at least one copy of the -217G allele developed a 7.23±1.55 and 5.38±1.12 mm Hg decrease ($p<0.0001$) in SBP and DBP respectively, following ACEI administration. Similarly, the -20A→C polymorphism predicted ABP responses to ACEI therapy ($p<0.01$) but not to nifedipine. Moreover, there was an interaction between the -217G→A

and the -20A→C polymorphisms. Patients who were AA genotype for both polymorphisms failed to develop an antihypertensive response to ACEIs (change in ABP; SBP= +1.06±3.05 mm Hg p=0.73; DBP= -0.39±1.83 mm Hg p=0.83); whereas patients with at least one copy of both the -217G allele and the -20C allele developed substantial decreases in ABP following ACEI therapy (change in ABP; SBP= -14.08±3.72; DBP= -9.62±2.74 mm Hg; P<0.0001). Patients with at least one copy of the –217G allele demonstrated a significant reduction in aldosterone-to-renin ratio (-0.098±0.035, p<0.01), whereas in those patients who were –217AA genotype the ratio was unchanged (-0.03±0.16, p=0.85).

Conclusion. These results suggest that functional polymorphisms of the AGT gene contribute to the variability of antihypertensive responses to ACEI therapy in subjects of African ancestry, with genotype determining whether or not responses occur.

INTRODUCTION

Blood pressure responses of patients to anti-hypertensive therapy are variable (Bidiville et al 1988), hence it has been suggested that treatment should be matched to individual responsiveness. Attempts to predict BP responses from physiological measurements have proved disappointing (Laragh 1993). However, as there is a strong genetic contribution to BP (Ward et al 1990), it is possible that genotyping of individuals may assist in predicting responses to antihypertensive therapy.

A striking example of heterogeneity of BP responses to antihypertensive therapy is the observation that angiotensin-converting enzyme inhibitors (ACEI) are less effective at lowering BP in subjects of African origins compared with Caucasians (Julius et al 2004). Indeed, this finding has been widely incorporated into guidelines for the management of hypertension (Chobanian et al 2003, Williams et al 2004). Yet a proportion of patients of African origins with hypertension can be successfully controlled on ACEI monotherapy (Cohn et al 2004). I hypothesized that this inter-individual variability may be genetically determined.

Among the genes of the renin-angiotensin-aldosterone system (RAAS), polymorphisms in the angiotensinogen (AGT) gene have been most consistently associated with hypertension and BP regulation (Jeunemaitre et al 1992, Sethi et al 2001). Recently, a number of functional polymorphisms in the promoter region of the AGT gene (-217G→A and –20A→C), which influence the transcription of the gene, have been characterized (Jain et al 2002, Wu et al 2003, Zhao et al 1999). As indicated in chapter II of this thesis, these variants interact to determine the risk for EHT and ambulatory BP in

subjects of African ancestry. Moreover, the –20A→C polymorphism is a strong determinant of body size on BP (Tiago et al 2002). In the present study I therefore investigated whether recently characterized functional polymorphisms of the AGT gene contribute independently or interactively to antihypertensive responses to ACEI therapy in subjects of African ancestry. I compared the impact of AGT genotype on ACEI responses to the influence of genotype noted in response to calcium channel blockade. Finally, I examined the effect of AGT genotype on plasma aldosterone-to-renin ratios after ACEI therapy.

METHODS

Study groups and BP measurements. A total of 194 hypertensive patients of African ancestry from district clinics in suburban areas of Johannesburg were recruited if they had office and mean daytime ambulatory diastolic BP (DBP) >90 mm Hg (Spacelabs model 90207) off medication. Inclusion and exclusion criteria, and the method as well as timing of ABP measurements are described in chapter II.

ABP responses to angiotensin-converting enzyme inhibitors and calcium channel blockade. Eighty consecutively recruited patients with a mean daytime DBP >90 mm Hg received 10 mg of either open-label enalapril or lisinopril monotherapy for a one-month period, which was subsequently uptitrated to 20 mg for a further month. After two months three patients had been withdrawn because of the development of an intolerable cough. Repeat ABP measurements were performed on the remaining 77 patients.

To ensure that any genotype effects on ABP in patients receiving an ACEI were specific to this class of agents, we also assessed the impact of genotype on ABP responses to long-acting nifedipine (Adalat XL). One hundred and fourteen patients received open-label nifedipine (30 mg) for one month and if daytime DBP was >90 mm Hg the dose was increased to 60 mg (n=61). At two months six patients were withdrawn because of the presence of ankle edema. The remaining 108 patients had repeat ABP measurements performed after two months.

Hormone measurements. In order to determine the effects of ACEI therapy on the RAS, venous blood samples were obtained after 20 minutes of rest in the supine position before and after two months of ACEI therapy. The latter samples were taken approximately 24 hours after the previous day's dose of AECI. Due to practical difficulties related to the timing of venesection, valid blood samples were obtained from 46 of the 77 patients receiving ACEI therapy. Plasma renin and aldosterone concentrations were determined as previously described (Norton et al 1999).

Genotyping. Deoxyribonucleic acid (DNA) was extracted as described in chapter II. Genotyping of the A-to-C substitution at nucleotide –20 and the G-to-A substitution at nucleotide -217 of the AGT gene was undertaken using polymerase chain reaction PCR-RFLP-based techniques employing the appropriate primer pairs and restriction enzymes as described in chapter II.

Data analysis. An ANCOVA with pretreatment ABP, type of ACEI (for the ACEI-treated group), dose of nifedipine (for the CCB-treated group), BMI, gender, age, recent cigarette smoking and alternative genotypes employed as covariates was used to determine the independent impact of polymorphisms on ABP responses to treatment. An

ANCOVA was also used to assess the effects of AGT genotypes on pretreatment and two month ABP. ABP responses to therapy within each genotype group were compared to a hypothetical mean of zero using univariate analysis. Formal interaction analyses between the -217G→A and -20A→C polymorphisms were performed using ANCOVA followed by Tukey *post hoc* test, with age, gender, BMI, pretreatment ABP, type of ACEI, recent cigarette smoking and alternative genotype included as covariates. The ACEI substudy was 80% powered to detect a 5 mm Hg difference in change in ABP with 15 patients in each of two genotype groups, with a correspondingly slightly greater power in the CCB sub-study given its larger size. Continuous data are expressed as mean ± SEM.

RESULTS

Demographic and clinical data. These are shown in Tables 18 and 19. Both the ACEI and the CCB groups had a preponderance of females and individuals with an increased BMI. As indicated in chapter II the high frequency of females reflects the gender distribution of patients attending district clinics, rather than a profoundly greater incidence of hypertension in African women compared to men. Notably, the ACEI and CCB groups were matched according to all demographic features. Furthermore, within the ACEI and CCB treatment groups the demographic and clinical data were comparable between the genotype groups of the -217G→A (Table 19) and the -20A→C polymorphisms (data not shown). Importantly, none of the AGT genotypes were associated with pretreatment ABP (data for -217G→A are shown in Table 20).

Table 18. Demographic and clinical characteristics of angiotensin-converting enzyme inhibitor (ACEI) and calcium channel blocker (CCB) therapy groups.

	ACEI	CCB
n =	77	108
Age (years)	51.8±1.1	52.5±0.8
Gender (% female)	70	79
Body mass index ($kg.m^{-2}$)	30.8±0.7	31.2±0.5
Recent cigarette smoking (%)	15	23
24 hour SBP/DBP (mm Hg)	150±2/97±1	151±1/96±1
Day SBP/DBP (mm Hg)	154±1/101±1	155±1/101±1
Night SBP/DBP (mm Hg)	145±2/91±1	145±1/90±1
Duration of hypertension (years)	2.6±0.4	2.6±0.4

SBP/DBP, systolic blood pressure, diastolic blood pressure.

Table 19. Demographic and clinical characteristics of -217G→A genotype groups receiving either angiotensin-converting enzyme inhibitor (ACEI) or calcium channel blocker (CCB) therapy. (see Table 20 for baseline ambulatory blood pressure values)

	ACEI		CCB	
-217G→A genotype	AA	AG+GG	AA	AG+GG
n =	19	58	25	83
Age (years)	54.3±2.1	51.0±1.2	52.0±1.9	52.7±0.8
Gender (% female)	74	68	88	78
Body mass index ($kg.m^{-2}$)	31.3±1.4	30.6±0.8	31.7±1.2	31.1±0.5
Recent cigarette smoking (%)	21	12	32	21
Duration of hypertension (years)	3.8±2.0	2.2±0.4	2.3±0.5	2.7±0.4

Independent genotype effects on ABP response to therapeutic agents. Genotype of the -217G→A and the -20A→C polymorphisms of the AGT gene predicted whether a decrease in ABP occurred in response to ACEI therapy. In patients with the -217AA genotype no change in 24 hour and night ABP occurred, whereas in those patients with at least one copy of the -217G allele, 24 hour and night ABP decreased in response to ACEI therapy (Figure 19). No effect of -217G→A polymorphism was noted on day ABP response to ACEI therapy (Figure 19). In contrast to the effect of the -217G→A polymorphism on ABP responses to ACEI therapy, AGT -217G→A genotype failed to predict changes in ABP in response to the long acting calcium channel blocker (CCB), nifedipine (Figure 19).

An effect of the -20A→C polymorphism was noted on changes in only day ABP in response to ACEI therapy. In patients with the -20AA genotype no change in day ABP occurred, whereas in those patients with at least one copy of the -20C allele day SBP decreased in response to ACEI therapy (Figure 20). Similar to the -217G→A polymorphism, the -20A→C polymorphism did not predict changes in ABP (24 hour, day or night) in response to nifedipine (Figure 20).

Genotype interactive effects on ABP response to therapeutic agents. An interactive effect of -217G→A and -20A→C polymorphisms on ABP responses to ACEI therapy was noted. Patients who were AA genotype for both the-217G→A and -20A→C polymorphisms failed to develop an antihypertensive response to ACEI monotherapy (Figure 21). In comparison, patients with at least one copy of the -217G allele as well as at least one copy of the -20C allele developed substantial reductions in ABP in response to ACEI therapy (Figure 21). A modest reduction in ABP was observed in those patients

with a combination of -217GA or GG plus -20AA genotype (Figure 21). Only one patient had a combination of -217AA plus -20AC genotype. This patient's ABP responses were similar to those observed in the -217GA or GG plus -20AA genotype group and were therefore included in this group (change in 24 hour ABP (mm Hg); SBP= -3.00; DBP= -2.00).

Hormonal measurements. With respect to ACEI-induced changes in plasma markers of activity of the RAAS, there was a significant increase in plasma renin in those patients with at least one -217G allele (Figure 22) and a similar trend toward an increase was observed in those patients who were -217AA genotype, but this failed to reach statistical significance (Figure 22). Minimal changes in plasma aldosterone concentrations were noted irrespective of genotype group (Figure 22). Importantly, however, those patients that had at least one -217G allele demonstrated a significant reduction in the aldosterone-to-renin ratio (Figure 22), whereas in those patients who were -217AA genotype the aldosterone-to-renin ratio was unchanged (Figure 22). The study was not statistically powered to assess -20A→C effects alone or via an interaction with -217G→A on hormonal measurements, as only 3 of the 46 patients with valid blood samples were -20AC or CC genotype.

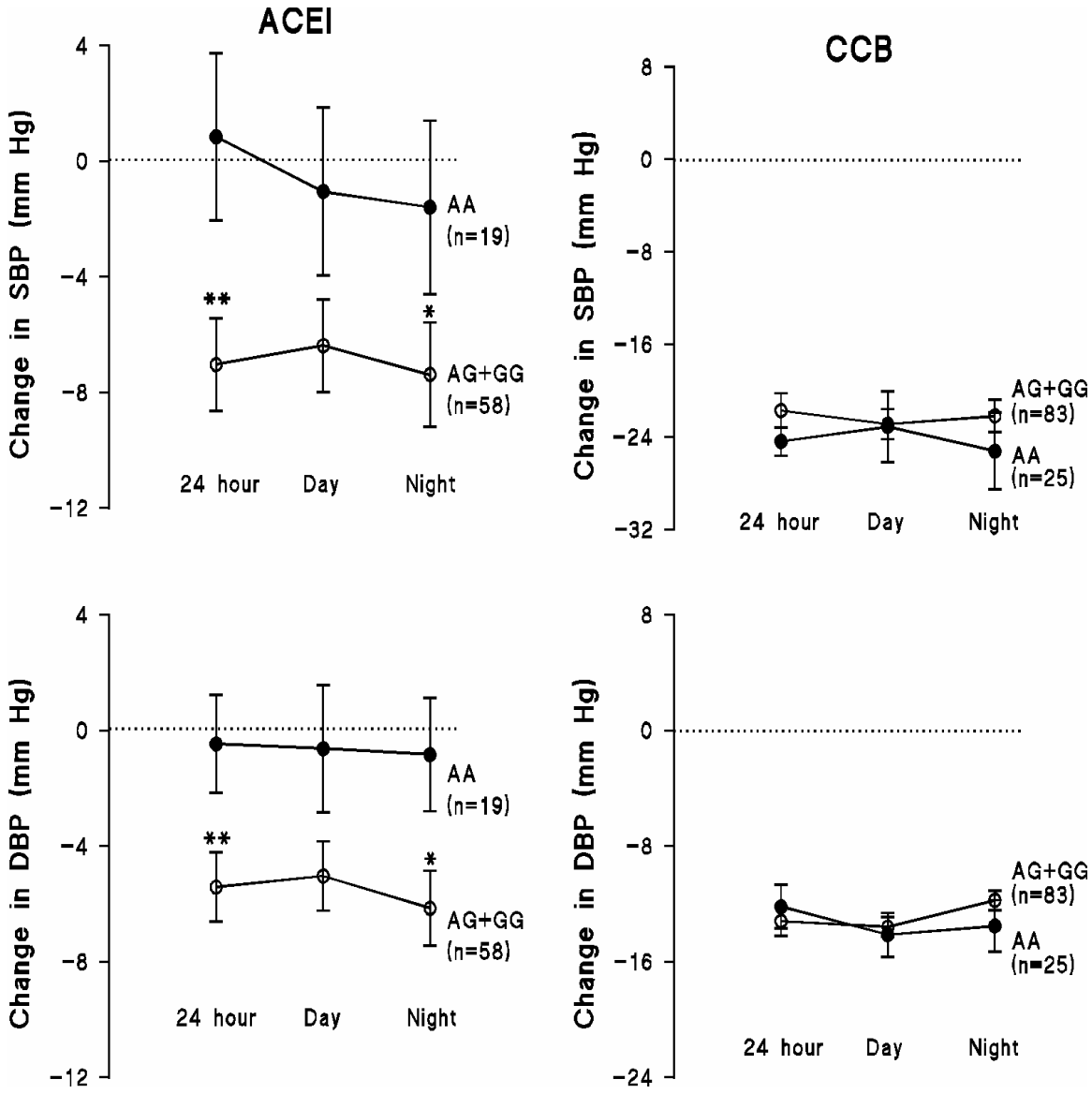

Figure 19. Impact of the angiotensinogen (AGT) gene -217G→A polymorphism on change in 24 hour, day and night ambulatory systolic (SBP) and diastolic (DBP) blood pressures following angiotensin-converting enzyme inhibitor (ACEI) or calcium channel blocker (CCB) therapy in hypertensives of African ancestry. ** $p<0.01$, *$p<0.05$ on ANCOVA.

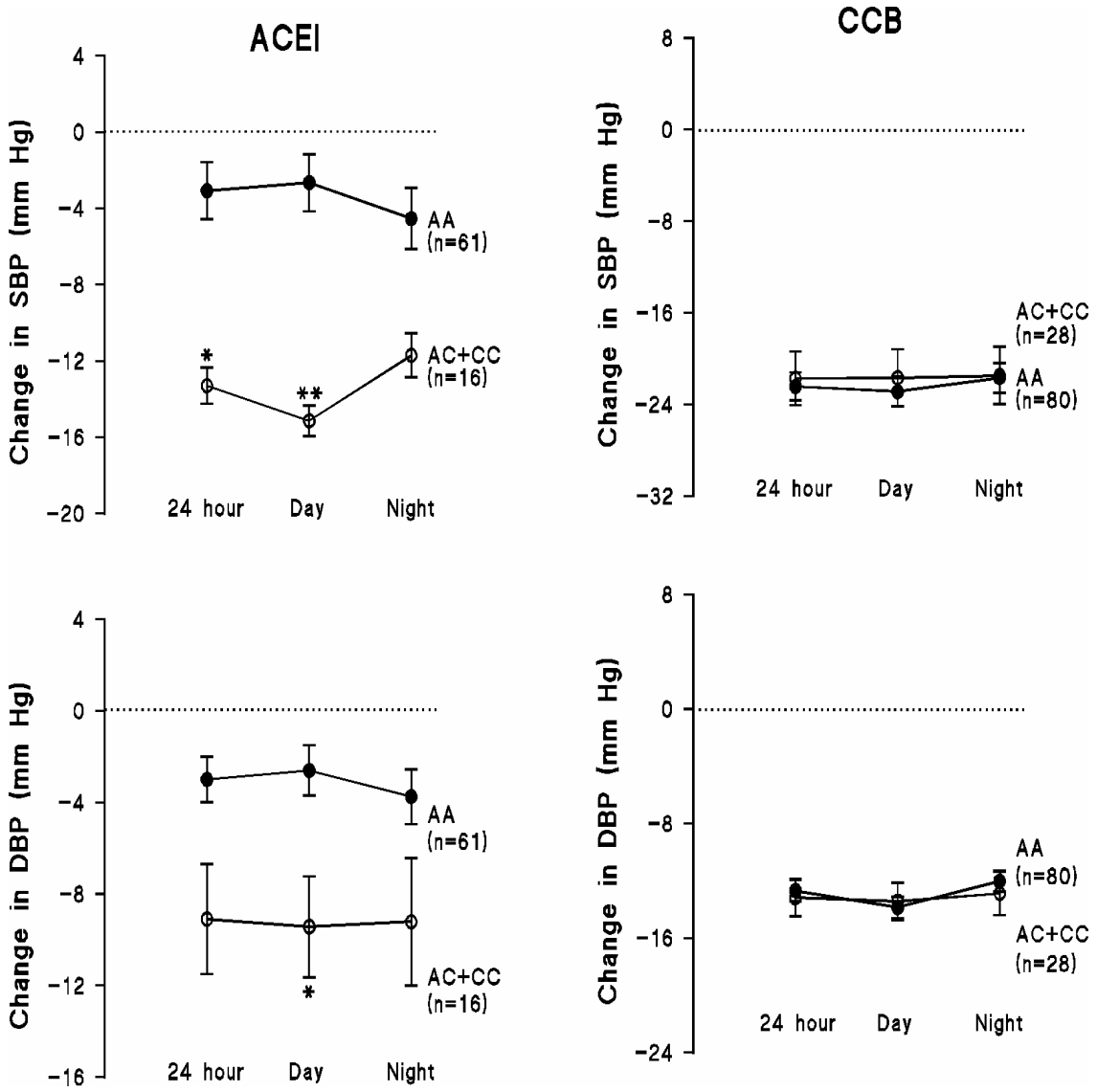

Figure 20. Impact of the angiotensinogen (AGT) gene -20A→C polymorphism on change in 24hour, day and night ambulatory systolic (SBP) and diastolic (DBP) blood pressures following angiotensin-converting enzyme inhibitor (ACEI) or calcium channel blocker (CCB) therapy in hypertensives of African ancestry. ** $p<0.01$, *$p<0.05$ on ANCOVA.

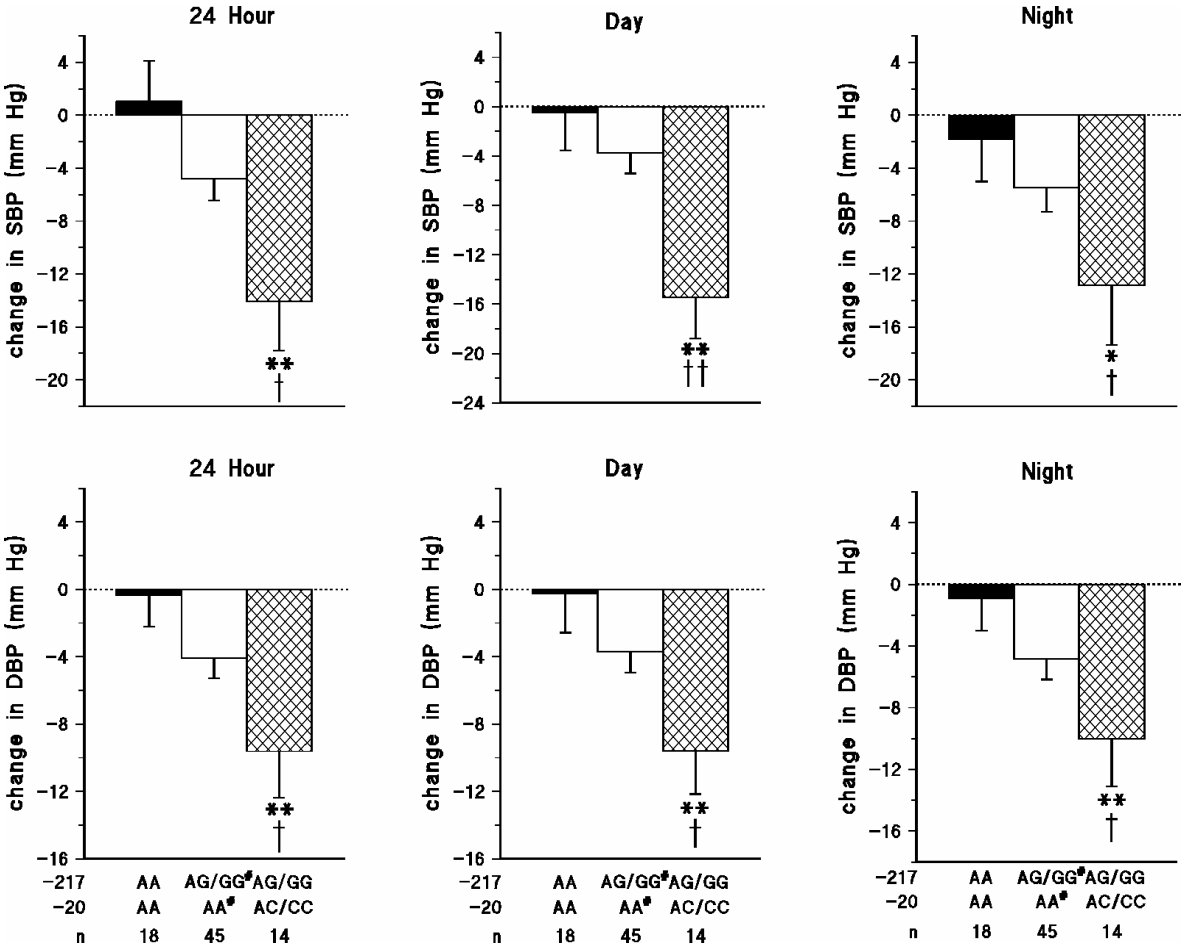

Figure 21. Impact of the interaction of angiotensinogen (AGT) gene -217G→A and -20A→C polymorphisms on change in 24 hour, day and night ambulatory systolic (SBP) and diastolic (DBP) blood pressures following angiotensin-converting enzyme inhibitor (ACEI) or calcium channel blocker (CCB) therapy in hypertensives of African ancestry. *p<0.05, **p<0.005 versus -217AA plus -20AA; †p<0.05, ††p<0.005 versus -217 AG/GG plus -20AA; # includes one patient who was -217AA plus -20AC genotype.

Table 20. Impact of angiotensinogen genotype on ambulatory blood pressure in hypertensives of African ancestry receiving either angiotensin-converting enzyme inhibitors (ACEI) or calcium channel blocker therapy (CCB).

	Mean 24 hour ambulatory blood pressure (mm Hg)			
Time (months)	0	2	0	2
	Patients receiving ACEIs			
Genotype group	-217AA (n=19)		-217GG+GA (n=58)	
24 hour SBP/DBP (mm Hg)	151±3/97±1	152±4†/96±2†	150±2/97±1	142±2*/91±1*
Day SBP/DBP (mm Hg)	157±3/102±1	155±4†/101±3†	154±2/101±1	147±2*/96±2*
Night SBP/DBP (mm Hg)	148±3/92±2	147±4†/91±2†	145±2/91±1	137±2*/85±1*
	Patients receiving CCBs			
Genotype group	-217AA (n=25)		-217GG+GA (n=83)	
24 hour SBP/DBP (mm Hg)	152±3/96±1	128±2*/88±2*	151±1/96±1	129±1*/83±1*
Day SBP/DBP (mm Hg)	153±2/101±1	130±3*/88±2*	156±1/102±1	133±1*/88±1*
Night SBP/DBP (mm Hg)	147±3/91±1	122±3*/78±2*	146±2/90±1	124±1*/78±1*

*p<0.05; **p<0.001 for 0 versus 2 months; †p<0.05 for -217AA group versus -217GG+GA group (see Figure 20 for differences in responses between -217AA group and -217GG+GA group); SBP, systolic blood pressure; DBP, diastolic blood pressure.

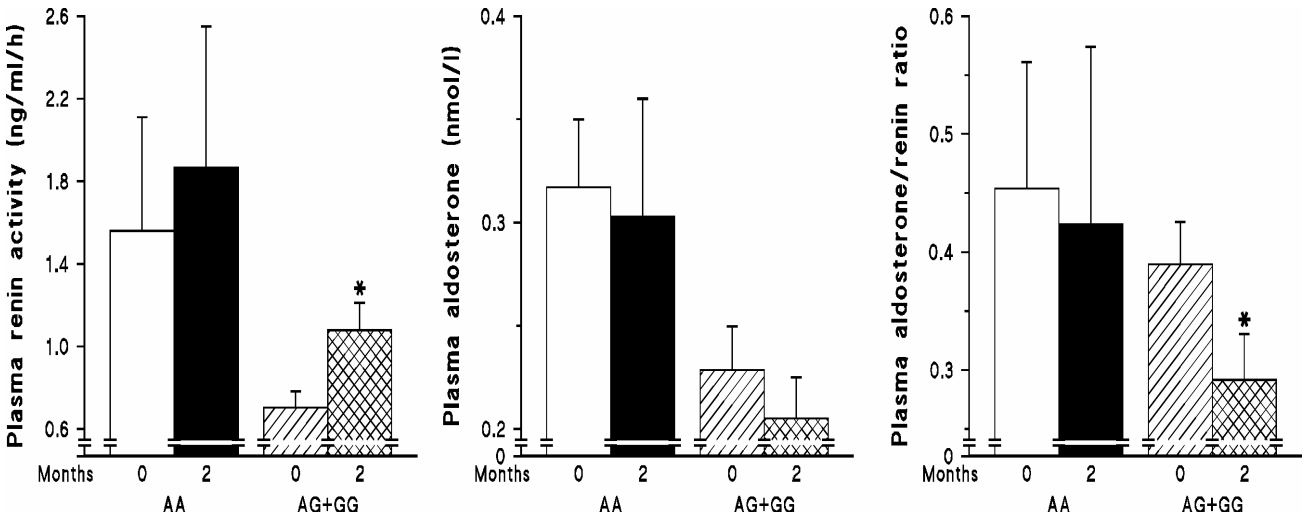

Figure 22. Impact of the angiotensinogen (AGT) gene -217G→A polymorphism on plasma renin, aldosterone and aldosterone-to-renin ratios following angiotensin-converting enzyme inhibitor (ACEI) therapy in hypertensives of African ancestry. * $p<0.01$ versus baseline values.

DISCUSSION

The main findings of the present study are that the -217G→A and -20A→C polymorphisms of the AGT gene are independent predictors of ABP responses to ACEI therapy, but not to calcium channel blocker therapy in subjects of African ancestry. Furthermore, these two polymorphisms have an interactive effect on ABP responses to ACEI therapy. The -217AA and the -20AA genotypes were associated with a lack of response, in contrast to patients with at least one copy of both the -217G and the -20C allele who developed a significant drop in ABP values.

The results are the first to indicate that functional polymorphisms of the AGT gene contribute toward the variability of ABP responses to ACEI therapy in patients of African origin.To-date only two studies conducted in Caucasians have investigated the role of the AGT gene as a predictor of BP responses to ACEI therapy (Hingorani et al 1995, Dudley et al 1996). Although Hingorani et al (1995) showed that the AGT gene 704T→C polymorphism was associated with BP responses to ACEI therapy in patients of European ancestry, this study was not conducted with ABP measurements. Indeed, a subsequent study using ABP measurements failed to confirm these results (Dudley et al 1996). Importantly, in contrast to the present study, neither of these studies investigated the impact of functional polymorphisms of the AGT gene. It therefore remains to be determined whether these functional polymorphisms also influence the response to ACEIs in Caucasian subjects. However, as the prevalence of the AGT genotype (-217AA) that largely prevents BP responses to ACEIs appears to be low in Caucasians, any clinical impact is likely to be diminished. This is in contrast to the considerable

importance that these genotypes could have in populations of African ancestry with a high prevalence of patients with either the –217AA or –20AA genotype (Jain et al 2002, Tiago et al 2002, chapter II). In this regard, it is estimated that about 20-to-30% of hypertensive patients of African ancestry respond well and can be controlled on ACEIs alone (Julius et al 2004, Sareli et al 2001). This concurs with the percentage patients in our study who were neither –217AA nor –20AA genotype and who developed a marked ambulatory BP response to ACEIs (Figure 21).

A potential mechanism that could explain the effects of the -217G→A and the-20A→C polymorphisms, as well as their interactions on ABP responses to ACEI therapy is their impact on tissue or circulating AGT production and consequently RAAS activity. The -217G→A polymorphism has been shown to increase binding of the CAAT/enhancer-binding protein, and subsequently enhance both basal and interleukin 6-stimulated AGT gene transcription (Jain et al 2002). Similarly, the -20A→C polymorphism has been found to influence basal transcription of angiotensinogen as well as stimulated transcription in response to receptor binding (Zhao et al 1999). Thus it is possible that despite ACEI therapy, subjects with the –217AA genotype and/or the –20AA genotype continue to produce greater amounts of angiotensin II compared to those with alternative genotypes, either because ACEI therapy does not completely abolish ACE activity, or through the activity of non-ACE tissue pathways for generating angiotensin II (Husain 1993). Such tissue non-ACE pathways have been shown to play a significant role in the presence of ACEI (Doggrell and Wanstall 2004) and high angiotensin I concentrations (Inoue et al 1999). The findings in the present study that the

aldosterone-to-renin ratio was not reduced in subjects with the –217AA genotype are consistent with this possibility.

The present study does not entirely explain the lower magnitude of BP responses to ACEIs in subjects of African ancestry as compared to other classes of pharmacological agents. Indeed, the present data indicate that calcium channel blockers still produce greater decreases in both systolic and diastolic BP as compared to ACEIs in even patients with the AGT genotype group that had the most pronounced BP response to ACEIs. Observations of this type have led to guidelines recommending avoidance of ACEIs as initial therapy in patients of African ancestry (Chobanian et al 2003, Williams et al 2004). Given the increasing benefits that have been shown to accrue from ACEI therapy in appropriate patients with cardiovascular disease (SOLVD Investigators 1992, Yusuf et al 2000, EUROPA Trial 2003), it is important that patients of African origin who are likely to benefit from such therapy, receive it. The present findings suggest that those patients with at least one copy of the –217G and/or the –20C allele may indeed benefit more from the use of ACEIs than those without a –217G and/or a –20C allele. Therefore, if the present findings are replicated, a potentially important clinical implication could be a reappraisal of current guidelines and possible use of genotype information to guide choice of therapy.

In summary, the present study provides novel data suggesting that the AGT gene plays an important role in ABP responses to ACEI therapy in subjects of African ancestry. It is conceivable that functional polymorphisms of the AGT gene could be used to predict ABP responses to ACEIs and hence to determine therapeutic choices in this population group.

Chapter VII

Conclusions

There is little question that blood pressure is an inherited trait. Consequently, EHT or primary hypertension is thought to be partly genetically determined. Yet the genetic basis of EHT has only begun to be unraveled. The high frequency of "salt-sensitivity" amongst hypertensives of African ancestry as compared to other groups suggests that genes that encode proteins responsible for renal salt handling could be involved in the pathogenesis of EHT in subjects of African descent. Although subjects of African ancestry are generally considered to have lower plasma renin levels than other groups, downstream products of renin production are not always suppressed, suggesting inappropriate activation of these products. In the present thesis I therefore explored whether gene candidates that encode proteins responsible for renal salt handling or activation of the renin-angotensin system downstream from renin could contribute to BP in subjects of African descent. Based on the present scientific literature I selected the AGT, GNB3, eNaC, and ANP genes as the most likely to be involved. Because most studies previously described have employed relatively small sample sizes and generally without ambulatory BP measurements, I conducted a relatively large study to assess the impact of polymorphisms within these genes on the risk of EHT (1325 subjects) and ambulatory BP in 626 patients off medication. With respect to the AGT gene I focused my efforts on a newly described functional polymorphism within the promoter region not previously assessed in the population studied (-217G→A).

In the present thesis, in addition to making use of the traditional case-control and association analysis I utilised a number of unique approaches. I designed my studies not only to assess independent gene effects, but also to account for potential interactions between polymorphisms within single loci that ultimately produce similar effects on

protein products. In this regard, the AGT gene is well recognized as having at least 3 functional polymorphisms within the promoter region, all of which influence angiotensinogen expression. Therefore, in addition to assessing the independent impact of a polymorphism whose function has only recently been described (-217G→A), I also explored the potential that in the absence of important independent gene effects, interactive effects between AGT gene polymorphisms could potentially have a profound impact on the risk for EHT and BP. Indeed, in the present thesis I was able to show using a case-control study design and association analysis in a large sample of subjects with 24 hour ambulatory BP in hypertensives off mediation, that interactions between the -217G→A and -20A→C polymorphisms account for a substantial portion of the risk for EHT and ambulatory BP in EHT.

A second unique aspect of the present thesis is that I designed my studies not only to assess independent gene effects (type III or IV gene actions), but also to account for type I or II genetic effects where genes influence the impact of alternative phenotypes. In this regard, having demonstrated that the 825T allele of the GNB3 gene is indeed not associated with EHT or ambulatory BP in subjects of African ancestry, I explored the possibility that to this variant influences the impact of body size on BP. This hypothesis developed from the fact that obesity influences the activity of the same cellular target as that produced by the GNB3 gene polymorphism, namely the Na^+/H+ exchanger. Indeed, in patients homozygous for the 825T allele, body size determined systolic BP, whereas in subjects with at least one copy of the 825C allele, the impact of body size on systolic BP was abolished. These effects were noted for ambulatory systolic BP in hypertensives as well as office systolic BP in controls. Thus, these data provide substantial evidence in

favor of the notion that type I or II effects rather than independent effects of functional gene polymorphisms have a clinically important role to play in BP control.

In a relatively large study (1033 subjects) conducted to attempt to substantiate the notion that a functional eNaC β-subunit gene polymorphism contributes to EHT and BP in subjects of African ancestry, I was unable to provide data to support this notion. However, a limitation of this study was the fact that although the polymorphism generally only occurs in notable proportions in subjects of African ancestry, the frequency of the risk variant is still very low in this population group. This obviously limits the outcome of genetic epidemiology studies. Nevertheless, the very low frequency with which the variant occurs should be seen as sufficient evidence to infer that if the variant contributes to BP control, its role in terms of population-attributable-risk of a cardiovascular event is limited at best.

Also described in the present thesis are data to suggest that the ANP locus could play a role in contributing to BP in subjects of African descent. The variant associated with the risk for EHT is in linkage disequilibrium with a functional variant in the promoter region. Hence, the relationship could be related to ANP expression. However, substantially more work is required to prove this notion. Indeed, the relationship between the promoter region variant and BP as well as ANP concentrations needs to be explored. Moreover, the study described in this thesis was not as rigorous as studies discussed in other chapters in that sample sizes were smaller and an association with ambulatory BP was not demonstrated probably because of the low frequency with which the protective allele occurs. Nevertheless, because the protective allele occurs with such a low

frequency in the population studied further work on the ANP locus was not pursued in this thesis.

Lastly, having demonstrated that an interaction between the novel -217G→A and a previously described -20A→C polymorphism in the AGT gene is a strong predictor of EHT and ambulatory BP, I evaluated whether these polymorphisms, which influence angiotensinogen expression, could determine the variable response to ACEIs in subjects of African descent. The hypothesis was that despite ACEI therapy, subjects with the –217AA genotype and/or the –20AA genotype may continue to produce greater amounts of angiotensin II compared to those with alternative genotypes, either because ACEI therapy does not completely abolish ACE activity, or through the activity of non-ACE tissue pathways for generating angiotensin II (Husain 1993). Such tissue non-ACE pathways have been shown to play a significant role in the presence of ACEI (Doggrell and Wanstall 2004) and high angiotensin I concentrations (Inoue et al 1999). Indeed, I was able to demonstrate that an interaction between the -217G→A and -20A→C polymorphisms produced a profound impact on the variable response to ACEIs, but not calcium channel blockers, with the -217AA and -20AA genotype associated with no response whilst other genotype groups developed a significant antihypertensive response. The findings that the aldosterone-to-renin ratio was not reduced in subjects with the –217AA genotype are consistent with the hypothesis that this genotype predisposes to enhanced production of angiotensin II because of excessive angiotensinogen expression. These data represent the first to suggest that gene variants may ultimately influence therapeutic choices in subjects of African ancestry. Further studies are required to either confirm or refute this notion.

Additional limitations

Besides all of those limitations acknowledged to exist when performing association analysis in genetic epidemiology (see chapter II for a description of these limitations), and those limitations indicated in the preceding section of this chapter, an important additional limitation of the present thesis needs to be highlighted. Assessing "salt-sensitivity" would have been an ideal goal in order to refine the phenotypic characteristic of interest, rather than relying on the high frequency of "salt-sensitivity" that exists in the population studied. However, the institution in which I carried out this research is not equipped with a metabolic unit for these purposes. Moreover, recruiting subjects to perform these studies would have been self-limiting as there is little chance that sufficient sample sizes would have been obtained if subjects had been asked to stay in a unit such as this for a two week period. Using lithium clearance as an index of salt sensitivity would have been a worthwhile exercise. However, accurate measures of lithium clearance were not available.

Although I acknowledge that given the low frequency of the functional exon 1 variant of ANP, it would have been better to test it directly (rather than its surrogate the intron 2 variant), at the time of conducting this study, the exon 1 variant had only been shown to have potential functional relevance and had not been investigated in salt-sensitive populations. In comparison, the intron 2 polymorphism, which is in complete linkage disequilibrium with the exon 1 variant, had been shown to be potentially associated with hypertension in a typical group of salt-sensitive hypertensive African-Americans; but not with salt-sensitivity in Caucasians. Hence, I chose to investigate the

intron 2 polymorphism in conjunction with the exon 3 polymorphism and the cis-acting promoter/enhancer element of the clearance receptor of the ANP gene in order to clarify the potential role of the ANP gene in hypertension in peoples of African ancestry.

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UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

COMMITTEE FOR RESEARCH ON HUMAN SUBJECTS (MEDICAL)
Ref: R14/49 Candy

CLEARANCE CERTIFICATE PROTOCOL NUMBER M 951122

PROJECT	Genetic Polymorphisms in the urban Black hypertensive population
INVESTIGATORS	Mr G P Candy
DEPARTMENT	Nuclear Medicine, Medical School
DATE CONSIDERED	951124

DECISION OF THE COMMITTEE *

Approved unconditionally

DATE 960223

CHAIRMAN. ..... (Professor P E Cleaton-Jones)

c c Supervisor: Dr G Norton
Dept of Physiology, Medical School

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DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and ONE COPY returned to the Secretary at Room 10001, 10th Floor, Senate House, University.

I/we fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee.

DATE 4/3/1996 SIGNATURE .....

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

COMMITTEE FOR RESEARCH ON HUMAN SUBJECTS (MEDICAL)
Ref: R14/49 Norton

CLEARANCE CERTIFICATE PROTOCOL NUMBER M980811

PROJECT Genome Search For Hypertensive Loci In Black South Africans

INVESTIGATORS Dr GR Norton

DEPARTMENT Physiology Dept, Wits Medical School

DATE CONSIDERED 980828

DECISION OF THE COMMITTEE *

Approved unconditionally

DATE 981012 CHAIRMAN .....(Professor P E Cleaton-Jones)

* Guidelines for written "informed consent" attached where applicable.

c c Supervisor:
Dept of ,

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DATE 20/11/1998 SIGNATURE .....

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

COMMITTEE FOR RESEARCH ON HUMAN SUBJECTS (MEDICAL)
Ref: R14/49 Tiago

CLEARANCE CERTIFICATE **PROTOCOL NUMBER** M01-01-11

PROJECT An Evaluation Of The Genetic Basis of Essential Hypertension In Black South Africans

INVESTIGATORS Dr A Tiago

DEPARTMENT Physiology Department, Wits Medical School

DATE CONSIDERED 01-01-26

DECISION OF THE COMMITTEE *

Protocols M951122 and M010111 Approved Unconditionally

DATE 03-03-06 **CHAIRMAN** .....(Professor P E Cleaton-Jones)

* Guidelines for written "informed consent" attached where applicable.

c c Supervisor: Dr A Woodiwiss
Dept of Physiology Department, Wits Medical School

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DATE 06/03/03 SIGNATURE .....

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES