INVESTIGATION OF THE ETHNIC DIFFERENCES AND GENETICS OF SALT SENSITIVITY AND SALT SENSITIVE HYPERTENSION IN SOUTH AFRICA

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DECLARATION

I, Brian Lindsay Rayner, hereby declare that the research described herein was performed by me with assistance as indicated in the acknowledgements. The dissertation was written by me and reviewed by my supervisor. Neither the whole thesis nor any part thereof has been, is being or will be submitted by me for any other degree at this or any other University.

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DEDICATION:

This thesis is dedicated to my late father, and mother, Arthur and Nancy Rayner, who inspired their children to achieve academically; to my wife, Yvonne Trinder, who has supported me in my long endeavours to complete this thesis, and to my children, Michael and Nandi Rayner, who understood that their father sometimes neglected them through the pressures of completing this thesis. Lastly I would like to dedicate this thesis to the late Prof Ralph Kirsch, who mentored my academic career over a long period.

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ABSTRACT:

This thesis is a culmination of work over 15 years of a clinician scientist investigating the causes of of severe hypertension and hypertension in indigeous populations in South Africa. It all started with the recognition that many South Africans of all races had uncontrolled hypertension due to primary aldosteronism. The implementation of spironolactone in our clinic led to dramatic improvements in hypertension control. Further investigation to establish normal ranges for aldosterone and renin in indigenous populations in South Africa found that normotensive black South Africans had markedly suppressed renin and aldosterone suggesting an underlying predisposition to sodium (Na) retention. Part of this explanation was the presence of the p.Arg563Gln mutation in ENaC *(*product of the *SCNN1β* gene*)* that also resulted in severe hypertension, responsive to amiloride.

Because the frequency of this mutation was low in the normal population, it could not account for numbers of normotensive subjects with suppressed renin and aldosterone. Our attention then focused on the *GRK4* single nucleotide polymorphisms (SNPs) which had been shown to be linked to salt sensitivity and blood pressure (BP) in rats (especially those variants leading to the p.Ala142Val and p.Arg65Leu amino acid substitutions). Initial studies showed a high frequency of the TT genotypes of these variants in indigenous populations suggesting that the respective T alleles may be associated with salt sensitivity and hypertension in humans.

It was then decided to test the the *GRK4* variants, p.Ala142Val and p.Arg65Leu, against intermediate phenotypes of salt sensitive hypertension. In the first experiment the ability of black and white lean subjects to excrete Na load and their BP responses was tested. It was found that black subjects had suppressed aldosterone and failed to incrementally increase Na excretion. The p.Ala142Val genotypes (CC, CT and TT) were predictive of the ability to excrete a Na load and responses of aldosterone to saline challenge. The p.Arg65Leu polymorphism had no effect.

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Furthermore, BP responses to Na restriction and increases in dietary K, Ca and Mg in black patients with mild to moderate hypertension were assessed. The low Na diet resulted in significant reductions in ambulatory BP which were predicted by both the p.Ala142Val and p.Arg65Leu polymorphisms.

Finally a computational bio-informatics approach was used to search for other candidate genes associated with salt sensitive hypertension. A novel search engine was developed, and predicted the top 2 candidate genes to be the *angiotensin type 1 receptor* and *PTH*. The latter was a unique association but a body of literature suggested a role of the gene product, PTH, in salt sensitive hypertension in the black population. Additionally, analysis of allele frequencies of *PTH* in Caucasians and indigenous African populations in South Africa revealed significant differences suggesting a potential role for SNPs in *PTH* in salt sensitive hypertension. A full sequence analysis of the *PTH* gene revealed several unique variants not previously described, but we were unable to find an association of these SNPs with either salt sensitive hypertension or essential hypertension.

In conclusion, in this thesis an important role of the *GRK4* SNPs in salt sensitivity in indigenous Southern Africans was demonstrated. They were not only predictors of natriuretic responses to saline challenge but also BP response to dietary intervention. *PTH* was found to be a strong candidate gene for salt sensitive hypertension using a bioinformatics approach, but this could not be confirmed in a full analysis of the *PTH* gene.

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ABBREVIATIONS:

i) BMI – body mass index
ii) BP – blood pressure
iii) Ca – calcium
iv) CV - cardiovascular
v) ENaC – epithelial sodium channel
vi) GLM – generalised linear model
vii) GRK4 – G-protein-coupled receptor Kinase-4
viii) K – potassium
ix) Mg – magnesium
x) MRC – Medical Research Council
xi) Na – sodium
xii) PA - primary aldosteronism
xiii) PCR – polymerase chain reaction
xiv) PRA - plasma renin activity
xv) PTH – parathyroid hormone
xvi) RAAS – renin-angiotensin-aldosterone system
xvii) SNP – single nucleotide polymorphism
xviii) UCT – University of Cape Town

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CHAPTER 1

KEY ETHNIC DIFFERENCES IN THE PATHOGENESIS OF ESSENTIAL HYPERTENSION

What is essential hypertension?

Essential hypertension is a chronic disorder with a poorly understood pathogenesis resulting from a complex interplay between genetic and environmental (including lifestyle) factors. Renal sodium (Na) handling, ionic transport mechanisms, the renin-angiotensin-aldosterone system (RAAS), vasoactive substances, the autonomic nervous system, diet, obesity, and stress are all potentially implicated. However BP is a heretable trait; a family history is common in patients with hypertension (Morrison et al. 2004; Wilk et al. 2004). Heritable estimates of systolic and diastolic BP for identical twins is reported to be as high as 0.40 and is similar between Caucasians and African Americans (Weder 1995). In the first 'Demographic and Health Survey' in SA, the risk factors for hypertension included education level (specifically less than tertiary level), older age, overweight and obesity, excess alcohol use, and a family history of hypertension and stroke, which supports the concept of genetic-environmental interaction (Steyn et al. 2001).

Epidemiology

Essential hypertension (and its complications) is a major health burden in Africa (Seedat 2001). It is an established risk factor for cardiovascular (CV) disease, including stroke, heart attack, coronary artery disease and kidney disease. In South Africa, the 1st 'Demographic and Health Survey' conducted on 13 802 randomly selected individuals revealed prevalence rates of 21% for hypertension, using a cut-off point of 140/90 mmHg (Steyn et al. 2001). Worldwide, hypertension affects 1 billion people and is implicated in 7.1 million deaths annually from stroke and ischaemic heart disease (www.who.int/en/index.html). A relatively recent major global publication has suggested that there is a hidden and burgeoning epidemic of CV disease in developing countries (Leeder et al. 2005). In South Africa, a cluster of CV diseases, namely stroke, ischaemic heart disease

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and hypertensive heart disease account for three of the top ten causes of mortality, and CV disease accounts for 17% of all deaths (Bradshaw et al. 2003).

Meta-analysis of findings from studies involving both American and non-American blacks and whites confirmed that blacks have a higher systolic and diastolic blood pressure (BP) than whites, both at night and during the day (Profant et al. 1999). In the USA and South Africa, blacks had a higher prevalence of hypertension than whites living in the same areas (Seedat 1996a). The Centres for Disease Control (CDC) published results from a study conducted from 1999-2002, which showed the prevalence of hypertension in the study group to be 28.6% (CDC 2005). Of this percentage of hypertensives, 40.5% were black and 27.4% were white. BP has also been reported to rise with age across all urban racial groups (CDC 2005; Seedat 1983).

Population specific differences

i) Renal Na handling

Epidemiological studies demonstrate that the prevalence of hypertension and its associated CV consequences are directly related to the level of dietary Na intake in societies throughout the world, in whom the intake is above a level of 50–100 mmol/day (MacGregor 1985). In societies where habitual intake is below that range, hypertension is rare. It is also evident that salt plays a role in the age-related increase in BP (Prior et al. 1968). Cross-sectional observations demonstrate that such age-related increases in BP are most commonly observed in 'industrialized' societies, i.e. those featuring habitual Na intake >120 mmol/day (MacGregor 1985). By comparison, in societies in which the usual salt intake is much lower, such age-related increases in BP have not been reported (MacGregor 1985).

In addition, it has been observed that a subgroup of individuals retains more Na and undergo a greater rise in BP, in response to high salt intake. This is termed salt-sensitivity. For both normotensives and hypertensives, the BP response of blacks to Na loading is more salt-sensitive (Dustan et al. 1998; Falkner et al. 1990; Parmer et al.

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1994), and there is a reduced ability to excrete a Na load as compared to whites (Luft et al. 1977; Brier and Luft 1994). Na retention is perhaps an adaptive mechanism in people who originally came from hot climates where salt was a scarce resource. As diets are now abundant in Na, this mechanism would be maladaptive and would result in an increased extracellular fluid volume and hypertension, but this has proved difficult to demonstrate definitively (Mitas et al. 1979; He et al. 1998).

In experimental models, kidney transplantation from hypertensive to normotensive rats caused hypertension in the recipient, and vice versa (Rettig et al. 1996). This strongly suggests that hypertension may stem from the kidneys since the previously normotensive rats became hypertensive. In humans undergoing renal transplantation, there is an increased chance of developing hypertension if there is a history of hypertension in the donor's family (Beevers et al. 2001). Since the kidney is the main site for Na handling, changes in Na handling by the kidney may well be a causal factor in the pathogenesis of essential hypertension.

According to the Guyton hypothesis, there is a relationship between Na intake and BP called the pressure-natriuresis relationship (Guyton 1987). He postulated that changes in the pressure-natriuresis relationship always participate in the genesis of hypertension regardless of the initiating event (Guyton 1989). Kimura and colleagues (1991) proposed 3 major renal mechanisms for the development of hypertension: an increase in pre-glomerular vascular resistance, a decrease in whole kidney ultrafiltration, and increased tubular Na reabsorbtion. The first mechanism would lead to salt-resistant hypertension, and the latter two to salt-sensitive hypertension.

Chiolero and co-workers (2000) investigated the relationship of proximal tubular Na reabsorbtion in 38 hypertensives and 27 young normotensives. They used the technique of fractional excretion of lithium (FE_{Li}), which has a close relationship to FE_{Na} and is a marker of proximal tubular reabsorbtion of Na. The subjects and patients were studied on low and high Na diets. In the control group, changing from a low to high Na diet resulted in no change in BP, but increased the glomerular filtration rate, renal blood flow, and

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FE_{Li}. In contrast, in hypertensives, comparable changes in Na intake induced an increase in BP with no changes in renal haemodynamics and FE_{Li}. BP responses correlated positively with age and negatively with FE_{Li}. These results suggest that proximal tubular Na reabsorbtion is an independent determinant of BP response to Na. Additionally, they found that renin and aldosterone levels were unrelated to FE_{Li}, and therefore other hormonal systems or Na reabsorbtion in the kidney *per se* must be responsible for these observations (Chiolero et al. 2000). In a family study of black South Africans and white Belgians, segmental Na reabsorbtion along the nephron was found to be highly heritable; it was also observed that after a Na load, black participants reabsorb more Na than whites in the proximal tubule, and less post proximally (Bochud et al. 2009).

Several other lines of evidence, however, support this hypothesis. There are differences in the RAAS between blacks and whites. For the majority of normotensive and hypertensive South African blacks, plasma levels of renin and aldosterone levels are significantly lower than in whites (Rayner et al. 2001a; Sagnella 2001), which is independent of salt intake. The lower plasma renin activity appears to be as a result of a reduction in renin release (Sagnella 2001) due to the inhibition of the RAAS through negative feedback.

Additionally, in black patients, plasma renin levels do not seem to increase in response to Na and volume depletion (He et al. 1998; Hoosen et al. 1985). Sagnella (2001) also found that black hypertensives have a greater reduction in BP in response to short-term Na restriction. These results suggest that the RAAS in black hypertensives (and normotensives) is suppressed in response to Na retention by the kidney. In addition suppressed plasma renin activity is an index of salt sensitivity in experimental models (Tang JI, et al. 2011). The most likely explanation for this is genetic differences between blacks and whites, although environmental factors cannot be excluded.

ii) Genetic factors

The heritability of BP is high in all population groups. In a genome wide scan undertaken in subjects with early onset hypertension (<35 years for African-Americans

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and < 45 years for whites) the heritability of 'age at diagnosis' was higher for African Americans compared to whites (0.42 versus 0.35) (Wilk et al. 2004). Genome wide association studies (GWAS) have identified candidate loci in non-obese African Americans, which include loci on chromosomes 2 (Morrison et al. 2004), 4 and 15 (Wilk et al. 2004). In addition, the same locus on chromosome 4, which predicted early onset hypertension (<35 years) in African Americans spans the *mineralocorticoid receptor* gene (Wilk et al. 2004. Kotchen et al. (2002) performed GWAS on 150 African American sib pairs concordant for hypertension. Significant linkage was found with BMI to loci on chromosomes 1 and 8, and for the ratio of extracellular water to total body water, to loci on chromosomes 3, 5, 6 and 7 (Kotchen et al. 2002). The ratio of extracellular to total body water correlated to ambulatory BP in both hypertensives and normotensives.

However, most efforts to identify genetic loci for hypertension-predisposition have focused on examining candidate genes that were tested for linkage and/or association with either BP levels or the diagnosis of hypertension. Numerous candidate genes have been investigated, with most belonging to the RAAS, which is principally involved in BP regulation and water-electrolyte homeostasis. Other candidates examined include the genes for the epithelial sodium channel (ENaC), the renal kallikrein-kinin system, catecholaminergic/adrenergic function, alpha-adducin, G-proteins, endothelin, atrial natriuretic peptide and nitric oxide (Worthington et al. 1993; Timberlake et al. 2001; Beeks et al 2004). These studies have yielded promising results suggesting a number of potential candidate gene/regions which may contribute to BP variation and ethnic differences.

1. Renal epithelial sodium channel (ENaC) and other Na channels in the kidney

The renal ENaC, located within the collecting duct is one of the most important transport mechanisms in the overall control of Na balance, even though it only accounts for 5% of renal Na absorbtion. It is responsible for the final adjustment of Na excretion, being the most distal site in the kidney for Na reabsorbtion.

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In the rare autosomal dominant disorder, Liddle's syndrome (OMIM: 177200), mutations in the genes encoding for the γ- subunits (*SCNN1G)* or β-subunits (*SCNN1β*) of the ENaC, result in increased channel activity. This leads to Na retention, hypokalaemia, hypertension, and suppression of both renin and aldosterone (Rossier and Schild 2008). This syndrome has similarities with hypertension in blacks, and mutations in the ENaC subunits are strong candidates to account for observed differences in hypertension between blacks and whites (Rossier and Schild 2008).

Genetic variants of the ENaC subunits have been shown to be far more frequent in patients of African descent (Persu 2003). The NM_000336.2:c.1781C>T (p.Thr594Met) variant (*rs1799979*) of *SCNN1β,* identified by Baker et al. (1998), is associated with low plasma renin activity (PRA) and has been shown to be significantly more common in hypertensive than normotensive blacks living in London. However, the prevalence of this polymorphism is very low, even in black subjects, and other studies in South Africa have found no significant role for this variant in the development and severity of hypertension or the RAAS profiles of black patients (Nkeh et al. 2003; Tiago et al. 2001).

The NM_000336.2:c.1325G>T (p.Gly442Val) polymorphism (*rs16940050*) in *SCNN1β* is also frequently found in black patients, and has been associated with a decreased urinary aldosterone/K^+ ratio – indicating increased ENaC activity. However no association has been demonstrated with hypertension (Ambrosius et al. 1999). In the GenSalt Study from China common variants of the ENaC were associated with salt sensitive hypertnension. (Zhao Q, et al. 2011). Furthermore our group described the NM_000336.2:c.1688G>A (p.Arg563Gln) polymorphism (*rs149868979*) of the *SCNN1β* gene, which was associated with low renin, low aldosterone hypertension in South African black and mixed ancestry patients, but not white patients (Rayner et al. 2003). This will be discussed in more detail, in Chapter 2.

On the other hand, Pratt et al. (2002) suggested that ENaC activity is actually lower in blacks due to Na retention occurring in the proximal tubule due to increased activity of the Na-K-2Cl co-transporter in the thick ascending limb of Henle or the thiazide sensitive

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Na-Cl transporter in the distal convoluted tubule. This suppresses aldosterone, which in turn suppresses ENaC activity (Pratt et al. 2002).

More recently, Tobin et al. (2008) investigated several monogenic forms of hypotension and hypertension, through the evaluation of common variants in the relevant genes, and their effect on BP in 2037 adults from 520 nuclear families. For instance, the renal outer-medullary potassium channel (ROMK) mediates potassium secretion and regulates NaCl reabsorbtion in the kidney. Reduced activity of the channel results in Barter's syndrome manifested at birth by severe Na wasting, hypotension, and markedly elevated renin and aldosterone levels (Gross and Henduchka 2010). Overactivity of this channel may conversely result in Na retention, and hypertension. Similarly, overactivity or underactivity of the ENaC results in Liddle's syndrome (hypertension and low potassium) and pseudohyperaldosteronism type 1 (hypotension and high potassium) (Gross and Henduchka 2010).

Five polymorphisms (namely, *rs2855800, rs2846679, rs2186832, rs675759, rs675388),* in *KCNJ1* coding for the ROMK channel were associated with mean 24 hour systolic and diastolic BP (Tobin al. 2008). Additional associations were seen with variations in *CASR* (Barter syndrome 5), *NR3C2* [autosomal dominant pseudohypoaldosteronism type 1 (PHA1) and hypertension exacerbated by pregnancy], *SCNN1β* and *SCNN1G* (Liddle's syndrome and autosomal recessive PHA1). All these genes are responsible for Na regulation within the kidney. These observations stress the importance of Na regulation by the kidney in the control of BP, and the effect of common variants on both hypertension and hypotension.

2. Renin-angiotensin-aldosterone system (RAAS*)*

The RAAS is a critically important endocrine system that regulates BP and Na balance, and mutations in certain genes are known to cause autosomal dominant Mendelian forms of hypertension (figure 1.1) through well understood physiological mechanisms, all related to Na retention. Variants in these genes are therefore potential candidates for the development of hypertension. Because of their potential influence on Na balance, renin and aldosterone could account for differences in hypertension between blacks and whites,

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especially if there are population specific differences in allele frequencies of polymorphisms in candidate genes comprising the RAAS.

Polymorphisms in *angiotensinogen* (*AGT)* have been associated with angiotensinogen expression in Nigerians, but not hypertension (Rotimi et al. 1994). Tiago et al. (2002) showed that the NM_000029.3:c.803T>C (p. Met268Thr) variant (*rs699*) in *AGT* associated only with hypertension in South African blacks with a BMI greater than 27 kg/m^2, possibly emphasising the importance of the environment on phenotypic expression. In contrast, Larson et al. (2000), found no association with the variant and hypertension in African Americans, even when stratified for gender and BMI. Thompson et al. (2004) showed that the *AGT*:c.803T>C (p. Met268Thr) variant had a marked geographical variation related to distance from the equator, suggesting that this variant is important in salt homeostasis and that it might be a target for specific environmental pressures.

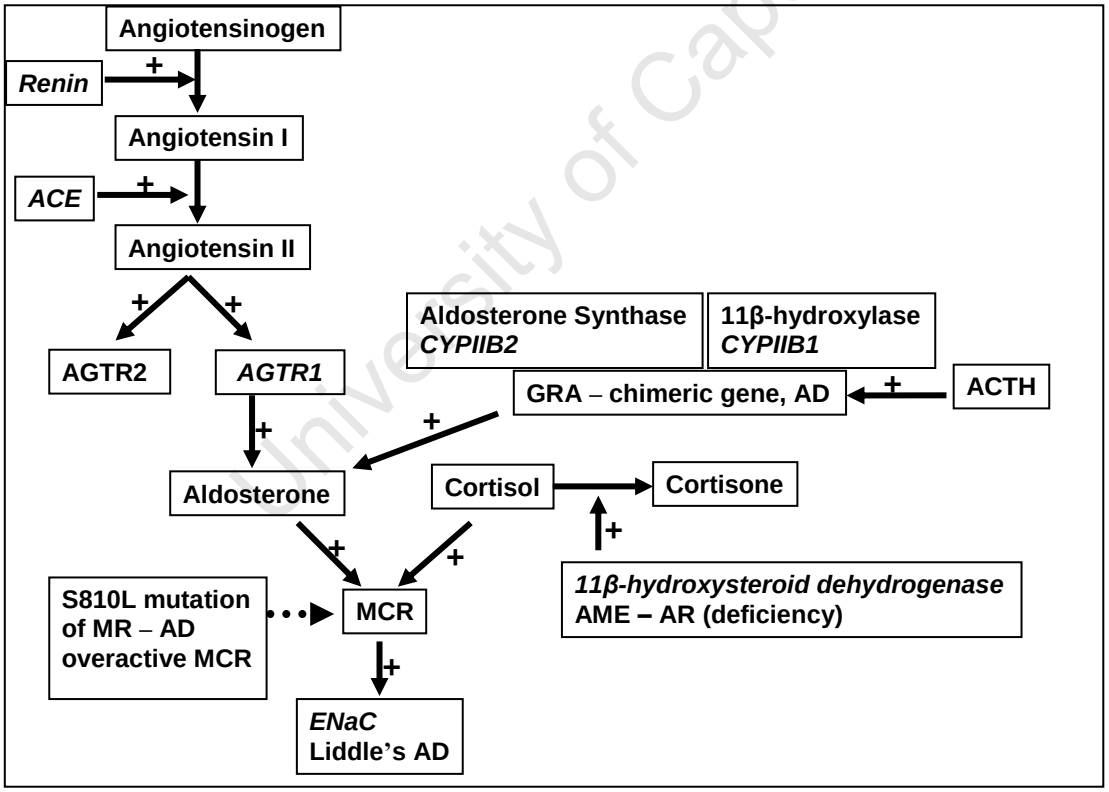

Figure 1.1 The RAAS and known Mendelian autosomal dominant causes of hypertension. ACE, angiotensin converting enzyme; AGTR1, angiotensin receptor 1; AD, autosomal dominant; AR, autosomal recessive; GRA, glucocorticoid remedial aldosteronism; ACTH, adrenocorticotropic hormone; MCR, mineralocorticoid receptor; AME, apparent mineralocorticoid excess; +, stimulation; - inhibition

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Zhu et al. (2003) analysed 192 African-American and 153 European-American families to evaluate the contribution of variations in genes that encode the RAAS components. There were significant associations between hypertension and haplotypes across the genes for the *renin angiotensin II type 1 (AGTR1)* receptor and to a lesser extent for *ACE* in African Americans. Henderson et al. (2004) found an association between the T allele of NM_000498.3:c.-344T>C (*rs1799998*) in *CYP11B2* (aldosterone synthetase) and the (-535) T allele of *AGTR*, and an increased risk of hypertension in African Americans. No association with polymorphisms of the *ACE* gene and hypertension was found in the study population.

Barbato et al. (2004) also linked the T allele of *CYP11B2:*c.-344T>C (*rs1799998*) with hypertension and plasma aldosterone levels in a multi-ethnic population, but not specifically to ethnic differences. However, the significantly lower frequency of the C allele in African Americans suggested the T allele would result in a greater propensity to hypertension in this population group.

Genotype and allele frequency of variants regulating the RAAS have recently been determined in African Americans, Caucasians, Japanese and Hispanic populations. Compared to Caucasians, African Americans had significant differences in frequency for variants in *AGT, Renin, ACE, AGTR1* and *CYP11B2* (Chapman et al. 2005). However, the manner in which these differences relate to suppressed renin and aldosterone in black normotensives and hypertensives is unknown. A systematic review of the genetic predisposition to salt sensitivity by Beeks et al. (2004) found only the NM_014189.3:c.1378G>T (p.Gly460Trp) variant (*rs4961*) of the *ADD1* gene (see below) linked to Na sensitivity, but none of the polymorphisms in the RAAS were linked to this phenotype. The review was hampered by the lack of standardisation for the testing of salt sensitivity. In an analysis of candidate genes *(AGT, ACE, AGTR1* and *alpha 1-antichymotrypsin)* causing hypertension in African American adults, Whitfield et al. (2009) reported only the *ACE* SNP NM_000789.3:c.-262T>A (*rs4291*) associated with hypertension.

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3. Atrial Natriuretic Peptide

Atrial Natriuretic Peptide (ANP) is produced mainly in the cardiac atria in response to increased blood volume and atrial stretch and causes renal vasodilatation, increased Na excretion and suppression of the RAAS (Oparil 1995). A reduction in ANP secretion or function could result in Na retention and salt-sensitive hypertension. In a study involving mice, disruption of the pro-*ANP* gene resulted in the development of salt-sensitive hypertension (John et al. 1995).

Human studies have shown that hypertensive subjects have low to normal plasma ANP levels (Schiffrin et al. 1988; Talartschik et al. 1990). Kohno et al. (1987) found that Na loading increased plasma ANP more in salt-sensitive than salt-resistant patients, but in other studies there was a smaller increase, or even a decrease, in plasma ANP levels in response to a high Na diet in salt-sensitive compared to salt-resistant patients (Schiffrin et al. 1988; Nimura et al. 1991). Reduced atrial secretion of ANP during Na loading could partly explain their reduced ability to excrete Na and the Na-induced rise in BP. Several studies have suggested association of polymorphisms in *ANP* genes with hypertension (Marteau et al. 2005), but linkage with salt-sensitive hypertension has not been established (Beeks et al. 2004). In contrast, Nkeh et al. (2002) showed that the C1364A polymorphism in intron 2 of *ANP* may confer protection against hypertension in a cohort of indigenous black South Africans. More recently, two SNPs, *rs5068* and *rs198358,* were associated with increased levels of atrial and B-type natriuretic peptides and with reduced systolic and diastolic BP in 29,717 subjects of European ancestry (Newton-Cheh et al. 2009).

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4. ADD1

Adducin is a heterodimeric protein composed of 3 different subunits - α, β, and γ, which are encoded by the *ADD1, ADD2* and *ADD3* genes, respectively (Marteau et al. 2005). Adducins are involved in cell membrane ion transport and mutations have been associated with salt-sensitive hypertension in a Milan hypertensive strain of rat (Marteau et al. 2005). However, the relationship to hypertension has been inconsistent. The *ADD1*: c.1378G>T polymorphism has been related to salt sensitivity and BP response, and

occurs more frequently in African American populations (Chapman et al. 2005). Larson et al. (2000) could find no association with hypertension in a study of 905 subjects of African American origin. In contrast, Barlassina et al. (2000) found the *ADD1*: c.1378G>Tpolymorphism at a very low frequency in black South Africans, although the odds ratio for hypertension was 2.68. In a functional study using saline loading, Manunta et al. (2008) showed that the *ADD1*: c.1378G>Tpolymorphism caused increased proximal tubular reabsorbtion of Na and caused a greater rise in BP.

5. Kallikrein

This enzyme is responsible for the synthesis of diuretic and renal vasodilating kinins (Symposium 1988) and there is evidence suggesting that it may directly promote the conversion of inactive renin to active renin (Sealey et al. 1978). Black normotensives and hypertensives have a lower urinary kallikrein than whites and may be the hint for a possible mechanism in salt sensitivity (Kailasam et al. 1998; Zinner et al. 1976). However there have been no studies linking genetic changes in the kallikrein system to hypertension.

6. Endothelin

Studies have shown that plasma concentrations of endothelin-1 (ET-1) are increased in hypertensive black patients (Ergul et al. 1996). ET-1 is a vasoconstrictor, but also promotes Na retention (Allcock et al. 1998; Ohichi et al. 1999). Campia et al. (2004) suggested that there is increased ET-dependent vasoconstriction in black hypertensives, compared to white hypertensives, and that there was either increased production or reduced clearance of ET-1. However, neither *endothelin*/*endothelin-converting enzyme* nor *endothelin-receptor* genes have been linked to salt-sensitive hypertension (Beeks et al. 2004).

7. Ionic transport mechanisms and endogenous digitalis-like factor

There are several lines of evidence to suggest that intracellular Na and Ca concentrations are raised in both normotensive and hypertensive blacks compared to whites (Blaustein 1977; Seedat 1983; Aviv and Gardener 1989; Touyz et al. 1993; Kaplan and Ram 1994;

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Milne 2003). However, these findings are tentative as Worthington et al. (1993) found that black hypertensives had normal levels of intracellular Ca. Blaustein proposed that the combination of raised intracellular Na and Ca concentrations cause vasoconstriction of vascular smooth muscle, and in turn raises peripheral vascular resistance and BP. Milne (2003), on the other hand, hypothesised that the Na and Ca overload would cause swelling of the vascular smooth muscle cells, reduction in luminal size and elevation of peripheral vascular resistance. The pathogenesis of the elevated intracellular Na and Ca is probably related to a reduction in Na-K ATPase activity in the cellular membrane. In several studies using erythrocytes and platelets from black and white normo- and hypertensives, reduced ATPase activity was demonstrated in black hypertensives (Milne 2003).

There is some evidence that a digitalis-like factor (or endogenous ouabain) may be responsible for reduced Na-K-ATPase activity in blacks, but its exact biological role is uncertain (Seedat 1997; Mc Dougal and Yates 1998). However, in experimental models of cultured renal cells, the Na-K–ATPase is the driving force of proximal renal tubular Na reabsorbtion (Goto et al.1988), and ouabain is likely to decrease this activity. In contrast, Manunta et al. (2008) showed that patients with high vs. low endogenous ouabain concentrations had increased proximal tubular reabsorbtion of Na.

Touyz et al. (1993 speculated that decreased intracellular Mg may cause depression of Na-K-ATPase activity, since ATP requires Mg in order to function. It has been reported that diets of urban blacks are generally low in Ca and Mg (Akinkugbe et al. 1985; Kaplan and Ram 1994). Other perturbations in transport mechanisms have been observed in black hypertensives. Reduced activity of the sodium-lithium countertransporter and increased Na-H countertransport has been demonstrated (Gillum 1979; Smith et al. 1988; Aviv and Gardener 1989). Increased Na-H countertransport has been associated with decreased expression of *SLC9A3R1* (an inhibitory protein of the Na-H countertransporter) (Kobayashi et al. 2004).

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It is implied that these differences may account for the differences in intracellular ionic concentrations and, thus, for the differences in BP between blacks and whites (Kaplan and Ram 1994). However, this has not yet been confirmed *in vivo* or *in vitro,* nor has the mechanism ever been sufficiently explained. Sagnella (2001) questioned the role of these pumps. Firstly, the Na-K-ATPase is also located in the basolateral membrane of the renal tubular epithelium, and is the driving force for Na reabsorbtion in the kidney. Reduced activity in the renal tubular epithelium is unlikely to cause increased Na reabsorbtion. Secondly, the functional effect of endogenous digitalis-like factor is conflicting and, thirdly, there is little known about the functional significance of the Na-Li countertransporter and the effects of reduced activity (Sagnella 2001). It is likely that these differences are related to genetic factors. However, there are no studies associating differences with polymorphisms in the genes coding for these channels in different ethnic groups.

8. Nitric oxide

Nitric oxide (NO) is an important regulator of endothelial function and vascular tone. Healthy black normotensives have reduced NO-mediated vasodilation, which could indicate impaired vascular smooth muscle relaxation, leading to increased vascular tone and possibly hypertension (Stein et al. 1997; Cardillo et al. 1999). In humans, the NM_001160109.1:c.894T>G (p. Asp298Glu) variation (*rs1799983*) in the *nitric oxide synthetase (NOS3)* gene has been associated with hypertension, but this has been inconsistent between Caucasian and Japanese populations (Marteau et al. 2005). As with genes that regulate the RAAS, common variants in the *NOS3* are frequently observed in African Americans, which could potentially contribute to endothelial dysfunction and hypertension (Chapman et al. 2005).

9. Autonomic nervous system (ANS)

The ANS is a critical regulator of cardiac output, vascular tone, Na retention and BP. To date there are no significant ethnic differences in circulating catecholamines (Rowlands et al. 1982; Pratt et al. 1992). However, it has been shown that blacks are more sensitive to the vasoconstrictor effects of noradrenaline and that this response is enhanced by a

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high Na diet (Yang et al. 1990). Studies have revealed lower levels of plasma dopamine β-hydroxylase in blacks, which may impair Na excretion in the kidney (Berenson et al. 1979a; O'Connor et al. 1983).

Blacks, however, have a reduction in β_2 receptor-mediated vasodilatation, which may lead to greater peripheral resistance and increased BP (Lang et al. 1995; Watkins et al. 1995). Maxwell (2005) studied 3 functional polymorphisms in codons 16, 27 and 164 of the *β_2 receptor* gene *(ADRB2)* in 726 individuals from 8 distinct ethnic populations (Chinese, Filipino, Southwest Asian, Saudi, Ghanaian, Kenyan, Sudanese, and European from Scotland). The results showed marked differences and frequencies of haplotypes. However, it is uncertain how these variants are linked to the activity of the receptor. In a study from South Africa, there was no association of the E27 *ADRB2* genotype with hypertension in black South Africans (Candy et al. 2000). In a study from the USA, the NM_000024.5:c.46A>G (p. Arg16Gly) variant (*rs1042713*) of the *ADRB2* gene were not associated with hypertension in African and European Americans (Xie et al. 2000). However Cui et al. (2003) showed that a SNP in the promoter region of the *phenylethanolamine N-methyl transferase* gene (which codes for an essential enzyme in the synthesis of noradrenaline) was associated with hypertension in African Americans, and not in European Americans.

10. Dopamine and G-protein-coupled receptors

i) Dopamine:

The catecholamines, dopamine and norepinephrine, are synthesized from the same precursors, the amino acid tyrosine and its hydroxylated product L-DOPA. In the kidney, dopamine is mainly synthesised in the proximal tubules by decarboxylation of L-DOPA (figure 1.2, Zeng et al. 2004) which is transported to the tubules from the circulation. The renal tubules are unable to synthesise L-DOPA due to lack of tyrosine hydroxylase. The proximal tubules lack dopamine β-hydroxylase, and there is no conversion to noradrenaline. L-DOPA is metabolised to homovallinic acid. Dopamine receptors are present on both the basolateral and apical membranes.

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Dopamine secreted by the tubules acts in an autocrine/paracrine fashion to regulate Na transport in the renal proximal tubule and thick ascending loop of Henle, and the jejenum (Aperia 2000). Under conditions of Na excess, locally generated dopamine acts on the renal tubular cells to reduce Na transport and promote natriuresis, and the jejenal cells to reduce Na absorbtion.

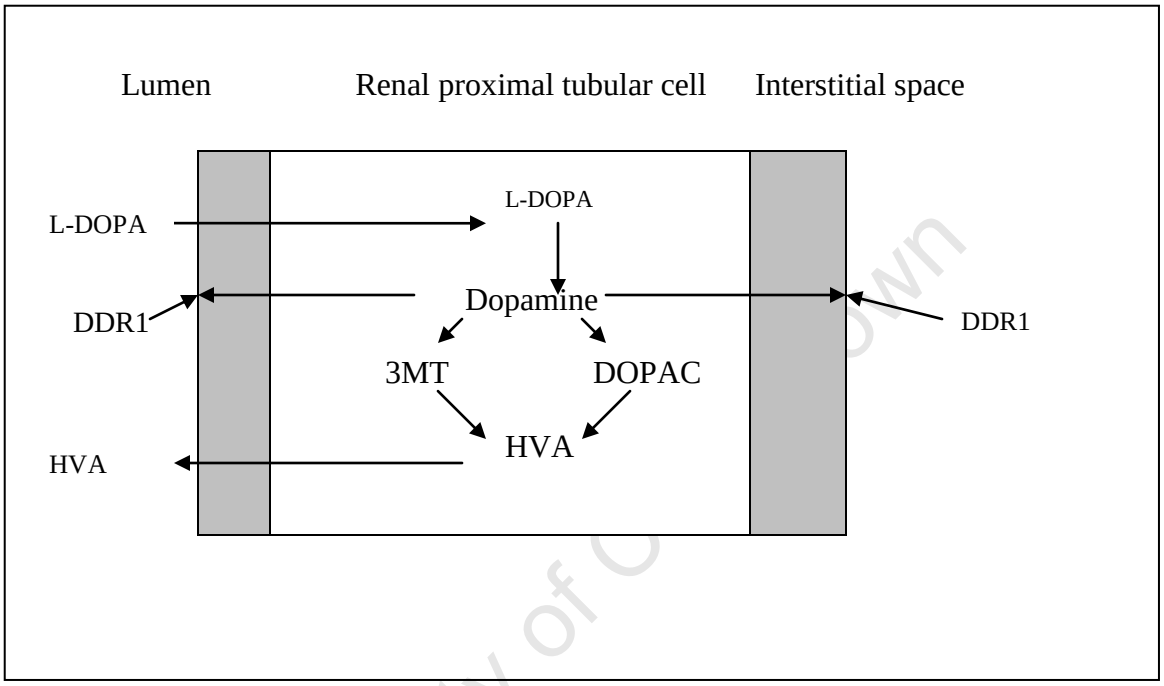

Figure 1.2 Metabolism of dopamine in the kidney (adapted from Zeng et al, 2004)

3MT = 3-methoxytyramine, DOPAC = dihydroxyphenol acetic acid, HVA = homovallinic acid, DDR1 = dopamine receptor 1

Dopamine inhibits renal Na transport by inhibiting the Na-H exchanger, 3 (NHE3) and Na/phosphate co-transporter in apical membranes, and Na/HCO_3^- co-transporter and NaK-ATPase in basolateral membranes in the proximal tubule and thick ascending loop of Henle (Figure 1.3). Renal dopamine is responsible for over 50% of the incremental Na excretion in response to increased intake (Jose et al. 2003).

ii) Dopamine receptors

The dopamine receptors are distributed widely in the kidney. They belong to a super family of G protein-coupled receptors divided into D_1-like (D_1 and D_5 subtypes) and D_2-like receptors (D_2, D_3 and D_4 subtypes). The former are linked to the stimulatory G-

protein, and stimulate adenyl cyclase and protein kinase A (PKA), which inhibits Na reabsorbtion (Figure 1.3). In contrast, D2-like receptors inhibit adenylyl cyclases, Ca channels and K channels (Aperia, 2000).

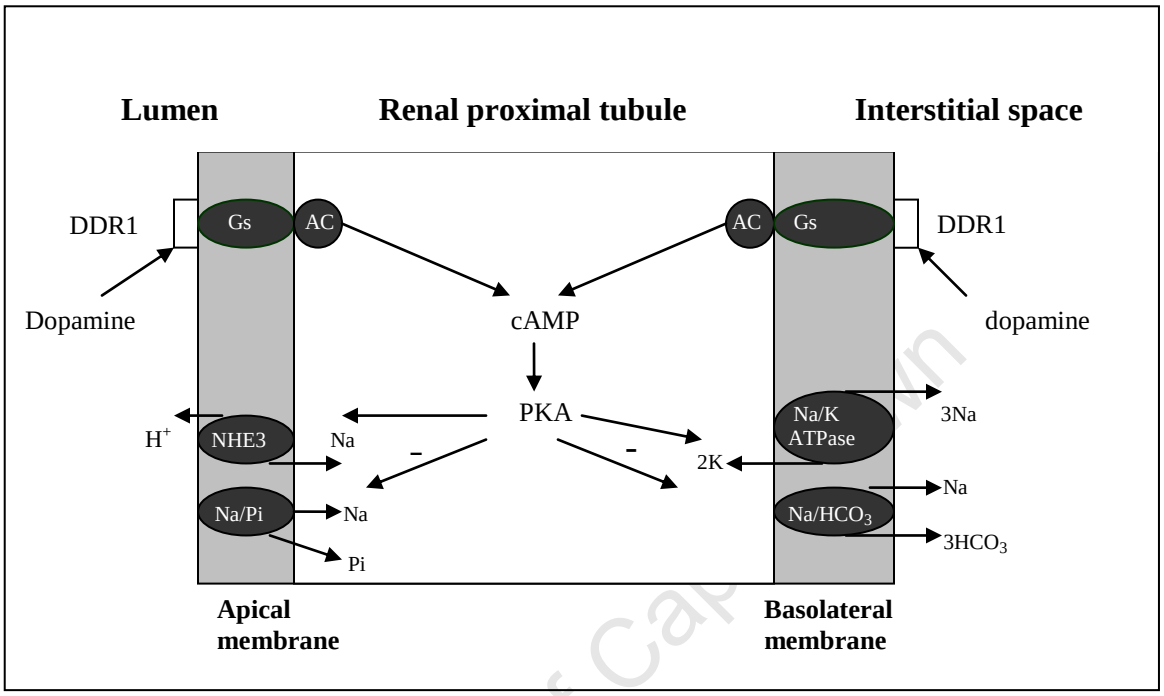

Figure 1.3. Effects of stimulation of D_1 receptors in the kidney (adapted Zheng et al. 2004)
DDR1 = dopamine receptor 1, Gs = stimulatory G protein, AC – adenylyl cyclise, cAMP = cyclic adenine monophosphate, NHE3 = Na-Hydrogen exchanger 3, Na/Pi = Na/phosphate cotransporter, Na/HCO3 = Na/HCO_3 cotransporter, PKA = protein kinase A

During conditions of Na loading, the D_1-like receptors act to increase renal Na excretion (Aperia 2000). In addition, water permeability in the proximal tubule is bi-directionally regulated. The antidiuretic effects of arginine vasopressin are opposed by several hormones, including dopamine, most likely via the DRD_4 receptor (Aperia, 2000).

iii) Regulation of Na in the proximal tubule

Dopamine interacts with ANP, angiotensin II and α adrenergic receptors in the proximal tubule to maintain Na homeostasis (Jose et al. 2003). The model for bidirectional regulation of tubular Na transport is shown in figure 1.4.

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There is evidence that dopamine opposes the anti-natriuretic effect of ANG II, both in the short and long term. It stimulates the activity of proximal tubular Na/K ATPase, which is abolished by dopamine or its messenger cAMP. In addition, dopamine reduces angiotensin 1 receptor mRNA synthesis (Aperia 2000). In fish, dopamine is the predominant catecholamine; but in the terrestrial environment, where Na is a relatively scarce resource, noradrenaline predominates and is essential in the regulation of the anti-natriuretic forces by stimulating the Na/K ATPase in the proximal tubule (figure 1.3). Dopamine opposes the action of noradrenaline. ANP is an important natriuretic hormone but it requires the presence of dopamine receptors for its full effect (figure 1.4). The inhibitory effect of dopamine on the Na/K ATPase is potentiated by ANP.

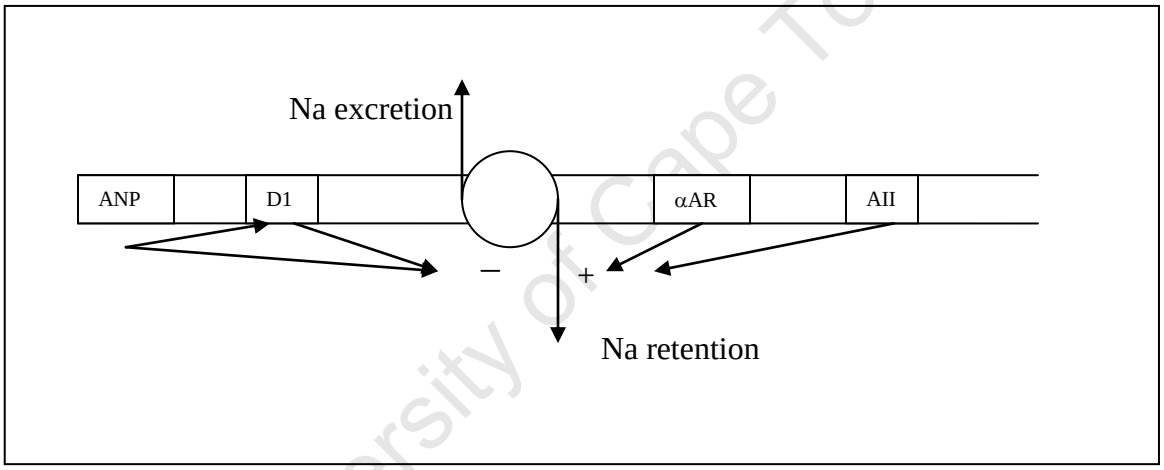

Figure 1.4. Bidirectional regulation of proximal tubular Na (adapted from Aperia (2000) ANP =atrial natriuretic peptide, D = dopamine, αAR = α adrenergic receptor, AII = angiotensin II

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The effects of dopamine in the kidney provide a physiological model for salt-sensitive hypertension. Any defect in the *DDR1* itself, or in the stimulatory G proteins will result in unopposed action of ANG II and noradrenaline on the proximal tubule, resulting in a pronounced antinatriuretic effect (Aperia 2000). In addition the effect of ANP will be impaired.

iv) Renal dopamine receptor defect in hypertension

The dopamine receptor 1 (DDR1) agonist, fenoldopam, increases cAMP in the proximal tubules of the kidney in normotensives, but is markedly impaired in cells from

hypertensive subjects, even though baseline levels are identical (Zeng et al. 2004). In mice, disruption of the *DDR1* increases both systolic and diastolic BP. In humans, a polymorphism in the non-coding region of *DRD1*, the NM_000794.3:c.-48a>g *DRD1* variant has been reported to be in association with essential hypertension in a Japanese population (Jose et al. 2003). However, there are no differences in mRNA or protein expression of the *DRD1* in renal proximal tubules in animal models of genetic hypertension, or in the proximal tubules from humans with essential hypertension (Jose et al. 2003). This suggests that a defect in the *DDR1* is not a likely candidate for salt-sensitive hypertension in humans.

v) Role of G protein-coupled receptor kinase and protein phosphatase

The uncoupling of the *DDR1* from its G protein effector complex is similar to a desensitisation process (Jose et al. 2002). This is a mechanism which protects the cell from repeated agonist exposure. Desensitisation involves several processes including phosphorylation, sequestration and degradation of receptor protein. The initial step in this process (phosphorylation) is mediated by a member of the G protein-coupled receptor kinase (GRK). GRKs are serine and threonine kinases that phosphorylate G protein-coupled receptors in response to agonist stimulation (Jose et al. 2002). The phosphorylation of G protein-coupled receptor kinases, including *DDR1*, leads to binding with members of the arrestin family of proteins that uncouple the receptor from its G protein, and reduction in the functional response (Jose et al. 2002).

vi) The role of GRK4

In hypertension there appears to be constitutive desensitisation of the renal *DDR1* but not the D_5R (Jose et al. 2003). This appears to be caused by GRK as decreasing its activity or expression in the proximal tubular cells in hypertensives normalises the ability of *DDR1* to increase cAMP. In the proximal tubule, GRK4 is the most important component, compared to other GRKs. GRK4 activity is increased in spontaneously hypertensive rats, and infusion of GRK4 antisense oligonucleotides attenuates the increase in BP (Sanada et al. 2006a). This functional defect is replicated in the proximal tubule by expression of *GRK4* variants in cell lines, and this is rectified by prevention of

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GRK4 expression (Felder et al. 2002). These three tested variants are the NM_001004057.1:c.194G>T, p.Arg65Leu (*rs2960306*), c.425C>T, p.Ala142Val (*rs1024323)*, and c.1457T>C, p.Val486Ala (*rs1801058*), with the p.Ala142Valshowing the most activity (Felder et al. 2002).

vii) *GRK4* Variants and Hypertension in Humans

Dopamine may reset the proximal tubule to ANG II. An impairment of dopaminergic function as evidenced by the *GRK4* variants in animal models would allow the antinatriuretic function of ANG II to be unopposed, which may then be responsible for salt sensitivity and rise in BP. It is therefore an attractive candidate gene for human hypertension.

In a study from Australia involving white subjects, *GRK4* polymorphisms were associated with essential hypertension in 168 unrelated patients and 312 normotensive controls (Speirs et al. 2004). Furthermore, a recent study linked the *GRK4* polymorphisms, in particular p.Arg65Leu, with BP regulation in adolescents and young adults (Zhu et al. 2006b). Whilst a study from Japan showed that the *GRK4* p.Ala142Val polymorphism was 78.4% predictive of low-renin hypertension, the combination of the p.Arg65Leu, p.Ala142Val and the p.Val486Ala was 94.4% predictive (Sanada et al. 2006b). The p.Arg65Leu allele was also associated with impaired stress-induced Na excretion in normotensive black men (Zhu et al. 2006a).

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iii) Individual and shared environment

1. Dietary factors

The salt content in a westernised diet is several times more than the body's physiological requirement (Charlton et al. 2005). In the 1960s, Dahl measured the prevalence of hypertension amongst several population groups and found that BP rises in direct proportion with salt consumption (Falkner et al. 1990). However, several studies analysing dietary history and 24-hour salt excretion have failed to show a significant ethnic difference in Na intake between blacks and whites (Berenson et al. 1979; Meade et al. 1983; Pratt el al. 1989). An ethnic difference in K intake however has been suggested

by several studies (Meade et al. 1983; Barlow et al. 1986; Pratt et al. 1989). However, this disparity was not noted in all comparative studies (Berenson et al. 1979b; Adams-Campbell et al. 1993). In particular, a study from South Africa found no difference in Na and K intake between blacks and whites (Charlton et al. 2005). Both groups had excessive Na and reduced K intake.

The "Dietary Approaches to Stop Hypertension" (DASH) study (Sacks et al. 2001) revealed that a diet rich in potassium (fruits and vegetables), Ca (low-fat dairy products) and decreased total fat, together with Na restriction, significantly reduces BP in blacks. An increase in K may lower BP (Cappuccio and MacGregor 1991) but the mechanism is unclear. The results seem to reflect an interaction between the dietary cations resulting in a decrease in BP. Therefore, the DASH study postulated that it is better to monitor salt intake together with the levels of the other cations, rather than salt alone, in order to determine the exact effects on BP. Similarly, in the Trials of Hypertension Prevention follow up study (Cook et al. 2009), a higher urinary Na to K ratio was associated with increased risk of subsequent CV events supporting the concept that K intake ameliorates the effects of increased dietary Na.

As mentioned earlier, the diets of South African urban blacks were found to be low in Ca and Mg (Charlton et al. 2005). Alterations in Ca uptake and metabolism have been implicated in the increased susceptibility to hypertension in blacks, and Ca supplementation has been known to cause a modest reduction in BP in some patients (ref)**.** Mg depletion may result in reduced Mg-ATPase activity (see above) (Milne 2003). Analysis of the National Health and /Nutrition Examination Survey (NHANES) database supports the suggestion that inadequate Ca, K and Mg intake is associated with hypertension (Townsend et al. 2005).

2. Obesity

Obesity is a major risk factor for hypertension (Hall 2003). Accordingly, people more than 20% over their ideal body weight have a two- to three-fold higher risk of becoming hypertensive. Obesity is also epidemic in the black community, especially among

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females and urban blacks in South Africa and the United States of America (Seedat 1996b). In the 2003 South African Health and Demographic Survey, obesity (BMI ≥30 kg/m^2) was recorded in 21% of rural Blacks compared to 33.8% of urban dwellers (http://70.84.171.10/etools/doh/sadhs/index.html). In a South African cross-sectional study by Sever et al. (1980), BP correlated positively with all measures of obesity. Various mechanisms for the impact of obesity on BP have been suggested. In a review by Good et al. (2008), the pathophysiology of obesity-related hypertension is recognised to be complex. There is growing evidence that adipose tissue is active in the neurohormonal axis, and is not a passive storage depot. Adipocyte-related hormonal activity, including the RAAS, and resistance to feedback mechanisms, are associated with Na retention, increased plasma volume and increased sympathetic activity (Good et al. 2008). For instance, the *AGT* gene is expressed in adipose tissue, and variants of *AGT* could influence BP (Good et al. 2008). In an elegant study in South African black hypertensives with a BMI greater than 27 kg/m^2, Tiago et al. (2002) demonstrated interactions between the environment and variants of the *AGT* gene.

3. The Barker hypothesis

The Barker Hypothesis proposes that birth weight and adult BP are reciprocally related (Lackland et al. 2003). The implication is that low birth weight babies are more prone to hypertension later in life (Barker et al. 1989). The proposed mechanisms for this hypothesis include reduced foetal kidney development, impaired endothelial development, increased sensitivity to glucocorticoids, and a higher tendency to retain Na due to reduced nephron number (Dodic et al. 2002). Low birth weight has been associated with salt sensitivity in a group of normotensive white adults from Switzerland (De Boer 2008). There is evidence, both in the USA and South Africa, that the prevalence of low birth weight in black babies is higher than in white babies (Bachman et al. 1996; Carlson 1984). Thus, the Barker Hypothesis is an attractive model for acquired salt sensitivity due to reduced nephron number (and other factors), and would account for the higher prevalence of hypertension in blacks vs. whites.

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4. Urbanisation and stress

The observation that the prevalence of hypertension is higher in urban black South Africans compared to their rural counterparts suggests a strong environmental influence on the pathogenesis of hypertension (Seedat 1983). Similar rural/urban trends have been described in the rest of Sub-Saharan Africa (Poulter et al 1985). The rise in BP is often seen within weeks of rural-urban migration, and the reasons for the change are likely to be multi-factorial, and attributed to the adoption of a Western style diet with high Na and low K content, obesity, sedentary lifestyle, and stress (Seedat 1983; Seedat 1996b).

iv) Complications of hypertension

Hypertension in black South Africans differs in its clinical presentation, frequency and type of target-organ damage, or complications to comparable hypertensives in developed countries, or in their white South-African counterparts (Lindhorst et al. 2007). Malignant hypertension is still frequently seen in young black hypertensives even in the absence of obesity or secondary causes, and is commonly complicated by renal failure (Seedat 1996b). In a survey in Bloemfontein (a city in South Africa), end stage renal disease (ESRD) was attributed to hypertensive nephrosclerosis in 51.2% of black patients (Van Rensburg et al. 2010). A similar high prevalence of ESRD due to hypertension has been reported in African Americans (United States Renal Data System http://www.usrds.org)

CV complications of hypertension are different between blacks and whites. There is a higher prevalence of stroke and hypertensive heart disease, and a lower incidence of coronary disease in indigenous black South Africans (Seedat 1996b). In the Heart of Soweto Study, cardiac heart failure was the most common primary diagnosis, where 68% were attributable to dilated cardiomyopathy and/or hypertensive heart disease (Stewart et al. 2011). Black Africans were more likely to have heart failure than were the rest of the cohort, but were less likely to have coronary artery disease (38% vs. 6%), despite the high prevalence of cardiovascular risk factors. In an analysis of deaths attributable to non-communicable diseases, from 1999 to 2006, Mayosi et al. (2009) found a sustained 20% and 23% increase in deaths due to hypertensive heart disease and ill-defined heart disease respectively. Stroke had the highest death rates per 100,000 population compared

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to other cardiovascular diseases, and the stroke mortality is twice as high in blacks compared to whites. The lower prevalence of atherosclerotic complications of hypertension in blacks may be attributed to lower fibrinogen and cholesterol, and higher HDL-cholesterol levels (Cappuccio 1997).

In a study examining determinants of target organ damage in 403 black hypertensives attending primary health clinics in Cape Town, renal impairment by any criteria was identified in 26% and ECG left-ventricular hypertrophy in 35% (Peer et al. 2008). Ischaemic ECG changes were seen in 49% of patients, but this is unexplained given the low prevalence of coronary disease in blacks, and may be attributable to repolarization changes due to hypertension. The higher prevalence of target organ damage in black hypertensives has also been reported in African Americans, and other studies in South Africa (Mayet et al. 1998). Norton et al. (2009) showed that the relationship between BP and left ventricular mass index (LVMI) depended on excess adiposity in black South Africans possibly explaining the excess risk of cardiac disease. LVMI increased by 1.61 g/m compared to 5.24 g/m in patients with normal or increased waist circumference for every 1 standard deviation in BP.

Norman et al. (2007) estimated that the burden of disease related to high BP in SA (based on the 1998 Health survey), caused 48,888 deaths, which represents 9% of all deaths and 2.4% of all disability–related life years.

Accessibility of health care, affordability and availability of drugs are limiting factors for black patients in South Africa. Many people living in urban informal settlements do not have ready access to hypertension screening and anti-hypertensive treatment. Many people are ignorant of the dangers of untreated hypertension (Steyn et al. 2003). These factors may partly explain some of the differences in complications between blacks and whites.

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v) Drug responses

The varying drug responses amongst hypertensive blacks and whites support the hypothesis that salt sensitivity is an important differentiating factor between the two ethnic groups.

Thiazide diuretics

Thiazide diuretics have been shown to cause a greater decrease in BP in blacks than in whites (Veterans Administration Cooperative Study 1982; Moser and Lunn 1982). Since diuretics cause an increase in urinary Na excretion, their effectiveness in blacks supports the claim that blacks have a salt-sensitive phenotype.

Ca Channel Blockers

Studies have found that black hypertensives respond better than whites to Ca channel blockers (M'Buyamba-Kabangu et al. 1988; Seedat and Naiker, 1993). This may be related to differences in intracellular Ca and Na concentrations and cellular Ca transport mechanisms, and the diuretic effect of Ca channel blockers.

Beta-adrenoreceptor Antagonists

Seedat (1989) found that black hypertensive patients have a poor response to β_1-blockers. A higher proportion of black hypertensives have low-renin hypertension, and an important mechanism of action of β_1-blockers is inhibition of renin release.

Angiotensin Converting Enzyme (ACE) Inhibitors

Black patients respond poorly to ACE inhibitors in montherapy (Savage et al. 1990). However when ACE inhibitors are combined with a diuretic, Blacks respond as well as whites (Sareli et al. 2001). The poor response to monotherapy with ACE inhibitors is presumably due to suppression of the RAAS. Additionally, black hypertensives have a nearly three- fold greater risk of angioedema compared to whites with the use of the ACE inhibitor, enalapril (Kostis et al. 2005).

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A meta-analysis by Brewster and Seedat (2013) supported these observations that blacks respond differently to antihypertensive medication. Black patients responded best to diuretics and Ca channel blockers.

METHODOLOGICAL APPROACHES FOR THE DELINEATION OF THE GENETIC CONTRIBUTION TO ESSENTIAL HYPERTENSION

BP is a hereditable trait and the estimates of systolic and diastolic BP for identical twins is as high as 0.40 (Weder 1995). Historically, the genetic basis of hypertension has been a controversial issue. Platt (1947) proposed that essential hypertension is a Mendelian dominant trait with a distinct division between normotension and hypertension. On the other hand, Pickering (1955) proposed that hypertension was the extreme of the normal distribution of BP and argued against having an arbitrary division between normotension and hypertension. He postulated that hypertension has a multifactorial genetic and environmental basis with each individual trait having a small effect on BP (Figure 1.5, Weder 1995).

The Pickering hypothesis has gained wide acceptance as the likely relationship between BP and inheritance, but there is substantial uncertainty about the kind of genetic diversity underlying complex traits (Zhang et al. 2010). Whether the bulk of contributing allelic variation to common genetically complex diseases, like hypertension, consists of common variants with weak effects (common disease/common variant) or rare variants with individual strong effects (common disease/rare variant), is generally unknown.

Emerging evidence from the HapMap project suggests that common genetic variants may only account for a small proportion of heritable trait variance, but testing for common disease/rare variant is extremely difficult unless a distinctive trait phenotype is produced e.g. Liddle's syndrome (Zhang et al. 2010). It is likely that both mechanisms are operative, but the common disease/rare variant is likely to have more clinical impact on the treatment of hypertension (Kurtz 2010).

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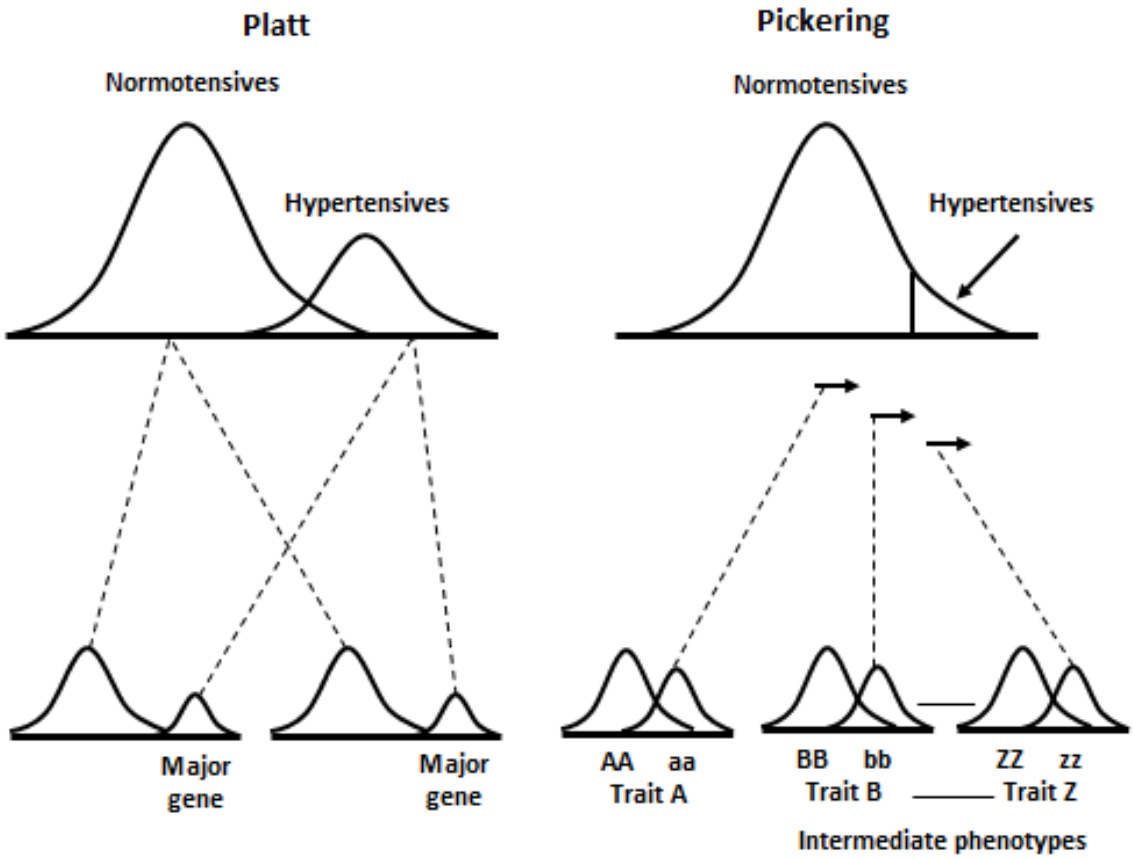

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Figure 1.5. The Platt and Pickering hypotheses for the genetic basis for hypertension

However proving the relationship between SNPs and the phenotype (hypertension) has been a very difficult task, and often with conflicting results. There are several reasons for this (summarised in Zhang et al. 2010):

1. Hypertension is, by definition, a dichotomous phenotype with arbitrary cut off levels, and the relationship between a SNP with a small effect on BP (with hypertension) is difficult to prove.

2. There are considerable difficulties in accurately defining the phenotype due to the inherent inaccuracies of BP measurement, and the often poor relationship between office/clinic BP measurement and hypertension; e.g. white coating and masked hypertension (Myers 2012).

3. The wide variety of candidate genes that may be associated with hypertension, and differing mechanisms for the development of hypertension.
4. Geographical/ population specific allele frequency distributions of certain SNPs may differ as a result of environmental factors e.g. availability of dietary Na.
5. As there are multiple pathways affecting BP, gene-gene interactions (epistasis) may mask the effects of a SNP.

GWAS are a relatively new automated and powerful technique to link genes to hypertension (Reviewed by Zhang et al, 2010). One important advantage is lack of *a priori* assumption about biological pathways (or essentially hypothesis-free testing). In addition, regions harbouring genes are smaller and easier to detect. However the success of GWAS in revealing the genetic underpinnings of chronic diseases has been exceedingly disappointing. In the Wellcome Trust Case Control Consortium (WTCCC), the study failed to show any significant association with hypertension (Welcome Trust Consortium, 2007). There was no phenotypic assessment of the controls, while unphenotyped population controls should not impair a case/control study for a rare disease, in a common trait such as hypertension, failure to do this would lead to an approximate 25% misclassification of hypertension. Similar results were reported by the Framingham Heart Study (Levy et al. 2007). Studies since 2009 have only accounted for < 1% of the heritability of BP (Zhang et al. 2010). This means that the majority of the BP heritability is still "missing" and remains 'at large' or unaccounted for (Zhang et al. 2010). The reasons for under performance of GWAS may be related to the following factors, which in part are similar to problems with candidate genes summarised in Zhang et al. (2010)]:

1. Misclassification of the phenotype as discussed above.
2. Studies are underpowered to detect common disease/rare variant
3. Epistasis (gene-gene interaction). This is likely given the number of genes involved in regulation in BP.
4. Gene by environment interactions. As discussed previously, genes enhancing Na reabsorbtion may only lead to hypertension in the presence of high Na diet.

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Kurtz (2010) argues that GWAS are unlikely to address the key questions regarding the genetic architecture of hypertension as they have limited ability to detect rare variants.

Another approach to defining the relationship of genes with hypertension is to use intermediate phenotypes that may be more accurately defined. For example, the genetic and environmental relationship with intermediate phenotype and hypertension is shown in figure 1.6 (Adapted from Weder 1995). In this figure, it is assumed that there are discrete hypertension-promoting genes that interact with environmental factors through a variety of pathophysiological mechanisms to produce hypertension. For example, *GRK4* regulates Na reabsorbtion in the proximal tubule in the kidney, and dysregulation of Na metabolism has been linked to hypertension. As previously discussed, defining the hypertension phenotype is fraught with difficulties, and its expression may be influenced by gene-gene, gene-environmental influences, and other factors. Thus it may be better to focus on the function of the gene and apply provocative tests like Na loading to determine if various *GRK4* SNPs are linked to differences in Na excretion and changes in renin and aldosterone.

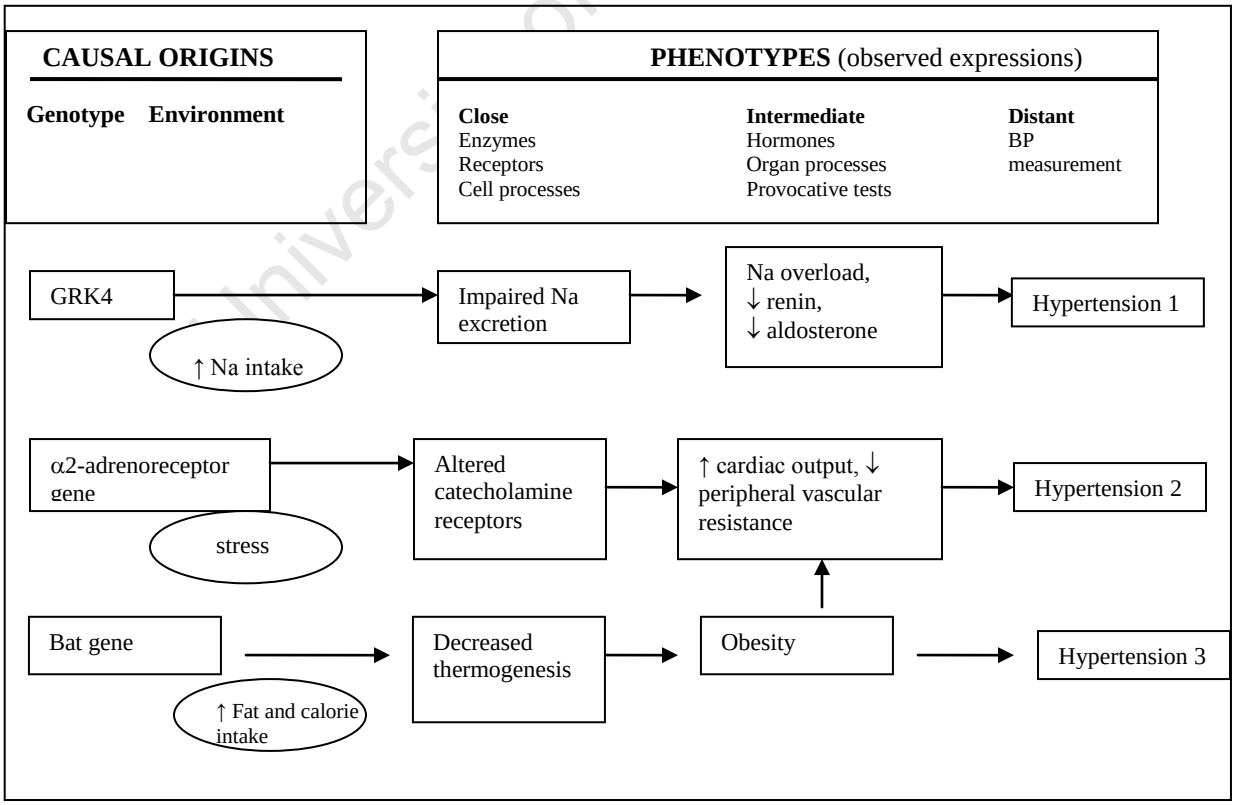

Figure 1.6. Flow of events from genes to ultimate phenotypic expression (hypertension)

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Conclusions

BP is under the control of haemodynamic, cellular, genetic and hormonal factors and this review has proposed that a number of these factors could account for differences observed between the disease indifferent ethnic populations. This study covers largely the indigenous African population in South Africa and Caucasians, generally referred to as 'blacks' and 'whites', respectively. The review also suggests that environmental factors may be important confounders in differences between blacks and whites, and Tiago et al. (2002) have recently demonstrated the interaction of obesity with an *angiotensinogen* mutation. Differences in the function of ionic transporters have been shown to be different between blacks and whites, but their significance is unknown. Causality with hypertension or salt sensitivity has not been established.

Currently it is reasonable to consider that an acquired or inherited predisposition toward Na retention, provides the basis for differences in BP between blacks and whites. In general, blacks are more salt sensitive than whites, and respond better to diuretics and Ca channel blockers than to ACE inhibitors or β blockers, as monotherapy. The mechanisms behind these differences are elusive, but are not due to dietary Na intake. Although mutations in the *ENaC subunit*, *angiotensinogen* and *aldosterone synthetase* genes could partly explain this, it only applies to a small percentage of hypertensives (Lindhorst et al. 2007). A far more common mutation, contributing to salt sensitivity, would be required to account for the population differences, but has yet to be found.

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However polymorhpisms in the *GRK4* gene provide attractive candidates for further investigation for the following reasons:

1. Na reabsorbtion in the proximal tubule in the kidney is greater in hypertensives compared to normotensives (Chiolero 2000).
2. In a family study of black South Africans and white Belgians, segmental Na reabsorbtion along the nephron was found to be highly heritable, and after a Na load, black participants reabsorb more Na than whites in the proximal tubule and less post proximally (Bochud et al. 2009).

3. Dopamine promotes natriuresis in the proximal tubule in combination with GRK.
4. Variants of the GRK4 receptor protein desensitise dopamine receptors, impairing natriuresis.
5. These variants are linked to human hypertension, and the p.Ala142Val polymorphism has been shown to cause salt sensitivity and hypertension in transfected mice.
6. Initial studies in our unit have shown that p.Ala142Val variant is common in indigenous black populations compared to whites (see chapter 2).

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CHAPTER 2

PRELIMINARY WORK

Primary aldosteronism, normal ranges for renin and aldosterone in South Africa, and the p.Arg563Gln mutation

In 1994, severe drug-resistant hypertension was noted to be a common problem in the Hypertension Clinic at Groote Schuur Hospital in Cape Town, despite good adherence with medication. Dr Richard Gordon from Brisbane in Australia found that the prevalence of primary aldosteronism (PA) in their locality was 12% (Gordon et al. 1994). There were also reports from other areas in the world of similar high prevalence (Conn et al. 1965; Anderson et al. 1994; Edwards 1998). Importantly, most of these cases occurred in the absence of hypokalaemia (Conn et al. 1965, Vallotton 1996). Since the literature suggested that the aldosterone/renin ratio was a reliable screening test for PA, a survey was performed to estimate the prevalence in the Hypertension Clinic at Groote Schuur Hospital. Thirty two percent of patients had evidence suggestive of PA, and 10.1% of patients had either adrenal adenoma or hyperplasia on CT scanning (Rayner et al. 2000).

Due to the higher prevalence of low-renin hypertension, it was decided to embark on a survey of normal ranges of aldosterone and renin in three South African population groups. In hypertensive patients, 7.1% had evidence suggestive of PA. However, the mean PRA in African normotensive subjects (0.95 ± 1.25 ng/mL/h; mean±SD) was significantly lower than in white (2.09 ± 1.12; $p<0.0001$) and Cape Mixed Ancestry (1.81±1.86; $p=0.013$) normotensives (Rayner et al. 2001a). Mean plasma aldosterone in African normotensives (306±147 pmol/L) was also significantly lower than in white (506 ± 324; $p=0.0002$) and Cape Mixed Ancestry (418 ± 304; $p=0.0148$) normotensives. In hypertensives, there were no significant differences in renin or aldosterone levels. None of the normotensives had an aldosterone/renin (A/R) ratio ≥ 1000 plus aldosterone ≥ 750, while 7.1% of hypertensives exceeded these levels, suggesting they are appropriate parameters for screening for PA.

The most important environmental factor that can influence renin and aldosterone levels is dietary Na intake. High Na intake would suppress, and low Na would stimulate renin

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to maintain homeostasis. However, analysis of urinary Na/creatinine ratios (an index of Na intake) between the three population groups showed that there were no significant differences (Rayner et al. 2001a). This provided support for the argument that genetic factors that predispose to Na retention by the kidney may be important.

The primary candidate gene for Na retention by the kidney was the ENaC, which is the final site of Na regulation in the kidney (Lindhorst et al. 2007). The SCNN1β: p.Thr594Met variant of β subunit of the ENaC was reported to be associated with hypertension in blacks living in London (Baker et al. 1998), but this has not been confirmed in a subsequent study in South Africa (Nkeh et al. 2003). It was hypothesised that another common yet undefined SNP of the β subunit of the ENaC may account for differences in hypertension between blacks and whites.

In collaboration with the Department of Chemical Pathology the entire gene for the *SCNN1*β subunit of the ENaC was sequenced and a novel SNP (p.Arg563Gln) was identified (Rayner et al. 2003). It was later shown to be associated with low renin and low aldosterone-related hypertension in indigenous southern African and Cape Mixed Ancestry population groups (Rayner et al. 2003). This formed the basis for the doctoral dissertation of Dr Erika Jones, (University of Cape Town), which was successfully completed in 2009. The key findings of her thesis were the following:

i) There was a strong association with hypertension and the p.Arg563Gln mutation in blacks living in urban areas (Jones et al. 2012).

ii) In a kindred study, the p.Arg563Gln mutation strongly associated with hypertension (Jones et al. 2010).

iii) The p.Arg563Gln mutation was capable of causing a full blown Liddle's syndrome in pregnancy.

iv) The prevalence of the p.Arg563Gln mutation was 20% in the Khoi-San population, who are hunter gatherer people living in the Kalahari desert. It is suggested that this was the population of origin (for this variant) and may

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have offered survival advantage in a Na restricted environment (Jones et al. 2012).

v) Patients with severe hypertension carrying the p.Arg563Gln mutation responded dramatically to amiloride, a specific inhibitor of the ENaC (Jones et al. 2012).

However, the p.Arg563Gln mutation was present in about 5% of hypertensives and < 1% of normotensives (Jones et al. 2012). Thus, it does not explain the suppressed aldosterone and renin levels in the vast majority of black normotensives.

As a result, the research study was extended to candidate genetic determinants of hypertension in indigenous black South Africans in the Division of Human Genetics, UCT. The first candidate chosen for investigation in this study was the *GRK4* gene on chromosome 4p16.3 (OMIM: 137026), which had not been studied in the South African population, previously.

The working hypothesis was that *GRK4* variants were associated with hypertension particularly in indigenous African patients, and the objective was to determine if p.Ala142Val, in particular, was associated with hypertension.

METHODOLOGY:

A case-control study was performed to investigate whether an association exists between DNA sequence variants in the *GRK4* gene and severe hypertension in the South African cohort.

Research participants:

The approach was to carefully phenotype patients with severe hypertension attending the Hypertension Clinic at Groote Schuur Hospital. Only individuals with severe or treatment-resistant hypertension without identifiable secondary causes were recruited for this study. Severe hypertension was defined as an office BP > 180/110 mmHg, or a patient with uncontrolled BP > 140/90 mmHg on at least three anti-hypertensive medications, as measured by standard mercury sphygmomanometer. The rationale for

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using severe hypertension as the phenotype of interest was based on the study published by the MRC BRIGHT (British Genetics of Hypertension) Study (Caulfield et al. 2003). Patients with severe hypertension are more likely to have identifiable genetic influences on the development of their hypertension. In patients with mild hypertension, environmental factors may be more dominant and much larger numbers of patients would be required to detect the genetic factors involved. Additionally, studying severe hypertension was expected to exclude patients with white coat hypertension, which may be a major confounding variable. All patients underwent extensive phenotypic investigation, and first and second degree relatives were traced, and recruited where possible. Venous blood samples were taken from all recruits, for DNA extraction and genetic analysis. The study was approved by the University Research Ethics Committee, UCT (003/2002).

Genomic DNA was extracted for each study subject and aliquots of the DNA were archived in the DNA Bank in the Molecular Genetics Laboratory in the Division of Human Genetics at UCT. In addition, all the detailed phenotyping data, including body mass index, echocardiography results and various biochemical blood tests were stored in a Microsoft Access database in the Division of Human Genetics Research Database. This database had been especially created to store the comprehensive phenotypic data on each research participant.

Genotyping

Primers were designed flanking the p.Ala142Val polymorphism and the polymerase chain (PCR) analysis was performed on all the DNA samples available for this study. Genotyping of the polymorphism was performed using denaturing high-performance liquid chromatography (dHPLC) analysis, and selected samples were sequenced to verify the dHPLC genotyping results. Chi-squared analysis was performed on the genotyping results within each ethnic group and between groups.

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RESULTS

A total of 440 patients and their relatives from 198 hypertensive families were recruited to this study. The ethnic breakdown was as follows: 80 indigenous African (black), 106 Cape Mixed Ancestry, and 12 Caucasian (white) South African families. These families were all recruited through a proband with severe, treatment-resistant hypertension. The large number of Cape Mixed Ancestry families reflects the demographics of the Western Cape Province, where the recruitment centre is situated. Normotensive ethnic-matched control samples for this study were obtained from Dr EP Owen (Division of Chemical Pathology, UCT). No phenotypic data was available on these patients.

For the data within each ethnic group, there was no significant difference in the allele frequencies between hypertensive and normotensive individuals (data not shown). However, when the allele frequencies were compared across ethnic groups highly significant differences were observed (Table 2.1) for the allele frequencies of the p.Ala142Val polymorphism.

When comparing the indigenous Black and White hypertensives, there was a significantly higher frequency of the c.425T (p.142Val) allele in the Black population (79% vs 21%, Table 2.1). At the genotype level, there are many more individuals who are T679T homozygotes in the Black population than in the Caucasian population (62% vs 14%, Table 2.1). When Blacks were compared with the Mixed Ancestry hypertensives, a smaller difference was detected between the two groups. However, the difference is still statistically significant. At the allele level, the frequency of the c.425T (p.142Val) allele is 79% vs. 55 % (Table 2.1), and at the genotype level, the frequency of individuals who are T679T homozygotes were 62% vs. 30% (Table 2.1).

In the normotensive populations the frequency of the c.425T (p.142Val) variant is 73% in the Black, 61% in the mixed ancestry and 27% in the Caucasian populations, respectively.

There was no association with hypertension in this small sample size.

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Table 2.1. Comparison of the allele and genotype frequencies of the c.425>T (p.Ala142Val) polymorphism in the *GRK4* gene between three South African population groups.

A. Allele distribution

	C	T	Total	Chi^2	dof*	p value
Black vs. Caucasian hypertensives:						
Black	33 (21%)	127 (79%)	160	69.3	1	0.0001
Caucasian	99 (68%)	47 (32%)	146			
Total	132	174	306			
Black vs. Mixed ancestry hypertensives:						
Black	33 (21%)	127 (79%)	160	19.7	1	0.0001
Mixed ancestry	60 (45%)	74 (55%)	134			
Total	93	201	294			

B. Genotype distribution

	C/C	C/T	T/T	Total	Chi^2	dof*	p value
Black vs. Caucasian hypertensives:							
Black	3 (4%)	27 (34%)	50 (62%)	80	54.4	2	0.0001
Caucasian	36 (49%)	27 (37%)	10 (14%)	73			
Total	39	54	60	153			
Black vs. Mixed ancestry hypertensives:							
Black	3 (4%)	27 (34%)	50 (62%)	80	18.9	2	0.0001
Mixed ancestry	13 (19%)	34 (51%)	20 (30%)	67			
Total	16	61	70	147			

* dof = degrees of freedom

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GRK4 allele frequencies in other populations

Speirs et al. (2004) recently published the frequencies for p.Arg65Leu, p.Ala142Val and p.Val486Ala (Table 2.2) variants of *GRK4* in Australia, and showed an association between p.Val486Ala polymorphisms and hypertension.

Table 2.2 Allele frequencies of the *GRK4* variants in hypertensives and normotensives from Australia

Group	N	Genetype frequencies			Chi^2	P
p.Arg65Leu		**RR (GG)**	**RL (GT)**	**LL (TT)**		
Normotensive	312	38%	53%	9%	5	0.08
Hypertensive	160	36%	48%	16%		
p.Ala142Val (c.425C>T)		**AA (CC)**	**AV (CT)**	**VV (TT)**		
Normotensive	189	40%	49%	11%	1.2	0.55
Hypertensive	168	36%	50%	14%		
p.Val486Ala		**AA**	**AV**	**VV**		
Normotensive	248	32%	54%	14%	6.2	0.04
Hypertensive	145	28%	55%	30%		

Using genotype and allele data from Ensembl (www.ensemble.org), the p.Arg65Leu (*rs2960306*) and p.Ala142Val (*rs2014323*) variants (obtained using the HapMap and Corriel Cell Repository) showed significant differences in the distribution of genotype frequencies in different population groups. (Table 2.3) The p.Ala142Val data in Caucasian and Asian vs. African populations showed that the frequencies of the T and C allele are significantly different with the T allele predominating in Africans whilst the C allele was found to predominate in Caucasians and Asians. This data mirrors the allele frequencies obtained in our black and white populations. We have no local data as yet for the distribution of the p.Arg65Leu polymorphism in South African populations, but the data from Ensembl shows that there is a significant difference between Caucasian and Asians vs. Africans. The G allele is far more frequent in Caucasians and Asians whereas the T allele predominantes in peoples of African origin.

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Table 2.3 Allele frequencies of the p.Ala142Val and p.Arg65Leu in differing populations.

Group	**Gene frequencies**				
p.Arg65Leu	**RR (GG)**	**RL (GT)**	**LL (TT)**	**R (G)**	**L (T)**
European	38.3%	46.7%	15%	61.7%	38.3%
Han Chinese	88.9%	11.1%	0	94.4%	5.6%
Japanese	84.1%	15.9%	0	92%	8%
Yoruba	25%	46.7%	28.3%	48.3%	51.7%
African American	26.1%	39.1%	34.8%	45.7%	54.3%
p.Ala142Val (c.425C>T)	**AA (CC)**	**AV (CT)**	**VV (TT)**	**A(C)**	**V (T)**
European	38.3%	45%	16.7%	60.8%	39.2%
Han Chinese	68.9%	31.1%		84.4%	15.5%
Japanese	65.9%	34.1%		83%	17%
Yoruba	10%	45%	45%	32.5%	67.5%
African American	-	-	-	39%	61%

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These significant differences in *GRK4* genotypes and allele distributions between populations suggest that there may be environmental factors influencing the selection of these genes or due to genetic drift. Although no association with hypertension could be demonstrated for these variants, it does not necessarily rule out a biological role for *GRK4*, as the variant may still have an epistatic effect on the observed phenotype in concert with other environmental influences. It has been previously argued that the demonstration of association between a SNP and hypertension can be extremely difficult. The fact that the p.Ala142Val has functional significance in rats leading to impaired salt excretion and hypertension supports this argument. It could lead to natural selection of different genotypes in differing environments. For example, in areas like Africa, where historically, Na was a scarce resource, the TT allele may give survival advantage to individuals carrying this trait because of enhanced ability to conserve Na. Conversely, in

environments adopting a Western style diet, with plentiful Na, the TT allele may result in Na retention and hypertension. Conversely the CC allele may compromise survival in areas of Na scarcity, but protect against hypertension with excess Na.

Conclusion:

Thus the concept of salt sensitivity or resistance due to genetic factors needs to be studied in concert with environmental risk-factors and population stratification

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CHAPTER 3

GRK4 SALT SENSITIVITY STUDY

HYPOTHESES

1. Normotensive and hypertensive indigenous black South Africans are more salt sensitive than their white counterparts.
2. Variants of the *GRK4* gene account for the differences in salt sensitivity.

INTRODUCTION:

As outlined in Chapter 1, the *GRK4* variants are strong candidates for 'causing' salt sensitivity. The research had two approaches (i) Na excretion was examined after acute saline challenge in young black and white normotensive subjects, (section 3.1) and (ii) BP responses to dietary Na restriction were examined in black hypertensives over an 8 week period (section 3.2).

DEFINING SALT SENSIVITY:

Although the term salt sensitivity is used widely in the literature, there is considerable confusion as to how we determine and define this phenotype. Most authors have defined this in terms of BP response to acute or chronic Na restriction or loading (Weinberger et al. 2001; Sanders 2009). In other words, in salt-sensitive individuals, BP drops to a greater extent during times of restriction, and rises to a greater extent during Na loading. In terms of feasibility, chronic Na loading, or restriction, over 2-4 weeks, will be fraught with problems in controlling Na intake outside the controlled premises of metabolic research/observation units, unless there is a very strict supervision of the diet.

Weinberger et al. (2001) proposed that salt sensitivity should be determined by the BP response to acute saline loading intravenously followed by acute deprivation and the administration of diuretics. Accordingly, salt sensitivity is seen as a decrease in mean arterial BP of 10 mm Hg or more, between the end of Na loading and the end of Na deprivation, whilst a difference of < 5mm Hg, is defined as reflecting Na resistance. Between 5-10 mmHg is considered indeterminate. The use of BP in defining salt sensitivity (while important) is fraught with problems related to measurement and

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variability of BP, unless 24 hour BP monitoring is performed (Myers 2012). Alternative to BP measurement, specific physiological processes related to Na metabolism in the kidney, especially in the acute setting, may be correlated with salt sensitivity (Weeder 1995). It has been proposed (but has never been formally proven in humans) that the impaired ability of the kidney to excrete Na is the underlying problem in salt sensitivity (Sanders 2009).

Aim:

In this thesis, determination of salt sensitivity was performed by two independent methods. The first approach (Section 3.1) will be novel, and has not been described previously in the literature. It assessed Na excretion, BP response, renin and aldosterone levels, after acute saline challenge. The aim of this approach had been to develop a reproducible, clinically practical and accurate test to define salt sensitivity. By analysing Na excretion in this model, the focus will be on the underlying pathophysiological mechanisms or robust intermediate phenotype, since the *GRK4* variants are expected to reduce the ability of the kidney to excrete Na as outlined above. A similar research design was used by Zhu (2006a) to show impaired stress induced Na excretion by *GRK4* variants.

The second approach (Section 3.2) used a more classical design to investigate BP responses to Na restriction over an 8 week period, to determine if *GRK4* variants predicted the magnitude of the BP response. To minimise the inherent inaccuracies of office or clinic BP measurements, all patients underwent ambulatory BP monitoring before and after 8 weeks of the diet.

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3.1 ACUTE SALT LOADING IN YOUNG NORMOTENSIVES

This was a prospective study designed to investigate salt sensitivity in indigenous South African black and white subjects, by acute saline challenge. It involved 60 healthy age matched black and white male subjects. The study was approved by the Research Ethics Committee of the University of Cape Town (ref 045/2006).

OBJECTIVES:

Primary:

1. To analyse Na excretion in indigenous black South African subjects compared to their white counterparts after acute saline loading.
2. To determine if differences in Na excretion were related to either the p.Arg65Leu and/or p.Ala142Val variants in the *GRK4* gene

Secondary:

1. To analyse BP responses in indigenous black South African subjects compared to their white counterparts after acute saline loading
2. To determine if BP responses were related to the p.Arg65Leu and p.Ala142Val *GRK4* variants

Tertiary:

1. To determine if changes in renin and aldosterone before and after saline loading was related to ethnicity
2. To determine if changes in renin and aldosterone was related to the p.Arg65Leu and A142V *GRK4* variants

ENDPOINTS:

1. The primary endpoint was the change in Na excretion in response to 2 litres of intravenous normal saline.
2. The secondary endpoint was the change BP between baseline and at the end of the 4 hour period.
3. The tertiary endpoint was the changes in renin and aldosterone between baseline and at the end of 4 hours.

SUBJECTS

Ethnic Xhosa (representing indigenous African or black) and white male subjects aged between 18 and 30 years with a BMI < 25 kg/m^2 were eligible for the study. Subjects were excluded if there was any previous significant illness, known hypertension or diabetes mellitus, regular use of medication, particularly antihypertensive drugs, NSAIDs or any drug influencing salt or water metabolism, any underlying disease that made salt loading hazardous e.g cardiac failure, recent weight loss or weight gain

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exceeding 2 kg, history of idiopathic oedema, failure to give informed consent, an elevated serum creatinine or abnormal electrolytes, alcohol or drug abuse, and use of cigarettes/cigars/pipes, within one week of the study.

Healthy young lean male subjects were chosen to avoid any acquired development of salt sensitivity due to subtle renal injury, age-related increase in salt sensitivity, the effects of hyperinsulinaemia on Na excretion, and the effects of the female menstrual cycle on Na and water excretion.

METHODS:

Subjects fasted overnight but were encouraged to maintain their hydration with water intake. After admission to the Metabolic Unit at Groote Schuur Hospital, the subjects signed informed consent, and underwent history and physical examination with measurement of BP, height, weight, waist circumference, and calculation of BMI. Baseline bloods for Na, K, creatinine, renin and aldosterone, and urine for urinalysis was performed after the bladder was emptied. An intravenous catheter was inserted into a suitable vein in the forearm and an automated BP monitor (Dynamap) was attached to the opposite arm. This was followed by an infusion of 2 litres of normal saline (308 mmols Na) over 2 hours. BP and pulse rate was measured every 60 minutes by the automated BP monitor, and blood was taken for Na, K and creatinine measures. Hourly urine output was measured, and urine was analysed for Na, K, and creatinine. The experiment was completed after 4 hours, and final bloods were taken for Na, K, creatinine, aldosterone and renin measures. The intravenous catheter was removed. Total volume of urine was recorded, and the urinary Na excretion calculated. Subjects remained fasting throughout the study, and no oral fluids were allowed for the four hour study period.

GENETIC ANALYSIS

Genotyping of *GRK*4 (p.Ala142Val)

DNA isolation from the peripheral blood leukocytes

Genomic DNA was extracted from peripheral blood lymphocytes for each research participant and isolated using the Gentra (Qiagen, Hilden, Germany) Puregene blood kit

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protocol for DNA purification from 300µl whole blood according to the manufacturer's protocol (Gentra Puregene Handbook 06/2011). The NanoDrop-1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) was used to determine the quality and yield of DNA obtained from the isolation procedure.

Genomic amplification of *GRK4* c.425C>T (p.Ala142Val) by polymerase chain reaction (PCR)

For amplification of the 737bp region flanking the *GRK4* c.425C>T (p.Ala142Val) variant, primers were designed using Primer3 (*http://frodo.wi.mit.edu/*). The resulting oligonucleotides were assessed for secondary structure formation in OligoAnalyser 3.1 (*http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/default.aspx),* whilst genomic target specificity was interrogated through Primer-BLAST (*http://www.ncbi.nlm.nih.gov/ tools/ primer-blast/).* The PCR was performed with 100ng/µl genomic DNA in a final reaction volume of 25µl, which contained 0.4µM of each primer (left primer 5`-CCT CCA GAT GTT GTG ACA-3`; right primer 5`-GTG CCT CGT GTG TAC ATG G-3`), 10mM Tris-HCl, 50mM KCl, 1.5mM $MgCl_2$, 0.2mM of each dNTP (Bioline, London, UK) and 0.5 units of GoTaq® DNA polymerase (Promega, Madison, WI, USA). The PCR was performed on the Multigene thermal cycler (Labnet, Edison, NJ, USA) and cycling conditions consisted of an initial denaturation step of 5min at 95°C and 35 cycles of amplification at 94°C for 30sec, 50°C for 30sec and 72°C for 40sec. This was followed by a final extension step at 72°C for 7min.

Direct cycle sequencing

For DNA sequence determination of the 737bp fragment containing the genomic region flanking the *GRK4* NM_182982.2_c.425C>T variant, either of the two PCR primers was used. Direct cycle sequencing was performed in a final volume of 20µl, which contained 2µl of the 1x BigDye® Terminator v3.1 (Applied Biosystems, Foster City, CA, USA) sequencing kit, 4ul 1x Sequencing Buffer (Life Technologies), 0.4µM of either the left or right primer and 150-300ng PCR template). DNA sequencing cycling parameters included an initial denaturation step at 98^{o}C for 15min, followed by 25 cycles consisting of denaturation at 96^{o}C for 15sec, annealing at 55^{o}C for 10sec and extension at 60^{o}C for

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4min. Sequencing reactions were performed on the GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems).

The sequencing products were re-suspended in 4µl dH_2O and 8µl of HiDi™formimide, and capillary electrophoresis (Figure 3.1.1) performed on an ABI Prism 3130*xl* Genetic Analyzer (Applied Biosystems). BioEdit (Hall 1999) was used to analyse and align each sample sequence with the *GRK4* reference sequence.

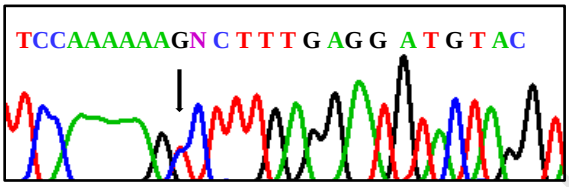

Figure 3.1.1 An electropherogram of the DNA sequence for exon 5 of *GRK4*. The black arrow indicates the position in the sequence that represents the *GRK4* NM_182982.2_c.425C>T variant in exon5. The y-axis represents relative fluorescent units (RFU) whilst each dNTP is represented at specific positions on the x-axis.

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Genotyping of *GRK*4 (p.Arg65Leu)

Genomic amplification of *GRK4*:c.425C>T (p.Arg65Leu) by PCR

For the amplification of the genomic sequence containing the *GRK4* p.Arg65Leu variant (*rs2690306*), primers were designed as described above to amplify a 670bp fragment. The target DNA fragment was amplified in a final reaction volume of 25µl consisting of 100ng/µl genomic DNA, 0.4µM of each primer (left primer 5`-AGT GAG CGG AGA TTG TAC CG-3`; right primer 5`-TGA GAC GGA GTC TTG CTG TG-3`), 1X colourless GoTaq® reaction buffer (Promega), 0.2mM of each dNTP (Bioline) and 0.5 units of GoTaq® DNA polymerase (Promega). The PCR was performed on the GeneAmp 9700 thermal cycler (Applied Biosystems) and cycling conditions consisted of an initial denaturation step of 5min at 95°C and 35 cycles of amplification at 94°C for 30sec, 67°C for 30sec and 72°C for 40sec. This was followed by a final extension step at 72°C for 7min.

Restriction Endonuclease Assay for the *GRK4* p.Arg65Leu variant

The 670bp fragment containing the *GRK4* p.Arg65Leu variant was digested with the restriction endonuclease *BsaHI* (New England Biolabs, Ipswich, MA, USA). The enzymatic reaction was incubated at 60°C for 3 hours and consisted of 16.8µl of the amplified DNA product, 1X reaction buffer 4 (New England Biolabs, where), and 10 units of *BsaHI* (New England Biolabs, where).

The G-allele of the variant (*rs2690306*) introduces a *BsaHI* endonuclease cutting site into the DNA sequence, whilst the DNA sequence containing the T-allele is not cut by the endonuclease. Thus, a PCR fragment containing a homozygous G/G genotype would, once digested by *BsaHI*, produce two restriction fragments (411 and 259bp fragments). For samples of heterozyogous G/T for the variant, three fragments are observed; the uncut 670bp of the T-allele and the two fragments resulting from the digestion of the G-allele (411 and 259bp fragments). Finally, samples homozygous, T/T, for the variant produce a single undigested fragment of 670bp.

Products of the *BsaHI* restriction endonuclease digest were separated on a 2% (w/v) agarose (Lonza, Rockland, ME,USA) gel containing 0.5µg/ml ethidium bromide (Sigma-Aldrich, St Louis, MO,USA) and visualised under UV illumination (UVItec, Cambridge, UK).

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STATISTICAL ANALYSIS

Sample Size: 60 healthy age and sex matched black and white normotensive controls were recruited into the study. In calculating the sample size needed, the formula below was used $N = \frac{(s.d_1^2 + s.d_2^2)(Z_1 - a + Z_1 - b)}{D^2}$

Where:

N= Minimum sample size

Z= Normal standard deviation for the required confidence level (95%)=1.96

$s.d_1$ =Standard deviation of first group

$s.d_2$ =Standard deviation of second group

D = Difference between two means

a= constant = 0.05

b = constant =80%

Using the above formula:

N= 30 for each group

The two study groups were compared with respect to their baseline characteristics. Group specific descriptive statistics included means, standard deviations, medians, minima, maxima and proportions. A formal comparison of the baseline profiles was done using independent Student's *t* tests for the continuous data, and the χ^2 test or Fisher's exact test for the categorical data which included the two genetic markers. The baseline characteristics were also compared by genetic markers using one-way analysis of variance (ANOVA) and Bonferroni post-hoc test was used where significant differences were found.

To supplement the descriptive statistics, graphical displays were used to depict the distributions of the two study groups. These included box plots, scatter plots, and bar charts. Since the biochemistry measurements were measured over time, the analysis of the study groups and genetic marker groups were analysed using repeated measure. This was performed using generalized linear models (GLM) with an identity link function and clustering indicator for each participant to enable the standard errors of the estimates to be adjusted for the repeated measurements. The regression models included interaction terms of time and genetic marker group since this was the main inference of the study. The biochemistry profiles over time were also graphically depicted using median profiles of the genetic marker groups. Throughout the analysis, two-sided statistical tests were used at α=0.05 after correction for multiple testing. STATA 10.0 for Windows (STATA Corporation, College Station, TX, USA) was used in the analysis of data.

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RESULTS:

Patient demographics at baseline

The baseline characteristics of the participants are provided in Table 3.1.1. Important baseline differences in subject demographics were present. White men were significantly heavier, taller with a slightly higher BMI and serum creatinine, and their plasma aldosterone levels were 3 fold higher. Renin levels were similar, but K levels were slightly but significantly lower. Diastolic BP was significantly higher in blacks than whites (77.0 vs. 71.2 mm Hg, $p<0.05$), despite the white subjects having a higher BMI.

Table 3.1.1. Baseline characteristics by ethnicity

Variable	***Black* Men (n=30)**	**White Men (n=31)**	**P value**
	Mean (SD)	Mean (SD)	
Age (years)	21.6 (3.2)	21.4 (2.3)	0.80
Height (cm)	171.5 (7.6)	182.2 (6.2)	<0.001
Weight (kg)	66.4 (11.8)	79.6 (10.9)	<0.001
BMI (kg/m^2)	22.5 (3.1)	24.0 (2.8)	0.05
Na (mmol/L)	140.6 (1.7)	141.1 (1.3)	0.23
K (mmol/L)	4.57 (0.41)	4.32 (0.37)	0.01
Creatinine (µmol/L)	78.0 (12.3)	86.0 (10.4)	0.05
Creatinine clearance ($mls/min/1.73m^2$)	99.69 (14.4)	95.3 (11.4)	0.19
Renin (u/ml)	24.6 (18.3)	22.9 (19.5)	0.72
Aldosterone (pmol/L)	132.6 (76.4)	298.3 (145)	<0.0001
Systolic BP (mmHg)	137.1 (13.7)	134.1 (12.7)	0.38
Diastolic BP (mmHg)	77.0 (8.2)	71.2 (6)	0.002
Pulse (beats/min)	71.3 (13.7)	65 (12.3)	0.11

The ethnic differences in the frequencies of the p.Ala142Val and p.Arg65Leu SNPs are shown in Table 3.1.2. The C/C, C/T and T/T genotyping frequency of the p.Ala142Val was significantly different between the two population groups ($p<0.001$), but the G/G,

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G/T, and T/T genotyping frequencies approached significance for the p.Arg65Leu variant (p=0.05). The allele frequencies were in Hardy Weinberg Equillibrium in both subject groups.

Table 3.1.2. Ethnic distribution of alleles (p.Ala142Val and p.Arg65Leu).

p.Ala142Val	**Black**	**White**	**P value**
CC	1	7	<0.0001*
CT	6	17	
TT	22	6	
p.Arg65Leu			
GG	1	5	0.05*
GT	11	16	
TT	18	10	

*Difference in allele frequency between Black and White subjects

Na Excretion by ethnicity

The mean hourly BP response to saline loading was similar between the two study groups (Table 3.1.3). Contrary to expectations, there was no rise in systolic or diastolic BP in either group. The higher diastolic BP in Black subjects was maintained throughout the study period. In Black subjects the mean total Na excretion was higher at 1 hour but only increased by 27% (95% CI: 2.5 to 51.8%, p = 0.03) from 1 to 4 hours. In contrast White patients had a lower initial Na excretion which increased by 150% (95% CI: 102.6 to 195.4%, p < 0.0001) from 1 to 4 hours. Using GLM analysis there were significant time dependent changes in Na excretion at 2, 3 and 4 hours but there were significant ethnic differences at time 3 (p=0.04) and 4 (p<0.0001).

Renin was not different between the groups at baseline or at 4 hours. In contrast aldosterone showed marked suppression at 4 hours after saline loading in both Blacks and Whites, but the Black subjects had highly significant lower aldosterone levels at baseline (132 vs 298 pmol/L, p<0.0001) and at 4 hours (32 vs 84 pmol/L, p<0.0001). Using GLM there was a significant time/ethnic interaction.

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Table 3.1.3. Mean values over time in BP, serum Na, renin, aldosterone and urinary Na in blacks and whites.

Parameter	Race	Baseline	1 hour	2 hours	3 hours	4 hours
SBP (mmHg)	Black	137.1	129.4	132.9	128.6	131.6
	White	134.1	128.5	129.1	128.7	128.8
DBP (mmHg)	Black	77.0	74.0	73.5	71.0	71.6
	White	71.2	68.3	68.2	66.8	68
Na (mmol/L	Black	140.6	-	142.2	-	-
	White	141.1	-	142.7	-	-
Renin (u/ml)	Black	24.6	-	-	-	17.9
	White	22.9	-	-	-	18.2
Aldosterone (pmol/L)	Black	132.6^{+}	-	-	-	32.4*
	White	298.3^{+}	-	-	-	83.1*
Urinary Na (mmol)	Black	-	20.5	27.5	27.4$^{\&}$	26.1$^{\#}$
	White	-	15.2	24.4	24.8$^{\&}$	37.9$^{\#}$

+ - p<0.0001, * - p<0.001, & - p=0.04, # - p< 0.0001

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Genetic differences

i) p.Arg65Leu

The baseline data for the p.Arg65Leu polymorphism and changes after saline loading are shown in Table 3.1.4. There were no significant differences between the G/G, G/T and T/T genotyping frequencies for the p.Arg65Leu polymorphism.

i) p.Ala142Val

The baseline data for the p.Ala142Val polymorphism and changes after saline loading are shown in Table 3.1.5. At baseline, aldosterone levels were lowest in subjects carrying the homozygous T/T genotype, intermediate in the subjects with the C/T heterozygous

genotype, and highest in subjects who were C/C homozygotes (p<0.002). However, renin was not different. In contrast, the systolic and diastolic BP was highest in the T/T subjects, intermediate in the C/T subjects, and lowest in the C/C group. This was not significant for systolic BP, and approached significance for diastolic BP (p=0.07). However, combining the C/T and T/T genotyping groups resulted in a significant difference in BP when compared to the C/C group (p=0.04).

Table 3.1.4. Mean values over time in BP, serum Na, renin, aldosterone and urinary Na related to p.Arg65Leu SNPs.

Parameter	Genotype	Baseline	1 hour	2 hours	3 hours	4 hours
SBP (mmHg)	GG	139.5	132.2	133.9	132.2	132.1
	GT	134.1	126.8	128.1	126.1	127.6
	TT	136.2	130.4	133.1	130.4	132.2
DBP (mmHg)	GG	73.8	68.2	69.8	67.3	68.1
	GT	72.0	69.6	68.7	67.3	67.5
	TT	76.0	73.1	73.1	70.7	72.3
Na (mmol/L)	GG	141.3	-	143.4	-	-
	GT	140.9	-	142.6	-	-
	TT	140.7	-	142.0	-	-
Renin (u/ml)	GG	16.2	-	-	-	15.0
	GT	23.67	-	-	-	24.6
	TT	25..4	-	-	-	11.9
Aldosterone	GG	260.5	-	-	-	75.8
(pmol/L)	GT	234.1	-	-	-	61.7
	TT	190.8	-	-	-	51.0
Urinary Na (mmol)	GG	-	14.3	18.6	24.7	34.1
	GT	-	17.1	25.5	24.6	33.6
	TT	-	19.3	27.9	27.8	30.3

Table 3.1.5. Mean values over time in BP, serum Na, renin, aldosterone and urinary Na related to p.Ala142Val SNPs.

Parameter	Genotype	Baseline	1 hour	2 hours	3 hours	4 hours
SBP (mmHg)	CC	130.9	125.4	125.9	127.1	127.9
	CT	136.8	130.2	131.7	129.6	130.67
	TT	137.8	130.1	133.5	129.7	132.2
DBP (mmHg)	CC	69.2^{+}	67.9	68.3	65.9	67.3
	CT	73.9^{+}	70.2	70.4	68.7	69.5
	TT	76.1^{+}	72.9	72.1	69.9	70.8
Na (mmol/L)	CC	141.3	-	143.4	-	-
	CT	140.9	-	142.6	-	-
	TT	140.7	-	142	-	-
Renin (u/ml)	CC	29.0	-	-	-	11.7
	CT	21.0	-	-	-	13.7
	TT	24.8	-	-	-	23.1
Aldosterone	CC	345.1*	-	-	-	91.5
(pmol/L)	CT	244.5*	-	-	-	71.2
	TT	161.5*	-	-	-	40.2
Urinary Na	CC	-	8.3	11	19.6	33
(mmol)	CT	-	19	29.3	27.7	35.6
	TT	-	20.6	26.8	26.6	29.3

+ - p=0.07, TT/CT vs. CC p=0.04, * p<0.002

Using GLM there was no marker effect for both diastolic and systolic BP after the saline challenge. Na excretion showed significant time and marker effects. C/C was significantly different from C/T ($p<0.004$) and T/T ($p<0.001$) genotyping frequencies. The increase in mean hourly Na excretion from 1 hour to 4 hours was 297% (95% CI: 56.6 to 536.1%, $p = 0.015$) for the C/C genotype, 87% (95% CI: 45.3 to 130%, $p = 0.0001$) for the C/T genotype and 42% (95% CI: 22.3 to 62.1%, $p < 0.0001$) for T/T genotype.

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Renin only showed a significant time effect at 4 hours compared to baseline, whereas aldosterone showed significant time and marker effect at 4 hours between T/T and C/C. There was also a graded aldosterone response at 4 hours similar to baseline levels (C/C>C/T>T/T).

DISCUSSION

As previously discussed, intermediate phenotypes were used to assess the importance of ethnicity and polymorphisms in the *GRK4* gene (namely the p.Ala142Val and p.Arg65Leu variants) on BP, Na excretion and renin and aldosterone responses to saline challenge.

The baseline ethnic data showed previously described differences between whites and blacks. Aldosterone was significantly suppressed in blacks under the same conditions of the experiment. Of interest is the lower K concentrations in whites which is probably related to the higher aldosterone levels as previous data from Charlton et al (2005) have shown that urban blacks and whites consume similar quantities of Na and K in their diets. Whites were taller, heavier, had a higher mean BMI and creatinine compared to blacks, probably reflecting better nutrition in whites. The lower creatinine in blacks did not translate into increased renal function as the calculated creatinine clearance was not different between blacks and whites (Table 3.1). The significantly higher diastolic BP at baseline and throughout the experiment in young Black men is unexplained.

The BP responses to saline challenge did not show any mean rise in BP. In fact, there was a slight lowering of BP in both blacks and whites. Nor did any *GRK*4 polymorphism correlate with any difference in BP response. This probably suggests that assessing acute BP responses to saline challenge is not a good model to assess salt sensitivity, and in retrospect we should have followed the experiment with intravenous furosemide as described by Weinberger et al. (1996) to induce Na depletion, and to investigate the relative rise and fall in BP after Na loading and depletion.

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The response of Na excretion following saline challenge showed significant ethnic trends and differences in the group of subjects with the p.Ala142Val polymorphism. In contrast, the group with the p.Arg65Leu polymorphism did not show any significant trends. In blacks, the initial Na excretion was higher but only showed a 27% increment in Na excretion from 1 hour to 4 hours, whereas in whites the initial Na excretion was lower but the increment in Na excretion from 1 to 4 hours was 150%. Taken together with the significantly lower aldosterone levels at baseline, suggests that black subjects had a tendency to retain Na, thus explaining the initial higher Na excretion, and the relative failure to incrementally excrete Na. In contrast, whites had significantly higher aldosterone levels at baseline suggesting they did not have a tendency to retain Na at baseline. Thus, their Na excretion was lower but showed a highly significant 150% increment. The relative tendency to retain Na in blacks was further confirmed at 4 hours, where their aldosterone levels remained significantly lower compared to Whites.

These ethnic differences in Na excretion and aldosterone levels suggest an underlying genetic difference, as both groups were investigated under the same experimental conditions. There was a significant incremental Na excretion in the C/C genotypic group (297%), which was significantly greater than the C/T group (87%), which in turn was greater than the T/T (42%) group. As dopamine in the proximal tubule is responsible for 50% of the incremental Na excretion in response to Na loading (Jose et al. 2003), these observations would support the hypothesis that the p.Ala142Val polymorphism leads to desensitisation of the agonist. Further support for this hypothesis comes from the aldosterone concentrations, where there was a highly significant graded effect between groups with the varying polymorphisms. The group with the C/C homozygous genotype was highest followed by the C/T heterozygous group, and then the T/T homozygous group. This suggests that research participants with the T/T genotype had the greatest tendency to retain Na and were less able to incrementally increase Na excretion. This contrasts with participants with the C/C genotype where subjects did not have a tendency to retain Na and had less initial Na excretion, but a marked increment with time. Research participants with the C/T genotype demonstrated an intermediate response.

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Although it was not our intention to demonstrate baseline BP difference as we selected young lean subjects without a preceding history of hypertension, the diastolic BP showed borderline statistical trends for the p.Ala142Val genotypes with T/T>C/T>C/C (p=0.07). Similar trends were seen for systolic BP but were not statistically significant. However, when we compared TT/CT vs. CC genotypes, there was a significant difference in diastolic BP. This is an important observation even within the normal ranges of BP as several genetic studies have been hampered by considering hypertension as a categorical variable with an arbitrary cut off level at 140/90 mm Hg. These findings are in agreement with those of Sanada et al (2006b), who demonstrated that the p.142Val genotype was 78.4% predictive of low renin hypertension.

As the C/T and T/T genotype frequencies are significantly higher in blacks compared to whites these observations may explain the predisposition to salt sensitivity and possibly to hypertension in black subjects. From a therapeutic perspective, these findings may lead to the development of a new class of diuretic for hypertension that enhances the effects of dopamine in the proximal renal tubule.

The strength of this study is that it is powered to detect the differences in Na excretion between blacks and whites, and possibly between the different SNPs. Additionally, the Na responses and aldosterone were graded according to the predicted function effects of the p.Ala142Val SNPs, and all subjects were under exactly the same experimental conditions, thereby reducing the chances of confounding factors. Mutations like the p.Arg563Gln in the *SCNN1β* gene cannot account for the differences in aldosterone because only 1.9% of normotensive indigenous Africans carry this variant (Jones et al, 2012).

Limitations of the study include the fact that we did not measure lithium excretion as a marker of proximal tubular excretion of Na, nor did we measure urinary excretion of dopamine. It could be argued that the p.Ala142Val polymorphisms are more common in blacks compared to whites, and merely reflect differences in Na excretion between these ‘groups’. However, this polymorphism has been shown to be functional in rats, resulting

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in increased Na reabsorbtion and suppression of aldosterone levels, which is in keeping with the results of our findings. In addition, the p.Arg65Leu polymorphism is also more common in blacks, but there was no relationship with Na excretion and/or aldosterone levels.

CONCLUSION

Na and aldosterone responses to saline challenge between Blacks and Whites are significantly different. This is seemingly related to the p.142Val genotype of the *GRK4*, which is more common in Black subjects. Additionally, even in the normotensive range, differences in BP were demonstrated in accordance with functional activity of the p.142Val genotype. This study probably explains, in part, the predisposition to salt sensitivity in blacks compared to whites, as the p.142Val genotype desensitises the dopamine receptor and limits Na excretion.

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3.2 DIETARY SALT RESTRICTION IN BLACK PATIENTS WITH MILD TO MODERATE HYPERTENSION

Introduction

As previously discussed a more classical approach to the determination of salt sensitivity will be used, i.e. patients with salt sensitivity are expected to show a greater BP response to salt restriction whereas salt resistant individuals are expected to show little change.

BP responses to dietary salt restriction in black hypertensives was examined in a randomized, double blind, controlled trial to investigate the impact of an 8-week feeding study (in which Na intake is decreased, and potassium, magnesium and Ca intake is increased) on BP in mild–to-moderate hypertensive black South Africans, and to investigate if the response was related to *GRK4* polymorphisms.

HYPOTHESIS:

BP response to dietary Na restriction is predicted by *GRK4* SNPs.

AIMS

To demonstrate that ambulatory BP response to Na restriction is predicted by the p.Ala142Val and p.Arg65Leu polymorphisms in *GRK4*.

METHODS

Research participants and sample size

The methods for this study have been previously published in detail. (Charlton et al, 2008) Black residents of a peri-urban suburb of Cape Town (i.e. Langa), aged 50 - 75 years, with drug-treated mild-to-moderate hypertension (Systolic BP ≤ 160 mmHg and BP Diastolic ≤ 95 mm Hg) were eligible for the study. Exclusion criteria included taking two or more diuretics; taking furosemide for cardiac failure; cerebral infarction or hemorrhage; renal impairment (serum creatinine > 114.4 umol/l or creatinine clearance < 50 ml/min) (Cockroft-Gault equation;Cockroft and Gault 1976), three or more alcoholic

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drinks per day; Type 1 diabetes mellitus; impaired cognitive function incontinence; and a Body Mass Index (BMI) > 45kgm^{-2}.

Sample size calculations were performed using the nQuery Advisor 5.0 program. Assuming a reduction in systolic BP of 6.3 mmHg, a common (SD) of 10 mmHg, and a two group t-test with a 0.05 two-sided significance level and 80 % power, a sample size of 41 subjects per arm (N = 82 in total) was required.

The intervention comprised 7 commonly consumed food items modified by reducing the Na content and increasing K, Mg and Ca content. The modified foods were a salt replacement (Solo™), bread, margarine, stock cubes, soup mixes and Aromat (monosodium glutamate-based flavour enhancer). *Maas* (fermented milk commonly eaten with the staple maize porridge) was used to further increase Ca intake. The control diet provided the same foods of standard commercial composition. Artificially-sweetened cold drink replaced *maas* in the control arm to ensure equivalent fluid intake between the two diets.

Subjects were instructed to consume their usual amounts of food and sufficient food was provided for the whole family. Based on laboratory-determined chemical food analyses, compared to control foods, the intervention foods provided 41% less Na (100.3 vs. 170.3 mmol/day, respectively), 826% more K (70.9 vs. 8.6 mmol/day), 388% more Ca (857 vs. 221 mg/day) and 368% more Mg (13.8 vs. 3.7 mmol/day).

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Outcomes

Primary outcome measures included resting office BP and 24-hr ambulatory BP. Resting BP was measured according to the American Heart Association Recommendations for Human BP Determination, using an automated Omron BP monitor (Omron Health Care Inc., Illinois) endorsed by the British Hypertension Association. A large cuff was used for arm circumferences ≥ 33 cm. BP was measured three times on each occasion and an average of the second and third measurements used for analyses. BP was measured weekly, four times before the initiation of the intervention, and the mean taken as

baseline BP ("Pre"). Thereafter, BP was measured on two occasions at weeks four and eight.

Average 24-hour ambulatory systolic, diastolic BP, awake and asleep BP were measured at baseline and during the final week of the intervention, using an Oscar 2 (SunTech Medical) Ambulatory BP Monitor (ABPM) which has been validated for use in clinical trials according to international protocol (Jones et al, 2004). Blood was taken at baseline for DNA analysis.

ETHICAL CONSIDERATIONS

The Research Ethics Committee of the University of Cape Town approved the study protocol. The trial was registered with the South African Department of Health National Research Register (DOH-27-0806-1394). Written informed consent was obtained from all participants who were closely monitored for adverse effects and had access to a back-up medical service throughout the trial.

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STATISTICAL ANALYSES

Intention-to-treat analyses were performed using a 5% significance level. For analysis of office (Omron) BP, linear regression was performed to assess the intervention effect at weeks 4 and 8. The model had indicators for diet group, phase and time and interaction effects for testing the consistency of the intervention effect over the two phases and the expected differential change in BP over time in the two diets. The analysis accounted for the repeated nature of the BP measurements within each subject by using the generalised estimation equation (GEE) approach. From this model, a contrast in Omron BP measurements between baseline (mean of four repeated measurements) and the intervention period (mean of two repeated measurements) was estimated.

For analysis of 24-hr ABPM, multivariate linear regression was used to assess the intervention effect. The vector of 24-hr ABPM measurements (systolic and diastolic BP for average, wake, sleep and mean arterial pressure (MAP)) obtained at the end of the intervention period was modeled on indicator variables for diet and phase, as well as the baseline values of average 24-hr systolic and diastolic BP and 24-hr MAP. For both the

Omron BP and 24-hr ABPM, the intervention effect was estimated for the pooled data over both phases. Confidence intervals (95%) were estimated to reflect the precision of those estimates.

Since the BP measurements were done over time, the analysis of the study groups and genetic marker groups were analysed using repeated measures analysis. This was performed using GLM with an identity link function and clustering indicator for each participant, to enable the standard errors of the estimates to be adjusted for the repeated measurements. The regression models included interaction terms of time and genetic marker group, since this was the main inference of the study.

GENETIC ANALYSIS

DNA was genotyped in the research participants for the *GRK4* polymorphisms, p.Ala142Val and p.Arg65Leu, as previously described in the chapter.

RESULTS

i) Overall

The control and intervention groups were well matched at baseline (Table 3.2.1) with no statistical difference. The parameters were normally distributed.

Table 3.2.1 Baseline characteristics of control and intervention group

Parameter	Control (n=41)	Intervention (n=41)
Age (years)	61.8 (6.6)	60.4 (7.4)
Male/female	33/7	34/6
Systolic BP (mmHg)	133.9 (14.6)	135.4 (16.7)
Diastolic BP	79.8 (8.6)	82.3 (7.5)
Mean 24 hour systolic BP	135.0 (13.5)	138.9 (170
Mean 24 hour diastolic BP	79.3 (8.7)	80.4 (8.9)
Weight (kg)	83.3 (13.7)	88.8 (15.5)

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In the overall study, there was a significant decline of BP in the intervention group. The intervention effect estimated as the contrast of the within-diet group changes in BP from baseline to post-intervention, demonstrated a significant reduction of 6.2 mm Hg (95% CI 0.9-11.4) for systolic BP. The largest intervention effect in 24 hour BP was observed for wake systolic BP with a reduction in 5.12 mm Hg (95% CI 0.4 – 9.9). For wake diastolic this was 2.7 mm Hg (95% CI -0.2 – 5.6). Based on 24 hour urine estimations, the difference in mean urinary Na, K, Mg and Ca between the control and intervention group was -8.7, +24.6, +0.68, and -0.5 mmol/24 hours, respectively.

ii) **Distribution of genetic markers**

The distribution of p.Ala142Val and p.Arg65Leu polymorphisms in *GRK4* is shown in Table 3.2.2 and 3.2.3. These were in Hardy Weinberg Equilibrium.

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Table 3.2.2. Genotying distribution of the p.Ala142Val variant

p.Ala142Val	Control (%)	Intervention (%)	Total (%)
C/C (%)	2 (5.26)	1 (2.6)	3 (3.85)
C/T (%)	9 (23.68)	10 (25)	19 (24.36)
T/T (%)	27 (71.05)	29 (72.5)	66 (71.79)
Total (%)	38 (100)	40 (100)	78 (100)

Table 3.2.3 Genotyping distribution of the p.Arg65Leu variant

p.Arg65Leu	Control (%)	Intervention (%)	Total (%)
G/G (%)	2 (5.71)	2 (5.13)	4 (5.41)
G/T (%)	14 (40)	16 (41.03)	30 (40.54)
T/T (%)	19 (54.29)	21 (53.85)	40 (54.05)
Total (%)	35 (100)	39 (100)	74 (100)

Unfortunately the numbers of C/C and G/G genotypes was very small, but this is in line with the genotype frequencies observed in the Black subjects in the saline challenge study. The C/C and C/T genotypes of the p.Ala142Val, and the G/G and G/T genotypes of the p.Arg65Leu were combined for statistical analysis, respectively.

iii) Baseline characteristics according to group assignment and *GRK4* polymorphisms

The baseline ambulatory BP characteristics of the groups according to genotype are shown in Table 3.2.4. There were no significant differences between the groups.

Table 3.2.4 Baseline ambulatory BP according to group assignment and *GRK4* genotypes

p.Ala142Val	**CONTROL**		**INTERVENTION**	
Genotype	**CC/CT (n=11)**	**T/T (n=27)**	**CC/CT (n=11)**	**T/T (n=29)**
Ave SBP	**135.7**	**140.1**	**140.5**	**132.9**
Ave DBP	**80.9**	**80.4**	**83.8**	**77.5**
p.Arg65Leu	**CONTROL**		**INTERVENTION**	
Genotype	**GG/GT (n=16)**	**T/T (n=19)**	**GG/GT (n=18)**	**T/T (n=21)**
Ave SBP	**140.1**	**139.3**	**138.1**	**132.1**
Ave DBP	**81.9**	**79.9**	**81.2**	**77.8**

Legend: Ave = average, SBP = systolic BP, DBP = diastolic BP, MAP = mean arterial pressure, units = mmHg

iv) BP responses according to genotype

a) Control group

In the control group there was no significant difference in mean 24 hour systolic BP or mean diastolic BP at baseline and at the end of the study, in the response according to genotype (Figures 3.2.1 and 3.2.2).

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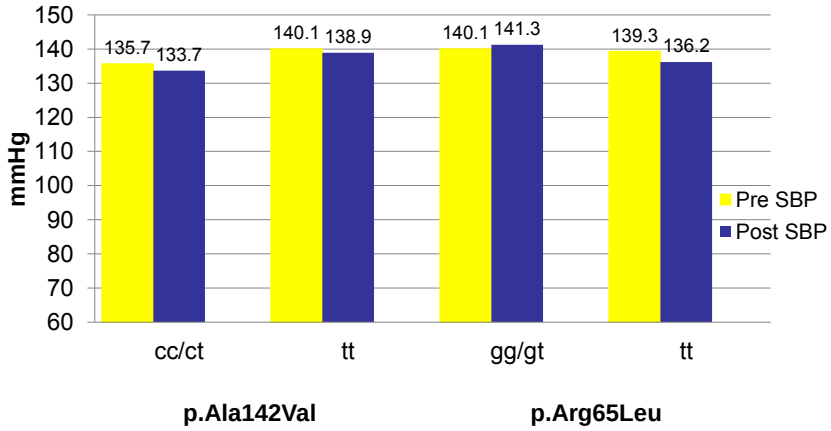

Figure 3..2.1. Changes in mean 24 systolic BP in the control group according to genotype

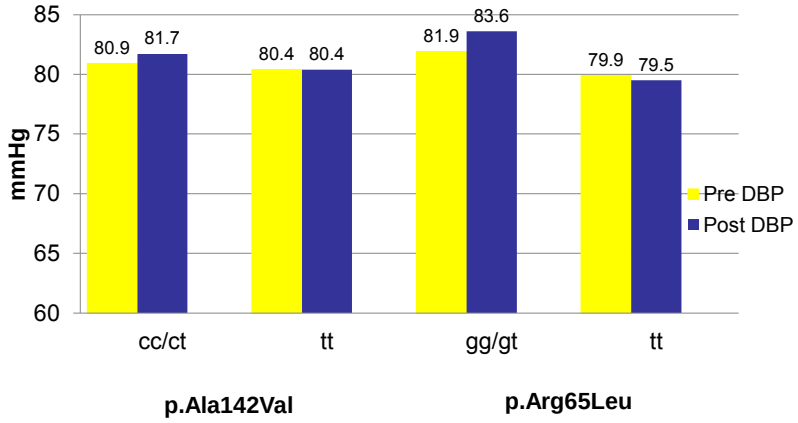

Figure 3..2.2. Changes in mean 24 diastolic BP in the control group according to genotype

b) Intervention group

In the combined group the 24 hour average systolic and diastolic BP is shown in figures 3.2.3 and 3.2.4.

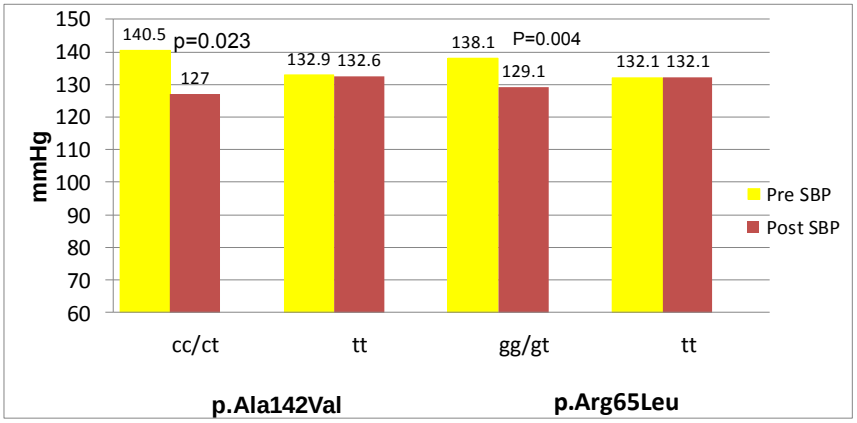

Figure 3.2.3. Changes in mean 24 systolic BP in the intervention group according to genotype

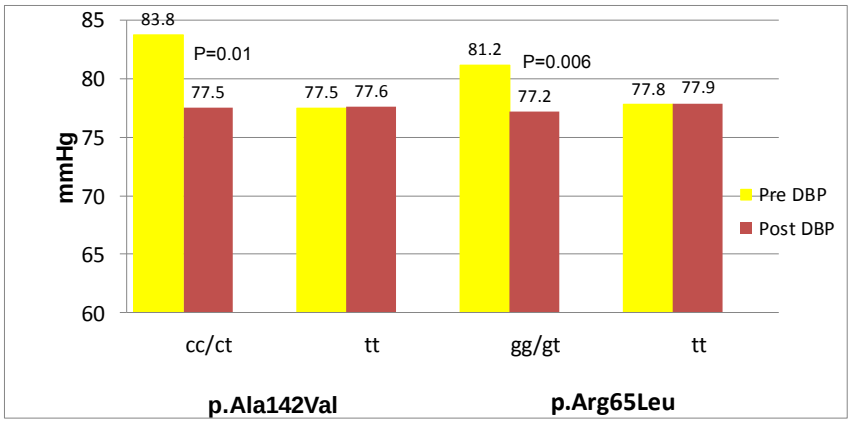

Figure 3..2.4. Changes in mean 24 diastolic BP in the intervention group according to genotype

Using GLMs in the combined CC/CT category of the p.Ala142Val variant, there was a significant response to dietary intervention for average systolic BP (p=0.023) and average diastolic BP (p=0.01). In contrast, there was no BP response to intervention for the T/T genotype. For diastolic BP, this was contrary to the overall intervention group (p=0.05).

With regard to the combined GG/GT category of the p.Arg65Leu variant, there was a highly significant BP response to dietary intervention for average systolic BP (p=0.004) and average diastolic BP (p=0.006). In contrast, the T/T genotype showed no BP response and there was a contrary effect for average systolic BP (p=0.024) and average diastolic BP (p=0.04) to the overall intervention group.

DISCUSSION

In this controlled study, which was similar to the DASH-Na study (Sacks et, 2001), a dietary intervention with Na reduction with increased K, Mg and Ca was associated with a significant reduction in BP (mainly systolic) and 24 hour ambulatory BP. The changes in diet were confirmed by 24 hour urine excretion of these electrolytes.

In a sub-study the dietary response was analysed according to *GRK4* genotypes. The main finding of the study was that subjects with the GG/GT genotype of the p.Arg56Leu polymorphism, responded to dietary intervention and there was no response in the TT group. Similar findings were evident in the combined CC/CT vs the TT genotypes of the p.Ala142Val polymorphism, but were less statistically robust (p=0.024). There was no influence on BP in the control group.

The therapeutic importance of the *GRK4* SNPs was emphasised in the African American Study of Kidney Disease (AASK) where African-American hypertensives with hypertensive nephrosclerosis were randomised to the antihypertensive agents, amlodipine, ramipril or metoprolol (Bhatnager et al, 2012). Sex specific responses to the β-blocker, metoprolol, were identified in this study. Men with the p.142Ala genotype were less likely to respond to metoprolol, especially if they had the p.65Leu variant. This may well be the reason why African-Americans respond less well to β-blockers, as this class of drug has very little influence on Na metabolism. Furthermore, in the analysis of response to treatment in two major hypertension studies, the 56Leu/142Val haplotype predicted a significantly decreased response to atenolol treatment, and the 56Leu/142Val and the 486Val homozygote were associated in an additive fashion with adverse CV outcomes independent of BP (Vandell et al. 2012).

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The strength of the present study is that BP responses were based on 24 hour ambulatory monitoring, which is the most accurate assessment of BP. In addition, as the food was supplied to the subjects, adherence to the study protocol was good (as further confirmed/validated by 24 hour urine collections). The limitations of the study were the relatively small numbers of subjects especially in the C/C and G/G allelic genotyping groups. The small numbers also did not allow us to investigate interactions between the p.Arg65Leu and p.Ala142Val variants.

This study provides further confirmation of the importance of *GRK4*. Previously it was shown that the p.142Val homozygous genotypes are linked to Na excretion in the kidney after acute saline challenge. Although the p.56Leu homozygous genotypes were not linked to Na excretion, it does not negate their strong prediction of response to dietary intervention as the mechanism/s underlying responses to Na over load; this may be disparate from responses of BP to Na restriction.

Of course it is important to note that this is not strictly a dietary Na restriction study, and involved supplementation of the diet, especially with K. However as elegantly outlined by Androgué and Madias (2007), deficiency in K in the diet enhances the adverse effects of high Na (figure 3.2.5), and fits with our current hypothesis: that lack of renal adaptation or other defects in the kidney results in maladaptation to the current Westernised diet, which is highly prevalent amongst urban South Africans. The thesis proposes that the *GRK4* polymorphisms in the proximal tubule of the kidney impair Na excretion in response to high Na/low K diet, and thus determines the BP response to dietary intervention. As these polymorphisms (T/T of the p.Ala142Val and T/T of the p.Arg65Leu) are more common in people of African descent, it may underpin the susceptibility to salt sensitivity.

However there is a major underlying paradox in our experiments that challenges definitions related to the salt-sensitive phenotype/genotype. The hypothesis correctly predicted that the C/T and T/T of the p.Ala142Val would have lower aldosterone levels (an indirect index of Na overload) and impaired ability to incrementally increase Na

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excretion after saline challenge, compared to the C/C genotype. However the expected increase in BP was not shown for either the C/T or T/T genotypes. The p.Arg65Leu polymorphism had no effect on either Na excretion or BP.

In contrast, in the dietary intervention our hypothesis would have predicted that the T/T genotype of the p.Ala142Val and the T/T genotype of p.Arg65Leu would have shown the greatest BP reduction, as these subjects are the "most salt sensitive". As discussed previously, this was not the case. In fact, the combined C/C and C/T of the p.Ala142Val and the combined G/G and G/T of the p.Arg65Leu showed the best response whilst both T/T groups exhibited no response. These two studies raise important issues around the definition of salt sensitivity. Is it based on BP response to Na restriction, low renin and/or aldosterone levels or impaired Na excretion by the kidney? These definitions appear to be used interchangeably in the literature.

Sanders (2009) states that salt sensitivity is either a hereditary or acquired defect of salt excretion by the kidney. Sanada et al. (2006a) use the term salt sensitivity and low-renin hypertension interchangeably, whilst Weinberger et al. (2001) defined it by BP response to Na overload and Na restriction i.e. Na loading leads to greater increases in BP and restriction of this cation results in greater reduction in BP. Our experiment showed that these terms cannot be used interchangeably. In fact, the polymorphisms associated with impaired Na excretion and low aldosterone were the subjects in the dietary intervention group that had no BP response. Those with polymorphisms associated with greater Na excretion had the best BP response to dietary intervention. Acute Na loading had no effect on BP in either group. To help understand these paradoxes it may be helpful to consider that there may be different sets of responses to Na loading or restriction. For instance, from first principles, in a subject who has genetic variants that promote Na retention, one would actually expect the renin and aldosterone to be suppressed.

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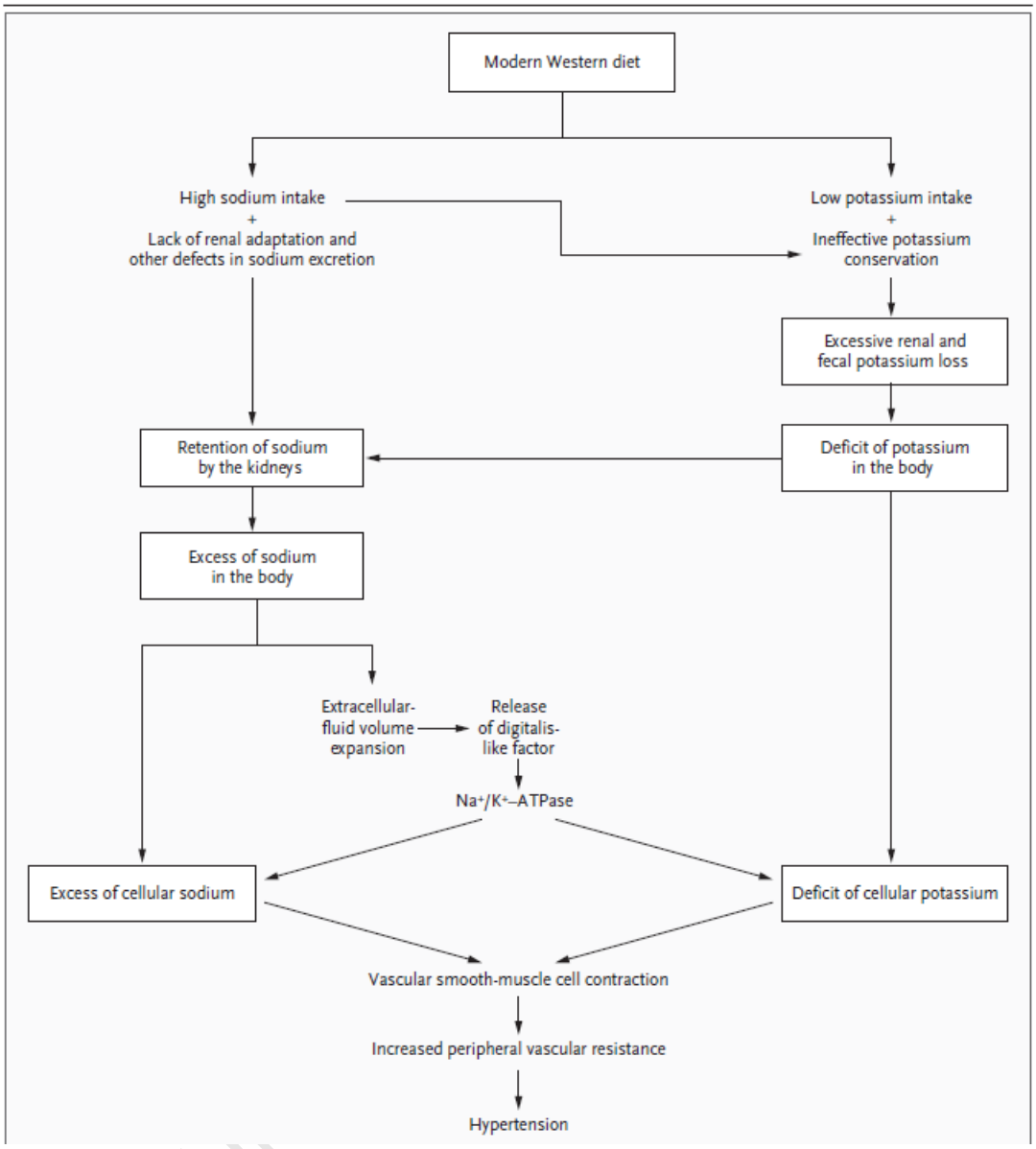

Figure 3.2.5. Renal adaptation to high Na/low K diet. (Androgué and Madias, 2007)

However, this subject may actually be better able to counter the effects of Na restriction on BP compared to a subject without Na-retaining genetic variants. Current dogma states the opposite, and our experiment challenges this. There needs to be an important rethink on this issue and salt sensitivity. It should probably be best defined by the inability to excrete Na after loading, and indirectly by evidence of suppressed renin and/or aldosterone, which may be hereditary or acquired.

Linking of BP to salt sensitivity is fraught with many problems as we have shown in our experiments. It has been clearly shown that salt sensitivity in terms of inability to excrete Na, and suppressed aldosterone, is present in normotensives and may only be an intermediary phenotype that may be just one factor in the ultimate pathogenesis of overt hypertension.

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CHAPTER 4
COMPUTATIONAL ANALYSIS OF CANDIDATE DISEASE GENES AND VARIANTS FOR SALT-SENSITIVE HYPERTENSION IN INDIGENOUS SOUTHERN AFRICANS – A GENOME WIDE APPROACH

INTRODUCTION:

In the previous chapter, the focus has been on a single candidate gene to investigate salt sensitivity. It is clear the *GRK4* polymorphisms are associated with responses to saline challenge and dietary effects of Na restriction, but no clear relationship to salt sensitive hypertension was demonstrated.

As previously discussed, hypertension is a typical example of a common complex disease where phenotypic association with individual candidate genes has been disappointing and difficult to prove. Computational identification of most likely etiological genes can facilitate more efficient identification of genes of diagnostic, prognostic and therapeutic value by presenting strong candidates for future empirical research. Regions of the genome implicated in susceptibility to multi-factorial disease by linkage analysis can be up to 30 Mb, often containing several hundreds of genes (Perez-Iratxeta et al. 2005). Comprehensive empirical analysis of so many candidate genes is prohibitively time-consuming and expensive, and thus there is a pertinent role for bio-informatic methodologies that mine and analyse biological data in existing databases to develop a subset of candidate genes for empirical validation.

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AIM

The aim of this study, therefore, was to use computational approaches to prioritize and present most likely candidate disease genes for salt-sensitive hypertension, for further empirical analysis.

METHODS

The analysis used a computational approach that combined text-mining of PubMed abstracts for potentially relevant genes, and extensive mining of gene annotation data. Affymetrix SNP data in a total of 126 South African individuals, spanning five distinct

indigenous South African population groups, was analysed for top-scoring candidate genes from the computational analysis. Allele frequencies at all SNPs assayed in indigenous Southern Africans, were compared with those in the Caucasian population assayed in the HapMap project. Variation in the copy number of candidate genes between the different population groups was analysed. The intention was to prioritise candidates that are sufficiently different between the indigenous South African and Caucasians to potentially underlie susceptibility to salt-sensitive hypertension in the former population group.

This project has not previously been undertaken. An approach was developed based on first principles related to genes and structures that influence Na reabsorbtion and excretion (Table 4.1).

Selection of candidate genes by Gene Ontology (GO) annotation

Data was accessed from the Ensembl database (Flicek et al. 2008). GO annotations were selected using AmiGO (www.geneontology.org) to reflect a range of pathways and functions. An overview of the functions and pathways included is shown in Table 4.1

Selection of candidate genes by text mining of PubMed abstracts:

Text-mining was used to identify gene names that most frequently occur in conjunction with a variety of salt-sensitive hypertension-related terms. These terms are described in Table 4.1. The PubMed queries were made on March 24, 2008. Six queries (Table 4.1) retrieved 22,966 PubMed abstracts, in total, that were analyzed by Dragon Explorer System (DES), a licensed tool of OrionCell (http://www.orioncell.org). DES uses proprietary and manually curated dictionaries of entities related to various topics and maps them to the text documents for extracting potentially relevant information from these documents. The functioning of the text-mining modules of DES is based on similar concepts as described by Pan et al. (2004) and Bajic et al. (2005), and has been

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Table 4.1. Criteria for terms used to identify candidate genes

Genes involved in regulatory pathways, and second messenger/signalling pathways, as annotated with GO terms;	**Genes**	**Score**
1. Renin-angiotensin-aldosterone pathway i. 'renin'-containing GO terms ii. 'angiotensin'-containing GO terms iii. 'aldosterone'-containing GO terms 2. Renin-angiotensin-aldosterone pathway second messengers i. cAMP/protein kinase- associated GO terms ii. Ca signalling – related GO terms iii. Protein Kinase A – related GO terms	183	1
3. Adrenergic or sympathetic nervous system i. "adrenergic"- containing GO terms ii. "sympathetic"-containing GO terms 4. Adrenergic or sympathetic nervous system second messengers i. GTP-binding – related GO terms	1707	1
5. Brain and atrial natriuretic peptide i. natriuretic peptide receptor activity –related GO terms ii. regulation of systemic arterial BP by atrial natriuretic peptide –related GO terms 6. Brain and atrial natriuretic peptide signalling-related terms i. cGMP/Protein Kinase G – related GO terms	17	1
7. Dopaminergic system i. 'dopamine receptor'– containing GO terms 8. Dopaminergic system signalling-related terms i. 'dopamine receptor signaling' – related GO terms ii. adenylate-cyclase/dopamine receptor/G-protein – related GO terms iii. cAMP-dependent protein kinase–related GO terms iv. phospholipase C-related GO terms	210	1
Gene product is part of sodium pump or channel		
9. "sodium channel"- containing GO terms	49	1
Gene influences sodium excretion or reabsorbtion in the kidney		
10. Gene names co-occurring with sodium excretion/reabsorbtion/kidney terms in PubMed abstracts		
i. Genes co-occurring with "sodium excretion" and "kidney" [4173 PubMed hits]	59	0.5
ii. Genes co-occurring with "sodium reabsorbtion" and "kidney" [1568 PubMed hits]		
iii. Genes co-occurring with "sodium" and "reabsorbtion" and "kidney" [4643 PubMed hits]	84	
iv. Genes co-occurring with "sodium" and "excretion" and "kidney" [9799 PubMed hits]	140	0.5
	99	
Activity of gene is associated with low renin and with variable aldosterone levels		
11. Gene names co-occurring with low renin/variable aldosterone levels in PubMed abstracts		
i. Genes co-occurring with "low renin" [997 PubMed hits]	24	1
ii. Genes co-occurring with "aldosterone levels" [1786 PubMed hits]	41	

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previously applied in the creation of a DDESC database of Na channels (Sagar et al. 2008) and for parts of the DDOC database (Kaur et al. 2009) and DDEC databases of esophageal cancer (Essack et al. 2009).

In this study, DES is applied with the dictionary of "human genes and proteins" that contains over 300,000 variants of names, symbols, aliases, previous names and previously used symbols of genes and proteins, compiled from the literature and public databases. In the study by Sagar et al (2008), the accuracy of DES systems to correctly identify human genes and proteins in PubMed abstracts was estimated to be with a sensitivity of 81%, a specificity of 96% and an F-measure of 88%. After gene and protein names had been identified, the respective EntrezGene IDs were determined, which eliminated naming redundancies. These genes have been used for further analysis in our study.

Scoring and ranking of candidate genes

For each included gene, a cumulative score was assigned for every category assayed that was met by the gene. For most categories, the gene was assigned a score of 1 if the category was met. However, for some of the terms found in PubMed abstracts, this score was divided such that a score of 0.5 was assigned if the gene co-occurred with the independent components of the given phrase. An additional score of 0.5 was subsequently only assigned if certain of these components occurred together as a complete phrase. For example a gene co-occurring with “sodium” and “reabsorbtion” and “kidney” will score 0.5, whereas a gene co-occurring with “sodium reabsorbtion” and “kidney” will score (0.5+0.5)=1.

Selection of SNPs by population-specific allele frequencies

This study was reviewed and approved by the Research Ethics Committee of the University of Cape Town (ethics approval REC REF 305/2009: "Genome Wide Microarray Analysis of Southern African Human Populations"). Data analysis was performed using a combination of Python scripting and MySQL databases, scripts and datasets. For the five indigenous South African population groups, allele frequencies in the genetic material from a total of 126 individuals was analysed using the Affymetrix

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GenomeWideSNP 6.0 Array (Homo sapiens, Genome assembly: NCBI Build 36, UCSC hg18), and covering 933684 SNPs. Population groups included are, with number of individuals in parentheses, Khoisan (22), Xhosa (34), Hererro (25), Setswana (25) and Zulu (20). For each candidate gene, all SNPs analysed using the Affymetrix array were selected (a total of 1079 SNPs), and the allele frequencies calculated across the South African populations. All of these South African allele frequencies for each South African population group were then compared to the allele frequencies for these SNPs as reported for Caucasians in the HapMap project (The International HapMap Consortium, 2005). Information regarding the nature of each SNP was downloaded from the Ensembl database (www.ensembl.org) wherever such data was available.

Analysis of Copy Number Variation

Copy number analysis was performed with the Birdsuite package (version 1.5.2; Korn et al. 2008), which uses hybridisation intensities of both SNP and CN probes to provide greater coverage and enable the detection of novel as well as known copy-number variations. Default settings, as described by Korn et al. (2008) were used, with the exception that copy number models were not limited to known variants. Reference CEL files for the HapMap CEU population were processed using the same configuration. In addition to the previously mentioned samples filtered due to low quality, two Zulu samples were identified as having high copy number variance and removed. For each gene and its flanking sequence, a heatmap was generated to indicate copy number of probes assayed.

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STATISTICAL ANALYSIS

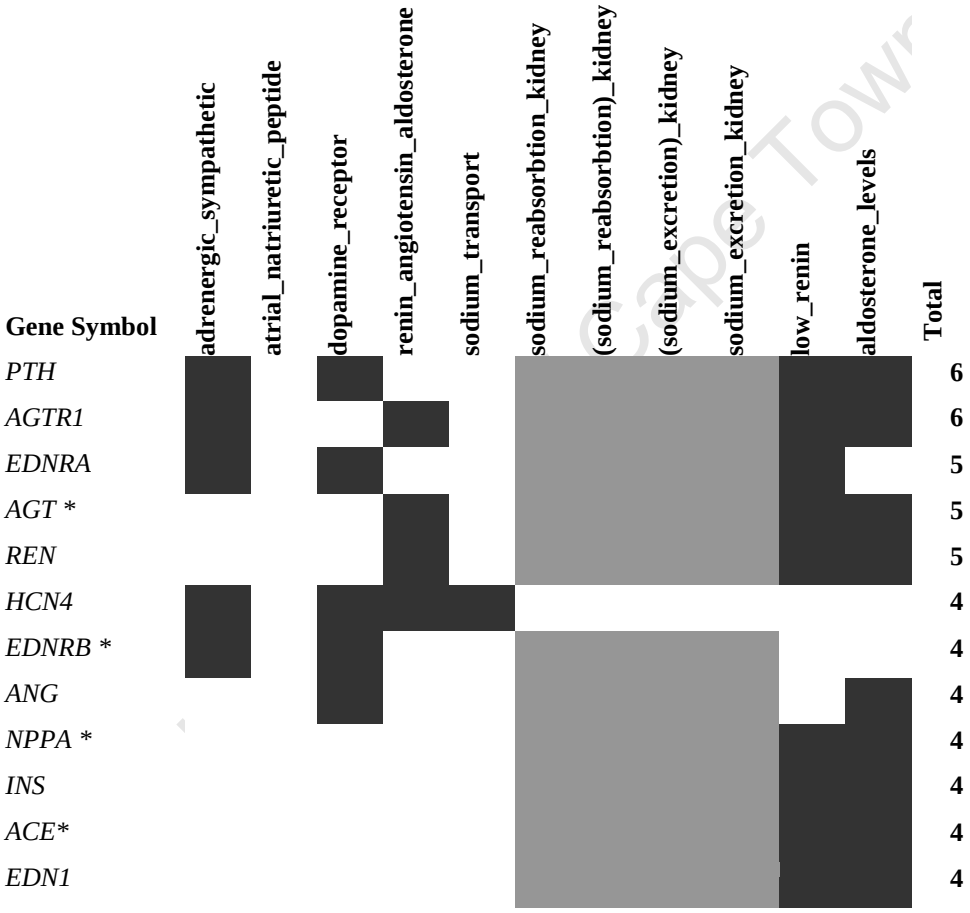
The significance level of differences in allele frequencies for the same SNPs between the different populations was calculated using the Fishers Exact Test, using Python scripting with the RPy module (http://rpy.sourceforge.net/) and R statistical software (Ihaka et al, 1996)

IDENTIFICATION OF CANDIDATE GENES

In total, 2057 unique genes were included across all lists, and were used as the primary set of candidate genes. Each of these was then assayed for the various characteristics (see

Table 4.1), by both text-mining and GO annotation, and assigned a cumulative score, shown in Table 4.2.

Table 4.2. Scoring of candidates selected for further analysis.
Clear cells – score = 0; grey cells – score = 0.5, black cells – score = 1.0. Genes with appropriate second-messenger annotations are included in each category. * Genes also annotated with hypertension-related GO terms.



The genes were ranked by this score, and the top scoring candidates prioritised as most likely candidate disease genes. The top scoring candidates, *PTH - Parathyroid hormone precursor* and *AGTR1 - Type-1 angiotensin II receptor*, were curated to exclude any

spurious results.. A selection of additional likely candidates was made from those ranked in the top twenty positions, as shown in Table 4.3.

Table 4.3. Candidate genes selected for further analysis.

Gene Symbol	Ensembl ID	Description
PTH	ENSG00000152266	Parathyroid hormone precursor (Parathyrin) (PTH) (Parathormone)
AGTR1	ENSG00000144891	Type-1 angiotensin II receptor (AGTR1) (AT1AR) (AT1BR).
EDNRA	ENSG00000151617	Endothelin-1 receptor precursor (Endothelin A receptor) (ET-A) (hET- AR) (ETA-R).
AGT	ENSG00000135744	Angiotensinogen precursor (Serpin A8) [Contains: Angiotensin-1 (Angiotensin I) (Ang I); Angiotensin-2 (Angiotensin II) (Ang II); Angiotensin-3 (Angiotensin III) (Ang III) (Des-Asp[1]-angiotensin II)].
REN	ENSG00000143839	Renin precursor (EC 3.4.23.15) (Angiotensinogenase)
HCN4	ENSG00000138622	Potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 4
EDNRB	ENSG00000136160	Endothelin B receptor precursor (ET-B) (Endothelin receptor Non- selective type).
HCN2	ENSG00000099822	Potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 2 (Brain cyclic nucleotide-gated channel 2) (BCNG-2).
ANG	ENSG00000214274	Angiogenin precursor (EC 3.1.27.-) (Ribonuclease 5) (RNase 5).
NPPA	ENSG00000175206	Atrial natriuretic factor precursor (ANF) (Atrial natriuretic peptide) (ANP) (Prepronatriodilatin) (CDD-ANF) [Contains: Cardiodilatin-related peptide (CDP)].
INS	ENSG00000129965	Insulin precursor [Contains: Insulin B chain; Insulin A chain]
ACE	ENSG00000159640	Angiotensin-converting enzyme
EDN1	ENSG00000078401	Endothelin-1 precursor (Preproendothelin-1) (PPET1) [Contains: Endothelin-1 (ET-1); Big endothelin-1].

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COMPARATIVE ANALYSIS OF SNP FREQUENCIES IN CANDIDATE GENES, BETWEEN POPULATIONS

A total of 1079 SNPs were selected from the Affymetrix data as associated with the prioritised candidate genes, falling either within the genes, or within 100 000 bp either upstream or downstream of them. Of these, 303 had allele frequencies that were significantly different between Caucasian and all indigenous Southern African populations at a threshold of $p<0.05$. There were 159 SNPs that had allele frequencies that were significantly different at a threshold of $p<0.001$. The categories and numbers of SNPs selected are shown in Table 4.4.

Table 4.4. The categories and numbers of all SNPs selected.

	With 100 000 bp flanking sequence		Without flanking sequence	
SNP Type	P < 0.05	P < 0.001	P < 0.05	P < 0.001
3' Downstream	18	13	1	1
3' UTR	5	3	1	0
5' Upstream	14	6	0	0
Coding synonymous	3	3	2	2
Coding nonsynonymous	1*	0	0	0
Intronic	104	43	43	22
Undefined	158	91	0	0

* This falls in a neighbouring gene, and not the candidate gene of interest.

Additionally, the numbers of SNPs falling within the coding region of the gene and not the flanking sequence are shown. These were distributed across the candidate genes as shown in Table 4.5, and results for a selection of genes are shown in Figure 4.1. The distribution of SNPs within the genes and excluded from the flanking sequence are also shown.

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Table 4.5 The numbers of SNPs selected for each candidate gene.

		Flanking sequence included			Gene sequence only		
Gene Name	Gene Length (bp)	Total SNPs Assayed	SNPs selected p<0.05	SNPs selected p<0.001	Total SNPs Assayed	SNPs selected p<0.05	SNPs selected p<0.001
HCN4	47242	49	7	2	8	0	0
HCN2	27264	17	4	3	1	1	1
INS	32100	46	9	3	9	1	1
NPPA	2633	59	11	4	3	0	0
EDNRB	24126	82	21	6	16	4	1
PTH	3965	48	14	9	1	1	1
AGT	11667	131	27	9	9	0	0
ACE	44769	45	16	9	19	4	2
ANG	9571	130	22	15	8	0	0
REN	11517	110	35	16	15	2	2
EDN1	6800	124	38	24	9	5	3
EDNRA	63980	101	42	27	42	18	9
AGTR1	45123	137	57	32	25	11	5

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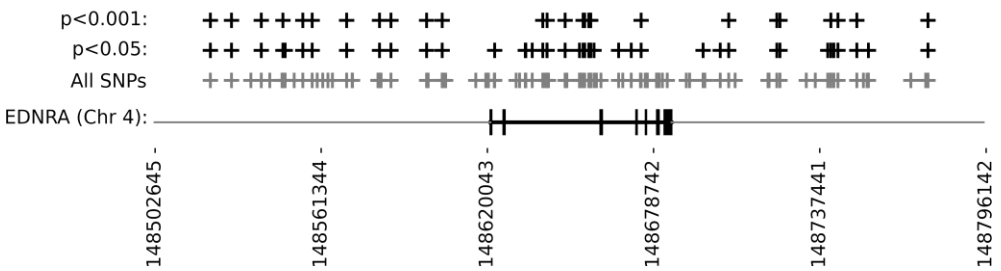

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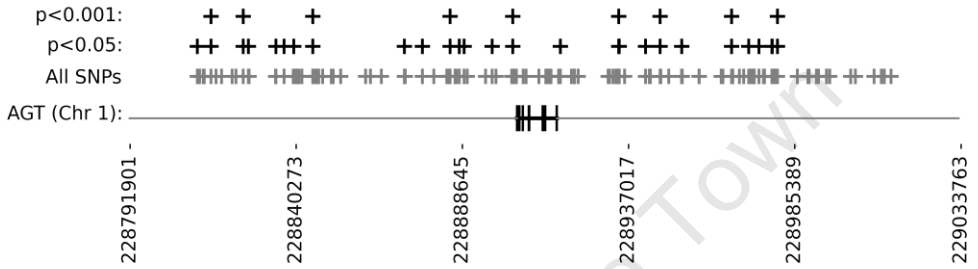

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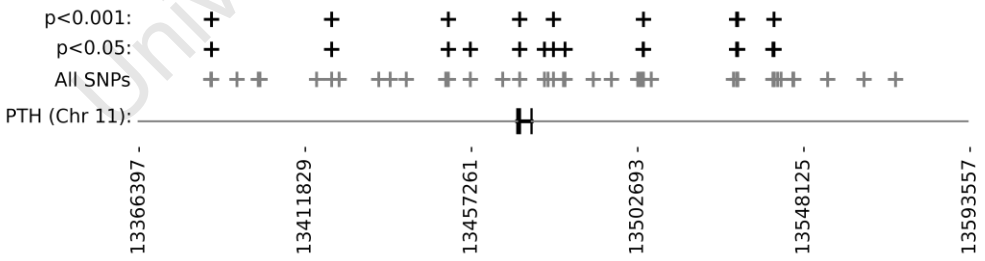

D.

Figure 4.1. Examples of distribution of SNPs with significantly different allele frequencies when comparing indigenous Southern African and Caucasian populations. Gene exon structure (bold vertical bars), chromosome and base pair position (horizontal axis) are shown. Each black cross represents a SNP with allele frequencies that differ between Caucasian and South African populations at the level of significance indicated, and the grey crosses represent all SNPs analysed. A. *EDNRA* (many SNPs within the gene) B. *AGT* (a few SNPs within the gene, most SNPs in flanking regions); C. *ANG* (no SNPs within the gene); D. *PTH* (few SNPs assayed within the gene)

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Analysis of Copy Number Variation

Variation in copy number of individual probes is evident, as shown on the heatmaps generated; however there was no evidence of a change in copy number at the gene level for any of the candidate genes analysed.

DISCUSSION

The high ranking genes in the candidate gene list included several previously identified genes associated with hypertension, and involved in Na metabolism. The top scoring *AGTR1* gene has been extensively implicated in controlling BP and volume in the cardiovascular system, and pharmacologic agents that interrupt the action of angiotensin II action by antagonizing the AGTR1 receptor are highly successful in the treatment of angiotensin II-dependent hypertension. *AGT*, *AGTR1*, *ACE* and *REN* are all members of the RAAS. ACE inhibitors and AGTR1-blocking drugs are already extensively exploited as therapy for hypertension. Given that these genes are well annotated and form the foundation of the characteristics used to select new candidates, it is unsurprising that they are highly scored in the analysis and act as an internal control showing that the selective process is accurate. The same is true of *NPPA*- Atrial natriuretic peptide, which is involved in Na excretion by the kidney. Interestingly, this gene was not selected by atrial natriuretic peptide-specific GO annotation, but rather by association in PubMed abstracts with terms including Na reabsorbtion/excretion, low renin and aldosterone levels.

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PTH was also top-scoring alongside *AGTR1*. It has a less defined role in essential hypertension, which makes it an interesting novel candidate for salt-sensitive hypertension. Several lines of research support a potential role for PTH in the pathogenesis of salt-sensitive hypertension. PTH may be raised as a secondary response to hypercalciuria due to excess Na intake in hypertensive patients (MacGregor and Cappuccio 1993; McCarron et al. 1980). It has, however, also been shown that administration of PTH to healthy volunteers under normal glycaemic conditions, in the presence of controlled circulating insulin levels, results in a significant and consistent

increase in BP, and concurrently in ionised Ca concentration. The increase in BP was strongly correlated to changes in Ca ion concentration (Fliser et al. 1997). Similarly, hypercalcaemia and hypertension were observed in response to administration of supraphysiological levels of PTH (Hulter et al. 1986). Further observations have been made previously that in uraemic patients with secondary hyperparathyroidism, in patients with secondary hyperparathyroidism, and in genetically pre-hypertensive patients, a positive correlation is found between BP and PTH/Ca ion concentrations (Erne et al. 1984; Van Hooft et al. 1993; Resnick et al. 1994). In humans, salt-induced increases in BP have been linked to increases in intracellular free Ca (Kurtz and Morris 1990). The Ca binding to the Ca-sensing receptor (which is expressed in the thick ascending limb) inhibits the Na-K-2Cl cotransporter, which decreases Na reabsorbtion, and thus influences BP (Jung et al. 2009). PTH may also induce endothelial dysfunction and aortic pulse wave velocity that may affect arterial function and predispose to hypertension (Bosworth et al. 2013). Finally, the prevalence of hypertension is known to be high in patients with primary hyperparathyroidism (Nyby et al. 1995).

PTH levels are elevated in African American individuals when compared to individuals of Caucasian descent (Aloia et al. 2006), which further supports a role for *PTH* as an underlying causative gene for salt-sensitive hypertension in indigenous Southern African patients. However, it is possible that this is an indirect association as these elevated levels may be the result of vitamin D insufficiency, due to less sun-stimulated vitamin D synthesis observed in dark-skinned people (Aloia et al. 2006; Harris 2006).

Finally, in a prospective study, Taylor et al. (2008) examined the association between plasma intact PTH levels and the risk of incident hypertension in 481 men without baseline hypertension, and suggested that plasma levels of intact PTH (even within ranges considered normal) are positively and independently associated with a higher risk of incident hypertension. These authors proposed that PTH could potentially serve as a novel target for the prevention of hypertension, supporting its ranking as a primary candidate gene in our study. This may also have implications for therapy as these patients could potentially respond better to Ca channel blockers.

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Of the additional high-scoring candidates, some have been previously directly associated with hypertension and salt-sensitivity. Candidates *EDNRA*, *EDN1* and *EDNRB* function together in a regulatory pathway with endothelin-converting enzyme *ECE1*, which has been implicated in essential hypertension. Additionally, Rothermund et al. (2001) proposed a role for *EDNRA* and *EDNRB* in salt-sensitive hypertension. Our study suggests that these genes are good candidates and should be revisited for a potential role in salt-sensitive hypertension. The association between the insulin gene, *INS*, and hypertension has been well documented. There is extensive cross-talk between insulin and angiotensin II regulating the metabolic and circulatory systems. The effects are at both, (i) the extracellular level whereby ACE controls angiotensin II synthesis and interferes with insulin signalling through angiotensin II regulation and accumulation of bradykinin; and at (ii) the intracellular level, whereby signal transduction pathways are affected (reviewed in Velloso et al. 2006; and Muscogiuri et al. 2008). Clinically, the association between insulin resistance and pre-hypertension has been demonstrated (Anan et al. 2008), and it has also been proposed that insulin promotes renal Na retention (Rocchini et al. 1989). Therefore, insulin is a good candidate for salt-sensitive hypertension, as it has been implicated in both hypertension and Na retention.

Other high-scoring candidates have not been shown previously to have a direct role in hypertension, although they do have roles in vascular and heart physiology, and have been associated with other diseases. *HCN2* and *HCN4* are hyperpolarisation-activated cation channels of the *HCN* gene family, and contribute to spontaneous rhythmic activity in both heart and brain (Baruscotti et al. 2010). Expression of *HCN4* is predominantly in the heart, as well as the thalamus and the developing central nervous system. Mutations in *HCN4* have been associated with the cardiac disease, sinus bradycardia (OMIM #163800). The gene product of *ANG*, the *angiogenin* gene, is an inducer of neovascularization, and the RNase activity of ANG is important for its angiogenic activity. It is induced by hypoxia to elicit angiogenesis and is expressed in motor neurons; mutations in *ANG* are associated with the neurodegenerative disease, amyotrophic lateral sclerosis in some populations (OMIM # 611895). In general, the

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ability of our method to identify a number of genes already implicated in hypertension indicates that the methodology is sound.

It is difficult to make a direct comparison between the numbers, positions and types of SNPs identified for each candidate gene, as the gene sizes and total number of SNPs are so varied. The distribution of SNPs within candidate genes can be described in several approximated categories, and examples are shown in Figure 4.1. In some genes, there are no selected SNPs falling within the gene itself, despite many SNPs being assayed in those regions, and those in flanking sequences are fairly distant from the gene. These are *AGT*, *ANG*, *HCN4* and *NPPA*. In some genes, a few SNPs were selected within and close to the gene, although many more SNPs were assayed than selected in these regions – these are *REN*, *ACE*, *EDNRB* and *INS*. These candidates that showed fewer differences in allele frequency are less likely to underlie the salt-sensitive hypertension in indigenous Southern African populations. In a few candidates, many of the assayed SNPs were selected falling within or close to the gene – these are *EDNRA*, *EDN1* and *AGTR1*.

Finally, in a few of the candidates, few SNPs were assayed within or close to the gene, although these were generally selected as having significantly different alleles between the populations – these are *HCN2*, and the primary candidate *PTH*. The two latter categories contain candidates that appear to have substantial differences in allele frequencies between Caucasian and the indigenous Southern African populations, and as such are more likely to contain variants that are responsible for the salt-sensitive hypertension that is prevalent in the indigenous Southern African population. Our analysis of copy number variation did not suggest that the candidate genes investigated here have any alteration in copy number at the gene level in either of the population groups. Copy number variation of these genes is therefore unlikely to underlie the salt-sensitive hypertension that is prevalent in the indigenous Southern African populations (although it is interesting to note that the heatmaps generated for the genes *AGTR1* and *INS* do show evidence of population-specific variation at sites within the genes).
The selection of top-scoring candidates in this study is inevitably affected by some inherent bias: appropriate genes that have more extensive annotations are more likely to

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be selected in the study than those that have not yet been analysed, so it is more difficult to select entirely novel candidates. However, we have aimed to combine existing knowledge about hypertension and salt sensitivity to draw new conclusions about candidate genes for salt-sensitive hypertension. Computational analysis thus allows the synthesis of existing information to make novel predictions.

Despite the previous description of the association of hypertension with the p.Arg563Gln variant, *SCNN1β* was not identified as a good candidate gene. It only fulfilled one of the characteristics that we used to prioritise candidates ("sodium transport"), and therefore was not assigned a high priority as a candidate gene. The focus of this computational analysis is to synthesise various sources of information to highlight less obvious good candidates, such as *PTH*, rather than selecting more obvious candidates such as *SCNN1β* based only on a one-dimensional analysis of the direct link between gene and disease. The limitations of detecting genetic associations with hypertension must also be noted, whereby detection of common variants with small effects is more likely than detection of a rare variant dominant effect like the p.Arg563Gln mutation (Zhang et al, 2010).

The *GRK4* gene (ENSG00000125388) was not a top scoring candidate and achieved a score of 2 with association with adrenergic sympathetic and atrial natriuretic peptide. As discussed previously, the object of computational analysis is to synthesise various sources to find less obvious candidate genes.

Due to the population-specific nature of salt-sensitive hypertension, the SNPs in the prioritised candidate genes that showed significantly different allele frequencies between Caucasian and indigenous African populations, provide good targets for further clinical and empirical research. These prioritized genes may have a significant contribution to the occurrence of salt-sensitive hypertension, and thus warrant further investigation.

CONCLUSIONS

In this study, candidate genes for salt-sensitive hypertension have been prioritized using computational approaches including text- and data-mining. *PTH* proved to a novel candidate gene and provides a basis for further empirical research.

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CHAPTER 5
LINKING SNPs OF THE *PTH* GENE TO SALT SENSITIVE HYPERTENSION

INTRODUCTION:

In the previous chapter it has been shown that *PTH* might be linked to salt-sensitive hypertension using bio-informatics methodology. However the results are hypothesis generating and demand further experimental work to link specific SNPs of the *PTH* gene to salt-sensitive hypertension.

The human PTH is a small gene, on chromosome 13, consisting of 3 exons, each with 2 transcripts (Figure 5.1). The gene is approximately 4,200 base pairs long, and the PTH protein contains 84 amino acids.

There are currently 16 known SNPs in the *PTH* gene, none of which have been linked to hypertension. Five of these are in the 3'untranslated region (UTR) and two in the 5'UTR. SNPs in *PTH* have been linked to Ca metabolism and height, in the Tromso Study (Jorde et al. 2012) and Ca homeostasis and bone density (Laaksonen et al. 2009; Guo et al. 2010).

HYPOTHESIS:

SNPs of the *PTH* gene in a population of ethnic Xhosas are associated with salt-sensitive hypertension.

AIM:

To identify genetic variants and their (SNP) frequencies in the *PTH* gene, for pooled samples from Xhosa patients with salt-sensitive hypertension and non-salt sensitive hypertension; compared to normotensive controls.

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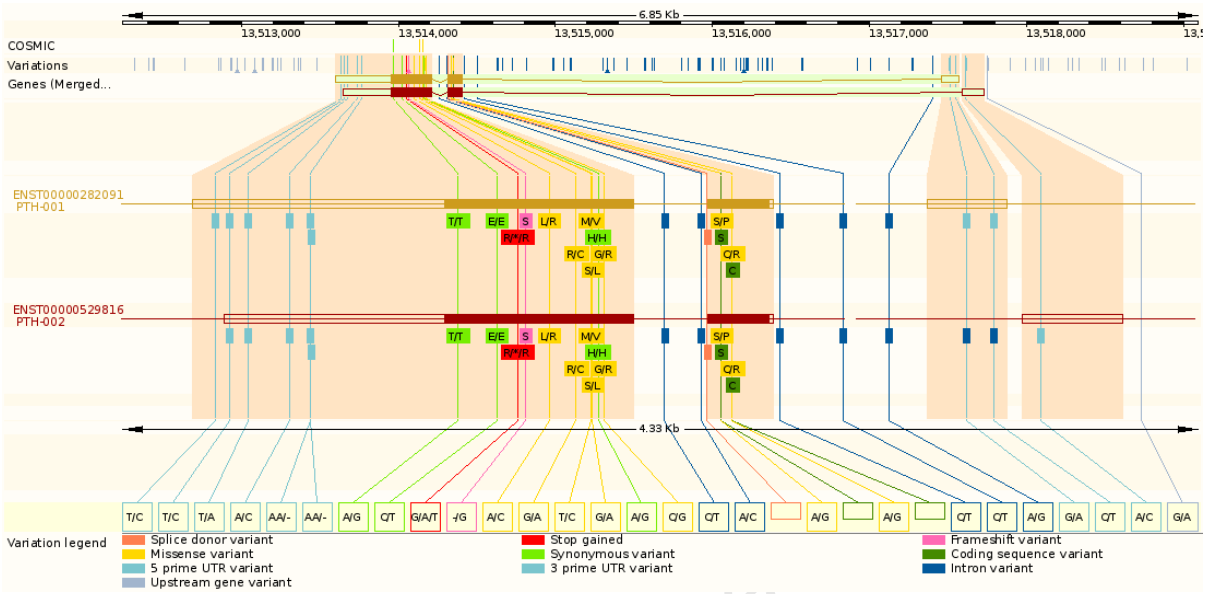
Figure 5.1. Structure of the *PTH* Gene **(www.ensemble.org)**



Exon Number	Transcript	Exon start	Exon End
Exon 1	Transcript 1 Transcript 2	13 517 567 13 517 728	13 517 458 13 517 589
Exon 2	Transcript 1&2	13 514 407	13 514 317
Exon 3	Transcript 1 Transcript 2	13 514 213	13 513 602 13 513 646

METHODS:

Subjects:

Genetic samples collected from Xhosa hypertensive patients attending the Groote Schuur Hospital hypertension clinic were identified from the archive in the Divisions of Human Genetics and Chemical Pathology. All subjects had plasma renin activity performed as part of their routine work up. Salt sensitivity was defined as direct renin assay < 10 units/ml and non-salt sensitivity as levels above this. Suppressed plasma renin activity is considered an indirect measure of salt sensitivity as described previously. Normotensive Xhosa controls were identified from the same archive.

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Laboratory Methods:

Genetic sequencing analysis was performed at the Central Analytical Facitilities at the University of Stellenbosch. Computational analysis of the sequencing data was undertaken at the South African National Bioinformatics Institute (SANBI) at the University of the Western Cape. Individual DNA samples were used for each Xhosa individual in the study, from existing cohorts. A set of primers was selected and optimised for long range PCR of the entire *PTH* gene in a single amplicon. Validation of the PCR product, to confirm correct and specific amplification, was undertaken by Sanger sequencing of the amplicon for a small subset of samples. For each individual, the entire *PTH* gene was amplified from the genomic DNA by long-range PCR in a single amplicon of 4.5kb.

The PCR product was purified and quantified, and equal amounts from each individual were combined to form the pooled samples: for (i) salt-sensitive hypertensives; (ii) non-salt sensitive hypertensives, and (iii) normotensive controls.

Each pooled sample was sequenced using Ion Torrent (Life Technologies http://www.iontorrent.com/) Next Generation Sequencing (NGS) technology, to an average depth of approximately 32x across the amplicon. The read data underwent quality control and statistical analysis. Reads were trimmed to remove lead sequence and inaccurate end sequence. The NGS-read data from each pool was aligned to a chromosome 11 reference sequence (human build 37) SNPs, insertions and deletions (I/D) were identified and allele frequencies were called at each SNP position. SNPs and their allele frequencies were compared between the pooled samples; and were mapped to the gene coordinates to determine position and function within the gene. Identified SNPs and insetion/deletions (I/D) were compared to known SNPs from the dbSNP database at the National Institute of Health. Novel SNPs were identified.

Bioinformatics analysis:

(i) *Alignment of sequencing reads to the PTH gene*: Fastq format files were generated for the reads for each of the pooled DNA samples from the sequence analysis. Novoalign

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software (www.novocraft.com) was used to align the reads to the indexed reference sequence file (human build 37, chromosome 11). The SAM format output file was validated using Picard-tools software and SAMtools software was used to convert the SAM output file to BAM format, and to index and sort the aligned read file (Li et al, 2009). GenomeAnalysisTK software was used to realign the reads around identified insertions and deletions ("indels") (McKenna et al. 2010).

(ii) *SNP calling from aligned reads*: The software SNVer was used to call the allele frequencies at each point where a SNP or indel was detected (Wei et al, 2011). This software is specifically designed to call allele frequencies from pooled samples taking into consideration read depth and number of alleles in the pooled sample. By calculating the number of sequencing reads per allele in the sample at each base position (the "depth", or "cover" of sequencing reads), the software identified whether low frequency base differences were likely to be the result of errors in sequencing – in which case the variation is discarded; or whether they are likely to be real rare allele changes that have been identified in the pooled sample.

Allele frequencies of the SNPs and Insertion/Delection (I/D) were calculated for each group, and analysed for differences by Chi^2 and Fisher's exact test.

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RESULTS:

There were 64 salt-sensitive hypertensives, 80 non-salt snesitive hypertensives, and 130 control samples that were successfully analysed. The mean age of the salt-sensitive hypertensives was 38.3 years and the non-salt sensitive hypertensives, 36.7 years. All were ethnic Xhosa.

Twenty five SNPs were found in the pooled samples, 12 of which have been previously described. 3 were in exon 3, none in exon 1 or 2, 17 in the introns, 1 downstream and 4 upstream (Table 5.1, see appendix 5).

One hundred and fifty two I/Ds were found, of which only 6 have been previously described (Table 5.2, see appendix 5). Ten of the I/Ds were downstream, 14 in 3'-UTR – exon 3-T1 and 3'UTR-exon3-T2, 7 in exon-3, 98 in introns, 3 in exon 2, 3 in 5'UTR exon 1 T-1 and intron, and 3 in 5'UTR exon 1T-2, and 14 upstream (Table 5.2, see

appendix 5).The allele frequencies of the individual SNPs and I/Ds were calculated, and analysed for significance using both Chi^2 and Fisher's exact test. There was no significant difference between normal controls, salt sensitive and non-salt sensitive hypertensives for any of the SNPs or I/Ds. In addition, comparing controls to a pooled sample of all hypertensives there were no significant differences.

DISCUSSION:

In this chapter, the SNPs and I/Ds in the *PTH* gene which was earlier identified as a good candidate for salt-senstive hypertension were investigated. Unfortunately there appeared to be no relationship with either salt sensitive or non-salt sensitive hypertension. It is possible, however, that the unique and extensive variation in the *PTH* gene in the entire Xhosa group, when compared to known SNPs from Caucasian ethnic groups, may be a predisposing factor that underlies susceptibility to salt-sensitive hypertension; however, additional environmental or genetic factors may also be required for salt-sensitive hypertension to develop.

It does raise the question as to why the bio-informatics analysis suggested a relationship with salt-sensitive hypertension. However, it is important to realise that PTH function can be influenced by other mechanisms. For instance, the parathyroid gland is exquisitely sensitive to Ca (Carrillo-López et al. 2010). This is because Ca needs to be controlled within a very narrow range, and PTH is the major regulator of Ca. Small changes in PTH in either direction may result in biological harm e.g. hypocalcaemia results in tetany and even seizures. Ca is sensed by the Ca-sensing receptor or CASR, and mediates PTH secretion in response to changes in Ca. Hypocalcemia increases PTH, and hypercalcemia reduces PTH to maintain Ca homoeostasis (Carrillo-López et al. 2010).

As the CASR is constantly exposed to Ca, there needs to be a mechanism to maintain responsiveness of the receptor. CASR belongs to a family of G-protein-coupled receptors (GPCRs) similar to that discussed in the previous chapters in regard to dopamine (Pi et al. 2005). The GPCRs are linked to the G-protein receptor kinases or

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GRKs, which are a family of serine/threonine kinases (GRK 1-7) that phosphorylate various serine and threonine residues that bind to members of the β-arrestin family (as described earlier in this thesis). This uncouples the receptor from its cognate G protein and targets the receptor for internalisation, thus maintaining sensitivity to Ca by the replenishment of the cell with new receptors.

The parathyroid gland has not been extensively studied with regard to uncoupling of the receptor, but a paper by Pi et al. (2005) shed new light on these mechanisms.
Thus, mutations in either *GRKs* or in *CASR* could lead to changes in PTH stimulation or inhibition, possibly leading to changes in BP mediated by PTH, as discussed in the preceding chapter.

LIMITATIONS:

The measure of salt sensitivity was indirect using direct renin levels, and the hypertensive patients could have been misclassified. However even if the hypertensives were pooled to help overcome this problem, there was no hint of an association with SNPs and I/Ds in *PTH*.

Pooled sample sequencing is able to produce allele frequency for the group of individuals in the pool. It is not, however, able to provide haplotype data for the individuals, so it cannot be determined whether alleles are heterozygous or homozygous within the individuals. Data cannot be phased, so it is impossible to determine which alleles may be inherited together in haploblocks, or which two alleles pair in an individual. For these reasons, the study aimed primarily to identify potentially relevant SNPs and I/Ds, based on their frequencies in the different sample pools, that could then be genotyped in the individuals in future studies.

FUTURE RESEARCH

Given the very high number of SNPs and I/Ds in this population it opens the way for further research into the phylogenetics of the *PTH* gene. The gene is highly conserved within species, as no mammalian species have known paralogues for this gene. It is also

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a gene with a crucial role in homeostasis that is tightly regulated, and highly sensitive to environmental changes. The appropriate functioning of the PTH is essential to the metabolic integrity of the organism and this makes this gene an interesting candidate to study in terms of divergent evolution between ethnic groups within different geographical and nutritional environments; as random changes in gene structure are unlikely to be well-tolerated, and variation in the gene is more likely to relate to adaptation of the organism to its environment.

Further studies could be designed to characterise some of the novel SNPs in the Xhosa *PTH* gene in conjunction with other candidate hypertension gene variations, and dietary factors including Na and Ca.

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CHAPTER 6

GENERAL OVERVIEW, IMPORTANCE AND RECOMMENDATIONS, AND FURTHER RESEARCH

General overview

In this thesis it has been argued that there are phenomenological differences in hypertension between blacks and whites. The former are more likely to be salt sensitive and this difference appears to be related to a primary problem in the ability to excrete Na that appears to have a genetic basis.

GRK4 polymorphisms cause Na retention and hypertension in genetically transformed rats. These polymorphisms are more common in indigenous Southern Africans and are attractive candidates to explain the underlying genetic predisposition to salt sensitivity in these population groups. Preliminary work confirmed that the c.425C>T SNP (p.Ala142Val) is more common in Blacks in South Africa but not associated with hypertension in a relatively small sample size.

To establish the importance of these *GRK4* polymorphisms (p.Ala142Val and p.Arg65Leu) two novel experiments were undertaken to determine if they were associated with salt sensitivity. In the first experiment, young lean males (black and white) were subjected to saline challenge to determine their ability to excrete Na and record their BP response.

Surprisingly, there was no increment in BP in either blacks or whites, but blacks failed to incrementally increase Na excretion in response to load. The ability to incrementally excrete Na was related incrementally to the C/C, C/T and T/T genotypes of the p.Ala142Val polymorphism, but not to that of the p.Arg65Leu variant. In addition, aldosterone levels (an indirect measure of salt sensitivity) were incrementally related to C/C, C/T and T/T genotypes of the p.Ala142Val variant.

In the second experiment, BP responses determined by 24 hour ambulatory BP to dietary intervention of Na restriction and increased K intake were investigated relation to *GRK4* polymorphisms. In the overall experiment the intervention was associated with

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significant BP response and this was predicted by both the p.Ala142Val and p.Arg65Leu variants.

Finally using a bioinformatics approach *PTH* was selected as a likely candidate for salt sensitivity. This was a novel finding. Additionally, there were significant differences in allele frequency in this gene between indigenous Southern African populations, making *PTH* a strong candidate for further research. However when the *PTH* gene was analysed for SNPs and insertion/deletions, there was no obvious link to salt-sensitive hypertension, or hypertension in general, in indigenous Xhosa subjects compared to normotensive subjects.

Importance and recommendations

The importance of the findings in this thesis is the following:

1. The observation that black patients appear to be more salt sensitive that whites after saline challenge and using indirect assessments like aldosterone, was confirmed.
2. The thesis suggested that GRK4 may be involved as one of the important underlying mechanisms for salt sensitivity in black patients.
3. *GRK4* polymorphisms were linked to ability to excrete Na after a saline challenge, and to predict the BP response to dietary intervention. This may help practitioners to determine which hypertensive patients best to target for dietary Na restriction. Dietary Na restriction is difficult to achieve in the real world due to the high Na content of processed foods, and requires expert dietary counselling. Focusing limited resources on patients most likely to respond may be more cost effective.
4. Genotyping patients for *GRK4* polymorphisms may also offer a pharmacogenetic approach to treating patients with hypertension, as the patients with these genotypes are more likely to respond to diuretic therapy, and less likely to β blockers.
5. This thesis indirectly re-establishes the importance of dopamine in excretion of Na. Previously, there has been an enormous focus on the RAAS in investigating the underlying genetic predisposition to hypertension. As an aside, an often

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neglected fact is that dopamine through GRKs limit Na absorbtion in the jejunum, offering a further protective mechanism against a high salt diet.

6. The findings of this thesis raise the possibility of developing novel dopamine agonists to overcome the uncoupling of the receptor from the second messengers to promote Na excretion and improve BP control in black patients.
7. The novel finding using computational analysis that *PTH* is one of the leading candidate genes for salt-sensitive hypertension in black populations has important implications for future research focus.

Further research

1. It is important to undertake studies to determine if the p.Ala142Val and p.Arg65Leu variants are associated with hypertension and salt sensitivity in a larger sample size.
2. A pharmacogenetic approach to treating hypertension in black people needs to be further explored especially in relation to *GRK4* SNPs.
3. Further work in larger sample sizes needs to consolidate the finding that *GRK4* polymorphisms predict BP response to dietary intervention with Na restriction and K supplementation.
4. Further research needs to be performed to determine if PTH and CASRs are involved in the pathogenesisis of hypertension in indigenous South Africans.
5. Dopamine receptors are a target for potential development of antihypertensive drugs in mainly the black population.
6. The *PTH* gene is highly conserved within species, as no mammalian species have known paralogues for this gene and it is highly conserved. Given the very high number of SNPs and I/Ds in this population it opens the way for further research into the phylogenetics of the PTH gene.

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APPENDICES

APENDIX 1

PEER REVIEWED PUBLICATIONS ARISING FROM THE THESIS

i. Rayner B, Musekiwa A, Lombard C, Ramesar R.The A142V polymorphism of the G protein-coupled receptor kinase 4 gene predicts natriuretic response to saline challenge in young normotensive lean Black and White South African men. Nephrology Reviews 2011; 3: e9

ii. Rayner B, Ramesar R, Steyn K, Levitt N, Lombard C, Charlton K. G-protein-coupled receptor kinase 4 (GRK-4) polymorphisms predict blood pressure response to dietary modification in black patients with mild to moderate hypertension. J Hum Hypertens 2011; 26: 334-9

iii. Tiffin N, Meintjies A, Ramesar R, Bajic V, Rayner B. Computational Analysis of Candidate Disease Genes and Variants for Salt-Sensitive Hypertension in Indigenous Southern Africans. PLoS One 2010; 5(9): e12989

iv. Lindhorst J, Alexander N, Blignaut J, Rayner B. Differences in hypertension between blacks and whites: an overview. Cardiovasc J S Afr 2007; 18: 241-7.

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APPENDIX 2.

PATIENT INFORMATION AND INFORMED CONSENT (SALINE CHALLENGE)

GRK-4 SALT SENSIVITY STUDY

INTRODUCTION

You are invited to participate in this study, which will be done in accordance with the Declaration of Helsinki, 2000. This information leaflet will help you decide if you would like to participate. Before you agree, you should fully understand what is involved. If you have any questions, which you do not understand, please do not hesitate to discuss this with the study doctor.

WHAT IS THE PURPOSE OF THE STUDY

The purpose of the study is to investigate the causes of high blood pressure in black and white people. We believe that salt sensitivity (inability to secrete salt properly by the kidneys) is the underlying difference between blacks and white hypertensives, and this is related to inherited factors or genes. By studying salt handling in subjects with normal blood pressure we hope to show this.

HOW MAY THE STUDY BENEFIT ME?

There will be no benefits for you personally to participate in this study.

WHAT IS THE DURATION OF THE STUDY AND WHAT PROCEDURES WILL BE PERFORMED

After an overnight fast you will be admitted to a metabolic unit. After informed consent you will be examined and blood and urine tests taken. Thereafter you will have an intravenous catheter inserted into a vein in your arm. 2 litres of saline will be administered over 2 hours by the catheter (saline test) and all urine will be collected over the next 4 hours. Blood pressure will be measured every 30 minutes, and at the end of the study more blood tests will be taken. The study will last 4-5 hours. Samples will be taken for genetic studies and a separate consent will be taken for this.

HAS THE STUDY RECEIVED ETHICAL APPROVAL?

The study protocol has been approved by the Research Ethics Committee of the University of Cape Town.

WHAT ARE MY RIGHTS AS A PARTICIPANT IN THE TRIAL?

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Your participation is entirely voluntary and you can refuse to participate or stop at any time without stating a reason. Your withdrawal will not affect your access to future medical care.

MAY THE TRIAL PROCEDURES RESULT IN DISCOMFORT OR INCONVENIENCE?

Blood tests may result in a bruise at the puncture site, swelling of the vein, infection, or bleeding. In experienced hands this is highly unlikely and only minor discomfort may occur.
The saline test will involve the insertion of a drip into a vein in the arm and as for the blood test may cause minor discomfort listed above.

WHAT ARE THE RISKS INVOLVED?

In rare circumstances the saline test may cause fluid overload and you may become breathless but this can be treated with an injection of a diuretic. In experienced hands this is very unlikely to occur and is easily treated. If you are known to have heart disease or kidney disease you should not have the saline test.

INSURANCE AND FINANCIAL ARRANGEMENTS

All the study doctors are covered by insurance for medical liability.
You will be paid R500.00 to participate in the study for transport costs and time spent at the study centre.

SOURCE OF ADDITIONAL INFORMATION

For the duration of the study you will be under the care of Prof. Rayner, and you are entitled to speak to him before entering the study.

CONFIDENTIALITY

All information during the course of the trial is strictly confidential. Data will be reported in scientific journals, but will not include information that identifies you.

It is important that the Research Ethics Committee of the University of Cape Town be able to review records of the trial, but only in relation to their regulatory obligations.

INFORMED CONSENT

I hereby confirm that I have been informed by the study doctor, Dr ……., about the nature, conduct, benefits and risks of this clinical protocol. I have also received, read, and understand the written Patient Information and Consent form.

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I am aware that the results of the trial will be anonymously processed into a trial report.

I may, at any stage, withdraw my consent and participation without prejudice.

I have had sufficient opportunity to ask questions declare myself prepared to participate in the trial.

Patient's Name**:** **…….**(print)

Patient's Signature:……Date: ……

Study Doctor's Name: …… (print)

Study Doctor's Signature: ……..Date: …….

Witness Name: …… (print)

Witness Signature: ……

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APPENDIX 3

PATIENT INFORMATION AND INFORMED CONSENT FOR GENETICS

Introduction:

We are conducting research into the causes of hypertension in South Africa, and need to take blood samples to help us analyse differences and activity of certain genes that may cause hypertension. We are specifically analysing the GRK-4 variants as a cause for salt sensitivity.

1. I, _____ , hereby consent that my blood cells be analysed for genetic changes in the GRK-4 variants.

2. I request that no portion of the sample be stored for later use. ☐ (MARK IF APPLICABLE)

Or

I request that a portion of the sample be stored indefinitely for (DELETE WHERE NOT APPLICABLE)

a) possible re-analysis
b) analysis for benefits of members of my immediate family
c) research purposes, subject to the approval of the University of Cape Town Research Ethics Committee, provided that any information from such research remain confidential.

3. The results of the analysis will be made known to me or my doctor if and when available. In addition I authorise that they be made known to the following: (DELETE WHERE NOT APPLICABLE)

a) other doctors involved in my care
b) the following family members _____
c) other _____

4. I have been informed that:

a) There are risks and benefits of genetic analysis of stored material
b) The analysis procedure is specific to this genetic condition mentioned above, and can not determine the complete genetic make up of an individual
c) The laboratory is under an obligation to maintain confidentiality
d) Genetic analysis may not be informative for myself or other family members
e) There may be no direct benefit to me
f) Even in the best of conditions, current technology is not perfect and there is a possibility of incorrect results

5. I understand I can withdraw my consent for any aspect of the above at any time without affecting my future medical care.

6. ALL OF THE ABOVE HAS BEEN EXPLAINED TO ME IN A LANGUAGE THAT I CAN UNDERSTAND AND MY QUESTIONS HAVE BEEN ANSWERED

Patient's Name: …….(print)

Patient's Signature:……Date: ……

Witness Signature: ……

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APPENDIX 4

GENETIC CONSENT FOR DIETARY STUDY

REQUEST FOR MOLECULAR STUDIES (DNA)

Molecular Laboratory
Division of Human Genetics
1st Floor, Anatomy Building
UCT Medical School, Observatory 7925

Tel: (021) 406 6425 Fax: (021) 448-0906

Blood should be drawn in 2 plastic EDTA Tubes (Purple top) +/- 10ml each using a yellow barrel. Each tube should be inverted to mix and should be clearly labelled with the patient's name and DOB
Keep blood in fridge at 4°C until able to send to laboratory

Please DO NOT send specimens on ice or frozen.

Please fill in all the information requested:

Surname:_____First Name(s):_____

New Family: Yes ☐ No ☐ (If no, please fill in family name) Family name: _____

Medical Aid: _____Medical Aid No: _____

Sex: M ☐ F ☐ Date of Birth: Year:_____ Month:_____ Day: _____

Number of children: _____

Ethnic Origin : (please indicate ancestry of both your mother and father)_____

Contact Address: _____ Town: _____ Fax: Tel: _____

Referring Doctor/Sister: _____ Town: _____ Fax: Tel: _____

Hospital or Address: _____ Town: _____ Fax: Tel: _____

Reason for Referral (Clinical diagnosis):

Affected ☐ At Risk ☐ Carrier ☐ Spouse ☐ Query ☐ Unaffected ☐

Becker Muscular Dys. ☐	Duchenne Muscular Dys ☐	Colonic Carcinoma ☐
Fragile-X Syndrome ☐	Bipolar Disorder ☐	Huntington Disease ☐
Retinitis Pigmentosa ☐	Spinocerebellar Ataxia ☐	Hypertension ☐

Additional disorders (apparent or previously treated): _____

Additional family history _____

Clinical Details:

Physical disability ☐ Mental retardation ☐ Deafness ☐ Impaired vision ☐ Night blindness ☐

Other: _____

Have samples from this patient been sent to a DNA lab before? (DELETE WHERE NOT APPLICABLE) YES / NO / Don't Know

If Yes, where: _____

For Laboratory use only:

DNA number: _____ *Vol.Blood:* _____*(ml)* *Other:* _____

Date Received: Year: _____ *Month:* _____ *Day:* _____ *Computer Index No:*_____

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Consent for DNA analysis and storage for study on essential hypertension

1. I, _____ , request that an attempt be made using genetic material to assess the probability that: I / my child / my unborn child (DELETE WHERE NOT APPLICABLE) might have inherited a disease-causing mutation in the gene for:_____

2. I understand that the genetic material for analysis is to be obtained from: blood cells/skin sample/other (specify) (DELETE WHERE NOT APPLICABLE) :

3. I request that **no** portion of the sample be stored for later use. ☐ **(**MARK IF APPLICABLE)
   Or
   I request that a portion of the sample be stored indefinitely for (DELETE WHERE NOT APPLICABLE):
   (a) possible re-analysis
   (b) analysis for the benefit of members of my immediate family
   (c) research purposes, subject to the approval of the University of Cape Town Research Ethics Committee, provided that any information from such research will remain confidential.

4. The results of the analysis carried out on this sample of stored biological material will be made known to me, via my doctor, in accordance with the relevant protocol, if and when available.
   In addition, I authorise that they may be made known to: (DELETE WHERE NOT APPLICABLE) :
   other doctors involved in my care
   the following family members: _____
   other: _____

5. I authorise / do not authorise my doctor(s) (DELETE WHERE NOT APPLICABLE) to provide relevant clinical details to the Division of Human Genetics, UCT.

6. I have been informed that:
   (a) there are risks and benefits associated with genetic analysis and storage of biological material and these have been explained to me.
   (b) the analysis procedure is specific to the genetic condition mentioned above and cannot determine the complete genetic makeup of an individual.
   (c) the genetics laboratory is under an obligation to respect medical confidentiality .
   (d) genetic analysis may not be informative for some families or family members.
   (e) even under the best conditions, current technology of this type is not perfect and could lead to incorrect results.
   (f) where biological material is used for research purposes, there may be no direct benefit to me.

7. I understand that I may withdraw my consent for any aspect of the above at any time without this affecting my future medical care.

8. **ALL OF THE ABOVE HAS BEEN EXPLAINED TO ME IN A LANGUAGE THAT I UNDERSTAND AND MY QUESTIONS ANSWERED BY:**

_____ DATE: _____

Patient signature _____ **Witnessed consent** _____

NOTE - PLEASE INSERT A FAMILY PEDIGREE DRAWING ON THE REVERSE OF THIS FORM

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APPENDIX 5

Table 5.1 Summary of SNPs of the PTH gene

SNPS

					total alleles:	160	128	260
						% Reference allele:		
Transcript 1	**Position**	**Known SNP**	**Reference**	**Alternative**		**Straight Hyp**	**Salt Hyp**	**Controls**
Downstream	13513478	.	G	A	30790	45.1	43.6	39.6
Exon 3	13513633	rs16912873	T	C	18804	25.6	23.4	30.8
Exon 3	13514024	rs76760723	C	T	19137	1.9	3.1	1.5
Exon 3	13514053	rs6256	G	T	17152	2.3	1.6	2.3
Intron	13514308	.	A	C	17872	N/A	3.5	N/A
Exon 2								
Intron	13514417	rs694	C	T	21557	8.2	5.3	7.9
Intron	13514505	rs177706	C	T	20926	68.7	65.9	67.6
Intron	13514526	.	C	T	17012	N/A	1.6	N/A
Intron	13514654	.	A	T	4993	2.1	2.5	2.0
Intron	13514656	.	T	A	5220	1.4	1.8	1.2
Intron	13514819	rs186788473	C	T	17559	0.5	1.6	NA
Intron	13515571	rs144726611	A	C	21530	1.2	0.8	2.3
Intron	13515621	.	A	T	23077	1.7	1.4	1.8
Intron	13516026	.	G	C	19065	5.2	3.6	4.7
Intron	13516027	.	C	G	19321	5.1	3.2	4.2
Intron	13516194	.	A	G	18687	42.7	46.6	38.6
Intron	13516229	rs192325615	G	T	17693	0.9	1.1	2.2
Intron	13516349	rs77839047	A	G	16196	17.9	14.6	21.2
Intron	13516379	rs751610	C	T	16179	7.2	8.7	4.7
Intron	13517125	rs116386346	T	G	17368	3.9	3.5	1.9
Intron	13517404	rs3099597	A	G	19351	46.7	44.4	42.4
exon 1								
Upstream	13517730	.	A	C	19960	4.7	6.7	5.8
Upstream	13517893	.	A	T	16569	1.9	1.6	1.6
Upstream	13517894	rs1380144	C	T	16189	56.9	51.5	55.5
Upstream	13518092	rs143237038	A	G	8554	0.9	1.2	3.0

Table 5.2. Insertion/Deletions of PTH gene

						total alleles:	160	128	260
							% Reference allele:		
Transcript 1	**Transcript 2**	**Position**	**Known SNP**	**Reference**	**Alternate**	**Read no**	**Str Hyp.**	**Salt Hyp**	**Controls**
Downstream	Downstream	13513418	.	A	-A	30092	5.56	3.78	4.83
Downstream	Downstream	13513463	.	T	-T	32638	4.20	4.09	3.91
Downstream	Downstream	13513476	.	A	-A	33021	7.98	5.59	6.85
Downstream	Downstream	13513491	.	A	-A	17412	5.17	5.54	3.18
Downstream	Downstream	13513506	.	A	-A	10105	3.87	4.70	4.59
Downstream	Downstream	13513522	.	A	-A	13153	15.60	18.44	15.10
Downstream	Downstream	13513523	.	A	+A	13326	5.19	6.04	6.77
Downstream	Downstream	13513544	.	A	-G	18015	4.28	6.94	5.01
Downstream	Downstream	13513568	.	G	-G	19734	6.95	5.94	6.51
Downstream	Downstream	13513587	.	A	-A	20034	21.84	21.80	21.28
3'-UTR - exon 3-T1	3'-UTR - exon 3-T2	13513648	.	T	-T	19376	10.71	10.85	10.13
3'-UTR - exon 3-T1	3'-UTR - exon 3-T2	13513649	.	T	-GT	19135	4.56	5.63	4.16
3'-UTR - exon 3-T1	3'-UTR - exon 3-T2	13513660	.	T	-T	19094	5.31	5.22	5.95
3'-UTR - exon 3-T1	3'-UTR - exon 3-T2	13513675	.	G	-G	16880	5.03	4.81	5.60
3'-UTR - exon 3-T1	3'-UTR - exon 3-T2	13513692	.	T	-T	13929	4.89	4.66	4.89
3'-UTR - exon 3-T1	3'-UTR - exon 3-T2	13513729	.	A	-A	8890	9.90	10.49	9.47
3'-UTR - exon 3-T1	3'-UTR - exon 3-T2	13513746	.	A	-A	9190	15.65	14.55	16.31
3'-UTR - exon 3-T1	3'-UTR - exon 3-T2	13513765	rs148300531	A	-AA	11691	23.84	19.94	28.41
3'-UTR - exon 3-T1	3'-UTR - exon 3-T2	13513775	.	A	-A	13904	6.62	7.12	7.34
3'-UTR - exon 3-T1	3'-UTR - exon 3-T2	13513800	.	A	-A	17087	6.72	6.52	6.24
3'-UTR - exon 3-T1	3'-UTR - exon 3-T2	13513815	.	G	-G	18475	21.96	24.75	22.18
3'-UTR - exon 3-T1	3'-UTR - exon 3-T2	13513946	.	T	-T	19675	9.49	10.91	9.26
3'-UTR - exon 3-T1	3'-UTR - exon 3-T2	13513952	.	T	-T	19833	4.64	4.69	4.08
3'-UTR - exon 3-T1	3'-UTR - exon 3-T2	13513959	.	G	-G	20183	6.07	6.52	5.42
Exon 3	Exon 3	13514016	.	T	-T	19802	16.66	16.65	16.51
Exon 3	Exon 3	13514017	.	T	+T	19586	4.19	4.48	4.18
Exon 3	Exon 3	13514051	.	T	-T	17731	21.55	20.21	20.73
Exon 3	Exon 3	13514052	.	T	-CG	17174	9.53	9.35	9.40

Exon 3	Exon 3	13514064	rs35645521	G	-G	17735	9.48	8.80	10.21
Exon 3	Exon 3	13514080	.	G	-G	19097	7.58	7.47	6.87
Exon 3	Exon 3	13514171	.	T	-T	22389	8.57	8.29	8.57
Intron	Intron	13514218	.	G	-G	22115	11.99	11.72	11.54
Intron	Intron	13514258	.	C	-C	18458	10.35	10.67	9.69
Intron	Intron	13514288	.	A	-A	17428	11.69	12.52	11.92
Intron	Intron	13514306	.	A	-A	17757	9.03	7.25	8.16
Exon 2	Exon 2	13514340	.	T	-T	19958	9.07	9.77	8.59
Exon 2	Exon 2	13514349	.	A	-A	20575	5.12	5.45	4.62
Exon 2	Exon 2	13514391	.	T	-T	22625	8.30	9.37	7.35
Intron	Intron	13514413	.	A	-A	22364	10.94	8.84	9.55
Intron	Intron	13514425	.	A	-A	20703	9.27	8.69	8.24
Intron	Intron	13514437	.	T	-T	19823	7.53	8.53	6.83
Intron	Intron	13514441	.	A	-A	19793	4.33	3.28	3.23
Intron	Intron	13514448	.	T	-T	19956	13.14	13.17	12.02
Intron	Intron	13514502	.	T	-T	21764	7.17	6.52	6.98
Intron	Intron	13514536	.	T	-T	15110	10.98	10.51	9.90
Intron	Intron	13514611	.	T	-T	9935	15.71	16.59	14.69
Intron	Intron	13514633	rs141879370	T	-T	8080	4.74	5.16	4.42
Intron	Intron	13514654	.	T	-ATTT	5447	41.05	40.93	44.49
Intron	Intron	13514664	.	T	-ATT	5826	13.69	10.29	14.86
Intron	Intron	13514671	.	T	-T	7333	9.25	11.03	7.46
Intron	Intron	13514686	.	T	-T	8738	11.34	12.12	11.55
Intron	Intron	13514687	.	T	+T	8806	3.88	4.38	5.52
Intron	Intron	13514793	.	A	-A	19577	7.23	7.56	7.57
Intron	Intron	13514834	.	T	-T	16983	5.43	4.54	4.26
Intron	Intron	13514908	.	T	-T	20232	15.87	16.08	15.26
Intron	Intron	13514922	.	A	-A	20158	5.17	4.91	4.17
Intron	Intron	13514945	.	A	-A	18858	7.03	7.29	6.20
Intron	Intron	13515003	.	A	-A	18623	9.58	8.14	8.07
Intron	Intron	13515064	.	A	-A	21695	12.30	12.46	12.06
Intron	Intron	13515089	.	A	-A	20460	8.62	9.19	8.87
Intron	Intron	13515158	.	G	-G	20015	6.00	6.16	5.56
Intron	Intron	13515201	.	A	-A	22314	4.69	4.61	4.86

Intron	Intron	13515300	.	A	-A	19223	3.84	4.36	4.75
Intron	Intron	13515310	.	T	-T	18734	9.39	10.69	8.66
Intron	Intron	13515344	.	T	-T	15950	10.20	9.04	9.21
Intron	Intron	13515368	.	T	-TA	16877	6.26	5.14	5.63
Intron	Intron	13515371	.	A	-ATT	16572	10.25	10.40	9.97
Intron	Intron	13515379	.	T	-T	16966	16.61	19.07	14.97
Intron	Intron	13515380	.	T	+T	17010	4.96	6.26	7.01
Intron	Intron	13515412	.	C	-C	21450	29.91	25.67	27.39
Intron	Intron	13515419	.	T	+C	21616	4.66	3.94	4.33
Intron	Intron	13515438	.	A	-A	21632	10.42	9.85	10.60
Intron	Intron	13515453	.	A	-A	20990	5.09	3.81	4.30
Intron	Intron	13515488	.	T	-T	17867	4.74	4.72	4.22
Intron	Intron	13515538	.	T	-T	19035	15.82	16.26	15.19
Intron	Intron	13515612	.	T	-T	23118	9.72	9.63	9.20
Intron	Intron	13515621	.	A	-A	23416	13.23	15.09	11.61
Intron	Intron	13515639	.	T	-TT	22418	13.48	15.40	12.56
Intron	Intron	13515640	.	T	-T	22056	35.26	37.49	34.21
Intron	Intron	13515641	.	T	+T	21629	5.77	6.24	6.40
Intron	Intron	13515669	.	A	-A	20378	13.53	15.00	12.69
Intron	Intron	13515756	.	T	-T	19628	4.48	4.19	4.37
Intron	Intron	13515785	.	C	-C	18474	4.19	5.04	3.89
Intron	Intron	13515802	.	C	-C	18591	20.03	21.28	20.36
Intron	Intron	13515839	.	C	-C	18846	12.85	12.82	12.03
Intron	Intron	13515859	.	C	-C	18911	14.25	12.35	13.17
Intron	Intron	13515873	.	T	-T	19360	5.69	4.88	5.06
Intron	Intron	13515886	.	T	-T	19061	5.94	5.99	6.36
Intron	Intron	13515920	.	A	-A	20346	6.64	7.57	6.51
Intron	Intron	13515977	.	T	-T	20017	8.33	7.57	8.00
Intron	Intron	13516030	.	C	-C	19510	14.70	13.20	13.59
Intron	Intron	13516075	.	C	-C	20128	5.19	5.32	5.14
Intron	Intron	13516108	.	A	-A	19430	11.77	10.39	12.35
Intron	Intron	13516124	.	T	-T	18055	9.50	11.12	8.62
Intron	Intron	13516136	.	T	-T	18187	14.22	14.88	12.81
Intron	Intron	13516150	.	C	-C	17906	5.73	4.70	5.09

Intron	Intron	13516205	.	T	-T	18769	6.39	6.82	8.03
Intron	Intron	13516211	.	G	-G	18350	5.97	5.16	5.95
Intron	Intron	13516254	.	A	-A	18005	5.86	5.38	5.46
Intron	Intron	13516366	.	T	-T	16073	21.25	21.72	19.56
Intron	Intron	13516379	rs751610	C	-C	16636	26.70	23.96	24.51
Intron	Intron	13516430	.	A	-A	19565	4.68	4.50	4.59
Intron	Intron	13516441	.	T	-T	19569	9.18	8.44	8.22
Intron	Intron	13516487	.	T	-T	19260	4.07	4.90	4.51
Intron	Intron	13516500	.	T	-T	18334	10.74	10.09	11.01
Intron	Intron	13516560	.	T	-T	18036	8.59	7.37	8.14
Intron	Intron	13516596	.	A	-A	17967	9.69	9.39	9.75
Intron	Intron	13516619	.	A	-A	17794	9.36	10.67	9.19
Intron	Intron	13516706	.	T	-T	16071	9.85	9.80	8.77
Intron	Intron	13516723	.	T	-T	15951	12.29	13.20	11.84
Intron	Intron	13516735	.	T	-T	16784	6.43	6.45	6.69
Intron	Intron	13516743	.	T	-T	17858	6.58	5.80	6.20
Intron	Intron	13516852	.	T	-T	17044	6.13	6.68	6.42
Intron	Intron	13516887	.	T	-T	17999	16.06	14.52	14.14
Intron	Intron	13516893	.	G	-G	18160	11.64	10.15	9.83
Intron	Intron	13516933	.	T	-T	18520	9.80	9.29	8.74
Intron	Intron	13516988	.	G	-G	16676	10.94	10.36	9.15
Intron	Intron	13517001	.	T	-T	17097	5.22	4.27	5.38
Intron	Intron	13517016	.	G	-G	17230	11.37	10.48	10.06
Intron	Intron	13517035	.	A	-A	16245	4.91	5.27	5.58
Intron	Intron	13517070	.	A	-A	14367	19.65	20.32	18.68
Intron	Intron	13517095	.	A	-A	14260	5.03	6.01	5.68
Intron	Intron	13517146	.	T	-T	16717	12.53	12.93	11.92
Intron	Intron	13517155	.	A	-AT	17018	15.14	12.22	14.45
Intron	Intron	13517159	.	T	-T	17228	5.02	3.66	4.20
Intron	Intron	13517182	.	A	-A	18658	5.77	4.07	4.45
Intron	Intron	13517212	.	T	-T	17305	19.98	20.08	19.22
Intron	Intron	13517239	.	A	-A	19133	3.37	4.98	3.16
Intron	Intron	13517271	rs3761861	A	-A	18465	12.60	10.19	10.90
Intron	Intron	13517319	.	T	-T	18192	4.90	4.93	4.31

Intron	Intron	13517418	.	A	-A	20699	11.74	13.09	11.52
Intron	Intron	13517445	.	T	-T	21200	10.55	10.96	9.86
5' UTR - exon 1-T1	Intron	13517483	.	T	-T	21106	8.20	7.11	8.86
5' UTR - exon 1-T1	Intron	13517551	.	C	-C	19409	11.08	9.98	9.00
5' UTR - exon 1-T1	Intron	13517567	.	T	-T	18197	5.46	5.80	5.45
Upstream	5' UTR - exon 1-T2	13517604	.	T	-T	17080	18.35	19.41	17.50
Upstream	5' UTR - exon 1-T2	13517628	.	C	-C	17003	4.88	5.15	4.62
Upstream	5' UTR - exon 1-T2	13517728	.	G	-GA	19423	4.32	4.76	4.04
Upstream	Upstream	13517732	.	A	-A	19971	14.10	13.94	12.88
Upstream	Upstream	13517754	rs141835866	G	-G	20640	17.38	16.87	15.60
Upstream	Upstream	13517771	.	T	-T	21034	4.40	4.35	3.51
Upstream	Upstream	13517802	.	A	-A	19374	7.15	7.43	6.56
Upstream	Upstream	13517842	.	A	-A	18608	20.43	19.15	19.14
Upstream	Upstream	13517888	.	A	-A	17053	3.85	4.73	4.05
Upstream	Upstream	13517896	.	C	-CA	16510	9.54	9.42	8.22
Upstream	Upstream	13517901	.	A	-A	16686	13.16	12.66	11.86
Upstream	Upstream	13517941	.	T	-T	18117	12.04	11.01	10.77
Upstream	Upstream	13518018	.	T	-CA	17259	4.58	5.46	4.27
Upstream	Upstream	13518024	.	A	-A	17060	33.83	31.98	29.37
Upstream	Upstream	13518122	.	T	-T	19245	4.34	2.92	3.27
Upstream	Upstream	13518126	.	A	-A	20635	4.79	3.78	4.34
Upstream	Upstream	13518145	.	T	-T	22107	10.51	7.49	7.54