Context-Dependent Effects of the Renin-Angiotensin-Aldosterone System on Blood Pressure in a Group of African Ancestry

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ABSTRACT

In groups of African ancestry, who have a high prevalence of "salt-sensitive, low-renin" hypertension, there is considerable uncertainty as to relevance of the renin-angiotensin-aldosterone system (RAAS) in the pathophysiology of primary hypertension. In the present thesis I explored the possibility that the RAAS, through interactions with environmental effects, contributes to blood pressure (BP) in this ethnic group.

After excluding participants with aldosterone-to-renin ratios (ARR) above the threshold for primary aldosteronism, in 575 participants of African ancestry, I demonstrated that with adjustments for confounders, an interaction between ARR and urinary Na^+/K^+ (and index of salt intake obtained from 24-hour urine samples) was independently associated with BP ($p<0.0001$). This effect was accounted for by interactions between serum aldosterone concentrations and urinary Na^+/K^+ ($p<0.0001$), but not between plasma renin concentrations and urinary Na^+/K^+ ($p=0.52$). The interaction between ARR and urinary Na^+/K^+ translated into a marked difference in the relationship between urinary Na^+/K^+ and BP in participants above and below the median for ARR ($p<0.0001$ for a comparison of the relationships).

Having demonstrated that circulating aldosterone concentrations may account for a substantial proportion of the relationship between salt intake and BP in this community sample, I subsequently assessed whether genetic factors contribute toward serum aldosterone concentrations. In 153 randomly selected nuclear families of African ancestry consisting of 448 participants without primary aldosteronism, with, but not without adjustments for plasma renin concentrations, independent correlations were noted for

serum aldosterone concentrations between parents and children ($p<0.05$), with parent-child partial correlation coefficients being greater than those for father-mother relationships ($p<0.05$). Furthermore, after, but not before adjustments for plasma renin concentrations, serum aldosterone concentrations showed significant heritability ($h^2=0.25\pm0.12$, $p<0.02$). No independent relationships between RAAS gene polymorphisms and serum aldosterone concentrations were observed.

I also aimed to assess whether RAAS genes modify the relationship between cigarette smoking and BP in groups of African descent. However, as the impact of mild smoking on BP is uncertain, and in the community studied only 14.5% smoked and the majority of smokers were mild smokers (mean=7.4±4.6 cigarettes per day) in 689 randomly participants I initially assessed the relationship between smoking habits and out-of-office BP. In this regard, current smokers had higher unadjusted and multivariate adjusted 24-hour systolic/diastolic BP (SBP/DBP in mm Hg) ($p<0.005$-$p<0.0005$) than non-smokers, effects that were replicated in sex-specific groups, non-drinkers, and in the overweight and obese. Current smoking was second only to age and at least equivalent to body mass index in the quantitative impact on out-of-office BP and the risk of uncontrolled out-of-office BP was increased in smokers as compared to non-smokers. Thus, despite minimal effects on in-office BP, predominantly mild current smoking was independently associated with an appreciable proportion of out-of-office BP in a community of African ancestry.

In 652 participants I subsequently assessed whether the angiotensin-converting enzyme (ACE) insertion/deletion (I/D) polymorphism accounts for the strong relationships between predominantly mild smoking and out-of-office BP. After

appropriate adjustments, an interaction between ACE DD genotype and current cigarette smoking, or the number of cigarettes smoked per day was independently associated with 24-hour and day diastolic BP (DBP) ($p<0.05$-0.005). This effect translated into a relationship between smoking and out-of-office BP or the risk for uncontrolled out-of-office BP only in participants with the DD as compared to the ID + II genotypes.

In conclusion therefore, I afford evidence to suggest that in groups of African ancestry, aldosterone, within ranges that cannot be accounted for by the presence of primary aldosteronism, modifies the relationship between salt intake and BP, and that genetic factors account for the variation in serum aldosterone concentrations in this group. Furthermore, I show that the ACE gene modifies the relationship between smoking and out-of-office BP and hence accounts for even predominantly mild smoking producing a marked and clinically important effect on out-of-office BP. The present thesis therefore provides further evidence in favour of an important pathophysiological role for the RAAS in contributing toward BP in groups of African ancestry.

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 Medicine, 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.

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LEON SCOTT

……day of ……., 2011

I certify that the studies contained in this thesis have the approval of the Committee for Research in Human Subjects of the University of the Witwatersrand, Johannesburg. The ethics numbers are M110243, M070469 and M020472.

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……day of ……., 2011

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GAVIN R. NORTON (Supervisor) ANGELA J. WOODIWISS (Supervisor)

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DEDICATION

This thesis is dedicated to John James Issel

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LIST OF ABBREVIATIONS

ACE	angiotensin-converting enzyme
AGT	angiotensinogen
Aldo	aldosterone concentration
ALLHAT	Antihypertensive and Lipid-Lowering treatment to prevent Heart Attack Trial
APOGH	African Program on Genes in Hypertension
ARR	aldosterone-to-renin ratio
AP-2	activator protein-2 transcription factors
BMI	body mass index
BP	blood pressure
CI	confidence intervals
cm	centimeters
conv.	conventional
DBP	diastolic blood pressure
dl	decilitre
DNA	nuclear deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DOCA	deoxycorticosterone
ECG	electrocardiogram
ERD	effective reflecting distance
h^2	heritability estimate

HbA1c	glycated haemoglobin
HCl	hydrochlorate
HDL	high density lipoprotein
I/D	insertion/deletion
^{125}I	^{125}iodine
K^+	potassium
KCl	potassium chloride
kg	kilogram
kg/m^2	kg per meter squared
l	litre
m/sec	meters per second
MAP	mean arterial pressure
mg	milligram
$MgCl_2$	magnesium chloride
ml	millilitre
mm Hg	millimetres of mercury
mmol	millimole
mmol/l	millimoles per litre
n	sample number
ng	nanogram
Na^+	sodium ion
NHLB	National Heart Lung and Blood
OR	Odds ratio

p	probability value
P1	forward or incident pressure wave
Paug	backward or reflective pressure wave
pc	parent-child pairs
PCR	polymerase chain reaction
pg	picogram
PP	pulse pressure
PWV	pulse wave velocity
r	correlation coefficient
r^2	coefficient of determination
RAAS	renin-angiotensin-aldosterone system
RDA	recommended daily allowance
RWTT	reflective wave transit time
S.A.G.E	Statistical Analysis for Genetic Epidemiology
SAS	statistical analyses software
SBP	systolic BP
SD	standard deviation
SEM	standard error of the mean
sib	sibling-sibling pairs
SOLVD	Studies of Left Ventricular Dysfunction
Soweto	South West Township
sp	spouse-spouse pairs
Tris-HCl	tris(hydroxymethyl)aminomethane-HCl

μl	microlitre
χ^2	chi-squared

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PREFACE

The studies described in the present thesis were prompted by recent concerns expressed regarding disease processes in urban, economically developing communities of black African ancestry in South Africa. In this regard, as highlighted in Chapter 1, contemporary evidence suggests that an epidemic of cardiovascular disease exists in these communities and that the dominant risk factor for these diseases is hypertension. The existing evidence also indicates that in these groups, hypertension is poorly controlled by current antihypertensive therapy, that these patients receive largely thiazide diuretic monotherapy and that few hypertensives receive blockers of the renin-angiotensin-aldosterone system (RAAS) even in combination with diuretic therapy, despite the high prevalence of left ventricular hypertrophy, the presence of which should normally compel clinicians to employ RAAS blockers.

The aforementioned therapeutic approaches may have arisen because in groups of black African descent, the dominant phenotype characterising hypertension is a “salt-sensitive, low-renin” state, a feature that casts doubt as to the role of the RAAS in contributing toward increases in blood pressure (BP) in groups of African ancestry. This approach has nevertheless failed to recognise that environmental factors that contribute toward increases in BP, such as a high salt intake, high calorie diets, a lack of physical activity and smoking may depend in-part on systems such as the RAAS to produce their effects. In this regard, our group have previously demonstrated an important interaction between obesity (which depends on high calorie diets and a lack of physical activity) and the RAAS to promote increases in BP. In the present thesis I have extended these notions

by exploring the possibility that various aspects of the RAAS interact with either salt intake or smoking to promote increases in BP.

In chapter 1 I have reviewed the literature that relates to the content of the present thesis and presented a series of arguments for the importance of conducting the studies included as part of the thesis. Chapters 2 to 5 are a series of semi-independent chapters each with an introduction, methods, results and discussion section, presenting arguments as to why the study described within each chapter could advance our current knowledge, describing the methods involved, showing the results of these studies and discussing how these studies advance our current understanding of each field as well the potential limitations of each study. In chapter 6 I provide a narrative that integrates each chapter in the context of our current understanding of the role of the RAAS in groups of African descent.

In support of the present thesis, the work contained within Chapters 2, 4 and 5 have been accepted for publication as full research papers in the *American Journal of Hypertension* (chapter 2) and the *Journal of Hypertension* (chapters 4 and 5).

Chapter 1

Current knowledge and outstanding evidence for a role of the renin-angiotensin-aldosterone system in blood pressure control with a specific focus on groups of African ancestry.

1.0 Introduction

The pathophysiological role of blood pressure (BP) as a cause of cardiovascular disease is well established. Indeed, as reviewed by guideline committees for the diagnosis and management of hypertension (Chobanian et al 2003, Mancia et al 2007, Williams et al 2004), numerous large clinical intervention studies have provided strong evidence to indicate that decreasing BP with antihypertensive agents reduces the risk for cardiovascular events. Although intervention studies provide the highest level of evidence, epidemiological data obtained from developed nations to support a role for BP in the pathophysiology of cardiovascular disease is equally as strong as the intervention data. In this regard, data obtained from economically developed countries show a continuous and positive relationship between BP and the risk of mortality from ischaemic heart disease and stroke (Lewington et al 2002). Moreover, in economically advanced countries an increased BP (hypertension) is the second most common risk factor for end stage renal disease (Chobanian et al 2003). In developed nations, hypertension is not only a risk factor for cardiovascular outcomes in the elderly but also in the young (18-39 year old) (Miura et al 2001). Despite the considerable evidence to indicate that hypertension plays an important role in the pathogenesis of cardiovascular disease in developed countries, the evidence for a role in emerging nations is by comparison considerably less substantial. Although some of these studies have been conducted in relatively small study samples, there is nevertheless some evidence to indicate that in economically emerging countries, which include populations of black African descent, hypertension is the most

prevalent risk factor for heart failure (Stewart et al 2008), coronary artery disease (Steyn et al 2005) and stroke (Mensah 2008, Connor et al 2009).

Despite the evidence to indicate that hypertension contributes to a substantial proportion of cardiovascular events in any country, in the United States of America, only ~34-35% of all hypertensives and only ~55% of treated hypertensives are controlled to target BP levels (Hertz et al 2005, Cutler et al 2008). By comparison, the control of BP in some European countries may be far worse (Wang et al 2007, Wolf-Maier et al 2004). Moreover, in economically emerging countries such as South Africa where the majority of the population are of black African ancestry, only ~14% of hypertensives at a national level (Steyn et al 2001) and ~33-44% of hypertensives in primary care settings (Steyn et al 2008, Dennison et al 2007) are controlled to target BP levels. Clearly the management of hypertension requires considerable improvement at a global level. However, some ethnic groups may be particularly at risk for the adverse effects of BP.

1.1 Ethnic disparities in the risk for cardiovascular disease.

In developed countries there is a greater prevalence of cardiovascular disease in groups of African as compared to European origins. Indeed, in the United States of America, the prevalence of strokes and major cardiovascular intermediate phenotypes for stroke, namely left ventricular hypertrophy and urinary albumin-to-creatinine ratios, are higher amongst groups of African as compared to European origins (Gillum 1999, Hollar et al 2004, McGruder et al 2004, Jamerson 2004, Howard 2001, Sacco et al 2001, Nunez et al 2005, Skelton et al 2003, Lorber et al 2003, Kizer et al 2004, Rodriguez et al 2004,

Drazner et al 2005, Murtaugh et al 2003, Bryson et al 2006). As strokes, left ventricular hypertrophy and urinary albumin-to-creatinine ratios are strongly determined by BP, these differences could be explained by the lack of BP control in groups of African ancestry. Is there evidence to show that BP control is worse in groups of African as compared to European ancestry?

In the United States of America, in contrast to the 59.7% of hypertensives of European ancestry that are controlled to target BP levels, 48.9% of hypertensives of African ancestry are controlled to target BP levels (Hertz et al 2005). Moreover, in economically emerging countries such as South Africa where the majority of the population are of black African ancestry, a marked ethnic disparity in BP control rates has also been reported on (Steyn et al 2001). Ethnic differences in BP control rates have been attributed to some extent to differences in the care given to hypertensives (Steyn et al 2001). However, this cannot be the only explanation as in developed nations, where gaps in care provision are generally not determined on racial or ethnic grounds, hypertensives of African descent are more likely than hypertensives of European descent to receive medication and reduce salt intake and to adhere to these interventions, whilst BP control is still worse (Natarajan et al 2009). What are the other possibilities that could explain the poor BP control rates in groups of African ancestry?

1.2 Ethnic disparities in the prevalence of hypertension: Could pathophysiological differences explain differences in prevalence rates?

There is a higher prevalence of hypertension in groups of African as compared to European ancestry, differences which are not fully accounted for by lifestyle or body size (Hajjir and Kotchen 2003, Hertz et al 2005). The exact reasons for the higher prevalence rates in this ethnic group have not been elucidated. Is it possible that groups of African ancestry have different pathophysiological processes causing their hypertension than other ethnic groups? In this regard, as will be reviewed in subsequent sections, there is now considerable evidence to indicate that salt intake is a major pathophysiological mechanism responsible for hypertension in groups of African descent whilst the activity of the renin-angiotensin-aldosterone system (RAAS), particularly renin (but not aldosterone), is markedly reduced. As a consequence, RAAS blockers may not decrease BP in a significant proportion of hypertensives (Woodiwiss et al 2006) and current guidelines do not recommend RAAS blockers as first line therapy in groups of African ancestry (Chobanian et al 2003, Williams et al 2004). Moreover, guidelines for the diagnosis and management of hypertension in the African context indicate that diuretics rather than alternative agents, such as RAAS blockers, should be used as first line therapy (Seedat et al 2006) and indeed RAAS blockers are used extremely infrequently in black South Africans (Maseko et al 2006, Maseko et al 2011).

Despite the limited use of RAAS blockers in groups of African descent (Maseko et al 2006, Maseko et al 2011), as groups of African descent have a high prevalence of left ventricular hypertrophy (Woodiwiss et al 2008), and a compelling indication for the

use of RAAS blockers is left ventricular hypertrophy (Dahlöf et al 2002), there is a distinct possibility that the lack of use of RAAS blockers in groups of African ancestry in South Africa may have deleterious consequences. Are there unequivocal arguments to indicate that RAAS blocker use is of little value in groups of African descent? In this regard a proportion of hypertensives of African ancestry will respond to RAAS blockade when used as monotherapy (Woodiwiss et al 2006). Moreover, when employed together with diuretics, in groups of African ancestry RAAS blockers are able to reduce BP to a similar extent as that noted in groups of European ancestry (Libhaber et al 2004). Therefore, the question of the role of the RAAS in BP control in groups of African ancestry has not been completely resolved. Further, whether combination diuretic and RAAS blocker use should be employed as first line therapy in hypertensives of African descent has not been established.

Consequently, as part of the present thesis I have explored a number of possible outstanding questions with regards to the role of the RAAS in BP control in a community sample of African ancestry. In the present chapter I will therefore outline the current evidence to support or refute a role of the RAAS in BP control in groups of African descent? In this process I will also underscore some of the outstanding questions which have not been addressed and indicate the questions that I have attempted to answer in the present thesis. However, before doing so, it is important to outline the evidence to support a role for the RAAS in BP control in general.

2.0 Evidence to support a role of the renin-angiotensin-aldosterone system in blood pressure control

There is no question that the RAAS plays an important role in BP control. This has been established in a number of preclinical animal studies as well as in human studies. In preclinical studies the role of the RAAS in BP control has been established at a variety of levels. These studies include numerous studies demonstrating antihypertensive effects of angiotensin-converting enzyme (ACE) inhibitors, angiotensin II receptor blockers and aldosterone receptor antagonists in animal models of hypertension. These studies also include animal studies where non-genetic manipulations have been designed to increase the activity of the RAAS and the consequence is hypertension. Examples of these non-genetic manipulations include “Goldblatt” hypertension (one or two-kidney) where renin release from the kidney is increased by decreases in renal blood flow and pressure, or manipulations that involve increases in mineralocorticoid effects, such as the deoxycorticosterone (DOCA)-salt model and aldosterone infusion. Furthermore, at a preclinical level, a number of studies have demonstrated not only that hypertension occurs in response to an increased circulating RAAS, but also that most forms of hypertension, irrespective of the cause, are associated with an increased tissue (vascular and renal) RAAS expression and activity. There are presently too many of these studies to review in the present thesis. More recently however, additional substantial support for a role for the RAAS in BP control at a physiological level has been obtained from preclinical (animal) studies involving genetic modulation of the system. As these studies

provide direct evidence to establish a physiological role for components of the RAAS in BP control, these studies warrant special mention.

2.1 Genetic manipulations of components of the renin-angiotensin-aldosterone system influence blood pressure.

Genetic manipulation in animals of almost all components of the RAAS has provided substantial direct evidence to support a role for the RAAS in BP control. In this regard, transgenic mice over-expressing the gene for renin have higher BP values than their littermates (Morimoto et al 2002). Furthermore, knockout (Tanimoto et al 1994) or decreasing the function with adenoviral vectors (Stec et al 2002) of the gene for angiotensinogen, the substrate upon which renin acts to form angiotensin I, causes hypotension. In addition, transgenic mice carrying the rat angiotensinogen gene develop hypertension (Kimura et al 1992) and inactivation, or duplication the angiotensinogen gene in mice, results in marked decreases or increases in BP respectively (Kim et al 1995). Moreover, a dramatic 34 mm Hg decrease in BP occurs in male, but not female mice in response to functional inactivation of the gene for angiotensin-converting enzyme (Krege et al 1995) the enzyme responsible for converting angiotensin I to angiotensin II. Last, genetic manipulation resulting in over expression of aldosterone is a necessary prerequisite to mediating the hypertensive effects of salt-intake (Makhanova et al 2008). As a consequence of these animal studies that have provided direct evidence to support a physiological role for the RAAS in BP control, there is little question as to the relevance

of the RAAS in mediating BP effects. However, what is the evidence in human studies to support a role for the RAAS in BP control?

2.2 Evidence from human studies to support a role for the renin-angiotensin-aldosterone system in blood pressure control

In human studies the strongest support for a role for the RAAS in BP control is derived from the numerous intervention studies demonstrating a BP lowering action of renin inhibitors, angiotensin-converting enzyme (ACE) inhibitors, angiotensin II receptor blockers and aldosterone receptor antagonists. A review of these numerous studies nevertheless goes beyond the scope of the present thesis, but it is important to highlight that this evidence is sufficiently well established that all of the more recent guidelines for the diagnosis and management of hypertension recommend RAAS blockers in the management of hypertension (Mancia et al 2007, Chobanian et al 2003, Williams et al 2004). Further evidence in favour of a role for the RAAS in human studies are the equally numerous studies that have established a role for this system as the primary cause of some forms of secondary hypertension. In this regard an increased renin release is responsible for increases in BP in renovascular hypertension (Laragh 1986) and an increased release of aldosterone from adrenal gland is responsible for an increase in BP in adrenal hyperplasia or adrenal adenomas, thus causing primary hyperaldosteronism (Conn's syndrome) (Stowasser et al 2001). However, apart from a role for the RAAS in secondary forms of hypertension, is there evidence beyond the antihypertensive effects of RAAS blockers, that the RAAS could play a significant role in the pathogenesis of

human essential hypertension? This question has an important context as there are many effective antihypertensive agents that have been developed that do not necessarily target the underlying pathophysiological mechanisms responsible for essential hypertension.

2.2.1 Circulating components of the renin-angiotensin-aldosterone system and blood pressure in primary hypertension or at a population level.

In early studies in patients with essential hypertension and their offspring, higher circulating angiotensin II concentrations were noted as compared to normotensive controls (Fasola et al 1968). Although subsequent evidence to support these data appears to be scarce, probably because of the difficulties encountered in accurately measuring circulating angiotensin II concentrations, a more recent study has nevertheless demonstrated that circulating angiotensin II concentrations are indeed associated with an increased risk for hypertension (Harrap et al 1996). There are also a number of lines of evidence to show that substrates or enzymes important in the synthesis of angiotensin II are also associated with BP. In this regard, a relationship between BP and circulating concentrations of the renin substrate and precursor of angiotensin I, namely angiotensinogen was first reported in hypertensives in 1979 (Walker et al 1979). This evidence was further supported by data published in 1980 (Ito et al 1980) and in later years (Bennet et al 1993, Bloem et al 1995). Moreover, hypertensive offspring of hypertensive parents have higher circulating angiotensinogen concentrations than normotensive offspring of hypertensive parents (Watt et al 1992). With respect to angiotensin-converting enzyme, the enzyme responsible for the conversion of angiotensin

I to angiotensin II, the activity of this enzyme has also been shown to be associated with BP at a population level (Schunkert et al 1996). With respect to substances produced as a consequence of the activity of angiotensin II, as noted in the Framingham Heart Study concentrations of circulating aldosterone have been shown to be associated with the incidence of hypertension (Vasan et al 2004) data which has subsequently been confirmed in alternative studies (Meneton et al 2008). Insights into the potential point in the pathway that may explain this relationship were provided by further data from the Framingham Heart Study demonstrating that the aldosterone-to-renin ratio is also associated with the incidence of hypertension (Newton-Cheh et al 2007), thus suggesting that abnormalities downstream from renin may explain the aldosterone-BP relationship.

Despite the data to show the aforementioned relationships between components of the RAAS and BP, in the majority of these studies the circulating concentrations of these substances were only modestly related to BP or related to BP in sub-groups of participants rather than in a cross-section of the population. Moreover, relationships between blood concentrations of components of the RAAS and BP do not necessarily indicate cause-effect relationships. Furthermore, relationships between circulating concentrations of the RAAS and BP do not necessarily account for the impact of the well-established presence of tissue (local autocrine and paracrine) RAAS in contributing toward increases in BP. Thus, in more recent years, to attempt to establish a cause-effect relationship between components of the RAAS and BP in human populations, a number of investigators have turned to genetic studies. In this regard, the underlying assumption is that associations between gene variants of the RAAS, which influence the expression of components of the RAAS, and BP are likely to reflect a cause-effect relationship and

could be accounted for by the actions of either circulating or tissue RAAS effects. Nevertheless, as shall be highlighted in subsequent discussion, genetic studies assessing relationships between RAAS genes and BP have unveiled a number of further problems inherent in the field of polygenic traits.

2.2.2 Gene variants of the renin-angiotensin system and blood pressure at a population level or in primary hypertension.

With respect to effects on BP at a population level or on the prevalence or incidence of primary hypertension, perhaps the best studied genes are the angiotensin-converting enzyme (ACE) and the angiotensinogen (AGT) genes. These were also the genes that I focussed on as part of my thesis. The following section provides an overview of the current evidence to support or refute a role for these genes in controlling angiotensin-converting enzyme activity and angiotensinogen expression or concentrations. The following section also reviews the current evidence to support or refute a role for these genes in controlling BP control at a population level or in primary hypertension.

2.2.2.1 Association between the angiotensin-converting enzyme gene and angiotensin-converting enzyme concentrations

Does the ACE gene influence circulating angiotensin-converting enzyme concentrations or activity? In this regard, in 1990, Rigat et al (1990), produced evidence

to show that an insertion/deletion (I/D) polymorphism (variant) in intron 16 of the ACE gene was strongly associated with circulating angiotensin-converting enzyme concentrations. In participants homozygous for the D allele (DD genotype), plasma angiotensin-converting enzyme concentrations were on average twice those of participants homozygous for the I allele (II genotype), whilst those heterozygous for the I/D variant had plasma angiotensin-converting enzyme concentrations intermediate between the two. These data suggest that the ACE gene I/D variant has a co-dominant effect. Subsequent combined segregation and linkage analysis further supported the notion that the ACE gene contributes toward plasma angiotensin-converting enzyme concentrations (Tiret et al 1992). However, the I/D polymorphism is found in a region that is not translated (intron). The relationship between the I/D variant and angiotensin-converting enzyme concentrations is therefore likely to be explained by the fact that the variant exists in linkage disequilibrium with a variant elsewhere in the ACE gene. Has the causal variant of the ACE gene been identified?

Further segregation and linkage analysis identified two quantitative trait loci (genetic regions related to a quantitative trait) that may account for the relationship between the I/D variant and plasma angiotensin-converting enzyme concentrations (McKenzie et al 1995) and that one of the two quantitative trait loci may involve either the I/D variant itself, despite the fact that it is not translated, or an alternative variant downstream from the I/D polymorphism (4656 $[CT]_{2/3}$) (Villard et al 1996). Moreover, mutations in exons 1-5 and introns 1-4 and part of intron 5 upstream from the I/D variant were excluded (Farrall et al 1999). However, despite the previous suggestion that the I/D polymorphism may be functional (Villard et al 1996), subsequent fine mapping excluded

the I/D variant, and suggested that a variant downstream from the I/D variant was likely to be involved (Zhu et al 2000). No further studies have emerged that point to the functional variant within the ACE gene that could account for relationships between the I/D polymorphism and circulating angiotensin-converting enzyme concentrations.

Although the functional mutation in the ACE gene has not as yet been identified, there is nevertheless currently strong data to show that the ACE gene controls angiotensin-converting enzyme concentrations. In this regard a recent meta-analysis of small and large studies has demonstrated consistent relationships between the I/D variant and plasma angiotensin-converting enzyme concentrations with an overall ~56% increase in angiotensin-converting enzyme concentrations in people with the DD as compared to people with the II genotype (Agerholm-Larsen et al 2000). Moreover, the 4656$[CT]_{2/3}$ variant has also been shown to have a strong relationship with plasma angiotensin-converting enzyme concentrations (Paillard et al 1999). What is the evidence for and against a role for the ACE gene in human BP control?

2.2.2.2 The angiotensin-converting enzyme gene and blood pressure

A number of approaches have been adopted to evaluate the hypothesis that the ACE gene plays a role in BP control and each will be considered in-turn. In this regard, linkage analysis, cross-sectional prevalence analysis, meta-analyses and prospective incidence analysis have all been performed in large studies to evaluate the role of the ACE gene. Although a number of genome-wide association or linkage studies have been performed which incorporate markers for the ACE gene as part of the “gene chips”, these

will not be discussed as to achieve statistical power when employing Bonferroni or modified Bonferroni corrections to adjust for what is often thousands of multiple comparisons in these studies, would require probability values which are so large that few loci contributing to a polygenic effect would achieve significance. In addition, as will be discussed later (see sections 2.2.2.3 to 2.2.2.5) these approaches fail to account for possible gene-environment and gene-phenotype interactions (including gender, body size, salt intake and smoking).With respect to cross-sectional data, although a number of small studies have been published, I will only review the outcomes of large population-based studies (>1000 participants), as these are less likely to produce false positive or false negative outcomes.

On the whole the linkage data better substantiate (Fornage et al 1998, O'Donnell et al 1998) than refute (Jeunemaitre et al 1992) a role for the ACE gene in BP control. This conclusion is based on the fact that linkage with the ACE gene has been shown in two independent study populations (Fornage et al 1998, O'Donnell et al 1998) consisting of a markedly greater sample sizes than the study that failed to show linkage (Jeunemaitre et al 1992). Furthermore, linkage analysis supporting a role for the ACE gene employed quantitative trait variance components based linkage analysis (allele sharing non-parametric based techniques) (Fornage et al 1998, O'Donnell et al 1998) which does not require a precise specification of the genetic models (unlike classical linkage analysis). The caveat is that the linkage data supporting a role for the ACE gene in BP control was not for BP in general and was not for both sexes, but was specifically for diastolic BP in males only (Fornage et al 1998, O'Donnell et al 1998). These findings are consistent with data showing a profound decrease in BP after functional inactivation of the ACE gene in

male, but not female mice (Krege et al 1995). Importantly, the study that failed to show linkage (Jeunemaitre et al 1992) of the ACE gene to hypertension was not statistically powered to assess sex-specific effects and did not employ a quantitative based approach, thus excluding the potential for analysis of diastolic separate from systolic BP.

Cross-sectional, population-based data also support a role for the ACE gene in BP control in men only (O'Donnell et al 1998, Higaki et al 2002). However, there are equally as strong data that suggest a lack of role for the ACE gene in BP control (Agerholm-Larsen et al 1997, Matsubara et al 2002, Zaman et al 2001, Castellano et al 2003). The sample sizes for all of these studies were particularly impressive with the total sample for cross-sectional data favouring a role for the ACE gene numbering 8109 (O'Donnell et al 1998, Higaki et al 2002) and the total sample for cross-sectional data (excluding data where possible population stratification exists) against a role for the ACE gene in BP control numbering 15196 (Agerholm-Larsen et al 1997, Matsubara et al 2002, Zaman et al 2001). There is no particular limitation in most of the cross-sectional data either favouring (O'Donnell et al 1998, Higaki et al 2002) or rebutting (Agerholm-Larsen et al 1997, Matsubara et al 2002, Zaman et al 2001, Castellano et al 2003) a role for the ACE gene in BP control or hypertension. There were nevertheless some differences in the population samples studied. In addition, the approaches employed to show an ACE gene effect on BP in studies with a positive outcome were not always employed in studies that failed to show a relationship between ACE genotype and BP. What were these differences?

In the largest study (10 150 participants) that failed to show a relationship between ACE genotype and BP (Agerholm-Larsen et al 1997), the diastolic BP values

appeared to be higher (~81-85 mm Hg) than in the populations (~78-81 mm Hg) where ACE genotype was related to BP (O'Donnell et al 1998, Higaki et al 2002). This suggests that different factors may have been contributing to diastolic BP in these populations. Furthermore, in a population study conducted at a national level in 2461 Italians (Castellano et al 2003), that similarly failed to show a relationship between ACE I/D genotype and hypertension, the proportion of hypertensives in this study (~75%) was exceptionally high for a cross-sectional study (Castellano et al 2003). In this same study (Castellano et al 2003), ACE I/D allele frequencies differed in the North as compared to the South of Italy, suggesting population stratification. Furthermore, in the study by Castellano et al (2003), effects on diastolic BP were not assessed separately from systolic BP. In the Ohasama Study, a large cross-sectional study conducted in 1245 Japanese participants (Matsubara et al 2002), although no significant association between ACE genotype and hypertension diagnosed with home BP measurements occurred, a trend effect in elderly males was noted (Odds ratio=2.20 in DD vs. II genotypes) which if the study had been of a larger sample size could have achieved statistical significance. Furthermore, in the Ohasama Study the impact of ACE genotype on hypertension diagnosed on the basis of diastolic BP measurements alone was not assessed (Matsubara et al 2002). In the Shibata Study, an alternative study conducted in 1340 Japanese (Zaman et al 2001), although the association between ACE I/D genotype and BP did not achieve statistical significance, again the probability values for the multivariate adjusted relations between ACE I/D genotype and diastolic BP in men was p=0.06. Thus, the Shibata Study (Zaman et al 2001) was similarly statistically underpowered to achieve a significant association for diastolic BP in men. Last, although Mondry et al (2005) in a

case-control study of 1358 people failed to show a relationship between ACE I/D genotype and hypertension, no analysis of diastolic BP or hypertension diagnosed on diastolic BP alone was performed, and no sex-specific analysis was conducted.

Meta-analyses conducted on a considerable number of small and large case-control studies do not support an association between the ACE gene I/D polymorphism and hypertension or variations in BP (Agerholm-Larsen et al 2000, Staessen et al 1997). However, there are a number of factors which favour a negative rather than a positive outcome in these meta-analyses. First, the analyses were performed on pooled data from studies with different designs (case-referent, case-control, cohort, twin, and family-based). Further, no sex-specific analyses were conducted and hence this approach precluded the possibility of detecting an ACE gene effect in males as reported on in other studies (Fornage et al 1998, O'Donnell et al 1998, Higaki et al 2000). In addition, one meta-analysis contesting a role for the ACE gene in BP control only showed data for systolic BP (Agerholm-Larsen et al 2000). Consequently, the potential impact of the ACE gene on diastolic BP, as suggested by other studies (Fornage et al 1998, O'Donnell et al 1998, Higaki et al 2000) was not evaluated.

Two relatively large (n=684 and 678) prospective studies have reported on a relationship between ACE genotype and the development of hypertension over 6-12 year follow up periods (Staessen et al 2001, Di Pasquale et al 2004). Importantly, cross-sectional data mostly rely on a diagnosis of hypertension being made by the presence of treatment and hence are subject to physician bias. In contrast, incidence data, which allow for an objective diagnosis of hypertension being made according to current guidelines (increased BP on three separate occasions and measured with trained observers), may

provide more insights than cross-sectional studies. There are presently no studies that have reported a lack of association between the ACE I/D variant and the incidence of hypertension.

2.2.2.3 Association of the angiotensinogen gene with angiotensinogen concentrations.

Does the AGT gene influence circulating angiotensinogen concentrations? In 1992 Jeunemaitre et al (1992) demonstrated a relationship between a T→C nucleotide substitution at position 704 in exon 2 of the AGT gene and plasma angiotensinogen concentrations in two independent study samples. Although this nucleotide substitution translates into a M→T amino acid change at position 235 of the angiotensi nogen protein (M235T polymorphism), the mechanism responsible for an influence of this polymorphism on angiotensinogen concentrations was not demonstrated. However, a number of subsequent studies provided further evidence to support a role for variations within the AGT gene as determinants of plasma angiotensinogen concentrations. In this regard, in more recent studies the AGT gene has been shown to account for ~5% of the variance of plasma angiotensinogen concentrations (Brand et al 2002). Furthermore, in a meta-analysis of seven studies with a total of 1085 participants, those participants homozygous for the T allele of the M235T variant were estimated to have plasma angiotensinogen concentrations ~11% higher than those participants homozygous for the M allele ($p<0.00001$) (Sethi et al 2003).

As there is no evidence to support the notion that the M235T variant is functional, the likely explanation for the strong relationship between this variant and plasma angiotensinogen concentrations is that it is in linkage disequilibrium with potentially functional variants elsewhere in the AGT gene. In this regard, linkage disequilibrium between the M235T variant and a number of variants within the highly polymorphic promoter region of the AGT gene, which could influence angiotensinogen expression, may explain the relationship between the AGT gene and circulating angiotensinogen concentrations. Indeed, a G→A substitution at position -6 is associated with increased basal angiotensinogen transcription rates (Inoue et al 1997). Furthermore, an A→C substitution at position -20, which determines whether a major late transcription factor rather than the oestrogen receptor (oestrogen is well known to determine angiotensinogen expression) binds to the promoter (Zhao et al 1999) accounts for ~10% of the variability of plasma angiotensinogen concentrations (Ishigami et al 1997). Moreover, a G→A substitution at position -217 is also associated with increased basal angiotensinogen transcription rates (Jain et al 2002). Last, although, there is no direct evidence for an effect on AGT gene transcription, a G→T substitution at position -532 of the AGT gene, which is located within a consensus sequence to the transcription factor AP-2, is strongly associated with plasma AGT concentrations (Paillard et al 1999). What is the evidence for and against a role for the AGT gene in human BP control?

2.2.2.4 The angiotensinogen gene and blood pressure.

As with the ACE gene, a number of approaches have been adopted to evaluate the hypothesis that the AGT gene plays a role in BP control and each will be considered in-turn. With respect to these approaches, linkage analysis, cross-sectional prevalence analysis and meta-analyses have all been performed in large studies to evaluate the role of the AGT gene. However, as yet, large prospective studies with incidence data have not been reported on. As with the ACE gene, although a number of genome-wide association or linkage studies have been performed which incorporate markers for the AGT gene as part of the "gene chips", these will not be discussed for the reasons previously indicated. That is, to achieve statistical power when employing Bonferroni or modified Bonferroni corrections to adjust for what is often thousands of comparisons in these studies, would require probability values which are so large that few loci contributing to a polygenic effect would achieve significance. As with the ACE gene, again with respect to cross-sectional data, although a number of small studies have been published, I will only review the outcomes of large population-based studies (>1000 participants), as these are less likely to produce false positive or false negative outcomes.

Strong linkage and family-based association data both verify and refute a role for the AGT gene in contributing toward BP. In this regard, there are four studies that support linkage of the AGT gene to hypertension (Jeunemaitre et al 1992, Caulfield et al 1994, Caulfield et al 1995, Baker et al 2007) and five studies that do not support linkage or family-based associations (Brand et al 1998, Niu et al 1999, Niu et al 1998, Niu et al 1999[b], Wang et al 1999). In linkage studies, a wide range of sample sizes have been

employed. In this regard, one of the studies that failed to show linkage (Brand et al 1998) had an impressive sample size (n=350 families and 630 affected sibling pairs) and most likely included data from a number of the groups that had previously reported on linkage or a lack thereof. A limitation of the large study that failed to show linkage (Brand et al 1998) was that the method of analysis employed is sensitive to allele frequency estimations when the parental genotypes are not known. Nevertheless, in that study (Brand et al 1998) the same analytical methods were employed as in most of the studies demonstrating linkage between the AGT gene and hypertension (Jeunemaitre et al 1992, Caulfield et al 1994, Caulfield et al 1995). Moreover, in support of this large study that failed to show linkage of the AGT gene to hypertension (Brand et al 1998), using analytical techniques which do not rely on precise specifications of the genetic models, in 335 hypertensives from 315 nuclear families, a second group failed to show excess transmission of AGT gene variants to hypertensive Chinese (Niu et al 1999, Niu et al 1999[b]). What should nevertheless be underscored is that unlike the ACE gene where linkage to diastolic, but not systolic BP was identified, presently there are no linkage studies that have attempted to assess the contribution of the AGT gene to either systolic or diastolic BP considered as separate traits and which pathophysiologically are to some extent driven by separate mechanisms.

Similar to the linkage data, large (>1000 participants) cross-sectional and association studies provide evidence both for and against a role for the AGT gene in contributing toward BP. Variants of the AGT gene have been shown to be a risk factor for hypertension in 9100 randomly selected participants of the Copenhagen Heart Study (this association was in women only) (Sethi et al 2001) and in 2461 participants sampled

from the general population in Italy (Castellano et al 2003). A role for the AGT gene in BP control is also supported by an association between AGT genotype and ambulatory pulse pressure in 1425 individuals from 248 families each with one proband (Baker et al 2007), and home BP measurements in 1245 individuals randomly selected from the general Japanese population (Matsubara et al 2003). A confounding characteristic of these studies is nevertheless that the authors used different AGT gene markers to show these effects. These markers included the T→M at amino acid position 174 [C→T substitution at nucleotide position +521 (Sethi et al 2001), the M235T variant (Castellano et al 2003), T+31C (Matsubara et al 2003), and the C→T substitution at nucleotide position -532 (Baker et al 2007) within the AGT gene. Thus, the exact causal variant of the AGT that contributes toward BP control still remains uncertain. Last, recent analysis of the Framingham data in 2155 participants shows an association between AGT genotype and BP using the general estimations equation (Levy et al 2007). However, this analysis was part of a much larger study intended to generate rather than test hypotheses and hence the outcomes were not corrected for multiple genotyping.

As with the major studies showing positive associations, there are similarly key studies which have failed to show a relationship between the AGT gene and BP. In 4322 participants of the NHLB Family Blood Pressure Program, (which nevertheless pooled data from different study designs including sib-pair, sib-ship and extended family designs)(Province et al 2000), and in 904 randomly selected African-Americans (Larson et al 2000), no relationship between G→A (-6) polymorphism and hypertension was noted. Furthermore, despite showing strong heritability estimates for the BP values obtained, in 1006 participants of a genetically isolated Dutch population sample, no

relationship between the M235T variant and BP was noted (van Rijn et al 2007). Last, in a large case-control study in 1358 participants, participants homozygous for the T allele of the AGT M235T variant even had a decreased risk for hypertension (Mondry et al 2005).

Three meta-analyses conducted on a substantial number of small and large case-control studies, with overall large total sample sizes (n=5493-18704), support a role for the AGT gene in contributing toward hypertension (Sethi et al 2003, Staessen et al 1999, Kunz et al 1997, Kato et al 1999). These meta-analyses nevertheless indicate that a publication bias in favour of positive outcomes may have occurred.

2.2.2.5 Potential explanations for discrepancies in data assessing the relationship between renin-angiotensin system genes and blood pressure.

As reviewed in the preceding paragraphs, studies assessing the genetic determinants of BP provide strong evidence both for and against a role for genetic variants of the ACE and AGT genes in BP control. These contrasting outcomes may characterise polygenic traits. In this regard, gene variant effects on BP should never be expected in all study samples. This point is analogous to the heterogeneous effects of environmental or phenotypic risk factors on BP, such as the marked BP effects of obesity or an excess salt intake in some individuals, whilst in others there is little or no effect on BP. This point may be illustrated by a preclinical example. Indeed, under basal conditions, mice with two or three copies of the ACE gene may have similar BP values as

mice with one copy of the ACE gene, despite marked differences in ACE concentrations (Krege et al 1997). However, when infusing angiotensin I, substantial increases in BP occur in mice with two or three copies as compared to one copy of the ACE gene (Krege et al 1997). Thus in this example of differences in ACE gene copy numbers (Krege et al 1997) the penetrance of the genetic variation depends entirely on whether upstream molecules (i.e., angiotensin I) are present in excess. To further support this argument with human data as an example, although the ACE 4556$(CT)_{2/3}$ genotype and the C→T(-532) variant of the AGT gene are strongly associated with plasma angiotensin-converting enzyme or angiotensinogen concentrations respectively in humans, in the same individuals this does not translate into increases in circulating angiotensin II or aldosterone concentrations (Paillard et al 1999). Presumably in the presence of excessive angiotensin I, people with the genotype that predisposes to increased circulating angiotensin-converting enzyme or angiotensinogen concentrations would develop higher BP levels. In short, the penetrance of RAAS genes should depend on a wide variety of factors. The obvious question therefore is under what conditions could we expect to note an ACE or AGT gene effect on BP?

Interactions between genotype and environmental factors or phenotypic traits are likely to characterise a polygenic trait. A good example is salt-sensitive hypertension, where a dramatic increase in BP may occur in some individuals in response to a salt load, whilst in salt-insensitive individuals who presumably lack the susceptibility gene(s), BP is maintained in response to a salt load. Is their evidence that the ACE or AGT genes interact with environmental or other phenotypic factors to influence BP?

2.2.2.5.1 Interaction between gender and renin-angiotensin system genes.

Probably the best evidence for an interaction between RAAS genes and phenotypic features influencing BP has previously been alluded to. That is, there appears to be a sex-specific effect of the ACE gene on BP in humans (Fornage et al 1998, O'Donnell et al 1998, Higaki et al 2000) and in genetically manipulated mice (Krege et al 1995). In this regard, the consistency of the relationship between ACE gene variations and BP in only males across all of these studies provides confidence that this interaction is real. Although, the mechanisms of the interactive effect of male gender and the ACE gene on BP are as yet unclear, and hence warrant further investigation, it has been suggested that an interaction between the Y chromosome and a locus that maps close to the ACE gene impacts on BP responses to salt loading in spontaneously hypertensive rats (Kreutz et al 1996). As discussed in the aforementioned sections, a sex-specific effect may also characterise AGT gene effects on BP. Indeed, in a large (n=9100) population-based study supporting a role for the AGT gene in hypertension, a relationship between the AGT gene and BP was noted (Sethi et al 2001), but this effect was noted in women only. This sex-specific effect is consistent with the well described effect of oestrogen on angiotensinogen expression. However, no other studies have explored a sex-specific effect of the AGT gene and thus, further studies are warranted to determine whether this genotype-gender interaction indeed exists. Are there other potential interactions that need to be considered?

2.2.2.5.2 Interaction between body-size and renin-angiotensin system genes.

There is evidence that body size interacts with both the ACE and the AGT genes to determine BP. With respect to the ACE gene, in a study conducted in 1875 persons, the ACE gene I/D variant was shown to interact with body size to influence body size effects on BP (Turner et al 1999). With regards to the AGT gene, in a study conducted by our group, the A→C(-20) variant of the AGT gene was demonstrated to produce a marked impact of body size on 24-hour systolic BP in 521 untreated hypertensives off medication (Tiago et al 2002). This interaction was explained by the fact that adipose tissue is a strong source of circulating angiotensinogen concentrations (Cassis et al 1988, Sharma et al 2001) and that the promoter region of the AGT gene may influence the expression and subsequently the release of angiotensinogen from adipose tissue. Indeed, AGT mRNA levels in adipose tissue have been shown to be about 60% of those found in the liver (Cassis et al 1988), and plasma leptin (produced from adipose tissue and hence a reflection of the degree of obesity) and plasma AGT concentrations are closely correlated (Schorr et al 1998). The notion that an interaction occurs between obesity and the AGT gene to influence BP are supported by a trend for a greater reduction in BP (-2.4 vs +0.3 mm Hg, p=0.05) in response to weight reduction in the Trials of Hypertension Prevention in people with the AA as compared to the GG genotype of the G→A(-6) variant of the AGT gene (Hunt et al 1998). Moreover, the concept that an interaction occurs between obesity and the AGT gene to influence BP is also supported by the interaction between obesity and the AGT gene to determine renal vascular responses to angiotensin II infusions (Hopkins et al 1996).

2.2.2.5.3 Interaction between salt intake and renin-angiotensin system genes.

As indicated in the aforementioned discussion, a good example of interactions between genetic and environmental factors as determinants of BP is salt-sensitive hypertension, where BP increases in some individuals in response to a salt load, whilst in salt-insensitive individuals who may lack the susceptibility gene(s), BP is maintained in response to a salt load. Is there evidence that RAAS genes may interact with salt intake to determine BP. Indeed, albeit evidence obtained in small study samples (n=71) (Poch et al 2001)(n=61)(Hiraga et al 1996) the ACE gene has been shown to determine the extent to which salt intake increases BP. However, in these studies (Poch et al 2001, Hiraga et al 1996) the I allele of the I/D polymorphism, which is related to reduced angiotensin-converting enzyme concentration, was associated with a greater BP response to a salt load. More impressive data has nevertheless also been obtained in the Trials of Hypertension Prevention study, where after a three year follow-up period in 1509 participants with diastolic BP values of 83-89 mm Hg, those persons homozygous for the A allele of the G→A(-6) variant of the AGT gene developed a greater response to a reduced salt intake than in those individuals homozygous for the G allele (-2.2 vs +1.1 mm Hg, p=0.01) (Hunt et al 1998). Considering that the overall systolic BP/diastolic BP response to ~77 mmol/l salt restriction in that study (Hunt et al 1998) was -4.8/-2.5 mm Hg, the genotype effect can indeed be considered to be of importance. Despite these data supporting a view that an interaction between RAAS genes and salt intake influences BP, considerably more evidence is required to validate this hypothesis.

2.2.2.5.4 Interaction between smoking and renin-angiotensin system genes.

Renin-angiotensin-aldosterone system genes may also interact with cigarette smoking to determine BP (Schut et al 2004, Xu et al 2004). Indeed, a modest effect of the ACE gene I/D polymorphism on BP in 1508 current smokers has been shown to occur in cigarette smokers, but not non-smokers (Schut et al 2004). Moreover, an interaction between smoking and ACE genotype was noted to modify the risk for hypertension in 1099 Mongolians (Xu et al 2004). Hence the effect of the ACE gene could depend on cigarette smoking or at least modify the impact of smoking on BP or the risk for hypertension. What are the potential mechanisms that explain how an interaction between cigarette smoking and ACE genotype contributes toward BP? In this regard, there is an additive effect of the ability of smoking and the ACE genotype to modify vascular responses to acetylcholine (Butler et al 1999). Indeed, both smoking and the risk genotype of the ACE gene are associated with a decreased vasodilator response to acetylcholine and the two effects together produce a greater impact (Butler et al 1999). This interactive effect may be explained by the fact that that nicotine influences endothelial cell angiotensin-converting enzyme expression (Saijonmaa et al 2005). As ACE genotype has a marked effect on endothelial-dependent vasodilation (Butler et al 1999), it is possible that ACE genotype may interact with nicotine to modify endothelial cell angiotensin-converting enzyme expression and hence endothelial cell function, the consequence being effects on BP.

The modest association ($p<0.05$) between ACE genotype and BP in 1508 current smokers (Schut et al 2004) nevertheless does not provide confidence in the outcomes of

that study. Moreover, in that study (Schut et al 2004), smoking effects on BP were difficult to identify and this confounds the outcomes of the study. Nevertheless, a lack of impact of smoking on in-office BP is not surprising considering the current scientific evidence for smoking effects on BP. Indeed, the concept that smoking influences BP is highly controversial. In this regard, although several studies indicate that cigarette smoking acutely increases systolic and diastolic blood pressure (BP) (Kool et al 1993, Mahmud et al 2003, Failla et al 1997, Rhee et al 2007, Gropelli et al 1999) the evidence for a significant impact of smoking on in-office BP at a community or population level has produced discrepant outcomes (Thuy et al 2010, Tesfaye et al 2008, Niskanen et al 2004, Halimi et al 2002, Mundal et al 1997, Okubo et al 2004, Primatesta et al 2001, Lee et al 2001, Hughes et al 1998, Green et al 1986, Bowmen et al 2007, Fogari et al 1996, Tsai et al 2005, John et al 2006). Although some studies suggest that cigarette smoking is associated with an increased in-office BP or the risk for hypertension (Thuy et al 2010, Tesfaye et al 2008, Niskanen et al 2004, Halimi et al 2002, Mundal et al 1997), other studies indicate that smoking is associated with a decreased in-office BP and a reduced risk for hypertension (Okubo et al 2004, Primatesta et al 2001, Lee et al 2001, Hughes et al 1998, Green et al 1986), or that smoking has modest (Okubo et al 2004), or clinically negligible (Primatesta et al 2001, Fogari et al 1996, Tsai et al 2005, John et al 2006) relationships with in-office BP or the risk for hypertension.

The controversy regarding the impact of smoking on in-office BP not only confounds the outcomes of the study by Schut et al (2004), whom although demonstrating a BP effect of an interaction between smoking and ACE genotype, failed to show increased BP values in smokers, but also casts doubt as to the validity of the

findings demonstrating an impact of an interaction between smoking and ACE genotype on the risk for hypertension (Xu et al 2004). Indeed, in that study (Xu et al 2004) smoking alone was related to the diagnosis of hypertension based on in-office BP, a finding that, as highlighted in previous discussion, has been questioned. Moreover, in that study (Xu et al 2004), the authors did not adjust for other lifestyle factors. Indeed, Xu et al (2004), demonstrated a similar association between ACE genotype-alcohol intake and ACE genotype-obesity interactions and the risk for hypertension. As these risk factors are known to aggregate in people who are less aware of the risks of a poor lifestyle, whether the ACE genotype-smoking interaction can be attributed to smoking *per se* is uncertain. Thus, further studies are required to evaluate interactions between smoking and ACE genotype as determinants of BP using a study design that clearly shows a smoking effect on BP consistent with the current understanding of this field and a study that accounts for the possibility that smoking-BP relationships aren't because of confounding relationships between associated lifestyle factors and BP. This issue will be further discussed below.

2.3 Summary of data obtained in human studies to support a role for the renin-angiotensin-aldosterone system in BP control.

In summary, there are a number of lines of evidence obtained in human studies to indicate that the RAAS is important in BP control. The strong data are derived from innumerable intervention studies demonstrating the capacity of RAAS blockers to decrease BP. In addition, a number of studies indicate that circulating components of the RAAS are related to BP. Furthermore, studies assessing the genetic determinants of BP

provide strong evidence both for and against a role for genetic variants of the ACE and AGT genes in BP control. In this regard, there are major studies demonstrating a role for both the ACE and AGT genes in hypertension and BP control. These studies have generally fulfilled a number of criteria that experts have previously recommended (Anonymous 1999, Corvol et al 1999, Sharma et al 2000, Gambaro et al 2000), particularly with respect to the use of large sample sizes, replicating data in independent samples, using family-based and population-based designs, employing BP as a continuous trait in the analysis and thus enabling diastolic and systolic BP to be considered separately, including multivariate adjustments in the analysis, and using informative genetic markers. However, there is also strong evidence that opposes a role for specific variants of the ACE and AGT gene in either hypertension or BP control which have similarly fulfilled the criteria that experts have previously recommended (Anonymous 1999, Corvol et al 1999, Sharma et al 2000, Gambaro et al 2000). These contrasting outcomes may characterise polygenic traits in that gene variant effects on BP should never be expected to occur in all samples and that the penetrance depends on a wide variety of factors, including salt intake, body size or smoking. Importantly, the bulk of the evidence described above has been obtained from studies performed in groups of European or Asian descent. What is the current evidence to support or refute a role of the RAAS in BP control in groups of African ancestry and what is the outstanding evidence that still requires further study?

3.0 Current and outstanding evidence for or against a role for the renin-angiotensin-aldosterone system in blood pressure control in groups of African ancestry

The critical question with respect to ethnic differences in factors that contribute toward BP is what is considered the major mechanism responsible for an increased BP in groups of African ancestry? This has been a question pondered for decades and the bulk of the evidence overwhelmingly points to a renal mechanism which predisposes a large proportion of persons of African descent to the BP effects of salt intake. In this regard, as a consequence of renal tubular changes, the exact mechanism which is still uncertain (Aviv et al 2004), groups of African ancestry are considered more likely to develop a greater increase in BP in response to salt intake than other ethnic groups (Luft et al 1979, Luft et al 1991, Campese et al 1991). As dietary Na^+ intake is increased, normotensive participants of African descent experience a greater rise in BP at a lower Na^+ intake than do their Caucasian counterparts and also achieve a higher BP at a lower Na^+ intake (Luft et al 1979). Approximately 50% of hypertensives of African descent whilst only 20% of their Caucasian counterparts respond to a salt load with an increased BP (Peters and Flack 2000). As a consequence of the higher prevalence of salt-sensitive hypertension, the impact of lowering Na^+ (He et al 2000) and increasing K^+ (Whelton et al 1997) intake on BP is substantially greater in hypertensives of African as opposed to European descent. Moreover, the high prevalence of salt-sensitive hypertension in groups of African ancestry is thought to sensitise this ethnic group to the antihypertensive effects of diuretic agents when used as monotherapy (Freis et al 1988).

With respect to the RAAS, it is important to note that salt intake markedly suppresses renin release from the kidney (Channik et al 1969, Brunner et al 1972, Kaplan et al 1976, Chrysant et al 1979, James et al 1986, Pratt et al 1999). Therefore, as groups of African descent are particularly sensitive to the effects of salt intake, the impact of salt intake on renin supression is considerably greater in groups of African as compared to European ancestry (Chrysant et al 1979, James et al 1986, Pratt et al 1999). Consequently, groups of African ancestry generally have lower plasma renin concentrations than groups of European descent (Fisher et al 1999). Indeed, up to 33% of people of African ancestry have been reported to have undetectable plasma renin levels even with stimulation (Helmer et al 1964, Voors et al 1976). The aforementioned evidence challenges a role for the RAAS in the pathogenesis of an increased BP in groups of African ancestry. Indeed, this "salt-sensitive, low-renin" state is associated with a poor BP response to antihypertensive therapy involving blockers of the RAAS (ALLHAT Collaborative Research Group 2002, Cushman et al 2000). Thus, despite the increasing benefits that accrue from RAAS blocker therapy in appropriate patients with cardiovascular disease (The SOLVD Investigators 1992, Yusuf et al 2000, The European Trial on Reduction of Cardiac Events with Perindopril in Stable Coronary Artery Disease Investigators 2003), these agents are generally not considered as the first line of antihypertensive therapy in patients of African descent (Chobanian et al 2003, Williams et al 2004). However, as shall be discussed whether the RAAS is appropriately diminished in salt-sensitive hypertension has been a matter of debate for some years. Indeed, as previously mentioned BP responses to combined diuretic and RAAS blocker therapy are similar in groups of African as compared to European descent (Libhaber et al

2004) suggesting that salt–sensitive hypertension is indeed a state that depends to some extent on RAAS activity. Is there evidence to suggest that the RAAS could still play a role in BP control in groups of African descent despite the high prevalence of salt-sensitive, low-renin hypertension?

3.1 Current and outstanding evidence for a role for circulating components of the renin-angiotensin-aldosterone system in blood pressure control in groups of African descent.

The possibility that the RAAS is involved in BP control in groups of African ancestry is supported by very few lines of evidence with respect to measures of circulating concentrations or activity of components of the RAAS. In accordance with studies conducted in other ethnic groups, in groups of African ancestry, a relationship exists between circulating angiotensinogen concentrations and BP (Forrester et al 1996). Furthermore, in groups of African descent circulating angiotensinogen concentrations may in fact be higher than in their Caucasian counterparts (Bloem et al 1997). However, with respect to circulating aldosterone concentrations, in contrast to the Framingham study Framingham Heart Study conducted largely in a population sample of European ancestry, where circulating aldosterone concentrations were associated with the incidence of hypertension (Vasan et al 2004), earlier studies conducted in groups of African ancestry have failed to show a relationship between circulating aldosterone concentrations and BP at a population level (Hoosen et al 1985, Hoosen et al 1990). More recent studies conducted in groups of African descent have produced conflicting results,

with one study conducted in the Seychelles reporting on a lack of relationship except in the context of an older age (Bochud et al 2006) whilst in studies where Na^+ intake has been standardised at relatively high levels (150 mmol/day), a relationship between aldosterone concentrations and BP has been reported on in African-Americans (Kidambi et al 2009, Grim et al 2005). The lack of relationship between aldosterone concentrations and BP in groups of African descent is consistent with the high prevalence of salt-sensitive, low renin hypertension, where a decrease in renin will result in a decrease in circulating aldosterone concentrations. However, whether circulating aldosterone concentrations are appropriately diminished in salt-sensitive, low renin states in groups of African or European descent has been a matter of debate for some years.

Despite a suppressed renin activity or concentrations in groups of African ancestry, plasma aldosterone concentrations are often normal or even elevated (Grim et al 2005). Indeed, although plasma renin activity tends to decrease with age in hypertensive patients of African descent, plasma aldosterone concentrations may remain normal (Sagnella et al 2001). Moreover, although plasma renin activity is suppressed as a consequence of the BP response to salt intake in salt-sensitive individuals, reductions in plasma aldosterone concentrations may be attenuated (Wisgerhof et al 1978, Marks et al 1979, Griffing et al 1990). Indeed, in patients with “low-renin” hypertension, although the adrenal response to angiotensin II infusion is normal on a high salt diet, it becomes progressively more sensitive on a low salt diet (Fisher et al 1999). This effect may be genetically predetermined as strong sibling correlations with the aldosterone response to angiotensin II have been reported on (Giacche et al 2000). Thus, in salt-sensitive individuals there is a possibility that salt-induced increases in BP may in-part depend on

the presence of an inability to appropriately reduce aldosterone secretion, despite marked decreases in renal renin release. This is entirely consistent with the finding in a preclinical study demonstrating that BP does not increase in response to a salt-load alone, but does increase in animals genetically modified to develop an attenuated decrease in plasma aldosterone concentrations (Makhanova et al 2008). Moreover, this hypothesis is reminiscent of the BP changes that occur in the well-described DOCA-salt model, where neither excessive salt intake nor administration of the mineralocorticoid (DOCA) alone produce BP changes, but the combination produces profound hypertensive effects (Ouchi et al 1987). However, this hypothesis has **not** been tested at a community or population level in any ethnic group. Thus the importance of the hypothesis that salt-sensitivity depends on an attenuated suppression of aldosterone in response to a salt load has not been substantiated at a community or population level. This hypothesis is particularly important to address in groups of African descent where current evidence is against a role for aldosterone in BP control at a population level in black African samples in Africa (Hoosen et al 1985, Hoosen et al 1990, Bochud et al 2006) except in the elderly (Bochud et al 2006), but where relationships may exist in African-Americans if Na^+ intake is standardised at relatively high levels (150 mmol/day) (Kidambi et al 2009, Grim et al 2005).

As part of the present thesis I therefore evaluated whether circulating aldosterone concentrations or the aldosterone-to-renin ratio (an index of the relationship between aldosterone release in response to renin) are associated with the relationship between salt intake, as assessed from 24-hour urinary electrolyte excretion rates, and BP in a community sample of African ancestry. These data are presented in chapter 2 and have

been accepted for publication in the *American Journal of Hypertension* (Scott et al, 2011) Having demonstrated the importance of this concept at a community level in a group of African descent, I subsequently went on to attempt to identify whether genetic factors may in-part account for circulating aldosterone concentrations in this community. Thus, in chapter 3 I describe data where I have taken advantage of the fact that in the community studied in this thesis, the study design required the random selection of nuclear families, and evaluated the familial aggregation and heritability of serum aldosterone concentrations and the relationship of serum aldosterone concentrations with clinically and functionally relevant variants of the ACE and AGT genes. This study was conceived in response to conflicting data on the heritability of circulating aldosterone concentrations in groups of European as compared African descent. In this regard, data obtained in the predominantly Caucasian community of the Framingham Heart Study demonstrated heritability of the circulating aldosterone concentrations either in the context of plasma renin concentrations (Newton-Cheh et al 2007) or without adjustments for plasma renin concentrations (Kathiresan et al 2005). In contrast, significant heritability of plasma aldosterone concentrations was not observed in African-Americans (Kotchen et al 2000). Obviously, as the role of the RAAS in groups of African descent may not be the same as in groups of European ancestry, the possibility that genetic factors play a role in contributing to the variation in circulating aldosterone concentrations remains in question in this ethnic group.

Although it is important to assess the relationships between circulating concentrations of components of the RAAS and BP in groups of African descent, as previously indicated there is also substantial evidence for a role for genetic variants of

components of the RAAS, particularly the ACE and AGT gene, in BP control in population groups of ethnic origins other than of African ancestry. The obvious question with respect to groups of African descent is therefore whether there is also any evidence for a role for the ACE and AGT gene in contributing to the development of primary hypertension or influencing BP at a population level in this ethnic group? If this evidence does exist, is it sufficient to draw definitive conclusions or is there outstanding evidence that is still requires further attention in this regard?

3.2 Current and outstanding evidence for a role for gene variants of the renin-angiotensin system in blood pressure control in groups of African ancestry

In contrast to the multitude of large and small studies conducted in groups of European descent, relatively few studies have evaluated the relationship between RAAS genes and circulating RAAS concentrations or activity and BP in groups of African ancestry. With respect to the ACE gene, an association with circulating ACE activity has been noted in groups of African ancestry (Bloem et al 1996). In addition, in two case-control studies with very small sample sizes (n=68-89) the ACE gene D allele was reported to be associated with hypertension and/or a higher BP in groups of African descent (Barley et al 1996, Duru et al 1994). However, in contrast to data with positive outcomes in groups of African ancestry (Barley et al 1996, Duru et al 1994), in three larger case-control studies no relationship between the ACE gene D allele and hypertension was noted in groups of African descent (Forrester et al 1997, Borecki et al 1997, Kamdar et al 1994). Thus, there is considerable uncertainty as to the contribution of

the ACE gene to BP in groups of African ancestry. What is the evidence for a role of the AGT gene in contributing toward either circulating angiotensinogen concentrations or BP in groups of African descent?

In groups of African ancestry variants of the AGT gene have been shown either to be associated with circulating angiotensinogen concentrations (Bloem et al 1997, Rotimi et al 1997) or have failed to show associations with circulating angiotensinogen concentrations (Bloem et al 1995, Forrester et al 1996). In a number of case-control studies with small study samples (Caulfield et al 1995, Borecki et al 1997, Forrester et al 1996, Rotimi et al 1994, Barley et al 1994) and in two case-control studies with large study samples (>1000 participants) (Larson et al 2000, Tiago et al 2002) with ambulatory BP measurements performed in the over 500 hypertensives studied in one of these studies (Tiago et al 2002), no relationships between variants of the AGT gene and hypertension were noted. Nevertheless, one small linkage study has provided evidence to indicate that the AGT gene is involved in the development of hypertension in groups of African ancestry (Caulfield et al 1995).

Clearly much larger population-based cross-sectional and prospective studies are required to either confirm or refute the notion that RAAS genes contribute toward a significant proportion of the variation in BP in populations of African ancestry. Importantly however, just as there are few studies conducted in general evaluating the contribution of RAAS genes to BP control in groups of African descent, there are similarly few studies that have pursued the notion that interactions between RAAS genes and either environmental or phenotypic factors may contribute toward BP in groups of African ancestry. These studies are nevertheless only likely to be of high priority if they

contribute toward a world-wide understanding of the role of RAAS genes in BP control. Indeed, as previously underscored, as compared to the number of studies that have sought an independent relationship between RAAS genes and BP or hypertension, there are few studies that have explored the possibility that RAAS gene effects are only likely to occur in the context of environmental or phenotypic effects on BP in any ethnic group. What is the current evidence that interactions between RAAS genes and either environmental or phenotypic factors may contribute toward BP in groups of African ancestry? Moreover, are there outstanding studies that could be conducted in groups of African ancestry that may be considered to be of high priority as they may contribute toward a world-wide understanding of the role of RAAS genes in BP control?

3.2.1 Current and some outstanding evidence for a role for interactions between gene variants of the renin-angiotensin system and either environmental or phenotypic features in groups of African ancestry

In the present thesis I considered the possibility that interactions between RAAS genes and a number of environmental or phenotypic changes could account for a significant proportion of the variability of BP at a population level in a group of African descent. In this regard however, despite having demonstrated in Chapter 2 that the aldosterone-to-renin ratio is associated with the relationship between salt intake and BP and in Chapter 3 that aldosterone aggregates in families, I was unable to show relationships between aldosterone concentrations and AGT or ACE genotypes (Chapter 3). I therefore did not seek to assess the impact of RAAS genotype on relationships

between salt intake and BP. Moreover, I failed to achieve sufficiently large sample sizes to evaluate whether interactions between gender and RAAS genotypes were independently related to BP. This was largely because, as with many other community-based studies, more females than males volunteer for studies. Furthermore, as our group have already demonstrated a strong relationship between an AGT gene-obesity interaction and BP in groups of African descent (Tiago et al 2002), I did not explore this possibility. Rather, I focused my efforts on contributing further to our knowledge of the possibility that interactions between RAAS genotypes and smoking could influence BP. The following underscores the advantages of such a study in the population evaluated and in the context of the study design.

As indicated in preceding sections, the influence of the ACE gene I/D polymorphism on BP has been shown to occur in cigarette smokers, but not non-smokers (Schut et al 2004) and hence the effect of the gene could depend on cigarette smoking. However, the ACE gene effect failed to produce a statistically striking effect on BP in that study ($p<0.05$) despite a sample of 1508 current smokers being studied (Schut et al 2004). In this regard, the study was performed in a Caucasian population sample where the risk genotype for the ACE gene (DD genotype) occurs with a lower prevalence than groups of African ancestry. Thus, it is possible that a study assessing the interactive effects of ACE genotype and smoking may produce more striking and clinically significant effects in African as opposed to European population samples. As part of the present thesis I therefore further explored this issue in a community sample of African ancestry.

Moreover, as previously highlighted in section 2.2.2.5.4, the study demonstrating a relationship between the ACE gene and BP in smokers, smoking effects on in-office BP in the group as a whole were not identified (Schut et al 2004), a finding that agrees with inconsistencies noted for smoking effects in many population-based studies (see section 2.2.2.5.4). Indeed, as pointed out in this aforementioned discussion (section 2.2.2.5.4), the concept that smoking influences in-office BP is highly controversial. The controversy regarding the impact of smoking on in-office BP not only confounds the outcomes of the study by Schut et al (2004), but also casts doubt as to the validity of the findings demonstrating an impact of an interaction between smoking and ACE genotype on the risk for hypertension diagnosed with in-office BP measurements (Xu et al 2004). Indeed, in that study (Xu et al 2004) smoking alone was related to the diagnosis of hypertension based on in-office BP, a finding that, as highlighted in previous discussion, has been questioned. Therefore, in the present thesis I first addressed the issue of whether smoking could indeed account for a significant proportion of the variability of BP at a community level. In this regard, I considered whether smoking mainly affects out-of-office (ambulatory) BP or central (aortic) BP at a community level, rather than in-office BP. The following outlines my arguments for exploring these possibilities.

As the effects of cigarette smoking on BP are relatively short-lived (Kool et al 1993, Mahmud et al 2003, Failla et al 1997, Rhee et al 2007, Gropelli et al 1999) with perhaps the exception of heavy smoking (Gropelli et al 1999), the ability to adequately capture the effects of smoking may best occur with out-of-office BP measurements. Indeed, in some (Verdecchia et al 1995, Mann et al 1991, Bang et al 2000, Bolinder et al 1998, Benowitz et al 2002), but not other (Stewart et al 1994, Green et al 1991,

Mikkelsen et al 1997) case-control (Verdecchia et al 1995, Mann et al 1991, Bang et al 2000, Bolinder et al 1998, Benowitz et al 2002) or cross-sectional (Green et al 1991, Mikkelsen et al 1997) studies conducted either in small study samples (Mann et al 1991, Bang et al 2000, Bolinder et al 1998, Benowitz et al 2002, Stewart et al 1994, Green et al 1991, Mikkelsen et al 1997), or where office BP was matched between cases and controls (Verdecchia et al 1995), medium (10-20 cigarettes a day, Minami et al 2009)-to-heavy (greater than 20 cigarettes a day, Verdecchia et al 1995) cigarette smoking was associated with increases in out-of-office, but not in-office BP. Furthermore, whilst smokers may have similar in-office brachial artery BP values as non-smokers, central (aortic) BP may be increased in smokers as compared to non-smokers (Mahmud and Feely 2003). Thus, before assessing whether an interaction between smoking and the ACE gene may influence BP at a community level in a group of African ancestry, I first evaluated whether clinically relevant smoking effects on 24-hour or central BP could be identified at a population level. These data are described and discussed in chapter 4 of the present thesis and have been accepted for publication in the *Journal of Hypertension* where I am second author and contributed equally to the publication as the first author (Woodiwiss, Scott et al, 2011). Having demonstrated a marked effect of smoking on out-of-office (ambulatory) BP in chapter 4, I then assessed the possibility that the ACE gene and smoking interact to contribute toward a significant variation in out-of-office BP at a community level. These data are in-turn described and discussed in chapter 5.

4.0 Summary of the aims of the dissertation

As a consequence of the uncertainty that surrounds a) the role of aldosterone as a determinant of BP in groups of African ancestry, an ethnic group that may have a suppressed renin-angiotensin-aldosterone system; b) the role of aldosterone in mediating the impact of salt intake on BP at a population level in any ethnic group; c) the role of genetic factors in influencing circulating aldosterone concentrations in groups of African ancestry; d) the impact of smoking on out-of-office and central BP at a population level; and e) the role of the ACE gene in modifying the impact of smoking on BP in any population group, **in the present dissertation I aimed to evaluate**:

1) Whether aldosterone-to-renin ratios are associated with the relationship between salt intake and blood pressure in a community sample of African ancestry. These data are described in chapter 2.

2) The genetic basis of circulating aldosterone concentrations in a community sample of African ancestry. These data are described in chapter 3.

3) The relationship between smoking and ambulatory or central BP in a community sample of African ancestry. These data are described in chapter 4.

4) The impact of the angiotensin-converting enzyme gene on the relationship between smoking and ambulatory blood pressure in a community sample of African ancestry. These data are described in chapter 5.

Chapter 2

Aldosterone-to-Renin Ratio and the Relationship Between Urinary Salt Excretion and Blood Pressure in a Community Sample of African Ancestry.

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Abstract

The factors that determine the heterogeneous relationship between salt intake and blood pressure (BP) at a community level are uncertain. I therefore aimed to determine, in a community of black African descent, whether in the absence of primary aldosteronism, the relationship between salt intake and BP is associated with circulating aldosterone concentrations. After excluding participants with aldosterone-to-renin ratios (ARR) above the threshold for primary aldosteronism (2.4% of the sample), in 575 participants of African ancestry (age>16years), I assessed whether the ARR is associated with the relationship between urinary Na^+-to-K^+ ratio (urinary Na^+/K^+)(from 24-hour urine samples), an index of salt intake, and BP. With adjustments for confounders, interactions between ARR and urinary Na^+/K^+ were independently associated with systolic BP (SBP) ($p<0.0001$), an effect that was accounted for by interactions between serum aldosterone concentrations and urinary Na^+/K^+ ($p<0.0001$), but not between plasma renin concentrations and urinary Na^+/K^+ ($p=0.52$). The interaction between ARR and urinary Na^+/K^+ translated into a marked difference in the relationship between urinary Na^+/K^+ and SBP in participants above and below the median for ARR (effect of one SD increase in urinary Na^+/K^+ on systolic BP: ARR above the median=4.2±0.6 mm Hg; ARR below the median=1.2±0.4 mm Hg, $p<0.0001$ for a comparison of the relationships). Participants with a urinary Na^+/K^+ above the median had a higher multivariate adjusted SBP ($p<0.001$) only if the ARR was also above the median for the sample. In conclusion, in groups of African descent, in the absence of primary aldosteronism an increased aldosterone relative to renin accounts for a substantial proportion of the relationship between urinary Na^+/K^+ and BP at a community level.

2.1 Introduction

Large cross-sectional studies indicate that dietary salt intake as indexed by increases in urinary sodium (Na^+), decreases in urinary potassium (K^+) excretion or increases in urinary Na^+/K^+, is associated with BP (Intersalt Cooperative Research Group 1988, Smith et al 1988, Hajjar et al 2001, Buyck et al 2009, Liu et al 2009). Moreover, intervention studies have demonstrated that reductions in Na^+ intake and increases in K^+ intake reduce BP and the risk for hypertension (The Trials of Hypertension Prevention Collaborative Research Group 1997, Whelton et al 1998, He et al 2000, Sacks et al 2001, Vollmer et al 2001, Jurgens and Graudal 2008). As a consequence of these findings guidelines for the diagnosis and management of hypertension recommend that changes in salt intake should constitute part of lifestyle changes for the management of hypertension (Mancia et al 2007, Chobanian et al 2003, Williams et al 2004). However, it is well accepted that some individuals are more sensitive to the BP effects of modifications in salt intake than others.

Early clinical studies (Wisgerhof and Brown 1978, Marks et al 1979, Griffing et al 1990, Fisher et al 1999) suggest that despite decreases in plasma renin activity that occur as a consequence of the BP response to salt intake, in salt-sensitive individuals reductions in plasma aldosterone concentrations may be attenuated. A recent pre-clinical study (Makhanova et al 2008) demonstrating increases in BP in response to a salt-load only in animals genetically modified to develop an attenuated decrease in plasma aldosterone concentrations, supports the notion that salt-induced increases in BP depend on an inability to appropriately reduce aldosterone secretion. However, whether this

effect contributes to an appreciable proportion of the variability in BP at a population level has not been determined.

The possibility that at a population level, salt-induced increases in BP may in-part depend on the presence of an inability to appropriately reduce aldosterone secretion, despite marked decreases in renal renin release, is a particularly important question to answer in groups of African ancestry. Indeed, groups of African ancestry are more likely to develop an increased BP in response to salt intake than other ethnic groups, an effect which suppresses renin release from the kidney (Channick et al 1969, Brunner et al 1972, Kaplan et al 1976, Chrysant et al 1979, James et al 1986, Pratt et al 1999). This "salt-sensitive, low-renin" state in groups of African descent is associated with a poor BP response to antihypertensive therapy involving blockers of the renin-angiotensin-aldosterone (RAAS) (ALLHAT Collaborative Research Group 2002, Cushman et al 2000). Thus, despite the increasing benefits that accrue from RAAS blocker therapy in appropriate patients with cardiovascular disease (The SOLVD Investigators 1992, Yusuf et al 2000, The European Trial on Reduction of Cardiac Events with Perindopril in Stable Coronary Artery Disease Investigators 2003), and the fact that combined with diuretic agents RAAS blockers may have similar effects on BP in groups of African as compared to European ancestry, these agents are generally not considered as the first line of antihypertensive therapy in patients of African descent (Chobanian et al 2003, Williams et al 2004). Indeed, in community studies, few hypertensives of African ancestry have been noted to receive RAAS blockers (Maseko et al 2006, Maseko et al 2011). To further evaluate the relative importance of the RAAS in BP control in groups of African ancestry and to assess whether an attenuated decrease in plasma aldosterone concentration

contributes toward an appreciable proportion of salt-induced increases in BP at a community level, in the present study I therefore aimed to evaluate whether the ratio between serum aldosterone and renin concentrations (ARR)(an index of the extent of aldosterone activation in the context of renin) is associated with the relationship between salt intake and BP in a community sample of African ancestry without primary aldosteronism.

2.2 Methods

2.2.1 Study group.

The present study was part of the ongoing African Project on Genes in Hypertension initiated in 2002 and conducted according to the principles outlined in the Helsinki declaration. The Committee for Research on Human Subjects of the University of the Witwatersrand approved the protocol (approval number: M02-04-72 and renewed as M07-04-69). Participants gave informed, written consent. The study design has recently been described (Norton et al 2008, 2009, Woodiwiss et al 2008, 2009, Majane et al 2007, 2008, Libhaber E et al 2004, Libhaber C et al 2009, Maseko et al 2006). Participants recruited were black-Africans of the Nguni, Sotho and Venda chiefdoms living in the South West Township (Soweto) of Johannesburg, South Africa. Soweto with a population of 1.1 million people predominantly of African descent, is one of the largest urban concentrations of Black Africans on the continent of Africa. Soweto has a population growth rate of about 2% per annum (1991-1996), with half of the population under 25 years. Gender distribution is evenly balanced (City of Johannesburg Regional

Spatial Development Framework 2003). There are about 301 000 households, with 76% of the population earning under R1 500 per month, 9% over R1 500 and only 2% earning over R3 500 per month (South African census conducted in 2001, City of Johannesburg Regional Spatial Development Framework 2003). Although 66% of the population is employed, only 40.5% aged 16 or over are employed full time. 50% of the population live in council houses, 11% in privately owned dwellings, 27% in backyard shacks, and 5% in informal settlements (City of Johannesburg Regional Spatial Development Framework, 2003). Nuclear families of African descent with siblings older than 16 years of age were randomly recruited using the population census figures of 2001.

Spouses and siblings living in households from formal dwellings (council and privately owned) represented in the last census conducted (2001) were randomly recruited. Street names and addresses of households with at least one parent and two siblings or two parents and one sibling were obtained from the Department of Home Affairs. These households were allocated numbers and random numbers were obtained by means of a random number generator. Of the participants approached, 62% agreed to participate (n=1029 participants up to the end of 2009). Of the 1029 participants sampled, 589 recruited in the sub-study had 24-hour urine samples that met with pre-specified quality control criteria previously described (Maseko et al 2006), as well as measurements of circulating renin and aldosterone concentrations. 2.38% of these participants were excluded from the analysis as they had an ARR that exceeded a previously defined threshold of 5.4 ng/dl per ng/l, which is suggestive of primary aldosteronism (Ferrari et al 2004).

2.2.2 Clinical, demographic and anthropometric measurements.

A standardized questionnaire was administered to obtain demographic and clinical data (Norton et al 2008, 2009, Woodiwiss et al 2008, 2009, Majane et al 2007, 2008, Libhaber E et al 2004, Libhaber C et al 2009, Maseko et al 2006). In order to avoid translational errors, the questionnaire was not translated into an African language, but trained study assistants familiar with all languages spoken in these townships and who either previously lived in Soweto or currently reside in Soweto assisted with the completion of each questionnaire. Only same sex assistants were used to assist each family member with the completion of the questionnaire. Support was only provided when requested. The majority of participants were reasonably proficient in English. Study assistants first visited homes of participants in order to develop a trusting relationship. The questionnaire was only completed at a subsequent clinic visit and then ambiguities checked by performing a follow-up home visit. If family members were absent at follow-up home visits, data was checked with them personally via telephonic conversations whenever possible. Ambiguities in answers to the questionnaire were detected by an independent observer prior to the second home visit. A pilot study was conducted in 20 participants to ensure that data obtained in the questionnaires were reproducible when obtained with the assistance of two separate study assistants.

The questionnaire requested specific answers to date of birth, gender, previous medical history, the presence of hypertension, diabetes mellitus and kidney disease, prior and current drug therapy (analgesic use included), smoking status (including the number of cigarettes smoked in the past and at the present time), daily alcohol consumption (beer, traditional beer or other forms of alcohol and the daily quantity), and family history of

hypertension and cardiovascular events. For females, menstrual history, history of pregnancies and oral contraceptive use was evaluated. If participants were unable to provide the name of medication taken these were obtained on the second home visit.

From height and weight measurements, body mass index (BMI) was calculated and participants were identified as being overweight if their BMI was ≥25 kg/m^2 and obese if their BMI was ≥30 kg/m^2. Standard laboratory blood tests of renal function, liver function, blood glucose, lipid profiles, haematological parameters, and percentage glycated haemoglobin (HbA_{1C}) (Roche Diagnostics, Mannheim, Germany) were performed. Blood samples were obtained on the day of the clinic visit and the tests included a full blood count and differential count, plasma urea, creatinine and electrolyte concentrations, alanine transaminase, aspartate transaminase, gamma gluteryl transaminase, alkaline phosphatase, albumin, total protein, total bilirubin, conjugated and unconjugated bilirubin, urate, total cholesterol, high density lipoprotein cholesterol, triglycerides, blood glucose, and a follicle stimulating hormone concentration (in females only to confirm menopausal status). Diabetes mellitus or an abnormal blood glucose control was defined as the use of insulin or oral hypoglycemic agents or an HbA_{1C}>6.1% (Bennett et al 2007).

2.2.3 Conventional blood pressure measurements.

Trained nurse-technicians measured BP using a standard mercury sphygmomanometer during a clinic visit as previously described (Woodiwiss et al 2009) within a half hour of obtaining blood samples for measurements of plasma renin and serum aldosterone concentrations in the opposite arm to that subjected to venesection. A

standard cuff with a 12 × 24 cm inflatable bladder was used, but if upper arm circumference exceeded 31 cm, larger cuffs with a 15 × 35 cm inflatable bladder were used. After 10 minutes of rest in the seating position, five consecutive BP readings were taken 30 to 60 seconds apart with the participants in a sitting position, followed by a pulse rate count. The cuff was deflated at approximately 2 mm Hg per second and phase I (systolic) and phase V (diastolic) Korotkoff sounds employed to determine BP to the nearest 2 mm Hg according to the recommendations of the European Society of Hypertension (O'Brien et al 2003). The average of the five readings was taken as the clinic BP. In the present study quality control of BP measurements was assessed as previously described (Majane et al 2007). Only 0.35% of visits had fewer than the planned BP recordings. The frequency of identical consecutive recordings was 0.17% for systolic BP and 1.04% for diastolic BP. The occurrence of BP values recorded as an odd number was 0.02%. Of the 5742 systolic and diastolic BP readings, 29.16% ended on a zero (expected =20%). A diagnosis of hypertension was made if subjects were receiving antihypertensive therapy and/or if the average of the mean values for the clinic readings was ≥140/90 mm Hg as defined by guidelines (Mancia et al 2007, Chobanian et al 2003, Williams et al 2004).

2.2.4 *Urinary electrolyte excretion rates*.

Timed urine samples were obtained over at least a 24-hour period on the same day as the BP measurements and the blood sampling. The mean (±SD) time period of urine collection was 24.5±1.5 hours (range=24.0-31.1 hours). Urine collections started at 13:30 hours (SD±3.4 hours) and ended at 14:00 hours (SD±3.4 hours) the next day. Urine

collection started after discarding urine obtained immediately prior to the collection period. Urine Na^+, K^+, and creatinine concentrations were measured and 24 hour urine Na^+ and K^+ excretion rates calculated from the product of 24-hour urine volumes and urine electrolyte concentrations. Creatinine clearance was determined from the product of urine volume and urine creatinine concentration/plasma creatinine concentration. The quality of urine samples was determined by constructing regression relations between 24-hour urine creatinine and body weight and 24-hour urine volume and age in gender-specific groups. Based upon the 95% confidence intervals for each group, a 24-hour urine sample was considered acceptable if 24-hour urine creatinine (mmol) was >3.5 and <35 for males and >3.5 and <30 for females. Samples with urine volumes <300 ml/day were also assumed to be incomplete urine collections.

2.2.5 Renin and aldosterone concentrations.

Blood samples were obtained in the supine position after 10 minutes of rest in the morning between 10:00 and 12:00 hours. Participants were allowed to continue taking all routine medications at the time as in the population sampled neither K^+ sparing diuretics, loop diuretics, nor aldosterone receptor blockers are employed as antihypertensive therapy. However, the majority of treated hypertensives were receiving low-dose thiazide diuretics as monotherapy and hence sensitivity analysis was conducted in untreated participants (n=441) (see data analysis). After centrifugation, samples were stored at -70^{o}C until the time of analysis. Plasma renin concentrations were measured using an immunoradiometric technique (Renin III Generation, Cisbio International, Ceze, France). The assay is sensitive with intra-assay coefficients of variation ranging from 1.1% for

high concentrations to 4.5% for low concentrations and with a mean inter-assay coefficient of variation of 5.3%. Serum aldosterone concentrations were measured using an ^{125}I radioimmunoassay (Coat-A-Count, Diagnostic Products Corporation, Los Angeles, CA). The assay is sensitive with intra-assay coefficients of variation ranging from 2.3% for high concentrations to 5.4% for low concentrations, and with a mean inter-assay coefficient of variation of 5.4%.

2.2.6 Data analysis.

For database management and statistical analysis, SAS software, version 9.1 (SAS Institute Inc., Cary, NC) was employed. Data are shown as mean±SD unless otherwise specified. As circulating renin and aldosterone concentrations and ARR were not normally distributed, in data analysis they were expressed as either log (renin) or square-root (aldosterone and ARR) values in order to achieve better distribution profiles. Independent relationships with BP were determined from multivariate regression models with age, sex, BMI, the presence of diabetes mellitus/abnormal blood glucose control, regular alcohol consumption, regular tobacco use, and antihypertensive treatment (in all participants) included in the regression models. The presence of diabetes mellitus/abnormal blood glucose control, regular alcohol consumption, regular tobacco use, and antihypertensive treatment were treated as discrete traits and assigned either a value of 0 (absence) or a value of 1 (presence) for the purposes of adjustments. Multivariate regression models including the same confounders were used to determine the significance of interactive terms independent of the individual terms. As there were 104 sibling pairs, and 304 parent-child pairs, the probability values derived from

multivariate models were adjusted for non-independence of family members using a mixed model of analysis as defined by SAS software. Using this approach, both random and fixed effects (relatedness) were included in the repression model. Following the demonstration of a significant interaction between ARR and urinary Na^+/K^+, the sample was subdivided according to the median for ARR (expressed as a square root value) and separate multivariate regression analysis was performed in participants with a square root of ARR either above or below the median for the sample.

As antihypertensive therapy may modify plasma renin and aldosterone concentrations, or urinary salt excretion, sensitivity analysis was conducted where the aforementioned relationships were assessed in participants not receiving therapy. In this regard, this approach excludes the potential effects of antihypertensive therapy on plasma renin and aldosterone concentrations, and urinary salt excretion and hence eliminates the possibility that antihypertensive therapy confounds relationships between these factors and BP. However, as the primary question was whether circulating ARR influences the relationship between salt intake and BP at a community level, the analysis was not confined to just participants not receiving therapy, as this subgroup would not necessarily represent a cross-section of the community.

2.3 Results

2.3.1 Characteristics of the participants.

Table 2.1 gives the characteristics of the whole study group including the treated participants. More women than men participated. In general participants had a high BMI with 67.5% being either overweight or obese. No participants were receiving hormone replacement therapy, but 45.8% of women were postmenopausal. A high proportion had hypertension and either diabetes mellitus or an abnormal blood glucose control. 6.0% of all participants were receiving oral agents for diabetes mellitus and 1% insulin. Of the total sample, 42.3% were hypertensive and only about half of these were receiving antihypertensive medication (23.3% of the total sample). Of the hypertensives receiving treatment, low-dose thiazide diuretic agents were the most commonly used antihypertensive agents (87%) and only 23%, 13% and 2% of treated participants were receiving angiotensin-converting enzyme inhibitors, dihydropyridine-type calcium channel blockers and β-blockers respectively. No participants were receiving centrally acting α_2 agonists, angiotensin receptor blockers, aldosterone receptor antagonists or K^+ sparing or loop diuretic agents. No participants were hypokalemic. Importantly, as compared to the group studied, those without appropriate 24-hour urine collections who were excluded from the study sample (n=454) were of a similar age (43.0±18.6 years), had a similar BMI (29.3±8.0 kg/m^2), and an equivalent proportion of these participants were overweight/obese (20.3/43.2%), smoked regularly (15.4%), consumed alcohol regularly (19.8%), received glucose lowering agents (6.6%) and had hypertension (42.1%). A modestly lower proportion of the sample excluded from the analysis had

Table 2.1 Characteristics of the population sample of African ancestry.

	Mean (±SD)
Number (% women)	575 (64.5)
Age (years)	44.6±18.1
Body mass index (kg/m^2)	29.6±8.0
% overweight/obese	25.6/41.9
Regular tobacco intake (% subjects)	13.9
Regular alcohol intake (% subjects)	23.7
% Females postmenopausal	45.8
% Diabetes mellitus or HbA_{1c}>6.1%	25.9
% receiving glucose lowering agents	6.6
% Hypertension	42.3
Current antihypertensive medication (%)	23.3
Total/HDL cholesterol	3.55±2.39
Serum K^+ (mmol/l)	3.90±0.38
Urinary Na^+ excretion (mmol/24 hours)	104.3±65.9
Urinary K^+ excretion (mmol/24 hours)	28.0±17.8
Urinary creatinine (mg/24 hours)	10.2±9.5
Urinary (Na^+/K^+)	4.2±2.2
Urinary Na^+/creatinine (mmol/l / mg/dl)	1.22±0.74
Urinary K^+/creatinine (mmol/l / mg/dl)	0.32±0.17
Plasma renin concentrations (pg/ml)	38.7±74.5 (range=0.20 to 814)
Serum aldosterone concentrations (ng/dl)	7.2±5.8 (range=0 to 40.3)
Log plasma renin concentrations (pg/ml)	1.18±0.58
Square root serum aldosterone concentrations (ng/dl)	2.41±1.16
Aldosterone-to-renin ratio (ARR) (ng/dl / ng/l)	0.74±1.05 (range=0 to 5.35)
Square root ARR (ng/dl / ng/l)	0.69±0.52 (range=0 to 2.31)
Conventional SBP/DBP (mm Hg)	131±23/85±12
Pulse rate (beats/minute)	65±12

HbA_{1c}, glycated haemoglobin; HDL, high density lipoprotein; SBP, systolic blood pressure; DBP, diastolic blood pressure.

diabetes mellitus or an HbA_{1c}>6.1% (18.9%, p<0.01 as compared to 25.9% of the study group).

The average 24-hour urinary Na^+ excretion rate was well above the recommended daily allowance (RDA) for Na^+ intake of 65 mmol/day (Appel et al 2006), with most of the study group (69%) ingesting more than the RDA for Na^+ intake (see Table 2.1 for mean values). All participants had 24-hour urinary K^+ excretion rates less than the RDA for K^+ intake of 120 mmol/day (see Table 2.1 for mean values).

2.3.2 Distribution of RAAS measurements.

Figure 2.1 shows the distribution of plasma renin and serum aldosterone concentrations and ARR. The distributions of all of these parameters departed from normality and were positively skewed (skewness: 5.15, 1.60, 2.35; kurtosis: 38.48, 4.53, 5.49; Shapiro-Wilk's statistic: 0.485, p<0.0001, 0.888, p<0.0001, 0.685, p<0.0001 respectively).

2.3.3 Factors other than BP associated with RAAS measurement.

In a multivariate model including age, sex, BMI, urinary Na^+/K^+, diuretic use, angiotensin-converting enzyme inhibitor use, β-blocker use, menopausal status, and total/HDL cholesterol as adjustors; diuretic use (partial r=0.17, p<0.0005), and urinary Na^+/K^+ (partial r=-0.31, p<0.0001) were independently associated with square root of serum aldosterone concentrations. With 24-hour urinary Na^+ or 24-hour urinary K^+ excretion replacing urinary Na^+/K^+ in separate models, 24-hour urinary K^+ excretion was positively related to the square root of serum aldosterone concentrations (partial r=0.12, p<0.005), but neither 24-hour urinary Na^+ (partial r=-0.08, p=0.06), nor an interaction between urinary Na^+ and K^+ excretion were significantly related to the square root of

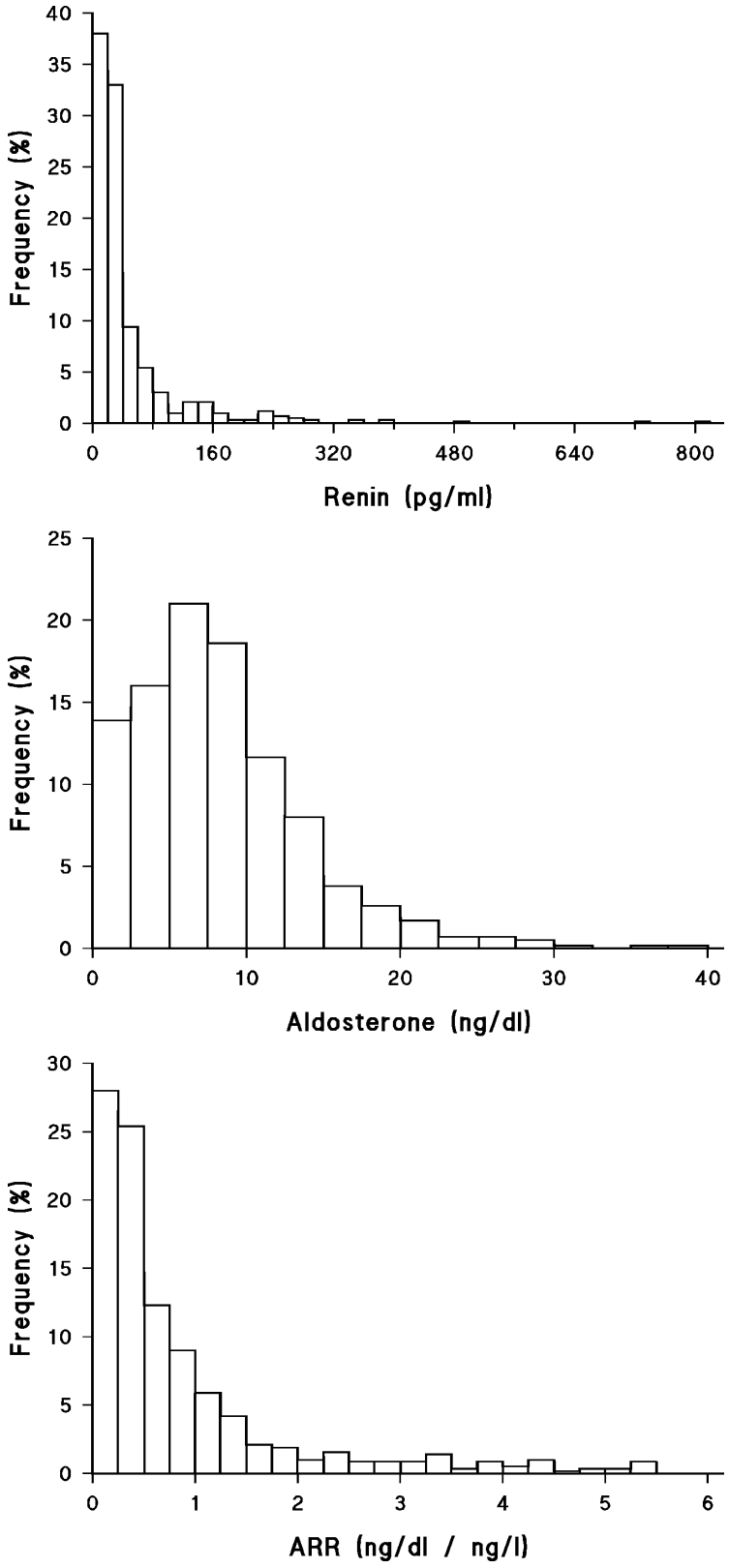


Figure 2.1. Distribution of plasma renin and serum aldosterone concentrations and the aldosterone-to renin ratio (ARR) in the community sampled.

serum aldosterone concentrations independent of confounders. In addition, age (partial r=-0.11, p<0.02), male gender (partial r=0.12, p<0.02), diuretic use (partial r=0.13, p=0.0001), angiotensin-converting enzyme inhibitor use (partial r=0.14, p<0.005) and urinary Na^+/K^+ (partial r=-0.15, p<0.0001) were associated with log plasma renin concentrations. However, none of the aforementioned factors were independently associated with the square root of ARR. Neither BMI, nor alternative adiposity indexes (waist circumference or waist-to-hip ratio) were associated with RAAS concentrations independent of age (data not shown). Moreover, the multivariate adjusted square root of serum aldosterone concentrations (ng/dl) and the multivariate adjusted square root of ARR (ng/dl / ng/l) were similar in those participants with DM or an HbA1c>6.1% (n=149) as compared to those without (n=426) (Aldosterone: participants with DM or an HbA1c>6.1%=2.48±1.22; participants without DM or an HbA1c>6.1%=2.39±1.24; p=0.45; ARR: participants with DM or an HbA1c>6.1%=0.63±0.57; participants without DM or an HbA1c>6.1%=0.71±0.52; p=0.15).

2.3.4 *Association between urinary electrolyte excretion and BP*.

Table 2.2 summarizes the multivariate adjusted relationships between urinary electrolyte excretion and BP. Urinary Na^+/K^+ was the only index of salt intake to consistently show modest independent associations with systolic BP in all participants and in participants not receiving antihypertensive therapy. No independent relationships between indexes of salt intake and diastolic BP were noted (Table 2.2).

Table 2.2. Multivariate adjusted relationships between urinary electrolyte excretion and blood pressure (BP) in a group of African ancestry.

	Systolic BP		Diastolic BP	
	partial r*	p value†	partial r*	p value†
	All participants (n=575)			
Urinary Na^+ (mmol/24 hours	0.02	0.49	-0.002	0.95
Urinary K^+ (mmol/24 hours)	-0.04	0.11	-0.06	0.07
Urinary Na^+/K^+	0.11	<0.005	0.05	0.11
Urinary Na^+/creatinine	0.05	0.10	0.03	0.34
Urinary K^+/creatinine	-0.05	0.27	-0.03	0.50
	Untreated participants (n=441)			
Urinary Na^+ (mmol/24 hours)	0.04	0.37	0.05	0.26
Urinary K^+ (mmol/24 hours)	0.04	0.28	0.02	0.48
Urinary Na^+/K^+	0.10	<0.01	0.05	0.19
Urinary Na^+/creatinine	0.07	<0.05	0.06	0.15
Urinary K^+/creatinine	0.003	0.94	0.01	0.73

*Adjustments were for age, sex, body mass index, the presence of diabetes mellitus/abnormal blood glucose control, regular alcohol consumption, regular tobacco use, and antihypertensive treatment. † Probability values were further adjusted for non-independence of family members.

2.3.5 *Associations between BP and either ARR or an interaction between ARR and urinary Na^+/K^+.*

The ARR and an interaction between ARR and urinary Na^+/K^+ were independently associated with BP independent of potential confounders (Table 2.3). The relationship between ARR and BP was largely attributed to the strong negative relationship between plasma renin and BP, whilst only a modest positive relationship between serum aldosterone concentrations and BP was noted (Table 2.3). However, the independent association between BP and the interaction between ARR and salt intake was driven only by a positive association between BP and an interaction between serum aldosterone concentrations and salt intake (Table 2.3). No independent negative association between BP and the interaction between plasma renin concentrations and salt intake was noted (Table 2.3). These results were noted in either all participants or in participants not receiving antihypertensive therapy (Table 2.3).

2.3.6 *Values of ARR and indices of salt excretion above and below the median*.

In order to evaluate the clinical significance of the interactive effects between ARR and urinary Na^+/K^+, relationships with BP were determined in participants with ARR and urinary Na^+/K^+ values above and below the median for the sample. In participants with the square root of ARR above the median (median of the square root of ARR=0.57 ng/dl / ng/l), the mean (±SD) ARR=1.08±0.45 ng/dl / ng/l, plasma renin concentrations=10.8±10.2 pg/ml and plasma aldosterone concentrations=9.2±6.0 ng/dl. In participants with the square root of ARR values below the median, the mean (±SD) ARR=0.30±0.18 ng/dl / ng/l, plasma renin concentrations=66.6±97.1 pg/ml and plasma aldosterone concentrations=5.1±4.8 ng/dl.

In participants with urinary Na^+/K^+ values above the median (median urinary Na^+/K^+=3.73), the mean (±SD) urinary Na^+/K^+=5.8±2.1, 24-hour urinary Na^+ excretion=122.8±75.6 mmol/day and 24-hour urinary K^+ excretion=22.6±14.6 mmol/day. In participants with urinary Na^+/K^+ values below the median, the mean (±SD) urinary

Table 2.3. Multivariate adjusted relationships and interactive (with urinary Na^+/K^+) relationships between circulating markers of the renin-angiotensin-aldosterone system (RAAS) and blood pressure (BP) in a group of African ancestry.

	All participants (n=575)		Untreated (n=441)	
Systolic BP vs	partial r*	p value†	partial r*	p value†
Relationships of the RAAS with BP				
ARR#	0.22	<0.0001	0.14	<0.005
Serum [aldosterone]#	0.08	<0.05	0.08	<0.05
Plasma [renin]#	-0.20	<0.0001	-0.14	<0.005
Relationships of interactions between the RAAS with indexes of salt intake and BP				
ARR# x urinary Na^+/K^+	0.19	<0.0001	0.17	<0.0001
Serum [aldosterone]# x urinary Na^+/K^+	0.13	<0.0001	0.11	<0.005
Plasma [renin]# x urinary Na^+/K^+	0.02	0.52	0.04	0.24

ARR, aldosterone-to-renin ratio. #Serum [aldosterone] and ARR refer to the square root of the values and plasma renin refers to log transformed data *Adjustments were for age, sex, body mass index, urinary Na^+/K^+ (when assessing independent effects), the presence of diabetes mellitus/abnormal blood glucose control, regular alcohol consumption, regular tobacco use, and antihypertensive treatment (in all participants). When assessing aldosterone effects, plasma renin concentrations and diuretic use were also included in the model. When assessing renin effects, diuretic use and angiotensin-converting enzyme inhibitor use were also included in the model. When assessing interactive effects, the independent terms were included in the model. † Probability values were further adjusted for non-independence of family members.

Na^+/K^+=2.7±0.7, 24-hour urinary Na^+ excretion=86.0±48.1 mmol/day and 24-hour urinary K^+ excretion=33.4±19.0 mmol/day.

2.3.7 ARR or serum aldosterone concentrations and the relationship between urinary Na^+/K^+ and BP.

Irrespective of whether all participants or only untreated participants were evaluated, urinary Na^+/K^+ was only significantly related to systolic BP in participants with a square root ARR above the median (Figure 2.2) or a square root serum aldosterone concentration above the median (Figure 2.3) for the group. Moreover, a urinary Na^+/K^+ above the median for the group was only significantly associated with an increased systolic BP in participants with a square root ARR above the median for the group (Figure 2.4). In participants with a square root ARR below the median for the group, urinary Na^+/K^+ was not significantly associated with systolic BP (Figure 2.4). In participants with the square root ARR (Figure 2.5) or square root serum aldosterone concentration (Figure 2.6) above as compared to below the median for the group, every one SD increase in urinary Na^+/K^+ was associated with an approximately three to four-fold greater difference in systolic BP.

2.4 Discussion

The novel findings of the present study are as follows: In 575 participants from a community sample of African ancestry without primary aldosteronism (based on ARR values), despite modest independent associations between urinary indices of salt intake and BP, this relationship was markedly enhanced by an interaction with ARR. In this regard, urinary Na^+/K^+ was only significantly associated with a higher systolic BP in participants with an ARR above the median for the sample and the difference in systolic BP for every one

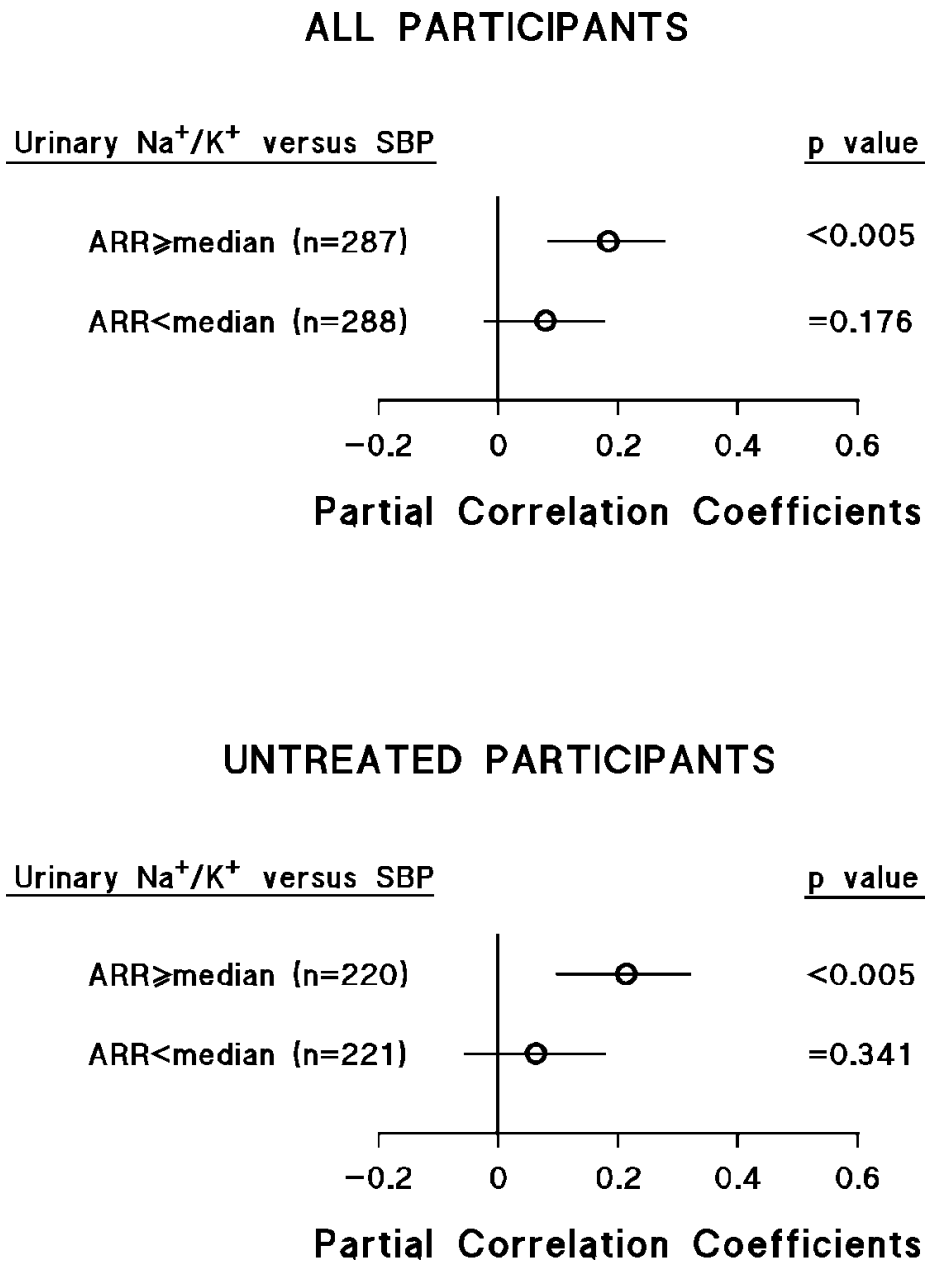
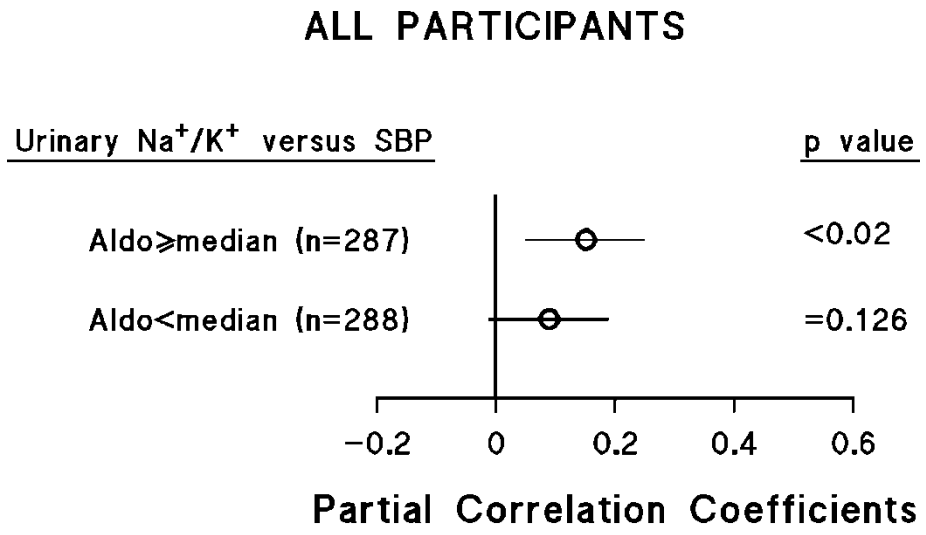


Figure 2.2. Multivariate adjusted partial correlation coefficients (partial r) and 95% confidence intervals for the relations between urinary Na^+/K^+ and systolic blood pressure (SBP) in participants with square root aldosterone-to-renin ratios (ARR) above or below the median for the sample (see text for values). The upper panel shows data in the whole group and the lower panel shows data in participants not receiving antihypertensive therapy (untreated). Adjustments were for age, sex, body mass index, the presence of diabetes mellitus/abnormal blood glucose control, regular alcohol consumption, regular tobacco use, and antihypertensive treatment (in the whole group). Probability values were further adjusted for non-independence of family members.



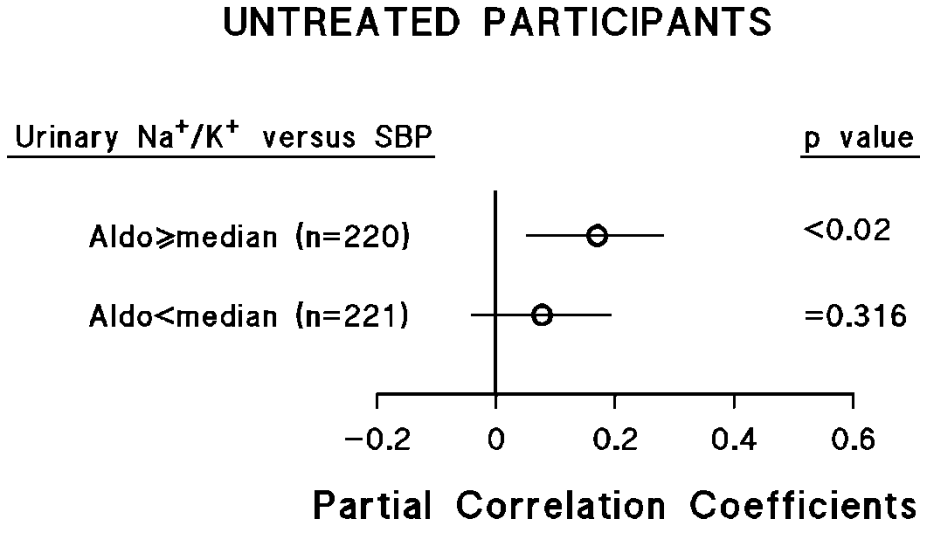


Figure 2.3. Multivariate adjusted partial correlation coefficients (partial r) and 95% confidence intervals for the relations between urinary Na^+/K^+ and systolic blood pressure (SBP) in participants with square root aldosterone concentrations (Aldo) above or below the median (13.1 ng/dl in all and 12.5 ng/dl in untreated) for the sample. The upper panel shows data in the whole group and the lower panel shows data in participants not receiving antihypertensive therapy (untreated). Adjustments are as given in Figure 2.2 with log plasma renin concentrations included.

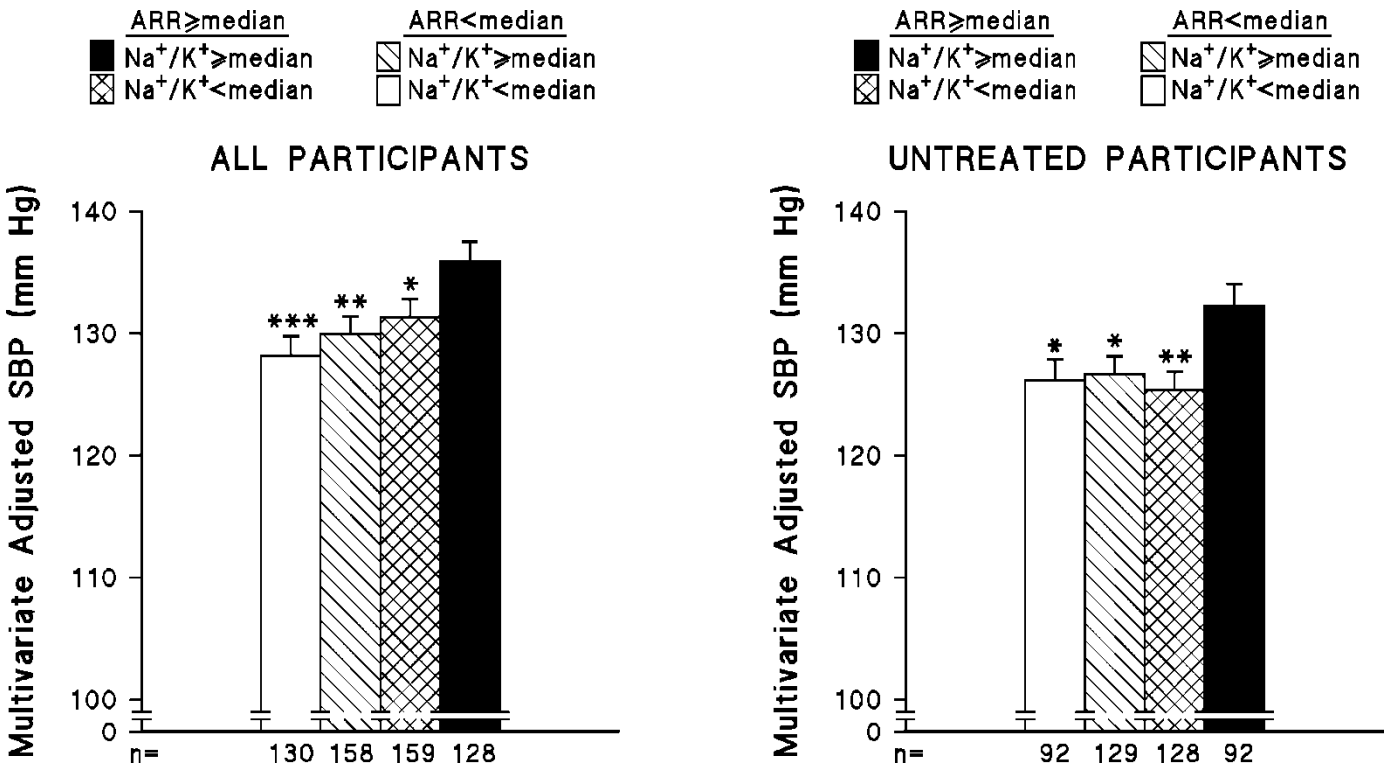


Figure 2.4. Multivariate adjusted systolic blood pressures (SBP) in participants with urinary Na^+/K^+ or a square root aldosterone-to-renin ratio (ARR) above and below the median for the sample (see text for values). The left panel shows data in the whole group and the right panel shows data in participants not receiving antihypertensive therapy (untreated). Adjustments were for age, sex, body mass index, the presence of diabetes mellitus/abnormal blood glucose control, regular alcohol consumption, regular tobacco use, and antihypertensive treatment (in the whole group). Probability values were further adjusted for non-independence of family members.*$p<0.05$, **$p<0.01$, ***$p<0.001$ versus ARR and urine Na^+/K^+ above the median.

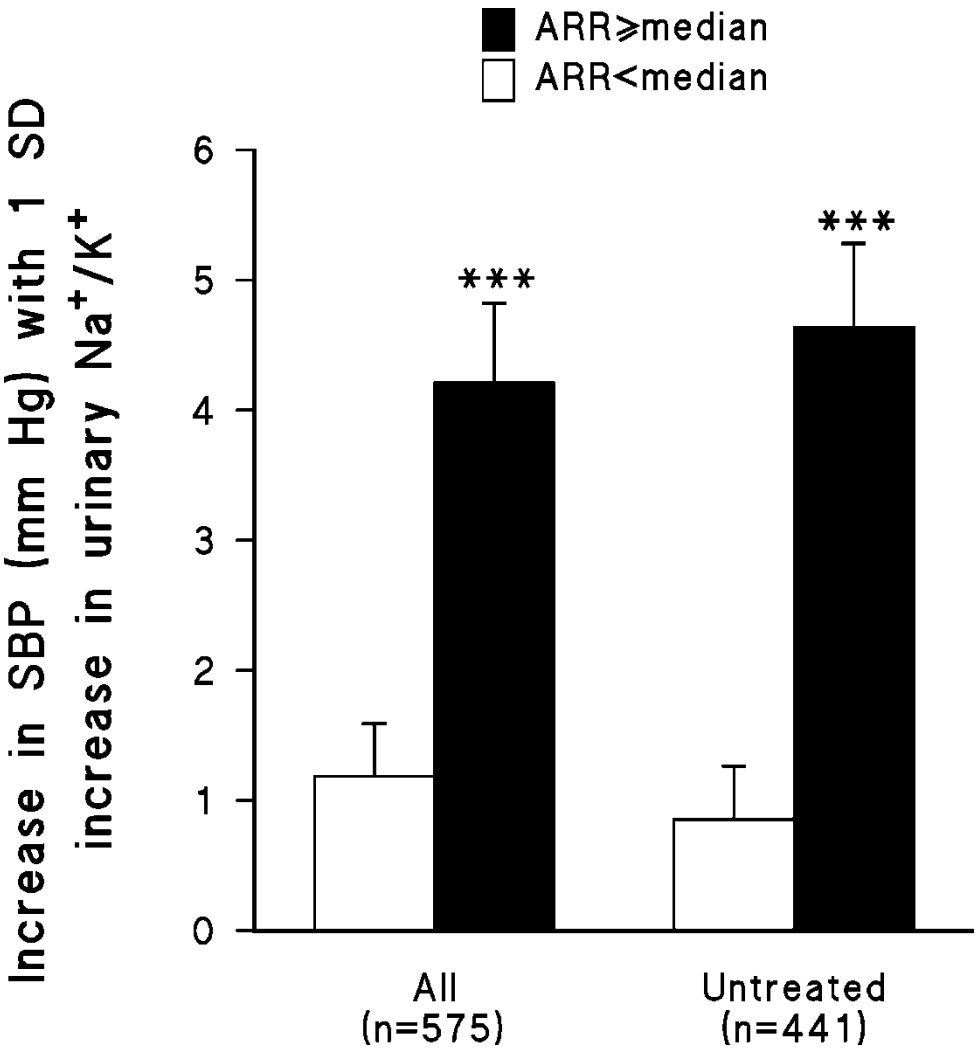


Figure 2.5. Relationship between one standard deviation (SD) increase in urinary Na^+/K^+ and systolic blood pressure (SBP)(±SEM) in participants with square root aldosterone-to-renin ratios (ARR) above or below the median for the sample (see text for values). The left panel shows data in the whole group and the right panel shows data in participants not receiving antihypertensive therapy (untreated). Adjustments were for age, sex, body mass index, the presence of diabetes mellitus/abnormal blood glucose control, regular alcohol consumption, regular tobacco use, and antihypertensive treatment (in the whole group). Probability values were further adjusted for non-independence of family members. ***$p<0.0001$ versus ARR below the median.

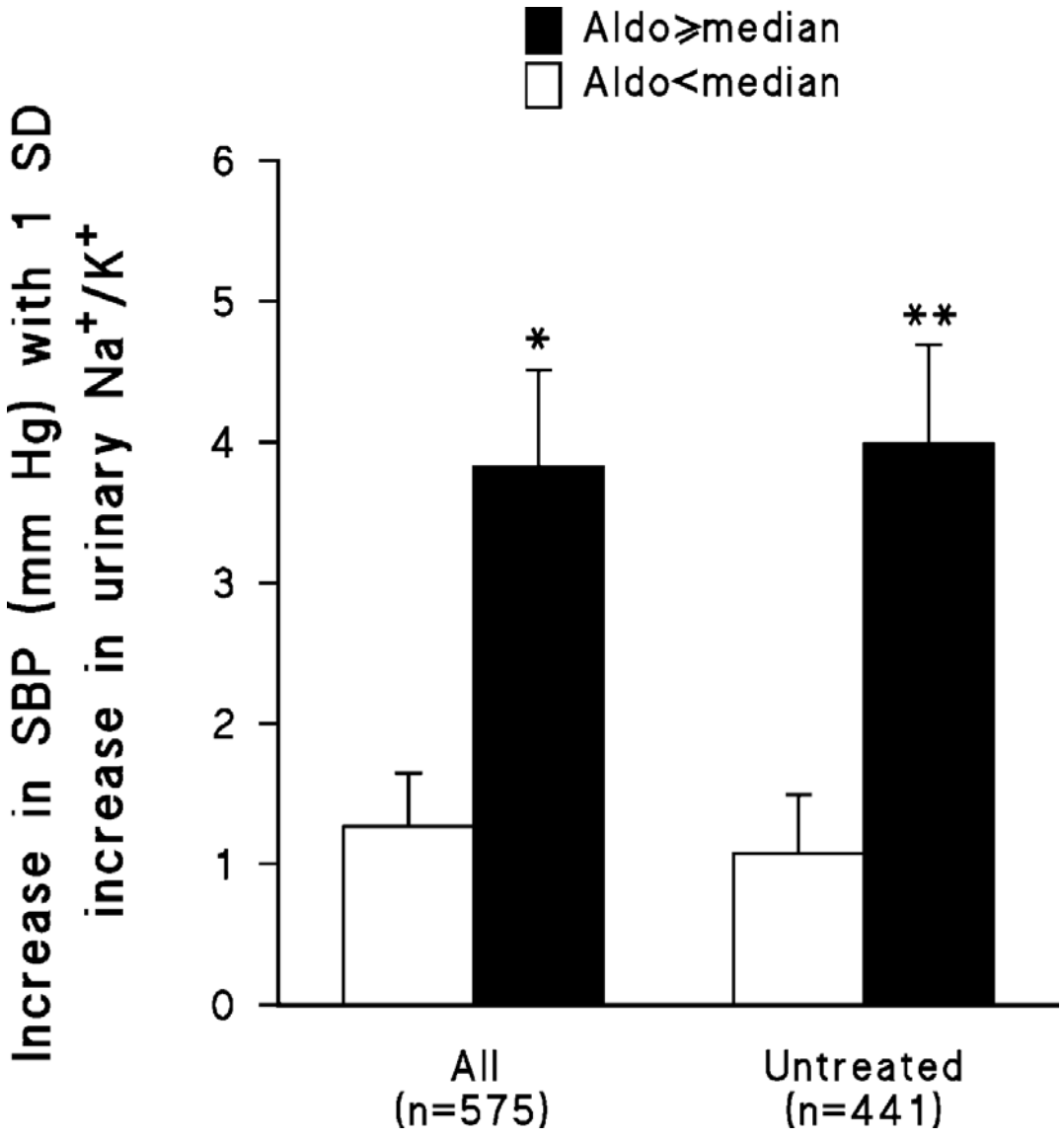


Figure 2.6. Relationship between one standard deviation (SD) increase in urinary Na^+/K^+ and systolic blood pressure (SBP)(±SEM) in participants with square root aldosterone concentrations (Aldo) above or below the median (13.1 ng/dl in all and 12.5 ng/dl in untreated) for the sample. The left panel shows data in the whole group and the right panel shows data in participants not receiving antihypertensive therapy (untreated). Adjustments are as given in Figure 2.5 with log plasma renin concentrations included. *p<0.005 **p<0.0005 versus serum aldosterone concentrations below the median.

SD increase in urinary Na^+/K^+ was four-fold greater in participants with an ARR above as compared to below the median for the sample.

The present study provides the first evidence to indicate that plasma aldosterone concentrations account for a substantial proportion of the variability of the relationship between salt intake and BP at a community level. In this regard, the outcome of the present study suggests that the attenuated decrease in aldosterone concentrations demonstrated to occur in low-renin, salt-sensitive hypertension in case-control studies (Wisgerhof and Brown 1978, Marks et al 1979, Griffing et al 1990, Fisher et al 1999) may indeed play an important role in contributing to salt effects on BP at a community level, at least in groups of African ancestry. The association between ARR or serum aldosterone concentrations and BP noted in the present study is in accordance with the relationship between ARR (Newton-Cheh et al 2007) or circulating aldosterone concentrations (Vasan et al 2004) and the incidence of hypertension reported on in the Framingham Heart Study, and in a French population sample (Meneton et al 2008), as well as the association between circulating aldosterone concentrations and BP in groups of African-descent in some studies (Kidambi et al 2009, Grim et al 2005). However, the present study extends these findings by suggesting that at a community level, the association between aldosterone and BP may be explained by an interaction with salt intake, a hypothesis not explored in incidence studies (Newton-Cheh et al 2007, Vasan et al 2004, Meneton et al 2008) or in cross-sectional studies conducted in groups of African descent (Kidambi et al 2009, Grim et al 2005).

The mean values for aldosterone concentrations reported on by us are similar to those described in alternative groups of African ancestry (Bochud et al 2006, Kidambi et al 2009). However, the mean aldosterone concentrations in groups of largely European descent (Newton-Cheh et al 2007, Vasan et al 2004) tend to be higher than those reported on in groups of African descent as shown in the present and previous (Bochud et al 2006, Kidambi

et al 2009) studies. This may reflect the higher prevalence of low renin, salt sensitive hypertension in groups of African descent, where aldosterone is also suppressed (Wisgerhof and Brown 1978, Marks et al 1979, Griffing et al 1990, Fisher et al 1999). The distribution of plasma aldosterone and plasma renin concentrations shown in the present study are consistent with the distribution of data reported on in an alternative group of African ancestry (Bochud et al 2006). Moreover, the distribution for ARR is very similar to that noted in a group of largely European ancestry (Newton-Cheh et al 2007).

In contrast to the very modest relations noted between aldosterone concentrations and systolic BP in our study ($r=0.08$, $r^2=0.0064$, $p<0.05$), one previous study conducted in African-Americans (Kidambi et al 2009) has demonstrated stronger relations between aldosterone and systolic BP ($r^2=0.047$-0.103). A possible explanation for these differences as suggested by the present study is that in the study conducted in African-Americans (Kidambi et al 2009) Na^+ intake was standardized to a relatively high value (150 mEq per day) over a prolonged period before blood for RAAS measurements was obtained, and hence is more likely to show relationships between aldosterone and BP than in our study where Na^+ intake was lower (mean=107 mEq/day). The results of the present and a previous study (Kidambi et al 2009) are in apparent contrast to the lack of independent relationship noted between aldosterone and BP ($r^2= -0.02$) in a group of East African descent (Bochud et al 2006). However, in that study (Bochud et al 2006) a relationship was noted in older age-groups.

Although no formal interactive analysis was conducted, one previous study (Meneton et al 2008) has assessed the relationship between aldosterone and the incidence of hypertension or an increased BP at different tertiles of salt intake. In contrast to our study which suggests that the relationship between aldosterone and BP is most evident in participants on a high salt diet (Figure 2.3), in this previous study the relationship between aldosterone and hypertension and an increased BP was most evident in participants with the

lowest tertile of salt intake (Meneton et al 2008). An explanation for this potential discrepancy is not evident. However, one possibility is that this previous study (Meneton et al 2008) was conducted in predominantly Caucasian participants who have a lower prevalence of salt-sensitivity than those of African ancestry (Aviv et al 2004). In addition, in this previous study only middle aged (45-60 years) participants were studied (Meneton et al 2008) whereas in the present study a wide age-range of participants were included in the sample.

An excess Na^+ intake is well recognized as decreasing renin release and hence the activity of the RAAS (Wisgerhof and Brown 1978, Marks et al 1979, Griffing et al 1990, Fisher et al 1999). A decreased RAAS activity in-turn promotes Na^+ excretion partly by decreasing aldosterone concentrations (Wisgerhof and Brown 1978, Marks et al 1979, Griffing et al 1990, Fisher et al 1999). Thus, in some measure through compensatory decreases in aldosterone concentrations, an excess Na^+ intake may not increase BP. However, as recently demonstrated in genetically modified animals (Makhanova et al 2008) in circumstances where aldosterone concentrations are insufficiently attenuated relative to renin in the presence of a Na^+ load, the Na^+ load subsequently increases BP. Similarly, in the present study a significant relationship between an index of salt intake (urinary Na^+/K^+) and BP was noted only in participants with an ARR above the median, where aldosterone concentrations are maintained at higher levels, despite Na^+ intake-induced decreases in renin concentrations.

The modest relationship between 24-hour urinary electrolyte excretion rates and BP when considered in the group as a whole (as opposed to in the context of ARR or serum aldosterone concentrations) is consistent with the variable relations noted to occur between urinary electrolyte excretion rates and BP in previous large epidemiological studies (Intersalt Cooperative Research Group 1988, Smith et al 1988) and the lack of relationship between salt intake and BP in epidemiological studies in Africa (Hoosen et al 1985, Charlton et al

2005). Previously suggested reasons for these apparent limited relationships include imprecision in urinary measurements because of considerable individual variability in salt intake and inaccuracies in urine collection. However, the present study suggests that modest relationships between 24-hour urinary electrolyte excretion rates and BP may in-part also be attributed to inter-individual differences in RAAS activity. Indeed, when assessing the relationship between salt intake and BP in participants with an ARR above the median for the sample a 4.2 mm Hg increase in conventional systolic BP was associated with a one standard deviation increase in urinary Na^+/K^+, a size effect that cannot be considered to be clinically negligible. In contrast, in participants with an ARR below the median no detectable relationship between urinary Na^+/K^+ and BP was noted.

In the present study urinary Na^+/K^+, but not 24-hour urinary Na^+ excretion was associated with BP, data that is consistent with the stronger relations noted between urinary Na^+/K^+ and BP than between 24-hour urinary Na^+ excretion rates and BP in previous large, epidemiological studies (Intersalt Cooperative Research Group 1988, Smith et al 1988). This finding could be explained by the decrease in urinary K^+ excretion on a high Na^+ diet in salt-sensitive individuals (Price et al 2002). This effect may occur as a consequence of an enhanced activity of the Na-K-2Cl co-transporter in the thick ascending limb of the renal tubule (Aviv et al 2004).

In the present study primary aldosteronism was excluded on the basis of thresholds for ARR (Ferrari et al 2004), which, in treated participants (23.3%) was measured whilst receiving antihypertensive treatment. In this regard, it is well recognised that circulating measures of the RAAS are influenced by antihypertensive therapy. Indeed, as noted in the present study circulating concentrations of renin and/or aldosterone were associated with diuretic and/or angiotensin-converting enzyme therapy. However, in the present study neither low-dose thiazide diuretic, nor angiotensin-converting enzyme inhibitor therapy was

associated with ARR and no other agents that could modify ARR to any great extent were being used to treat hypertension. Nevertheless, to exclude the possibility that antihypertensive therapy may have produced a false negative result for primary aldosteronism, or influenced relationships between ARR or urinary Na^+/K^+ measurements and BP, sensitivity analysis was conducted by assessing relationships in untreated participants and the primary results of the present study were reproduced. Thus, the results of the present study cannot be attributed to the inadvertent inclusion of individuals with primary aldosteronism or to the effects of antihypertensive therapy on study variables.

A limitation of the present study is the cross-sectional nature of the study design. Thus, the modifying effect of aldosterone on the relationships between salt intake and BP may be a marker rather than a determinant. Longitudinal and intervention studies are required to address this question. Moreover, in the present study 24-hour urinary excretion rates were assessed only once and this is subject to inaccuracies in urine collection despite quality control measures, and does not account for daily variations in salt intake. However, the mean 24-hour urine volumes noted in the present study are higher than those reported on in 23 of 52 sites of the Intersalt study (Intersalt Cooperative Research Group 1988). Furthermore, the electrolyte excretion rates in the present study are the same as that reported on in an alternative study conducted in the same population group and region (Barlow et al 1982) as the present study. In addition, whether 10 minutes of rest was sufficient to achieve steady state renin and aldosterone concentrations is uncertain. However, this is likely to have biased against the results of the present study.

In conclusion, the present study suggests that at a community level aldosterone may modify the association of salt intake with BP in groups of African ancestry. These findings lend insights into the mechanisms that explain the variable relationship between salt intake and BP at a population level and suggest that aldosterone receptor blockade may be an

important modality of therapy at least in groups of African descent. Intervention studies conducted at a population level are required to further evaluate these hypotheses. Moreover, the mechanism of inappropriate suppression of aldosterone relative to plasma renin concentrations requires further study. The contribution of genetic factors was further explored as part of the present thesis and these results are shown in Chapter 3.

Chapter 3

Do Genetic Factors Contribute Toward Circulating Aldosterone Concentrations in a Community Sample of African Ancestry?

Abstract

Although in the absence of primary aldosteronism, circulating aldosterone concentrations are associated with the relationship between salt intake and BP at a community level in participants of African ancestry, whether genetic factors determine excessive circulating aldosterone concentrations in this ethnic group requires elucidation. The familial aggregation and heritability of circulating aldosterone concentrations was therefore assessed in 153 randomly selected nuclear families of African ancestry consisting of 448 participants without primary aldosteronism. Moreover, the relationship between the angiotensin-converting enzyme (ACE) gene insertion/deletion (I/D) polymorphism, the A→C substitution at position -20 of the angiotensinogen (AGT) gene, or the G→A substitution at position -217 of the AGT gene and serum aldosterone concentrations was evaluated. In multivariate models including a number of potential confounders, log renin ($p<0.0001$) and urinary Na^+/K^+ ($p<0.0001$) were independently associated with serum aldosterone concentrations. With, but not without adjustments for plasma renin concentrations, independent correlations were noted for serum aldosterone concentrations between parents and children ($p<0.05$), with parent-child partial correlation coefficients being greater than those for father-mother relationships ($p<0.05$). After, but not before adjustments for plasma renin concentrations, serum aldosterone concentrations showed significant heritability ($h^2=0.25\pm0.12$(SEM), $p<0.02$). No independent relationships between the ACE or AGT gene polymorphisms and serum aldosterone concentrations were observed. In conclusion, the present study highlights the need to consider genetic modulators of serum aldosterone concentrations in the context of plasma renin concentrations in groups of African descent, and supports the notion that considerably more work is required to identify the renin-angiotensin-aldosterone system genes involved.

3.1 Introduction

Although primary aldosteronism may occur with an estimated frequency of 5-15% (Gordon et al 1994, Lim et al 1999, Lim and MacDonald 2003, Mulatero P et al 2004, Olivieri et al 2004), there is increasing evidence to suggest a role at a population level, for increased circulating aldosterone concentrations in the pathogenesis of primary rather than secondary hypertension. In this regard, in the Framingham Heart Study circulating aldosterone concentrations (Vasan et al 2004) and the aldosterone-to-renin ratio (ARR)(Newton-Cheh et al 2007) in ranges that cannot be attributed to primary aldosteronism, were associated with the incidence of hypertension, albeit that the relationship (Vasan et al 2004) was modest at best. These data were supported by the relationship between circulating aldosterone concentrations and the incidence of hypertension in a French population sample (Meneton et al 2008) and the association between aldosterone concentrations and BP in groups of African descent on a high Na^+ diet (Kidambi et al 2009, Grim et al 2005). Furthermore, in chapter 2 of the present thesis I provide evidence to show that although circulating aldosterone concentrations may make only a minor independent contribution to the variability of BP at a community level in a group of African ancestry, serum aldosterone concentrations in the context of renin concentrations were associated with relationship between an index of salt intake and BP (Chapter 2. Scott et al 2011). This association was sufficiently strong in that the relationship between salt intake and BP was 3-4 times greater in participants with an ARR or serum aldosterone concentrations higher when compared to those lower than the median (Chapter 2. Scott et al 2011). Importantly, the association of serum aldosterone concentrations and ARR with the salt intake-BP relationship described in Chapter 2 was noted in participants with an ARR that failed to fulfil the criteria for primary

aldosteronism. Under these circumstances it is important to understand the mechanisms that may drive increased circulating aldosterone concentrations at a community level.

Investigators of the Framingham Heart Study have identified a number of clinical and other correlates with circulating aldosterone concentrations (Kathiresan et al 2005, Newton-Cheh et al 2007) in a predominantly Caucasian group. One notable finding of the Framingham data is the high degree of heritability of circulating aldosterone concentrations in the context of plasma renin, noted in 2271 participants of 998 extended families, the responsible genetic defect which has nevertheless not been identified (Newton-Cheh et al 2007). However, because the impact of salt intake on renin suppression is considerably greater in groups of African as compared to European ancestry (Chrysant et al 1979, James et al 1986, Pratt et al 1999), groups of African ancestry generally have lower plasma renin concentrations than groups of European descent (Fisher et al 1999). Indeed, up to 33% of people of African ancestry have been reported to have undetectable plasma renin levels even with stimulation (Helmer et al 1964, Voors et al 1976). As circulating aldosterone is strongly related to renin release, it is therefore possible that different factors contribute toward the variability of circulating aldosterone concentrations in groups of African ancestry. Indeed, in 32 African-American sibling pairs, no significant heritability of plasma aldosterone concentrations was previously noted (Kotchen et al 2000), a result which could nevertheless be attributed to a small sample size or to an inability to account for the low plasma renin concentrations that may occur in this ethnic group.

To attempt to resolve the issue of the role of genetic factors in contributing toward variations in circulating aldosterone concentrations in groups of African ancestry, in the present study the intrafamilial aggregation and heritability of serum aldosterone concentrations in the context of plasma renin was assessed in nuclear families of African ancestry from a community sample. To identify the potential genetic factors involved,

relationships between serum aldosterone concentrations and a number of gene variants that may influence the production of angiotensin II, and hence aldosterone, was also assessed. These gene variants included the angiotensin-converting enzyme (ACE) gene insertion/deletion (I/D) polymorphism, a A→C substitution at position -20 of the angiotensinogen (AGT) gene which accounts for ~10% of the variability of plasma angiotensinogen concentrations (Ishigami et al 1997), and a G→A substitution at position -217 of the AGT gene, which is associated with increased basal angiotensinogen transcription rates (Jain et al 2002).

3.2 Methods

3.1.1 Study group

The study group has been described in section 2.2 on pages 50-51 of the present thesis. Nuclear families consisting of at least two parents and one sibling or two siblings and one parent were recruited. Of the 538 participants that originally fulfilled the criteria for the study described in Chapter 2 (24-hour urine samples that met with pre-specified quality control criteria [Maseko et al 2006, Redelinghuys et al 2010], measurements of circulating renin and aldosterone concentrations, and whom had an ARR that did not exceed a previously defined threshold of 5.4 ng/dl per ng/l suggestive of primary aldosteronism (Ferrari et al 2004)], in 90 participants incomplete data were obtained in at least one other member of the original nuclear family recruited, thus excluding these 90 participants from the familial aggregation and heritability analysis. Thus, 448 participants were studied in the present analysis. For these analyses 153 families comprising 67 spouse-spouse (father-mother) pairs, 304 parent-child pairs and 104 sibling pairs were included. Of the 448 participants studied, 11 did not consent to genotyping and hence 437 participants were genotyped.

3.2.2 Clinical, demographic and anthropometric measurements.

A standardized questionnaire was administered to obtain demographic and clinical data as described in section 2.2 (pages 51-52) of the present thesis.

3.2.3 Conventional blood pressure measurements.

Trained nurse-technicians measured BP using a standard mercury sphygmomanometer during a clinic visit as described in section 2.2 (page 53) of the present thesis.

3.2.4 Urinary electrolyte excretion rates.

Timed urine samples were obtained over at least a 24-hour period on the same day as the BP measurements as described in section 2.2 (page 54) of the present thesis. The quality of urine samples was determined as described in section 2.2 (page 54) of the present thesis.

3.2.5 Renin and aldosterone concentrations.

Blood samples were obtained in the supine position after 10 minutes of rest in the morning between 10:00 and 12:00 hours. Participants were taking all routine medications at the time. After centrifugation, samples were stored at -70^{o}C until the time of analysis. Plasma renin and serum aldosterone concentrations were determined as described in section 2.2 (pages 54-55) of the present thesis.

3.2.6 Angiotensin-converting enzyme and angiotensinogen genotypes

The restriction fragment length polymorphism (RFLP) method was selected as the primary experimental method, since direct sequencing is cost prohibitive. Deoxyribonucleic acid (DNA) was extracted from whole blood by lysing red blood cells and digesting the remaining white cell pellet with proteinase K. The ACE gene I/D polymorphism was detected by the polymerase chain reaction (PCR) technique using the oligonucleotide primers 5'- CTG GAG ACC ACT CCC ATC CTT TCT -3' and 5'- GAT GTG GCC ATC ACA TTC GTC AGA T -3' flanking the insertion sequence (Rigat et al 1992). The PCR mix contained extracted DNA (25-50ng; 3µl/50µl reaction mixture); 10 x PCR buffer (Tris-HCl [10mM; pH

8.3]; KCl [50mM])(Takara); dNTP mixture (50µM each); $MgCl_2$ (3mM); Taq polymerase (1U/50µl reaction mixture); and primers (25pM each). PCR reaction volumes were 10-50µl and PCR was performed with a denaturing step at 90^{o}C for 1minute; an annealing step at 58^{o}C for 1minute and an extension step at 72^{o}C for 1 minute for 30 cycles. PCR was repeated on DNA from patients homozygous for the D allele with insertion-specific primer pairs, as the deletion sequence is preferentially amplified (Shanmugan et al 1993, Hunley et al 1996). The 170 base pair (D allele) and/or 490 base pair (I allele) PCR products were visualised on a 2% agarose gel. A typical example of the gel electrophoresis patterns obtained is given in Figure 3.1.

Genotyping of an A→C transition at nucleotide -20 of the 5' upstream promoter region of the AGT gene (Ishigami et al 1997), was undertaken using PCR-restriction fragment length polymorphism-based techniques employing the appropriate primer pairs and restriction enzymes. Of the 437 participants genotyped, 3 participants could not be genotyped for the A→C transition at nucleotide -20 of the 5' upstream promoter region of the AGT gene. At least 50% of samples had repeat genotyping performed on them to ensure reproducibility. The standard PCR mix used to genotype for the -20A→C gene variant was 20 µl containing ~50 ng DNA, 10 x PCR buffer (Tris-HCl [10mM; pH 8.3]; KCl [50mM]) (Takara); 2 mM $MgCl_2$, 0.2 mM dNTP, 2.5 mM forward and reverse primers and 1 unit Taq polymerase (Takara). DNA amplification 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. PCR resulted in a 342 bp product. Restriction enzyme digestion was performed by incubating 8µl of the amplicon with 1U of the *EcoOR 109I* restriction endonuclease overnight at 37^{o}C. Restriction enzyme products 205bp and 137bp (A) and 342bp (C) were visualized on 2% agarose gel under UV light (Figure 3.2).

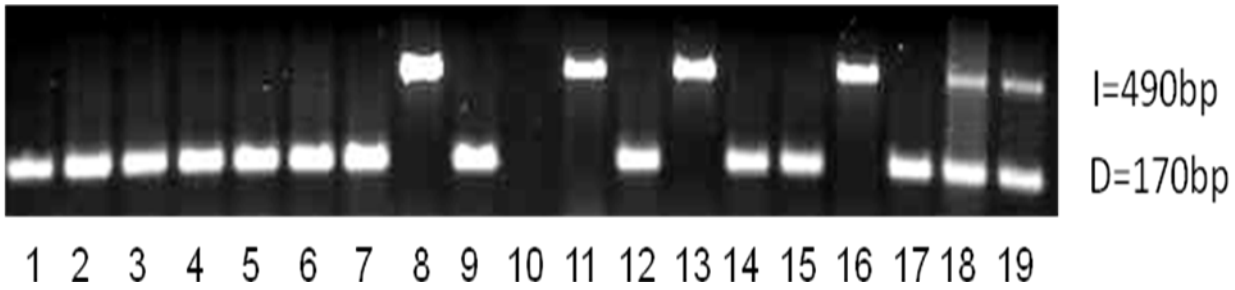


Figure 3.1. Typical example of of a 2% agarose gel with electrophoresis patterns of the angiotensin-converting enzyme (ACE) gene insertion/deletion (I/D) polymorphism. Subjects homozygous for the D and I alleles of the ACE gene are in lanes 1-7, 9, 12, 14, 15, 17 and lanes 8, 11, 13, 16 respectively, and subjects heterozygous for the I/D variant are in lanes 18 and 19.

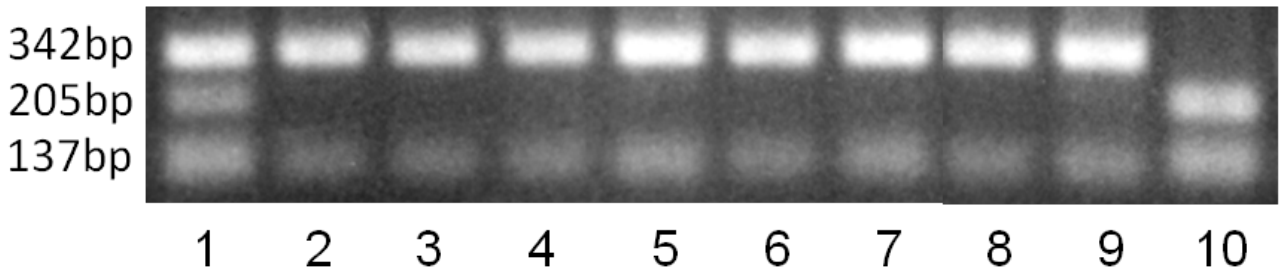


Figure 3.2. Typical example of a 2% agarose gel with electrophoretic patterns of the angiotensinogen (AGT) gene -20C→A polymorphism. Subjects homozygous for the risk allele (AA) are in lanes 2-9, while a heterozygote (-20AC) is in lanes 1. Lane 10 represents a subject homozygous for the C allele (-20CC).

Genotyping of a G→A substitution at position -217 of the AGT gene was undertaken also using PCR-restriction fragment length polymorphism-based techniques employing the appropriate primer pairs and restriction enzymes. Of the 437 participants genotyped, 5 participants could not be genotyped for the G→A substitution at position -217 of the AGT gene. The PCR mix used to genotype for the -217G→A polymorphism was 20 µl containing ~50 ng DNA, 1 x PCR buffer (Tris-HCl [10mM; pH 8.3]; KCl [50mM]) (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). DNA amplification was performed 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. PCR resulted in a 593bp 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 participants with a -217G allele an additional *Msp*I restriction site is recognized and 206, 182, 130, 40 and 37bp fragments are produced (Figure 3.3).

Since this technique was developed in our laboratory, the accuracy of genotyping of the G→A substitution at position -217 of the AGT gene was confirmed with direct sequencing techniques in 300 samples. In this regard, the DNA fragment containing the angiotensinogen gene polymorphism (AGT-217) 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 8µl

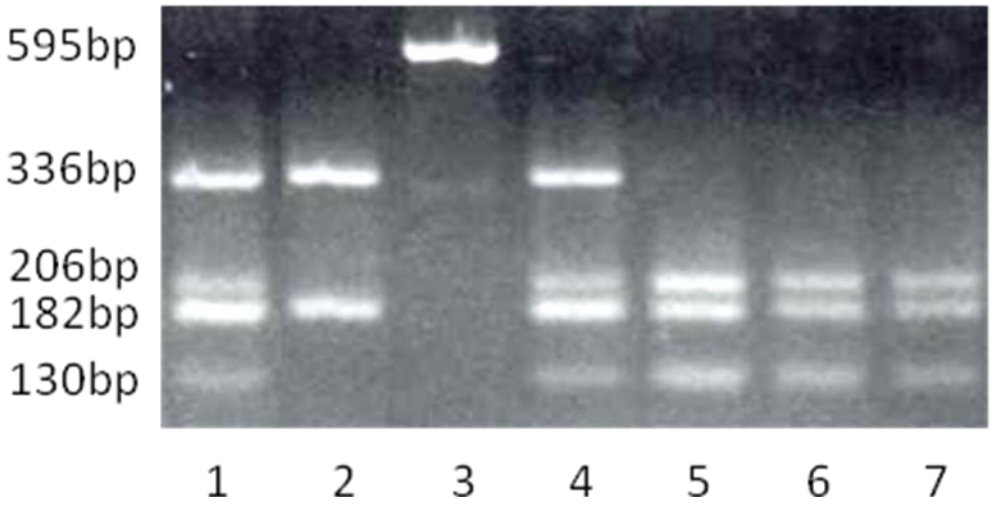


Figure 3.3. Typical example of a 3% agarose gel image showing electrophoretic patterns obtained for the angiotensinogen (AGT) gene -217G→A polymorphism. Lane 2 represents pattern for the -217AA genotype, whilst lanes 1 and 4 are patterns for the -217GA genotype. Lanes 5, 6 and 7 are patterns obtained for the -217GG genotype, whilst lane 3 is a sample of undigested PCR product for the AGT -217G→A gene variant.

Terminator Ready Reaction Mix (containing A-Dye Terminator labelled with dichloro[R6G], C-Dye Terminator labelled with dichloro[ROX], G-Dye Terminator labelled with dichloro[R110], T-Dye Terminator labelled with dichloro[TAMRA], deoxynucleoside triphosphates (dATP, dCTP, dITP, dUTP), AmpliTaq DNA polymerase, FS, with thermally stable pyrophosphatase, $MgCl_2$ and Tris-HCl buffer, pH9.0) and 3.2 pmol sequencing primer to 100ng 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 (AGT-217) Genetic Analyzer. A typical example of a spectrophotograph obtained is indicated in figure 3.4.

3.2.7 Data analysis.

For database management and statistical analysis, SAS software, version 9.1 (SAS Institute Inc., Cary, NC) was employed. Data are shown as mean±SD. As in chapter 2, circulating renin and aldosterone concentrations were expressed as either log (renin) or square-root (aldosterone) values, depending on which provided a better distribution profile. To test for Hardy-Weinberg equilibrium the expected genotype numbers were calculated from the allele frequencies and deviation from the observed genotype numbers was determined using a χ^2 test. Independent relationships with the square root of serum aldosterone concentrations were determined from multivariate regression models with log plasma renin concentrations, age, sex, BMI, the presence of diabetes mellitus/abnormal blood glucose control, regular alcohol consumption, regular tobacco use, and diuretic therapy included in the regression models. As adjustments for urinary Na^+/K^+ did not significantly change the results of the analysis, it was not included as an adjustor in the final analysis. For the derivation of probability values, further adjustments were made for non-independence of family members using the mixed procedure as outlined in the SAS package.

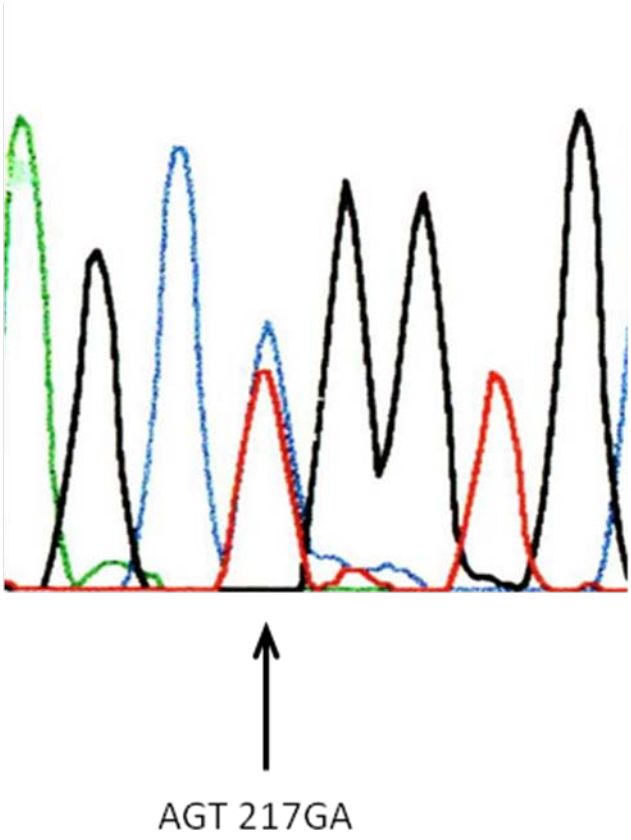


Figure 3.4. Typical example of spectrophotograph obtained whilst genotyping for the AGT -217G→A polymorphism. This spectrophotograph is for a subject with the -217GA genotype. It should be noted that sequencing occurred in the reverse direction and therefore the T/C corresponds to the GA genotype.

To assess the overall genetic contribution to the variability of the square root of serum aldosterone concentrations, intrafamilial aggregation and heritability analyses were performed. For intrafamilial aggregation analysis, intrafamilial correlations were determined from the PROC GENMOD procedure of the SAS package. Unadjusted and adjusted correlation coefficients for the square root of serum aldosterone concentrations were determined in spouse-spouse (sp), parent-child (pc) and sibling-sibling (sib) pairs and the adjusted correlations between related pairs (parent-child and sibling-sibling) compared to those noted between unrelated pairs (spouse-spouse). Narrow-sense heritability estimates were determined from Statistical Analysis for Genetic Epidemiology (S.A.G.E) software (version 6.0) (Department of Epidemiology and Statistics, Case Western Reserve, University of Cleveland, Ohio) using the Marker-Trait Associations in Pedigree Data (ASSOC) programme (S.A.G.E. 6.x [2008]). The ASSOC programme estimates by maximum likelihood, assuming a generalization of multivariate normality, familial variance components and hence heritability, assuming correlation structures described by Elston et al (1992) and the regression model described by George and Elston (1987). The ASSOC programme uses a linear regression model, in which the residual variance is partitioned into the sum of an additive polygenic component and a subject-specific random component. Heritability is the polygenic component divided by the total residual variance. For both the intrafamilial aggregation analysis and the determination of heritability estimates, adjustments were made for log plasma renin concentrations, age, sex, BMI, the presence of diabetes mellitus/abnormal blood glucose control, regular alcohol consumption, regular tobacco use, and diuretic therapy included in the regression models. As antihypertensive therapy may modify plasma renin and serum aldosterone concentrations, to identify the genetic contribution toward the square root of serum aldosterone concentrations, sensitivity analysis was conducted in participants not receiving therapy. However, as the question at hand is the

factors that determine circulating serum aldosterone concentrations at a population level, the analysis was not confined to just participants not receiving therapy, as this would not represent a cross-section of the community.

3.3 Results

3.3.1 Characteristics of the participants.

Table 3.1 gives the characteristics of the parents and siblings in the study. As would be expected with the parents being older, parents had a higher mean BMI than siblings, a greater proportion of parents were obese, were postmenopausal ($p<0.0001$), had hypertension ($p<0.0001$) and had either diabetes mellitus or an abnormal blood glucose control ($p<0.0001$), were receiving glucose lowering agents ($p<0.0001$) or antihypertensive therapy ($p<0.0001$). Both the conventional systolic and diastolic blood pressure of parent were higher than siblings ($p<0.0001$). Of the hypertensives receiving treatment, low-dose thiazide diuretic agents were the most commonly used antihypertensive agents (89.4%) and only 30.6%, 12.9% and 1.2% of treated participants were receiving angiotensin-converting enzyme inhibitors, dihydropyridine-type calcium channel blockers and β-blockers respectively. No participants were receiving centrally acting α_2 agonists, angiotensin receptor blockers, aldosterone receptor antagonists or K^+ sparing or loop diuretic agents. No participants were hypokalemic. As compared to the siblings, the parents had a lower average 24-hour urinary Na^+ excretion rate ($p<0.005$), but no difference in 24-hour urinary K^+ excretion rates ($p=0.20$). The siblings and parents had similar average plasma renin ($p=0.87$) and serum aldosterone ($p=0.11$) concentrations.

Table 3.1. Characteristics of the parents and siblings in randomly selected nuclear families of African ancestry.

	Parents (n=211)	Siblings (n=237*)
Number (% women)	211(63.5)	237 (64.1)
Age (years)	59.9±10.3	30.2±9.9
Body mass index (kg/m^2)	32.8±7.4	26.7±7.7
% overweight/obese	28.9/58.3	22.4/26.2
Regular tobacco intake (% subjects)	27(12.8)	42(17.2)
Regular alcohol intake (% subjects)	53(25.1)	62(26.1)
% Females postmenopausal	88.1	8.6
% Diabetes mellitus or HbA_{1c}>6.1%	43.6	10.3
% receiving glucose lowering agents	14.7	0.4
% Hypertension	63.5	18.6
Current antihypertensive medication (%)	40.3	6.3
Total/HDL cholesterol	3.9±1.2	3.3±3.4
Urinary Na^+ excretion (mmol/24 hours)	96.9±55.1	114±70.4
Urinary K^+ excretion (mmol/24 hours)	27.2±15.9	29.3±18.4
Urinary Na^+/K^+	4.0±2.0	4.4±2.4
Plasma renin concentrations (pg/ml)	39.4±67.6	38.3±71.5
Serum aldosterone concentrations (ng/dl)	7.5±6.1	6.6±5.5
Log plasma renin (pg/ml)	1.14±0.62	1.22±0.55
Square root serum aldosterone (ng/dl)	13.0±6.3	12.1±6.1
Conventional SBP/DBP (mm Hg)	143±23/89±12	121±17/81±12
Pulse rate (beats/minute)	65±13	64±12

HbA_{1c}, glycated haemoglobin; HDL, high density lipoprotein; SBP, systolic blood pressure; DBP, diastolic blood pressure.

As compared to the parents studied, parents who were excluded from the study sample (n=160) were of a similar age (60.3±11.3 years), had a similar BMI (33.0±7.7 kg/m^2), and an equivalent proportion of these participants were overweight/obese (20.7/63.6%), smoked regularly (11.4%), and consumed alcohol regularly (12.1%). However, a greater proportion had diabetes mellitus or an HbA_{1c}>6.1% (37.1%)($p<0.005$ as compared to the parents studied, and had hypertension (74.3%)($p<0.05$ as compared to the parents studied). As compared to the siblings studied, siblings without appropriate 24-hour urine collections who were excluded from the study sample (n=264) were of a similar age (31.7±12.1 years), had a similar BMI (27.2±7.1 kg/m^2), and an equivalent proportion of these participants were overweight/obese (19.8/31.7%), smoked regularly (16.0%), consumed alcohol regularly (21.1%), had diabetes mellitus or an HbA_{1c}>6.1% (8.4%), or had hypertension (21.9%).

3.3.2 Distribution of RAAS measurements in parents and siblings.

Figure 3.5 compares the distribution of plasma renin [renin] and serum aldosterone [aldosterone] concentrations in parents and siblings. The distributions of these parameters departed from normality and were positively skewed in both the parents (skewness: [renin]=2.82, [aldosterone]=1.55; kurtosis: [renin]=8.48, [aldosterone]=4.28; Shapiro-Wilk's statistic: [renin]=0.59, [aldosterone]=0.89) and in the children (skewness: [renin]=5.28, [aldosterone]=1.60; kurtosis: [renin]=40.2, [aldosterone]=4.2; Shapiro-Wilk's statistic: [renin]=0.48, [aldosterone]=0.88).

3.3.3 Clinical and phenotypic correlates with serum aldosterone concentrations.

As shown in Table 3.2, in a multivariate model including age, gender, BMI, log renin, urinary Na^+/K^+, diabetes mellitus or an abnormal blood glucose control, diuretic use, angiotensin-converting enzyme inhibitor use, β-blocker use, menopausal status, and total/HDL cholesterol in the model; as partly reported on in Chapter 2, log renin, diuretic use, urinary Na^+/K^+ and male gender were associated with the square root of serum aldosterone

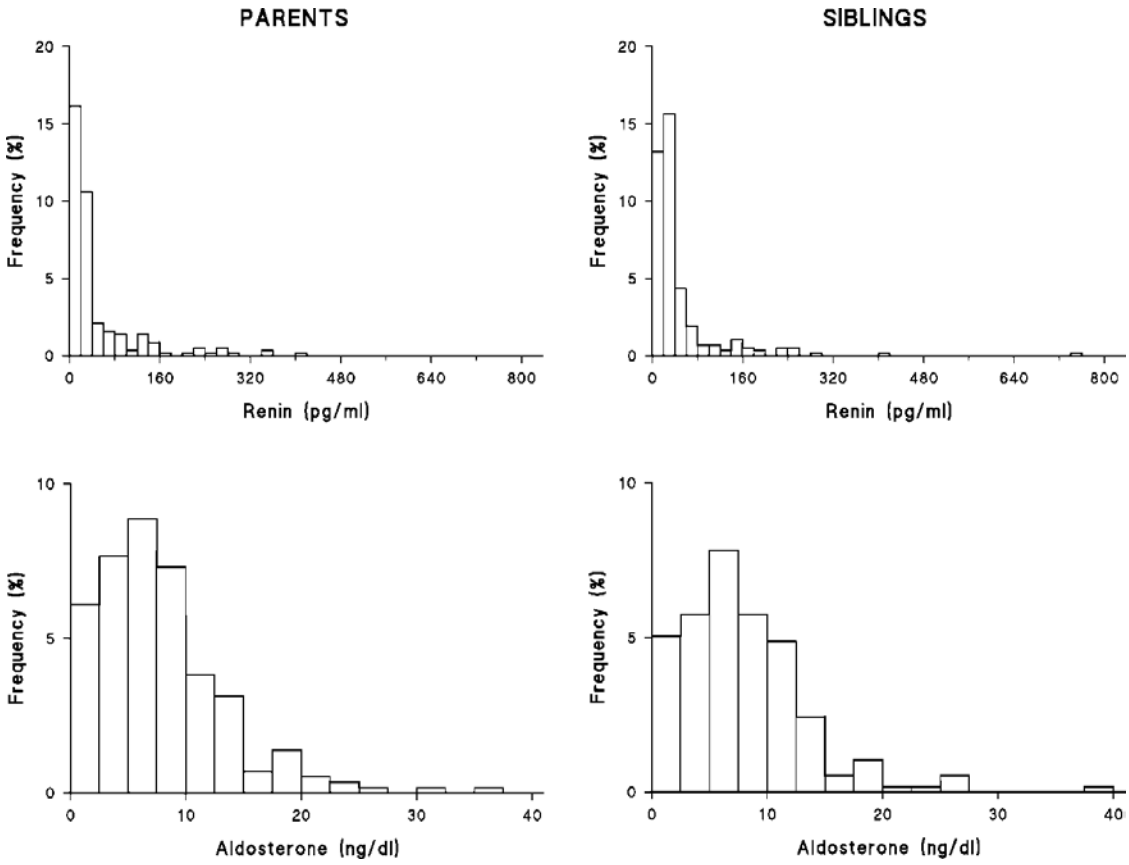


Figure 3.5. Distribution of plasma renin and serum aldosterone concentrations in the parents and siblings of the community sampled.

Table 3.2. Factors independently related to the square root of serum aldosterone concentrations in a multivariate model.

	Total sample (n= 448)		Untreated participants (n= 348)	
	β-coefficient±SEM	p value	β-coefficient±SEM	p value
Log renin	2.34±0.49	**<0.0001**	2.04±0.56	<**0.0005**
Age	0.0063±0.0192	=0.74	-0.01±0.02	=0.63
Male gender	1.60±0.63	**=0.01**	-1.43±0.71	<**0.05**
Body mass index	0.03±0.04	=0.42	0.07±0.05	=0.15
DM or HbA_{1c}>6.1%	0.08±0.72	=0.91	-0.11±0.86	=0.90
Urinary Na^+/K^+	-0.74±0.12	**<0.0001**	-0.74±0.13	**<0.0001**
Postmenopausal	0.24±1.24	=0.85	-0.42±1.35	=0.76
Total/HDL cholesterol	0.022±0.103	=0.8	0.03±0.10	0.77
Diuretic use	1.98±0.82	=**0.02**	-	-
ACEI use	0.38±1.20	=0.75	-	-
β-blocker use	6.05±5.81	=0.30	-	-

DM, diabetes mellitus, ACEI, angiotensin-converting enzyme inhibitor.

concentrations. In participants not receiving antihypertensive therapy, log renin, male gender and urinary Na^+/K^+ were similarly associated with the square root of serum aldosterone concentrations. In separate models, with plasma Na^+ and plasma K^+ concentrations in the model, neither parameter was independently associated with the square root of serum aldosterone concentrations (data not shown).

3.3.4 Familial aggregation of serum aldosterone concentrations.

Figures 3.6 and 3.7 show the unadjusted and multivariate adjusted correlation coefficients for the square root of serum aldosterone concentrations noted between spouse-spouse, parent-offspring, and sibling-sibling pairs. Figure 3.6 shows intrafamilial correlations in all participants and Figure 3.7 intrafamilial correlations in participants not receiving antihypertensive therapy. As a comparator of the sensitivity for detecting genetic effects using this approach, intrafamilial correlation coefficients for body height are shown. As one would predict, body height was strongly correlated between parents and children ($p<0.0001$) and between sibling pairs ($p<0.0001$), but not between mothers and fathers. Moreover, the correlations coefficients for height between sibling-sibling pairs was greater than that between mothers and fathers ($p<0.05$ for comparison of multivariate adjusted correlation coefficients).

In the whole group, before adjustments, no correlations of the square root serum aldosterone were noted between father-mother, parent-child or sibling-sibling (model 1, Figure 3.6). Moreover, no significant relationships in the square root serum aldosterone concentrations were noted when adjusted for age, gender, body mass index, diabetes mellitus or an HbA_{1c}>6.1%, regular alcohol, regular smoking and diuretic use (model 2, Figure 3.6). Adjustments for urinary Na^+/K^+ did not significantly modify the results (model 3). However, after adjustments for the aforementioned and log renin, but not urinary Na^+/K^+, positive correlations were noted in the square root serum aldosterone concentrations between parents

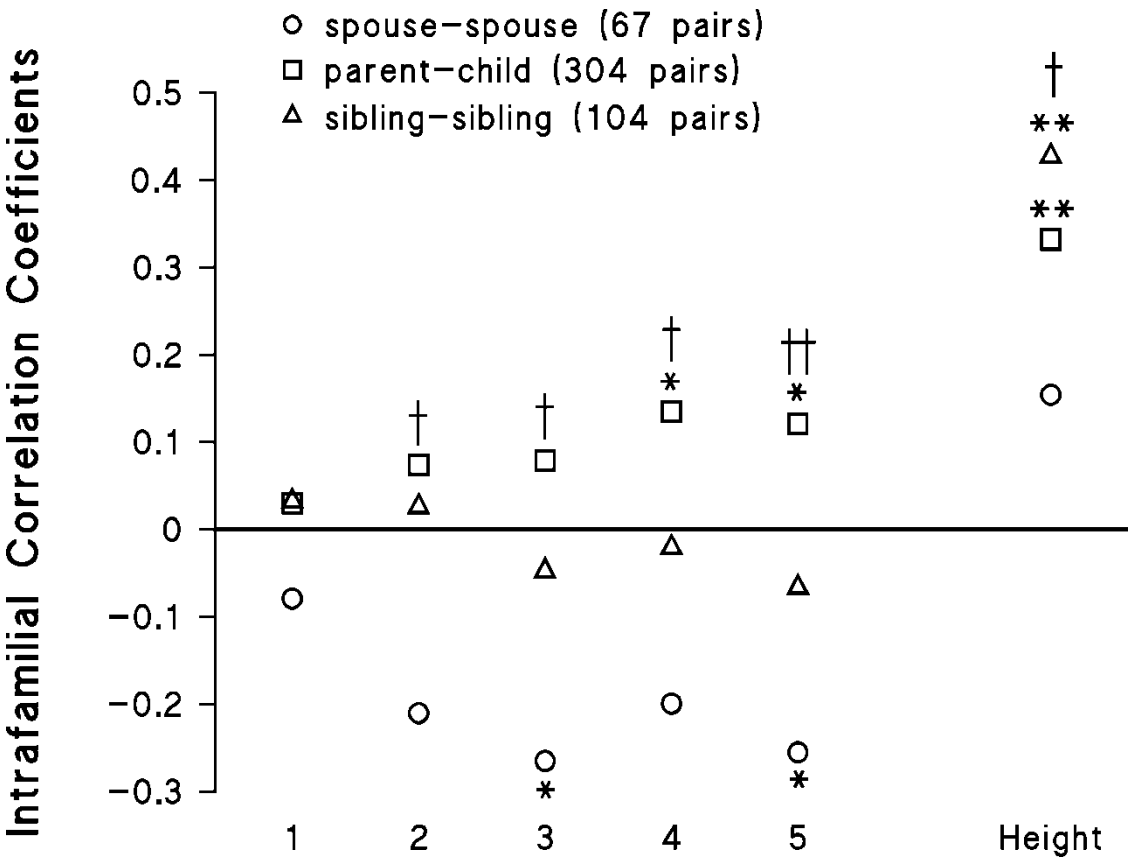


Figure 3.6. Intrafamilial correlation coefficients for the square root serum aldosterone concentrations in nuclear families of African origin (models 1 to 5). As a comparator of the sensitivity of detecting genetic effects using this approach, intrafamilial correlation coefficients for body height are also shown. Model 1 is unadjusted. Model 2 is adjusted for age, gender, body mass index, diabetes mellitus or an HbA_{1c}>6.1%, regular smoking, regular alcohol and diuretic use. Model 3 is adjusted for all of the aforementioned and urinary Na^+/K^+. Model 4 is adjusted for the aforementioned and log renin, but not urinary Na^+/K^+. Model 5 includes both log renin and urinary Na^+/K^+ in the same model. Sib, sibling. *p<0.05, ** p<0.001 versus 0; † p<0.05, †† p<0.01 versus spouse-spouse.

and offspring ($p<0.05$) (model 4, Figure 3.6). Moreover, differences were noted when comparing the correlations coefficients for the square root serum aldosterone concentrations between spouse-spouse and parent-child ($p<0.05$ for comparison of multivariate adjusted correlation coefficients) (model 4, Figure 3.6). The further addition of urinary Na^+/K^+ to model 4, did not change the results (model 5, Figure 3.6). In sensitivity analysis conducted in untreated participants similar trends were noted, but I was underpowered to show statistically significant differences (Figure 3.7).

3.3.5 Heritability of serum aldosterone concentrations.

Before adjustments the heritability (h^2) estimate for the square root of serum aldosterone concentrations was 0.08±0.11(SEM) ($p=0.24$). The heritability (h^2) estimate for the square root of serum aldosterone concentrations after adjustments for age, male gender, body mass index, diabetes mellitus or an $HbA_{1c}>6.1\%$, regular smoking, regular alcohol, and diuretic use was 0.14±0.11(SEM) ($p=0.11$). However, after including log renin as an additional adjustor, the heritability (h^2) estimate for the square root of serum aldosterone concentrations was 0.25±0.12(SEM) ($p<0.02$). In participants not receiving therapy the heritability (h^2) estimate for the square root serum aldosterone concentrations after adjustments for the same confounders except antihypertensive therapy or log renin was 0.15±0.14(SEM) ($p=0.14$). After including log renin as an additional adjustor, the heritability (h^2) estimate for the square root of serum aldosterone concentrations in untreated participants was 0.21±0.14(SEM) ($p=0.07$).

3.3.6 Genotype and allele frequencies and relationships between genotype and serum aldosterone concentrations.

The genotype frequencies of the ACE gene I/D and AGT gene -20A→C, and -217G→A variants were in Hardy-Weinberg equilibrium (Table 3.3). Neither ACE nor AGT genotypes were associated with serum aldosterone concentrations either before or after

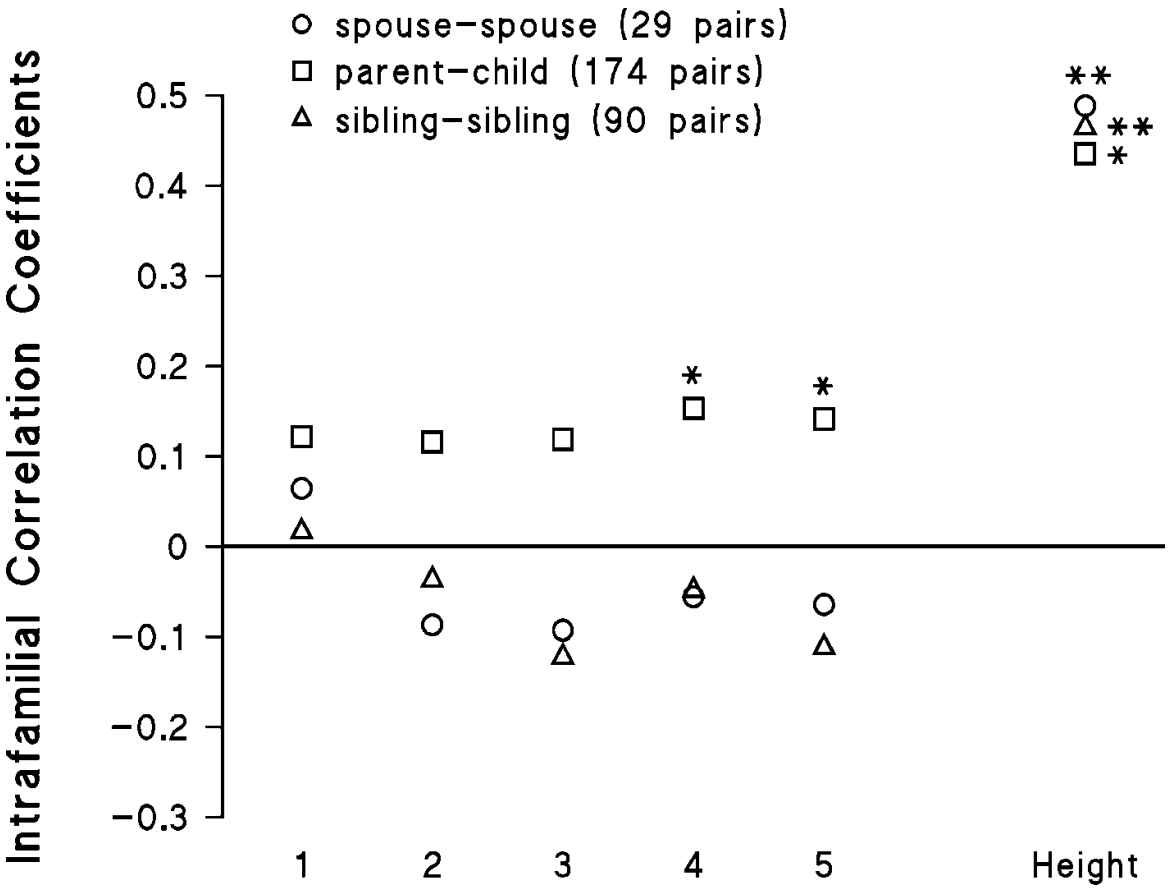


Figure 3.7. Intrafamilial correlation coefficients for the square root of serum aldosterone concentrations in participants of nuclear families of African origin not receiving antihypertensive therapy (models 1 to 5). As a comparator of the sensitivity of detecting genetic effects using this approach, intrafamilial correlation coefficients for body height are shown. Model 1 is unadjusted. Model 2 is adjusted for age, gender, body mass index, diabetes mellitus or an HbA_{1c}>6.1%, regular smoking, regular alcohol and diuretic use. Model 3 is adjusted for all of the aforementioned and urinary Na^+/K^+. Model 4 is adjusted for the aforementioned and log renin, but not urinary Na^+/K^+. Model 5 includes both log renin and urinary Na^+/K^+ in the same model. Sib, sibling. *p<0.05, ** p<0.001 versus 0.

Table 3.3. Genotype and allele frequencies of angiotensin-converting enzyme and angiotensinogen gene variants in participants of African origins.

All participants

Genotype Allele

Angiotensin-converting enzyme gene insertion/deletion (I/D) variant

II n=43(9.8%) ID n=164(37.5%) DD n=230(52.6%) I n=250(28.6%) D n=624(71.4%)

Angiotensinogen gene -20A→C variant

AA n=321(74.0%) AC n=105(24.2%) CC n=8(1.8%) A n=747(86.1%) C n=121(13.9%)

Angiotensinogen gene -217G→A

AA n=97(22.5%) AG n=199(46.1%) GG n=136(31.5%) A n=393(45.5%) G n=471(54.5%)

Unrelated participants

Genotype Allele

Angiotensin-converting enzyme gene insertion/deletion (I/D) variant

II n=15(7.7%) ID n=75(38.5%) DD n=105(53.8%) I n=105(26.9%) D n=285(73.1%)

Angiotensinogen gene -20A→C variant

AA n=137(72.9%) AC n=46(24.5%) CC n=5(2.7%) A n=320(85.1%) C n=56(14.9%)

Angiotensinogen gene -217G→A

AA n=39(21.1%) AG n=84(45.4%) GG n=62(33.5%) A n=162(43.8%) G n=208(56.2%)

Numbers are sample numbers (%).

Table 3.4. Serum aldosterone concentrations (ng/dl) in genotype-specific groups in participants of African origins.

	Unadjusted mean±SEM	Adjusted mean±SEM*	p values for comparisons of adjusted data
Angiotensin-converting enzyme gene insertion/deletion (I/D) variant			
II (n=43)	12.8±0.9	13.3±0.9	II vs DD=0.43
ID (n=164)	12.2±0.5	12.3±0.5	II vs ID =0.36
DD (n=230)	12.6±0.4	12.5±0.4	ID vs DD=0.75
Angiotensinogen gene -20A→C variant			
AA (n=321)	12.2±0.3	12.3±0.3	AA vs AC=0.30
AC (n=105)	12.9±0.6	13.0±0.6	AC vs CC=0.40
CC (n=8)	15.1±2.2	14.9±2.0	CC vs AA=0.18
Angiotensinogen gene -217G→A variant			
AA (n=97)	11.8±0.6	12.0±0.6	AA vs GG=0.31
AG (n=199)	12.8±0.4	12.6±0.4	AA vs AG=0.40
GG (n=136)	12.6±0.5	12.8±0.5	AG vs GG=0.75

* Adjustments are for log renin, age, gender, body mass index, diabetes mellitus or an HbA_{1c}>6.1%, regular smoking, regular alcohol, and diuretic use.

Table 3.5. Serum aldosterone concentrations (ng/dl) in genotype-specific groups in participants of African origins not receiving antihypertensive therapy.

	Unadjusted mean±SEM	Adjusted mean±SEM*	p values for comparisons of adjusted data
<u>Angiotensin-converting enzyme gene insertion/deletion (I/D) variant</u>			
II (n=39)	12.5±1.0	12.7±0.9	II vs DD=0.40
ID (n=124)	11.3±0.5	11.5±0.5	II vs ID =0.24
DD (n=177)	12.1±0.4	11.9±0.4	ID vs DD=0.53
<u>Angiotensinogen gene -20A→C variant</u>			
AA (n=248)	11.5±0.4	11.5±0.4	AA vs AC=0.19
AC (n=85)	12.5±0.6	12.5±0.6	AC vs CC=0.15
CC (n=4)	15.3±2.9	16.6±2.8	CC vs AA=0.11
<u>Angiotensinogen gene -217G→A variant</u>			
AA (n=76)	11.1±0.7	11.2±0.6	AA vs GG=0.26
AG (n=151)	12.1±0.5	11.9±0.5	AA vs AG=0.40
GG (n=108)	12.0±0.6	12.2±0.6	AG vs GG=0.70

* Adjustments are for log renin (in the case of serum aldosterone), age, gender, body mass index, diabetes mellitus or an HbA_{1c}>6.1%, regular smoking, and regular alcohol.

adjustments for confounders either in the whole group (Table 3.4) or in participants not receiving antihypertensive therapy (Table 3.5). Similar results were noted when assessing the relationship between ACE or AGT genotype and the square root serum aldosterone concentrations (data not shown).

3.4 Discussion

The main findings of the present study are that in nuclear families of African ancestry, before adjustments for plasma renin concentrations, the square root serum aldosterone concentrations showed no significant intrafamilial aggregation. However, after adjustments for plasma renin concentrations and additional confounders, serum aldosterone concentrations were related in parents and offspring, but not between spouse pairs. Importantly, the multivariate adjusted correlations for the square root serum aldosterone concentrations between parents and offspring (related individuals) were greater than the multivariate adjusted correlations for the square root serum aldosterone between spouse pairs (unrelated individuals). Moreover serum aldosterone concentrations showed significant heritability (h^2=25±0.12) after adjustments for plasma renin concentrations and additional confounders. Despite the evidence for a genetic contribution to the variance in serum aldosterone concentrations, with similar adjustments (log renin and other confounders), no relationships between previously described ACE and AGT gene variants and serum aldosterone concentrations were noted.

The present study conducted in 448 participants and 153 families is the first to show familial aggregation and heritability of circulating aldosterone concentrations in a community of African ancestry. This finding is in contrast to the lack of heritability of plasma aldosterone concentrations noted on 32 African-American sib-pairs previously reported on

(Kotchen et al 2000). However, in that study (Kotchen et al 2000), the heritability estimate for plasma aldosterone concentrations was 0.19, which is not that much lower than the heritability estimate of 0.25 for serum aldosterone concentrations reported on in the present study. Thus, this previous study (Kotchen et al 2000) is likely to have had insufficient statistical power to show significant heritability. Alternatively, in the study by Kotchen et al (2000), heritability estimates were not determined after adjustments for circulating renin activity or concentrations. In this regard, it is well recognised that groups of African ancestry have a high prevalence of low plasma renin activity and hence often low serum aldosterone concentrations relative to other populations (Bochud et al 2006, Helmer et al 1964, Voors et al 1976). Thus it is likely that the heritability of circulating aldosterone concentrations will only become evident if one accounts for circulating renin activity or concentrations. Indeed, in the present study significant heritability and intrafamilial correlations for serum aldosterone concentrations were noted only after adjustments for plasma renin concentrations. Without these adjustments, neither familial aggregation nor heritability of serum aldosterone concentrations was noted.

The familial aggregation and heritability of serum aldosterone concentrations noted in a group of black African descent in the present study is consistent with the heritability of serum aldosterone concentrations noted in a largely Caucasian group in the Framingham Heart Study (Kathiresan et al 2005, Newton-Cheh et al 2007). However, in contrast to the heritability estimate of 0.25 noted in the present study, the Framingham Heart Study only reported on heritability estimates of 0.10-0.11 (Kathiresan et al 2005, Newton-Cheh et al 2007). In one of these studies (Kathiresan et al 2005), no adjustments for plasma renin concentrations or activity were made, and in the later study (Newton-Cheh et al 2007) it is uncertain whether adjustments for plasma renin concentrations or activity were included in the multivariate models. In the present study before adjustments for plasma renin

concentrations, neither familial aggregation nor significant heritability of serum aldosterone concentrations was noted and the heritability estimates of serum aldosterone concentrations was 0.14, which is similar to the heritability estimates of the Framingham Study (0.10-0.11) (Kathiresan et al 2005, Newton-Cheh et al 2007). The present study therefore underscores the importance in genetic studies of carefully considering genotype-phenotype relationships in the context of the complexity of the determinants of specific phenotypes. In this regard, the penetrance of genetic factors that modulate serum aldosterone concentrations, at least in the African context is likely to be strongly influenced by plasma renin concentrations.

The lack of relationship between ACE or AGT genotypes and serum aldosterone concentrations noted in the present study is consistent with the lack of relationship between ACE and AGT gene variants previously reported on in a large study sample of Caucasians (Paillard et al 1999). Nevertheless, as compared to the present study, alternative genetic variants (ACE 4556$(CT)_{2/3}$ and AGT C→T(-532) gene variants) were also evaluated in the study by Paillard et al (1999). The strong modifying influence of plasma renin concentrations on the penetrance of genetic effects on serum aldosterone concentrations as suggested by the present study, may explain the previously reported lack of relationship between ACE and AGT genes and circulating aldosterone concentrations (Paillard et al 1999). Indeed, the lack of relationship between ACE and AGT genes and circulating aldosterone concentrations was noted despite relationships between genotypes and plasma angiotensin-converting enzyme or angiotensinogen concentrations (Paillard et al 1999). It is possible therefore that in the study by Paillard et al (1999), relationships between genotype and aldosterone concentrations could have occurred if adjustments had been made for the effects of plasma renin activity. However, the present study suggests that even with these adjustments, neither ACE not AGT gene variants are associated with serum aldosterone concentrations, at least for the variants studied.

The inability to show a relationship between two functional variants of the AGT gene and serum aldosterone concentrations in a group of African descent in the present study, even after adjustments for plasma renin concentrations, is in apparent contrast to one previous study demonstrating a relationship between the -6G→A variant of the AGT gene and plasma aldosterone concentrations in a Caucasian sample (Fardella et al 1999). In the present study I did not assess the relationship between the -6G→A variant of the AGT gene and serum aldosterone concentrations as our group have previously shown (Tiago et al 2002) that the -6G→A variant allele associated with plasma aldosterone concentrations in the one previous study (Fardella et al 1999) occurs with a frequency in groups of African ancestry in South Africa that would require a very large study sample to achieve statistical power. Nevertheless, our group have also previously shown linkage disequilibrium between one of the functional variants assessed in the present study (-20A→C) and the -6G→A variant of the AGT gene (Tiago et al 2002).

As recently pointed out (Norton et al 2010) the current approach to evaluating associations between single nucleotide polymorphisms and phenotypes does not account for the variability across the gene. The more modern approach is to assess the effect of genotypes based on haplotype bins of single nucleotide polymorphisms, where each bin is considered as a genotype, thus accounting for the full variability across the gene. Further studies with haplotype constructs are therefore required to exclude the possibility that the ACE and the AGT locus are associated with serum aldosterone concentrations in groups of African descent. These studies are presently underway in our laboratory.

In keeping with previous studies (Kathiresan et al 2005, Newton-Cheh et al 2007, Brunner et al 1972), in the present study an index of salt intake (urinary Na^+/K^+) was associated with decreased serum aldosterone concentrations, perhaps through suppression of the renin-angiotensin-aldosterone system. Moreover, as with the studies by Kathiresan et al

(2005), and Brunner et al (1972), in the present study diuretic use was positively associated with serum aldosterone concentrations, possibly because volume depletion activates the renin-angiotensin-aldosterone system.

In contrast to the findings of a relationship between female gender and plasma aldosterone concentrations by Kathiresan et al (2005) and Newton-Cheh et al (2007), in the present study I showed a relationship between male gender and serum aldosterone concentrations. Moreover, although in the present study a high proportion of mothers (88%) were postmenopausal as confirmed with FSH measurements, and none were receiving hormone replacement therapy, unlike in the study by Kathiresan et al (2005), I could show no relationship between serum aldosterone concentrations and menopause. In agreement with Kathiresan et al (2005), in the present study no relationships between serum aldosterone concentrations and either age, body mass index or diabetes mellitus were noted. However, this is in contrast to previously reported relationships between age (Weidman et al 1975); diabetes mellitus (Perez et al 1977); features of the metabolic syndrome (Bochud et al 2006); or obesity (Goodfriend et al 1998, Rocchini et al 1986) and aldosterone concentrations in alternative studies. Moreover, in contrast to what has previously been shown (Kathiresan et al 2005, Newton-Cheh et al 2007), where circulating aldosterone concentrations were positively associated with total/high-density lipoprotein cholesterol ratio, a similar independent relationship was not observed in the present study. There is no obvious explanation for any of these apparent discrepancies between studies.

In contrast to what has previously been shown (Newton-Cheh et al 2007) where in the context of renin concentrations, circulating aldosterone concentrations were inversely associated with angiotensin-converting enzyme inhibitor use and positively associated with β-blocker use, in the present study no relations were noted between angiotensin-converting enzyme inhibitor or β-blocker use and serum aldosterone concentrations. However, the

proportion of participants receiving these agents in the present study was very low and hence the lack of these relationships in the present study is likely to be attributed to a lack of statistical power to show relationships.

The limitations of the present study have largely been highlighted in previous discussion. In this regard, a significant limitation of the present study is that relationships between haplotype constructs and serum aldosterone concentrations were not assessed. Importantly however, in the present study I also did not evaluate the relationship between the -344C→T polymorphism of the aldosterone synthase gene and serum aldosterone concentrations, a variant that in previous studies has been associated with plasma aldosterone concentrations and urinary aldosterone excretion rates (an index of aldosterone synthesis) (Paillard et al 1999, Hautanen et al 1998, Pojoga et al 1998, Davies et al 1999). The -344C→T polymorphism of the aldosterone synthase gene or alternative gene variants that could modify the impact of angiotensin II on aldosterone are important gene candidates to consider as strong sibling correlations with the aldosterone response to angiotensin II have been reported on (Giacche et al 2000). However, in the study by Kathiresan et al (2005), no relationship between the -344C→T polymorphism of the aldosterone synthase gene and serum aldosterone concentrations was noted, despite this being the largest study evaluating this relationship (n=2418). However, in this study (Kathiresan et al 2005) serum aldosterone concentrations were not adjusted for plasma renin activity or concentrations. Thus further studies are required to evaluate this hypothesis.

A further limitation of the present study is that although with adjustments for diuretic use, heritability and familial aggregation for serum aldosterone concentrations was demonstrated, I was not statistically powered to show significant familial aggregation of serum aldosterone concentrations in untreated participants (sensitivity analysis). The results of the present study therefore still require confirmation by showing familial aggregation of

serum aldosterone concentrations in untreated participants. It could also be further argued that the heritability of aldosterone was relatively weak given the comparison with intrafamilial correlations for height. Nevertheless the heritability estimates obtained in the present study (h^2=0.25) were greater than those obtained in a previous study in which adjustments for renin was also made (h^2=0.19, Kotchen et al 2000).

In conclusion, through familial aggregation and heritability analysis, the present study suggests that genetic factors indeed contribute to serum aldosterone concentrations in groups of African ancestry, but that this contribution is only evident in the context of plasma renin concentrations. The genetic factors involved may not relate to the ACE or AGT genes. Further work is required to confirm a lack of involvement of the ACE and AGT genes and to identify alternative renin-angiotensin-aldosterone system genes that influence serum aldosterone concentrations in groups of African ancestry.

Chapter 4

Relationship of Predominantly Mild Current Smoking to Out-of-Office Blood Pressure in a Community Sample of African Ancestry

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Abstract

As the impact of mild smoking on blood pressure (BP) is uncertain, I aimed to assess the relationship between predominantly mild current smoking and out-of-office BP in a community sample of black African ancestry. In 689 randomly recruited participants of an urban, developing community of African descent in South Africa, smoking habits, out-of-office (24-hour monitoring) as well as in-office conventional and central (applanation tonometry) BP were assessed. Of the study sample, 14.5% (n=100) were current smokers, the majority being mild (72%) smokers (mean=7.4±4.6 cigarettes per day). Despite current smokers having a lower body mass index (BMI), only modest increases in in-office BP ($p<0.05$) and similar central aortic BP values as compared to non-smokers, current smokers had higher unadjusted ($p<0.005$-$p<0.0005$) and multivariate adjusted 24-hour systolic/diastolic BP (SBP/DBP in mm Hg) (smokers=123±15/76±10 and non-smokers=118±14/72±10, $p<0.005$-$p<0.0005$) than non-smokers, effects that were replicated in sex-specific groups, non-drinkers, and in the overweight and obese. Current smoking was second only to age and at least equivalent to body mass index in the quantitative impact on 24-hour (standardized β-coefficient for smoking effects=0.14±0.04 $p<0.0005$) and day (standardized β-coefficient for smoking effects=0.16±0.04, $p<0.0001$) DBP. Smoking 4.6 cigarettes (one standard deviation) per day translated into a 2.12 (CI=1.77 to 2.47) mm Hg increase in 24-hour SBP. The risk of uncontrolled out-of-office BP was increased in smokers as compared to non-smokers (day BP: adjusted odds ratio=2.98, CI=1.70 to 5.22, $p<0.0005$, 24-hour BP: adjusted odds ratio=1.87, CI=1.02 to 3.41, $p<0.05$). In conclusion, despite minimal effects on in-office conventional or central BP, predominantly mild current smoking is independently associated with an appreciable proportion of out-of-office BP in a community of African ancestry.

4.1 Introduction

As indicated in chapter 1 of the present thesis, currently the impact of the angiotensin-converting enzyme (ACE) gene on the relationship between smoking and BP is uncertain. The limitation of the previous study demonstrating this potential interaction was that smoking produced no discernable increase in BP in that study (Schut et al 2004). Thus, an important aim of the present thesis was to test whether an interaction between smoking and the ACE gene contribute to the variability of BP at a population level. However, before engaging in this hypothesis it was necessary to first clarify whether smoking was indeed a significant independent determinant of BP at a population level. In this regard, although several studies indicate that cigarette smoking acutely increases systolic and diastolic blood pressure (BP) (Kool et al 1993, Mahmud and Feely 2003, Failla et al 1997, Rhee et al 2007, Gropelli et al 1999), the evidence for a clinically important impact of smoking on BP at a community level is controversial. Although some studies suggest that cigarette smoking is associated with an increased in-office BP or the risk for hypertension (Thuy et al 2009, Tesfaye et al 2008, Niskanen et al 2004, Halimi et al 2002, Mundal et al 1997), other studies indicate that smoking is associated with a decreased in-office BP and a reduced risk for hypertension (Okubo et al 2004, Primatesta et al 2001, Lee et al 2001, Hughes et al 1998, Green et al 1986), or that smoking has modest (Bowmen et al 2007) or clinically negligible (Primatesta et al 2001, Fogari et al 1996, Tsai et al 2005, John et al 2006) relationships with in-office BP or the risk for hypertension. Therefore, current guidelines for the diagnosis and management of hypertension do not advocate the cessation of smoking to improve in-office BP control unless participants are heavy smokers (Mancia et al 2007, Chobanian et al 2003). However, present guidelines (Mancia et al 2007, Chobanian et al 2003) are less clear on the potential impact of cigarette smoking on out-of-office or central BP.

As the effects of cigarette smoking on BP are relatively short-lived (Kool et al 1993, Mahmud and Feely 2003, Failla et al 1997, Rhee et al 2007, Gropelli et al 1999), with perhaps the exception of heavy smoking (Gropelli et al 1999), the ability to adequately capture the effects of smoking may best occur with out-of-office BP measurements. Indeed, in some (Verdecchia et al 1995, Mann et al 1991, Bang et al 2000, Bolinder et al 1998, Benowitz et al 2002) but not other (Stewart et al 1994, Green et al 1991, Mikkelsen et al 1997) case-control (Verdecchia et al 1995, Mann et al 1991, Bang et al 2000, Bolinder et al 1998, Benowitz et al 2002, Stewart et al 1994) or cross-sectional (Green et al 1991, Mikkelsen et al 1997) studies conducted either in small study samples (Mann et al 1991, Bang et al 2000, Bolinder et al 1998, Benowitz et al 2002, Stewart et al 1994, Green et al 1991, Mikkelsen et al 1997) or where office BP was matched between cases and controls (Verdecchia et al 1995), medium-to-heavy cigarette smoking was associated with increases in out-of-office, but not in-office BP. Furthermore, whilst smokers may have similar brachial artery BP values as non-smokers, central (aortic) BP may be increased in smokers as compared to non-smokers (Mahmud and Feely 2003). Despite the possibility that smoking effects on BP may mainly influence out-of-office or central BP, the impact of predominantly mild-to-moderate smoking on out-of-office or central BP at a population or community level is uncertain. As in economically emerging nations, which now face the dual health burden of diseases of poverty and of affluence, cigarette smokers may predominantly partake in the mild-to-moderate range, it is important to identify whether even mild-to-moderate smoking has clinically important effects on out-of-office BP. Consequently, in the present study I aimed to evaluate the relationship between the prevalent smoking habits (predominantly mild) that occur in economically emerging communities in Africa and 24-hour BP in a randomly selected community sample of African ancestry in South Africa.

4.2 Methods

4.2.1 Study group

Details of the selection of the participants that made up the study group have been described in section 2.2 on pages 50-51 of the present thesis. Of the 1029 participants recruited from the population (for details see section 2.2 on pages 50 and 51) up until the end of 2009, 689 (67%) participants had both central hemodynamic measurements and 24-hour ambulatory BP values that met with pre-specified quality control criteria (longer than 20 hours and more than 10 and 5 readings for the computation of day and night means, respectively). Aortic pulse wave velocity could not be determined in 94 participants whom were too obese.

4.2.2 Clinical, demographic and anthropometric measurements.

A standardized questionnaire was administered to obtain demographic and clinical data as described in section 2.2 (pages 51-52) of the present thesis. With respect to smoking effects, the number of cigarettes currently smoked or previously smoked (if a previous smoker) per day, the age of starting and ending (if a previous smoker), and whether when partaking of the habit, smoke is inhaled, were recorded. The smoking of greater than 20 cigarettes a day was considered heavy smoking (Verdecchia et al 1995), 10-20 cigarettes a day was considered moderate smoking (Minami et al 2009) and <10 cigarettes a day was therefore considered as mild smoking.

4.2.3 Conventional blood pressure measurements.

Trained nurse-technicians measured BP using a standard mercury sphygmomanometer during a clinic visit as described in section 2.2 (page 53) of the present thesis.

4.2.4 Ambulatory blood pressure measurements.

24-Hour ambulatory BP monitoring was performed on the same day as conventional BP measurements using oscillometric monitors (SpaceLabs, model 90207), of which the calibration was checked monthly against a mercury manometer. The size of the cuff was the same as that used for conventional BP measurements. The study monitors were programmed to measure BP at 15-minute intervals from 06:00 to 22:00 hours and at 30-minute intervals from 22:00 to 06:00 hours. Participants kept a diary card for the duration of the recordings to note the time of going to bed in the evening and getting up in the morning. Diary cards were employed to identify the actual in-bed and out-of-bed periods. These periods were used to calculate the average in-bed and out-of-bed periods and thus the average transition periods during which BP changes rapidly in most participants. These average transition periods were then eliminated. The remaining periods were considered to be the night or day fixed-clock time periods. Fixed-clock time periods rather than actual in bed and out of bed periods were statistically analysed to ensure that similar day and night time periods were selected for comparisons between individuals. Day and night periods ranged from 09:00 to 19:00 hours and from 23:00 to 05:00 hours, respectively. No participants reported on daytime “naps”. Intra-individual means of the ambulatory measurements were weighted by the time-interval between successive recordings.

4.2.5 *Pulse wave analysis and central blood pressures.*

Central haemodynamics and carotid-femoral (aortic) pulse wave velocity (PWV) were estimated using techniques previously described (Shiburi et al 2006, Nichols and O’Rourke 1998). After participants had rested for 15 minutes in the supine position, arterial waveforms at the radial (dominant arm), carotid and femoral pulses were recorded by applanation tonometry, each during an 8-second period using a high-fidelity SPC-301 micromanometer (Millar Instrument, Inc., Houston, Texas) interfaced with a computer employing SphygmoCor, version 6.21 software (AtCor Medical Pty. Ltd., West Ryde, New South

Wales, Australia). A photograph of the hardware employed to perform the recordings is shown in Figure 4.1. Typical recordings obtained are shown in Figure 4.2. Recordings where the systolic or diastolic variability of consecutive waveforms exceed 5% or the amplitude of the pulse wave signal is less than 80 mV were discarded. The pulse wave was calibrated by manual measurement (auscultation) of BP taken immediately before the recordings. All measurements were made by a single experienced trained technician unaware of the clinical history of the participants. From an inbuilt validated (Nichols and O'Rourke 1998) transfer function an aortic waveform was generated from which central systolic, diastolic and mean arterial BP were derived. The magnitude of the forward wave (P1) was determined as the difference between the inflection point at the end of the first systolic shoulder and central diastolic BP (Figure 4.2). The magnitude of the augmented pressure wave (ΔPaug) was determined as the difference between central systolic blood pressure and the inflection point at the end of the first systolic shoulder. The reflected wave transit time was determined from the beginning of the incident wave to the end of the first systolic shoulder. The effective reflecting distance was calculated as (reflected wave transit time x PWV)/2 (Murgo et al 1980).

4.2.6. Urinary electrolyte excretion rates.

In order to adjust for the potential confounding effects of excessive salt intake, timed urine samples were obtained over at least a 24-hour period on the same say as the BP measurements as described in section 2.2 (page 54) of the present thesis. The quality of urine samples was determined as described in section 2.2 (page 54) of the present thesis.

4.2.7 Statistical analysis.

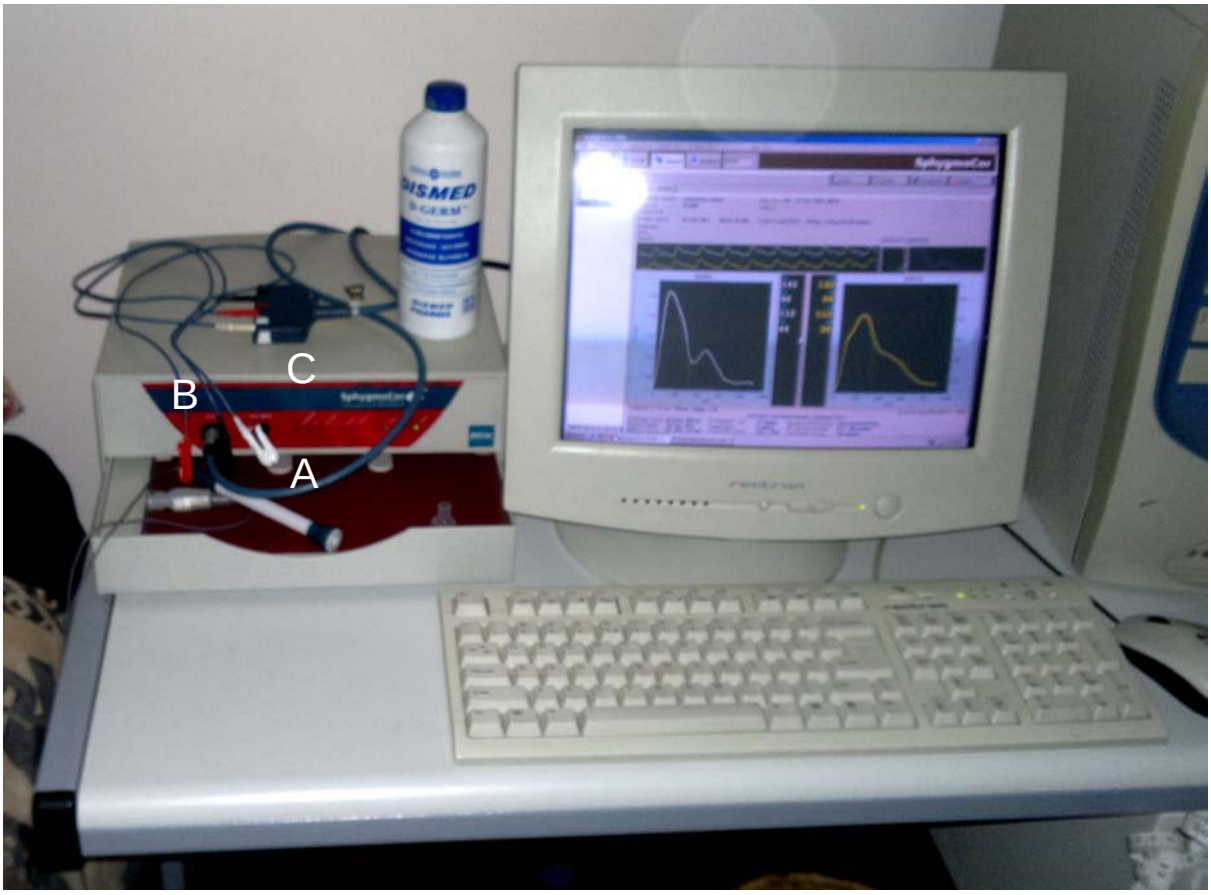
For database management and statistical analysis, SAS software, version 9.1 (SAS Institute Inc., Cary, NC) was employed. Data are shown as mean±SD. Multiple regression analysis was used to determine independent relationships. Relationships between current

smoking and BP were all adjusted for age, sex, BMI, diabetes mellitus or an HbA1c>6.1%, regular alcohol intake, and treatment for hypertension. As smokers tended to walk more than non-smokers and no differences in the overall physical activity score was noted between smokers and non-smokers, BP was not adjusted for walking or for overall physical activity. In secondary analysis, adjustments were also made for 24-hour urinary Na^+/K^+ in the 500 participants whom had data that met with pre-specified quality control criteria. Primary analysis included all participants to ensure that the study sample represented the community at large. However, as current smokers differed from non-smokers in a number of characteristics, sensitivity analyses were conducted in sex-specific groups, in participants whom did not drink alcohol, in participants whom were not receiving antihypertensive therapy, in overweight and obese participants, and in non-diabetics. For the derivation of probability values, further adjustments were made for non-independence of family members using the mixed procedure as outlined in the SAS package. Using this approach, both random (non-related) and fixed effects (relatedness), were included in the models.

4.3 Results

4.3.1 *Characteristics of the participants.*

Table 4.1 gives the demographic and clinical characteristics of the smokers and the non-smokers. More women (64.4%) than men participated. In general the study group had a high BMI, with ~64% of participants being either overweight (~24%) or obese (~40%). Of the participants 14.5% were current smokers and the majority of smokers (72%) were mild smokers (<10 cigarettes per day). No participants reported having previously smoked and subsequently having given up the habit. More men than women were current smokers.



A Applanation tonometer

B Electrocardiograph electrodes

C SphygmoCor device

Figure 4.1 Illustrates the hardware used to determine central haemodynamics and aortic pulse wave velocity.

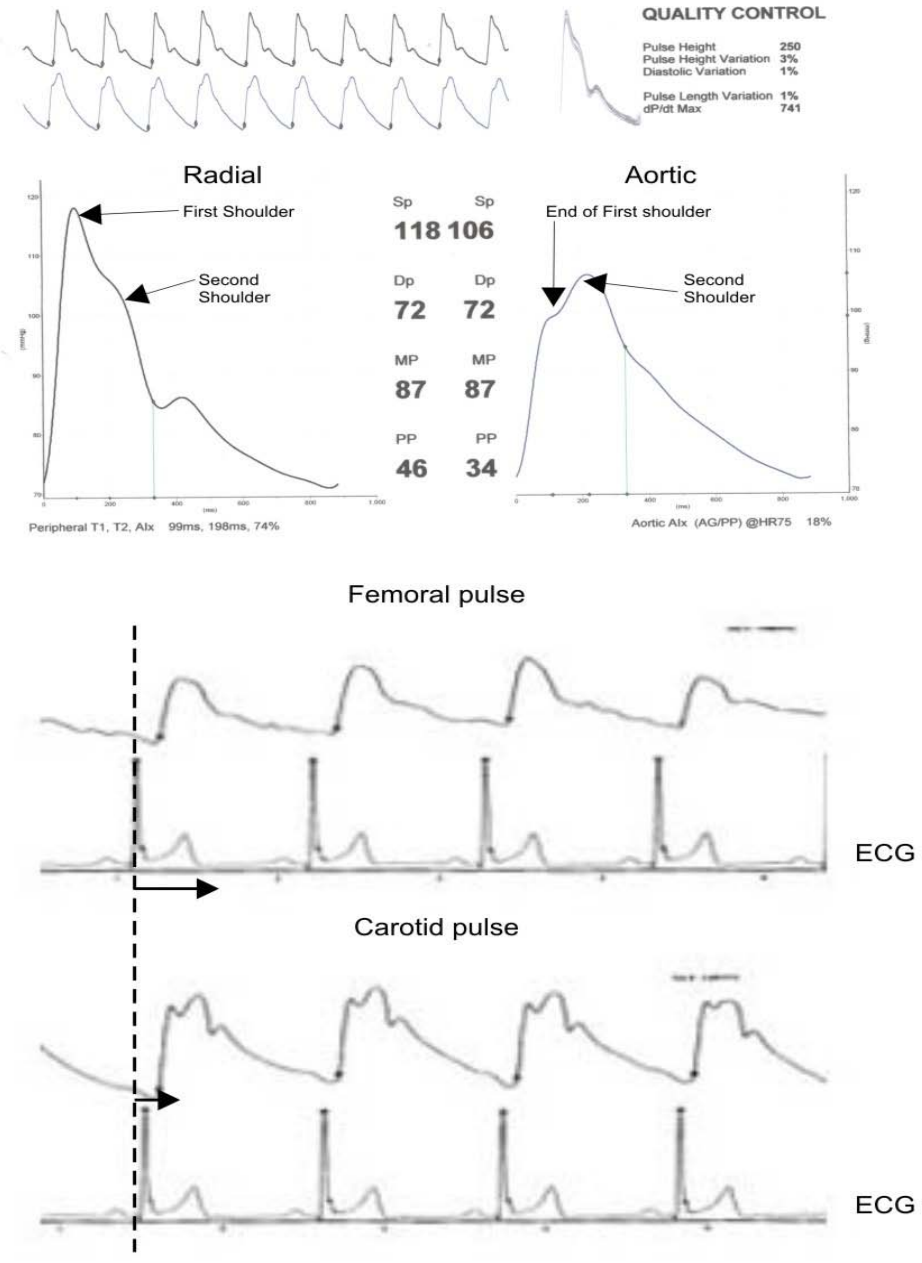


Figure 4.2. Illustrates an example of the applanation tonometry recordings obtained to determine central haemodynamics (upper panel) and pulse wave velocity (PWV)(lower panel). The figures in the upper panel show pulse waves obtained from the radial and carotid artery indicating the points (first and second shoulders) of importance. The arrows in the lower panel indicate the time differences between electrical events and the arterial pressure changes in the carotid and femoral arteries used to calculate PWV. See text for a further description.

Table 4.1. Characteristics of study participants.

	Non-smoker (n=589)	Smokers (n=100)
Sex (% female)	72.2	19.0
Age (years)	44.4±18.5	41.4±15.3
Current number of cigarettes per day	0	7.4±4.6
Number of years smoked	0	18.3±13.1
Pack years smoked	0	7.9±8.4
Body mass index (kg/m^2)	29.7±7.6	24.6±6.8***
% Overweight/obese	23.9/44.1	26.0/15.0***
Regular alcohol intake (%)	15.6	57.0***
Regular walking (%)	52.8	69.0**
Kilometres/day walking (%)	3.81±3.41	5.48±5.84**
Physical activity score (out of 10)	5.59±2.16	5.41±2.21
% with diabetes mellitus or HbA1c>6.1%	25.6	11.0**
% with hypertension	43.0	30.0*
% treated for hypertension	24.6	11.0**
24-hour pulse rate (beats/minute)	77.7±9.9	75.8±9.3
Urinary Na^+/K^+ (n=500)	4.15±3.24 (n=431)	4.70±2.48 (n=69)

HbA_{1C}, glycosylated haemoglobin. *p<0.05, **p<0.005, ***p<0.0001 for comparison with non-smokers.

Current smokers had a lower BMI, a lower proportion were obese, a higher proportion regularly consumed alcohol, a higher proportion had diabetes mellitus or an abnormal blood glucose control (HbA1c>6.1%), and a lower proportion had hypertension or were less likely to be treated for hypertension. More current smokers walked regularly and on average walked further per day than non-smokers. However, the overall physical activity score did not differ between current smokers and non-smokers. No differences in 24-hour pulse rate or urinary Na^+/K^+ were noted between current smokers and non-smokers. No differences were noted in the demographic and clinical characteristics between participants with and those without high quality 24-hour ambulatory BP recordings. In this regard, as compared to the participants studied, participants who were excluded from the study sample (n=340) were of a similar age (43.9±18.9 years), had a higher BMI (30.5±8.6 kg/m^2, $p<0.005$ versus study participants), a higher proportion of these participants were obese (47.7%, $p<0.05$ versus study participants). A similar proportion smoked regularly (15.0%), consumed alcohol regularly (22.7%), had diabetes mellitus or an HbA_{1c}>6.1% (21.5%), and had hypertension (44.4%).

4.3.2 *Relationship between current smoking and conventional, central and ambulatory BP values.*

Figure 4.3 and Table 4.2 show the conventional, and ambulatory BP values in current smokers and non-smokers. Before and after adjustments, current smokers had higher 24-hour (Table 4.2 and Figure 4.3) and day (Table 4.2) BP values than non-smokers, with highly significant differences in BP noted for day systolic (SBP) and diastolic (DBP) BP (Table 4.2) as well as 24-hour DBP (Figure 4.3)($p<0.0005$ for all). Only a modest association between current smoking and either conventional or night DBP was noted (Table 4.2) and current smoking was not independently associated with night SBP (Table 4.2). The multivariate adjusted relationships between current smoking and day SBP (partial r=0.18, CI=0.10 to 0.25,

Table 4.2. Association of smoking with unadjusted and multivariate adjusted conventional and ambulatory blood pressures (BP) in a community sample (see Figure 4.1 for 24-hour BP).

	Unadjusted			Multivariate adjusted*		
	Non-smokers (n=589)	Smokers (n=100)	p-value	Non-smokers (n=589)	Smokers (n=100)	p-value
Conventional SBP (mm Hg)	129±22	131±21	=0.45	129±18	132±20	=0.12
Conventional DBP (mm Hg)	84±12	86±12	=0.07	84±11	87±13	<0.05
Conventional MAP (mm Hg)	99±15	101±14	=0.17	99±13	102±14	<0.05
24hour SBP (mm Hg)	118±15	122±15	<0.005	118±14	123±15	<0.005
24hour DBP (mm Hg)	72±10	76±10	<0.0005	72±9	76±10	<0.0005
24 hour MAP (mm Hg)	88±11	92±11	<0.0001	88±10	92±11	<0.001
Day SBP (mm Hg)	122±15	128±15	<0.0001	122±14	128±15	=0.0001
Day DBP (mm Hg)	77±10	82±10	<0.0001	77±10	82±11	<0.0001
Day MAP (mm Hg)	92±12	98±12	<0.0001	93±11	97±12	<0.0005
Night SBP (mm Hg)	111±17	113±17	=0.22	111±16	114±17	=0.09
Night DBP (mm Hg)	65±11	67±12	=0.07	65±11	67±12	<0.05
Night MAP (mm Hg)	81±13	83±13	=0.16	81±12	84±13	=0.07

SBP, systolic BP; DBP, diastolic BP. *Adjusted for age, sex, body mass index, diabetes mellitus or an HbA1c>6.1%, regular alcohol intake, and treatment for hypertension. Probability values are further adjusted for non-independence of family members.

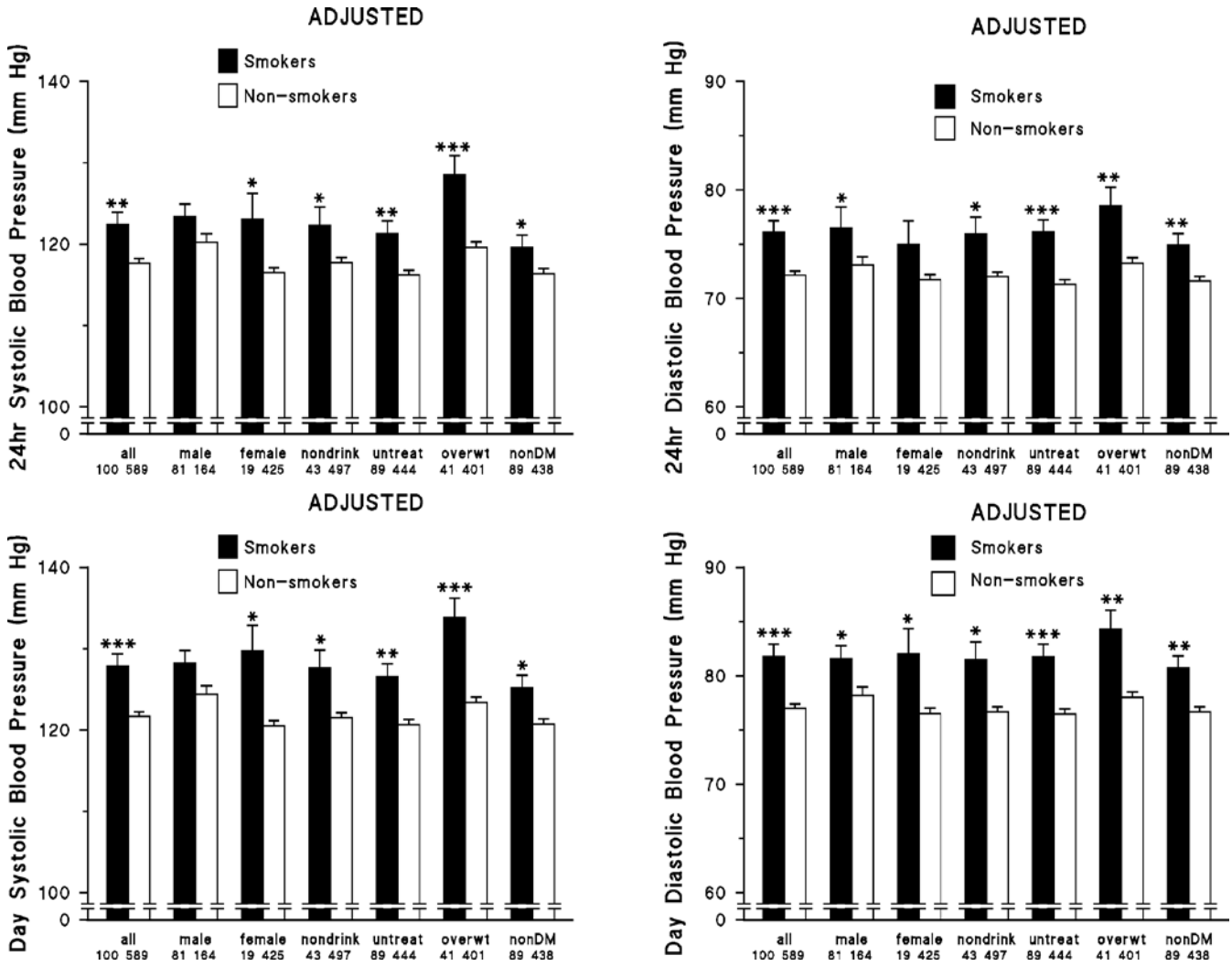


Figure 4.3. Comparison of multivariate adjusted 24-hour systolic (SBP) and diastolic (DBP) blood pressures (BP) between smokers and non-smokers in the whole group, and in a variety of subgroups. Where appropriate, adjustments are for age, sex, body mass index, diabetes mellitus or an HbA1c>6.1%, regular alcohol intake, and treatment for hypertension. Probability values are further adjusted for non-independence of family members. Overwt, overweight and obese; DM, diabetes mellitus or an HbA1c>6.1%. * $p<0.05$, ** $p<0.005$, *** $p<0.0005$ vs non-smokers.

p=0.0001) or DBP (partial r=0.19, CI=0.12 to 0.26, p<0.0001) was greater than the multivariate adjusted relationships between current smoking and conventional SBP (partial r=0.06, CI=-0.01 to 0.14, p<0.08) or DBP (partial r=0.09, CI=0.01 to 0.16, p<0.05) (p<0.05 for comparison of the partial r values). Although pack-years of smoking were independently associated with BP (data not shown) the impact was not as striking as that of current smoking and the number of cigarettes smoked. Moreover, the duration of smoking was not associated with BP (data not shown).

In sensitivity analysis, similar differences in out-of-office BP between current smokers and non-smokers were noted in sex-specific groups, participants whom did not drink alcohol, participants whom were not receiving antihypertensive therapy, in overweight and obese participants, and in non-diabetics showed (Figure 4.3). Moreover, in participants with complete 24-hour urinary collections, even with further adjustments for urinary Na^+/K^+, current smokers similarly had higher multivariate adjusted 24 hour and day DBP values (in mm Hg) than non-smokers (24 hour DBP: smokers=75±10, non-smokers=72±9, p<0.05; day DBP: smokers=81±11, non-smokers=77±10, p<0.02).

In separate analysis, where smokers were excluded, I also assessed the effects of second-hand smoking on BP. No differences in multivariate adjusted conventional, 24-hour, day or night BP (mm Hg) were noted between participants who co-habited with smokers and participants who co-habited with non-smokers (24 hour SBP: co-habited with smokers=117±13, co-habited with non-smokers=117±13, p=0.80; 24 hour DBP: co-habited with smokers=71±9, co-habited with non-smokers=72±9, p=0.57; day SBP: co-habited with smokers=121±13, co-habited with non-smokers=122±13, p=0.81; day DBP: co-habited with smokers=76±10, co-habited with non-smokers=77±9, p=0.27).

4.3.3 Quantitative effect of current smoking on out-of-office BP.

Smoking only 4.6 cigarettes (one standard deviation) per day translated into a 1.98 (CI=1.74 to 2.23) mm Hg increase in 24-hour DBP ($p<0.001$), a 2.34 (CI=2.08 to 2.6) mm Hg increase in day DBP ($p<0.0005$), a 2.12 (CI=1.77 to 2.47) mm Hg increase in 24-hour SBP ($p<0.05$), and a 2.75 (CI=2.39 to 3.10) mm Hg increase in day SBP ($p<0.005$) (Figure 4.4). Similar quantitative effects of current smoking were noted in sex-specific groups, participants whom did not drink alcohol, participants whom were not receiving antihypertensive therapy, in overweight and obese participants and in non-diabetics (Figure 4.4). Moreover, even after adjustments for urinary Na^+/K^+ in participants with complete urine collections, smoking 4.6 cigarettes (one standard deviation) per day translated into a 1.55 (CI=1.26 to 1.84) mm Hg increase in 24-hour DBP ($p<0.05$) and a 1.80 (CI=1.49 to 2.1) mm Hg increase in day DBP ($p<0.02$).

4.3.4 Comparison of the impact on out-of-office BP of current smoking and alternative factors associated with BP.

Tables 4.3a and 4.3b compare the standardized β-coefficients for the factors related to ambulatory BP, including current smoking, in multivariate regression models. The independent relationship between the presence of current smoking or the number of cigarettes smoked per day and 24-hour and day SBP and DBP was equivalent to the impact of BMI or male gender and second only to age (Tables 4.3a and 4.3b). The relationship between pack years smoked and 24-hour and day SBP and DBP was similarly equivalent to the impact of BMI or male gender and second only to age (Table 4.4).

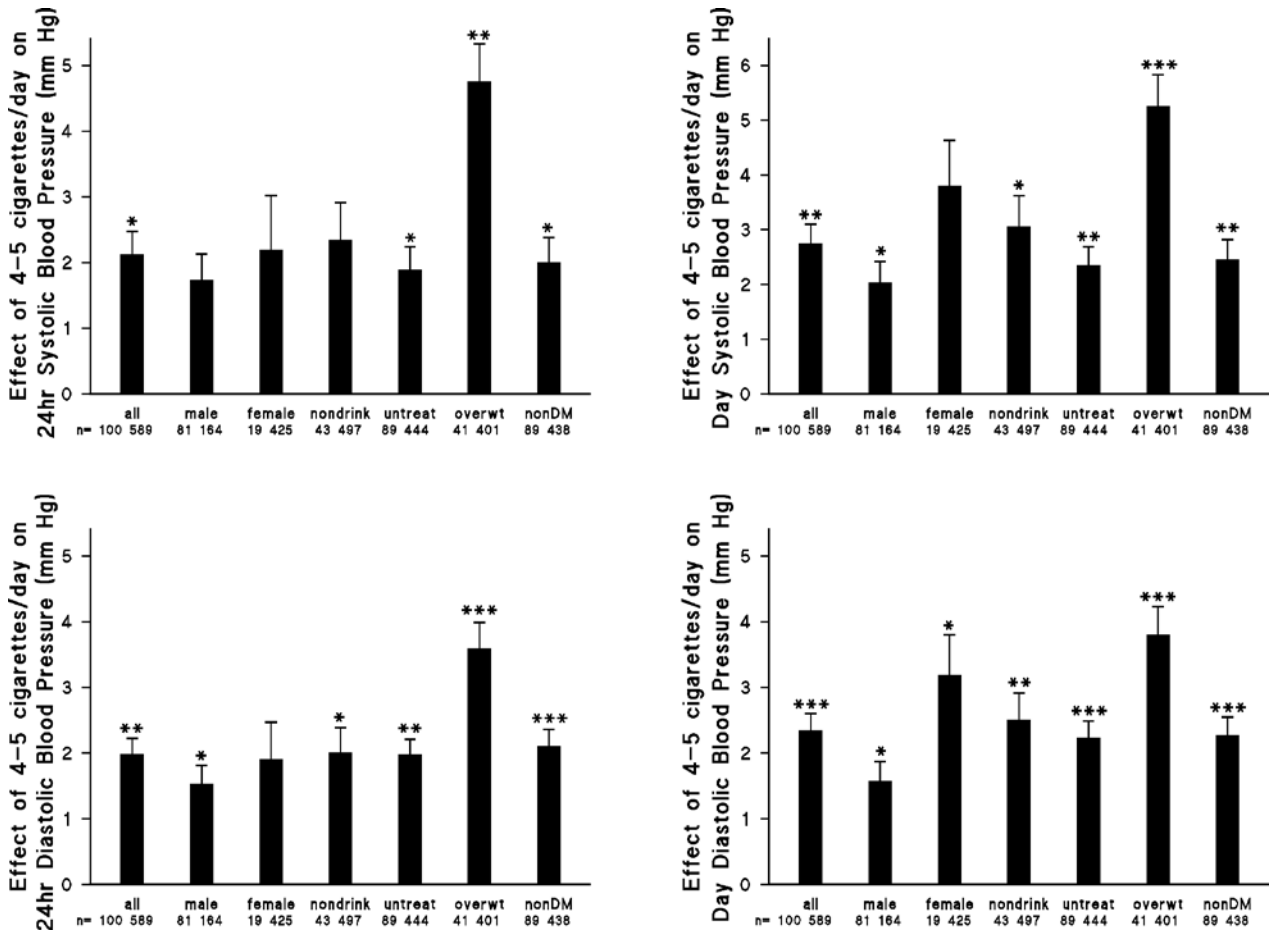


Figure 4.4. Estimated effect of 4.6 cigarettes smoked per day (one standard deviation) on 24-hour and day systolic (SBP) and diastolic (DBP) blood pressure (BP) in the whole group, and in a variety of subgroups. See Figure 4.3 legend for the details of multivariate adjustments and abbreviations.

4.3.5 Effect of current cigarette smoking on conventional and 24-hour BP control rates.

Using standard criteria for BP control of <140/90 mm Hg for conventional BP, <130/80 mm Hg for 24-hour BP and <135/85 mm Hg for day BP, after adjustments current smoking was independently associated with uncontrolled 24-hour and day BP (Figure 4.5) but not with uncontrolled conventional BP (multivariate adjusted odds ratio=1.31, CI=0.73 to 2.33, p=0.36). The relationship between current smoking and uncontrolled 24-hour BP was noted in participants whom were not receiving antihypertensive therapy, and in overweight and obese participants (Figure 4.5). Moreover, even after adjustments for urinary Na^+/K^+ in participants with complete urine collections, smoking was associated with an increased risk for uncontrolled day DBP (multivariate adjusted odds ratio=2.42, p<0.02).

4.3.6 Relationships between smoking and pulse pressure (PP) or its determinants.

Table 4.5 shows the unadjusted and multivariate adjusted values for conventional, 24-hour and central PP and central haemodynamic values. Other than a modest increase in aortic augmentation pressure after multivariate adjustments, no differences were noted in these variables between smokers and non-smokers. Table 4.6 summarises the relationships between current smoking or the number of cigarettes smoked per day and conventional, 24-hour or central PP or its determinants. Although current smoking was independently related to aortic augmentation pressure (Paug), this failed to translate into a relationship with central PP. Moreover, smoking was not associated with conventional, day, night, or 24-hour PP or with aortic PWV, the effective reflecting distance or the reflective wave transit time. Pack-years smoked was also not independently related to any of the haemodynamic variables listed in Tables 4.5 and 4.6 (data not shown).

Table 4.3a. Effects of smoking as compared to the effects of alternative risk factors on ambulatory (out-of-office) and conventional (in-office) systolic blood pressure (BP) in multivariate relationships in 689 participants.

	Models with			
	Current smoking		Number of cigarettes	
	β-coefficient*	p value†	β-coefficient*	p value†
		24-hour systolic BP		
Age	0.37±0.04	<0.0001	0.36±0.04	<0.0001
Smoking	0.11±0.04	**=0.002**	0.09±0.04	**=0.017**
Body mass index	0.18±0.04	<0.0001	0.18±0.04	<0.0001
Male gender	0.16±0.04	<0.0001	0.17±0.04	<0.0001
		Day systolic BP		
Age	0.33±0.04	<0.0001	0.32±0.04	<0.0001
Smoking	0.15±0.04	**=0.0001**	0.12±0.04	**=0.002**
Body mass index	0.18±0.04	<0.0001	0.18±0.04	<0.0001
Male gender	0.15±0.04	=0.0002	0.16±0.04	<0.0001
		Night systolic BP		
Age	0.38±0.04	<0.0001	0.38±0.04	<0.0001
Smoking	0.06±0.04	=0.09	0.05±0.04	=0.24
Body mass index	0.14±0.04	=0.0006	0.14±0.04	=0.0006
Male gender	0.14±0.04	=0.0007	0.15±0.04	=0.0003
		Conventional systolic BP		
Age	0.50±0.04	<0.0001	0.50±0.04	<0.0001
Smoking	0.05±0.04	=0.12	0.04±0.04	=0.23
Body mass index	0.15±0.04	<0.0001	0.16±0.04	<0.0001
Male gender	0.09±0.04	=0.012	0.10±0.04	=0.008

*Standardized β-coefficients with age, smoking, body mass index, sex, diabetes mellitus or an HbA1c>6.1%, regular alcohol intake, and treatment for hypertension included in the regression analysis. † Probability values are further adjusted for non-independence of family members. Significant smoking effects are highlighted in bold.

Table 4.3b. Effects of smoking as compared to the effects of alternative risk factors on ambulatory (out-of-office) and conventional (in-office) diastolic blood pressure (BP) in multivariate relationships in 689 participants.

	Models with Current smoking		Models with Number of cigarettes	
	β-coefficient*	p value†	β-coefficient*	p value†
		24-hour diastolic BP		
Age	0.36±0.04	<0.0001	0.36±0.04	<0.0001
Smoking	0.14±0.04	**=0.0003**	0.13±0.04	**=0.001**
Body mass index	0.09±0.04	=0.04	0.09±0.04	=0.03
Male gender	0.07±0.04	=0.07	0.08±0.04	=0.04
		Day diastolic BP		
Age	0.28±0.04	<0.0001	0.28±0.04	<0.0001
Smoking	0.16±0.04	**<0.0001**	0.15±0.04	**<0.0005**
Body mass index	0.11±0.04	=0.01	0.11±0.04	<0.01
Male gender	0.06±0.04	=0.11	0.07±0.04	=0.054
		Night diastolic BP		
Age	0.40±0.04	<0.0001	0.40±0.04	<0.0001
Smoking	0.08±0.04	**=0.033**	0.09±0.04	**=0.027**
Body mass index	0.06±0.04	=0.17	0.06±0.04	=0.14
Male gender	0.06±0.04	=0.14	0.06±0.04	=0.12
		Conventional diastolic BP		
Age	0.27±0.04	<0.0001	0.27±0.04	<0.0001
Smoking	0.08±0.04	**=0.030**	0.10±0.04	**=0.015**
Body mass index	0.22±0.04	<0.0001	0.23±0.04	<0.0001
Male gender	0.09±0.04	=0.031	0.09±0.04	=0.03

*Standardized β-coefficients with age, smoking, body mass index, sex, diabetes mellitus or an HbA1c>6.1%, regular alcohol intake, and treatment for hypertension included in the regression analysis. † Probability values are further adjusted for non-independence of family members. Significant smoking effects are highlighted in bold.

Table 4.4. Effect of pack years of smoking as compared to the effects of alternative risk factors on ambulatory (out-of-office) and conventional (in-office) blood pressure (BP) in multivariate relationships in 689 participants.

	Systolic BP		Diastolic BP	
	β-coefficient*	p value†	β-coefficient*	p value†
		24-hour BP		
Age	0.36±0.04	<0.0001	0.35±0.04	<0.0001
Smoking	0.11±0.04	**=0.003**	0.09±0.04	**=0.023**
Body mass index	0.17±0.04	<0.0001	0.17±0.04	<0.0001
Male gender	0.15±0.04	=0.0002	0.16±0.04	<0.0001
		24-hour diastolic BP		
Age	0.34±0.05	<0.0001	0.33±0.04	<0.0001
Smoking	0.14±0.04	**=0.0004**	0.13±0.04	**=0.001**
Body mass index	0.09±0.04	=0.04	0.09±0.04	=0.03
Male gender	0.08±0.04	=0.06	0.09±0.04	=0.03
		Day systolic BP		
Age	0.32±0.05	<0.0001	0.31±0.05	<0.0001
Smoking	0.15±0.04	**=0.0001**	0.12±0.04	**=0.003**
Body mass index	0.18±0.04	<0.0001	0.18±0.04	<0.0001
Male gender	0.14±0.04	=0.001	0.15±0.04	=0.0003
		Day diastolic BP		
Age	0.26±0.05	<0.0001	0.26±0.05	<0.0001
Smoking	0.17±0.04	**<0.0001**	0.15±0.04	**<0.0005**
Body mass index	0.11±0.04	=0.01	0.11±0.04	=0.01
Male gender	0.07±0.04	=0.10	0.08±0.04	=0.053

*Standardized β-coefficients with age, smoking, body mass index, sex, diabetes mellitus or an HbA1c>6.1%, regular alcohol intake, and treatment for hypertension included in the regression analysis. † Probability values are further adjusted for non-independence of family members. Significant smoking effects are highlighted in bold.

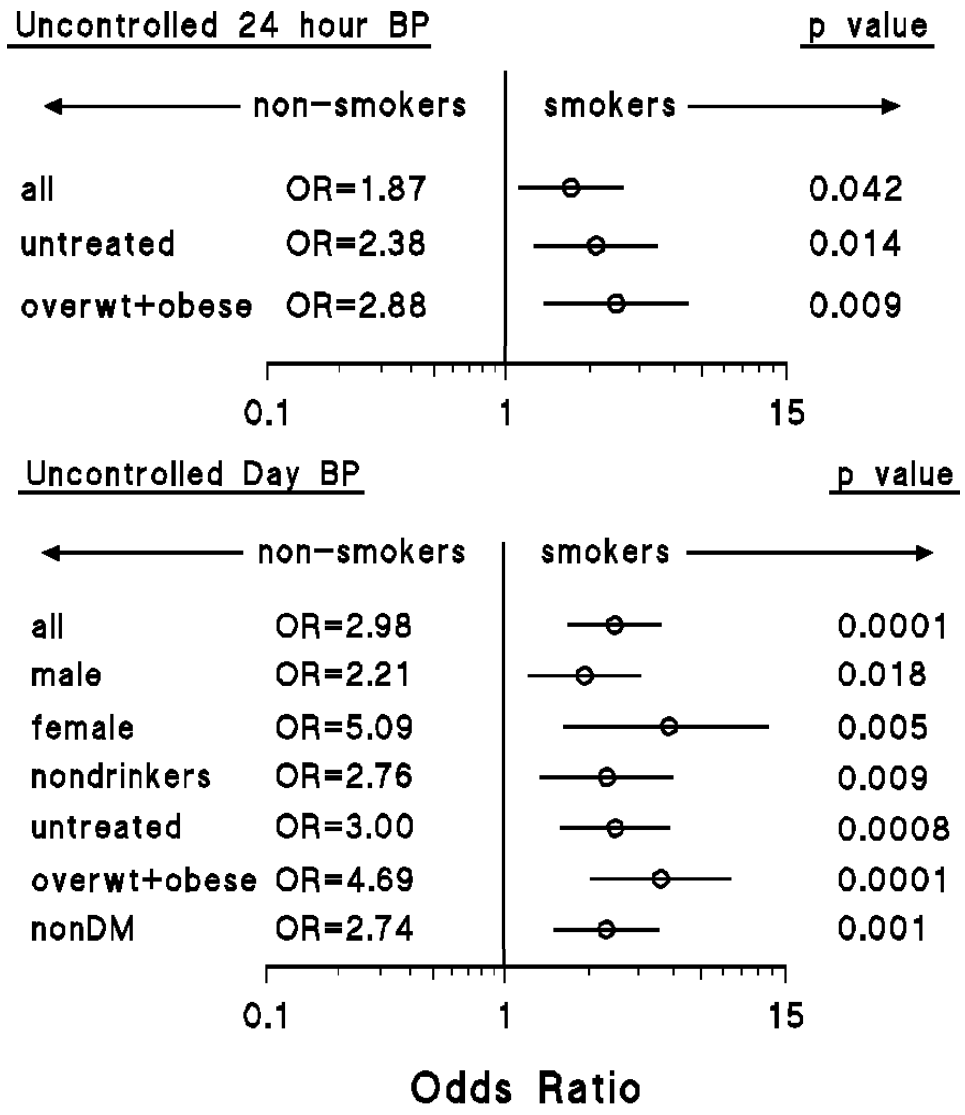


Figure 4.5. Association of current smoking with the risk of uncontrolled 24-hour and day blood pressure (BP) in the whole group, and in a variety of subgroups of the study sample. See Figure 4.1 legend for the details of multivariate adjustments.

Table 4.5. Unadjusted and multivariate adjusted conventional (in-office), ambulatory (out-of-office), or central pulse pressure (PP) and their potential determinants in smokers and non-smokers in a community sample.

	Unadjusted			Multivariate adjusted*		
	Non-smokers (n=589)	Smokers (n=100)	p-value	Non-smokers (n=589)	Smokers (n=100)	p-value
Conventional PP (mm Hg)	45±15	45±15	=0.73	45±13	46±14	=0.75
24-hour PP (mm Hg)	46±9	46±9	=0.54	46±9	46±9	=0.40
Day PP (mm Hg)	45±9	46±10	=0.18	45±9	46±9	=0.19
Night PP (mm Hg)	46±10	46±10	=1.00	46±10	47±11	=0.76
Central SBP (mm Hg)	121±23	122±23	=0.57	120±19	123±21	=0.13
Central DBP (mm Hg)	85±13	86±13	=0.20	84±12	87±13	=0.09
Central PP (mm Hg)	36±15	36±15	=0.84	36±13	37±14	=0.49
P1 (mm Hg)	25±9	25±9	=0.44	25±8	24±9	=0.34
Paug (mm Hg)	11±8	11±8	=0.67	10±7	12±7	**=0.01**
ERD (cm)	36±16†	35±15†	=0.41	36±12	35±12	=0.36
RWTT (msec)	105±13	107±13	=0.13	105±13	106±14	=0.72
Aortic PWV (m/sec)	6.86±2.87†	6.46±2.28†	=0.20	6.84±2.18	6.51±2.35	=0.21

SBP, systolic BP; DBP, diastolic BP; P1, forward pressure wave; Paug, augmentation pressure; ERD, effective reflecting distance; RWTT, reflective wave transit time; PWV, pulse wave velocity. *Adjusted for age, sex, body mass index, diabetes mellitus or an HbA1c>6.1%, regular alcohol intake, and treatment for hypertension. Probability values are further adjusted for non-independence of family members. † sample sizes for non-smokers=502 and smokers=93. Significant smoking effects are highlighted in bold.

4.4 Discussion.

The present study indicates that in an economically emerging community of African descent, predominantly mild current smoking is independently associated with an increased out-of-office BP and poor out-of-office BP control, an effect that was second only to age and at least equivalent to BMI or male gender in the impact on BP. The relationship between current smoking and out-of-office BP was noted in both males and in females, in participants who did not drink alcohol, in participants not receiving antihypertensive therapy, in overweight and obese participants and in non-diabetics. Moreover, the effect of current smoking was independent of a number of potential additional confounders including urinary Na^+/K^+ (an index of salt intake). The magnitude of the effect of smoking is exemplified by the fact that only 4.6 cigarettes (one standard deviation) smoked per day translated into a 1.96 mm Hg increase in 24-hour DBP, and a 2.10 mm Hg increase in 24-hour SBP and a 2.3 mm Hg increase in day DBP and a 2.7 mm Hg increase in day SBP. Moreover, smoking was associated with a 1.98 and 3.0 times greater risk of uncontrolled 24-hour and day BP respectively than non-smokers. In contrast, current smoking was only modestly associated with in-office conventional or central BP.

Although some previous small and/or case-control studies have demonstrated a relationship between current smoking and out-of-office BP whilst in-office BP may remain unchanged (Gropelli et al 1999, Verdecchia et al 1995, Mann et al 1991, Bang et al 2000, Bolinder et al 1998, Benowitz et al 2002), these studies were conducted in samples where the predominant smoking habit was moderate-to-severe smoking. In the present study, a marked quantitative effect of predominantly mild cigarette smoking on out-of-office BP was noted and a considerable proportion of uncontrolled BP at a community level was attributed to mild current smoking. In contrast to the majority of previous studies demonstrating an association

between smoking and out-of-office BP, which were case-control designs (Gropelli et al 1999, Verdecchia et al 1995, Mann et al 1991, Bang et al 2000, Bolinder et al 1998, Benowitz et al 2002) and where relatively small study samples were employed (n=12-177) (Mann et al 1991, Bang et al 2000, Bolinder et al 1998, Benowitz et al 2002), the strengths of the present study are the random recruitment procedures and the fact that this is the largest study reporting on smoking effects on out-of-office BP (n=689). One previous case-control study (Verdecchia et al 1995) assessing the impact of smoking on ambulatory BP employed a similar sample size (n=575) as the present study, but in that study (Verdecchia et al 1995) only hypertensives were evaluated, smokers and non-smokers were matched for in-office BP values thus excluding the possibility of random selection approaches, the effect of only heavy smoking was assessed, the impact on out-of-office BP control was not calculated, the size-effect of smoking on BP was not compared to alternative determinants of BP, such as body size and the impact of smoking was not confirmed in participants who did not drink alcohol, in overweight and obese participants, or after adjustments for alternative lifestyle factors such as salt intake. In the present study conducted in randomly selected participants, a marked impact of predominantly mild current smoking on out-of-office BP was noted, an effect that was at least equivalent to BMI and was confirmed after accounting for a number of alternative lifestyle factors. The relationship between smoking and out-of-office (where patients smoke at will) BP but not office (where patients refrain from smoking) is likely to reflect the short-lived effect of smoking on blood pressure.

Not all studies support the findings that smokers have increased out-of-office BP values (Stewart et al 1994, Green et al 1991, Mikkelsen et al 1997). In two cross-sectional studies with sample sizes ranging from 270-352 (Green et al 1991, Mikkelsen et al 1997), in which the majority of smokers smoked in the moderate-to-heavy range, smokers had lower out-of-office BP values than non-smokers. However, in these studies (Green et al 1991,

Table 4.6. Independent relationship between smoking and conventional (in-office), ambulatory (out-of-office), or central pulse pressure (PP) and their potential determinants in 689 participants.

	Models with			
	Current smoking		Number of cigarettes	
	partial r*	p value*	partial r*	p value*
Conventional PP (n=689)	-0.02	=0.56	-0.02	=0.68
24-hour PP (n=689)	-0.04	=0.35	0.01	=0.79
Day PP (n=689)	-0.06	=0.15	0.03	=0.44
Night PP (n=689)	-0.02	=0.70	-0.02	=0.65
Central PP (n=689)	-0.05	=0.25	-0.01	=0.74
P1 (n=689)	0.01	=0.71	-0.06	=0.10
Paug (n=689)	-0.10	**=0.02**	0.05	=0.20
Aortic PWV (n=595)	0.04	=0.30	-0.07	=0.08
ERD (n=595)	0.03	=0.45	-0.07	=0.12
RWTT (n=689)	-0.02	=0.66	-0.003	=0.94

* Determined from regression analysis with adjustments for age, body mass index, sex, diabetes mellitus or an HbA1c>6.1%, regular alcohol intake, and treatment for hypertension. Probability values are further adjusted for non-independence of family members. P1, central forward pressure wave; Paug, augmentation pressure; PWV, pulse wave velocity; ERD, effective reflecting distance; RWTT, reflective wave transit time. Significant smoking effects are highlighted in bold.

Mikkelsen et al 1997) in-office BP values were also lower in smokers as compared to non-smokers. The discrepancies between the aforementioned studies (Stewart et al 1994, Green et al 1991, Mikkelsen et al 1997) and the present and previous (Gropelli et al 1999, Verdecchia et al 1995, Mann et al 1991, Bang et al 2000, Bolinder et al 1998, Benowitz et al 2002) studies could also be attributed to differences in other characteristics of the study samples. In this regard, although in the present study as compared to non-smokers, more current smokers were male, a higher proportion consumed alcohol, a lower proportion were obese, and fewer were receiving antihypertensive medication or were diabetic, the outcomes of the study were reproduced in both genders, in participant's not drinking alcohol, in overweight and obese participants, in untreated participants and in non-diabetics. Moreover, in the present sample, smokers had modestly greater levels of regular walking, which would have biased against the outcomes of the study. In addition, in the present study no differences in salt intake were noted between current smokers and non-smokers as assessed from urinary indices of salt excretion determined from 24-hour urine collections, and the impact of smoking also persisted with adjustments for these indices of salt intake.

A previous study indicates that central (aortic) SBP may be higher in smokers as compared to non-smokers, despite similar brachial artery SBP values and that smoking increases aortic PWV (Mahmud and Feely 2003). In this regard the lack of relationship between current smoking and aortic SBP in the present study is not surprising as the mean number of cigarettes smoked per day (=7.4) in the present study was considerably less than that reported on in this prior study (=15 cigarettes per day) (Mahmud and Feely 2003). Moreover, the lack of relationship between current smoking and aortic PWV in the present study is similarly not surprising as in the present study participants did not smoke on the morning of the clinic visit and aortic PWV may only last for a short period after inhaling one cigarette (~15 minutes) (Mahmud and Feely 2003). Because the technology has only recently

been developed, we were unable to assess out-of-office (ambulatory) central aortic BP and hence it is likely that we missed this effect. Therefore, further studies are required to assess whether predominantly mild cigarette smoking is associated with substantial increases in out-of-office aortic SBP at a population level.

Although no relationship between smoking and a number of central haemodynamic measurements was noted in the present study, an independent association between smoking and the central (aortic) augmentation pressure was observed, although this was not quantitatively large enough to account for increases in aortic pulse pressure. As smoking was not associated with aortic PWV, the effective reflective distance or the reflective wave transit time, I assume that the impact of smoking on aortic augmentation pressure is attributed to the magnitude of wave reflection which may be determined by medium sized artery tone. Irrespective of the mechanism of the increased reflected pressure wave in smokers as compared to non-smokers, the importance of wave reflection in mediating cardiovascular damage independent of pulse pressure or peripheral BP is nevertheless still uncertain.

Although the proportion of hypertensives who were smokers was less than the proportion of non-smokers with hypertension, this would have biased against our results. Although through both statistical adjustments as well as sensitivity analysis conducted in sub-groups, care has been taken to account for a number of confounders, a limitation of the study is the cross-sectional design which prevents conclusions being drawn regarding causality. In this regard I did not account for the possibility that smokers have greater life stressors or more pronounced physiological responses to life stressors, an effect that could account for the increased BP. However, 24-hour heart rates and night blood pressures, which are frequently elevated in response to life stressors, were remarkably similar between smokers and non-smokers. Moreover, the present study was conducted in participants of African ancestry, and hence may apply only to one ethnic group. Furthermore, as with most previous studies

evaluating the relationship between smoking and out-of-office BP, smoking was assessed from self-reports. Thus, the presence or extent of smoking may have been underestimated. In addition, the lack of moderate and heavy smoking in the study sample prevented us from assessing a dose-response relationship.

In conclusion, the present study indicates that even mild smoking is associated with a quantitatively significant increase in out-of-office BP and risk for uncontrolled out-of-office BP in urban, developing communities of African ancestry. These data suggest that in economically emerging communities, encouraging the cessation of even mild smoking may produce considerable benefits in the management of out-of-office BP, at least in groups of African ancestry. Further large studies are required in urban, developing communities of African ancestry to assess the impact of the cessation of mild smoking on out-of-office BP.

Chapter 5

The Angiotensin-Converting Enzyme Insertion/Deletion Polymorphism and the Relationship Between Smoking and Ambulatory Blood Pressure in a Community Sample of African ancestry.

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Abstract

Whether genetic factors moderate the relationship between cigarette smoking and blood pressure (BP) is uncertain. I aimed to assess whether the relationship between predominantly mild-to-moderate smoking and out-of-office (24-hour ambulatory) BP is associated with the angiotensin-converting enzyme (ACE) insertion/deletion (I/D) polymorphism in 652 randomly recruited participants of African descent. The ACE I/D genotype was not independently associated with 24-hour, day or night BP. Current smoking was independently associated with 24-hour and day DBP and SBP. However, independent of age, sex, body mass index (BMI), treatment for hypertension, regular alcohol intake, diabetes mellitus or an HbA1c>6.1%, and the individual terms for smoking and ACE genotype, an interaction between ACE DD genotype and current cigarette smoking, or the number of cigarettes smoked per day was independently associated with 24-hour and day diastolic BP (DBP) ($p<0.05$-0.005). This smoking-ACE genotype interactive effect translated into a relationship between smoking and BP only in participants with the DD genotype of the ACE gene. In participants with the DD genotype, the adjusted day DBP in smokers was 83.7±1.6 mm Hg, whilst in non-smokers it was 76.0±0.6 mm Hg ($p<0.0005$). In contrast, in participants with the I allele, the adjusted day DBP in smokers was 80.5±1.5 mm Hg, whilst in non-smokers it was 77.5±0.6 mm Hg ($p=0.08$). Smoking was also independently associated with uncontrolled 24-hour (multivariate adjusted odds ratio=4.01, CI=1.59 to 10.09, $p<0.005$), and day (multivariate adjusted odds ratio=5.54, CI=2.33 to 13.21, $p=0.0001$) BP in participants with the ACE DD genotype. In contrast, in participants carrying the I allele, smoking was unrelated to uncontrolled 24-hour (multivariate adjusted odds ratio=1.13, CI=0.47 to 2.71, $p=0.79$) and day (multivariate adjusted odds ratio=2.05, CI=0.91 to 4.65, $p=0.085$) BP. In conclusion, these data suggest that ACE genotype markedly modifies the relationship between current mild-to-moderate smoking and BP.

5.1 Introduction

As highlighted in chapter 4, although several studies indicate that cigarette smoking acutely increases systolic and diastolic blood pressure (BP) (Kool et al 1993, Mahmud and Feely 2003, Failla et al 1997, Rhee et al 2007), the evidence for a clinically important impact of smoking on in-office BP at a community or population level is controversial. Although some studies suggest that cigarette smoking is associated with an increased in-office BP or the risk for hypertension (Thuy et al 2010, Tesfaye et al 2008, Niskanen et al 2004, Halimi et al 2002, Mundal et al 1997), other studies indicate that smoking is associated with a decreased in-office BP and a reduced risk for hypertension (Okubo et al 2004, Primatesta et al 2001, Lee et al 2001, Hughes et al 1998, Green et al 1986), or that smoking has modest (Bowmen et al 2007) or clinically negligible (Primatesta et al 2001, Fogari et al 1996, Tsai et al 2005, John et al 2006) relationships with in-office BP or the risk for hypertension. Adequate explanations for these discrepancies have not been forthcoming in the current scientific literature.

In chapter 4 and accepted for publication (Woodiwiss et al 2011, in-press) I provide the evidence to suggest that in-office BP measurements may result in an inability to adequately capture the clinically important effects of current smoking, possibly because smokers refrain from the habit prior to office visits. Indeed, in that study (chapter 4) I show that although predominantly mild-to-moderate smoking is only weakly related to in-office BP measurements, there is nevertheless a strong relationship between predominantly mild-to-moderate smoking and out-of-office BP measurements, presumably when smokers partake of the habit. However, alternative explanations should also be sought for the aforementioned discrepancies in the literature regarding the effects of smoking on BP. An alternative possible explanation is that the impact of smoking on BP may depend on genetic background.

With respect to studies demonstrating that a potential interaction between smoking and genetic background may determine BP, an interaction between smoking and the angiotensin-converting enzyme (ACE) gene insertion-deletion (ID) variant has been associated with in-office BP or the risk for hypertension (Schut et al 2004, Xu et al 2004). However, the extent to which this interaction modifies the relationship between smoking and BP at a population level is nevertheless uncertain. Indeed, although an interaction was identified in the study by Xu et al (2004) in 1099 participants, the ACE gene ID variant and smoking were both related to hypertension diagnosed on the basis of in-office BP. The controversy regarding the impact of smoking on in-office BP and that surrounding the independent role of the ACE gene in BP control (see Chapter 1, pages 14-18) casts some doubt on the validity of the findings by Xu et al (2004). Furthermore, in this study (Xu et al 2004) the relationship between ACE genotype-smoking interaction and the risk for hypertension was not multivariate adjusted. Indeed, in this study (Xu et al 2004), interactions occurred between ACE genotype and a number of risk factors for hypertension other than cigarette smoking, including alcohol intake, and indices of excess adiposity. As these risk factors are known to aggregate in people who are less aware of the risks of a poor lifestyle, whether the ACE genotype-smoking interaction can be attributed to smoking *per se* is uncertain. Moreover, in the study by Schut et al (2004), no discernable effect of smoking on BP was noted and the interaction translated into an effect of ACE genotype on BP in smokers, rather than an effect of smoking on BP in genotype-specific groups (Schut et al 2004). Despite the study by Schut et al (2004) being conducted in 1508 current smokers, the ACE gene effect on BP in smokers was modest at best ($p<0.05$). The modest ACE gene effect in the study by Schut et al (2004) may nevertheless have been driven by the lack of out-of-office BP measurements when smoking effects are noted (chapter 4).

Therefore the aim of the present study was to evaluate at a community level whether an interaction between ACE genotype and current smoking translates into a greater effect of current smoking on out-of-office BP in ACE genotype-specific groups after adjustments for other lifestyle factors. In this regard, I evaluated this question in the African Program on Genes in Hypertension conducted in a community sample of black-African ancestry, an ethnic group with a high prevalence of the DD genotype (Candy et al 1999).

5.2 Methods

5.2.1 Study group

Details of the selection of the participants that made up the study group have been described in section 2.2 (pages 50-51) and section 4.2 (page 114) of the present thesis. The present study was conducted in 689 participants with 24-hour ambulatory BP values that met with pre-specified quality control criteria (longer than 20 hours and more than 10 and 5 readings for the computation of day and night means, respectively).

5.2.2 Clinical, demographic and anthropometric measurements.

A standardized questionnaire was administered to obtain demographic and clinical data as described in section 2.2 (pages 51-52) of the present thesis. With respect to smoking effects, the number of cigarettes smoked per day, and the age of starting (and ending if a previous smoker) were recorded.

5.2.3 Conventional blood pressure measurements.

Trained nurse-technicians measured BP using a standard mercury sphygmomanometer during a clinic visit as described in section 2.2 (page 53) of the present thesis.

5.2.4 Ambulatory blood pressure measurements.

24-Hour ambulatory BP monitoring was performed on the same day as conventional BP measurements using oscillometric monitors (SpaceLabs, model 90207) as described in section 4.2 (pages 114-115) of the present thesis. The identification of day and night periods were similarly described in section 4.2 (page 115) of the present thesis.

5.2.5. Angiotensin-converting enzyme genotype

Deoxyribonucleic acid (DNA) was extracted from whole blood (652 participants [or 95%] consented to genotyping) by lysing red blood cells and digesting the remaining white cell pellet with proteinase K. The ACE gene I/D polymorphism was detected by the polymerase chain reaction (PCR) technique as described in section 3.2 (page 82-83) of the present thesis.

5.2.6 Statistical analysis.

For database management and statistical analysis, SAS software, version 9.1 (SAS Institute Inc., Cary, NC) was employed. Data are shown as mean±SD. To test for Hardy-Weinberg equilibrium the expected genotype numbers were calculated from the allele frequencies and deviation from the observed genotype numbers was determined using a χ^2 test. Regression analysis with relevant confounders included in the regression models was used to determine independent relationships. Relationships between smoking and BP were all adjusted for age, sex, BMI, diabetes mellitus or an HbA1c>6.1%, regular alcohol intake and treatment for hypertension. Multivariate regression models including the same confounders were used to determine the significance of interactive terms independent of the individual terms. Following the demonstration of significant interactions, the sample was subdivided according to ACE genotype or according to whether participants smoked or did not smoke and separate multivariate regression analysis was performed in subgroups. For the derivation of probability values, further adjustments were made for non-independence of family

members using the mixed procedure as outlined in the SAS package. For the assessment of ACE genotype effects on BP, sensitivity analysis was conducted in participants not receiving therapy. Furthermore, secondary data analysis was performed in the participants with urinary sodium excretion data (n=500), to assess the impact of sodium excretion on the effects of ACE genotype on the relationship between smoking and blood pressure.

5.3 Results

5.3.1 Characteristics of the participants.

Differences in the general and clinical characteristics of smokers as compared to non-smokers have been discussed in section 4.3 on page 118 of the present thesis.

5.3.2 Allele and genotype frequencies

The ACE gene I/D polymorphism was in Hardy-Weinberg equilibrium (for all participants DD=342[52.4%], ID=249[38.2%] and II=61[9.4%]; for unrelated participants (DD=189[53.7%], ID=136[38.6%] and II=27[7.7%]). No differences in ACE gene I/D allele or genotype frequencies were noted in smokers and non-smokers with 48% of smokers and 53% of non-smokers being homozygous for the risk D allele.

5.3.3 Smoking and genotype effects on BP.

The relationship between smoking and BP has been described in section 4.3 on pages 118-124 of the present thesis. No relationship between the ACE gene I/D polymorphism and either unadjusted or multivariate adjusted conventional or ambulatory BP was noted in either the whole study sample (Table 5.1), or participants not receiving antihypertensive therapy (Table 5.2). Indeed, with adjustments for age, sex, BMI, diabetes mellitus or an HbA1c>6.1%, regular alcohol intake, cigarette smoking and treatment for hypertension, no independent relationship between the ACE gene DD genotype and either conventional

Table 5.1. Association of the angiotensin-converting enzyme gene insertion/deletion (I/D) polymorphism with unadjusted and multivariate adjusted conventional and ambulatory blood pressures in **all** participants of African origins.

Genotype→	DD (n=342) mean±SD	ID (n=249) mean±SD	II (n=61) mean±SD
	Unadjusted data		
Conventional SBP (mm Hg)	130±21	130±23	126±22
Conventional DBP (mm Hg)	84±12	84±13	83±12
Conventional PP (mm Hg)	45±14	45±16	43±14
24 hour SBP (mm Hg)	118±15	118±15	118±15
24hour DBP (mm Hg)	72±10	72±10	73±10
24-hour PP (mm Hg)	46±9	45±9	45±9
Day SBP (mm Hg)	122±15	123±15	122±15
Day DBP (mm Hg)	77±10	78±11	78±10
Day PP (mm Hg)	45±9	45±9	44±9
Night SBP (mm Hg)	112±17	111±17	110±17
Night DBP (mm Hg)	65±12	65±11	64±11
Night PP (mm Hg)	47±10	46±10	46±10
	Multivariate adjusted data*		
Conventional SBP (mm Hg)	129±18	130±20	129±46
Conventional DBP (mm Hg)	84±11	85±11	85±11
Conventional PP (mm Hg)	45±12	45±12	45±12
24 hour SBP (mm Hg)	118±13	118±13	119±13
24hour DBP (mm Hg)	72±9	73±9	73±9
24-hour PP (mm Hg)	46±8	45±8	46±8
Day SBP (mm Hg)	122±13	123±13	124±13
Day DBP (mm Hg)	77±10	78±10	78±10
Day PP (mm Hg)	45±9	45±9	45±9
Night SBP (mm Hg)	111±15	111±15	112±16
Night DBP (mm Hg)	65±11	65±11	66±11
Night PP (mm Hg)	46±10	46±10	46±10

SBP, systolic blood pressure; DBP, diastolic blood pressure; PP, pulse pressure.

* Adjustments are for age, gender, body mass index, diabetes mellitus or an HbA_{1c}>6.1%, regular alcohol intake, cigarette smoking and treatment for hypertension. No significant differences in BP were noted between genotype groups (see text).

Table 5.2. Association of the angiotensin-converting enzyme gene insertion/deletion (I/D) polymorphism with unadjusted and multivariate adjusted conventional and ambulatory blood pressures in participants of African origins **not receiving antihypertensive treatment**.

Genotype→	DD (n=264) mean±SD	ID (n=188) mean±SD	II (n=50) mean±SD
	Unadjusted data		
Conventional SBP (mm Hg)	127±21	124±21	123±20
Conventional DBP (mm Hg)	83±12	82±12	82±12
Conventional PP (mm Hg)	43±14	43±14	41±12
24 hour SBP (mm Hg)	117±15	116±14	115±14
24hour DBP (mm Hg)	72±10	72±9	72±10
24-hour PP (mm Hg)	45±9	45±8	45±7
Day SBP (mm Hg)	122±15	121±14	121±14
Day DBP (mm Hg)	77±10	78±10	77±11
Day PP (mm Hg)	45±9	44±8	44±7
Night SBP (mm Hg)	111±17	108±16	108±15
Night DBP (mm Hg)	65±12	63±11	63±11
Night PP (mm Hg)	46±10	45±9	45±8
	Multivariate adjusted data*		
Conventional SBP (mm Hg)	126±17	125±17	126±17
Conventional DBP (mm Hg)	83±11	83±11	84±11
Conventional PP (mm Hg)	43±12	43±12	42±12
24 hour SBP (mm Hg)	117±13	116±13	117±13
24hour DBP (mm Hg)	72±9	72±9	72±9
24-hour PP (mm Hg)	45±8	45±8	45±8
Day SBP (mm Hg)	121±13	122±13	122±13
Day DBP (mm Hg)	77±9	77±9	78±9
Day PP (mm Hg)	45±8	44±8	44±8
Night SBP (mm Hg)	110±14	108±14	109±14
Night DBP (mm Hg)	65±10	63±10	64±10
Night PP (mm Hg)	46±9	45±9	45±9

SBP, systolic blood pressure; DBP, diastolic blood pressure; PP, pulse pressure.

* Adjustments are for age, gender, body mass index, diabetes mellitus or an HbA_{1c}>6.1%, regular alcohol intake and cigarette smoking. No significant differences in BP were noted between genotype groups (see text).

(p=0.34 to 0.64), or ambulatory (p=0.11 to 0.92) BP was noted. Similar results were observed in males and in females (data not shown).

5.3.4 ACE genotype effect on the relationship between smoking and conventional or ambulatory BP.

An interaction between ACE genotype and current smoking, or the number of cigarettes smoked per day was independently associated with BP (Table 5.3). This effect translated into greater multivariate adjusted conventional and ambulatory BP values in current-smokers than in non-smokers in participants with the DD genotype only (Figure 5.1). In contrast, in participants carrying the I allele, conventional and ambulatory BP values were similar in current smokers as compared to non-smokers (Figure 5.1).

Even after adjusting for sodium excretion, out-of-office BP (in mm Hg) was significantly increased in current smokers compared to non-smokers in only those participants with the ACE DD genotype (24 hour DBP: smokers=78±11, non-smokers=71±9, p<0.005; day DBP: smokers=82±11, non-smokers=76±10, p<0.005). No differences were noted in those participants with the I allele (24 hour DBP: smokers=73±10, non-smokers=73±9, p=0.76; day DBP: smokers=80±11, non-smokers=78±10, p=0.39).

5.3.5. Impact if ACE genotype on the quantitative effect of current smoking on out-of-office BP.

In participants with the DD genotype of the ACE gene, smoking 4.6 cigarettes (one standard deviation) per day translated into a 3.5 mm Hg increase in 24-hour DBP (p<.0.0001), a 3.6 mm Hg increase in day DBP (p<0.0001), a 3.6 mm Hg increase in 24-hour SBP (p<0.005) and a 3.8 mm Hg increase in day SBP (p<0.005) (Figure 5.2). In contrast, smoking 4.6 cigarettes (one standard deviation) per day translated into a 0.6 mm Hg increase in 24-hour DBP (p=0.50), a 1.1 mm Hg increase in day DBP (p=0.212), a 0.8 mm Hg increase in 24-hour SBP (p=0.488) and a 1.9 mm Hg increase in day SBP (p=0.104) in

Table 5.3. Association between blood pressure (BP) and an interactive effect between the angiotensin-converting enzyme (ACE) insertion/deletion (I/D) polymorphism and smoking in 689 participants.

	Interaction between ACE DD genotype and			
	Current smoking		Number of cigarettes	
	partial r*	p values	partial r*	p value*
24-Hour SBP	0.08	=0.05	-0.07	0.09
24-Hour DBP	0.12	**<0.005**	-0.10	**<0.05**
Day SBP	0.06	=0.14	-0.05	0.25
Day DBP	0.10	**<0.05**	-0.08	**<0.05**
Night SBP	0.05	=0.17	-0.07	0.10
Night DBP	0.08	**<0.05**	-0.09	**<0.05**
Conventional SBP	0.03	=0.40	-0.06	0.14
Conventional DBP	0.02	=0.68	-0.06	0.14

* Determined from multivariate regression analysis with adjustments for ACE genotype and smoking assessed as individual terms, as well as age, body mass index, sex, diabetes mellitus or an HbA1c>6.1%, regular alcohol intake, and treatment for hypertension. Probability values are further adjusted for non-independence of family members. SBP, systolic BP; DBP, diastolic BP. Significant p-values are indicated in bold type.

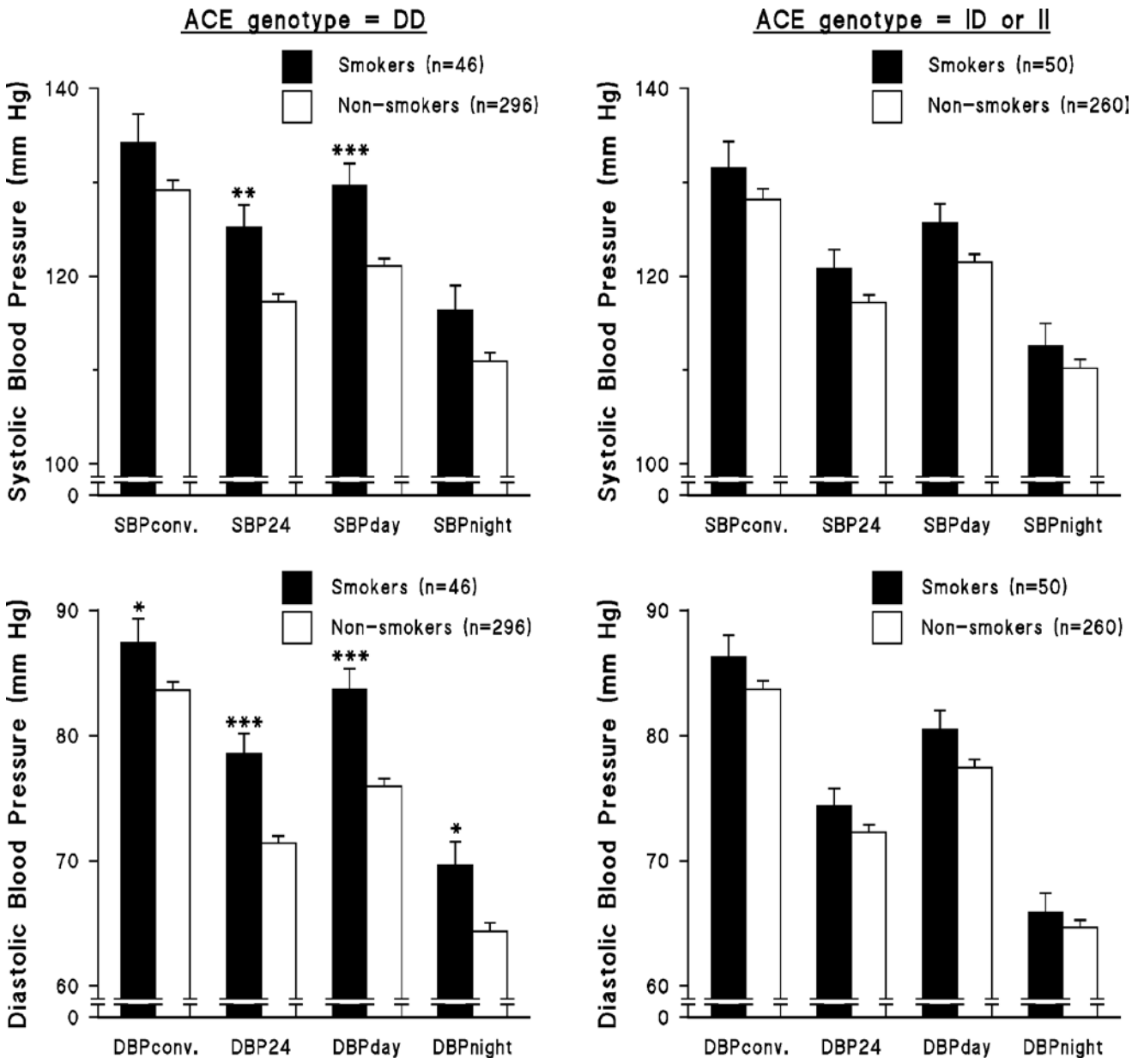

Figure 5.1. Relationship between current smoking and multivariate adjusted conventional (conv.), 24-hour (24), day and night systolic (SBP) and diastolic (DBP) blood pressures in participants grouped according to the angiotensin-converting enzyme (ACE) insertion/deletion (I/D) variant genotype. Adjustments in all analyses are for age, sex, body mass index, diabetes mellitus or an HbA1c>6.1%, regular alcohol intake, and treatment for hypertension. Probability values are further adjusted for non-independence of family members. * $p<0.05$, ** $p<0.005$, *** $p<0.0005$.

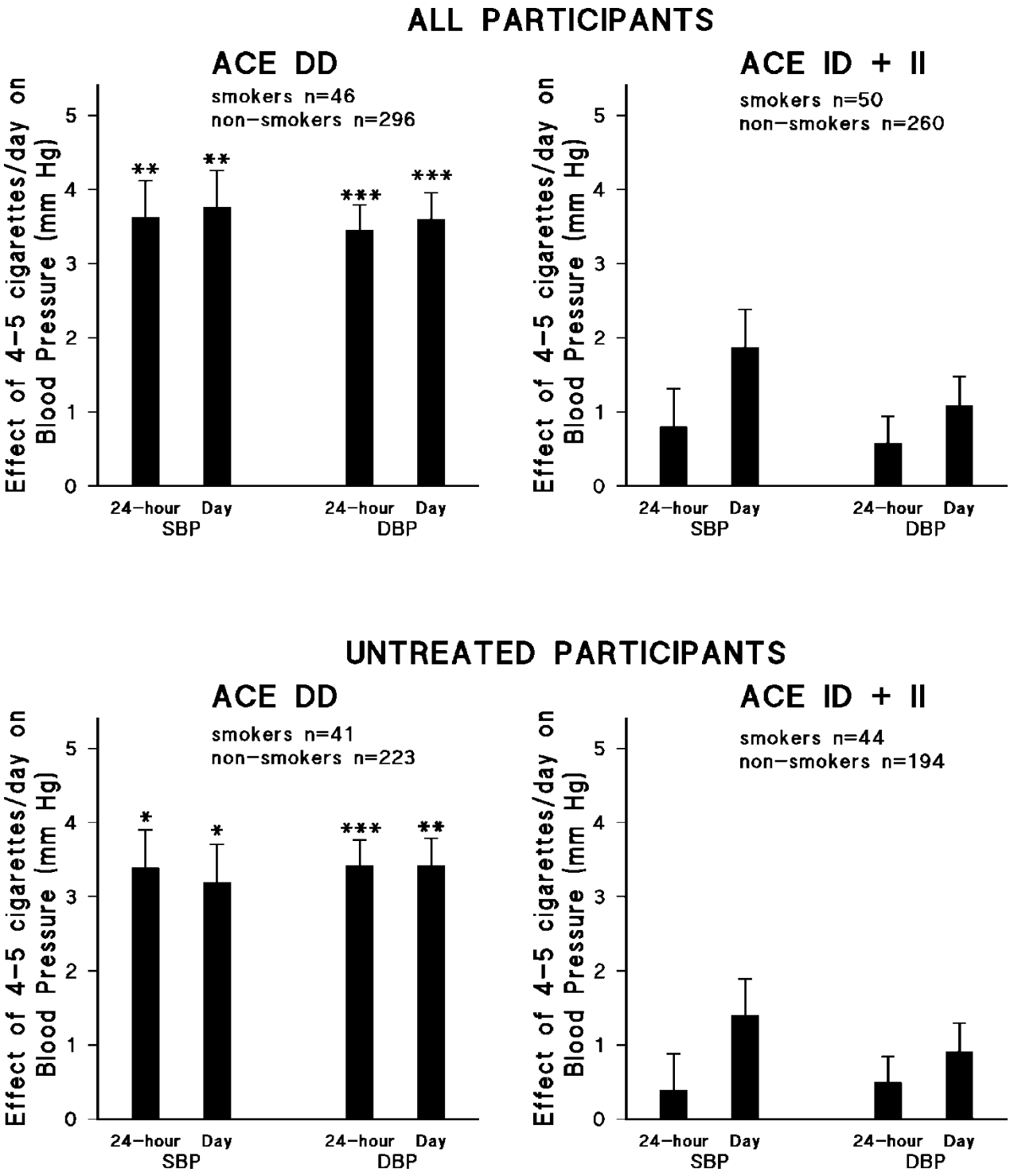

Figure 5.2. Estimated effect of 4.6 cigarettes smoked per day (one standard deviation) on 24-hour and day systolic (SBP) and diastolic (DBP) blood pressure (BP) in all participants and in untreated participants grouped according to the angiotensin-converting enzyme (ACE) gene insertion/deletion (I/D) polymorphism. Adjustments are for age, sex, body mass index, diabetes mellitus or an HbA1c>6.1%, regular alcohol intake, and treatment for hypertension (in the whole group). Probability values are further adjusted for non-independence of family members. *p<0.05, **p<0.005, ***p<0.0001.

participants with the ID or II genotype of the ACE gene (Figure 5.2). Similar results were noted in untreated participants (Figure 5.2).

The effects of ACE genotype on the impact of smoking 4.6 cigarettes per day on BP were not altered by adjusting for urinary sodium excretion. In participants with the DD genotype, smoking 4.6 cigarettes per day translated into a 3.58 (CI=3.15 to 4.01) mm Hg increase in 24-hour DBP ($p<0.001$), a 3.25 (CI=2.81 to 3.69) mm Hg increase in day DBP ($p<0.005$), a 2.90 (CI=2.27 to 3.52) mm Hg increase in 24-hour SBP ($p<0.05$) and a 2.66 (CI=2.03 to 3.28) mm Hg increase in day SBP ($p<0.05$) were noted. In comparison, in participants with the I allele, smoking 4.6 cigarettes per day translated into a 0.11 (CI=-0.51 to 0.28) mm Hg decrease in 24-hour DBP ($p=0.91$), a 0.53 (CI=0.10 to 0.96) mm Hg increase in day DBP ($p=0.61$), a 0.34 (CI=-0.90 to 0.22) mm Hg decrease in 24-hour SBP ($p=0.80$) and a 0.79 (CI=0.23 to 1.35) mm Hg increase in day SBP ($p=0.56$).

5.3.6. Impact of ACE genotype on the relationship between current cigarette smoking and conventional and 24-hour BP control rates.

Current smoking was also independently associated with uncontrolled 24-hour (multivariate adjusted odds ratio=4.01, $p<0.005$), and day (multivariate adjusted odds ratio=5.54, $p=0.0001$), but not with uncontrolled conventional (multivariate adjusted odds ratio=1.00,$p=0.99$) BP in participants with the ACE gene DD genotype (Figure 5.3). In contrast, in participants carrying the I allele, smoking was unrelated to either uncontrolled 24-hour (multivariate adjusted odds ratio=1.13, $p=0.79$) and day (multivariate adjusted odds ratio=2.05, $p=0.09$) or conventional (multivariate adjusted odds ratio=1.49, $p=0.34$) BP (Figure 5.3). Similar results were noted in untreated participants (Figure 5.3).

The impact of current smoking on out-of-office BP control was considerably modified by ACE genotype even after adjustments for urinary sodium excretion. In participants with the DD genotype, smoking was independently associated with uncontrolled 24-hour

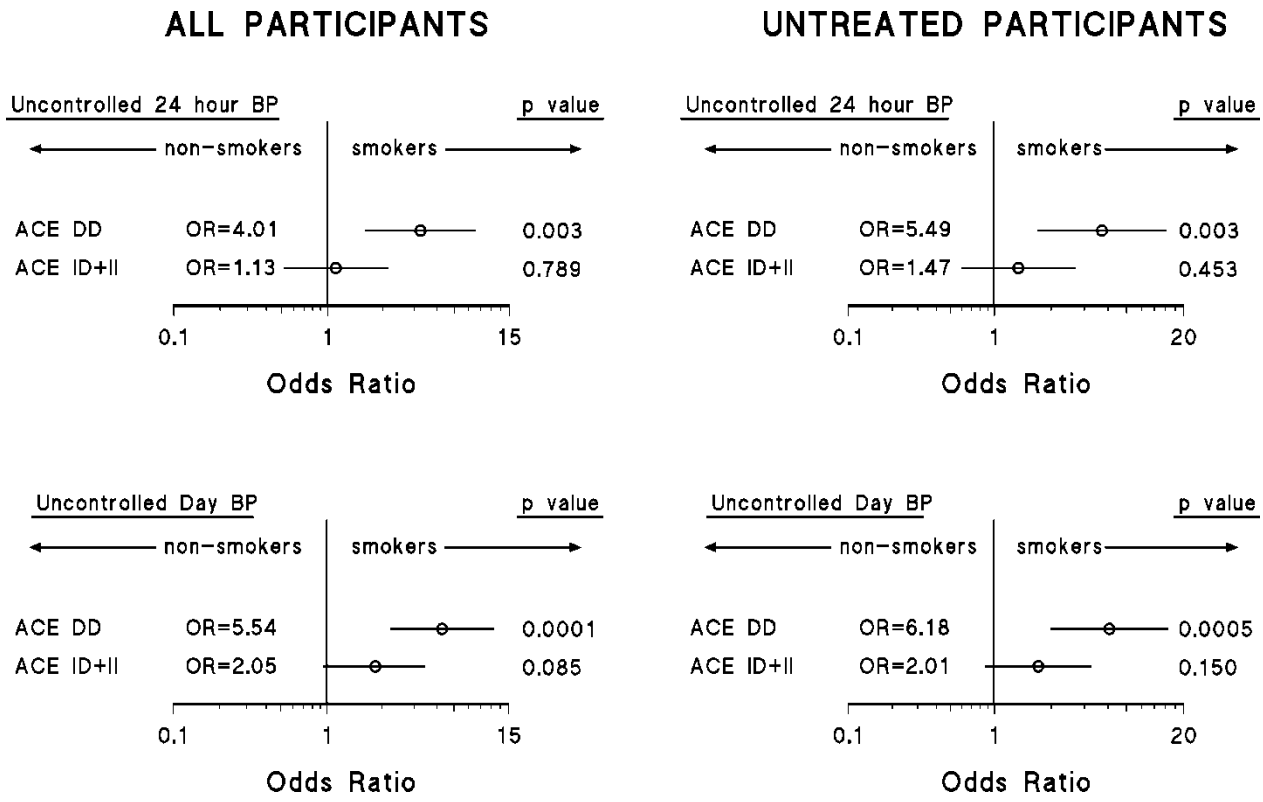

Figure 5.3. Relationship between current smoking and the risk of uncontrolled 24-hour and day blood pressure (BP) in the whole group, and in untreated participants grouped according to the angiotensin-converting enzyme (ACE) gene insertion/deletion (I/D) polymorphism. Adjustments are for age, sex, body mass index, diabetes mellitus or an HbA1c>6.1%, regular alcohol intake, and treatment for hypertension. Probability values are further adjusted for non-independence of family members.

(multivariate adjusted odds ratio=3.79, $p<0.05$) and day BP (multivariate adjusted odds ratio=4.54, $p<0.005$). In contrast, in participants carrying the I allele, smoking was unrelated to uncontrolled 24-hour and day BP (multivariate adjusted odds ratio=0.64, p=0.42 and odds ratio=1.47, p=0.45 respectively).

5.3.7 *Smoking and the impact of ACE genotype on BP.*

In contrast to the modifying effect of ACE genotype on the impact of smoking on BP (Figure 5.1), smoking failed to significantly influence the impact of ACE genotype on BP (Table 5.4). However, to achieve statistical significance at 80% power and $p<0.05$ for 24-hour BP I would have required a sample size of 107 in each of the three genotype groups to show differences in 24-hour SBP, and a sample size of 99 in each of the three genotype groups to show differences in 24-hour DBP.

5.4 Discussion

The present study is the first to demonstrate that the association between cigarette smoking and out-of-office BP may depend in-part on ACE genotype. In this regard, a significant interaction was noted between current cigarette smoking and ACE genotype. This translated into a marked association between smoking and out-of-office BP in participants with the DD genotype of the ACE gene. In this regard, in participants with the DD genotype, smokers had a 7.7 mm Hg increase in day DBP, only 4.6 cigarettes (one standard deviation) smoked per day translated into a 3 to 4 mm Hg increase in day and 24-hour BP, and smoking was associated with a 4.0 times greater risk of uncontrolled 24-hour BP and a 5.5 times greater risk of uncontrolled day BP than in non-smokers. In contrast, in participants with the ID or II genotypes, smokers had a 2.9 mm Hg increase in day DBP, 4.6 cigarettes (one standard deviation) smoked per day translated into a 0.58-1.87 mm Hg increase in day and

Table 5.4. Effect of smoking on the relationship between the angiotensin-converting enzyme gene insertion/deletion (I/D) polymorphism and multivariate adjusted conventional and ambulatory blood pressures in participants of African origins.

	mean±SD	mean±SD	mean±SD
	Smokers		
Genotype→	DD (n=46)	ID (n=38)	II (n=12)
Conventional SBP (mm Hg)	134±20	129±20	129±20
Conventional DBP (mm Hg)	87±13	87±12	85±13
Conventional PP (mm Hg)	47±14	43±14	43±14
24 hour SBP (mm Hg)	125±13	119±13	124±14
24hour DBP (mm Hg)	79±10	75±10	75±10
24-hour PP (mm Hg)	47±9	44±9	49±9
Day SBP (mm Hg)	130±14	126±14	130±14
Day DBP (mm Hg)	84±11	81±11	82±11
Day PP (mm Hg)	46±10	45±10	48±10
Night SBP (mm Hg)	116±15	111±15	115±15
Night DBP (mm Hg)	69±11	66±11	65±11
Night PP (mm Hg)	47±9	44±9	49±9
	Non-smokers		
Genotype→	DD (n=296)	ID (n=211)	II (n=49)
Conventional SBP (mm Hg)	128±18	130±18	130±18
Conventional DBP (mm Hg)	83±11	84±11	85±11
Conventional PP (mm Hg)	45±12	45±12	45±12
24 hour SBP (mm Hg)	117±13	118±13	118±13
24hour DBP (mm Hg)	71±9	72±9	73±9
24-hour PP (mm Hg)	46±8	45±8	45±8
Day SBP (mm Hg)	121±13	122±13	122±13
Day DBP (mm Hg)	76±10	78±10	78±10
Day PP (mm Hg)	45±8	44±8	45±8
Night SBP (mm Hg)	111±15	111±15	111±15
Night DBP (mm Hg)	64±11	65±11	66±11
Night PP (mm Hg)	46±10	46±10	46±10

SBP, systolic blood pressure; DBP, diastolic blood pressure; PP, pulse pressure. All data are adjusted for age, gender, body mass index, diabetes mellitus or an HbA_{1c}>6.1%, treatment for hypertension, relatedness and regular alcohol intake.

24-hour BP, and smoking was associated with only a 1.13 times greater risk of uncontrolled 24-hour BP and a 2.05 times greater risk of uncontrolled day BP than in non-smokers.

The present study suggests that the clinically significant quantitative impact of current mild-to-moderate smoking on out-of-office BP at a community level, as reported on in Chapter 4 (Woodiwiss et al 2011), may be explained by the high prevalence of the ACE gene D allele that occurs in groups of African ancestry (Candy et al 1999). Indeed, the effect of smoking on out-of-office BP was only evident in the 48% of smokers homozygous for the D allele. It is therefore possible that in population or community samples without a high prevalence of this genotype, the effects of mild-to-moderate smoking may not be as marked as those effects reported on in the present study.

Importantly, an association between smoking-ACE genotype interactions and BP or the risk for hypertension has previously been described (Schut et al 2004, Xu et al 2004). However, unlike in the present study where I show an unequivocal moderating influence of ACE genotype on the impact of smoking on BP, in a previous study conducted in a large population sample (Schut et al 2004) it was unclear whether this interaction translated into a greater impact of smoking on BP in genotype-specific groups. Indeed, in this previous study (Schut et al 2004) smoking effects on BP were difficult to identify, possibly because conventional rather than ambulatory BP measurements were employed.

In an alternative study conducted in 1099 participants, although an interaction between smoking and ACE genotype was associated with the risk for hypertension (Xu et al 2004), this increased risk was not multivariate adjusted. Indeed, in this study (Xu et al 2004), the risk for hypertension was also associated with interactions between ACE genotype and a number of risk factors for hypertension other than cigarette smoking, including alcohol intake, and indices of an excess adiposity. As these risk factors are known to aggregate in people who are less aware of the risks of a poor lifestyle, whether the association between the

ACE genotype-smoking interaction and BP can be attributed to smoking *per se* is uncertain. In contrast, in the present study, the association of ACE genotype-smoking interactive effects with out-of-office BP were adjusted for alcohol intake and BMI. In addition, the impact of ACE genotype on the relationship between smoking and out-of-office BP was also observed after adjustments for urinary Na^+/K^+ in participants who had complete 24-hour urine samples.

A potential mechanism that may explain the relationship between the cigarette smoking-ACE genotype interaction and BP is the additive effect of the ability of smoking and the ACE genotype to modify vascular responses to acetylcholine (Butler et al 1999). Indeed, both smoking and the risk genotype of the ACE gene are associated with a decreased vasodilator response to acetylcholine and the two effects together produce a greater impact (Butler et al 1999). This interactive effect may be explained by the fact that that nicotine influences endothelial cell angiotensin-converting enzyme expression (Saijonmaa et al 2005). As ACE genotype is strongly related to endothelial-dependent vasodilation (Butler et al 1999), it is possible that ACE genotype may interact with nicotine to modify endothelial cell ACE expression and hence endothelial cell function, the consequence being effects on BP.

As reviewed in section 2.2.2.2 of Chapter 1 (pages 14-18) linkage analysis, cross-sectional prevalence analysis, meta-analyses and prospective incidence analysis have all been performed in large, well conducted studies to evaluate the role of the ACE gene in BP control. Many of these studies have demonstrated linkage or an association of the ACE gene ID polymorphism with BP (see Chapter 1, pages 14-18). However, there are equally as many well done studies that have failed to show similar relationships (see Chapter 1, pages 14-18) and as argued in Chapter 1 (section 2.2.2.5, pages 24-31), these apparent discrepancies are potentially because the penetrance of the genotype is low as the ACE gene produces effects in the context of environmental and phenotypic characteristics of the study sample. Hence, although not a primary aim of the present study, the lack of association between ACE I/D

genotype and BP in the present study does not exclude the possibility of a role for the ACE gene in BP control in groups of African ancestry. Indeed, in the present study, although I could not show an association between ACE genotype and either ambulatory or conventional BP in the whole group, I was not statistically powered to evaluate the relationship between ACE genotype and ambulatory BP in smokers only. The lack of statistical power was largely due to the low prevalence of smoking in this community sample (14.5%, n=100). Hence, a significantly larger study sample is required to evaluate the relationship between ACE genotype and BP in the context of current smoking.

The effect of the ACE gene on BP may be sex-specific in that it has only been strongly related to BP in males (Fornage et al 1998, O'Donnell et al 1998, Higaki et al 2000, Krege et al 1995). Because more women than men volunteered for the present study, I was also not statistically powered to evaluate the impact of gender on the relationship between ACE genotype and either conventional or ambulatory BP. Hence, a significantly larger study sample is also required to evaluate the relationship between ACE genotype in sex-specific groups.

In a study conducted in 1875 persons, the ACE gene I/D variant was shown to interact with body size to influence body size effects on BP (Turner et al 1999). Moreover, albeit evidence obtained in small study samples (n=71)(Poch et al 2001)(n=61)(Hiraga et al 1996) the ACE gene has been shown to determine the extent to which salt intake increases BP. Nevertheless, in these studies (Poch et al 2001, Hiraga et al 1996) the I allele of the I/D polymorphism was associated with a greater BP response to a salt load, findings which are out of keeping with the association of the D allele with increases in ACE activity (see Chapter 1, section 2.2.2.1, pages 12-14). Although these data have not been shown in the results section, in an analysis of the current database I was unable to show a relationship between body size-ACE genotype or salt intake-ACE genotype interactions and either

conventional or ambulatory BP. However, it is possible that these effects may also be sex-specific and depend on the presence of smoking. Hence as indicated in the aforementioned discussion, body size-ACE genotype or salt intake-ACE genotype interactions may only occur in males and/or current smokers. Because more women than men volunteered for the present study and only 14.5% of the community sample smoked (n=100), I was also not statistically powered to evaluate these possibilities.

As indicated in previous chapters of the present thesis, a limitation of the present study is the cross-sectional design which does not allow for conclusions to be drawn regarding causality. Further prospective intervention studies are required to assess whether alterations in smoking habits influence out-of-office BP in an ACE genotype-dependent fashion.

Although speculative, a further clinical implication of the present results warrants consideration. It is possible that in contrast to the well recognised poor clinical response to ACE inhibitors when employed as first line therapy in groups of African ancestry (ALLHAT Collaborative Research Group 2002, Cushman et al 2000), cigarette smokers who are of African descent and who are unable to abstain from the habit, may benefit from ACE inhibitors as first line therapy. Whether ACE inhibitors are more effective antihypertensive agents in smokers with the DD genotype than in non-smokers or participants without the DD genotype requires further study.

In conclusion, the present study provides the first evidence to indicate that ACE genotype in-part accounts for the relationship between mild-to-moderate current smoking and out-of-office BP at a community level. These data provide a potential explanation for the clinically significant associations that occur between even mild smoking and out-of-office BP in groups of African descent who have a high prevalence of the ACE gene DD genotype. In addition these data provide further evidence to suggest that in populations of African

ancestry, the renin-angiotensin-aldosterone system may play a substantial role in contributing toward variations in BP, but that these effects are through a moderating influence on lifestyle factors, in the present case a moderating influence on smoking effects on BP. The present study further suggests that blockers of the renin-angiotensin-aldosterone system could be an important modality of therapy in people of African descent who smoke and carry the DD genotype of the ACE gene. Intervention studies aimed at encouraging cigarette smokers to quite or to employ ACE inhibitors as first line therapy in cigarette smokers who carry the DD genotype are required to further evaluate these hypotheses. Moreover, the mechanisms responsible for the association between ACE genotype-smoking interactions and BP require further study.

Chapter 6

Summary and conclusions

As highlighted in chapter 1 of the present thesis, the phenotypic features that characterise hypertension in groups of African ancestry is that of a “salt-sensitive, low-renin state” (Aviv et al 2004, Luft et al 1979, Luft et al 1991, Campese et al 1991, Peters and Flack 2000, Channik et al 1969, Brunner et al 1972, Kaplan et al 1976, Chrysant et al 1979, James et al 1986, Pratt et al 1999, Fisher et al 1999, Helmer et al 1964, Voors et al 1976). This notion has challenged a role for the renin-angiotensin-aldosterone system (RAAS) in the pathogenesis of an increased BP in groups of African ancestry. Indeed, this “salt-sensitive, low-renin” state is associated with a poor blood pressure (BP) response to antihypertensive therapy involving some blockers of the RAAS (ALLHAT Collaborative Research Group 2002, Cushman et al 2000) including angiotensin-converting enzyme inhibitors and angiotensin receptor blockers. Thus, despite the increasing benefits that accrue from RAAS blocker therapy in appropriate patients with cardiovascular disease (The SOLVD Investigators 1992, Yusuf et al 2000, The European Trial on Reduction of Cardiac Events with Perindopril in Stable Coronary Artery Disease Investigators 2003), these agents are generally not considered as the first line of antihypertensive therapy in patients of African descent (Chobanian et al 2003, Williams et al 2004).

In South Africa, at a community level, the evidence against a role for the RAAS in BP control in groups of African descent has translated into the dominant antihypertensive agent employed being thiazide diuretic agents, whilst very few patients receive RAAS blockers even as a second agent in combination with diuretics (Maseko et al 2006, 2011). This is despite the high prevalence of concentric cardiac hypertrophy that exists in these communities (Norton et al 2008, 2009, Woodiwiss et al 2008, 2009), a compelling indication for the use of RAAS blockers (Dahlöf et al 2002). However, BP responses to combined diuretic and RAAS blocker therapy may be similar in groups of African as compared to European descent (Libhaber et al 2004) suggesting that “salt–sensitive, low-renin”

hypertension is indeed a state that depends to some extent on RAAS activity. Furthermore, a clinically relevant BP response to angiotensin-converting enzyme inhibitors (ACEIs) when used as monotherapy may occur in hypertensives of African ancestry who have a particular angiotensinogen genotype (Woodiwiss et al 2006). Therefore, whether the RAAS is appropriately diminished in "salt-sensitive, low-renin" hypertension has been a matter of debate for some years. What is the current evidence either for or against this notion and how does the present thesis add to this information?

In accordance with studies conducted in other ethnic groups, in groups of African ancestry, a relationship exists between circulating angiotensinogen concentrations and BP (Forrester et al 1996) and angiotensinogen concentrations may in fact be higher than in their Caucasian counterparts (Bloem et al 1997). However, this information has not translated into the notion that ACE inhibitors may be particularly important to employ as antihypertensive agents in patients with angiotensinogen concentrations above a specific threshold. Indeed, the exact reason for high circulating angiotensinogen concentrations in groups of African descent has not been identified, although genetic variation could play a significant role, and how these high concentrations translate into increases in BP in "salt-sensitive" groups with low plasma renin concentrations has not been explained. Is there evidence for alternative components of the RAAS contributing toward hypertension in groups of African descent?

In contrast to the Framingham Heart study conducted largely in a population sample of European ancestry, where circulating aldosterone concentrations were associated with the incidence of hypertension (Vasan et al 2004), earlier studies conducted in groups of African ancestry failed to show a relationship between circulating aldosterone concentrations and BP at a community level (Hoosen et al 1985, Hoosen et al 1990). Moreover, recent studies conducted in groups of African descent have produced conflicting results, with one study conducted in the Seychelles reporting on a lack of relationship except in the context of an

older age (Bochud et al 2006) whilst in studies where Na^+ intake has been standardised at relatively high levels (150 mmol/day), a relationship between aldosterone concentrations and BP has been reported on in African-Americans irrespective of age (Kidambi et al 2009, Grim et al 2005). A potential lack of relationship between aldosterone concentrations and BP in groups of African descent is more consistent with the high prevalence of "salt-sensitive, low-renin" hypertension, where a decrease in renin will result in a decrease in circulating aldosterone concentrations. However, there are a number of lines of evidence to suggest that circulating aldosterone concentrations are inappropriately diminished in "salt-sensitive, low-renin" states.

Despite a suppressed renin activity or concentrations in groups of African ancestry, plasma aldosterone concentrations are often normal or even elevated (Grim et al 2005). Indeed, although plasma renin activity tends to decrease with age in hypertensive patients of African descent, plasma aldosterone concentrations may remain normal (Sagnella et al 2001). Moreover, although plasma renin activity is suppressed as a consequence of the BP response to salt intake in "salt-sensitive" individuals, reductions in plasma aldosterone concentrations may be attenuated (Wisgerhof et al 1978, Marks et al 1979, Griffing et al 1990). Indeed, in patients with "low-renin" hypertension, although the adrenal response to angiotensin II infusion is normal on a high salt diet, it becomes progressively more sensitive on a low salt diet (Fisher et al 1999). This effect may be genetically predetermined as strong sibling correlations with the aldosterone response to angiotensin II have been reported on (Giacche et al 2000). Thus, in "salt-sensitive" individuals there is a possibility that salt-induced increases in BP may in-part depend on the presence of an inability to appropriately reduce aldosterone secretion, despite marked decreases in renal renin release. This is entirely consistent with the finding in a preclinical study demonstrating that BP does not increase in response to a salt-load alone, but does increase in animals genetically modified to develop an attenuated

decrease in plasma aldosterone concentrations (Makhanova et al 2008). Moreover, this hypothesis is reminiscent of the BP changes that occur in the well-described deoxycorticosterone (DOCA)-salt model, where neither excessive salt intake nor administration of the mineralocorticoid (DOCA) alone produce BP changes, but the combination produces profound hypertensive effects (Ouchi et al 1987). However, prior to the present thesis this hypothesis had **not** been tested at a population or community level in any ethnic group. Thus the importance of the hypothesis that "salt-sensitivity" depends on an attenuated suppression of aldosterone in response to a salt load had not been substantiated at a population or community level. This hypothesis is particularly important to address in community samples of African descent where current evidence is against a role for aldosterone in BP control at a population level in black African samples in Africa (Hoosen et al 1985, Hoosen et al 1990, Bochud et al 2006) except in the elderly (Bochud et al 2006), but where strong relationships may exist in African-Americans if Na^+ intake is standardised at relatively high levels (150 mmol/day) (Kidambi et al 2009).

As part of the present thesis I therefore tested the aforementioned hypothesis and was able to show that aldosterone concentrations or the aldosterone-to-renin ratio (an index of the relationship between aldosterone release in response to renin) are indeed associated with the relationship between salt intake, as assessed from 24-hour urinary electrolyte excretion rates, and BP in a community sample of African ancestry. These data are presented in Chapter 2 and have been accepted for publication in the *American Journal of Hypertension* (Scott et al, 2011). Having demonstrated the importance of this concept at a community level in a group of African descent, I subsequently went on to identify whether genetic factors contribute to serum aldosterone concentrations in this community sample. In this regard, the role of genetic factors as determinants of the variability of circulating aldosterone concentrations in groups of African ancestry is uncertain. Indeed, despite significant heritability reported on for serum

aldosterone concentrations in a largely Caucasian community sample (Kathiresan et al 2005, Newton-Cheh et al 2007), no significant heritability has been noted for serum aldosterone concentrations in African-Americans (Kotchen et al 2000). Therefore, in Chapter 3, I have taken advantage of the nuclear family structure of participants and evaluated the familial aggregation and heritability of serum aldosterone concentrations in a community of African ancestry and subsequently the relationship between some variants of clinical and functional relevance the ACE and AGT genes. How have the results of the latter study described in Chapter 3 extended our knowledge of the mechanisms responsible for the variability of circulating aldosterone concentrations at a community level?

In keeping with the heritability of serum aldosterone concentrations in the context of plasma renin reported on in community samples of largely European ancestry (Newton-Cheh et al 2007), I was able to show significant familial aggregation and heritability of serum aldosterone concentrations in a community sample of African ancestry. The present study obviously extends the previous study by Newton-Cheh et al (2007) in that it is the first to show a genetic contribution of serum aldosterone concentrations in a group of black African ancestry, well recognised as having a high prevalence of low plasma renin activity and hence often low serum aldosterone concentrations relative to other populations (Bochud et al 2006, Helmer et al 1964, Voors et al 1976). The present study also extends this previous study (Newton-Cheh et al 2007) by demonstrating that significant heritability and intrafamilial correlations for serum aldosterone concentrations were noted only after adjustments for plasma renin concentrations. Without this adjustment, neither familial aggregation nor heritability of serum aldosterone concentrations were noted. The present study therefore underscores the importance in genetic studies of carefully considering genotype-phenotype relationships in the context of the complexity of the determinants of specific phenotypes. In this regard, the penetrance of genetic factors that modulate serum aldosterone concentrations,

at least in the African context is likely to be strongly influenced by plasma renin concentrations. Having demonstrated that genetic factors are likely to contribute toward the variation in serum aldosterone concentrations I subsequently set out to assess whether specific candidate genes may account for these relations.

In the present study I was unable to show relationships between clinically and functionally important ACE or AGT gene variants and serum aldosterone concentrations (Chapter 3). These results are consistent with the lack of relationship between ACE and AGT gene variants previously reported on in a large study sample of Caucasians (Paillard et al 1999) but in apparent contrast to another previous study demonstrating a relationship between the -6G→A variant of the AGT gene and plasma aldosterone concentrations in a Caucasian sample (Fardella et al 1999). The results of the present study nevertheless do not preclude a role of either the ACE or the AGT gene as factors that may modulate serum aldosterone concentrations in groups of African ancestry as further work is still required to account for variations across the whole of the AGT or ACE genes. This work is presently being performed by our group. However, in addition, we are also evaluating a potential role for the aldosterone synthase gene and other genes that may modify the activity of the RAAS downstream from renin.

Although it is important to assess the relationships between circulating concentrations of components of the RAAS and BP in groups of African descent, as previously indicated there is also substantial evidence for a role for genetic variants of components of the RAAS, particularly the ACE and AGT gene, in BP control in population groups of ethnic origins other than of African ancestry. However, in contrast to the multitude of large and small studies conducted in groups of European descent, relatively few studies have evaluated the relationship between RAAS genes and circulating RAAS concentrations or activity and BP in groups of African ancestry. As reviewed in Chapter 1 of the present thesis, in two case-

control studies with very small sample sizes (n=68-89) the ACE gene D allele was reported to be associated with hypertension and/or a higher BP in groups of African descent (Barley et al 1996, Duru et al 1994). However, in contrast to data with positive outcomes in groups of African ancestry (Barley et al 1996, Duru et al 1994), in three larger case-control studies no relationship between the ACE gene D allele and hypertension was noted in groups of African descent (Forrester et al 1997, Borecki et al 1997, Kamdar et al 1994). Thus, there is considerable uncertainty as to the contribution of the ACE gene to BP in groups of African ancestry. Moreover, in a number of case-control studies with small study samples (Caulfield et al 1995, Borecki et al 1997, Forrester et al 1996, Rotimi et al 1994, Barley et al 1994) and in two case-control studies with large study samples (>1000 participants) (Larson et al 2000, Tiago et al 2002) with ambulatory BP measurements performed in the over 500 hypertensives studied by our group in one of these studies (Tiago et al 2002), no relationships between variants of the AGT gene and hypertension were noted. Nevertheless, one small linkage study has provided evidence to indicate that the AGT gene is involved in the development of hypertension in groups of African ancestry (Caulfield et al 1995).

Clearly much larger population-based cross-sectional and prospective studies are required to either confirm or refute the notion that RAAS genes contribute toward a significant proportion of the variation in BP in populations of African ancestry. Importantly however, few studies have pursued the notion that interactions between RAAS genes and either environmental or phenotypic factors may contribute toward BP in groups of African ancestry. One possibility is that the ACE gene interacts with the effects of smoking on the vasculature and promotes increases in BP. Indeed, an influence of the ACE gene I/D polymorphism on BP has been shown to occur in cigarette smokers, but not non-smokers in a Caucasian population sample (Schut et al 2004) and an interaction between smoking and ACE genotype was associated with the risk for hypertension diagnosed with in-office BP

measurements in a Mongolian population sample (Xu et al 2004). However, in the study by Schut et al (2004) the ACE gene effect failed to produce a statistically striking effect on BP in that study ($p<0.05$) despite a sample of 1508 current smokers being studied. Moreover, smoking effects on in-office BP in the group as a whole were not identified (Schut et al 2004). This finding agrees with the considerable inconsistencies noted for smoking effects on in-office BP in many population-based studies (see Chapters 1 and 4 for a review of this literature). The controversy regarding the impact of smoking on in-office BP not only confounds the outcomes of the study by Schut et al (2004), but also casts doubt as to the validity of the findings demonstrating an impact of an interaction between smoking and ACE genotype on the risk for hypertension diagnosed with in-office BP measurements (Xu et al 2004). Indeed, in that study (Xu et al 2004) smoking alone was related to the diagnosis of hypertension based on in-office BP. Moreover, the study by Xu et al (2004), also demonstrated that the risk for hypertension was associated with interactions between ACE genotype and a number of risk factors for hypertension other than cigarette smoking, including alcohol intake, and indices of an excess adiposity. As these risk factors are known to aggregate in people who are less aware of the risks of a poor lifestyle, whether the association between the ACE genotype-smoking interaction and BP can be attributed to smoking *per se* is uncertain. In this regard, Xu et al (2004) failed to adjust for alternative lifestyle-related factors when reporting on the relationship between smoking-ACE genotype interaction and the risk for hypertension. Thus, in the present thesis to further evaluate whether an association exists between an ACE gene-smoking interaction and BP, I had to first further pursue the possibility that smoking indeed accounts for a significant proportion of the variability of BP and subsequently I evaluated whether an association between an ACE gene-smoking interaction and BP occurs independent of alternative lifestyle risk factors for hypertension.

As the effects of cigarette smoking on BP are relatively short-lived (Kool et al 1993, Mahmud and Feely 2003, Failla et al 1997, Rhee et al 2007, Gropelli et al 1999), with perhaps the exception of heavy smoking (Gropelli et al 1999), the ability to adequately capture the effects of smoking may best occur with out-of-office BP measurements. Indeed, in some (Verdecchia et al 1995, Mann et al 1991, Bang et al 2000, Bolinder et al 1998, Benowitz et al 2002) but not other (Stewart et al 1994, Green et al 1991, Mikkelsen et al 1997) case-control (Verdecchia et al 1995, Mann et al 1991, Bang et al 2000, Bolinder et al 1998, Benowitz et al 2002, Stewart et al 1994) or cross-sectional (Green et al 1991, Mikkelsen et al 1997) studies conducted either in small study samples (Mann et al 1991, Bang et al 2000, Bolinder et al 1998, Benowitz et al 2002, Stewart et al 1994, Green et al 1991, Mikkelsen et al 1997) or where office BP was matched between cases and controls (Verdecchia et al 1995), medium-to-heavy cigarette smoking was associated with increases in out-of-office, but not in-office BP. Furthermore, whilst smokers may have similar brachial artery BP values as non-smokers, central (aortic) BP may be increased in smokers as compared to non-smokers (Mahmud and Feely 2003). Despite the possibility that smoking effects on BP may mainly influence out-of-office or central BP, the impact of predominantly mild-to-moderate smoking on out-of-office or central BP at a population or community level is uncertain. As in economically emerging communities such as groups of African descent in South Africa, which now face the dual health burden of diseases of poverty and of affluence, cigarette smokers may predominantly partake in the mild-to-moderate range, it is important to identify whether even mild-to-moderate smoking has clinically important effects on out-of-office BP.

In chapter 4 of the present thesis and accepted for publication in the *Journal of Hypertension* I was able to show that that even mild smoking is associated with a quantitatively significant increase in out-of-office BP and risk for uncontrolled out-of-office

BP in urban, developing communities of African ancestry. Moreover, the smoking effect on BP was second only to age and at least equivalent to BMI or male gender. The relationship between current smoking and out-of-office BP was noted in both males and in females, in participants who did not drink alcohol, in participants not receiving antihypertensive therapy, in overweight and obese participants and in non-diabetics. Moreover, the effect of current smoking was independent of a number of potential additional confounders including urinary Na^+/K^+ (an index of salt intake). The magnitude of the effect of smoking was exemplified by the fact that only 4.6 cigarettes (one standard deviation) smoked per day translated into a 1.96 mm Hg increase in 24-hour DBP, and a 2.10 mm Hg increase in 24-hour SBP and a 2.3 mm Hg increase in day DBP and a 2.7 mm Hg increase in day SBP. Moreover, smoking was associated with a 1.98 and 3.0 times greater risk of uncontrolled 24-hour and day BP respectively than non-smokers. In contrast, current smoking was only modestly associated with in-office conventional or central BP. Having established that even mild smoking is associated with a significant variability of out-of-office BP, I subsequently evaluated whether this marked effect of predominantly mild smoking on out-of-office BP could be accounted for by the ACE gene I/D polymorphism.

In chapter 5 of the present thesis and also included in the paper accepted for publications in the *Journal of Hypertension*, I demonstrate for the first time that the association between cigarette smoking with out-of-office BP may depend in-part on ACE genotype. In this regard, a significant interaction was noted between current cigarette smoking and ACE genotype. This translated into a marked association between predominantly mild smoking and out-of-office BP in participants with the DD genotype of the ACE gene. In this regard, in participants with the DD genotype, smokers had a 7.7 mm Hg increase in day DBP, only 4.6 cigarettes (one standard deviation) smoked per day translated into a 3 to 4 mm Hg increase in day and 24-hour BP, and smoking was associated

with a 4.0 times greater risk of uncontrolled 24-hour BP and a 5.5 times greater risk of uncontrolled day BP than in non-smokers. In contrast, in participants with the ID or II genotypes, smokers had no significant changes in BP, 4.6 cigarettes (one standard deviation) smoked per day translated into a 1 to 2 mm Hg increase in day and 24-hour BP, and smoking was associated with only a 1.13 times greater risk of uncontrolled 24-hour BP and a 2.05 times greater risk of uncontrolled day BP than in non-smokers.

The study described in Chapter 5 therefore suggests that the clinically significant quantitative impact of current mild-to-moderate smoking on out-of-office BP at a community level, as reported on in Chapter 4, may be explained by the high prevalence of the ACE gene D allele that occurs in groups of African ancestry (Candy et al 1999). These findings extend the findings of Schut et al (2004) where an interaction between smoking and ACE genotype failed to translate into a clinically important effect of smoking on BP even in participants with the DD genotype, possibly because conventional rather than ambulatory BP measurements were employed in that study. These findings also extend the findings of Xu et al (2004), where although an interaction between smoking and ACE genotype translated into a clinically important effect of smoking on the risk for hypertension, hypertension was diagnosed on the basis of conventional BP, which as previously pointed out is a controversial result. These findings also extend the findings of Xu et al (2004), where although an interaction between smoking and ACE genotype was associated with an increased risk for hypertension, this increased risk was not multivariate adjusted and hence could have been attributed to similar interactions between ACE genotype and a number of risk factors for hypertension other than cigarette smoking, including alcohol intake, and indices of an excess adiposity. In this regard, in the present study, the association of ACE genotype-smoking interactive effects with out-of-office BP were adjusted for alcohol intake and BMI. In addition, the impact of ACE genotype on the relationship between smoking and out-of-office

BP was also observed after adjustments for urinary Na^+/K^+ in participants who had complete 24-hour urine samples.

In conclusion therefore, the findings of the present thesis provide evidence to suggest that in groups of African ancestry, aldosterone within ranges that cannot be accounted for by the presence of primary aldosteronism, modifies the relationship between salt intake and BP in groups of African descent. Moreover, the results of the present thesis suggest that genetic factors account for the variation in serum aldosterone concentrations in groups of African ancestry, but that this effect can only be evaluated in the context of plasma renin concentrations and that these effects may not occur because of genetic variations with in the ACE or AGT genes. Furthermore, the results of the present thesis suggest that the ACE gene modifies the relationship between smoking and out-of-office BP and hence accounts for even predominantly mild smoking producing a marked and clinically important effect on out-of-office BP. These findings therefore add to the present evidence produced by our group that indicates that the RAAS does indeed influence BP in groups of African descent, but that these effects are in the context of environmental influences. In this regard, our group have previously demonstrated that the relationship between obesity and BP in hypertensive patients of African ancestry is dependent on the angiotensinogen gene (Tiago et al 2002). The results of the present thesis therefore further support the notion that in conjunction with diuretic agents, the use of blockers of the RAAS may be an important therapeutic approach to appropriately targeting the pathophysiological mechanisms responsible for the condition when managing hypertension in this ethnic group. This is a particularly important issue as the use of diuretic agents alone does not produce adequate BP control in groups of African descent and RAAS blockers are seldom employed to treat hypertension in this ethnic group (Maseko et al 2011).

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UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Woodiwiss/Norton

CLEARANCE CERTIFICATE	**PROTOCOL NUMBER MO70469**
PROJECT	Gene Candidates As Determinants of Blood Pressure and Intermediary Phenotypes in Pathogenesis of Hypertension in Black S Africans
INVESTIGATORS	Profs A/G Woodiwiss/Norton
DEPARTMENT	School of Physiology
DATE CONSIDERED	07.05.09
DECISION OF THE COMMITTEE*	Approved unconditionally (refer M020472)

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 07.05.09 **CHAIRPERSON** .....
(Professors PE Cleaton-Jones, A Dhai, M Vorster, C Feldman, A Woodiwiss)

*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor : Woodiwiss A Prof

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10005, 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. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

M110243

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG
Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Mr Leon Scott

CLEARANCE CERTIFICATE	**M110243**
PROJECT	context-Dependent Effects of the Renin-Angiotensin-Aldosterone System and Their Impact on Ambulatory Blood Pressure in Subjects of African Descent (part of M070469/R14/49/Woodiwiss/Norton)
INVESTIGATORS	Mr Leon Scott.
DEPARTMENT	School of Physiology
DATE CONSIDERED	14/02/2011
DECISION OF THE COMMITTEE*	

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 14/02/2011 **CHAIRPERSON** .....
(Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable
cc: Supervisor : Professor Woodiwiss

DECLARATION OF INVESTIGATOR(S)

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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 Woodiwiss/Norton et al

CLEARANCE CERTIFICATE **PROTOCOL NUMBER** M02-04-72

PROJECT Gene Candidates As Determinants of Blood Pressure And Intermediary Phenotypes In Pathogenesis of Hypertension In Black South Africans

INVESTIGATORS Prof's AJ/G et al Woodiwiss/Norton et al

DEPARTMENT School of Physiology, Wits Medical School

DATE CONSIDERED 02-04-26

UNIVERSITY OF THE WITWATERSRAND
PROF PE CLEATON - JONES
HREC (MEDICAL)
2007 -05- 09
JOHANNESBURG

This clearance is valid and within the 5-year validity

DECISION OF THE COMMITTEE *

Approved unconditionally

DATE 02-05-14 **CHAIRMAN** .....(Professor P E Cleaton-Jones)

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

c c Supervisor: Prof AJ Woodiwiss
Dept of School of Physiology, Wits 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.

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