Development and application of a Real-time PCR method to detect selected single nucleotide polymorphisms associated with hypertension in a black South African population

By

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DECLARATION

I certify that the dissertation hereby submitted by me for the M.Med.Sc. (Molecular Biology) degree at the University of the Free State is my independent effort and has not previously been submitted for a degree at another university/faculty. I furthermore waive copyright of the dissertation in favour of the University of the Free State.

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List of Abbreviations

A	Adenine
ADD1	α-Subunit of adducin gene
ADRB1	β1-Adrenergic receptor gene
AGT	Angiotensinogen gene
AHA-FS	Assuring Health for All in the Free State
bp	Base pair
C	Cytosine
cAMP	Cyclic adenosine monophosphate
CI	Confidence interval
CYP11B2	Aldosterone synthase
CYP3A5	Cytochrome P-450 3A5 gene
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
ECUFS	Ethics Committee of the University of the Free State
EDTA	Ethylenediaminetetra-acetic acid
et al.	*et alia* (and others)
FAM	6-carboxyfluoresceine
FTA	Fast technology for analysis of nucleic acids
G	Guanine

GRK4	G protein-coupled receptor kinase 4 gene
in vitro	Outside the living organism
K^+	Potassium ion
kg	Kilogram
kg/m^2	Kilogram per square metre
M	Molar
m	Metre
ml	Millilitre
mm Hg	Millimetre of mercury
mM	Millimolar
mm	Millimetre
mRNA	Messenger ribonucleic acid
n	Number
Na^+	Sodium ion
N/A	Not applicable
ng	Nanogram
NIH	National Institute of Health
nM	Nanomolar
nmol	Nanomole
OR	Odds ratio
P	Probability value
PCR	Polymerase chain reaction

pH	Potential of hydrogen
RAAS	Renin-angiotensin-aldosterone system
rpm	Revolutions per minute
SNP	Single nucleotide polymorphism
T	Thymine
TAE	Tris(hydroxymethyl)aminomethane-acetate-ethylenediaminetetra-acetic acid
TE	Tris(hydroxymethyl)aminomethane-ethylenediaminetetra-acetic acid
Tris	Tris(hydroxymethyl)aminomethane
U	Units
µg	Microgram
µl	Microlitre
µM	Micromolar
UV	Ultraviolet
VIC	a proprietary fluorescent dye produced by Applied Biosystems
WHO	World Health Organization
www	World wide web
3’	3 prime end
5’	5 prime end
˚C	Degree Celsius
%	Percentage

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Preface

Hypertension is one of the leading causes of death and disability in the world. Hypertension is characterized by blood pressure ≥ 140/90 mm Hg. Undiagnosed hypertension can lead to damage of cerebral and coronary tissues, as well as the kidneys, which in turn can result in cerebrovascular, cardiovascular and renal disease. Hypertension is responsible for approximately 51% of global cerebrovascular disease (strokes) and is estimated to contribute to approximately 50% of the global cardiovascular disease burden. In 95% of individuals with hypertension, the condition arises from the interaction of multiple environmental factors with physiological systems. Environmental factors that have been found to increase blood pressure include obesity, aging, high salt and alcohol consumption, low potassium and calcium intake, stress and insulin resistance. Physiological systems that regulate blood pressure include the autonomic nervous system, the renal system, hormonal system and the cardiovascular system. Various genes in these systems, including the β1-adrenergic receptor (*ADRB1*), α-adducin (*ADD1*), angiotensinogen (*AGT*), aldosterone synthase (*CYP11B2*), cytochrome P-450 3A5 (*CYP3A5*), and G protein-coupled receptor kinase 4 (*GRK4*), have been implicated in causing hypertensive blood pressure as a result of single nucleotide polymorphisms (SNPs) found to be associated with the hypertensive phenotype. The occurrence of such SNPs is thought to result in altered gene expression or protein function, which can affect the ability of blood pressure regulatory systems to contend with hypertensive changes in blood pressure.

In South Africa, the prevalence of hypertension has been determined to be approximately 39.9% in males and 34.9% in females. A survey in rural areas of the Free State and Mangaung (Bloemfontein) has shown that the prevalence of hypertension in these areas was approximately 62.6% and 48.3%, respectively. It was also estimated that 37.6% and 51.2% of the Mangaung population was overweight or obese, respectively. As such, being overweight or obese could be an

important risk factor for hypertension in the Mangaung population. In addition to obesity, various genetic factors may also account for the high prevalence of hypertension in the Mangaung population. Thus, the aim of this study was to determine whether selected SNPs in genes associated with hypertension in the literature, especially in meta-analyses, were associated with hypertension in the Mangaung population.

The dissertation consists of five chapters, which include a literature review (chapter one), methodology section (chapter two) and three research chapters (chapter three to five). The research chapters consist of a section on method optimization (chapter three), SNP association analysis (chapter four) and sequence analysis of samples that failed genotyping (chapter five). The literature review provides background on hypertension and selected SNPs that have been implicated in the condition and also includes the aim of the study. Chapter two describes the methodology of the study. Chapter three explains the optimization of DNA extraction from FTA paper, as well as the optimization of Real-time PCR genotyping assays. In chapter four, the results of SNP genotyping as well as the association analysis of the SNPs with hypertension is given and discussed. Chapter five includes the sequencing analysis of samples that could not be successfully genotyped. The dissertation is summarized in the summary section, bringing together all the main findings from this study.

Chapter One

Literature Review

1.1 Introduction to hypertension

Hypertension is a major public health problem worldwide (Kearney *et al*., 2005). Hypertension is a chronic disorder that is characterized as blood pressure ≥ 140/90 mm Hg (Lupton *et al*., 2011). The condition is generally asymptomatic in the majority of affected individuals causing it to be under diagnosed (Chalmers *et al*., 1999). The global frequency of hypertension in males and females 25 years and older, is approximately 29.2% and 24.8%, respectively (WHO, 2014). In South Africa, the prevalence of hypertension has been determined to be approximately 39.9% in males and 34.9% in females (WHO, 2014). The Assuring Health for All in the Free State (AHA-FS) study was conducted to assess chronic lifestyle diseases, including hypertension. The study focused on black populations in rural communities in the Free State and Mangaung area (Bloemfontein). It was found that hypertension was the chronic disorder with the highest prevalence, affecting approximately 62.6% and 48.3% of the population groups assessed in the rural communities of the Free State and Mangaung, respectively (Van Zyl *et al*., 2012). The aforementioned features of hypertension have earned it the status of a 'silent epidemic' (Steyn, 2006).

Undiagnosed hypertension can lead to damage of various organs (Steyn, 2006). These include damage to cerebral and coronary tissues, as well as the kidneys. Damage to these tissues can result in the development of cerebrovascular, cardiovascular and renal disease (Lupton *et al*., 2011). Hypertension is responsible for approximately 51% of global cerebrovascular disease (strokes) and is estimated to contribute to approximately 50% of the global cardiovascular disease burden

(Caulfield *et al*., 2003; WHO, 2009). Hypertension is considered the third largest cause for disability-adjusted life years and is one of the major risk factors for mortality (Kearney *et al*., 2005).

Hypertension can be classified on the basis of underlying pathology (Tanira and Al Balushi, 2005). Hypertension that occurs as a consequence of disease conditions, such as aldosteronism, pheochromocytoma, renovascular disease and renal failure, is referred to as secondary hypertension (Carretero and Oparil, 2000). If hypertension is the result of known rare mutations that affect specific genes important in the regulation of blood pressure, it is referred to as monogenic hypertension (Ambler and Brown, 1999; Carretero and Oparil, 2000). Secondary and monogenic hypertension constitutes approximately 5% of all cases of hypertension (Carretero and Oparil, 2000). However, in approximately 95% of hypertension cases, no underlying clinical cause is apparent, and the hypertension is thought to have a multifactorial pathogenesis. This common form of hypertension is referred to as essential hypertension (Carretero and Oparil, 2000). Factors contributing to the development of essential hypertension include the interaction of environmental and physiological factors (Oparil *et al*., 2003). Environmental factors that have been found to increase blood pressure include obesity, aging, high salt and alcohol consumption, low potassium (K^+) and calcium intake, stress as well as insulin resistance (Carretero and Oparil, 2000). Physiological systems responsible for counteracting deviations from normal blood pressure include the autonomic nervous system, renal system, hormonal system and the cardiovascular system (Ambler and Brown, 1999). These systems maintain normal blood pressure by adjusting the physiological pathways that determine blood pressure, namely vascular resistance, fluid volume and cardiac output (Coffman and Crowley, 2008). Single nucleotide polymorphisms (SNPs) may result in nucleotide substitutions within a gene, which could alter the function of the resulting protein and prevent optimal functioning of physiological systems that regulate blood pressure. As a result, blood pressure changes induced by environmental factors may not be properly contended, resulting in hypertensive blood pressure (Saavedra, 2007; Kunes and Zicha, 2009; Lupton *et al*., 2011). Thus, in the vast majority of hypertensive cases, the high blood pressure

is the product of the additive effect of several SNPs in various genes and the environment.

A particularly important risk factor for hypertension in South Africa is being overweight or obese. In South Africa, the prevalence of obesity has been determined to be 42.8% in females and 23.2% in males (WHO, 2014). Findings of the AHA-FS study indicated that overweight and obesity had a prevalence of approximately 37.6% and 51.2% in the black Mangaung population, respectively (Van Zyl *et al*., 2010). It was further shown that hypertension was significantly more prevalent in overweight to obese individuals, compared to individuals of low to normal weight, with a prevalence of 76.5% in the overweight to obese group, compared to 47.4% in the low to normal weight group (Lategan *et al*., 2014). The results from the AHA-FS study suggest that the Mangaung population has a significantly higher than average incidence of hypertension, especially in overweight and obese individuals.

1.2 Blood pressure regulation by the autonomic nervous system

The autonomic nervous system is responsible for the moment-to-moment control of blood pressure (Cowley, 1992). It exerts control over blood circulation via the efferent sympathetic and parasympathetic systems (Guyenet, 2006). The efferent parasympathetic nervous system primarily acts through the neurotransmitter acetylcholine which binds to muscarinic acetylcholine receptors, which induces a functional change in cardiovascular tissues. The main consequence of parasympathetic activity on the blood circulatory system is decreased cardiac output, which lowers blood pressure (Thomas, 2011). Compared to this, the efferent sympathetic activity results in the up regulation of blood pressure, through the neuronal release of the neurotransmitter, norepinephrine (Goodfriend and Calhoun, 2004; Thomas, 2011). Norepinephrine activates α- and β-adrenergic receptors that induce hormonal systems to modify the regulation of renal fluid and this induces increased cardiac output and vasoconstriction (Thomas, 2011). Abnormal activity of

the sympathetic nervous system may result in the maintenance of high blood pressure, although the cause of this is not fully understood (Schlaich *et al*., 2004; Parati and Esler, 2012). While the coordinated activities of the sympathetic and parasympathetic nervous systems are essential for maintaining systemic blood pressure homeostasis in the face of different physiological and environmental challenges, the increase of sympathetic activity can play an important initiating role in the pathogenesis of hypertension (Schlaich *et al*., 2004; Thomas, 2011).

1.2.1 The role of polymorphisms in the β1-adrenergic receptor gene in hypertension

The β1-adrenergic receptor (ADRB1) affects blood pressure by relaying sympathetic signals to the heart and kidneys (Fung *et al*., 2009). The G protein-coupled ADRB1 relays sympathetic signals via a cascade in which adenlyl cyclase produces the secondary messenger cAMP, which induces renin release from the kidneys as well as increased cardiac contraction (Mason *et al*., 1999; Beierwaltes, 2010; Vidal *et al*., 2012). These cardiorenal effects of ADRB1 lead to increased blood pressure. Sustained ADRB1 activity results in abnormally increased renin release and cardiac output.

Various studies have investigated the effect of A145G (rs1801252) and G1165C (rs1801253) SNPs on *ADRB1* activity.

- A145G: The A145G SNP results in a serine to glycine substitution at amino acid position 49, which has been found to correlate with alterations in blood pressure (Ranade *et al*., 2002). The 145G allele has been shown to be associated with decreased receptor activity *in vitro*, as well as decreased heart rate in Japanese and Chinese cohorts (Levin *et al*., 2002; Ranade *et al*., 2002; Rathz *et al*., 2002). This allele has also been associated with hypertension in a cohort of Tamil Indians (Ramu *et al*., 2009). However, no association was found in Swedish and Italian Caucasian and Chinese cohorts

(Bengtsson *et al*., 2001; Filigheddu *et al*., 2004; Peng *et al*., 2009). A meta-analysis on studies that investigated the role of the A145G SNP in hypertension concluded that the A allele was associated with a 24% increased risk of developing hypertension (Kitsios and Zintzaras, 2010).

- G1165C: The G1165C SNP results in a glycine to arginine substitution at amino acid 389. Mason *et al*. (1999) found that this SNP increased G protein coupling and agonist-stimulated adenylyl cyclase activation *in vitro*. The 1165C allele has been found to be associated with an increased risk of developing hypertension in Swedish and Chinese cohorts (Bengtsson *et al*., 2001; Peng *et al*., 2009). Although no association was found between the G1165C SNP and hypertension in a second study on a Chinese cohort, as well as studies on Japanese, Italian Caucasian and Tamil Indian populations, a meta-analysis of studies on East Asians and Caucasians found that the 1165C allele was marginally associated with a 16% risk reduction for hypertension in East Asians (Ranade *et al*., 2002; Filigheddu *et al*., 2004; Ramu *et al*., 2009; Kitsios and Zintzaras, 2010).

A factor that may affect the association patterns of A145G and G1165C with hypertension is the linkage reported between these SNPs (Forleo *et al*., 2004). A145G and G1165C SNPs have been found to be in linkage disequilibrium in cohorts of American Caucasians and African Americans, as well as Tamil Indians, meaning that the true effect of each SNP on the hypertensive phenotype may be obfuscated by that of the other SNP (Belfer *et al*., 2005; Ramu *et al*., 2009). Ramu *et al*. (2009) determined that the haplotype combination of 145G and 1165G had the highest frequency in the hypertensive subgroup, and that this combination correlated with a near twofold higher risk of developing hypertension, even though no individual association was found between the 1165G allele and hypertension. It therefore appears that both *ADRB1* SNPs may play a role in hypertension. It has been suggested that the contradictory results found in association studies may arise from population variation in environmental factors, as well as differences between cohorts with regards to patient age and body mass index and the degree to which related patients are included in studies (Ramu *et al*., 2009).

1.3 Renal system involvement in hypertension

Long term control of blood pressure is mediated through the kidneys (Blaustein *et al*., 2006). This is accomplished through the regulation of blood volume, which influences cardiac performance and consequently blood pressure. The kidneys maintain the electrolyte balance by regulating sodium (Na^+) and water excretion and reabsorption, relative to what is ingested (Guyton, 1991; Atherton, 2006). An increase in renal perfusion as a result of increased blood pressure, results in the inhibition of Na^+ transporters that leads to enhanced Na^+ and water excretion and blood volume reduction, to achieve normal blood pressure (Guyton, 1991; McDonough, 2010). The excretion of Na^+ in response to increased blood pressure, beyond the normal set point, is referred to as pressure natriuresis (Granger *et al*., 2002; Guyenet, 2006). Pressure natriuresis can be impaired by increased tubular reabsorption of Na^+, which can decrease the capability of the kidneys to excrete Na^+ during pressure natriuresis (Hall *et al*., 1996). Impaired natriuresis results in Na^+ retention and blood volume expansion, which leads to increased blood pressure, a relationship referred to as salt-sensitive blood pressure (Rodriguez-Iturbe and Vziri, 2007). Thus, renal control of blood pressure can be perturbed by impaired pressure natriuresis, which ultimately can result in salt-sensitivity of blood pressure.

The cytoskeletal protein adducin has been implicated in salt-sensitivity of blood pressure. Adducin is involved in the organization of the actin-spectrin cytoskeleton, which facilitates correct membrane anchoring of transmembrane ion transporters, as well as regulation of transporter activity (Tripodi *et al*., 1996). Torielli *et al*. (2008) conducted *in vitro* studies on renal cell lines transfected with different genetic variants of adducin to determine the effect on Na^+/K^+ pump function. They found that the G217T SNP (rs4961) in the gene for the α- subunit of adducin (*ADD1*), which results in an amino acid substitution of a glycine to a tryptophan at residue 460, was associated with reduced endocytosis and increased membrane expression and activity of the Na^+/K^+ pump (Torielli *et al*., 2008). The Na^+/K^+ pump plays a major role in Na^+ reabsorption, by being the driving mechanism of Na^+ reabsorption throughout the renal nephron. Enhanced activity of the Na^+/K^+ pump may promote

salt-sensitivity of blood pressure (Bianchi, 2005). Grant *et al*. (2002) determined that there was an association between the G217T SNP in *ADD1* and salt-sensitive blood pressure in a multi-ethnic cohort.

Various studies have assessed the G217T SNP in *ADD1* for its role in hypertension. An association between the G217T SNP and hypertension has been found in Caucasian, Japanese and Chinese cohorts (Cusi *et al*., 1997; Iwai *et al*., 1997; Tamaki *et al*., 1998; Province *et al*., 2000; Sugimoto *et al*., 2002; Ju *et al*., 2003). In one of the few studies on a black population in South Africa it was found that the G217T SNP was associated with hypertension, even though the overall frequency of the 217T allele was low in the cohort (Barlassina *et al*., 2000). Although the findings of certain meta-analyses have been contradictory, an association between G217T and hypertension was confirmed by meta-analyses on Chinese populations (Liu *et al*., 2010; Ramu *et al*., 2010; Liu *et al*., 2011; Niu and Qi, 2011; Li, 2012). In the latter meta-analyses it was suggested that the 217T allele was recessive (Liu *et al*., 2010; Li, 2012). The frequency of the 217T allele differs markedly between ethnic groups, with frequencies ranging from 52.6% to 60.4% in Asians, to 14.4% to 24.4% in Caucasians and approximately 6% in a South African black population (Barlassina *et al*., 2000; Niu *et al*., 2010). The general low prevalence of the 217T allele in some of the ethnic groups can result in cohorts that are under representative of 217T homozygotes. This possibility, together with the recessive mode of action of the 217T allele, can contribute to the conflicting results found in association studies.

1.4 Hormonal systems involved in blood pressure regulation

There are several hormonal systems that can act on the physiological components that determine blood pressure. For example, vasodilation hormones such as bradykinin, prostaglandins, acetylcholine and serotonin lower blood pressure by decreasing vascular resistance. Compared to this, blood pressure can also be increased through the vasoconstrictive action of hormones such as vasopressin, endothelin and histamine (Ambler and Brown, 1999). In addition, the renal excretion

of Na^+ can also be enhanced through the action of the natriuretic peptide hormone (Schmitt *et al*., 2003). Other hormonal systems that are central in the regulation of blood pressure include the renin-angiotensin-aldosterone system (RAAS) and the dopaminergic system (Zhu *et al*., 2005; Thomas, 2011). Dysfunction of any of these hormonal systems can result in the abnormal elevation of blood pressure (Ambler and Brown, 1999).

1.4.1 Renin-angiotensin-aldosterone system control of blood pressure

The RAAS is responsible for maintaining electrolyte balance and increasing blood pressure when it becomes too low (Wang and Staessen, 2000). The RAAS is initiated when renin is released from the kidneys' juxtaglomerular cells. Renin release is stimulated by sympathetic nerve activity, reduced sodium chloride delivery to the macula densa cells in the kidneys, and/or decreased renal perfusion pressure (Atlas, 2007). Renin catalyses the hydrolysis of angiotensinogen (AGT), which is constitutively produced by the liver, which in turn results in the release of angiotensin I (Corvol and Jeunemaitre, 1997). Hydrolysis of angiotensin I, by the angiotensin-converting enzyme, produces angiotensin II (Atlas, 2007). Angiotensin II mediates the generation of aldosterone from the adrenal cortex, by stimulating the expression of the *CYP11B2* gene that encodes for aldosterone synthase. Aldosterone synthase catalyses the production of aldosterone from 11-deoxycorticosterone (Brand *et al*., 1998; Holloway *et al*., 2009). Angiotensin II and aldosterone bind to angiotensin II type 1 and mineralocorticoid receptors, respectively, which stimulates Na^+ reabsorption, increased cardiac function, and vasoconstriction, as well as sympathetic activity, resulting in structural changes in cardiovascular tissue. These actions increase Na^+ retention that in turn increases blood pressure, in an attempt to rectify homeostatic deficiencies (Weir and Dzau, 1999, McFarlane and Sowers, 2003). However, abnormally increased angiotensin II and aldosterone activity can result in the inappropriate initiation of sympathetic activity and impairment of pressure natriuresis that can lead to a pathological increase in blood pressure (Sealey *et al*., 1988; Perondi *et al*., 1992; Weir and Dzau, 1999). Thus, up regulation

of blood pressure conferred by the RAAS is essential for homeostasis, but can result in a hypertensive elevation of blood pressure if RAAS activity is too high.

1.4.1.1 The role of polymorphisms in the angiotensinogen gene in hypertension

Increased levels of AGT have been found to enhance the output of RAAS and the degree to which blood pressure is elevated (Klett and Granger, 2001). It has also been found that hypertensive individuals tended to have higher levels of plasma AGT compared to normotensive individuals (Walker *et al*., 1979). SNPs in the *AGT* gene have subsequently been investigated for a relationship with increased plasma levels of AGT and hypertension. Examples of SNPs in *AGT* that have been investigated include G-217A (rs5049), C521T (rs4762) and T704C (rs699).

- G-217A: The G-217A SNP is in a transcription factor binding region and has been shown *in vitro* to increase *AGT* expression due to enhanced binding of transcription factors (Jain *et al*., 2002). This SNP has also been positively associated with hypertension in African-American and Taiwanese cohorts (Jain *et al*., 2002; Wu *et al*., 2004). Although no association was found between G-217A and hypertension in cohorts of American Caucasian and Chinese Han individuals, a meta-analysis of studies on Chinese, Taiwanese, African American and American Caucasian populations determined that the G-217A SNP was associated with hypertension (Jain *et al*., 2002; Liu *et al*., 2004; Pereira *et al*., 2008).

- C521T: A correlation between C521T, which results in a threonine to methionine substitution at amino acid position 174, and plasma AGT levels has been observed in a cohort of Mexican individuals (Balam-Ortiz *et al*., 2011). The T allele of the C521T SNP has been associated with hypertension in different populations, including Hutterite and Russian and Tatar populations (Hegele *et al*., 1994; Mustafina *et al*., 2002). A multi-locus study which focused on polymorphisms in genes for *AGT*, the angiotensin II type 1

receptor, angiotensin converting enzyme and locus that was named FJ, indicated that an association between the C521T SNP and hypertension could not be distinguished independent of the other polymorphisms (Williams *et al*., 2000). However, several studies on European Caucasian and Asian population groups did not find any association between C521T and hypertension (Caulfield *et al*., 1994; Fernandez-Llama *et al*., 1998; Sato *et al*., 2000; Liu *et al*., 2004). A meta-analysis did, however, find an association between the C521T SNP and hypertension in Asians and mixed race groups, although no association was evident in European-derived groups (Pereira *et al*., 2008).

- T704C: The T704C SNP, resulting in a substitution of a methionine to a threonine at amino acid position 235, has been associated with elevated levels of AGT in hypertensive individuals (Jeunemaitre *et al*., 1992). The association of T704C with hypertension has, however, been inconsistent between population studies. For example, both positive and negative associations have been reported by studies on Japanese individuals and European Caucasians (Jeunemaitre *et al*., 1992; Caulfield *et al*., 1994; Schmidt *et al*., 1995; Jeunemaitre *et al*., 1997; Fernandez-Llama *et al*., 1998; Kato *et al*., 1999). Meta-analyses on Asian and Caucasian groups determined that T704C is associated with hypertension in these populations (Kato *et al*., 1999; Staessen *et al*., 1999; Sethi *et al*., 2003). Compared to this, meta-analyses of studies on populations of Jamaicans, African Americans, African Caribbeans, Nigerians and black individuals from Britain did not find any association between T704C and hypertension (Staessen *et al*., 1999; Sethi *et al*., 2003).

The G-217A, C521T and T704C SNPs have been found to be in linkage disequilibrium with other polymorphisms in *AGT* (Sethi *et al*., 2003; Wu *et al*., 2004). Some studies have suggested that other *AGT* polymorphisms, for example the A-20C and G-6A promoter SNPs that have been found to alter *AGT* transcription, may affect the association of G-217A, C521T and T704C with hypertension (Inoue *et al*.,

1997; Zhao *et al*., 1999). Thus, allelic variation at other polymorphic sites in *AGT* may therefore possibly confound association studies and contribute to the seemingly conflicting results.

1.4.1.2 Role of CYP11B2 in hypertension

It has been found that abnormal aldosterone activity and high blood pressure can arise from aberrant *CYP11B2* expression. Elevated plasma levels of aldosterone have been correlated to the C-344T SNP (rs1799998) in the *CYP11B2* promoter (Pojoga *et al*., 1998; Barbato *et al.,* 2004). The C allele of this SNP has been found to enhance binding of the steroidogenic factor-1 transcription factor to the upstream -351/-343 element, compared to the T allele (White and Slutsker, 1995). In a study that assessed regulatory elements of the *CYP11B2* gene, deletion of the -351/-343 element did not appear to impede transcription, and the element may as such not be pivotal in gene transcription (Clyne *et al*., 1997). Brand *et al*. (1998) suggested that the varying affinity of steroidogenic factor-1 for the different alleles of the C-344T SNP could impact steroidogenic factor-1 availability and consequently transcription rate of *CYP11B2*. In such an event, the -344C allele may result in a decrease in available steroidogenic factor-1 compared to the -344T allele, which could result in a higher transcription rate of the -344T allele. An increase in *CYP11B2* transcription would result in increased aldosterone synthase that could consequently enhance aldosterone synthesis (Brand *et al.,* 1998). Confoundedly, both the -344T and -344C alleles have been associated with increased aldosterone levels (Pojoga *et al*., 1998; Barbato *et al.,* 2004). In a South African cohort of black hypertensive individuals it was found that the -344T allele was associated with increased blood pressure (Tiago *et al*., 2003). Contrary to this, no association was found between C-344T and hypertension in German Caucasians and Japanese cohorts (Brand *et al*., 1999; Kato *et al*., 2000; Tsujita *et al*., 2001). However, a meta-analysis of studies on Chinese cohorts determined that the -344C allele correlated with a predisposition to developing hypertension (Cheng and Xu, 2009). In contrast, another meta-analysis found that homozygosity for the -344T allele was associated with a 17% increased risk of developing hypertension (Sookoian *et al*., 2007). Several factors have been

proposed to explain the discrepant associations of the C-344T SNP with levels of aldosterone, including epistatic interactions with other polymorphisms that may result in a similar phenotype, as well as ethnicity, gender and age effects on the phenotypic expression of the C-344T (Cheng and Xu, 2009).

1.4.2 Role of CYP3A5 in hypertension

Another member of the cytochrome P450 family, CYP3A5, has also been implicated in hypertension. CYP3A5 is involved in the metabolism of various drugs and steroids (Thompson *et al*., 2004; Eap *et al*., 2007). One such a steroid, corticosterone, is converted by CYP3A5 to 6β-hydroxycorticosterone which has been shown to stimulate Na^+ reabsorption in kidney cells (Duncan *et al*., 1988). An intronic SNP, A6986G (rs776746), in *CYP3A5* has been found to alter CYP3A5 activity and subsequently Na^+ reabsorption (Kuehl *et al*., 2001; Bochud *et al*., 2006). The A6986G SNP results in a transcript splice variant with a premature stop codon, which encodes for a truncated non-functional protein (Kuehl *et al*., 2001). While some studies have suggested that the A allele may influence blood pressure, other studies have found that the G allele may influence blood pressure (Givens *et al*., 2003; Fromm *et al*., 2005; Ho *et al*., 2005; Kreutz *et al*., 2005). In contrast to this, other studies have found no relation between either of these alleles and blood pressure (Langaee *et al*., 2004; Lieb *et al*., 2006; Langaee *et al*., 2007). With regards to hypertension, the 6986A allele was found to be associated with hypertension in cohorts of African-Americans and elder Finnish Caucasians (Ho *et al*., 2005; Kivisto *et al*., 2005). However, a meta-analysis did not find an association between A6986G and hypertension (Xi *et al*., 2011). It has been suggested that the contradictory results regarding the influence of the A6986G SNP on hypertension may be attributed to inter-ethnic differences in allele frequency and genetic and environmental factors that can influence the phenotypic effects of the different *CYP3A5* variants (Zhang *et al*., 2010).

1.4.3 Role of the dopaminergic system in blood pressure regulation

The dopaminergic system primarily acts as a negative modulator of blood pressure via dopamine (Zhu *et al*., 2005). Dopamine is generated from dihydroxyphenylalanine in dopaminergic and noradrenergic nerves, as well as extraneural tissues, such as the kidneys and gastrointestinal tract (Zeng *et al*., 2007; Zhang *et al*., 2011). Dopamine acts through different receptor subtypes, the D1-like receptor class, which includes the stimulatory G protein-coupled receptors D1 and D5, and the D2-like class, which includes the inhibitory G protein-coupled receptors D2, D3 and D4 (Zeng *et al*., 2007). The D1- and D2-like receptors are expressed in various renal and cardiovascular tissues, while D2-like receptors are additionally expressed in neural regions, such as the brain and nerve terminals (Zhu *et al*., 2005; Zeng *et al*., 2007). The major physiological effect of dopamine includes natriuresis, vasodilation and inhibition of sympathetic activity. However, D1 and D2 receptor stimulation also results in the release of renin, and reduction of renal blood flow and Na^+ excretion, respectively, which causes blood pressure to rise. Despite the latter, dopamine receptor stimulation primarily results in the lowering of blood pressure (Kuchel and Kuchel, 1991).

Defective dopamine receptor functioning has been associated with hypertension. The ability of D1-like dopamine receptors to mobilize secondary messengers and inhibit renal proximal Na^+ reabsorption, in response to agonist stimulation, has been found to be deficient in individuals with hypertension (O'Connell *et al*., 1997; Sanada *et al*., 1999). Sanada *et al*. (1999) determined that the diminished dopaminergic response that may be concurrent with hypertension could be attributed to a deficit in the coupling of D1 receptors to the G protein/effector enzyme complex. G protein-coupled receptor kinase 4 (GRK4) is one of the serine/threonine protein kinases responsible for mediating D1 receptor phosphorylation and uncoupling in response to repeated agonist exposure (Premont *et al*., 1999; Jose *et al*., 2010). It has been suggested that defective D1 receptor uncoupling may be the result of ligand-independent receptor phosphorylation (Sanada *et al*., 1999). Functional defects in D2-like receptors have also been found in individuals with hypertension, and have

been associated with increased sympathetic nervous system mediated vasoconstriction in mouse models (Li *et al*., 2001; Zeng *et al*., 2005). Thus, abnormalities in the receptors of the dopaminergic system may contribute to the development of hypertension through both renal and neural pathogenic mechanisms (Jose *et al*., 2003).

1.4.3.1 Role of the G protein-coupled receptor kinase 4 in hypertension

Defective D1 receptor uncoupling in hypertension has been attributed to abnormal GRK4 function. Studies conducted by Felder *et al*. (2002) on Chinese hamster ovary cells and renal proximal tubule cells from hypertensive Caucasian patients showed that the G448T (rs2960306), C679T (rs1024323) and C1711T (rs1801058) SNPs in *GRK4* were associated with enhanced GRK4 activity and decreased D1 receptor function. Several studies have investigated the association of these SNPs with hypertension:

- G448T: The G448T SNP, resulting in an arginine to leucine substitution at amino acid position 65, has been associated with hypertension, but mostly in conjunction with other polymorphisms. For example, G448T together with the angiotensin-converting enzyme insertion/deletion variant has been found to be 70.5% predictive of hypertension in a Ghanaian cohort (Williams *et al*., 2004). Haplotypes of different combinations of G448T, C679T and C1711T SNP alleles have also been associated with hypertension in different population groups. In a Japanese cohort the G448T, C679T and C1711T SNP combination was found to be predictive of salt-sensitive hypertension 94.4% of the time, and in an Australian Caucasian cohort the haplotype of 448G, 679T and 1711T alleles were associated with hypertension (Speirs *et al*., 2004; Sanada *et al*., 2006). However, most studies have not found an association between G448T, on its own, and hypertension (Speirs *et al*., 2004; Zeng *et al*., 2008; Martinez Cantarin *et al*., 2010).

- C679T: As for an association between C679T (alanine to valine amino acid substitution at position 142) and hypertension, the findings from studies have been contradictory. Although no association was found between C679T and hypertension in various population groups, a study on a Japanese population did establish an association with low-renin hypertension (Speirs *et al*., 2004; Sanada *et al.,* 2006; Zeng *et al*., 2008; Martinez Cantarin *et al*., 2010). C679T has also been associated with hypertension in combination with the *GRK4* SNPs, G448T and C1711T, in the studies of Speirs *et al*. (2004) and Sanada *et al*. (2006) on Australian Caucasian and Japanese cohorts, respectively.

- C1711T: The findings of studies investigating the association of the C1711T SNP (alanine to valine amino acid substitution at position 486) with hypertension have also been disparate. Studies on northern Han Chinese and African-American groups found that the C allele was associated with hypertension, while a study on another Chinese cohort found that the T allele correlated with hypertension instead (Gu *et al*., 2006; Wang *et al*., 2006; Martinez Cantarin *et al*., 2010). A meta-analysis on Caucasian and black population groups, however, determined that the 1711T allele was implicated in hypertension (Zeng *et al*., 2008).

The variability in the ethnic prevalence of G448T, C679T and C1711T could influence the findings of association studies (Lohmueller *et al*., 2005). In ethnic groups with a low SNP prevalence, cohorts may be selected that do not accurately represent the SNP carriers of a population, and the true effect of a SNP on hypertension may thus be obscured. This may contribute to the discordance that exists between the results of association studies.

1.5 Confounding factors of association studies

Determining whether a particular SNP is a definite risk factor for hypertension has been confounded by the heterogeneity in the results of association studies. Various

factors have been proposed that can confound the findings of association studies, including epistatic interactions between polymorphisms, and age, body mass index and ethnicity of the study participants (Ioannidis *et al*., 2004, Ramu *et al*., 2009). In this regard, perhaps the foremost problem faced by association studies is that the focus is often limited to the isolated hypertensive effects of individual genetic factors, not taking into account the possible interplay with other risk factors. Thus, controlling for known environmental risk factors may not result in an unbiased association, due to the presence of other genetic risk factors that also can influence the association of the target SNP. The observed effect that a particular polymorphism can have on a disease phenotype has been shown to be subject to variation in allele frequency and epistatic interactions with other polymorphic loci (Ioannidis *et al*., 2004). Furthermore, exclusion of environmental risk factors may cause the risk effect of a candidate SNP to be diminished and consequently missed in a study. The latter was demonstrated in the study by Tiago *et al*. (2002), which found that instead of having an independent hypertensive effect, the A-20C SNP of *AGT* moderated the effect of body mass index on blood pressure. Presence of the C allele diminished the effect of body mass index on blood pressure, whereas the A allele in homozygous form resulted in a strong relationship between body mass index and blood pressure. Thus, before the individual contribution of a SNP to hypertension risk can be determined, it is necessary to establish the interplay between the target SNP and other risk factors that may be essential for the target SNP to promote hypertension development. By examining the combined contributions of hypertension risk factors, the pathological mechanisms underlying hypertension may be eventually pieced together.

1.6 Aim

The primary aim of this study was to establish a method for detecting several SNPs, known to be associated with hypertension in various populations, including black South Africans, in the *ADRB1* (A145G and G1165C), *ADD1* (G217T), *AGT* (G-217A, C521T and T704C), *CYP11B2* (C-344T), *CYP3A5* (A6986G) and *GRK4* (G448T, C679T and C1711T) genes. The secondary aim was to use this method to assess

whether SNPs that have been associated with hypertension in different population groups were associated with hypertension in a black population from Mangaung, Free State.

Chapter Two

Methodology

2.1 Population

The current study was conducted in collaboration with the Department of Nutrition and Dietetics, University of the Free State, as an amendment to the AHA-FS study. The collaborators were responsible for selecting the study cohort from the urban baseline study population of the AHA-FS study (Van Zyl *et al*., 2012). Participants of the urban baseline study population of the AHA-FS study were recruited from black Sotho speaking households that were selected by means of stratified proportional cluster sampling from urban areas of Mangaung. The Department of Nutrition and Dietetics, University of the Free State was responsible for selecting the participants, informing them of the study and gathering information on the socio-demography, individual health and diet of the participants (Lategan, 2011).

2.1.1 Source of cohort genetic material

The collaborators were responsible for the collection of the clinical information and blood samples that were used in this study. The inclusion criteria used included participants 25 to 65 years of age, who provided written informed consent, with complete data sets for gender, age, body mass index and blood pressure, as well as blood samples for genetic testing. Blood pressure was measured using the guidelines in the Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation and Treatment of High Blood Pressure (NIH, 2004). Before blood pressure was measured, participants were instructed to avoid exercise, caffeine intake and cigarette smoking for a minimum of 30 minutes. After being

seated for at least 5 minutes, feet on the floor and arm supported at heart level, a registered medical practitioner proceeded to take two blood pressure measurements. Height and body weight measurements were obtained using the WHO guidelines (WHO, 2008). The body mass index of the participants was determined by dividing their weight (kg) with their height squared (m^2) (WHO, 2011). Body mass index was classified according to the WHO (2011) guidelines. Body mass index < 18.5 kg/m^2 was considered as underweight, between 18.5 and 24.9 kg/m^2 as normal, and between 25 and 29.9 kg/m^2 as overweight, and ≥ 30 kg/m^2 as obese. Participants with blood pressure exceeding 140/90 mm Hg and/or on hypertension treatment were classified as hypertensive. Whole blood was collected from study participants and blotted onto FTA paper. Ethics approval for this study was obtained from the Ethics Committee of the University of the Free State, Faculty of Health Sciences (ECUFS 99/2013). An amendment for the current study was obtained under the same ethics approval.

2.1.2 Cohort characteristics

The cohort for the current study was selected by the Department of Nutrition and Dietetics, University of the Free State and consisted of 339 black Sotho speaking individuals. Of the participants, 124 were normotensive and 215 were hypertensive. The normotensive individuals served as the controls for the current study. The majority of individuals that qualified and were willing to participate in this study were middle aged. The mean age for the normotensive group was 38.75 years, and for the hypertensive group 47.72 years. Although it is preferable to select cohorts of a younger average age in order to limit the effect that age can have on blood pressure, the cohort would not have been of adequate size if exclusions pertaining to age were made. In the normotensive group, 16.94% of the participants were obese (≥ 30 kg/m^2), whereas 40.93% of the hypertensive group was obese. The percentage of female and male participants in the normotensive group was 74.19% and 25.81%, respectively, while in the hypertensive group 79.07% of the participants were female and 20.93% male.

2.1.3 Volunteer samples for optimization experiments

Blood samples for the optimization of the DNA extraction method and SNP assays were obtained from staff and students in the Department of Haematology and Cell Biology, University of the Free State. Whole blood samples were collected by means of venepuncture of the finger and blood was blotted onto FTA paper (Whatman) and stored at room temperature until used.

2.2 Methodology

2.2.1 DNA isolation

The DNA was isolated from the blood that was blotted onto FTA paper. A 2 mm punch was used to obtain discs from FTA paper. Between samples the punch was thoroughly rinsed with 70% ethanol to remove debris from the punch tip, and clean FTA paper was punched thrice to remove residual ethanol from the tip. DNA was isolated from six FTA discs, using an optimized version of the methanol DNA extraction method described by Lebea and Pretorius (2008). Methanol solubilizes haemoglobin and was used to purify the FTA discs of blood residue, which can have an inhibitory effect on PCR. The FTA discs were incubated in 50 µl of 100% methanol for 10 minutes at room temperature. The methanol was removed from the FTA discs, and the discs dried at 28˚C for 15 minutes in an incubator. The FTA discs were then incubated in 60 µl of 0.1 x TE (10 mM Tris and 0.1 mM EDTA, pH 8) for 15 minutes at 95˚C to remove the DNA from the FTA paper. The 0.1 x TE solution containing the DNA was then removed from the FTA discs, and a further 60 µl of 0.1 x TE was added to the discs, which were again incubated for 15 minutes at 95˚C. The extracted DNA was centrifuged for 5 minutes at 13,400 rpm (revolutions per minute) so that any undissolved debris would form into a pellet from which the DNA solution could be removed. The concentration of the extracted DNA was determined during the optimization of the methanol extraction method using the

Qubit Fluorometer (Applied Biosystems), to assess the effect of extraction method on DNA yield. Extracted DNA was stored at 4˚C until used.

2.2.2 Real-time PCR

Genotypic analysis of the samples was done using Real-time PCR. Genomic DNA sequences containing the target SNPs were selectively amplified using unlabelled forward and reverse primers. The normotensive and hypertensive alleles for each SNP were defined according to what was considered to be normotensive and hypertensive in the literature. The alleles of a specific locus were detected simultaneously during Real-time PCR, using normotensive allele- and hypertensive allele-specific TaqMan probes that were labelled with different fluorescent dyes. The normotensive allele-specific TaqMan probes were labelled with VIC fluorescent dye, whereas the hypertensive allele-specific TaqMan probes were labelled with FAM fluorescent dye. However, one exception was the TaqMan probes that were used in the Real-time PCR assay for the C-344T SNP of *CYP11B2*, where the normotensive allele-specific probe was labelled with the FAM dye and the hypertensive allele-specific probe with the VIC dye. The primers were synthesized at a 10 nmol scale, and the TaqMan probes at 100 µM scale. Sequences of the primers and TaqMan probes that were used in the Real-time PCR assays for the SNPs in *ADRB1* (A145G and G1165C), *ADD1* (G217T), *AGT* (C521T) and *CYP3A5* (A6986G) were obtained from the publications of Yuan *et al.* (2006), Balkestein *et al.* (2001), Van der Net *et al*. (2008) and Eap *et al.* (2004) respectively (Table 2.1). For the Real-time PCR assays for *AGT* (T704C) and *CYP11B2* (C-344T), the primers and TaqMan probes were designed as part of this study using Primer3Plus (Table 2.1) (Untergasser and Nijveen, 2007). The primers and TaqMan probes were synthesized by Applied Biosystems (Warrington, England). The lyophilized primers were reconstituted with 0.1 x TE to a 10 µM stock. The TaqMan probes were diluted with 0.1 x TE to prepare 5 µM stock solutions of each probe.

Table 2.1: Primers and probes that were used in the PCR assays for each of the respective SNPs

Gene SNPs	Primer/ probe	Sequence (5' – 3')	Reference
***ADRB1* A145G**	Forward primer	GTCGCCGCCCGCCTCGTT	Yuan *et al*. (2006)
	Reverse primer	CCATGCCCGCTGTCCACTGCT	
	Normotensive probe	CCAGCGAAAGCCCCGAGCC (VIC)	
	Hypertensive probe	CCAGCGAAGGCCCCGAGCC (FAM)	
***ADRB1* G1165C**	Forward primer	GGCCTTCAACCCCATCATCTA	Yuan *et al*. (2006)
	Reverse primer	CCGGTCTCCGTGGGTCGCGT	
	Normotensive probe	AGGCCTTCCAGGGACTGCTCTGCT (VIC)	
	Hypertensive probe	AGGCCTTCCAGCGACTGCTCTGCT (FAM)	

***ADD1* G217T**	Forward primer	GGAGAAGACAAGATGGCTGAACTC	Balkestein *et al*. (2001)
	Reverse primer	CGTCCACACCTTAGTCTTCGACTT	
	Normotensive probe	TTCCGAGGAAGGGCAGAATGGAA (VIC)	
	Hypertensive probe	TTCCGAGGAATGGCAGAATGGAA (FAM)	
***AGT* G-217A**	Forward primer	TCCTGCAAACTTCGGTAAATGTGT	du Toit and Viljoen
	Reverse primer	GAAGTCTTAGTGATCGATGCAGAGT	
	Normotensive probe	CTGCACCGGCTCAC (VIC)	
	Hypertensive probe	CTGCACCAGCTCAC (FAM)	

***AGT* C521T**	Forward primer	CAGGGCAGGGCTGATAGC	Van der Net *et al*. (2008)
	Reverse primer	GCACAAACGGCTGCTTCAG	
	Normotensive probe	CACGGTGGTGGGCG (VIC)	
	Hypertensive probe	CATGGTGGTGGGCG (FAM)	
***AGT* T704C**	Forward primer	AGGCTGTGACAGGATGGAAGA	du Toit and Viljoen
	Reverse primer	CCAGGGTGCTGTCCACACT	
	Normotensive probe	TGCTCCCTGATGGGA (VIC)	
	Hypertensive probe	TGCTCCCTGACGGGA (FAM)	

***CYP11B2* C-344T**	Forward primer	ATCAATTTTGCAATGAACTAAATCTGTGGTATAAAA	du Toit and Viljoen
	Reverse primer	AGGGCTGAGAGGAGTAAAATGGAT	
	Normotensive probe	TCCAAGGCTCCCTCTC (FAM)	
	Hypertensive probe	TCCAAGGCCCCCTCTC (VIC)	
***CYP3A5* A6986G**	Forward primer	CCACCCAGCTTAACGAATGC	Eap *et al*. (2004)
	Reverse primer	GAAGGGTAATGTGGTCCAAACAG	
	Normotensive probe	TGTCTTTCAATATCTCT (VIC)	
	Hypertensive probe	TGTCTTTCAGTATCTCT (FAM)	
***GRK4* G448T**	Forward primer	CACCCAGAAAAGGATTATAGCAGTCT	du Toit and Viljoen
	Reverse primer	GAGTGGGTTTGGTATCACAGAACT	
	Normotensive probe	CGATAGGAAGACGTCTCTT (VIC)	

	Hypertensive probe	CGATAGGAAGACTTCTCTT (FAM)	
***GRK4* C679T**	Forward primer	GATTGGGACTGAAGGAGGAGAAC	du Toit and Viljoen
	Reverse primer	CTCAGCATGAACCACTTACCTAGT	
	Normotensive probe	TCCTCAAAGGCTTTTT (VIC)	
	Hypertensive probe	TCCTCAAAGACTTTTT (FAM)	
***GRK4* C1711T**	Forward primer	TGTAAGGACGTCCTGGATATCGA	du Toit and Viljoen
	Reverse primer	CTGCGGTGTCCAGGTAGATC	
	Normotensive probe	TTTCACCGCCGAGAAC (VIC)	
	Hypertensive probe	CTTTCACCACCGAGAAC (FAM)	

ADRB1 = β1-adrenergic receptor gene; *ADD1* = α-subunit of the adducin gene; *AGT* = angiotensinogen gene; *CYP11B2* = aldosterone synthase gene; *CYP3A5* = Cytochrome P-450 3A5 gene; GRK4 = G protein-coupled receptor kinase 4 gene; FAM = 6-carboxyfluoresceine; VIC = a proprietary fluorescent dye produced by Applied Biosystems

All the Real-time PCR assays were performed in reaction volumes of 22 µl, which consisted of 7.3 µl of 2x TaqMan Fast Advanced Master Mix (Applied Biosystems), forward and reverse primer at the optimized concentrations (Table 3.1), VIC- and FAM-labelled TaqMan probes at the optimized concentrations (Table 3.1), and 2 µl of the DNA template, sterile water was further added to obtain a final volume of 22 µl. The PCR assays involved a two-step cycle, of which the cycling conditions were assay specific. The Real-time PCR cycling conditions for the SNPs in *ADRB1* (A145G and G1165C), *ADD1* (G217T), *AGT* (C521T) and *CYP3A5* (A6986G) included 1 cycle at 95˚C for 10 minutes, followed by 50 cycles at 95˚C for 15 seconds and 58˚C for 1 minute. For the *AGT* (G-217A, T704C), *CYP11B2* (C-344T) and *GRK4* (G448T, C679T and C1711T) Real-time PCR SNP assays, the cycling conditions involved an initial cycle at 95˚C for 10 minutes, 50 cycles at 95˚C for 15 seconds and 60˚C for 1 minute. A no template control was included with each SNP assay that was performed. Real-time PCR was performed on the Mx3005P (Stratagene) thermal cycler. Optimization of the Real-time PCR assays for the respective SNPs were performed by evaluating different primer and TaqMan probe concentrations in the reactions and determining which concentration ratio resulted in the best SNP detection (chapter 3).

2.2.3 Conventional PCR

Sequencing was performed on samples for which Real-time PCR genotyping failed. Prior to sequencing, conventional PCR was performed on the samples to be sequenced. PCR was carried out with 2.5 µl of 10 x PCR Gold buffer (Applied Biosystems), 2.5 µl of 25 nM magnesium chloride (Applied Biosystems), 1.0 µl of 10 mM deoxynucleotide triphosphate mix (Thermo Scientific), 0.16 µl of 5 U/µl AmpliTaq Gold DNA polymerase (Applied Biosystems), forward and reverse primer at the optimized concentrations (Table 5.1), and 2 µl of DNA template, sterile water was further added to obtain a final volume of 22 µl. The cycling conditions that were applied were assay specific. For the *ADRB1* (A145G and G1165C), *ADD1* (G217T), *AGT* (C521T) and *CYP3A5* (A6986G) SNP assays, the cycling conditions were as

follows: 95˚C for 10 minutes, followed by 50 cycles at 95˚C for 15 seconds and 58˚C for 1 minute. For the *AGT* (G-217A, T704C), *CYP11B2* (C-344T) and *GRK4* (G448T, C679T and C1711T) SNP assays, cycling conditions entailed a single cycle of 95˚C for 10 minutes, followed by 50 cycles at 95˚C for 15 seconds and 60˚C for 1 minute. To confirm amplification of target DNA regions, the resulting PCR product was resolved on a 2% agarose gel submerged in 1 x TAE (40 mM Tris-acetate and 1 mM EDTA, pH 8) at 200 volts for 40 minutes. Following gel electrophoresis, gels were stained in a solution of ethidium bromide (0.5 µg/ml) for 10 minutes at a rotational speed of 50 rpm. Visualization of the separated DNA fragments in the stained gel was achieved under ultraviolet (UV) light with the Kodak Gel Logic 200 Imaging System and the Kodak ID 3.6 program.

2.2.4 Sequencing analysis

The PCR product of samples that yielded DNA fragments detectable by gel electrophoresis was further prepared for sequencing. Purification of the PCR product was performed with the USB ExoSAP-IT PCR Product Cleanup (Affymetix) kit. Following the protocol of the USB ExoSAP-IT PCR Product Cleanup (Affymetix) kit, 5 µl of PCR product was added to 2 µl of the ExoSAP-IT reagent and incubated at 37˚C for 15 minutes, followed by 80˚C for 15 minutes. The ABI Prism Big Dye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems) was used for the sequencing reaction on the purified PCR product. Separate sequencing reactions were performed using 2 µl of 0.8 µM forward and reverse primers (Table 2.1), respectively, with 1 µl buffer, 2 µl terminator mix, and 5 µl of the PCR product as template. The optimized cycling conditions for the sequencing reaction included a single cycle of 96˚C for 1 minute, followed by 25 cycles of 96˚C for 10 seconds, 58˚C for 5 seconds and 60˚C for 4 minutes, with a final temperature hold at 25˚C. The resulting cycle sequence product was subsequently purified with an ethanol/sodium acetate precipitation procedure. In this procedure, 3 µl of a 3 M sodium acetate solution, 14.5 µl sterile water, and 63 µl of a 95% ethanol solution was added to 10 µl of the sequencing product. The mixture was vortexed for 30 seconds and centrifuged for 10 seconds, before incubation at room temperature in the dark for 30

minutes. Following incubation, the mixture was centrifuged for 30 minutes at 13,400 rpm. The supernatant was carefully aspirated, so that the DNA pellet was left undisturbed afterwards. The DNA pellet was then washed by adding 250 µl of 70% ethanol, followed by a 2 minute vortex and 10 minute centrifugation at 13,400 rpm. Thereafter, the supernatant was carefully aspirated and the DNA pellet air dried at 90˚C for approximately 1 minute. Finally 25 µl Hi-Di formamide (Applied Biosystems) was added to the pellet and vortexed for 10 seconds and centrifuged at 13,400 rpm for 30 seconds. The sequencing product was denatured at 95˚C for 2 minutes, after which the sample was incubated on ice for 5 minutes. Before storage in the dark at 4˚C, the sample was shortly vortexed for 10 seconds and centrifuged for 30 seconds. Sequencing of the prepared samples was performed on the ABI prism 3130 Genetic Analyser. Sequence results were analysed using the Sequence Analysis Program 3.1 and Chromas 2.31. Sequences were aligned to the reference sequence using LALIGN (Huang and Muller, 1991). The sequence was confirmed by comparing the sequence of the forward and reverse primer reactions of each sample, and by performing duplicate sequencing reactions for each sample.

2.2.5 Data analysis and SNP association with hypertension

Single nucleotide polymorphism data was statistically analysed to determine if the selected SNP was associated with hypertension in the study population. Statistical analysis was performed with SNP and Variation Suite v8.x (Golden Helix, Inc., Bozeman, MT, www.goldenhelix.com), VassarStats (http://vassarstats.net/index.html), Statpages (http://statpages.org/index.html) and Microsoft Excel with Daniel's XL ToolBox add-in (http://xltoolbox.sourceforge.net/index.html). The frequencies of the alleles and genotypes for each SNP were calculated to determine SNP prevalence. Hardy-Weinberg equilibrium was also determined and deviations from Hardy-Weinberg equilibrium were assessed using the Chi-square test. Statistical comparisons were performed using the analysis of variance test, and where the data did not comply with the assumptions of the analysis of variance test the Mann-Whitney U test was performed. P-values were one-tailed and considered statistically significant at P <

0.05. The risk associated with hypertension for each of the respective SNPs was calculated using odds ratio (OR) analysis. All individuals in the cohort that met the inclusion criteria were considered in the statistical analyses, including individuals that were being treated for hypertension.

Chapter Three

Results and discussion

Optimization of experimental Methods

The procedure that was employed to genotype the samples in this study was optimized at the level of DNA extraction and Real-time PCR. The DNA extraction method was modified to maximize DNA yield, and the Real-time PCR assays were optimized in terms of primer and probe concentration to achieve optimal SNP detection.

3.1 Optimization of the methanol DNA extraction method

The methanol DNA extraction method of Lebea and Pretorius (2008) was modified to maximize the DNA yield for extraction from whole blood spotted onto FTA paper. The original DNA extraction procedure involved purification of a 1.2 mm disc in 200 µl methanol for 30 minutes, after which the disc was air-dried for 30 minutes and finally boiled in 50 µl distilled water to extract the DNA. However, the latter approach resulted in a low DNA yield (< 0.1 ng/µl). It was decided to optimize the method in terms of FTA disc number and size, incubation time and the amount of methanol used. It was also decided to maximize DNA yield by using two-step 0.1 x TE incubation, after which the DNA containing 0.1 x TE was pooled. The modified method resulted in higher DNA yield.

The modified method of extracting DNA from FTA paper used in the current study resulted in a yield of approximately 0.720 ng/µl. Compared to this Miles and Saul

used a more complicated methanol DNA extraction method with and without proteinase K to extract DNA from FTA paper, which yielded DNA of 0.004 ng/µl and 0.018 ng/µl, respectively (Miles and Saul, 2014). The extraction time for the method of Miles and Saul was 145 minutes (Miles and Saul, n.d.). In the modified method used in the current study the estimated extraction time was less than 60 minutes.

3.2 Optimization of the Real-time PCR assays of candidate SNPs

In this study Real-time PCR assays were optimized for the detection of alleles for *AGT* (G-217A and T704C), *CYP11B2* (C-344T) and *GRK4* (G448T, C679T and C1711T). Real-time PCR assays for genotyping alleles in *ADRB1* (A145G and G1165C), *ADD1* (G217T), *AGT* (C521T) and *CYP3A5* (A6986G) were optimized in a previous B.Med.Sc.Hons. project by T. Smith (data not shown).

The preliminary Real-time PCR assays were performed using 0.20 µM of the forward and reverse primer, respectively, and 0.10 µM of the normotensive and hypertensive probes, respectively. The Real-time PCR cycling conditions included an initial step of 95˚C for 10 minutes, followed by 50 cycles of 95˚C for 15 seconds and 60˚C for 1 minute. The preliminary assay conditions resulted in differences in the detection levels of the normotensive and hypertensive alleles in the Real-time PCR assays (Figure 3.1A and 3.2A). An adjustment of the probe concentrations for *AGT* (G-217A and T704C), *GRK4* (G448T, C679T and C1711T) and *CYP11B2* (C-344T) produced comparative allele detection levels for these assays (Table 3.1). However, for *AGT* (T704C) and *CYP11B2* (C-344T) a further adjustment of primer concentration was required to improve amplification and allele detection (Table 3.1).

Table 3.1: Optimized primer and probe amounts and concentrations in the final reaction mixture for each of the respective SNP Real-time PCR assays

Gene	SNP	Primer		Probe	
		Forward	Reverse	Normotensive	Hypertensive
ADRB1	A145G	0.44 µl (0.20 µM)	0.44 µl (0.20 µM)	1.54 µl (0.35 µM)	1.10 µl (0.25 µM)
	G1165C	0.44 µl (0.20 µM)	0.44 µl (0.20 µM)	1.10 µl (0.25 µM)	1.98 µl (0.45 µM)
ADD1	G217T	0.44 µl (0.20 µM)	0.44 µl (0.20 µM)	0.22 µl (0.05 µM)	0.22 µl (0.05 µM)
AGT	G-217A	0.44 µl (0.20 µM)	0.44 µl (0.20 µM)	0.66 µl (0.15 µM)	0.44 µl (0.10 µM)
	C521T	0.44 µl (0.20 µM)	0.44 µl (0.20 µM)	1.54 µl (0.35 µM)	1.10 µl (0.25 µM)
	T704C	1.76 µl (0.80 µM)	1.76 µl (0.80 µM)	1.54 µl (0.35 µM)	1.32 µl (0.30 µM)
CYP11B2	C-344T	0.88 µl (0.40 µM)	0.88 µl (0.40 µM)	0.88 µl (0.20 µM)	0.88 µl (0.20 µM)
CYP3A5	A6986G	0.44 µl (0.20 µM)	0.44 µl (0.20 µM)	1.54 µl (0.35 µM)	1.10 µl (0.25 µM)
GRK4	G448T	0.44 µl (0.20 µM)	0.44 µl (0.20 µM)	0.88 µl (0.20 µM)	0.44 µl (0.10 µM)
	C679T	0.44 µl (0.20 µM)	0.44 µl (0.20 µM)	0.66 µl (0.15 µM)	0.66 µl (0.15 µM)
	C1711T	0.44 µl (0.20 µM)	0.44 µl (0.20 µM)	0.66 µl (0.15 µM)	0.44 µl (0.10 µM)

SNP = single nucleotide polymorphism; *ADRB1* = β1-adrenergic receptor gene; *ADD1* = α-subunit of the adducin gene; *AGT* = angiotensinogen gene; *CYP11B2* = aldosterone synthase gene; *CYP3A5* = Cytochrome P-450 3A5 gene; GRK4 = G protein-coupled receptor kinase 4 gene; µM **=** microlitre; µM = micromolar

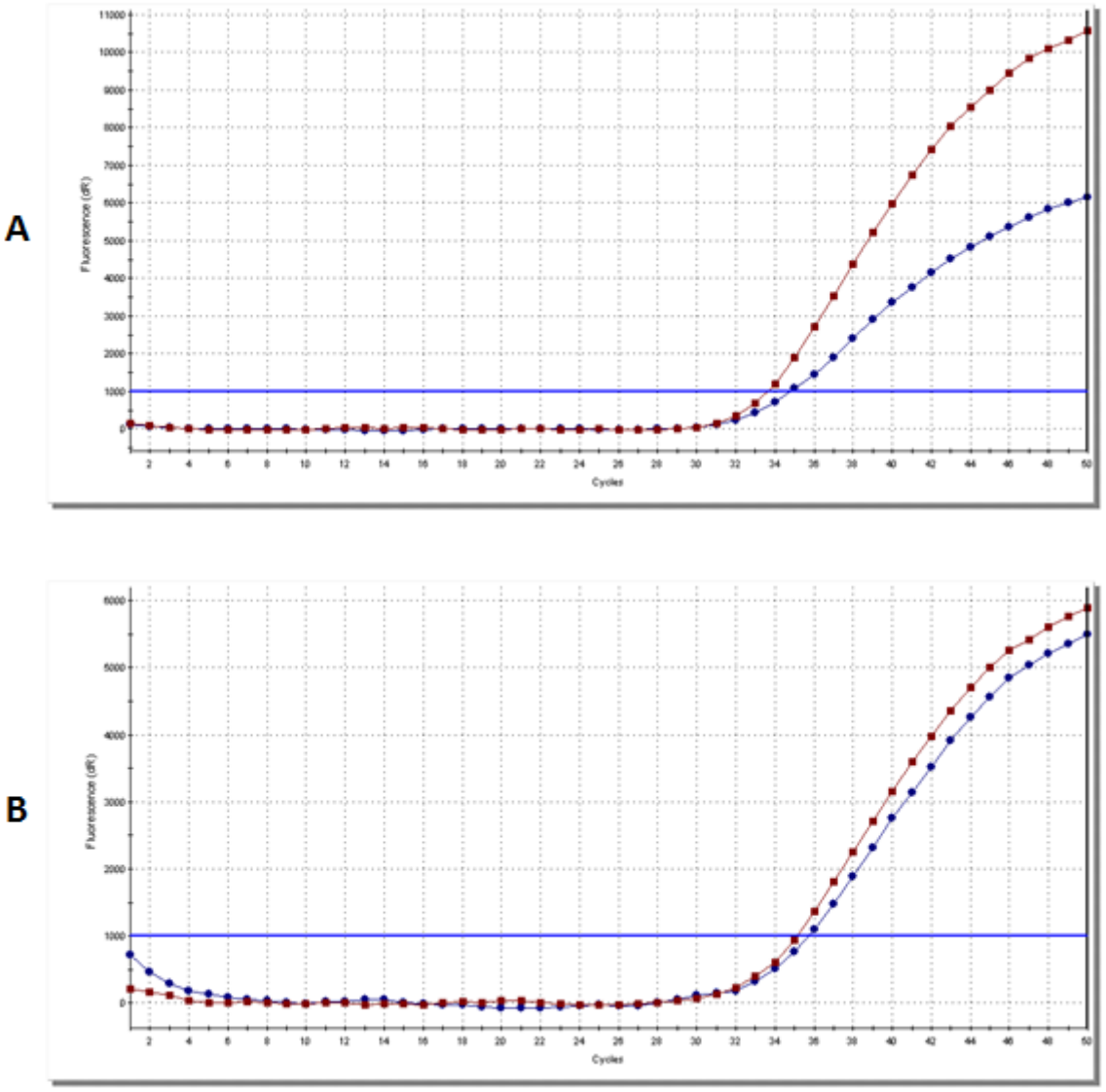

Figure 3.1: An example of the manner in which allele detection was improved by the optimization of probe concentrations for the SNP assay for G448T (*GRK4*). A: Amplification plot obtained with 0.20 µM forward and reverse primer, and 0.10 µM normotensive and hypertensive probe. B: Amplification plot obtained with 0.20 µM forward and reverse primer, and optimized hypertensive and normotensive probe concentrations of 0.10 µM and 0.20 µM, respectively. The no template control for the respective SNP assays was negative (data not shown).

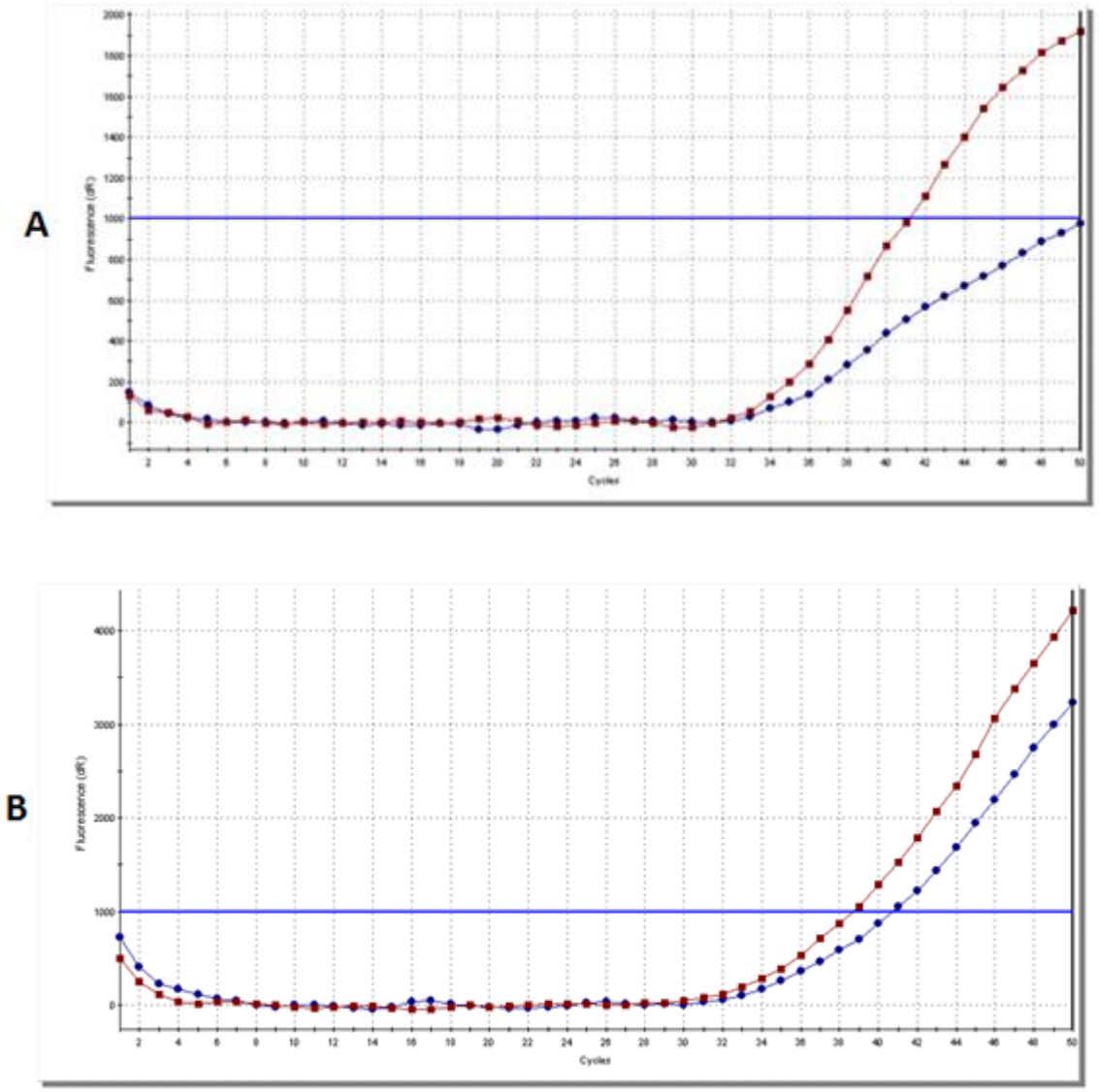

Figure 3.2: An example of the manner in which amplification and allele detection was improved by the optimization of probe and primer concentrations for the SNP assay for T704C (*AGT*). A: Amplification plot obtained with 0.20 µM forward and reverse primer, and 0.10 µM normotensive and hypertensive probe. B: Amplification plot obtained with optimized forward and reverse primer concentrations of 0.80 µM, respectively, and optimized hypertensive and normotensive probe concentrations of 0.30 µM and 0.35 µM, respectively. The no template control for the respective SNP assays was negative (data not shown).

3.3 Conclusion

In this study the methanol DNA extraction method described by Lebea and Pretorius (2008) was modified to maximize DNA yield and to reduce extraction time. The modified method used in this study was both time and cost effective to obtain maximum DNA yield from FTA paper. Furthermore, the optimization of primer and probe concentrations to produce similar amplification and detection levels resulted in a more accurate assessment of normotensive and hypertensive alleles in this study.

Chapter Four

Results and discussion

Single nucleotide polymorphism association analysis

4.1 General description of the study cohort

Of the total cohort 63.42% were hypertensive (≥ 140/90 mm Hg). The prevalence of hypertension in the female group was 65% and 58% in the male group of the cohort. Hypertension risk was not significantly increased for males (OR = 0.761, CI(95%): 0.45 – 1.28, P = 0.346) or females (OR = 1.314, CI(95%): 0.78 – 2.21, P = 0.346). Subsequently no statistically significant difference in systolic blood pressure (P = 0.460[‡]) or diastolic blood pressure (P = 0.087[‡]) was found between males and females. Thus, gender was not considered a factor affecting hypertension in this cohort and was not taken into account with further statistical analysis. According to body mass index, 54.28% of the participants were overweight to obese. A significant correlation was found between body mass index and systolic blood pressure (P < 0.001[§]), diastolic blood pressure (P < 0.001[§]) and hypertension (P < 0.001[§]) in the cohort. The risk of hypertension was 3.589 times higher in the overweight to obese subgroup compared to the underweight to normal weight subgroup (P < 0.001). Thus, the significant association between body mass index and hypertension was taken into account with SNP association analysis. Body mass was significantly higher in females than males (P < 0.001[‡]). Despite this, risk of hypertension was not significantly different between males and females.

§ = Linear regression; † = Analysis of variance F test; ‡ = Mann-Whitney U test

A total of 339 study participants of the Mangaung population were genotyped for eleven hypertension related SNPs. The genotypic data was assessed to determine whether any of the SNPs were associated with hypertension. SNP association analysis was performed using the analysis of variance test and the Mann-Whitney U test instead of using linear regression, as recommended by a statistician.

4.2 SNP analysis

The genotypic data obtained for each SNP is summarized in Table 4.2. All the SNPs were in Hardy-Weinberg equilibrium with the exception of C521T in *AGT*. Analysis of the genotypic distribution of SNPs in the cohort revealed a complete absence of the homozygous form of the normotensive T allele of T704C (*AGT*), and the hypertensive T allele of G217T (*ADD1*). In addition, the hypertensive T allele of C1711T (*GRK4*) was only present in the heterozygous state within the hypertensive group of the cohort, while the hypertensive A allele of A6986G (*CYP3A5*) was only present in the heterozygous form in the normotensive group. For the remaining SNPs, all genotypic forms were present in the hypertensive and normotensive groups of the cohort.

4.2.1 SNPs not associated with hypertension

Six of the 11 candidate SNPs did not appear to be associated with hypertension in the black population of Mangaung. These included G1165C (*ADRB1*), G-217A and T704C (*AGT*), G448T, C679T and C1711T (*GRK4*). None of the latter SNPs were associated with statistically significant elevations in either systolic or diastolic blood pressure (Appendix A, Table A6.1 to A6.3). Furthermore, no significant hypertension risk could be ascribed to any of the six SNPs (Appendix A, Table A7.1 and A7.2). The lack of association between these SNPs and hypertensive blood pressures in the black population group in Mangaung compared to other population groups could be the result of a too small cohort size, environmental factors, ethnicity and/or epistasis (Ioannidis *et al*., 2004; Ramu *et al*., 2009).

Table 4.1: General description of the study cohort in terms of gender, average systolic and diastolic blood pressure, body mass index and age

	Overall	Normotensive	Hypertensive	Underweight to normal weight (< 25 kg/m^2)	Overweight to obese (≥ 25 kg/m^2)
Participants (n)	339	124	215	155	184
Female (n)	262	92	170	93	169
Male (n)	77	32	45	62	15
Systolic blood pressure (mm Hg)	135.50 ± 23.67•	115.76 ± 13.79•	146.80 ± 20.59•	130.25 ± 22.82•	139.92 ± 23.54•
Diastolic blood pressure (mm Hg)	89.79 ± 17.57•	75.94 ± 8.66•	97.76 ± 16.42•	85.35 ± 16.38•	93.52 ± 17.71•
Body mass index (kg/m^2)	27.80 ± 8.80•	24.70 ± 8.10•	29.40 ± 8.70•	20.46 ± 2.73•	33.90 ± 7.31•
Age (years)	44.32 ± 10.60•	38.28 ± 9.19•	47.72 ± 9.78•	43.51 ± 10.76•	45.01 ± 10.45•

n = number; mm Hg = millimetre of mercury; kg/m^2 = kilogram per square metre; • = standard deviation

Table 4.2: Genotypic distribution of candidate SNPs in the study cohort for the hypertensive allele and the normotensive allele

***ADRB1* A145G (HT A; NT G)**								
Overall			Normotensive			Hypertensive		
AA	AG	GG	AA	AG	GG	AA	AG	GG
12.34% (n = 39)	44.99% (n = 139)	43.67% (n = 138)	9.32% (n = 11)	47.46% (n = 56)	43.22% (n = 51)	14.14% (n = 28)	41.92% (n = 83)	43.94% (n = 87)
***ADRB1* G1165C (HT G; NT C)**								
Overall			Normotensive			Hypertensive		
GG	CG	CC	GG	CG	CC	GG	CG	CC
49.20% (n = 154)	42.17% (n = 132)	8.63% (n = 27)	49.14% (n = 57)	43.97% (n = 51)	6.90% (n = 8)	49.24% (n = 97)	41.12% (n = 81)	9.64% (n = 19)
***ADD1* G217T (HT T; NT G)**								
Overall			Normotensive			Hypertensive		
GG	GT	TT	GG	GT	TT	GG	GT	TT
92.28% (n = 299)	7.72% (n = 25)	0	93.33% (n = 112)	6.67% (n = 8)	0	91.67% (n = 187)	8.33% (n = 17)	0

***AGT* G-217A**								
(HT A; NT G)								
Overall			Normotensive			Hypertensive		
GG	GA	AA	GG	GA	AA	GG	GA	AA
30.60% (n = 97)	50.79% (n = 161)	18.61% (n = 59)	36.21% (n = 42)	47.41% (n = 55)	16.38% (n = 19)	27.36% (n = 55)	52.74% (n = 106)	19.90% (n = 40)
***AGT* C521T**								
(HT T; NT C)								
Overall			Normotensive			Hypertensive		
CC	CT	TT	CC	CT	TT	CC	CT	TT
1.24% (n = 4)	88.82% (n = 286)	9.94% (n = 32)	1.65% (n = 2)	90.08% (n = 109)	8.27% (n = 10)	1.0% (n = 2)	88.06% (n = 177)	10.94% (n = 22)
***AGT* T704C**								
(HT C; NT T)								
Overall			Normotensive			Hypertensive		
TT	TC	CC	TT	TC	CC	TT	TC	CC
0	15.46% (n = 49)	84.54% (n = 268)	0	15.13% (n = 18)	84.87% (n = 101)	0	15.66% (n = 31)	84.34% (n = 167)

***CYP11B2* C-334T**								
(HT T; NT C)								
Overall			Normotensive			Hypertensive		
CC	CT	TT	CC	CT	TT	CC	CT	TT
5.64%	31.66%	62.70%	7.50%	33.33%	59.17%	4.52%	30.65%	64.82%
(n = 18)	(n = 101)	(n= 200)	(n = 9)	(n = 40)	(n = 71)	(n = 9)	(n = 61)	(n = 129)
***CYP3A5* A6986G**								
(HT A; NT G)								
Overall			Normotensive			Hypertensive		
AA	AG	GG	AA	AG	GG	AA	AG	GG
2.17%	26.32%	71.52%	0	24.17%	75.83%	3.45%	27.59%	68.97%
(n = 7)	(n = 85)	(n = 231)		(n = 29)	(n = 91)	(n = 7)	(n = 56)	(n = 140)
***GRK4* G448T**								
(HT T; NT G)								
Overall			Normotensive			Hypertensive		
GG	GT	TT	GG	GT	TT	GG	GT	TT
9.54%	37.54%	52.92%	6.56%	38.52%	54.92%	11.33%	36.95%	51.72%
(n = 31)	(n = 122)	(n = 172)	(n = 8)	(n = 47)	(n = 67)	(n = 23)	(n = 75)	(n = 105)

***GRK4* C679T (HT T; NT C)**								
Overall			Normotensive			Hypertensive		
CC	CT	TT	CC	CT	TT	CC	CT	TT
5.90% (n = 19)	35.40% (n = 114)	58.70% (n = 189)	6.61% (n = 8)	33.89% (n = 41)	59.50% (n = 72)	5.47% (n = 11)	36.32% (n = 73)	58.21% (n = 117)
***GRK4* C1711T (HT T; NT C)**								
Overall			Normotensive			Hypertensive		
CC	CT	TT	CC	CT	TT	CC	CT	TT
90.65% (n = 291)	9.04% (n = 29)	0.31% (n = 1)	92.44% (n = 110)	6.72% (n = 8)	0.84% (n = 1)	89.60% (n = 181)	10.40% (n = 21)	0

ADRB1 = β1-adrenergic receptor gene; *ADD1* = α-subunit of the adducin gene; *AGT* = angiotensinogen gene; *CYP11B2* = aldosterone synthase gene; *CYP3A5* = Cytochrome P-450 3A5 gene; GRK4 = G protein-coupled receptor kinase 4 gene; HT = hypertensive allele; NT = normotensive allele; n = number

4.2.2 Association of the A145G SNP of *ADRB1* with hypertensive blood pressure

An association was found between the hypertensive A allele of the A145G SNP in *ADRB1* and hypertensive systolic blood pressure in homozygotic form, but not in heterozygotes (Table 4.3). However, homozygosity for the A allele was not significantly associated with an increased risk of developing hypertension (Appendix A, Table A1.1). Stratification of the cohort revealed that there was an increased risk factor (OR = 11.250, P = 0.004) for hypertension in overweight to obese individuals homozygous for the A allele of the A145G SNP (Appendix A, Table A1.2). Thus it appears as if the A145G SNP is associated with hypertension especially in overweight to obese individuals in the Mangaung cohort.

Table 4.3: Genotypic association of the A145G SNP of *ADRB1* with blood pressure in the total cohort

Genotypic comparisons	Mean systolic blood pressure (mm Hg)	P	Mean diastolic blood pressure (mm Hg)	P
AA	140.41 ± 24.71•		90.90 ± 18.48•	
		0.035‡		0.248‡
AG	133.67 ± 21.57•		88.79 ± 15.07•	
GG	134.45 ± 24.87•		89.80 ± 19.30•	
		0.781†		0.629†
AG	133.67 ± 21.57•		88.79 ± 15.07•	
AA	140.41 ± 24.71•		90.90 ± 18.48•	
		0.062‡		0.233‡
GG	134.45 ± 24.87•		89.80 ± 19.30•	

A hypertensive allele; G normotensive allele; † Analysis of variance F test; ‡ Mann-Whitney U test; mm Hg = millimetre of mercury, P = probability value; • = standard deviation

A meta-analysis determined that the A allele of A145G was associated with increased risk of developing hypertension (Kitsios and Zintzaras, 2010). The findings of the current study further indicated that the A145G SNP may be of particular importance in hypertension related to overweight and obese body mass. In contrast to these findings, Ramu *et al*. (2009) found that the normotensive G allele and not the hypertensive A allele of A145G was associated with hypertension in Tamil Indians (882 study participants). Other studies on cohorts of Swedish (557 study participants), Italian (718 study participants) and Chinese individuals (1547 study participants) did not, however, find an association between the A145G SNP of *ADRB1* and hypertension (Bengtsson *et al*., 2001; Filigheddu *et al*., 2004; Peng *et al*., 2009). The relationship between the A145G SNP and hypertension risk in overweight to obese individuals reported here could account for the discrepant findings of the latter studies, which investigated cohorts with an average body mass below the obesity limit (30 kg/m^2).

4.2.3 Association of the G217T SNP of *ADD1* with hypertensive blood pressure

In this study, the G allele of the G217T SNP which is normally associated with normotension, was found to be associated with hypertensive systolic and diastolic blood pressure, but only in the homozygous form of the allele (Table 4.4). Stratification of the cohort according to body mass index revealed that the homozygous form of the G allele was associated with a significant risk for hypertension in overweight to obese individuals (Appendix A, Table A2.2). However, association between the homozygous form of the G allele and hypertensive blood pressure may be incidental, due to its high prevalence in the cohort and the absence of homozygotes for the T allele.

Table 4.4: Genotypic association of the G217T SNP of *ADD1* with blood pressure in hypertensive individuals

Genotypic comparisons	Mean systolic blood pressure (mm Hg)	P	Mean diastolic blood pressure (mm Hg)	P
GG	147.32 ± 20.43•	0.001‡	98.02 ± 16.64•	0.020‡
GT	133.35 ± 11.61•		91.18 ± 9.51•	
TT	-	N/A	-	N/A
GT	133.35 ± 11.61•		91.18 ± 9.51•	
GG	147.32 ± 20.43•	N/A	98.02 ± 16.64•	N/A
TT	-		-	

T hypertensive allele; G normotensive allele; ‡ Mann-Whitney U test; mm Hg = millimetre of mercury, P = probability value; • = standard deviation; N/A = not applicable, due to inadequate size of groups being compared

In contrast to the current study, Barlassina *et al*. (2000) found an association between the T allele of the G217T SNP of *ADD1* and hypertension in black South Africans (331 study participants). However, a meta-analysis by Ramu *et al*. (2010) determined that the T allele was not associated with hypertension in black individuals from different population groups. In an earlier study, Province *et al*. (2000) even suggested that the T allele may be a protective allele against high blood pressure in black African Americans (572 study participants). Thus, the role of the G217T SNP in hypertension in African ethnic groups is not as distinct as it is in other population groups. For example, several studies indicated that the G217T SNP plays a role in hypertension in Chinese and Japanese individuals (Iwai *et al*., 1997; Tamaki *et al*., 1998; Sugimoto *et al*., 2002; Ju *et al*., 2003; Li, 2012). It has been proposed that other unidentified polymorphisms, which also could affect hypertension susceptibility, could be in linkage disequilibrium with the

G217T SNP and that allelic variation of the other polymorphic loci could contribute to the inconsistent findings of association studies (Ramu *et al*., 2010).

4.2.4 Association of the C521T SNP of *AGT* with hypertensive blood pressure

The hypertensive T allele of the C521T SNP of *AGT* was associated with hypertensive diastolic blood pressure, but only in the homozygous state (Table 4.5). However, the risk of hypertension for the homozygous form of the T allele was not statistically significant in the cohort (Appendix A, Table A3.1). Stratification of the cohort according to body mass index determined that the risk of hypertension associated with the T allele in the homozygous form was significantly increased in overweight to obese individuals compared to underweight to normal weight individuals (Appendix A, Table A3.2). Thus, the T allele appears to be associated with increased risk of hypertensive diastolic blood pressure in individuals with overweight to obese body mass.

Table 4.5: Genotypic association of the C521T SNP of *AGT* with blood pressure in the total cohort

Genotypic comparisons	Mean systolic blood pressure (mm Hg)	P	Mean diastolic blood pressure (mm Hg)	P
CC	132.75 ± 19.97[•]		77.25 ± 8.77[•]	
		N/A		N/A
CT	134.05 ± 23.13[•]		88.91 ± 17.51[•]	
TT	141.34 ± 24.74[•]		94.44 ± 14.85[•]	
		0.072[‡]		0.012[‡]
CT	134.05 ± 23.13[•]		88.91 ± 17.51[•]	

CC	132.75 ± 19.97•		77.25 ± 8.77•	
		0.510†		0.031†
TT	141.34 ± 24.74•		94.44 ± 14.85•	

T hypertensive allele; C normotensive allele; † Analysis of variance F test; ‡ Mann-Whitney U test; mm Hg = millimetre of mercury, P = probability value; • = standard deviation; N/A = not applicable, due to inadequate size of groups being compared

Different studies have found an association between the C521T SNP and hypertension (Hegele *et al*., 1994; Mustafina *et al*., 2002). Results from one study on black Ghanaian individuals (177 study participants) suggested that C521T is not independently associated with hypertension (Williams *et al*., 2000). Thus, it appears as if the hypertensive effect of C521T only becomes apparent in the presence of other hypertension risk factors. The results from the current study confirm that the T allele is not independently associated with an increased risk of hypertension but that overweight to obese body mass index levels are a contributing risk factor in the Mangaung population.

4.2.5 Association of the C-344T SNP of *CYP11B2* with hypertensive blood pressure

An association was found between the hypertensive T allele of the C-344T SNP of *CYP11B2* and hypertensive systolic and diastolic blood pressure in overweight to obese individuals, for both the homozygous and heterozygous forms of the allele (Table 4.6). However, no significant hypertensive risk could be attributed to the T allele in the case-control cohort (Appendix A, Table A4.1). Thus, an association between the T allele and hypertensive systolic and diastolic blood pressure was only observed in the presence of overweight to obese body mass index levels, indicating that the T allele is not an independent risk factor for hypertension in the cohort.

Table 4.6: Genotypic association of the C-344T SNP of *CYP11B2* with blood pressure in overweight to obese individuals

Genotypic comparisons	Mean systolic blood pressure (mm Hg)	P	Mean diastolic blood pressure (mm Hg)	P
CC	125.17 ± 14.68•		84.83 ± 12.78•	
		0.018‡		0.020‡
CT	140.98 ± 26.47•		96.40 ± 20.23•	
TT	141.01 ± 22.68•		93.05 ± 16.18•	
		0.472‡		0.149‡
CT	140.98 ± 26.47•		96.40 ± 20.23•	
CC	125.17 ± 14.68•		84.83 ± 12.78•	
		0.005‡		0.023‡
TT	141.01 ± 22.68•		93.05 ± 16.18•	

T hypertensive allele; C normotensive allele; ‡ Mann-Whitney U test; mm Hg = millimetre of mercury, P = probability value; • = standard deviation

A study conducted by Tiago *et al*. (2003) on hypertensive black South African individuals (231 study participants) from Johannesburg also found an association between the C-344T SNP of *CYP11B2* and hypertensive blood pressure. In the latter study the homozygous form of the T allele was significantly associated with higher hypertensive systolic blood pressure in hypertensive individuals (Tiago *et al*., 2003). Furthermore, the study of Tiago *et al*. (2003) was comprised of individuals that had an overweight to obese body mass. Thus, based on this, the C-344T SNP appears to be associated with hypertension in overweight to obese black South Africans.

4.2.6 Association of the A6986G SNP of *CYP3A5* with hypertensive blood pressure

An association between the hypertensive A allele of the A6986G SNP and hypertensive systolic blood pressure was found in the cohort, for the homozygous form of the allele but not the heterozygous form (P = 0.0384[‡]) (Table 4.7). An explanation for this is that all the homozygous individuals for the A allele were hypertensive. Furthermore, the majority of the homozygous A allele individuals (85.71%) were also overweight to obese (Table A5.3). However, the effect of body mass index levels on the association of the homozygous form of the A allele and hypertension could not be assessed since only one homozygous individual was in the underweight to normal weight class, whereas the rest were overweight to obese. Thus, an association between the homozygous state of the A allele and hypertensive blood pressure appears to be present in the cohort, although overweight and obese body mass index may also play a role in the association.

Table 4.7: Genotypic association of the A6986G SNP of *CYP3A5* with blood pressure in the total cohort

Genotypic comparisons	Mean systolic blood pressure (mm Hg)	P	Mean diastolic blood pressure (mm Hg)	P
AA	145.86 ± 13.84[•]		97.29 ± 15.53[•]	
		0.138[‡]		0.129[‡]
AG	139.79 ± 21.76[•]		90.55 ± 15.91[•]	
GG	132.77 ± 23.92[•]		89.01 ± 17.99[•]	
		0.011[‡]		0.227[‡]
AG	139.79 ± 21.76[•]		90.55 ± 15.91[•]	

AA	145.86 ± 13.84•		97.29 ± 15.53•	
		0.038‡		0.087‡
GG	132.77 ± 23.92•		89.01 ± 17.99•	

A hypertensive allele; G normotensive allele; ‡ Mann-Whitney U test; mm Hg = millimetre of mercury; P = probability value; • = standard deviation

The A allele of the A6986G SNP, which results in expression of functional CYP3A5, has also been implicated in hypertension in African-Americans (683 study participants) and elderly Finnish Caucasians (373 study participants) (Ho *et al*., 2005; Kivisto *et al*., 2005). CYP3A5 activity results in the conversion of corticosterone to 6β-hydroxycorticosterone, which has been associated with increased sodium reabsorption and increased blood pressure (Duncan *et al*., 1988). In contrast, the G allele results in a premature stop codon that results in the translation of a truncated, non-functional form of the CYP3A5 enzyme. The truncated form of the enzyme does not lead to elevations in sodium reabsorption and blood pressure, hence the association between the G allele and normotensive blood pressure (Kuehl *et al*., 2001). In a study on African Americans, Ho *et al*. (2005) found that there was a significantly higher proportion of homozygous and heterozygous individuals for the A allele in the hypertensive group of the cohort. Similar results were found in a Finnish cohort where hypertension was associated with the heterozygous expression of the A allele (Kivisto *et al*., 2005). In the current study, the frequency of the hypertensive A allele was low (0.15) and this may explain why an association with hypertension was only observed for homozygotes of the A allele in the cohort. The average systolic blood pressure (139.79 ± 21.76 mm Hg) of the heterozygotes in this study bordered on the hypertension limit (140 mm Hg), and it is therefore likely that a larger cohort may reveal an association between heterozygotes for the A allele and hypertensive blood pressure in the Mangaung population. In conclusion, research indicated that hypertension susceptibility appears to be increased if the A allele of the A6986G SNP is expressed, especially if A allele expression is homozygous with regards to the Mangaung population.

4.3 Conclusion

In this study, five SNPs in *CYP3A5* (A6986G), *ADRB1* (A145G), *AGT* (C521T), *CYP11B2* (C-344T) and *ADD1* (G217T) were associated with an increased risk of hypertension. Except for the A6986G SNP of *CYP3A5*, none of the SNPs were associated with hypertensive blood pressure independent of overweight to obese body mass levels. The results of Ho *et al*. (2005) and Kivisto *et al*. (2005) are consistent with an independent role for the A6986G SNP in hypertension. Regarding *ADRB1* (A145G), *AGT* (C521T) and *CYP11B2* (C-344T), these SNPs were associated with a significant increase in blood pressure in overweight to obese individuals. However, the results for the SNP in *ADD1* (G217T) appeared to contradict that which has been found in the literature. A possible explanation for this incongruence is that the normotensive allele of the G217T SNP may be in linkage disequilibrium with other yet unidentified hypertension related polymorphism(s) as previously suggested (Ramu *et al*., 2010). The results from this study suggest that A6986G in *CYP3A5* could be an independent risk factor for hypertension in the Mangaung cohort, whereas A145G in *ADRB1*, C521T in *AGT* and C-344T in *CYP11B2* appear to be associated with increased risk of hypertension in only overweight to obese individuals in the cohort.

4.4 Limitations of this study

A limitation of the current study was that the effect of salt intake and diabetes on hypertension related SNPs could not be determined. Data was not available for the 24 hour urine sodium levels and diabetic status of the study participants. The present study only looked at preselected SNPs in specific genes and since this is a multifactorial trait other SNPs could play a role in hypertension of the Mangaung population. Furthermore, the current study was limited to a cohort of 339 individuals and larger cohort sizes could attain better coverage of the distribution of SNP alleles in the Mangaung population, which could provide a better grasp of the hypertensive effects of the SNPs that were implicated in hypertension in this study.

Chapter Five

Results and discussion

Analysis of samples for which Real-time PCR genotyping failed

Out of the 339 study participants, 21 could not be fully genotyped for all 11 of the candidate SNPs with Real-time PCR (Table 5.2). To ascertain whether the failed genotyping in specific samples could have resulted from sequence alterations in the target probe region, the samples were amplified using the primers in Table 2.1. Amplicon was assessed visually by agarose gel electrophoresis to determine if amplification was successful. Samples for which amplicon could be detected, sequencing was performed using both primers.

5.1 Conventional PCR amplification of samples for which genotyping failed

Gel electrophoresis based PCR assays were performed on the samples for which genotyping of target SNP regions failed. The PCR assays were optimized in terms of primer concentration to enhance the DNA yield and amplification specificity of the assays (Table 5.1).

Table 5.1: Optimized primer amounts and concentrations in the final reaction mixture for the conventional PCR assays for each of the respective SNPs

Gene	SNP	Primer	
		Forward	**Reverse**
ADRB1	A145G	1.0 µl (0.45 µM)	1.0 µl (0.45 µM)
	G1165C	4.62 µl (2.1 µM)	4.62 µl (2.1 µM)
ADD1	G217T	4.62 µl (2.1 µM)	4.62 µl (2.1 µM)
AGT	G-217A	1.0 µl (0.45 µM)	1.0 µl (0.45 µM)
	C521T	1.0 µl (0.45 µM)	1.0 µl (0.45 µM)
	T704C	1.0 µl (0.45 µM)	1.0 µl (0.45 µM)
CYP11B2	C-344T	4.62 µl (2.1 µM)	4.62 µl (2.1 µM)
CYP3A5	A6986G	1.0 µl (0.45 µM)	1.0 µl (0.45 µM)
GRK4	G448T	4.62 µl (2.1 µM)	4.62 µl (2.1 µM)
	C679T	4.62 µl (2.1 µM)	4.62 µl (2.1 µM)
	C1711T	1.0 µl (0.45 µM)	1.0 µl (0.45 µM)

SNP = single nucleotide polymorphism; *ADRB1* = β1-adrenergic receptor gene; *ADD1* = α-subunit of the adducin gene; *AGT* = angiotensinogen gene; *CYP11B2* = aldosterone synthase gene; *CYP3A5* = Cytochrome P-450 3A5 gene; GRK4 = G protein-coupled receptor kinase 4 gene; µM = microlitre; µM = micromolar

Table 5.2: Samples which failed to be genotyped for all 11 of the candidate SNPs

Gene	SNP	Samples not successfully genotyped
ADRB1	A145G	265.1; 270.1; 277.1; 314.1; 315.1; 328.1; 329.1; 330.1; 332.1; 338.1; 340.1
	C1165G	270.1; 277.1; 288.1; 294.1; 296.1; 300.1; 306.1; 310.1; 315.1; 328.1; 332.1; 338.1; 340.1; 343.1
ADD1	G217T	314.1; 329.1; 338.1
AGT	G-217A	270.1; 277.1; 296.1; 310.1; 315.1; 329.1; 332.1; 338.1; 343.1; 346.1
	C521T	271.1; 277.1; 288.1; 296.1; 310.1; 314.1; 315.1; 338.1; 346.1
	T704C	265.1; 271.1; 277.1; 287.1; 288.1; 296.1; 306.1; 310.1; 315.1; 329.1; 340.1
CYP11B2	C-344T	271.1; 277.1; 288.1; 296.1; 310.1; 314.1; 315.1; 328.1
CYP3A5	A6986G	270.1; 277.1; 315.1; 332.1
GRK4	G448T	310.1; 340.1
	C679T	287.1; 296.1; 314.1; 315.1; 329.1; 332.1; 346.1
	C1711T	288.1; 296.1; 314.1; 315.1; 328.1; 332.1

SNP = single nucleotide polymorphism; *ADRB1* = β1-adrenergic receptor gene; *ADD1* = α-subunit of the adducin gene; *AGT* = angiotensinogen gene; *CYP11B2* = aldosterone synthase gene; *CYP3A5* = Cytochrome P-450 3A5 gene; GRK4 = G protein-coupled receptor kinase 4 gene

Of the 21 samples that were re-assessed with conventional PCR, only 10 samples yielded reproducible PCR product that was detectable by gel electrophoresis (Table 5.3). Where conventional re-PCR amplification was successful, the correct size amplicon was amplified in all of the samples, with the exception of sample 338.1 for the C521T SNP in *AGT* (Figure 5.1). The latter repeatedly yielded two amplicon, a ~75 base pair (bp) and ~140 bp fragment, respectively, that differed in size from the expected fragment of 94 bp. The remainder of the samples was repeatedly negative for any detectable PCR product. The failure to obtain PCR amplification for the latter samples was not considered an issue of insufficient quality or quantity of DNA, since the same DNA extracts were successfully used in the Real-time PCR assays for other target SNPs. These results suggest that the samples that failed to produce PCR amplicon may have additional SNPs in the primer binding region, especially towards the 3' end of the primer, which could have prevented amplification despite using a lower annealing temperature of 50˚C.

Table 5.3: Samples re-assessed with conventional PCR that yielded amplicon detectable by gel electrophoresis

Gene	SNP	Fragment size (bp)	Samples
ADRB1	A145G	77	328.1; 340.1
AGT	C521T	94	277.1; 288.1; 296.1; 338.1; 346.1
	T704C	63	340.1
CYP3A5	A6986G	105	270.1; 332.1
GRK4	C679T	74	329.1; 332.1
	C1711T	64	296.1; 328.1

SNP = single nucleotide polymorphism; bp = base pair; *ADRB1* = β1-adrenergic receptor gene; *AGT* = angiotensinogen gene; *CYP3A5* = Cytochrome P-450 3A5 gene; GRK4 = G protein-coupled receptor kinase 4 gene

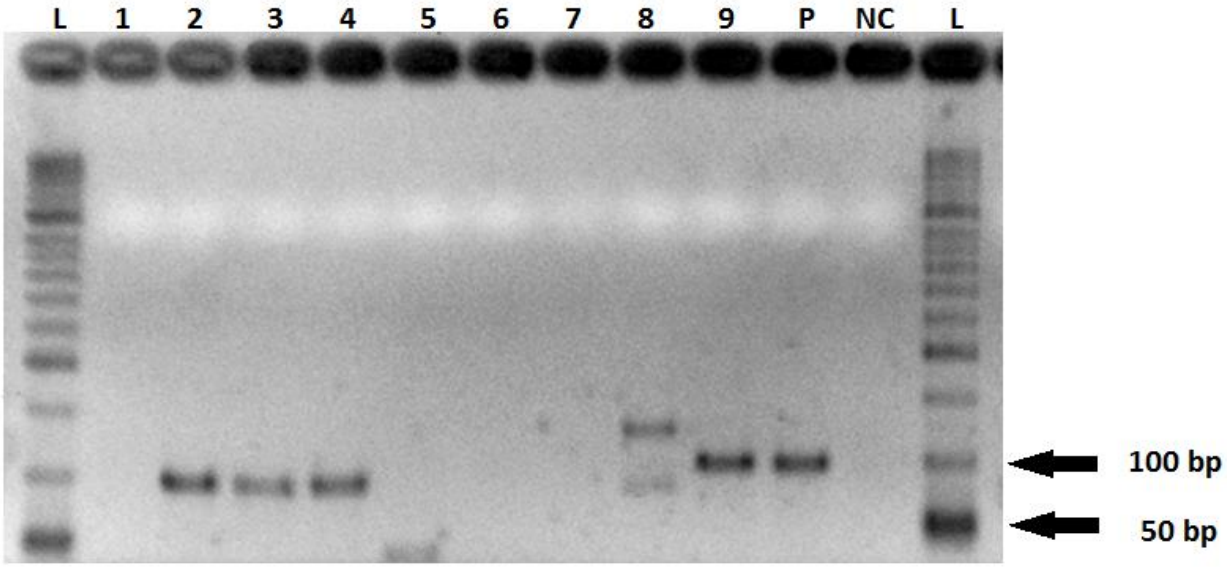

Figure 5.1: An example of a negative inverted gel image of a 2% agarose gel stained with ethidium bromide and visualized under UV light for the amplicon of the SNP assay for C521T of the *AGT* gene (94 bp). Lane L – DNA ladder (Hyperladder II), lane 1 - sample 271.1, lane 2 - sample 277.1, lane 3 - sample 288.1, lane 4 - sample 296.1, lane 5 - sample 310.1, lane 6 - sample 314.1, lane 7 - sample 315.1, lane 8 - sample 338.1, lane 9 - sample 346.1, lane P - positive control, lane NC - no template control.

5.2 Sequencing of amplicon from conventional PCR for samples for which genotyping failed

To determine if Real-time PCR genotyping failed as a result of additional SNPs in the probe binding site, sequencing was performed on the conventional re-PCR amplification product. Sequencing of the amplicon proved difficult due to the small size of the amplicon. Initial sequencing reactions failed due to a high noise to sequence ratio. The PCR sequencing cycle reaction was modified and the extension time was reduced from 4 minutes to 30 seconds and the extension temperature increased from 60˚C to 72˚C. The amount of template used in the sequencing reactions was also empirically reduced by approximately five fold to the sequencing kit insert recommendations (ABI Prism Big Dye Terminator v.3.1 Cycle Sequencing

Kit (Applied Biosystems)). Genotypes could only be resolved from the sequence data for four of the six SNP assays. The sequence data of the samples for the A145G (*ADRB1*) and C1711T (*GRK4*) SNPs was not of sufficient quality to allow accurate base calling. Genotypic data was derived from only the sequence data for C521T (*AGT*), T704C (*AGT*), A6986G (*CYP3A5*) and C679T (*GRK4*) (Table 5.4). Where the target SNP could clearly be deduced, the genotype was included in statistical analysis in chapter 4. However, where a non-target mutation was detected which had unknown phenotypic implications, for example by resulting in an amino acid substitution or a truncation of the resulting peptide, that target genotype was excluded from the statistical analysis (Table 5.4).

Table 5.4: Genotypes and non-target SNPs deduced from sequence data of samples for which Real-time PCR based genotyping failed

SNP	Sample	Genotype	Non-target SNPs in probe binding site
AGT C521T	277.1	CC	-
	288.1	CC	-
	296.1	CC	-
	338.1	TT	Multiple base changes
	346.1	CC	G base deletion
AGT T704C	340.1	CC	-
CYP3A5 A6986G	270.1	AA	A to C
	332.1	AA	A to C
GRK4 C679T	329.1	CC	-
	332.1	TT	-

SNP = single nucleotide polymorphism; *AGT* = angiotensinogen gene; *CYP3A5* = Cytochrome P-450 3A5 gene; *GRK4* = G protein-coupled receptor kinase 4 gene

5.2.1 A6986G SNP region in *CYP3A5*

In addition to the target SNP (Table 5.4), an additional mismatched nucleotide was found in the probe binding site for the A6986G SNP of *CYP3A5* in samples 270.1 and 332.1 (Figure 5.3 and 5.4). In both samples the mismatch appeared to have resulted from a substitution mutation of an A to a C, which could account for the failed genotyping with Real-time PCR. The latter substitution mutation occurred at the 5' end of exon 4 of the *CYP3A5* gene. Both samples were furthermore homozygous for the A allele of the A6986G SNP, which results in complete transcription of the *CYP3A5* gene. Transcription of the *CYP3A5* gene is, however, also influenced by alternative splicing, and several isoforms can consequently be expressed. It was found that the single base variant in exon 4 could only be transcribed into the mRNA transcript for isoform 3 of *CYP3A5*, due to alternative splicing. However, the single base variant did not appear to form part of the coding sequence in the mRNA transcript for isoform 3 of *CYP3A5*, and as such the substitution mutation may consequently not result in an amino acid substitution.

CCACCCAGCTTAACGAATGCTCTACTGTCATTTCTAACCATAATCTCTTTAAA
GAGCTCTTTTGTCTTTCA**G**TATCTCTTCCCTGTTTGGACCACATTACCCTTC

Figure 5.2: Reference sequence of the *CYP3A5* gene target region containing the A6986G SNP (Ensembl). The probe binding site is underlined and the target SNP is indicated in bold (hypertensive G allele shown).

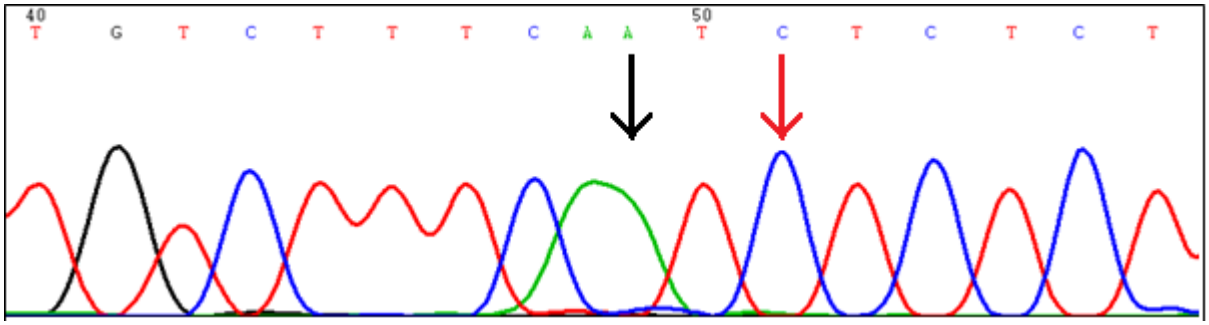

Figure 5.3: Sequence electropherogram of the probe binding site for the A6986G SNP of *CYP3A5*, in sample 270.1. The target SNP is present at position 49 in the electropherogram (black arrow). The substitution mutation of an A to a C was found at position 51 in the electropherogram (red arrow).

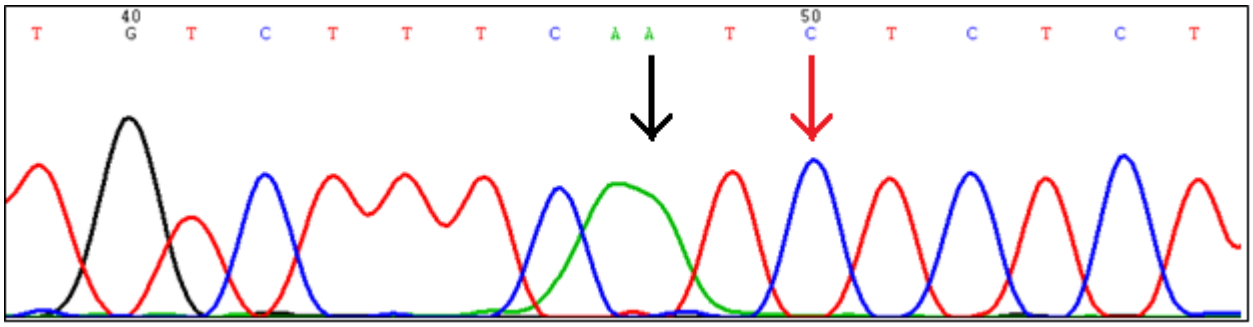

Figure 5.4: Sequence electropherogram of the probe binding site for the A6986G SNP of *CYP3A5*, in sample 332.1. The target SNP is present at position 48 in the electropherogram (black arrow). The substitution mutation of an A to a C was found at position 50 in the electropherogram (red arrow).

5.2.2 C521T SNP region in *AGT*

The conventional PCR of sample 338.1 for the C521T SNP of *AGT* yielded amplicon of ~140 bp and ~75 bp, instead of the expected 94 bp amplicon. Sequence determination was only possible for the ~140 bp fragment, because the sequence data for the ~75 bp fragment was not of sufficient quality to allow accurate base calling. In addition to the target SNP (Table 5.4), several non-target mismatched nucleotides were found in the probe binding site that could account for the failed

genotyping of the C521T SNP in sample 338.1 (Figure 5.6). Also found in the ~140 bp fragment was an inserted segment of approximately 32 bases that resembled a sequence region upstream of the forward primer binding site. The inserted segment was found in a head to tail orientation downstream of the probe binding site. The single base mutations and insertion mutation that was found in sample 338.1 occurred in exon 2 of the *AGT* gene and could as such have resulted in amino acid substitutions. However, the non-target single base changes and the insertion found in sample 338.1 needs to be confirmed and further assessed for any possible functional effects on the AGT protein. For sample 346.1 it appeared as if Real-time PCR assessment of the C521T SNP could have failed as a result of a possible single base deletion. The nucleotide which appeared to be deleted was a G nucleotide at the 3' end of the probe binding site. However, base calling of the probe binding site at the 3' end (Figure 5.7) was not conclusive and the apparent base deletion could not be confirmed with certainty. As for samples 277.1, 288.1 and 296.1 no unexpected sequence variants were detected in the probe binding site for the C521T SNP (Figure 5.8 – 5.10) in addition to the target SNP (Table 5.4). As such, the failed Real-time PCR assessment of the latter samples does not seem to be related to a failure in probe binding. Since the conventional amplification of these samples proved difficult, it is possible that a mutation in the primer binding region could have resulted in inadequate Real-time PCR amplification and thus lack of sufficiently bound probe to genotype these samples. Under such circumstances, sequencing would not detect a primer mismatch since the primer is incorporated into the amplicon.

CAGGGCAGGGCTGATAGCCAGGCCCAGCTGCTGCTGTCCA**C**GGTGGTGG
GCGTGTTCACAGCCCCAGGCCTGCACCTGAAGCAGCCGTTTGTGC

Figure 5.5: Reference sequence of the *AGT* gene target region containing the C521T SNP (Ensembl). The probe binding site is underlined and the target SNP is indicated in bold (normotensive C allele shown).

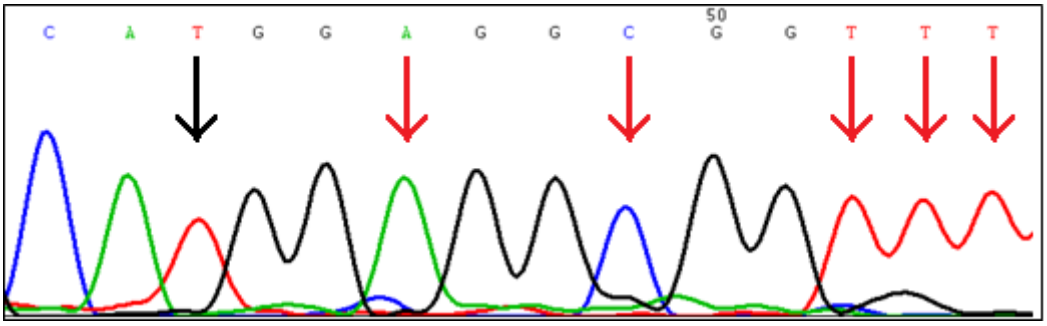

Figure 5.6: Sequence electropherogram of the probe binding site for the C521T SNP in *AGT* in sample 338.1 (~140 bp fragment). The target SNP is present at position 43 in the electropherogram (black arrow). Single base substitution mutations were found at position 46 (T to a A), position 49 (T to a C), position 52 (G to a T), position 53 (C to a T) and position 54 (G to a T) in the electropherogram (red arrows).

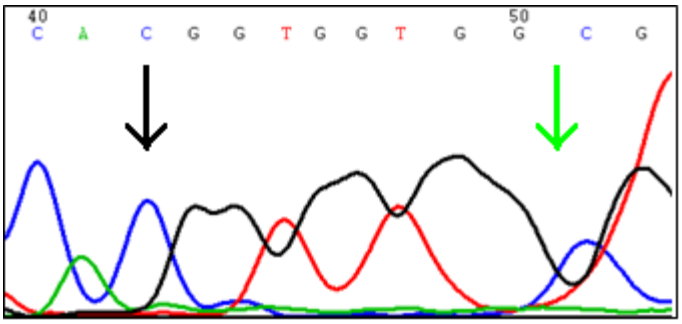

Figure 5.7: Sequence electropherogram of the probe binding site for the C521T SNP in *AGT* in sample 346.1. The target SNP is present at position 42 in the electropherogram (black arrow). The position of the G nucleotide that appeared to be deleted is indicated by the green arrow.

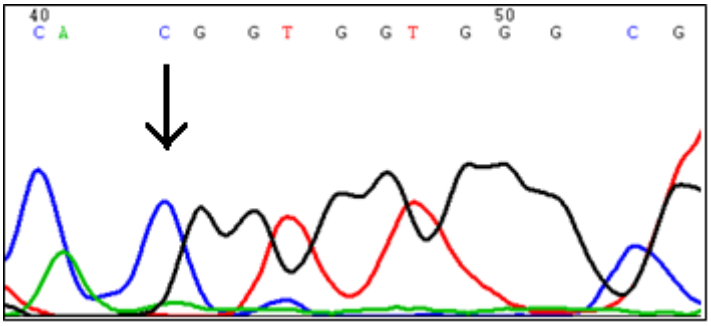

Figure 5.8: Sequence electropherogram of the probe binding site for the C521T SNP in *AGT* in sample 277.1. The target SNP is present at position 42 in the electropherogram (black arrow).

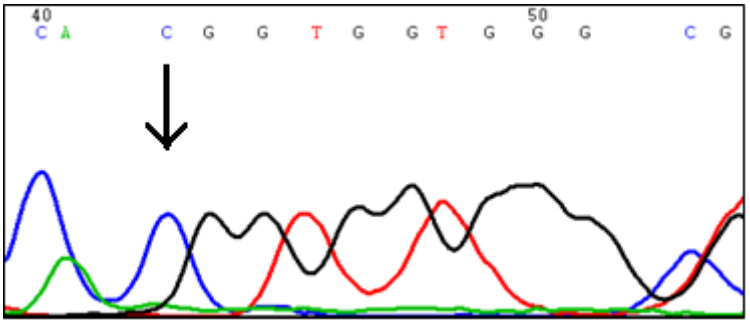

Figure 5.9: Sequence electropherogram of the probe binding site for the C521T SNP in *AGT* in sample 288.1. The target SNP is present at position 42 in the electropherogram (black arrow).

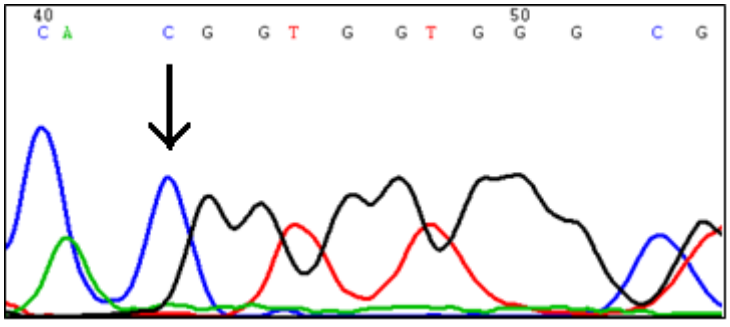

Figure 5.10: Sequence electropherogram of the probe binding site for the C521T SNP in *AGT* in sample 296.1. The target SNP is present at position 42 in the electropherogram (black arrow).

5.2.3 T704C SNP region in *AGT*

In addition to the target SNP (Table 5.4), no unexpected sequence variants were detected in the probe binding site for the T704C SNP in *AGT* in sample 340.1 (Figure 5.12). Failed Real-time PCR detection of the T704C SNP could also have resulted from deficient primer binding due to the presence of a mutation in a primer binding site, since PCR amplification of the sample also proved difficult. However, due to incorporation of the primers into the amplicon during PCR, sequencing would not detect mutations in the priming sites.

AGGCTGTGACAGGATGGAAGACTGGCTGCTCCCTGA**T**GGGAGCCAGTGTG
GACAGCACCCTGG

Figure 5.11: Reference sequence of the *AGT* gene target region containing the T704C SNP (Ensembl). The probe binding site is underlined and the target SNP is indicated in bold (hypertensive T allele shown).

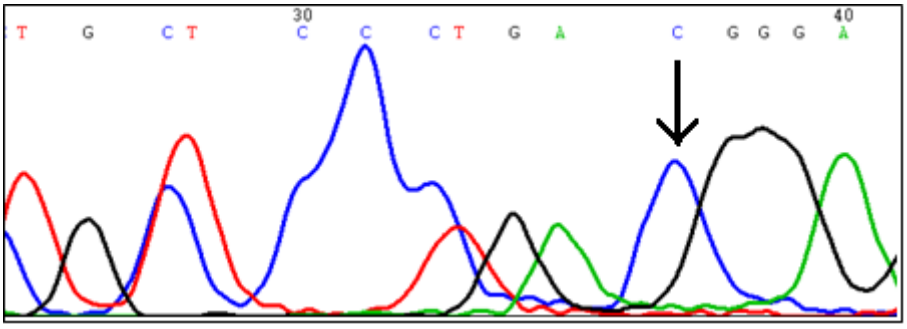

Figure 5.12: Sequence electropherogram of the probe binding site for the T704C SNP in *AGT* in sample 340.1. The target SNP is present at position 36 in the electropherogram (black arrow).

5.2.4 C679T SNP region in *GRK4*

Unexpected sequence variants in the probe binding site for the C679T SNP of *GRK4* could not be identified in the available sequence data for samples 329.1 and 332.1. For both of the samples the expected bases at the 5' end of the probe binding site could not be called from the sequence data, due to deterioration of the quality of terminal sequence data (Figure 5.14 and 5.15). As such, the possibility of unexpected sequence variation in the probe binding site for the C679T SNP, which could explain the failed Real-time PCR genotyping of samples 329.1 and 332.1, could not be established or ruled out.

GATTGGGACTGAAGGAGGAGAACCCTTCCAAAAAAGCCTTTGAGGAATGTA
CTAGGTAAGTGGTTCATGCTGAG

Figure 5.13: Reference sequence of the *GRK4* gene target region containing the C679T SNP (Ensembl). The probe binding site is underlined and the target SNP is indicated in bold (normotensive C allele shown).

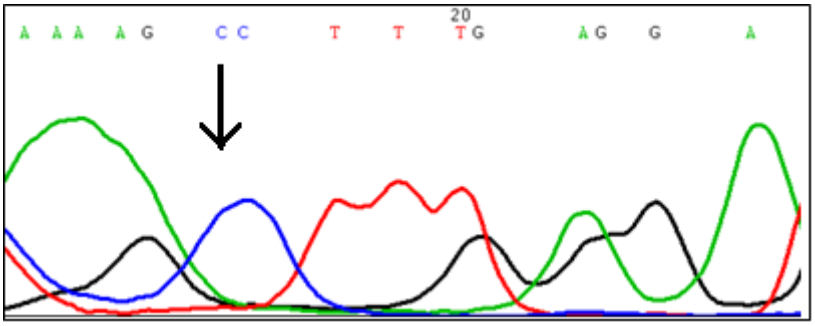

Figure 5.14: Sequence electropherogram of the probe binding site for the C679T SNP of *GRK4* in sample 329.1. The target SNP is present at position 16 in the electropherogram (black arrow). The given electropherogram does not cover the entire length of the probe binding site, because the expected A nucleotide at position 10 could not be called.

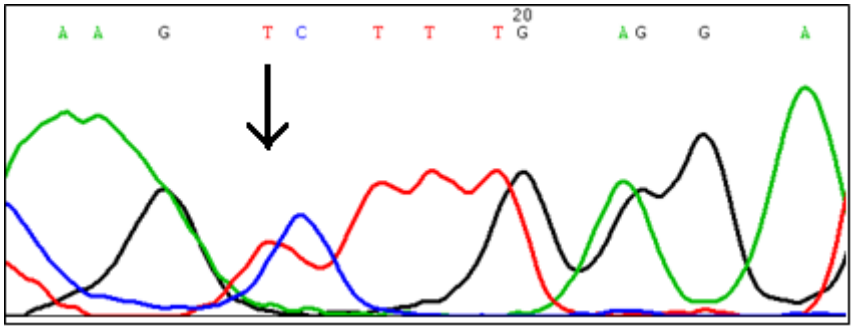

Figure 5.15: Sequence electropherogram of the probe binding site for the C679T SNP of *GRK4* in sample 332.1. The target SNP is present at position 15 in the electropherogram (black arrow). The given electropherogram does not cover the entire length of the probe binding site, because the expected A nucleotides at position 9 - 11 could not be called.

5.2.5 SNP regions of the A145G SNP in *ADRB1* and the C1711T SNP in *GRK4*

Sequence determination of the probe binding sites for the A145G (*ADRB1*) and C1711T (*GRK4*) SNPs was not possible with the available sequence data. Genotypes and possible sequence related causes for defective probe binding could consequently not be deduced. The inability to obtain readable sequence data for the latter SNP regions was likely the result of the short length of the amplicon for the respective SNPs, which were 77 bp for the A145G SNP and 64 bp for the C1711T SNP.

5.3 Conclusion

Unexpected sequence variants, which may have affected probe binding, could only be detected in the probe binding sites for the A6986G SNP in *CYP3A5* and the C521T SNP in *AGT*. The sequence variants that were found in the C521T SNP probe region of samples 338.1 and 346.1 could have resulted in amino acid substitutions in AGT. Compared to this, the substitution SNP found in exon 4 of

CYP3A5 in samples 270.1 and 332.1 was outside the coding region of CYP3A5 and is thus not expected to affect protein function. Previous reports of the *AGT* and *CYP3A5* gene variants were not found in the literature search that was performed and these variants may be novel. Future studies could explore the functional and possible clinical consequences of the variants that were found in *AGT* and *CYP3A5*, and whether they are isolated incidents or more widespread in the larger Mangaung population.

No sequence variants were, however, found in the probe binding sites of the other samples that were sequenced. The possibility of other mutations that could have interfered with SNP region amplification in the Real-time PCR assays could not be explored, because adequate sequence reads of the entire target region could not be generated. Proper sequencing of the target SNP regions was limited by the short lengths of the amplicon of the respective SNP regions (Table 5.3). Future studies could analyze larger fragment lengths of the SNP regions in these samples to determine whether novel SNPs are also present.

Summary

Hypertension is one of the leading causes of death and disability in the world. In 95% of individuals with hypertension, the condition arises from the interaction of multiple environmental factors with physiological systems. Environmental factors that have been found to increase blood pressure include obesity, aging, high salt and alcohol consumption, low potassium and calcium intake, stress and insulin resistance. Physiological systems that regulate blood pressure include the autonomic nervous system, the renal system, hormonal system and the cardiovascular system. Various genes in these systems including the β1-adrenergic receptor (*ADRB1*), α-adducin (*ADD1*), angiotensinogen (*AGT*), aldosterone synthase (*CYP11B2*), *CYP3A5* and G protein-coupled receptor kinase 4 (*GRK4*) have been implicated in hypertensive blood pressure due to the presence of single nucleotide polymorphisms (SNPs). The occurrence of such SNPs in blood pressure regulatory systems is thought to result in altered gene expression or protein function.

In South Africa, the prevalence of hypertension has been determined to be approximately 39.9% in males and 34.9% in females. The Assuring Health for all in the Free State (AHA-FS) study determined that the prevalence of hypertension was approximately 48.3% in the Mangaung population. The AHA-FS study also found that 37.6% and 51.2% of individuals in the study cohort were overweight or obese, respectively, and that high body mass could be an important risk factor for hypertension.

The aim of this study was to determine whether genes in the sympathetic nervous system, renal system and hormonal systems could contribute to the high prevalence of hypertension in the Mangaung population. Previously identified SNPs associated

with hypertension in *ADRB1* (A145G and G1165C), *ADD1* (G217T), *AGT* (G-217A, C521T and T704C), *CYP11B2* (C-344T), *CYP3A5* (A6986G) and *GRK4* (G448T, C679T and C1711T) were genotyped in a cohort of the AHA-FS study, which comprised black individuals from Mangaung, Free State.

Six of the 11 candidate SNPs did not appear to be associated with hypertension in the black population of Mangaung. These included G1165C (*ADRB1*), G-217A and T704C (*AGT*), G448T, C679T and C1711T (*GRK4*). None of the latter SNPs were associated with statistically significant elevations in either systolic or diastolic blood pressure. The association remained negative even after the cohort was stratified into underweight to normal weight and overweight to obese groups. The lack of association between these SNPs and hypertensive blood pressure in the black population group in Mangaung compared to other population groups could be attributed to population differences in environmental factors, ethnicity, cohort size and/or epistasis. Five SNPs were associated with hypertension in black individuals from Mangaung. The latter included SNPs in *CYP3A5* (A6986G), *ADRB1* (A145G), *AGT* (C521T), *CYP11B2* (C-344T) and *ADD1* (G217T).

The hypertensive A allele of the A6986G SNP of *CYP3A5* has been associated with systolic hypertension in homozygous individuals of the study. Similarly, an association between the A allele and hypertension in African-Americans and Swedish Caucasians has also been found. Thus, the A allele of the A6986G SNP seems to cause hypertension susceptibility in different populations, including the Mangaung population group.

As for the A145G SNP of *ADRB1*, hypertensive systolic blood pressure was associated with individuals that were homozygous for the hypertensive A allele of the A145G SNP. A meta-analysis determined that individuals expressing the A allele of A145G had a 24% higher risk for developing hypertension. In the Mangaung

population, hypertension risk associated with the A allele of A145G was especially increased in overweight to obese individuals.

The presence of the hypertensive T allele of the C521T SNP of *AGT* was associated with hypertensive diastolic blood pressure in the Mangaung population. Similar results were found in Hutterite, Russian and Tartar population groups. Furthermore, in the Mangaung population the hypertension risk conferred by the T allele was significantly increased in overweight and obese individuals. This suggests that the T allele of C521T may be involved in particularly overweight and obesity related hypertension.

The hypertensive T allele of the C-344T SNP of *CYP11B2* was only associated with hypertensive systolic and diastolic blood pressure in the overweight to obese individuals of the Mangaung population. In another study conducted on black South African individuals the T allele was also associated with hypertension. The cohort of the latter study furthermore had an overweight to obese body mass index average. It therefore appears that the T allele of C-344T could primarily be a risk factor for overweight and obesity related hypertension.

Interestingly the normotensive G allele of *ADD1* was implicated in obesity related hypertension, instead of the hypertensive T allele. In contrast, another study on black South Africans found that the T allele was associated with hypertension. However, results from a previous study on African-Americans suggested that the T allele may be protective against hypertension. It has been proposed that other unidentified polymorphisms, which also could affect hypertension susceptibility, could be in linkage disequilibrium with the G217T SNP and that allelic variation of the other polymorphic loci could contribute to the inconsistent findings of association studies.

Several individuals in the cohort from the AHA-FS study could not be genotyped for the candidate SNPs. Attempts to obtain conventional PCR product for individuals where genotyping failed did not prove entirely successful. In the cases where no PCR amplicon could be amplified even after attempts at assay optimization, it was concluded that primer mismatch, especially at the 3' end of the primer may be the likely cause. Where PCR amplicon was successfully amplified, sequencing proved difficult due to short amplicon size. However, partial sequence data revealed additional SNPs in some individuals in the probe binding region that would account for failed genotyping.

A limitation of this study was that only selective SNPs in genes associated with hypertension were genotyped in the cohort. Furthermore, since the study did not investigate the role of potentially novel SNPs in candidate genes, it is possible that additional unidentified SNPs in these genes may also contribute to hypertension. Despite these limitations, this study is currently the most comprehensive of its kind on the Mangaung population. Future research could focus on additional genes as well as screening these genes for novel SNPs, especially in the genes where the SNPs investigated were not associated with hypertension in the Mangaung cohort.

In conclusion, the A6986G SNP in *CYP3A5* appears to be an independent risk factor for hypertension, whereas A145G in *ADRB1*, C521T in *AGT*, C-344T in *CYP11B2* and G217T in *ADD1* may be associated with hypertension related to overweight and obese body weights. To our knowledge, this is the most comprehensive study investigating a combination of several gene SNPs associated with hypertension in a black Mangaung population.

Opsomming

Hipertensie is een van die grootste oorsake van sterftes en gestremdheid in die wêreld. In 95% van individue met hipertensie ontstaan die siektetoestand uit die interaksie tussen verskeie omgewingsfaktore en fisiologiese stelsels. Omgewingsfaktore wat tot 'n verhoging in bloeddruk kan lei, sluit in vetsug, veroudering, hoë sout en alkohol inname, lae kalium en kalsium inname, stres, en insulien weerstandigheid. Fisiologiese stelsels wat bloeddruk reguleer sluit in die outonomiese senuweestelsel, die nierstelsel, hormonale stelsel en die kardiovaskulêre stelsel. Verskeie gene in hierdie stelsels, byvoorbeeld β1-adrenergiese reseptor (*ADRB1*), α-addusien (*ADD1*), angiotensinogeen (*AGT*), aldosteroon sintase (*CYP11B2*), *CYP3A5* en G proteïen-gekoppelde kinase 4 (*GRK4*) is al geïmpliseer in hipertensie weens die teenwoordigheid van enkel nukleotied polimorfismes (SNPs). Die voorkoms van sulke SNPs in bloeddruk regulatoriese stelsels mag veranderde geenuitdrukking en proteïenfunksie veroorsaak.

Die voorkoms van hipertensie in Suid-Afrika is ongeveer 39.9% in mans en 34.9% in vrouens. Die "Assuring Health for All in the Free State" (AHA-FS) studie het bepaal dat hipertensie in ongeveer 48.3% van die Mangaung populasie voorkom. Die AHA-FS studie het ook gevind dat 37.6% en 51.2% van die individue in die studiegroep onderskeidelik oorgewig of vetsugtig was en dat hoë liggaamsmassa 'n belangrike risikofaktor vir hipertensie is. Die doel van die studie was om te bepaal of gene in die simpatiese senuweestelsel, nierstelsel en hormonale stelsel 'n bydrae lewer tot die hoë voorkoms van hipertensie in die Mangaung populasie. Voorheen geïdentifiseerde SNPs wat met hipertensie geassosieer is, naamlik *ADRB1* (A145G en G1165C), *ADD1* (G217T), *AGT* (G-217A, C521T en T704C), *CYP11B2* (C-344T),

CYP3A5 (A6986G), *GRK4* (G448T, C679T en C1711T), is gegenotipeer in die AHA-FS studiegroep, wat saamgestel was uit swart individue van Mangaung, Vrystaat.

Ses van die 11 kandidaat SNPs was nie geassosieer met hipertensie in die swart populasie van Mangaung nie. Hierdie sluit in G1165C (*ADRB1*), G-217A en T704C (*AGT*), G448T, C679T en C1711T (*GRK4*). Nie een van die laasgenoemde SNPs is geassosieer met ‘n statistiese noemenswaardige verhoging in sistoliese of diastoliese bloeddruk nie. Die assosiasie het negatief gebly te midde van subverdeling van die studiegroep in subgroepe van ondergewig tot normale gewig en oorgewig tot vetsug. Die gebrek aan ‘n assosiasie tussen hierdie SNPs en hipertensiewe bloeddruk in die swart populasiegroep in Mangaung in vergelyking met ander populasiegroepe, kan toegeskryf word aan populasie verskille met betrekking tot omgewingsfaktore, etnisiteit en epistatiese patrone. Vyf SNPs is geassosieer met hipertensie in swart individue van Mangaung. Laasgenoemde behels SNPs in *CYP3A5* (A6986G), *ADRB1* (A145G), *AGT* (C521T), *CYP11B2* (C-344T) en *ADD1* (G217T).

Die hipertensiewe A alleel van die A6986G SNP van *CYP3A5* is geassosieer met sistoliese hipertensie in homosigotiese individue in die studie. Soortgelyke bevindings van ‘n assosiasie tussen die A alleel en hipertensie in swart Amerikaners en Sweedse blankes bestaan. Dus lyk dit of die A alleel van die A6986G SNP kan bydra tot vatbaarheid vir hipertensie in verskillende populasies, insluitend die Mangaung populasiegroep.

‘n Assosiasie tussen die A145G SNP in *ADRB1* is gevind tussen hipertensiewe sistoliese bloeddruk en homosigote vir die A alleel van die SNP. ‘n Meta-analise het bepaal dat individue wat die A alleel van die A145G SNP uitdruk, ‘n 24% hoër risiko het om hipertensie te ontwikkel. Die hipertensie risiko geassosieer met die A alleel van die A145G SNP was veral verhoog in oorgewig tot vetsugtige individue.

Die teenwoordigheid van die hipertensiewe T alleel van die C521T SNP in *AGT* was geassosieer met hipertensiewe diastoliese bloeddruk in die Mangaung populasie. Soortgelyke resultate was gevind in Hutteriet, Russiese en Tartaarse populasiegroepe. Verder was die hipertensie risiko veroorsaak deur die T alleel statisties hoër in oorgewig en vetsugtige individue in die Mangaung populasie. Die resultate dui daarop dat die T alleel van C521T betrokke kan wees in veral hipertensie geassosieer met oorgewigtigheid en vetsug.

Die hipertensiewe T alleel van die C-344T SNP in *CYP11B2* was slegs geassosieer met hipertensiewe sistoliese en diastoliese bloeddruk in oorgewig tot vetsugtige individue van die Mangaung populasie. Die T alleel was ook geassosieer met hipertensie in ‘n ander studie op swart Suid-Afrikaanse individue. Die laasgenoemde studiegroep het ‘n oorgewig en vetsugtige liggaamsmassa gemiddeld gehad. Dit wil dus voorkom asof die T alleel van C-344T hoofsaaklik ‘n risikofaktor vir oorgewig en vetsug verwante hipertensie is.

Interessant was die normotensiewe G alleel van *ADD1* wat geïmpliseer was in vetsug verwante hipertensie, in plaas van die hipertensiewe T alleel. In teenstelling, het ‘n ander studie op swart Suid-Afrikaners gevind dat die T alleel geassosieer was met hipertensie. Alhoewel resultate van ‘n vorige studie op swart Amerikaners voorgestel het dat die T alleel beskermend teen hipertensie kan wees. Daar was ook ‘n voorstel dat ongeïdentifiseerde polimorfismes wat vatbaarheid vir hipertensie kan beïnvloed, in koppelingsonewewigtigheid met die G217T SNP kan wees. Indien wel, kan alleel variasie van die ander polimorfismes bydrae tot die nie-konsekwente bevindings van assosiasie studies.

Verskeie individue in die populasiegroep van die AHA-FS studie kon nie gegenotipeer word vir die kandidaat SNPs nie. Pogings om konvensionele polimerase ketting reaksie (PKR) produkte te verkry van individue waar genotipering

misluk het, was nie volkome suksesvol nie. In die gevalle waar PKR amplikon nie geamplifiseer kon word nie, selfs na PKR optimisering, was die gevolgtrekking dat die oorsaak nie-komplementering van veral die 3'-kant van die priemstuk kon wees. Waar PKR amplikon suksesvol geamplifiseer was, was volgordebepaling wel moeilik as gevolg van kort fragmentgrootte. Alhoewel, gedeeltelike volgorde data het addisionele SNPs in die peilstuk bindingsareas in sommige individue aangetoon wat verantwoordelik kon wees vir die mislukte genotipering.

'n Beperking van die studie was dat slegs geselekteerde SNPs, in gene geassosieer met hipertensie, gegenotipeer is in die studiegroep. Aangesien die studie nie die rol van potensiële nuwe SNPs in kandidaat gene ondersoek het nie, is dit moontlik dat addisionele ongeïdentifiseerde SNPs in die gene wel mag bydra tot hipertensie. Ten spyte van hierdie beperkings, is hierdie studie die mees omvangryke studie van sy soort op die Mangaung populasie. Toekomstige navorsing kan fokus op addisionele gene, asook die sifting vir nuwe SNPs, veral in die gene waar die ondersoekte SNPs nie met hipertensie geassosieer was in die Mangaung studie groep nie.

Samevattend het die A6986G SNP in *CYP3A5* geblyk om 'n onafhanklike risikofaktor vir hipertensie te wees, terwyl A145G in *ADRB1*, C521T in *AGT*, C-344T in *CYP11B2* en G217T in *ADD1* geassosieer is met oorgewig en vetsug verwante hipertensie. Tot ons wete, is hierdie studie die mees omvattende ten opsigte van die kombinasie SNPs in gene geassosieerd met hipertensie in die swart Mangaung populasie.

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Appendix A

ADRB1 A145G

Table A1.1: Hypertension risk associated with the genotypes of the A145G SNP of *ADRB1* in the total cohort

Genotype (Hypertensive A; Normotensive G)	OR	95% CI	P
AA	1.602	0.728 – 3.586	0.222
AG	0.799	0.492 – 1.297	0.351
GG	1.030	0.633 – 1.675	0.907

OR = odds ratio; CI = confidence interval; P = probability value

Table A1.2: Hypertension risk associated with A145G SNP genotypes across categorical body mass index subgroups

Genotype (Hypertensive A; Normotensive G)	Normal to Underweight vs. Overweight to obese			Overweight to obese vs. Normal to Underweight		
	OR	**95% CI**	**P**	**OR**	**95% CI**	**P**
AA	0.089	0.010 – 0.609	0.004	11.250	1.642 - 97.750	0.004
AG	0.253	0.116 – 0.550	< 0.001	3.946	1.817 - 8.643	< 0.001
GG	0.367	0.168 – 0.797	0.008	2.724	1.255 - 5.954	0.008

OR = odds ratio; CI = confidence interval; P = probability value

Table A1.3: Genotypic association of A145G SNP with systolic blood pressure across categorical body mass index subgroups

Genotype (Hypertensive A; Normotensive G)	Body mass index subgroup	n	Mean systolic blood pressure (mm Hg)	P
AA	Normal to Underweight (< 25 kg/m^2)	17	132.06 ± 25.54•	0.0436‡
	Overweight to obese (≥ 25 kg/m^2)	22	146.86 ± 22.54•	
AG	Normal to Underweight (< 25 kg/m^2)	62	129.10 ± 20.22•	0.0139‡
	Overweight to obese (≥ 25 kg/m^2)	77	137.35 ± 22.03•	
GG	Normal to Underweight (< 25 kg/m^2)	68	128.10 ± 22.08•	0.0028†
	Overweight to obese (≥ 25 kg/m^2)	70	140.61 ± 26.01•	

kg/m^2 = kilogram per square metre; n = number; mm Hg = millimetre of mercury; P = probability value; • = standard deviation; † Analysis of variance F test; ‡ Mann-Whitney U test

ADD1 G217T

Table A2.1: Hypertension risk associated with the genotypes of the G217T SNP of *ADD1* in the total cohort

Genotype (Hypertensive T; Normotensive G)	OR	95% CI	P
GG	0.786	0.328 – 1.880	0.588
GT	1.273	0.532 – 3.045	0.588
TT	N/A	N/A	N/A

OR = odds ratio; CI = confidence interval; P = probability value; N/A = not applicable, due to inadequate size of a group

Table A2.2: Hypertension risk associated with G217T SNP genotypes across categorical body mass index subgroups

Genotype (Hypertensive T; Normotensive G)	Normal to Underweight vs. Overweight to obese			Overweight to obese vs. Normal to Underweight		
	OR	**95% CI**	**P**	**OR**	**95% CI**	**P**
GG	0.294	0.181–0.479	< 0.001	3.396	2.088 – 5.524	< 0.001
GT	0.10	0.010 – 1.005	0.051	10.0	0.995 – 100.467	0.051
TT	N/A	N/A	N/A	N/A	N/A	N/A

OR = odds ratio; CI = confidence interval; P = probability value; N/A = not applicable, due to inadequate size of a group

Table A2.3: Genotypic association of G217T SNP with systolic blood pressure across categorical body mass index subgroups

Genotype (Hypertensive T; Normotensive G)	Body mass index subgroup	n	Mean systolic blood pressure (mm Hg)	P
GG	Normal to Underweight (< 25 kg/m^2)	136	129.82 ± 22.26•	0.0001‡
	Overweight to obese (≥ 25 kg/m^2)	163	140.53 ± 23.98•	
GT	Normal to Underweight (< 25 kg/m^2)	14	123.93 ± 12.95•	0.2351†
	Overweight to obese (≥ 25 kg/m^2)	11	131.09 ± 16.46•	
TT	Normal to Underweight (< 25 kg/m^2)	0	-	N/A
	Overweight to obese (≥ 25 kg/m^2)	0	-	

kg/m^2 = kilogram per square metre; n = number; mm Hg = millimetre of mercury; P = probability value; • = standard deviation; N/A = not applicable, due to inadequate size of groups being compared; † Analysis of variance F test; ‡ Mann-Whitney U test

Table A2.4: Genotypic association of G217T SNP with diastolic blood pressure across categorical body mass index subgroups

Genotype (Hypertensive T; Normotensive G)	Body mass index subgroup	n	Mean diastolic blood pressure (mm Hg)	P
GG	Normal to Underweight (< 25 kg/m^2)	136	84.71 ± 15.98•	< 0.0001‡
	Overweight to obese (≥ 25 kg/m^2)	163	94.01 ± 18.07•	
GT	Normal to Underweight (< 25 kg/m^2)	14	85.93 ± 12.62•	0.8534†
	Overweight to obese (≥ 25 kg/m^2)	11	86.82 ± 10.69•	
TT	Normal to Underweight (< 25 kg/m^2)	0	-	N/A
	Overweight to obese (≥ 25 kg/m^2)	0	-	

kg/m^2 = kilogram per square metre; n = number; mm Hg = millimetre of mercury; P = probability value; • = standard deviation; N/A = not applicable, due to inadequate size of groups being compared; † Analysis of variance F test; ‡ Mann-Whitney U test

AGT C521T

Table A3.1: Hypertension risk associated with the genotypes of the C521T SNP of *AGT* in the total cohort

Genotype (Hypertensive T; Normotensive C)	OR	95% CI	P
CC	0.598	0.083 – 4.301	0.610
CT	0.812	0.390 – 1.690	0.577
TT	1.364	0.623 – 2.988	0.438

OR = odds ratio; CI = confidence interval; P = probability value

Table A3.2: Hypertension risk associated with C521T SNP genotypes across categorical body mass index subgroups

Genotype (Hypertensive T; Normotensive C)	Normal to Underweight vs. Overweight to obese			Overweight to obese vs. Normal to Underweight		
	OR	**95% CI**	**P**	**OR**	**95% CI**	**P**
CC	1.0	0.002 – 50.40	1.0	1.0	0.002 – 50.40	1.0
CT	0.284	0.172 – 0.469	< 0.001	3.521	2.132 – 5.816	< 0.001
TT	0.161	0.031 – 0.834	0.030	6.222	1.20 – 32.273	0.030

OR = odds ratio; CI = confidence interval; P = probability value

Table A3.3: Genotypic association of C521T SNP with diastolic blood pressure across categorical body mass index subgroups

Genotype (Hypertensive T; Normotensive C)	Body mass index subgroup	n	Mean diastolic blood pressure (mm Hg)	P
CC	Normal to Underweight (< 25 kg/m^2)	2	79.50 ± 10.61•	0.7038†
	Overweight to obese (≥ 25 kg/m^2)	2	75.0 ± 9.90•	
CT	Normal to Underweight (< 25 kg/m^2)	135	84.21 ± 15.43•	<0.0001‡
	Overweight to obese (≥ 25 kg/m^2)	151	93.12 ± 18.23•	
TT	Normal to Underweight (< 25 kg/m^2)	13	90.0 ± 17.16•	0.1657†
	Overweight to obese (≥ 25 kg/m^2)	19	97.47 ± 12.63•	

kg/m^2 = kilogram per square metre; n = number; mm Hg = millimetre of mercury; P = probability value; • = standard deviation; † Analysis of variance F test; ‡ Mann-Whitney U test

CYP11B2 C-344T

Table A4.1: Hypertension risk associated with the genotypes of the C-344T SNP of *CYP11B2* in the total cohort

Genotype (Hypertensive T; Normotensive C)	OR	95% CI	P
CC	0.584	0.206 – 1.660	0.318
CT	0.862	0.515 – 1.443	0.618
TT	1.272	0.777 – 2.082	0.340

OR = odds ratio; CI = confidence interval; P = probability value

Table A4.2: Hypertension risk associated with C-344T SNP genotypes across categorical body mass index subgroups

Genotype (Hypertensive T; Normotensive C)	Normal to Underweight vs. Overweight to obese			Overweight to obese vs. Normal to Underweight		
	OR	**95% CI**	**P**	**OR**	**95% CI**	**P**
CC	0.357	0.028 – 3.972	0.620	2.80	0.252 – 36.20	0.620
CT	0.140	0.051 – 0.372	< 0.001	7.156	2.691 – 19.458	< 0.001
TT	0.345	0.180 – 0.658	0.001	2.90	1.519 - 5.563	0.001

OR = odds ratio; CI = confidence interval; P = probability value

Table A4.3: Genotypic association of C-344T SNP with systolic blood pressure across categorical body mass index subgroups

Genotype (Hypertensive T; Normotensive C)	Body mass index subgroup	n	Mean systolic blood pressure (mm Hg)	P
CC	Normal to Underweight (< 25 kg/m^2)	6	131.5 ± 26.61•	0.4801‡
	Overweight to obese (≥ 25 kg/m^2)	12	125.17 ± 14.68•	
CT	Normal to Underweight (< 25 kg/m^2)	43	124.19 ± 22.26•	0.0011†
	Overweight to obese (≥ 25 kg/m^2)	58	140.98 ± 26.47•	
TT	Normal to Underweight (< 25 kg/m^2)	99	130.65 ± 20.74•	0.0009†
	Overweight to obese (≥ 25 kg/m^2)	101	141.01 ± 22.68•	

kg/m^2 = kilogram per square metre; n = number; mm Hg = millimetre of mercury; P = probability value; • = standard deviation; † Analysis of variance F test; ‡ Mann-Whitney U test

Table A4.4: Genotypic association of C-344T SNP with diastolic blood pressure across categorical body mass index subgroups

Genotype (Hypertensive T; Normotensive C)	Body mass index subgroup	n	Mean diastolic blood pressure (mm Hg)	P
CC	Normal to Underweight (< 25 kg/m^2)	6	89.0 ± 17.23•	0.4247‡
	Overweight to obese (≥ 25 kg/m^2)	12	84.83 ± 12.78•	
CT	Normal to Underweight (< 25 kg/m^2)	43	80.53 ± 15.37•	< 0.0001†
	Overweight to obese (≥ 25 kg/m^2)	58	96.40 ± 20.23•	
TT	Normal to Underweight (< 25 kg/m^2)	99	86.27 ± 15.43•	0.0028†
	Overweight to obese (≥ 25 kg/m^2)	101	93.05 ± 16.18•	

kg/m^2 = kilogram per square metre; n = number; mm Hg = millimetre of mercury; P = probability value; • = standard deviation; † Analysis of variance F test; ‡ Mann-Whitney U test

CYP3A5 A6986G

Table A5.1: Hypertension risk associated with the genotypes of the A6986G SNP of *CYP3A5* in the total cohort

Genotype (Hypertensive A; Normotensive G)	OR	95% CI	P
AA	N/A	N/A	N/A
AG	1.195	0.690 – 2.077	0.517
GG	0.708	0.410 – 1.219	0.204

OR = odds ratio; CI = confidence interval; P = probability value; N/A = not applicable, due to inadequate size of a group

Table A5.2: Hypertension risk associated with A6986G SNP genotypes across categorical body mass index subgroups

Genotype (Hypertensive A; Normotensive G)	Normal to Underweight vs. Overweight to obese			Overweight to obese vs. Normal to Underweight		
	OR	**95% CI**	**P**	**OR**	**95% CI**	**P**
AA	N/A	N/A	N/A	N/A	N/A	N/A
AG	0.425	0.155 - 1.50	0.077	2.352	0.870 - 6.447	0.077
GG	0.455	0.254 - 0.815	0.006	2.196	1.227 - 3.942	0.006

OR = odds ratio; CI = confidence interval; P = probability value; N/A = not applicable, due to inadequate size of a group

Table A5.3: Genotypic association of A6986G SNP with systolic blood pressure across categorical body mass index subgroups

Genotype (Hypertensive A; Normotensive G)	Body mass index subgroup	n	Mean systolic blood pressure (mm Hg)	P
AA	Normal to Underweight (< 25 kg/m^2)	1	127.0	N/A
	Overweight to obese (≥ 25 kg/m^2)	6	149.0 ± 12.12•	
AG	Normal to Underweight (< 25 kg/m^2)	34	132.76 ± 21.95•	0.0142†
	Overweight to obese (≥ 25 kg/m^2)	51	144.47 ± 20.53•	
GG	Normal to Underweight (< 25 kg/m^2)	114	128.03 ± 21.60•	0.0028†
	Overweight to obese (≥ 25 kg/m^2)	117	137.38 ± 25.23•	

kg/m^2 = kilogram per square metre; n = number; mm Hg = millimetre of mercury; P = probability value; • = standard deviation; N/A = not applicable, due to inadequate size of groups being compared; † Analysis of variance F test

SNPs not associated with hypertension

Table A6.1: Genotypic association of *ADRB1* (G1165C) and *AGT* (G-217A) SNPs with blood pressure in the total cohort

Gene SNP	Genotypic comparisons	Mean systolic blood pressure (mm Hg)	P	Mean diastolic blood pressure (mm Hg)	P
***ADRB1* G1165C (Hypertensive G; Normotensive C)**	**GG**	134.54 ± 24.35•	0.763‡	89.69 ± 17.67•	0.802‡
	GC	135.37 ± 21.98•		90.20 ± 17.04•	
	CC	134.37 ± 26.06•	0.460‡	85.74 ± 16.98•	0.134‡
	GC	135.37 ± 21.98•		90.20 ± 17.04•	
	GG	134.54 ± 24.35•	0.444‡	89.69 ± 17.67•	0.149‡
	CC	134.37 ± 26.06•		85.74 ± 16.98•	
***AGT* G-217A (Hypertensive A; Normotensive G)**	**GG**	130.34 ± 24.55•	0.359‡	87.79 ± 17.27•	0.390‡
	GA	136.45 ± 22.48•		90.99 ± 18.57•	
	AA	138.95 ± 23.71•	0.017‡	88.98 ± 14.27•	0.111‡
	GA	136.45 ± 22.48•		90.99 ± 18.57•	
	GG	130.34 ± 24.55•	0.023‡	87.79 ± 17.27•	0.236‡
	AA	138.95 ± 23.71•		88.98 ± 14.27•	

SNP = single nucleotide polymorphism; *ADRB1* = β1-adrenergic receptor gene; *AGT* = angiotensinogen gene; mm Hg = millimetre of mercury; P = probability value; • = standard deviation; ‡ Mann-Whitney U test

Table A6.2: Genotypic association of *AGT* (T704C) and *GRK4* (G448T) SNPs with blood pressure in the total cohort

Gene SNP	Genotypic comparisons	Mean systolic blood pressure (mm Hg)	P	Mean diastolic blood pressure (mm Hg)	P
***AGT* T704C (Hypertensive C; Normotensive T)**	**TT**	-	N/A	-	N/A
	TC	135.61 ± 20.87•		91.41 ± 16.84•	
	CC	134.57 ± 23.93•	0.375‡	89.15 ± 17.51•	0.224‡
	TC	135.61 ± 20.87•		91.41 ± 16.84•	
	TT	-	N/A	-	N/A
	CC	134.57 ± 23.93•		89.15 ± 17.51•	
***GRK4* G448T (Hypertensive T; Normotensive G)**	**GG**	138.48 ± 21.43•	0.087‡	94.12 ± 18.47•	0.089‡
	GT	132.78 ± 23.22•		88.39 ± 15.34•	
	TT	135.61 ± 23.78•	0.106‡	89.20 ± 18.25•	0.440‡
	GT	132.78 ± 23.22•		88.39 ± 15.34•	
	GG	138.48 ± 21.43•	0.239‡	94.12 ± 18.47•	0.079‡
	TT	135.61 ± 23.78•		89.20 ± 18.25•	

SNP = single nucleotide polymorphism; *AGT* = angiotensinogen gene; *GRK4* = G protein-coupled receptor kinase 4 gene; mm Hg = millimetre of mercury; P = probability value; • = standard deviation; N/A = not applicable, due to inadequate size of groups being compared; ‡ Mann-Whitney U test

Table A6.3: Genotypic association of *GRK4* SNPs (C679T and C1711T) with blood pressure in the total cohort

Gene SNP	Genotypic comparisons	Mean systolic blood pressure (mm Hg)	P	Mean diastolic blood pressure (mm Hg)	P
***GRK4* C679T (Hypertensive T; Normotensive C)**	**CC**	131.0 ± 18.65•	0.386‡	91.0 ± 15.06•	0.323‡
	CT	133.68 ± 24.24•		89.08 ± 16.86•	
	TT	135.76 ± 23.38•	0.169‡	89.59 ± 17.95•	0.468‡
	CT	133.68 ± 24.24•		89.08 ± 16.86•	
	CC	131.0 ± 18.65•	0.221‡	91.0 ± 15.06•	0.323‡
	TT	135.76 ± 23.38•		89.59 ± 17.95•	
***GRK4* C1711T (Hypertensive T; Normotensive C)**	**CC**	134.71 ± 23.27•	0.291‡	89.25 ± 17.02•	0.055‡
	CT	138.55 ± 24.53•		94.44 ± 19.85•	
	TT	103	N/A	80	N/A
	CT	138.55 ± 24.53•		94.44 ± 19.85•	
	CC	134.71 ± 23.27•	N/A	89.25 ± 17.02•	N/A
	TT	103		80	

SNP = single nucleotide polymorphism; *GRK4* = G protein-coupled receptor kinase 4 gene; mm Hg = millimetre of mercury; P = probability value; • = standard deviation; N/A = not applicable, due to inadequate size of groups being compared; † Analysis of variance F test; ‡ Mann-Whitney U test

Table A7.1: Hypertension risk associated with the genotypes of the *ADRB1* (G1165C), *AGT* (G-217A and T704C) SNPs in the total cohort

Gene SNP	Genotype	OR	95% CI	P-value
***ADRB1* G1165C (Hypertensive G; Normotensive C)**	GG	1.004	0.618 – 1.631	1.0
	GC	0.890	0.545 – 1.453	0.637
	CC	1.441	0.573 – 3.727	0.533
***AGT* G-217A (Hypertensive A; Normotensive G)**	GG	0.664	0.395 – 1.115	0.102
	GA	1.238	0.763 – 2.008	0.414
	AA	1.268	0.669 – 2.420	0.459
***AGT* T704C (Hypertensive C; Normotensive T)**	TT	N/A	N/A	N/A
	TC	1.042	0.531 – 2.054	1.0
	CC	0.960	0.487 – 1.883	1.0

SNP = single nucleotide polymorphism; *ADRB1* = β1-adrenergic receptor gene; *AGT* = angiotensinogen gene; OR = odds ratio; CI = confidence interval; P = probability value; N/A = not applicable, due to inadequate size of a group

Table A7.2: Hypertension risk associated with *GRK4* SNPs (G448T, C679T and C1711T) in the total cohort

Gene SNP	Genotype	OR	95% CI	P-value
***GRK4* G448T (Hypertensive T; Normotensive G)**	GG	1.821	0.743 – 4.598	0.177
	GT	0.935	0.573 – 1.526	0.813
	TT	0.880	0.546 – 1.415	0.646
***GRK4* C679T (Hypertensive T; Normotensive C)**	CC	0.943	0.327 – 2.782	1.0
	CT	1.113	0.674 – 1.838	0.719
	TT	0.916	0.563 – 1.488	0.727
***GRK4* C1711T (Hypertensive T; Normotensive C)**	CC	0.705	0.287 – 1.692	0.435
	CT	1.610	0.649 – 4.109	0.317
	TT	N/A	N/A	N/A

SNP = single nucleotide polymorphism; *GRK4* = G protein-coupled receptor kinase 4 gene; OR = odds ratio; CI = confidence interval; P = probability value; N/A = not applicable, due to inadequate size of a group