Screening for genetic variants implicated in monogenic forms of hypertension in a hypertensive urban black Free State cohort

By

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Declaration

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

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"Fear not, for I am with you;

Be not dismayed, for I am your God.

I will strengthen you, yes I will help you,

I will uphold you with My righteous right hand."

Isaiah 41:10

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List of Abbreviations and Acronyms

’	Prime
^{o}C	Degree Celsius
%	Percentage
A	Adenine
ACTH	Adrenocorticotropic hormone
AHA-FS	Assuring Health for All in the Free State
AIDS	Acquired immune deficiency syndrome
Ala	Alanine
AME	Apparent mineralocorticoid excess
AP	Alkaline phosphatase
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
BMI	Body mass index
BP	Blood pressure
bp	Base pairs

C	Cytosine
CAH	Congenital adrenal hyperplasia
CCT	Cortical collecting tubule
Cl	Chloride
cm	centimetre
CRH	Corticotropin releasing hormone
C_t	Threshold cycle
CTAB	Cetyl trimethyl ammonium bromide
CVD	Cardiovascular disease
Cys	Cysteine
DBP	Diastolic blood pressure
dbSNP	Database of single nucleotide polymorphisms
DCT	Distal convoluted tubule
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DOC	Deoxycorticosterone
EDTA	Ethylenediamine tetra acetic acid
e.g.	*exempli gratia* (for example)
ENaC	Epithelial sodium channel

LIST OF ABBREVIATIONS AND ACRONYMS

et al.	*et alia* (and others)
ECUFS	Ethics Committee of the University of the Free State
FTA	Fast technology for analysis of nucleic acid
G	Guanine
Gln	Glutamine
Glu	Glutamic acid
Gly	Glycine
GR	Glucocorticoid receptor
GRA	Glucocorticoid remediable aldosteronism
His	Histidine
HIV	Human immunodeficiency virus
HPA	Hypothalamic pituitary adrenal
HRM	High resolution melting
HSD11B2	11β-hydroxysteroid dehydrogenase type 2
HT	Hypertension
HWE	Hardy-Weinberg equilibrium
Ile	Isoleucine
K^+	Potassium ion
kbp	Kilo base pairs

kg	kilogram
Leu	Leucine
LiCl	Lithium chloride
Lys	Lysine
M	Molar
m	metre
ml	Millilitre
mM	Millimolar
mm	Millimetre
mmHg	Millimetre of mercury
Met	Methionine
$MgCl_2$	Magnesium chloride
MR	Mineralocorticoid receptor
Na^+	Sodium ion
ng	Nanogram
NaOH	Sodium hydroxide
NCBI	National Center for Biotechnology Information
NCCT	Sodium-chloride co-transporter
NCDs	Non-communicable diseases

NIH	National Institute of Health
PA	Primary aldosteronism
PCR	Polymerase chain reaction
pH	Potential hydrogen
Phe	Phenylalanine
pmol	Piccomole
Pro	Proline
rpm	Revolutions per minute
rs	Reference single nucleotide polymorphisms number
Ser	Serine
SB	Sodium borate
SBP	Systolic blood pressure
T	Thymine
T_a	Annealing temperature
Taq	*Thermus aquaticus*
TE	Tris EDTA
Thr	Threonine
Tris	Tris hydroxymethyl aminomethane
Thr	Threonine

Trp	Tryptophan
Tyr	Tyrosine
UFS	University of the Free State
USA	United States of America
UV	Ultraviolet
V	Volt
Val	Valine
WHO	World Health Organisation
WT	Wild type
www	World wide web
α	Alpha
β	Beta
γ	Gamma
µg	Microgram
µl	Microlitre

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Preface

Non-communicable diseases (NCDs), also known as chronic diseases of lifestyle, are the leading cause of death worldwide. Amongst the risk factors for NCDs, hypertension (blood pressure (BP) ≥140/90 mmHg) is one of the leading causes of death in South Africa. Hypertension is either considered to be primary or secondary. Primary hypertension is reported to be the most common form, and develops as a result of the contribution of several genetic, environmental, and demographic factors. Secondary hypertension is considered to be the result of an underlying, identifiable cause. Included in secondary hypertension is a group of syndromes associated with monogenic hypertension, where the elevated BP is thought to be primarily due to a genetic component. Monogenic forms of hypertension are characterized by increased sodium reabsorption in the distal nephron, and several genes that play a role in the sodium reabsorption pathway have been implicated in these disorders.

The prevalence of hypertension in an urban black population in Mangaung in the Free State is reported to be much higher than the average for South Africa. In a previous study of the Mangaung population, the association of several factors with the prevalence of hypertension was investigated, including weight, physical activity levels, sodium intake and genetic factors. It was found that BP correlated positively with adiposity, as well as with sodium intake. In addition, genetic analysis indicated that a genetic variant implicated in primary hypertension could be an independent risk factor for hypertension in 2% of the Mangaung population. It is not known, however, if genetic variants implicated in monogenic forms of hypertension could play a role in the Mangaung cohort. The aim of this study was to screen for genetic variants in genes that have previously been implicated in monogenic forms of hypertension, to determine if hypertension in the Mangaung population could have a monogenic component.

This master's dissertation contains five chapters: Chapter one is a literature review and presents the background to the genes involved in monogenic forms of hypertension. Chapter two describes the materials and methods that were used in this study. Chapter three contains the optimization of DNA isolation from blood spotted onto FTA® paper, as well as the optimization of the conventional polymerase chain reaction (PCR) assays for *NR3C1* (exons 6, 7, 9, 10, and 11), *HSD11B2* (exons 3, 4, and 5), *SCNN1B* (exon 13), *SCNN1G* (exon 13), and *WNK4* (exons 7 and 17), high resolution melting (HRM) analysis for all of the assays with the exception of *HSD11B2* exon 4, and long range PCR assay to screen for the presence of the chimeric *CYP11B1/CYP11B2* gene. In Chapter four, the results for the long range PCR assay, as well as the sequence variants identified using DNA sequencing, is discussed. Finally, a general discussion and conclusion is included as Chapter five. The tables and figures are numbered according to the chapter in which they occur, and have been included within the text where applicable. A summary in both English and Afrikaans is included after Chapter five. A full reference list is included after the summary, followed by Appendices A and B. Appendix A contains tables wherein previously reported genetic variants are summarized for genes implicated in monogenic forms of hypertension. Appendix B contains the demographical data for the 90 hypertensive participants of this study.

In this study, I initially wanted to screen a cohort of hypertensive and normotensive individuals from Mangaung to identify sequence variants that have previously been implicated in monogenic forms of hypertension. However, due to the challenges that were encountered during PCR and HRM optimization, it was decided to reduce the sample size and only focus on hypertensive individuals. The cohort for this study consisted of 90 hypertensive individuals from the Mangaung population. A limitation of this study is that it is not known at which frequency the sequence variants identified in this hypertensive cohort are present in normotensive individuals of the Mangaung population. The association between BP and the sequence variants identified in this study could, therefore, not be determined.

Chapter 1

Literature Review

1.1 Introduction to non-communicable diseases (NCDs)

Non-communicable diseases (NCDs), also known as chronic diseases of lifestyle, are the leading cause of death worldwide. The four main NCDs are cardiovascular disease (CVD), cancer, respiratory disease, and diabetes (World Health Organisation (WHO) 2014a). According to projections by Mathers and Loncar (2006), the proportion of deaths attributed to NCDs will increase from 59% in 2002 to 69% in 2030. However, in 2012 NCDs resulted in an estimated 67% of deaths (WHO 2014a). Thus, it is likely that the deaths attributed to NCDs will exceed the projected 69% in the near future. The effect of NCDs is particularly severe in low- and middle-income countries due to the high cost of treatment and limited healthcare resources (Pestana *et al.* 1996; Yusuf *et al.* 2001; Kearney *et al.* 2004). According to statistics from the WHO, more than 73% of the deaths attributed to NCDs in 2012 occurred in low-and middle-income countries (WHO 2014a). South Africa is classified by the WHO as a middle-income country. According to statistics from the WHO, an estimated 44% of deaths in South Africa were due to NCDs in 2012 (WHO 2014b). The recognition of NCDs as a major threat to societies and economies has lead to the adoption of a global action plan for the prevention and control of NCDs (WHO 2013).

NCDs share similar risk factors, most of which are considered to be modifiable through changes in lifestyle. Behavioural risk factors for NCDs include physical inactivity, unhealthy diet, tobacco smoking and harmful use of alcohol (Bradshaw *et al.* 2011). An unhealthy lifestyle can, in turn, lead to elevated blood pressure (BP), being overweight, raised blood glucose as well as increased cholesterol, all of which are considered to be metabolic risk factors for NCDs. As part of the “Assuring Health for All in the Free State” (AHA-FS) study, Van Zyl *et al.* (2012) investigated the risk-factor profile for NCDs

in an urban black community in Mangaung in the Free State, South Africa. Van Zyl *et al.* (2012) investigated the prevalence of physical inactivity, overweight, elevated BP, tobacco smoking, cholesterol, and diabetes. The study found that at least three or more of the investigated risk factors were present in 34% of the Mangaung study population (Van Zyl *et al.* 2012). Due to the high prevalence of communicable diseases such as HIV/AIDS and tuberculosis, the prevention and treatment of NCDs are currently considered to be marginalized in South Africa (Mayosi *et al*. 2009). However, if measures are not taken to prevent and treat NCDs effectively, the disease burden is estimated to increase substantially in future (Abegunde *et al*. 2007; Bradshaw *et al.* 2011; World Economic Forum 2011).

CVD is responsible for the biggest proportion of deaths attributed to NCDs throughout the world. CVD is a group of diseases that includes stroke, coronary heart disease, heart failure and end-stage renal disease (Flack *et al.* 1995; Kannel *et al.* 1996; Klag *et al.* 1996; Levy *et al.* 1996; Van der Hoogen *et al.* 2000; Tozawa *et al.* 2003). In 2012 CVD accounted for an estimated 46% of deaths due to NCDs in the world and 41% of deaths due to NCDs in South Africa (WHO 2014a; WHO 2014b). The annual number of deaths due to CVD is projected to increase from 16.7 million in 2002 to 23.3 million in 2030 (Mathers and Loncar 2006). A higher incidence of CVD has been reported in black individuals compared to white individuals in the United States of America (USA) (Yusuf *et al.* 2001; Go *et al*. 2013). CVD was reported in 37% and 32% of Caucasian men and women, respectively, compared to 44% and 49% of African American men and women, respectively (Go *et al*. 2013). An estimated 45% of CVD is due to elevated BP (hypertension) in adults over the age of 30 years (WHO 2012). The WHO defines hypertension as sustained systolic BP ≥ 140 mmHg and/or diastolic BP ≥ 90 mmHg. Sheats *et al.* (2005) has proposed that the higher incidence of CVD in the black population could be ascribed to the higher prevalence of hypertension, as well as reduced control of elevated BP in black individuals. A higher prevalence of hypertension and a reduced control of hypertension have been reported in African Americans and black South Africans (Pavlin *et al*. 1996; Sowers *et al.* 2002; Kramer *et*

al. 2004; Connor *et al.* 2005; Hertz *et al.* 2005; Sheats *et al.* 2005; Cutler *et al.* 2008; Umscheid *et al*. 2010). Furthermore, a higher prevalence of severe hypertension (BP ≥180/110 mmHg) and a high frequency of co-morbid conditions such as diabetes mellitus and chronic kidney disease have been reported in African American individuals (Flack *et al*. 2010). A high prevalence of hypertension, poor control of BP levels despite anti-hypertensive treatment, and a high prevalence of diabetes has also been reported in black individuals from Mangaung in the Free State (Van Zyl *et al*. 2012). Early detection and management of hypertension is of great importance to try to minimize the occurrence of CVD, especially in the black population.

Amongst the risk factors for NCDs, hypertension (BP ≥140/90 mmHg) was the leading cause of death in South Africa in 2000 (Norman *et al.* 2007). In 2014, the prevalence of hypertension in the world was estimated to be 22% (WHO 2014a). Across all WHO regions, the prevalence of hypertension was estimated to be highest in Africa (30%). The prevalence of hypertension in South Africa was estimated to be approximately 25% for both men and women in 2014 (WHO 2014a). The prevalence of hypertension in an urban black population in Mangaung in the Free State was reported to be approximately 51% in men and 59% in women (Van Zyl *et al.* 2012). Thus, there is a much higher prevalence of hypertension in the urban black Mangaung population of the Free State compared to the rest of South Africa.

1.2 Different forms of hypertension

Hypertension (BP ≥140/90 mmHg) is classified as either primary or secondary. In primary or essential hypertension, a particular cause for the elevated BP cannot be identified. Instead, several genetic, environmental and demographic factors contribute to the development of elevated BP in affected individuals (Tanira and Al Balushi 2005). Primary hypertension is reported to account for approximately 90% to 95% of hypertension cases (Tanira and Al Balushi 2005). Secondary hypertension is described as elevated BP due to an underlying, identifiable cause. Secondary hypertension is

estimated to account for approximately 5% to 10% of hypertension cases (O'Rorke and Richardson 2001). Secondary hypertension comprises several physiological disorders, including a group syndromes associated with monogenic hypertension. In monogenic forms of hypertension, the elevated BP is primarily due to a genetic component (Lifton *et al.* 2001). However, there is a range in the severity of the syndromes associated with monogenic hypertension, often resulting in a variable phenotype in affected individuals (Rosler *et al.* 1982; Findling *et al.* 1997; Connell *et al.* 2001). Due to the wide range in phenotype in affected individuals, it has been suggested that some patients could be misdiagnosed as having primary hypertension instead of the monogenic form (Gates *et al.* 1996; Li *et al.* 1997; Li *et al.* 1998; O'Shaughnessy *et al.* 1998; Gates *et al.* 2001; Hassan-Smith and Stewart 2011). As a result of the wide range in phenotype, it has been suggested that monogenic forms of hypertension could be more common in the general population than is currently thought (Gates *et al.* 1996; Takeda *et al.* 1996; Findling *et al.* 1997; Li *et al.* 1998; O'Shaughnessy *et al.* 1998; Ferrari and Krozowski 2000; Huizenga *et al.* 2000; Wilson *et al*. 2001a; Wilson *et al*. 2001b; Morineau *et al.* 2006; Rossi *et al*. 2008). According to Persu (2003), the division between monogenic and polygenic hypertension might not be as definite as previously thought.

1.3 Monogenic forms of hypertension

The kidney plays a crucial role in regulating BP. The nephron is the structural and functional unit of the kidney. The primary function of the kidney is to regulate the concentration of water soluble substances (e.g. sodium ions (Na^+)), by filtering the blood, reabsorbing what is needed, and excreting the rest as urine (Guyton and Hall 2006). Sodium is the most abundant cation in the extracellular fluid and is central to fluid and electrolyte balance. Sodium exerts significant osmotic pressure, which means that water will move in the same direction as sodium flow in the nephron (Johnson and Criddle 2004). Therefore, changes in the plasma sodium concentration affect the plasma volume and consequently blood pressure (Lifton *et al.* 2001). If sodium homeostasis cannot be maintained, hypo- or hypertension can result (Lifton *et al.* 2001; Guyton and Hall 2006).

In cases of monogenic forms of hypertension, sodium reabsorption in the distal nephron is affected (Lifton *et al.* 2001). In response to a low plasma sodium concentration, renin is secreted (Young 1999). Renin cleaves angiotensinogen to angiotensin I, which is in turn cleaved to angiotensin II via the angiotensin-converting enzyme (Lifton *et al.* 2001). Angiotensin II binds to vascular and adrenal angiotensin receptors, leading to vasoconstriction and the secretion of aldosterone, respectively (Lifton *et al.* 2001). Aldosterone is the principal mineralocorticoid steroid hormone and binds to the mineralocorticoid receptor (MR) (Kim *et al.* 1998; Masilamani *et al.* 1999). Other hormones, including cortisol and deoxycorticosterone (DOC), can also bind to and activate the MR. DOC is a precursor to aldosterone, and since its mineralocorticoid potency is only about 2% that of aldosterone, it is thought to be unlikely that DOC contributes significantly to electrolyte and blood pressure homeostasis (Connell *et al*. 2001). However, there are syndromes associated with monogenic hypertension in which DOC secretion is increased to an extent that it has a significant effect on the activity of the MR (Connell *et al*. 2001). Upon binding of an agonist to the MR in the distal nephron, a series of events is initiated that results in increased transport of sodium (Figure 1.1) (Lifton *et al.* 2001). There are two types of sodium transporters in the distal nephron, namely the sodium-chloride co-transporter (NCCT) situated in the distal convoluted tubule, and the epithelial sodium channel (ENaC) located in the cortical collecting tubule (Lifton *et al.* 2001). Sodium transported through the NCCT and/or the ENaC is subsequently reabsorbed into the blood via the sodium-potassium pump (Na^+/K^+ ATPase) (Connell *et al.* 2001; Hassan-Smith and Stewart, 2011). Water reabsorption accompanies sodium reabsorption to maintain the correct plasma sodium concentration (Lifton *et al.* 2001). The resulting increase in extracellular fluid volume increases the amount of blood returning to the heart, which raises the cardiac output and subsequently BP (Lifton *et al.* 2001). The increase in extracellular fluid volume leads to the suppression of renin secretion and reduced production of aldosterone (Lifton 1996). Thus, sodium reabsorption in the distal nephron is achieved through an intricate pathway and ultimately affects BP through plasma volume expansion.

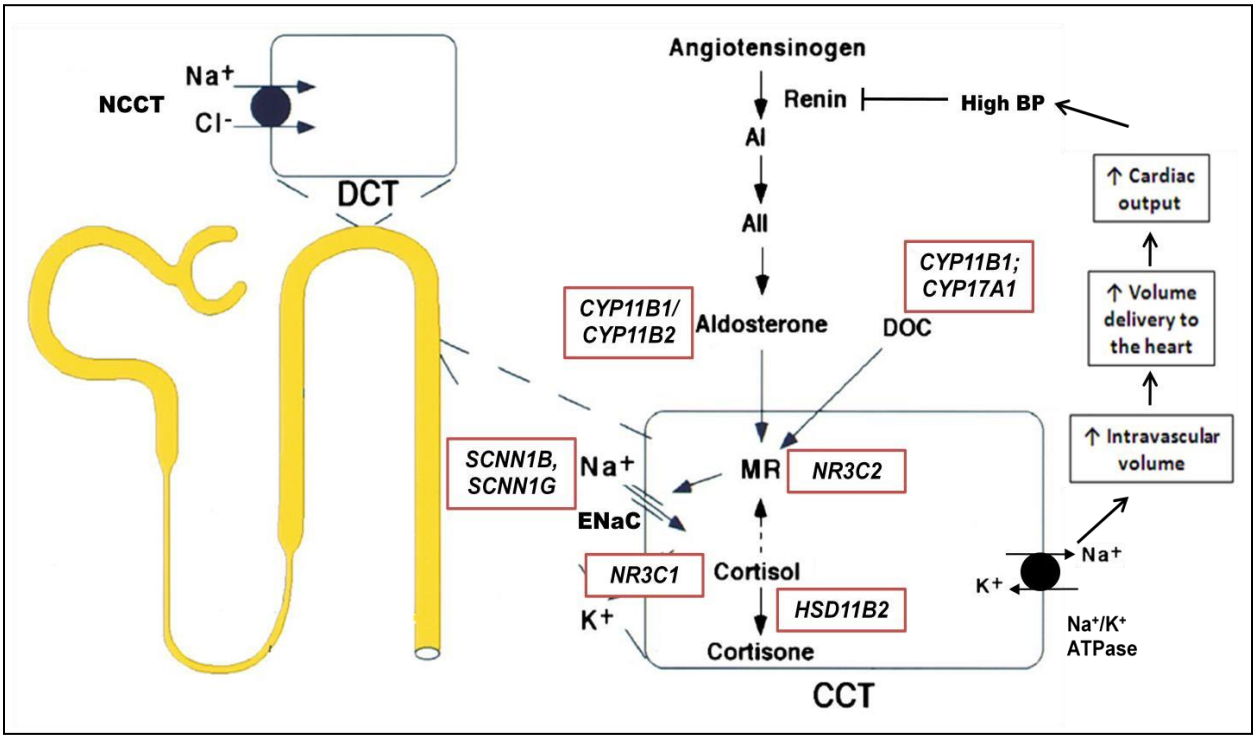

Figure 1.1: A schematic representation of sodium reabsorption in the distal nephron and where the genes implicated in monogenic forms of hypertension play a role. Sodium (Na^+) reabsorption can be increased indirectly as a result of enhanced activation of the mineralocorticoid receptor (MR) via aldosterone, cortisol or deoxycorticosterone (DOC), or directly as a result of enhanced sodium transport through the sodium-chloride co-transporter (NCCT) or the epithelial sodium channel (ENaC). Genetic variation in the following genes can result in increased activity of the MR: the chimeric *CYP11B1/CYP11B2*, *HSD11B2, NR3C1, CYP11B1*, *CYP17A1* and *NR3C2*. Genetic variation in *SCNN1B* and *SNN1G* can result in increased activity of the ENaC, while genetic variation in *WNK1* and *WNK4* can result in increased activity of the NCCT. The enhanced sodium transport through the NCCT and ENaC results in increased sodium reabsorption through the sodium-potassium pump (Na^+/K^+ ATPase) into the blood, ultimately leading to elevated blood pressure (BP) through plasma volume expansion [Copied and adapted from Lifton *et al.*(2001)].

Several genes that play a role in the sodium reabsorption pathway have been implicated in monogenic forms of hypertension (Lifton *et al.* 2001). These genes affect sodium transport either indirectly through their effect on the activity of the MR, or directly through their effect on sodium transport via the NCCT or the ENaC (Figure 1.1; Table 1.1). The genes that indirectly affect sodium transport include the chimeric *CYP11B1/CYP11B2, CYP11B1*, *CYP17A1, HSD11B2, NR3C1,* and *NR3C2.* These genes play a role in the production of MR agonists (aldosterone, DOC or cortisol), while *NR3C2* encodes the MR. Increased activity of the MR will result in increased sodium transport through the ENaC and/or the NCCT. The genes that directly affect sodium transport are *SCNN1B*, *SCNN1G, WNK1,* and *WNK4*. *SCNN1B* and *SCNN1G* encode the beta- and gamma-subunits of the ENaC, respectively, while *WNK1* and *WNK4* play a role in regulating the activity of the NCCT. Enhanced activity of the NCCT and/or the ENaC results in increased sodium reabsorption through the sodium-potassium pump. Water is reabsorbed along with sodium and the resulting increase in plasma volume ultimately leads to elevated BP (Lifton *et al.* 2001). High BP results in the suppression of renin and low renin levels are, therefore, characteristic of monogenic forms of hypertension (Chrousos *et al.* 1993; Hassan-Smith and Stewart 2011). Several of the candidate genes that have been implicated in monogenic forms of hypertension have also been associated with primary hypertension (Ferrari and Krozowski 2000; Connell *et al.* 2001; Quinkler and Stewart 2003; Tobin *et al.* 2008; Martinez *et al.* 2009; Ferrari 2010; McCormick and Ellison 2011; Hassan-Smith and Stewart 2011; Zhao *et al.* 2011). Therefore, it is possible that monogenic forms of hypertension could be more common than previously thought (Gates *et al.* 1996; Findling *et al.* 1997; Li *et al*. 1997; Li *et al.* 1998; O'Shaughnessy *et al.* 1998; Ferrari and Krozowski 2000; Huizenga *et al.* 2000; Wilson *et al*. 2001a; Wilson *et al*. 2001b; Morineau *et al.* 2006; Rossi *et al*. 2008).

Table 1.1: A summary of genes that have been implicated in monogenic forms of hypertension.

Candidate gene(s)	Function of the protein	Effect of genetic variation on the protein	Reference
Candidate genes that affect renal sodium reabsorption via the MR			
CYP11B1/ CYP11B2 chimeric gene	Misalignment and unequal crossing over of *CYP11B1* and *CYP11B2* give rise to a chimeric gene that encodes a protein with aldosterone activity that is regulated by the adrenocorticotropic hormone (ACTH). ACTH is involved in the regulation of cortisol biosynthesis.	The secretion of aldosterone is regulated by the ACTH hormone, which is not responsive to sodium levels. As a result, aldosterone is constitutively expressed. The high concentration of aldosterone increases the activity of the MR and can lead to elevated blood pressure (BP).	Lifton *et al.* (1992a); Lifton *et al.* (1992b); Lifton *et al.* (2001)
HSD11B2	The HSD11B2 enzyme converts cortisol to cortisone. Cortisone is not able to activate the MR, and the receptor is thereby protected from inappropriate activation.	Genetic variation in *HSD11B2* that results in reduced or abolished activity of the enzyme enables circulating cortisol to bind to and activate the MR. The increase in MR activity can result in elevated BP.	Funder *et al.* (1988); Mune *et al.* (1995); Wilson *et al.* (1995a)
NR3C1	The glucocorticoid receptor (GR) regulates circulating cortisol levels.	Genetic variation in *NR3C1* that renders the GR partially unresponsive to cortisol leads to continuous stimulation to secrete cortisol. The continuous secretion of deoxycorticosterone (DOC) and cortisol increases the activity of the MR and can lead to elevated BP.	Hurley *et al.* (1991); Van Rossum (2006)
CYP11B1	The CYP11B1 enzyme converts DOC to corticosterone and 11-deoxycortisol to cortisol, respectively.	Genetic variation in *CYP11B1* that results in reduced activity of the enzyme leads to 1) the accumulation of DOC and 11-deoxycortisol and 2) increased production of androgenic sex hormone precursors, which leads to abnormal sexual development in girls and boys. Excessive levels of DOC increases the activity of the MR and can result in elevated BP.	White *et al.* (1994a); Milford (1999); Connell *et al.* (2001)

(Table 1.1 continued)

Candidate gene(s)	Function of the protein	Effect of genetic variation on the protein	Reference
CYP17A1	CYP17 is an enzyme with both 17α-hydroxylase and 17, 20-lyase activities, which are essential for the synthesis of cortisol and gonadal hormones, respectively.	Genetic variation in *CYP17A1* that results in reduced activity of CYP17 leads to a deficiency of cortisol and sex hormones. The resulting continuous secretion of DOC and cortisol leads to increased activity of the MR and can result in elevated BP. The deficiency of sex hormones leads to abnormal sexual development.	Biglieri *et al.* (1966); Milford (1999); Connell *et al.* (2001); Garovic *et al.* (2006)
NR3C2	The mineralocorticoid receptor (MR) plays a crucial role in renal sodium reabsorption.	Genetic variation in *NR3C2* that results in reduced binding selectivity of the receptor leads to activation of the MR by steroids that are normally not able to. The increase in MR activity results in enhanced sodium reabsorption and can lead to elevated BP.	Geller *et al.* (2000)
Candidate genes that affect renal sodium reabsorption through sodium transporters			
SCNN1B	Encodes the beta-subunit of the epithelial sodium channel (ENaC). The ENaC transports sodium into the cells of the distal nephron.	Genetic variation in *SCNN1B* or *SCNN1G* that results in an altered or absent PY-motif (which is critical for the internalization and degradation of the ENaC) leads to the extension of the half-life of the ENaC. Channel activity is thereby increased several-fold, which increases sodium reabsorption and can lead to elevated BP.	Shimkets *et al*. (1994); Hansson *et al*. (1995a)
SCNN1G	Encodes the gamma-subunit of the ENaC. The ENaC transports sodium into the cells of the distal nephron.		

(Table 1.1 continued)

Candidate gene(s)	Function of the protein	Effect of genetic variation on the protein	Reference
WNK1	WNK1 exerts an inhibitory effect on WNK4.	Genetic variation in *WNK1* that results in increased expression of WNK1 leads to the inhibition of the activity of WNK4. Consequently, the sodium-chloride co-transporter (NCCT) is no longer inhibited by WNK4. The resulting increased in sodium transport can result in elevated BP.	Wilson *et al.* (2001a); Huang *et al.* (2008)
WNK4	WNK4 inhibits the activity of the NCCT, which transports sodium and chloride into the distal nephron.	Genetic variation in *WNK4* that results in reduced activity of WNK4 leads to increased activity of the NCCT. The increased transport of sodium through the NCCT can lead to elevated BP through plasma volume expansion.	Wilson *et al.* (2001a); Huang *et al.* (2008)

ACTH: adrenocorticotropic hormone; BP: blood pressure; DOC: deoxycorticosterone; ENaC: epithelial sodium channel; GR: glucocorticoid receptor; MR: mineralocorticoid receptor; NCCT: sodium-chloride co-transporter.

1.3.1. Candidate genes that affect sodium reabsorption via the MR

1.3.1.1 The role of the chimeric *CYP11B1/CYP11B2* gene in hypertension

The *CYP11B1* gene encodes 11β-hydroxylase while *CYP11B2* encodes aldosterone synthase. The enzyme 11β-hydroxylase plays a role in cortisol biosynthesis and aldosterone biosynthesis, and is regulated by the adrenocorticotropic hormone (ACTH) (Lifton *et al.* 1992a; Lifton *et al.* 1992b). Aldosterone synthase plays a role in the rate-limiting step for aldosterone biosynthesis and is regulated by the renin-angiotensin-aldosterone system (Lifton *et al.* 1992a; Lifton *et al.* 1992b). *CYP11B1* and *CYP11B2* are localized in close proximity on chromosome 8 (8q21-22), and share more than 90% homology (Chua *et al*. 1987; Mornet *et al*. 1989). Due to the localization and the high degree of similarity between the genes, *CYP11B1* and *CYP11B2* can misalign during meiosis (Lifton *et al.* 1992a; Lifton *et al.* 1992b). This results in unequal crossing over, that leads to the formation of a chimeric gene, in addition to the normal copy of *CYP11B1* and *CYP11B2* (Figure 1.2). The chimeric *CYP11B1/CYP11B2* encodes a protein with aldosterone activity, but the production of which is regulated by ACTH (Lifton *et al.* 1992a). Unlike with the renin-angiotensin-aldosterone system that responds to a low plasma sodium concentration, ACTH is not responsive to sodium levels. As a result, in individuals with the chimeric *CYP11B1/CYP11B2,* aldosterone is constitutively secreted, which leads to increased activity of the MR (Lifton *et al.* 1992a). The enhanced activity of the MR results in increased sodium reabsorption and can lead to elevated BP through plasma volume expansion (Lifton *et al.* 1992a).

The chimeric *CYP11B1/CYP11B2* gene has been implicated in glucocorticoid-remediable aldosteronism (GRA). GRA, also known as familial hyperaldosteronism type 1, is an autosomal dominant disorder (Sutherland *et al.* 1966). It is thought that GRA may account for approximately 1% of patients with primary aldosteronism (PA) (Rayner *et al.* 2000). PA is considered to be the most common cause of secondary hypertension, and it has therefore been suggested that GRA could be the most common

syndrome associated with monogenic hypertension (Young 1999; McMohan and Dluhy 2004; Mulatero *et al.* 2011). PA is estimated to be the causal agent in 5% to 12% of hypertensive cases, although a frequency of 32% was reported after screening for PA in a hypertension clinic in South Africa (Gordon *et al*. 1993; Gordon *et al.* 1994; Fardella *et al.* 2000; Lim *et al.* 2000; Loh *et al.* 2000; Rayner *et al.* 2000; Rayner *et al.* 2001; Mosso *et al.* 2003; Schwartz and Turner 2005). Typical features of individuals with GRA include low renin levels, elevated aldosterone levels and moderate to severe salt-sensitive hypertension that usually develops early in life (Sutherland *et al.* 1966; Lifton 1992a; Rich *et al*. 1992). However, variable phenotypes have been documented in individuals with this disorder (Dluhy and Lifton 1995; Stowasser *et al.* 1999; Fallo *et al.* 2004). The presence and severity of hypertension has been found to vary between individuals with GRA, even between affected individuals within a family (Dluhy and Lifton 1995; Jamieson *et al.* 1995; Stowasser *et al.* 1995; Gates *et al.* 1996; Gordon and Stowasser 1998; Fallo *et al.* 2004; Lee *et al*. 2010; Mulatero *et al.* 2011). Several factors have been associated with the variation and severity of hypertension, including multiple genetic factors that affect BP, gender, level of kallikrein excretion, parental origin of the chimeric gene, position of the crossover point, level of aldosterone production and environmental factors (e.g. sodium intake) (Dluhy and Lifton 1995; Jamieson *et al.* 1995; Stowasser *et al.* 2000; Stowasser *et al.* 2001). Due to the variable phenotype in affected individuals, it has been suggested that GRA is a hypertension-predisposing syndrome, and that other BP regulation systems could influence the presentation of hypertension in individuals with this disorder (Dluhy and Lifton 1995; Stowasser *et al.* 1999). According to Stowasser *et al*. (2005), individuals with GRA who are normotensive may still be at an increased risk for CVD. Therefore, it is important to determine if the chimeric gene is present in individuals who are suspected of having this disorder, since the occurrence and severity of hypertension may change in individuals over time.

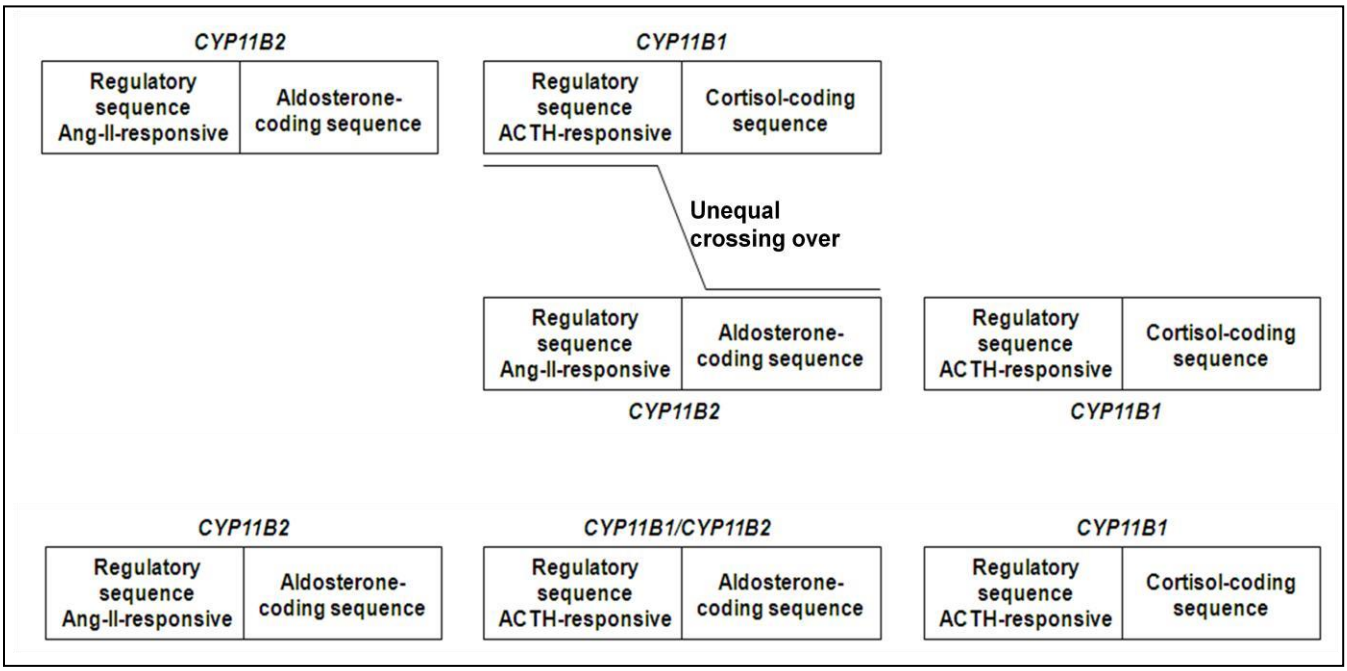

Figure 1.2: A schematic representation of the formation of the chimeric *CYP11B1/CYP11B2*. Unequal crossing over between *CYP11B1* and *CYP11B2* gives rise to a chimeric gene with aldosterone activity that is regulated by the adrenocorticotropic hormone (ACTH) [Adapted from Blanchard *et al.* (2002)].

1.3.1.2 The role of *HSD11B2* in hypertension

The *HSD11B2* gene encodes the enzyme 11β-hydroxysteroid dehydrogenase type 2. In the kidney, HSD11B2 is responsible for the conversion of cortisol to cortisone (Funder *et al.* 1988; Edwards *et al.* 1988). Cortisol is a potent activator of the MR. By metabolizing cortisol to its inactive form, namely cortisone, the MR is protected from being inappropriately activated by cortisol (Edwards *et al.* 1988; Ferrari and Krozowski 2000). Genetic variation in *HSD11B2* has been found to result in reduced enzyme activity (Appendix A, Table 1), which enables cortisol to bind to and activate the MR. The resulting increase in MR activity leads to enhanced sodium reabsorption and subsequently elevated BP through plasma volume expansion (Mune *et al.* 1995; Wilson *et al.* 1995a). Most of the known genetic variants that have been associated with elevated BP have been found in exons three to five of *HSD11B2* (Ferrari 2010).

Genetic variation in *HSD11B2* that results in reduced enzyme activity is the causal mechanism of hypertension in apparent mineralocorticoid excess (AME). This disorder is characterized by an increase in the ratio of cortisol-to-cortisone metabolites in the urine (Ulick *et al.* 1979; Monder *et al.* 1986). This monogenic form of hypertension is an autosomal recessive disorder that usually presents during childhood (Wilson *et al.* 2001b). AME typically features salt-sensitive hypertension, hypokalemia, low renin and low aldosterone levels (White *et al.* 1997; Ferrari and Krozowski 2000). The severity of this disorder depends on the degree to which the genetic variant causes a loss of HSD11B2 activity. As a result, the severity of AME in affected individuals can range from mild to severe (Li *et al.* 1998; Ferrari and Krozowski 2000). Several studies have suggested that there could be a link between a mild reduction in HSD11B2 activity and primary hypertension (Soro *et al.* 1995; Takeda *et al.* 1996; Li *et al*. 1997; Li *et al.* 1998; Ferrari and Krozowski 2000; Wilson *et al*. 2001b; Morineau *et al.* 2006). Therefore, although AME is considered to be rare, genetic variants that result in reduced HSD11B2 activity could be more common than previously thought and contribute to the development of hypertension.

1.3.1.3 The role of *NR3C1* in hypertension

The *NR3C1* gene encodes the glucocorticoid receptor, which plays a role in regulating the level of circulating cortisol. Glucocorticoids, primarily cortisol, are involved in the regulation of physiological systems and are critical for maintaining cardiovascular and metabolic homeostasis (Chrousos *et al*. 1993). Consequences of cortisol excess include elevated BP, truncal obesity, hyperinsulinemia, hyperglycemia, insulin resistance and dyslipidemia (Whitworth *et al.* 2005). Genetic variants in *NR3C1* that result in reduced binding affinity of the glucocorticoid receptor for cortisol, which in turn decreases the ability of the glucocorticoid receptor to transactivate target genes, has been associated with elevated BP (Appendix A, Table 2). When the glucocorticoid receptor becomes partially unresponsive to cortisol, cortisol levels are underestimated by the receptor and continuously perceived to be low. Thus, there is no feedback inhibition of the hypothalamus and pituitary gland to prevent the secretion of

corticotropin-releasing hormone (CRH) and ACTH, respectively. As a result, cortisol is overproduced, along with DOC and androgens (Figure 1.3) (Van Rossum 2006). Elevated levels of cortisol and DOC lead to increased activation of the MR, which in turn enhances sodium reabsorption and results in elevated BP through plasma volume expansion (Hurley *et al.* 1991).

Genetic variation in *NR3C1* is the underlying cause of hypertension in glucocorticoid resistance (Vehaskari 2009). Glucocorticoid resistance is a familial or sporadic disorder that can have either an autosomal recessive or dominant pattern of inheritance (Vehaskari 2009). The disorder is characterized by a partial insensitivity to cortisol, with corresponding increases in the production of circulating cortisol and androgenic steroids (Chrousos *et al.* 1993). Individuals with glucocorticoid resistance present with a wide range in phenotype. Some individuals affected by glucocorticoid resistance are asymptomatic while others present with fatigue, signs of mineralocorticoid excess (e.g. hypertension), and/or signs of androgen excess in females (Chrousos *et al.* 1993; Van Rossum *et al.* 2006). Signs of androgen excess in females can include hirsutism, male pattern hair loss and menstrual irregularities (Chrousos *et al.* 1993; Van Rossum *et al.* 2006). Possible reasons for the variation in the clinical manifestation of glucocorticoid resistance include variability in the degree of resistance, variability in the sensitivity of target tissues to mineralocorticoids and androgens, other genetic and epigenetic factors (Chrousos *et al.* 1993). As a result of the range in phenotype, it has been suggested that glucocorticoid resistance may be more common in individuals with hypertension than currently thought (Lamberts *et al.* 1992; Huizenga *et al.* 2000).

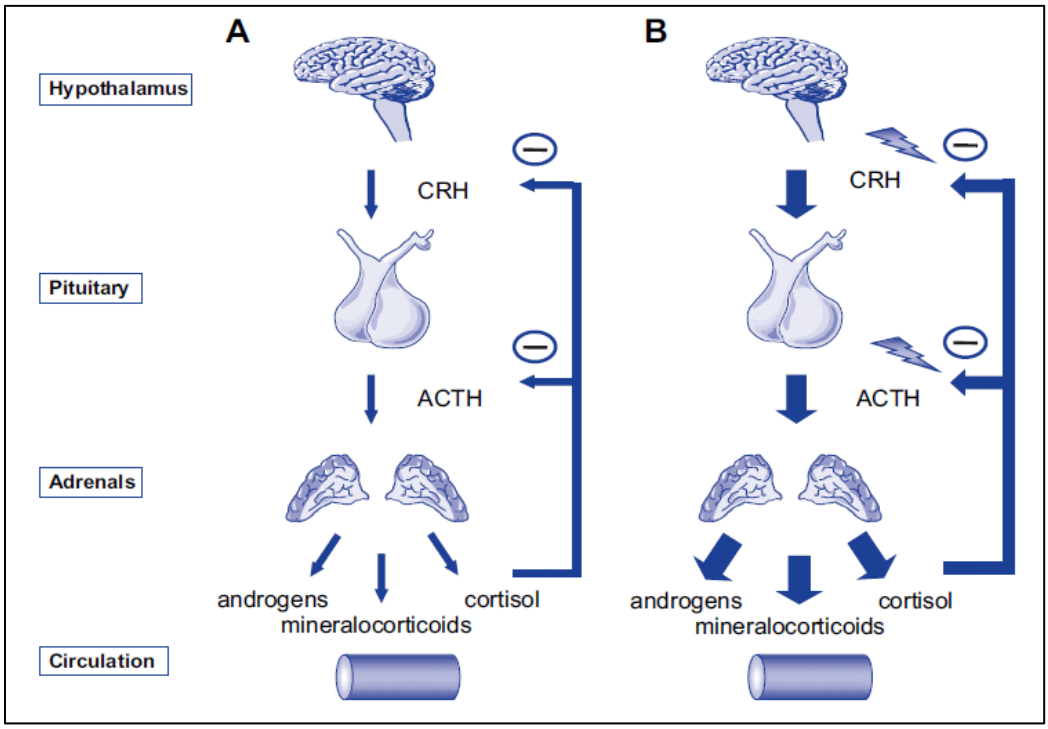

Figure 1.3: A schematic diagram of the regulation of the hypothalamic-pituitary-adrenal (HPA) axis. A) When the glucocorticoid receptor (GR) registers sufficient cortisol levels, feedback inhibition occurs and the secretion of corticotropin releasing hormone (CRH) and adrenocorticotropic hormone (ACTH) is inhibited; B) When the GR is rendered partially unresponsive to cortisol, the perceived low levels of cortisol prevents feedback inhibition of the HPA axis, leading to an overproduction of mineralocorticoids, androgens and cortisol [Copied from Van Rossum (2006)].

1.3.1.4 The role of *CYP11B1* in hypertension

The *CYP11B1* gene encodes 11β-hydroxylase, the enzyme that converts DOC to corticosterone and 11-deoxycortisol to cortisol (Figure 1.4). Genetic variants in *CYP11B1* that result in reduced enzyme activity have been associated with elevated BP (Appendix A, Table 3). Reduced activity of CYP11B1 leads to the accumulation of DOC and 11-deoxycortisol (White *et al.* 1994b; Milford 1999; Connell *et al.* 2001). In the majority of individuals with CYP11B1 deficiency, the excessive level of DOC results in

hypertension (Curnow *et al*. 1993; White *et al.* 1994b; Geley *et al.* 1996; Krone *et al*. 2005; Kuribayashi *et al.* 2005; Krone *et al*. 2006; Riedl *et al*. 2008). The accumulation of DOC and 11-deoxycortisol also results in the increased conversion of progesterone to 17α-hydroxyprogesteron, and 17α-hydroxyprogesterone to androstenedione, respectively (Figure 1.4). In individuals with CYP11B1 deficiency, androgen excess can manifest as masculinization in girls and precocious puberty in boys (Joehrer *et al.* 1997; Krone *et al.* 2009). In cases of severe CYP11B1 deficiency, early diagnosis and effective therapy are important to ensure normal growth and sexual development (Connell *et al.* 2001).

Genetic variation in *CYP11B1* has been implicated in congenital adrenal hyperplasia (CAH) due to 11β-hydroxylase deficiency. CAH refers to a group of inherited disorders of the adrenal gland, which produces steroid hormones (Vehaskari 2009). CAH as a result of 11β-hydroxylase deficiency is the second most common form of CAH (Vehaskari 2009). CAH due to 11β-hydroxylase deficiency is an autosomal recessive disorder and affected individuals usually present with hypertension during childhood, low plasma renin activity, decreased aldosterone levels and signs of androgen excess (Krone *et al.* 2009; Vehaskari 2009). However, a wide range in the phenotype has been reported for this disorder, with affected individuals presenting with mild to severe symptoms (Connell *et al.* 2001; Hassan-Smith and Stewart 2011). Studies have found that the severity of hypertension and the degree of androgen excess in individuals with 11β-hydroxylase deficiency varies significantly, even within families (Rosler *et al*. 1982; White *et al.* 1991; Curnow *et al*. 1993; Zhu *et al.* 2003). Furthermore, several studies have shown that there is a poor correlation between hypertension and hormone levels, including DOC (Rosler *et al*. 1982; Zachmann *et al.* 1983; Curnow *et al*. 1993). As a result, several authors have suggested that other epigenetic or non-genetic factors influence the clinical phenotype of this disorder (White *et al.* 1991; Curnow *et al*. 1993; Nakagawa *et al.* 1995).

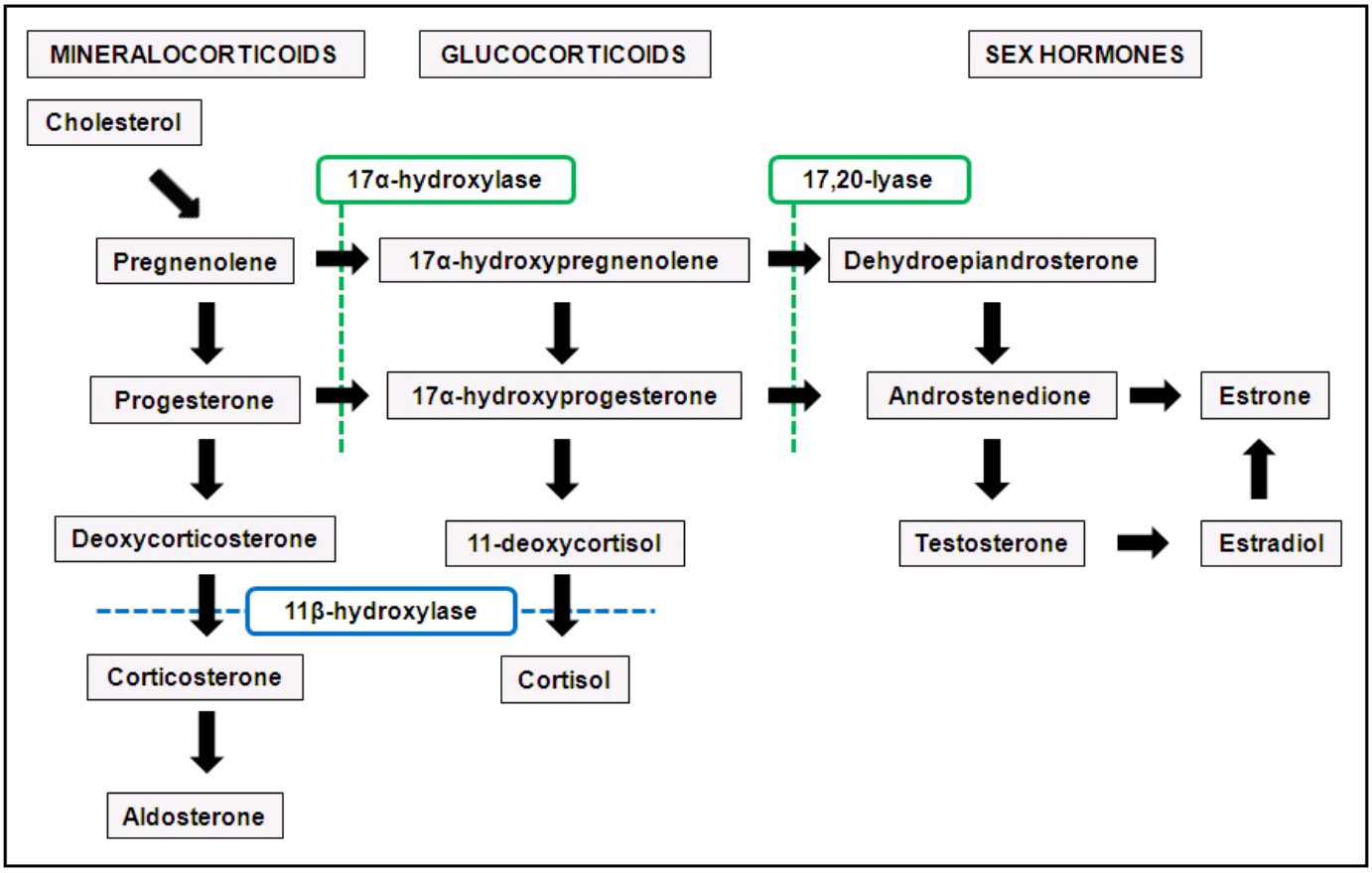

Figure 1.4: A schematic representation of steroid biosynthesis and the steps where 11β-hydroxylase, 17α-hydroxylase and 17, 20-lyase play a role [Adapted from New and Wilson (1999)].

1.3.1.5 The role of *CYP17A1* in hypertension

CYP17A1 encodes CYP17, an enzyme with both 17α-hydroxylase and 17, 20-lyase activity. 17α-hydroxylase is essential for the synthesis of cortisol, while 17, 20-lyase is necessary for the synthesis of sex hormones (Figure 1.4) (Biglieri *et al.* 1966; Chung *et al.* 1987). Genetic variation in *CYP17A1* that results in combined 17α-hydroxylase/17, 20-lyase deficiency has been associated with elevated BP (Appendix A, Table 4). Combined 17α-hydroxylase/17, 20-lyase deficiency results in the inadequate synthesis of androgens and a decreased concentration of cortisol. Low cortisol levels stimulate cortisol production and leads to feedback overproduction of DOC. The excessive

production of DOC results in increased activity of the MR and can ultimately lead to elevated BP through plasma volume expansion (Biglieri *et al.* 1966; Yanase *et al.* 1995). Biason-Lauber *et al*. (2000) suggested that the catalytic activity of CYP17 must be above 25% to prevent the onset of hypertension.

Genetic variation in *CYP17A1* that results in reduced catalytic activity has been found to be the cause of elevated BP in one form of CAH. CAH resulting from a CYP17 deficiency is an autosomal recessive disorder and affected individuals usually present with hypertension in childhood, low plasma renin activity, hypokalemia, decreased aldosterone levels, and signs of reduced androgen production (Dhir *et al.* 2009; Vehaskari 2009). However, variable phenotypes have been reported for this disorder. It has been found that some individuals are normotensive at the time of diagnosis, despite a complete lack of CYP17 activity with correspondingly high levels of DOC (Mussig *et al.* 2005). Individuals with the more severe form of 17α-hydroxylase/17, 20-lyase deficiency where hypertension is expected (< 25% CYP17 catalytic activity), signs of inadequate production of sex hormones are often apparent during puberty (lack of secondary sex characteristics in girls and female or ambiguous genitalia in boys).

1.3.1.6 The role of *NR3C2* in hypertension

NR3C2 encodes the MR, which plays a critical role in sodium homeostasis in the distal nephron. Molecular studies have identified a single base change in *NR3C2* that is associated with elevated BP (Geller *et al.* 2000). This sequence variant leads to an amino acid substitution at codon 810 (Ser810Leu) and results in the MR being activated by cortisone and progesterone, in addition to normal activation by aldosterone and cortisol (Geller *et al.* 2000; Williams 2007). The increased MR activity leads to enhanced sodium transport through the ENaC and/or NCCT. The subsequent increase in sodium reabsorption can lead to elevated BP through plasma volume expansion (Geller *et al.* 2000; Sahay and Sahay 2012).

The Ser810Leu amino acid substitution in *NR3C2* has been shown to be the underlying cause of Geller syndrome (Geller *et al.* 2000). Geller syndrome is an autosomal dominant disorder and individuals affected by this disease usually present with hypokalemia, low aldosterone levels, low renin levels and hypertension (Vehaskari 2009). Geller *et al.* (2000) described this disorder in a family affected by high blood pressure. They found that the Ser810Leu polymorphism was present in all eight of the family members who had severe hypertension before the age of 20, while the polymorphism was absent in normotensive family members (Geller *et al.* 2000; Sahay and Sahay 2012). Compared to this, Ramirez-Salazar *et al*. (2011) reported similar genotypic frequencies of Ser810Leu in normotensive (9%) and hypertensive (12%) individuals. Genetic variation in the MR was investigated in hypertensive individuals in three studies in Germany, Japan, and Switzerland, but the Ser810Leu variant was not found to be present (Schmider-Ross *et al.* 2004; Kamide *et al.* 2005; Escher *et al.* 2009). The authors of these concluded that the Ser810Leu variant does not appear to play a major role in the development of hypertension in the studied populations (Schmider-Ross *et al.* 2004; Kamide *et al.* 2005; Escher *et al.* 2009)

1.3.2. Candidate genes that affect sodium reabsorption via ion transporters

1.3.2.1. The role of *SCNN1B* and *SCNN1G* in hypertension

The *SCNN1B* and *SCNN1G* genes encode the beta- and gamma-subunits of the ENaC, respectively. The ENaC plays a critical role in maintaining sodium homeostasis, blood volume and BP, and is the primary site where the net sodium balance is usually determined (Lifton *et al.* 2001). Each of the ENaC subunits has a critical proline-rich region (PY motif) in the C-terminus that is necessary for the internalization and degradation of the ENaC by Nedd4 (Snyder *et al.* 1995; Staub *et al*. 1996). Genetic variation in *SCNN1B* and *SCNN1G* that result in either an amino acid substitution in the critical PY motif, or in a truncated protein that lacks the C-terminus containing the PY motif, have been associated with elevated BP (Appendix A, Table 5) (Shimkets *et al*.

1994, Hansson *et al*. 1995a; Freundlich and Ludwig 2005). It has been found that if the PY motif in one of the ENaC subunits is either altered or absent, the half-life of the channel is prolonged (Snyder *et al.* 1995; Uehara *et al*. 1998). The activity of the ENaC is consequently increased, leading to elevated BP through plasma volume expansion (Snyder *et al.* 1995; Uehara *et al*. 1998).

Genetic variants in *SCNN1B* and *SCNN1G* have been implicated in Liddle syndrome (Shimkets *et al.* 1994; Hansson *et al*. 1995a; Hansson *et al*. 1995b; Tamura *et al.* 1996). It has been suggested that Liddle syndrome could be the most common syndrome associated with hypertension (Vehaskari *et al.* 2009). Liddle syndrome is an autosomal dominant disorder that is characterized by hypertension, hypokalemia, suppressed renin activity and suppressed secretion of aldosterone (Liddle *et al.* 1963; Botero-Velez *et al.* 1994). This disorder is usually diagnosed in individuals between the age of 10 and 30 years, although it has been diagnosed in infants (Vania *et al.* 1997; Warnock 1998; Assadi *et al.* 2002). A wide range in phenotype has been reported in individuals with Liddle syndrome and, as a result, is has been suggested that the disorder is underdiagnosed among hypertensive individuals (Botero-Velez *et al.* 1994; Gadallah *et al.* 1995; Findling *et al.* 1997; Rossi *et al*. 2008). The phenotypic variation in the presentation of Liddle syndrome in affected individuals has been attributed to several factors, including variable penetrance of the gene(s), the extent to which the genetic variant increases ENaC activity, other genetic factors, as well as environmental factors such as salt intake (Botero-Velez *et al.* 1994; Palmer *et al*. 1998; Sawathiparnich *et al.* 2009). Pradervand *et al.* (1999) developed a mouse model for Liddle syndrome with a deleted C-terminus in the βENaC. Mice that were heterozygous or homozygous for the deleted βENaC C-terminus only developed hypertension when a high salt diet was maintained (Pradervand *et al.* 1999). Therefore, individuals with genetic variation in *SCNN1B* or *SCNN1G* that are normotensive at the time of genetic testing, could develop hypertension later in life as a result of environmental factors such as high salt intake (Rayner *et al.* 2003; Zhao *et al.* 2011).

1.3.2.2 The role of *WNK4* and *WNK1* in hypertension

The *WNK1* and *WNK4* genes play a role in the transport of sodium and potassium in the distal nephron (Kahle *et al.* 2003; Subramanya *et al.* 2006). WNK1 exerts an inhibitory effect on WNK4 while WNK4 inhibits the activity of the NCCT (Yang *et al.* 2003; Wilson *et al.* 2003). Genetic variation in *WNK1* and *WNK4* that results in increased activity of the NCCT has been associated with elevated BP. Two deletions in intron 1 of *WNK1,* both of which lead to increased expression of WNK1, have been described (Wilson *et al.* 2001a; Delaloy *et al.* 2008). The increased expression of WNK1 results in enhanced inhibition of WNK4. Consequently, WNK4 can no longer inhibit the activity of the NCCT, and the transport of sodium and chloride is increased (Figure 1.5) (Huang *et al.* 2008). Several polymorphisms in *WNK4* that results in reduced inhibition of the activity of the NCCT have also been described (Appendix A, Table 6). Increased NCCT activity enhances net renal sodium reabsorption and ultimately leads to elevated BP through plasma volume expansion (Lifton *et al.* 2001; Wilson *et al.* 2003).

The *WNK1* and *WNK4* genes have been implicated in Gordon's syndrome (Wilson *et al.* 2001a). Gordon's syndrome, also known as familial hyperkalemic hypertension or pseudohypoaldosteronism type 2, follows an autosomal dominant form of inheritance. Features of Gordon's syndrome include salt-sensitive hypertension (which usually occurs in the second or third decade of life), low renin levels, normal or elevated aldosterone levels and hyperkalemia (Mayan *et al.* 2004; Sahay and Sahay 2012). However, the clinical presentation of this disorder in affected individuals can vary and the age at which Gordon's syndrome is diagnosed ranges from the first weeks of life to late adulthood (Brautbar *et al.* 1978; Gereda *et al.* 1996, Achard *et al.* 2001). Due to the variable phenotype, it has been suggested that Gordon's syndrome could be indistinguishable from primary hypertension in some cases and that this disorder could be underdiagnosed in hypertensive individuals (O'Shaughnessy *et al.* 1998). According to Mayan *et al.* (2004), all individuals with Gordon's syndrome due to variants in *WNK4* will develop hypertension over time. Studies suggest that *WNK4* could also contribute to BP variation in individuals not affected by this disorder. The *WNK4* gene is located in

a chromosomal region that has been linked to BP variation in three studies (Julier *et al.* 1997; Baima *et al.* 1999; Levy *et al.* 2000; Wilson *et al.* 2001a). More recently, two other genes that also affect the activity of the NCCT have been implicated in Gordon's syndrome. Boyden *et al.* (2012) identified variants in *CUL3* and *KLHL3* in individuals with Gordon's syndrome that do not have genetic variants in either *WNK1 or WNK4.* CUL3 and KLHL3 form a complex that plays an important role in ubiquitylation and the stability of WNK isoforms. Thus, the CUL3 - KLHL3 complex plays an important role in BP regulation through its effect on the activity of the NCCT (Ohta *et al.* 2013).

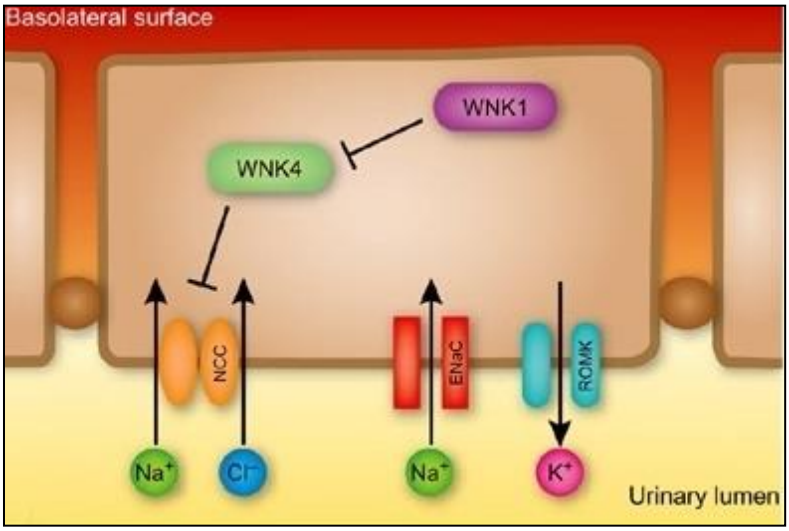

Figure 1.5: A schematic diagram of the sodium-chloride co-transporter (NCCT) and how WNK1 and WNK4 play a role. WNK1 inhibits the activity of WNK4, while WNK4 inhibits the activity of the NCCT. If the expression of WNK1 is enhanced, inhibition of WNK4 activity will increase. WNK4 will no longer be able to inhibit the activity of the NCCT, leading to increased sodium transport. Genetic variation in *WNK4* that result in reduced activity of the protein will also lead to increased sodium transport through the NCCT and consequently elevated blood pressure as a result of plasma volume expansion [Copied from Coffman (2006)].

1.4 Salt sensitive hypertension

Several features are indicative of increased sodium reabsorption, one of which is sensitivity to salt. Several studies have suggested that high dietary salt intake may be associated with hypertension (Humphries 1957; Dahl 1972; He and MacGregor 2002; He *et al.* 2013). BP response to salt in individuals is heterogeneous and in some cases reducing dietary salt intake results in a significant decrease in BP (Geleijnse *et al*. 2003; He *et al.* 2009; Tiffin *et al.* 2010). Salt sensitivity is described as individual differences in BP in response to salt intake and is the result of either hereditary or acquired defects in renal function (Strazzulo and Galletti 2007; Sanders 2009). Several genes have been implicated in salt sensitivity, including the genes that have been associated with monogenic forms of hypertension (Lovati *et al.* 1999; Poch *et al.* 2001; Beeks *et al.* 2004; Strazzulo and Galletti 2007; Zhao *et al.* 2011). In a computational study by Tiffin *et al.* (2010), candidate genes for salt-sensitive hypertension were identified. The PubMed database was used to identify the genes that most frequently occur with terms related to salt-sensitive hypertension (e.g. “angiotensin”, “low renin”, “sodium channel”, “sodium reabsorption + kidney”, etc). Thereafter, the genes were scored and ranked based on the terms that co-occur with it (Tiffin *et al.* 2010). The genes that have previously been implicated in monogenic forms of hypertension were not amongst the highest scored genes and they were, therefore, not considered to be the most likely candidates for salt-sensitivity (Tiffin *et al.* 2010). However, common genetic variants with small effects are more likely to be detected in association studies than rare alleles (Zhang *et al.* 2010) and would therefore be described more often in literature. As a result, the genes wherein common genetic variants occur could feature more often in the PubMed database and could therefore have been more likely to be considered as candidate genes for salt-sensitive hypertension in the study by Tiffin *et al.* (2010).

Apart from salt sensitivity, mild plasma volume expansion, low renin levels and increased activity of the ENaC are also indicative of increased sodium reabsorption. According to several studies, features indicative of increased sodium reabsorption (e.g.

salt sensitivity, mild plasma volume expansion, low renin levels and increased activity of the ENaC) are more frequently encountered in black individuals compared to Caucasians (Luft *et al.* 1991; Weinberger 1996; Morris *et al.* 1999; Baker *et al.* 2001; Franco and Oparil 2006; He *et al.* 2013). Increased sensitivity to sodium intake and low renin levels has also been reported for black South Africans (Rayner *et al*. 2001; Sagnella *et al*. 2001). Gadallah *et al.* (1995) suggested that the possibility of abnormalities in the ENaC should be investigated in a subset of black hypertensive individuals who had low renin levels and were considered to be salt-sensitive. Since monogenic forms of hypertension are characterized by increased sodium reabsorption with corresponding low renin levels, the increased sodium reabsorption that has been reported in studies on black individuals could indicate that monogenic forms of hypertension may be more common in black individuals.

1.5 Hypertension in an urban black population in Mangaung

The prevalence of hypertension in an urban black population in Mangaung in the Free State is very high compared to the average for South Africa (Lategan 2011; Van Zyl *et al.* 2012; WHO 2014a). According to Van Zyl *et al.* (2012), an estimated 51% of men and 59% of women in the Mangaung population are affected by hypertension. Furthermore, 67% of hypertensive individuals on anti-hypertensive medication had BP levels ≥ 140/90 mmHg (Van Zyl *et al.* 2012). Other risk factors for chronic diseases of lifestyle, including physical inactivity, overweight, diabetes and high cholesterol, were also present in the Mangaung population (Van Zyl *et al.* 2012). In a study by Lategan (2011), the association of several factors with the prevalence of hypertension in the Mangaung population was investigated. The factors investigated by Lategan (2011) included body weight, physical activity, sodium intake and genetic factors. Lategan (2011) found that the BP levels of the study participants correlated positively with adiposity, as well as with sodium intake. Polymorphisms in *ADRB1* (A145G and G1165C), *ADD1* (G217T), *AGT* (G-217A, C521T and T704C), *CYP11B2* (C-344T), *CYP3A5* (A6986G) and *GRK4* (G448T, C679T and C1711T) were selected for genetic analysis, all of which have previously been implicated in primary hypertension (Lategan

2011; du Toit 2014). Of these, only polymorphism (A6986G in *CYP3A5*) was identified as a possible independent risk factor for hypertension in 2% of the Mangaung population (du Toit 2014). Although diet and lifestyle appear to be contributing factors to the high incidence of hypertension in a black population in Mangaung, it is not known if genetic variation in genes implicated in monogenic forms of hypertension could also play a role.

1.6 Conclusion

Monogenic forms of hypertension are characterized by increased sodium reabsorption in the distal nephron with corresponding low renin levels. A wide range in phenotype has been described in individuals affected by the syndromes associated with monogenic forms of hypertension. As a result, several authors have suggested that monogenic forms of hypertension could be underdiagnosed in hypertensive patients (Gates *et al.* 1996; Takeda *et al.* 1996; Findling *et al.* 1997; Li *et al.* 1998; O'Shaughnessy *et al.* 1998; Ferrari and Krozowski 2000; Huizenga *et al.* 2000; Wilson *et al*. 2001a; Wilson *et al*. 2001b; Morineau *et al.* 2006; Rossi *et al*. 2008). Physiological characteristics indicative of increased sodium reabsorption, appear to be more commonly found in black hypertensive individuals, including black South Africans cmpared to Caucasians (Luft *et al.* 1991; Weinberger 1996; Morris *et al.* 1999; Baker *et al.* 2001; Rayner *et al*. 2001; Sagnella 2001; Franco and Oparil 2006). Previous studies on the black population in Mangaung, Free State, have shown a much higher prevalence of hypertension compared to the average for South Africa (Lategan 2011; Van Zyl *et al.* 2012; WHO 2014a). Although it has been shown that diet and lifestyle contribute to the high prevalence of hypertension in the Mangaung population (Lategan 2011), no studies have investigated whether genetic variation in genes implicated in monogenic forms of hypertension could be a contributing factor.

From the literature, several candidate genes have been consistently associated with monogenic forms of hypertension, including the chimeric *CYP11B1/CYP11B2,*

HSD11B2, NR3C1, CYP11B1, CYP17A1, NR3C2, *SCNN1B, SCNN1G, WNK1 and WNK4* (Lifton *et al.* 2001, Vehaskari 2009). Of these, *CYP11B1*, *CYP17A1, NR3C2,* and *WNK1* were not selected for the current study. The *CYP11B1* and *CYP17A1* genes were excluded since individuals with a severe form of CYP11B1 or CYP17 deficiency where hypertension is expected, should present with signs of abnormal sexual development during puberty (Joehrer *et al.* 1997; Biason-Lauber *et al*. 2000; Dhir *et al.* 2009; Krone *et al.* 2009; Vehaskari 2009). Furthermore, it has been shown that there is a weak correlation between BP and DOC, the hormone that is thought to be responsible for the elevated BP in individuals affected by CYP11B1 and CYP17 deficiency (Rosler *et al*. 1982; Zachmann *et al.* 1983; Curnow *et al*. 1993; Mussig *et al.* 2005). The polymorphism in *NR3C2* (Ser810Leu) that has been associated with elevated BP in one family, has subsequently been found to be present in normotensive individuals and is not considered to play an important role in monogenic forms of hypertension (Geller *et al.* 2000; Schmider-Ross *et al.* 2004; Kamide *et al.* 2005; Escher *et al.* 2009; Ramirez-Salazar *et al*. 2011). Lastly, *WNK1* was also not included in the current study, since *WNK4* was considered to be a better candidate to screen for genetic variants that previously have been associated with elevated BP. According to Mayan *et al.* (2004), all individuals with Gordon's syndrome due to genetic variation in *WNK4* will develop hypertension over time. In the current study, *NR3C1, HSD11B2, SCNN1B, SCNN1G,* and *WNK4* were selected to be screened for variants that have previously been associated with elevated BP. The chimeric *CYP11B1/CYP11B2* gene was also selected for the current study. The aim of this study was to screen for genetic variants implicated in monogenic forms of hypertension in *NR3C1, HSD11B2*, *SCNN1B, SCNN1G* and *WNK4,* and to screen for the presence of the chimeric *CYP11B1/CYP11B2* gene in a black hypertensive cohort in Mangaung in the Free State.

Chapter 2

Materials and Methods

2.1 Rationale

Previous studies of an urban black population in Mangaung in the Free State, revealed a much higher prevalence of hypertension compared to the average for South Africa (Lategan 2011; Van Zyl *et al.* 2012; WHO 2014a). Studies have shown that physiological characteristics indicative of increased sodium reabsorption, which are typical of monogenic forms of hypertension, are more commonly found in black hypertensive individuals compared to Caucasians (Luft *et al.* 1991; Weinberger 1996; Morris *et al.* 1999; Baker *et al.* 2001; Rayner *et al*. 2001; Sagnella 2001; Franco and Oparil 2006). Increased sensitivity to sodium and low renin levels, which are indicative of increased sodium reabsorption, have also been reported to be prevalent in black South Africans (Rayner *et al*. 2001; Sagnella *et al*. 2001). Although studies have shown that diet and lifestyle contribute to the high prevalence of hypertension in a black population from Mangaung, no studies have investigated whether monogenic forms of hypertension could be a contributing factor.

High resolution melting curve (HRM) analysis is a pre-sequencing technique that has been found to be a sensitive and reliable method to screen for sequence variants (Wittwer *et al.* 2003; Reed and Wittwer 2004; Audrezet *et al.* 2008; Polakova *et al.* 2008). HRM analysis has a number of limitations, however, including the need for optimization and validation, and requires the use of short PCR amplicon to achieve the necessary sensitivity (Audrezet *et al.* 2008; Polakova *et al.* 2008; Tindall *et al.* 2009). The limitations of HRM analysis needs to be weighed against the advantage of being less costly due to a reduction in the number of samples that need to be sequenced (Polakova *et al.* 2008; Tindall *et al.* 2009). HRM screening followed by sequencing of segments of the candidate genes *NR3C1, HSD11B2*, *SCNN1B, SCNN1G* and *WNK4* for previously identified genetic variants, and screening for the presence of the chimeric

CYP11B1/CYP11B2 gene could indicate if hypertension in an urban black population in Mangaung has a monogenic component.

2.2 Aim

The aim of the study was to screen for genetic variants *in NR3C1, HSD11B2, SCNN1B, SCNN1G* and *WNK4*, and to screen for the presence of the chimeric *CYP11B1/CYP11B2*, which have previously been implicated in monogenic forms of hypertension, in a hypertensive urban black Free State cohort.

2.3 Objectives of the study

The primary objective of the current study was to determine whether genetic variants that have previously been associated with elevated blood pressure (BP) were present in a hypertensive black cohort from Mangaung. A secondary objective was to standardize and implement HRM analysis to identify potential sequence variants in *NR3C1, HSD11B2*, *SCNN1B, SCNN1G* and *WNK,* in order to reduce the number of samples required to be sequenced. Lastly, long range Polymerase Chain Reaction (PCR) was used to detect the presence of the chimeric *CYP11B1/CYP11B2* gene in the study cohort.

2.4 Study population

The current study formed part of the Assuring Health for All in the Free State (AHA-FS) study and was undertaken in collaboration with the Department of Nutrition and Dietetics, University of the Free State (UFS). AHA-FS is a prospective and longitudinal epidemiological study in which baseline data was collected from an urban and a rural population (Van Zyl *et al.* 2012). The Department of Nutrition and Dietetics, UFS, was responsible for the following: selecting the study cohort from the urban baseline study population of the AHA-FS study, obtaining informed consent from study participants,

gathering epidemiological data, and collecting blood samples from the study participants (Table 2.1; Appendix B, Table 1) after informed consent was obtained (Lategan 2011).

2.4.1 Cohort selection and epidemiological data

The urban population of the AHA-FS study comprised of adults from black households in the Freedom Square, Turflaagte, Namibia, Kagisanong, Chris Hani and Rocklands Buffer area of Mangaung, South Africa. Participants were recruited by means of stratified proportional cluster sampling. The cluster was stratified by area and formal plot or squatter households, after which 100 starting points were randomly selected in this area. From each starting point five adjacent households were approached and invited to participate in the study (Lategan 2011). Study participants were between 25 and 64 years of age. Complete data was obtained for BP, gender, body weight, height and activity level, and blood samples were collected for genetic testing and 25-Hydroxy vitamin D analysis (Lategan 2011). BP was measured by a registered medical practitioner using an electronic blood pressure monitor, according to the Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation and Treatment of High Blood pressure (National Institute of Health (NIH) 2004; Lategan 2011). Participants were considered to be hypertensive if the average of two BP readings was equal to or exceeded 140/90 mmHg, or if individuals were using prescription medication for the management of hypertension (Lategan 2011). Anthropometry measurements were taken according to WHO guidelines (WHO 2008) and the Body Mass Index (BMI) of the participants was determined by the Department of Nutrition and Dietetics, UFS. A BMI between 18.5 and 24.9 kg/m^2 was considered to be normal, between 25 and 29.9 kg/m^2 was considered to be overweight, and equal to or exceeding 30 kg/m^2 was considered to be obese (Lategan 2011).

2.4.2 Cohort characteristics of current study

The current study cohort comprised of 90 hypertensive adults that were randomly selected from the urban baseline population of the AHA-FS study. The cohort comprised of 18 males and 72 females with a mean age of 46 years (Table 2.1; Appendix B, Table 1). The average systolic and diastolic blood BP for this cohort was estimated to be 151.06 mmHg and 100.53 mmHg, respectively (Table 2.1). Of the 60 individuals who were using prescription medication for the management of hypertension, only two had systolic BP below 140 mmHg and/or diastolic BP below 90 mmHg (Table 2.1; Appendix B, Table 1). The average BMI for this cohort was estimated to be 29.9 kg/m^2 (Table 2.1). An estimated 27% of the study participants were considered to be overweight (25 ≤ BMI < 30) while an estimated 43% were considered to be obese (BMI ≥ 30) (Appendix B, Table 1). Females in this hypertensive study group had a higher average BMI and BP readings compared to males (Table 2.1). However, the numbers of hypertensive males that were included in the study were too few to be able to draw any conclusions in terms of sex. Ethics approval for this study was obtained from the Ethics Committee of the Faculty of Health Sciences, UFS (ECUFS: 184/2013).

Table 2.1: Characteristics of the 90 hypertensive study participants, including demographic factors, anthropometric factors, hemodynamic factors and whether the individuals are on antihypertensive medication.

Variable	Average[a]
Demographic factors	
Age	**46.30 (± 10.0)**
Males (n = 18)	43.44 (± 9.72)
Females (n= 72)	46.43 (± 10.53)
Anthropometric factors	
Body mass index (kg/m^2)	**29.90 (± 8.45)**
Males	23.9 (± 8.55)
Females	31.38 (± 7.79)
Waist circumference (cm)	**92.28 (± 16.42)**
Males	82.44 (± 18.01)
Females	94.74 (± 15.16)
Hip circumference (cm)	**109.13 (± 17.25)**
Males	96.53 (± 16.12)
Females	112.29 (± 16.13)
Hemodynamic factors	
Systolic blood pressure (mmHg)	**151.06 (± 18.46)**
Males	145.5 (± 14.71)
Females	152.44 (± 19.13)
Diastolic blood pressure (mmHg)	**100.53 (± 16.98)**
Males	96.5 (± 12.06)
Females	101.54 (± 17.92)
Medication	
On antihypertensive medication, n	**60 (66.66%)**
Males	10 (58.82%)
Females	50 (69.44%)

[a] The standard deviation is given in brackets.

2.5 Methods

2.5.1 DNA isolation

The Department of Nutrition and Dietetics, UFS, collected whole blood from the study participants, after which they removed the plasma for 25-Hydroxy vitamin D analysis. Thereafter, blood samples (the buffy coat and red blood cells) were blotted onto fast technology for analysis of nucleic acid (FTA®) paper (Whatman®) for genetic analysis. The DNA is captured in the fibres of the FTA® paper and remains stabilized (GE Healthcare Life Sciences). The DNA on FTA® paper can be stored for several years between 15°C and 25°C (GE Healthcare Life Sciences).

2.5.1.1 Purification of FTA® discs

Purification of the FTA® discs was performed according to the Whatman® FTA® protocol (GE Healthcare Life Sciences). Discs of 2 mm were punched into the FTA® paper spotted with blood (the buffy coat and red blood cells). The FTA® discs were incubated in 200 µl FTA® Purification Reagent (Whatman®) for 5 minutes to remove the haemoglobin. The FTA® Purification Reagent was removed and the wash step repeated twice. Thereafter, the discs were washed twice with 0.1 x TE buffer (10 mM Tris and 0.1 mM EDTA, pH 8.0). The FTA® discs were air-dried and subsequently used to amplify target regions using polymerase chain reaction (PCR).

2.5.1.2 DNA isolation from FTA® discs using a modified methanol method

The methanol DNA extraction method (Lebea and Pretorius 2008) was used to extract DNA from the blood (the buffy coat and red blood cells) that was spotted onto FTA® paper. Methanol was used to remove substances such as haemoglobin from the blood on the FTA® paper, since it could inhibit the PCR reaction. The methanol DNA extraction method was optimized to obtain maximum DNA yield. For each sample, six discs of 2 mm were punched from the FTA® paper. After the addition of 50 µl methanol, the discs were incubated for 10 minutes at room temperature. The methanol was discarded and the discs dried for 15 minutes at 28°C in a heating block. Thereafter, the

FTA® discs were incubated in 60 µl of 0.1 x TE buffer for 15 minutes at 95°C to solubilise the DNA. The 0.1 X TE solution containing the DNA was removed from the discs, after which a further 60 µl of 0.1 x TE buffer was added to the FTA® paper discs for a second incubation step for 15 minutes at 95°C. The 0.1 x TE solution from the second incubation step was added to that of the first and the combined solution was vortexed and centrifuged for 5 minutes at 13,400 rpm. The extracted DNA was removed after centrifugation, and stored at 4°C for later use.

2.5.1.3 DNA isolation from FTA® paper using a modified CTAB method

DNA was extracted from the blood samples (the buffy coat and red blood cells) that were blotted onto FTA® paper using a modified CTAB (Cetyl Trimethyl Ammonium Bromide, pH 8.0) method. FTA® paper spotted with blood was incubated in 2 ml of CTAB buffer with 20 µl of proteinase K (20 mg/ml) (Roche) for two hours at 60°C, followed by centrifugation for 5 minutes at 13,000 rpm. Thereafter, the solution was transferred to a 2 ml eppendorf tube containing 600 µl chloroform, mixed by inversion and centrifuged for 10 minutes at 13,000 rpm. The chloroform step was repeated on the supernatant. Thereafter, the supernatant was transferred to a 2 ml tube containing 1 ml isopropanol and 2 µl glycogen (Sigma-Aldrich) to precipitate the DNA. The solution was mixed by inversion and incubated for an hour at 4°C, followed by centrifugation for 10 minutes at 13,000 rpm. The supernatant was subsequently discarded and the pellet washed with 500 µl of 70% ethanol by vortexing and centrifugation for 10 minutes at 13,000 rpm. The 70% ethanol wash step was repeated. The DNA pellet was air-dried for 30 minutes. Thereafter, the DNA pellet was dissolved in 100 µl of 0.1 x TE buffer. The DNA solution was stored at 4°C for later use.

2.5.2 Conventional PCR

PCR was performed to amplify selected target regions in *HSD11B2* (exons 3, 4 and 5), *NR3C1* (exons 6, 7, 9, 10 and 11), *SCNN1B* (exon 13), *SCNN1G* (exon 13), and *WNK4* (exons 7 and 17) in which variants have previously been associated with elevated BP.

As far as possible, primers were selected or designed to span the entire exon to minimize the risk of genetic variation occurring in a primer binding region (Table 2.2). Primer3Plus, an online primer design program, was used to design primers where necessary (Untergasser and Nijveen 2007). Due to the large size of *HSD11B2* exon 5, two primer pairs (referred to as *HSD11B2* exon 5-1 and exon 5-2; Table 2.2) were used. For *HSD11B2* exon 4, semi-nested PCR was used in order to increase the amplification specificity. For all of the primer pairs the cycling conditions of the PCR reactions were optimized individually, with the exception of *HSD11B2* exon 4. All of the PCR reactions were performed on the Veriti 96 well Thermal Cycler (Applied Biosystems).

PCR reactions for *HSD11B2* (exons 3 and 5), *NR3C1* (exons 6, 7, 9, 10 and 11), *SCNN1B* (exon 13), *SCNN1G* (exon 13), and *WNK4* (exons 7 and 17) were optimized in terms of annealing temperature and annealing and extension times. The PCR reactions consisted of the following: 2.5 µl of 10 x buffer (Applied Biosystems), 1.5 µl $MgCl_2$ (25 mM) (Applied Biosystems), 0.16 µl of 5 U/µl AmpliTaq Gold DNA polymerase (Applied Biosystems), 0.5 µl dNTPs (10 mM) (Thermo Scientific), 0.5 µl of 10 pmol/µl forward and reverse primer (Table 2.2), respectively, 2 µl DNA and sterile nuclease free water to a final volume of 25 µl. For primer pairs *NR3C1* exon 6, *NR3C1* exon 7, *NR3C1* exon 9 and *NR3C1* exon 10, 1 µl of 10 pmol/µl forward and reverse primer was used in the PCR reaction mixture, respectively. Cycling conditions included the following: 1 cycle at 95^{o}C for 10 minutes, 50 cycles of 95^{o}C for 30 seconds, the optimal annealing temperature and time, followed by 72^{o}C for the optimal extension time, and one final cycle at 72^{o}C for 5 minutes.

For *HSD11B2* exon 4, successful amplification was achieved with the semi-nested PCR and further optimization was not required. Two forward primers (referred to as F1 and F2) and a common reverse primer (referred to as R) (Table 2.2) were used to perform the semi-nested PCR. The expected fragment size of the first PCR product (using forward primer F1) is 335 bp while the fragment size of the second PCR product (using

forward primer F2) is 300 bp. The cycling conditions for the first and second PCR reactions was as follows: 1 cycle at 95^{o}C for 10 minutes, 50 cycles of 95^{o}C for 30 seconds, 60^{o}C for 1 minute and 72^{o}C for 2 minutes, and 1 cycle at 72^{o}C for 5 minutes. The first and second PCR reactions consisted of 2.5 µl of 10 x buffer (Applied Biosystems), 1.5 µl $MgCl_2$ (25 mM) (Applied Biosystems), 0.16 µl of 5 U/µl AmpliTaq Gold DNA polymerase (Applied Biosystems), 0.5 µl dNTPs (10 mM) (Thermo Scientific), 0.5 µl of 10 pmol/µl forward and reverse primer (Table 2.2), respectively, 2 µl DNA and sterile nuclease free water to a final volume of 25 µl. The amplicon of the first PCR reaction was diluted (1:100,000) and purified using FastAP (Thermosensitive Alkaline Phosphatase) in conjunction with Exo 1 (Thermo Scientific) according to the manufacturer's instructions. For every 5 µl PCR amplicon, 1 µl FastAP and 0.5 µl Exo 1 were added. The combined solution was incubated at 37^{o}C for 15 minutes, followed by incubation at 85^{o}C for 15 minutes. Thereafter, the FastAP treated PCR product (1:100,000) was used as template in the subsequent PCR reaction.

2.5.3 Gel electrophoresis

PCR amplicon was resolved on a 2% agarose gel in sodium borate (SB) buffer (0.2 mM NaOH and 1 mM LiCl, pH 8.0) at approximately 200 V for 50 minutes. A 100 bp molecular weight marker (Bioline) was also resolved on the gel to confirm the fragment size of the PCR products. The gel was stained in ethidium bromide (0.5 µg/ml) for 15 minutes on a rotary shaker (Gyrotory Shaker Model G2). Thereafter, the gel was visualised under UV light and documented using the Kodak ID 3.6 Program of the Gel Logic 200 Imaging System.

Table 2.2: Primers used to amplify selected target regions in *NR3C1* (exons 6, 7, 9, 10 and 11), *HSD11B2* (exons 3, 4 and 5), *SCNN1B* (exon 13), *SCNN1G* (exon 13), and *WNK4* (exons 7 and 17).

Primer pair	Sequence (5' to 3')[a]	Fragment size (bp)	Reference
NR3C1 exon 6	F: GGCTGTCCTTATAAAATATGT R: ATTTTTATAAGCTACAGAGACA	232	Feng *et al*. (2000)
NR3C1 exon 7	F: TACCACAACTCACCCCTAC R: ATAGTTGCTCTTTTATGTTTTG	204	Feng *et al*. (2000)
NR3C1 exon 9	F: GGAAGTAGCAGTATTTCTAAC R: TTCATATTTCATGCTTTTGACA	226	Feng *et al*. (2000)
NR3C1 exon 10	F: AATCCTTTAACTGACTTCATC R: ATACTTTGTCCCAGAAAACTC	241	Feng *et al*. (2000)
NR3C1 exon 11	F: CACTGACCAATTTGGAAGCC R: CTCTACAGGACAAACTGATAG	281	Ruiz *et al.* (2001)
HSD11B2 exon 3	F: AGGACACGGGGACTGGAAG R: GGGGGCTCCTTTTTGCTCC	292	Morineau *et al.* (2006)
HSD11B2 exon 4	F1: ACTGGAGCAAAAAGGAGCC F2: GGCTTAGGGAGCCCCTTG R: ACACCCCCAACCAAAACCAT	F1+R: 335; F2+R: 300	Coeli *et al.* (2008); Smith (2016)[b]
HSD11B2 exon 5-1	F: CTCCTTCCCCAGAGTCAGTG R: GGCATCTACAACTGGGGTGA	182	Smith (2016)[b]
HSD11B2 exon 5-2	F: CAAGGACTACATCGAGCACTTGCA R: TTTTCGGGGAGGAACCAAGG	401	Dave-Sharma *et al.* (1998)
SCNN1B exon 13	F: AGTTTGGCTTCTGGATGGGG R: ATGGCATCACCCTCACTGTC	346	Kyuma *et al.* (2001)
SCNN1G exon 13	F: TTGATGGTGTGGCTTGGCCTG R: GATCTGTCTTCTCAACCCTGC	432	Edelheit *et al*. (2005)
WNK4 exon 7	F: GCAAGGGAATTGGAGGCACTC R: CTGGGACCTACGTCTCAAGATCAG	198	Wilson *et al.* (2001a)
WNK4 exon 17	F: CCTGGGAGAGCAAGGTGTGT R: CCTGGAGAAAAACAGTCGACAGAA	323	Mendes *et al.* (2011)

[a] F and R refers to the forward and reverse primers, respectively; [b] Primers were designed for this study with Primer3Plus using NG_016549.1 as reference sequence.

2.5.4 HRM analysis

HRM analysis was used to screen for sequence variants in *NR3C1* (exons 6, 7, 8, 10 and 11), *HSD11B2* (exons 3 and 5), *SCNN1B* (exon 13), *SCNN1G* (exon 13), and *WNK4* (exons 7 and 17). HRM analysis is a pre-sequencing technique that is used to characterize DNA samples according to their dissociation behaviour as they transition from double to single stranded DNA with an increase in temperature (Wittwer *et al.* 2003; Tindall *et al.* 2009). The HRM PCR reactions were performed on the ABI 7500 Fast (Applied Biosystems) using the MeltDoctor HRM master mix (Applied Biosystems).

Conventional PCR amplicon was diluted 1:100,000 in 0.1 x TE buffer in order to obtain a threshold cycle (C_t) value between 15 and 25. The HRM PCR reactions were optimized in terms of the annealing and extension times. The HRM PCR reaction mixture consisted of 10 µl of MeltDoctor HRM master mix (Applied Biosystems), 0.5 µl (10 mM) forward and reverse primer (Table 2.2), respectively, 2 µl diluted amplicon and sterile nuclease free water to a final volume of 20 µl. The cycling conditions were as follows: 95^{o}C for 10 minutes, followed by 40 cycles of 95^{o}C for 15 seconds and the optimized annealing and extension times. The temperature profile for the melting curve was 95^{o}C for 10 seconds, 60^{o}C for 1 min, followed by an increase to 95^{o}C at a ramp rate of 0.03^{o}C/second and finally 60^{o}C for 15 seconds. For each primer pair, a sample previously sequenced and shown to have no variants when compared to the reference sequence obtained from NCBI, was used as the wild type (WT) in the HRM reaction. The samples where variation from the WT occurred, which would indicate the presence of a genetic variant, were sequenced to confirm the results.

2.5.5 DNA Sequencing

Preliminary sequencing was performed in the laboratory, after which the bulk of the sequencing was performed by Macrogen USA. Prior to sending PCR amplicon to Macrogen USA for the bulk of the sequencing, the PCR product was purified using FastAP in conjunction with Exo 1 (Thermo Scientific), according to the manufacturer's

instructions. For every 5 µl PCR amplicon, 1 µl FastAP and 0.5 µl Exo 1 were added. The combined solution was incubated at 37^{o}C for 15 minutes, followed by incubation at 85^{o}C for 15 minutes. Prior to preliminary sequencing in the laboratory, the PCR amplicon was purified using USB® ExoSAP-IT® (Affymetrix) according to the manufacturer's instructions. For every 10 µl PCR product, 4 µl ExoSAP-IT® was added. The 14 µl reaction volume was incubated at 37^{o}C for 15 minutes, after which it was incubated at 80^{o}C for 15 minutes. The ExoSAP-IT® treated PCR product was sequenced using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems). The sequencing reaction consisted of 2 µl BigDye Terminator mix, 1 µl sequencing buffer, 5 µl ExoSAP-IT® treated PCR product and 2 µl of either the forward or the reverse primer. The cycling conditions were as follows: 1 minute at 95ºC; followed by 25 cycles of 10 seconds at 95ºC, 5 seconds at the optimized annealing temperature and 4minutes at 60ºC. The sequencing product was purified using the ethanol/sodium acetate precipitation method. The mixture consisted of 3 µl (3 M) sodium acetate, 62.5 µl absolute ethanol, 14.5 µl sterile nuclease free water, and 10 µl sequencing product. The mixture was incubated in the dark for 30 minutes, after which it was centrifuged for 30 minutes at 13,000 rpm. After the supernatant was discarded, the pellet was washed with 250 µl of 70% ethanol and centrifuged for 10 minutes at 13,000 rpm. The supernatant was discarded and the pellet dried at 90ºC for 1 minute. Thereafter, 25 µl Hi-Di Formamide was added to the sequencing product. The sequencing product was incubated at 95ºC for 2 minutes and cooled on ice for 5 minutes, followed by vortexing for 10 seconds and centrifugation for 30 seconds. Thereafter, the sample was stored in the dark until capillary electrophoresis was performed. The sequencing samples were run on the ABI Prism 3130 Genetic Analyzer (Applied Biosystems). The sequencing data were edited using Chromas Lite 2.3.1 (www.technelysium.com.au/chromas_lite.html). Thereafter, the online program LALIGN (Huang and Muller, 1991) was used to compare the alignment of the sequence data to the reference sequence obtained from NCBI (http://www.ncbi.nlm.nih.gov/) to identify sequence variants. Numbering of amino acids is according to Ensembl genome

browser 83 (2015) (www.ensembl.org/index.html). Hardy-Weinberg equilibrium (HWE) was determined using the equation $p^2 + 2pq + q^2 = 1$, where p is the frequency of the major allele and q is the frequency of the minor allele. However, where two alleles were found in addition to the wild type allele at a specific locus, HWE was determined using the equation $p^2 + 2pq + q^2 + r^2 + 2pr + 2qr = 1$, where p is the frequency of the major allele, and q and r is the frequency of the two minor alleles, respectively.

2.5.6 Long range PCR

Long range PCR was used to test for the presence of the chimeric *CYP11B1/CYP11B2* gene. The amplification of *CYP11B2* (exons one to five) served as an endogenous amplification control. The expected fragment size of the chimeric target region is approximately 3.9 kilo base pairs (kbp) (Table 2.3). The expected fragment size of the *CYP11B2* target region is 4.1 kbp, which was estimated using NG_008374.1 as reference sequence (http://www.ncbi.nlm.nih.gov/) (Table 2.3). Three primers were used: two forward primers binding to the 5' end of the chimeric *CYP11B1/CYP11B2* and *CYP11B2*, respectively, and a common reverse primer binding to the 3' end of exon 5 of *CYP11B2* (Table 2.3). Two kits, namely the Expand High Fidelity PCR system (Roche) and the Q5 High Fidelity PCR kit (New England Biolabs), were used to amplify the target regions. The PCR reactions were performed on the Veriti 96 well Thermal Cycler (Applied Biosystems).

The long range PCR using the Expand High Fidelity PCR system (Roche) was optimized in terms of annealing temperature, cycle number, and volume of DNA used as template. PCR amplification of the target control was successful using the Q5® High-Fidelity PCR kit (New England Biolabs), and further optimization was, therefore, not performed. The PCR reactions using the Expand High Fidelity PCR system (Roche) each consisted of the following components supplied with the kit: 5 µl buffer, 1 µl nucleotides, 0.75 µl High Fidelity PCR system enzyme mix, as well as 1.5 µl (10 pmol/µl) forward primer (Table 2.3), 1.5 µl (10 pmol/µl) reverse primer (Table 2.3), 4 µl

of DNA, and sterile nuclease free water to a final volume of 50 µl. The PCR conditions using the Expand High Fidelity PCR system (Roche) were 95^{o}C for 1 minute, 12 cycles at 95^{o}C for 1 minute and 68^{o}C for 5 minutes, followed by 40 cycles at 95^{o}C for 1 minute and 68^{o}C for 5 minutes with the combined annealing and extension time increasing by 30 seconds for each cycle. This was followed by 1 cycle at 72^{o}C for 15 minutes. For the Q5 High Fidelity PCR kit (New England Biolabs) the PCR reactions each consisted of 12.5 µl of 2 x Q5 master mix, 1.25 µl (10 pmol/µl) forward primer (Table 2.3), 1.25 µl (10 pmol/µl) reverse primer (Table 2.3), 2 µl DNA and sterile nuclease free water to a final volume of 25 µl. The PCR conditions using the Q5 High Fidelity PCR kit (New England Biolabs) were as follows: 1 cycle at 98^{o}C for 30 seconds, 50 cycles at 98^{o}C for 10 seconds, 68^{o}C for 30 seconds and 72^{o}C for 3.5 minutes, followed by 1 cycle at 72^{o}C for 2 minutes. PCR amplicon was resolved on a 0.7% agarose gel in SB buffer, using a molecular weight marker ranging from 100 bp to 10,000 bp (KAPA Biosystems).

Table 2.3: Primers used to detect the chimeric *CYP11B1/CYP11B2* gene and the endogenous control (*CYP11B2)* in long range PCR.

Target region	Primer Sequence (5' to 3')[a]	Fragment size (kbp)	Reference
Chimeric *CYP11B1/CYP11B2*	F: TCATGCACCCCCAATGAGTCCCTG R: GAGTCCTCCAGCTGCCTCTCAACC	3.9	Johnsson *et al.* (1995)
CYP11B2	F: TCCTTCATCTACCTTTGGCTGGGG R: GAGTCCTCCAGCTGCCTCTCAACC	4.1[b]	

[a] F refers to the forward primer and R refers to the reverse primer; [b] Fragment size estimated using NG_008374.1 as reference sequence.

Chapter 3

Results and discussion: Optimization of DNA extraction, long range PCR, conventional PCR, and HRM analysis

3.1. Optimization of DNA extraction

PCR amplification using 10 1.2 mm FTA® discs prepared according to the protocol of Whatman® FTA® for blood DNA (GE Healthcare Life Sciences) did not result in successful PCR amplification of all samples (Figure 3.1 A). It is suspected that, since the FTA® paper was saturated with buffy coat and red blood cells, inhibitory factors were present and resulted in PCR inhibition.

An alternative approach was employed, whereby the DNA was extracted from the blood spotted onto FTA® paper and the resulting DNA solution was used as template in the PCR reaction. PCR amplification using DNA extracted from 10 1.2 mm FTA® discs using the methanol extraction method by Lebea and Pretorius (2008) also did not result in successful amplification of all samples (data not shown). It is suspected that low DNA concentration or inhibitory factors that were present contributed to the low success rate of PCR amplification. In order to increase the DNA yield using the methanol DNA extraction method, the protocol was modified in terms of the volume of methanol, the volume of 0.1 x TE, and the number of 0.1 x TE incubation steps. Decreasing the amount of methanol did not make a considerable difference in the yield of DNA (Table 3.1). However, increasing the volume of 0.1 x TE from 50 µl to 100 µl resulted in a dilution of extracted DNA (Table 3.1). It was suspected that using only one 0.1 x TE incubation step may not have removed the maximum amount of DNA from the FTA® discs. To test this theory a second 0.1 x TE incubation step was introduced using 40 µl of 0.1 x TE, after which both incubation aliquots were combined. The inclusion of a second 0.1 x TE incubation step resulted in an increased yield of DNA, with the

exception of the combination of 100 µl and 40 µl 0.1 x TE (Table 3.1). A further optimization was tried using 50 µl of methanol with two incubation steps of 60 µl of 0.1 x TE each. Although using 60 µl of 0.1 x TE in two incubation steps did not increase the yield of DNA compared to the use of 50µl and 40 µl of 0.1 x TE (Table 3.1), it did increase the volume of the DNA extracted. A final optimization was attempted using six 2 mm FTA® discs with two 60 µl 0.1 x TE incubation steps. The latter produced the highest yield of DNA compared to using 10 1.2 mm discs (Table 3.1). As a result, it was decided to standardize the methanol DNA extraction method for six 2 mm FTA® discs using 50 µl of methanol and two 60 µl 0.1 x TE incubation steps. Using the modified methanol method, DNA was extracted from 18 samples. The concentration of extracted DNA ranged from 0.19 ng/µl to 2.12 ng/µl (Table 3.2). Unfortunately, the extracted DNA did not result in successful amplification in most of the samples (Figure 3.1 B). Since the DNA concentration was within an acceptable range for PCR, it is suspected that the modified methanol DNA extraction method also resulted in PCR inhibition.

Since the modified methanol method proved unsuccessful, it was decided to use a modified CTAB extraction method to extract DNA from FTA® paper. The whole area of FTA® paper spotted with buffy coat and red blood cells was used in the CTAB extraction. Although the CTAB extraction resulted in a wide range of DNA concentration (< 0.1 ng/µl to 114.0 ng/µl) compared to the methanol method, a 100% PCR success rate was obtained for 18 samples (Table 3.2; Figure 3.1 C). As a result, the CTAB method was used for all further DNA extractions.

Table 3.1: Optimization of the methanol DNA extraction method with regard to the volume of methanol, the volume of 0.1 x TE, the number of 0.1 x TE incubation steps, the amount of FTA® discs, and the size of FTA® discs.

Disc amount	Size of discs (mm)	Volume of methanol (µl)	Volume of 0.1 x TE in the 1st incubation step (µl)	Volume of 0.1 x TE in the 2nd incubation step (µl)	DNA concentration (ng/µl)
10	1.2	200[a]	50[a]	-	0.37
10	1.2	50	50	-	0.426
10	1.2	200	100	-	0.15
10	1.2	200	50	40	0.584
10	1.2	50	50	40	0.736
10	1.2	200	100	40	0.15
10	1.2	50[b]	60	60	0.39 (± 0.02)
6	2.0	50[b]	60	60	0.91 (± 0.27)

[a] According to the method of Lebea and Pretorius (2008); [b] This approach was followed using two samples and the average DNA concentration of the two samples is given with the standard deviation in brackets.

Table 3.2: The DNA concentration obtained for 18 samples using the optimized methanol DNA extraction method and the modified CTAB DNA extraction method, respectively.

Sample	Optimized methanol DNA extraction protocol (ng/µl)	Modified CTAB DNA extraction protocol (ng/µl)
1	0.68	5.60
2	0.35	95.40
3	0.68	0.62
4	2.12	0.69
5	0.60	0.12
6	0.72	114.0
7	0.37	<0.1
8	0.62	1.59
9	1.46	2.58
10	2.02	36.20
11	0.68	0.26
12	0.19	0.70
13	0.45	1.13
14	2.06	21.40
15	0.53	83.20
16	1.45	29.60
17	0.69	4.38
18	0.41	0.61
Average [a]	**0.89 (± 0.63)**	**22.12 (± 36.72)**

[a] The average DNA concentration of the 18 samples is given with the standard deviation in brackets.

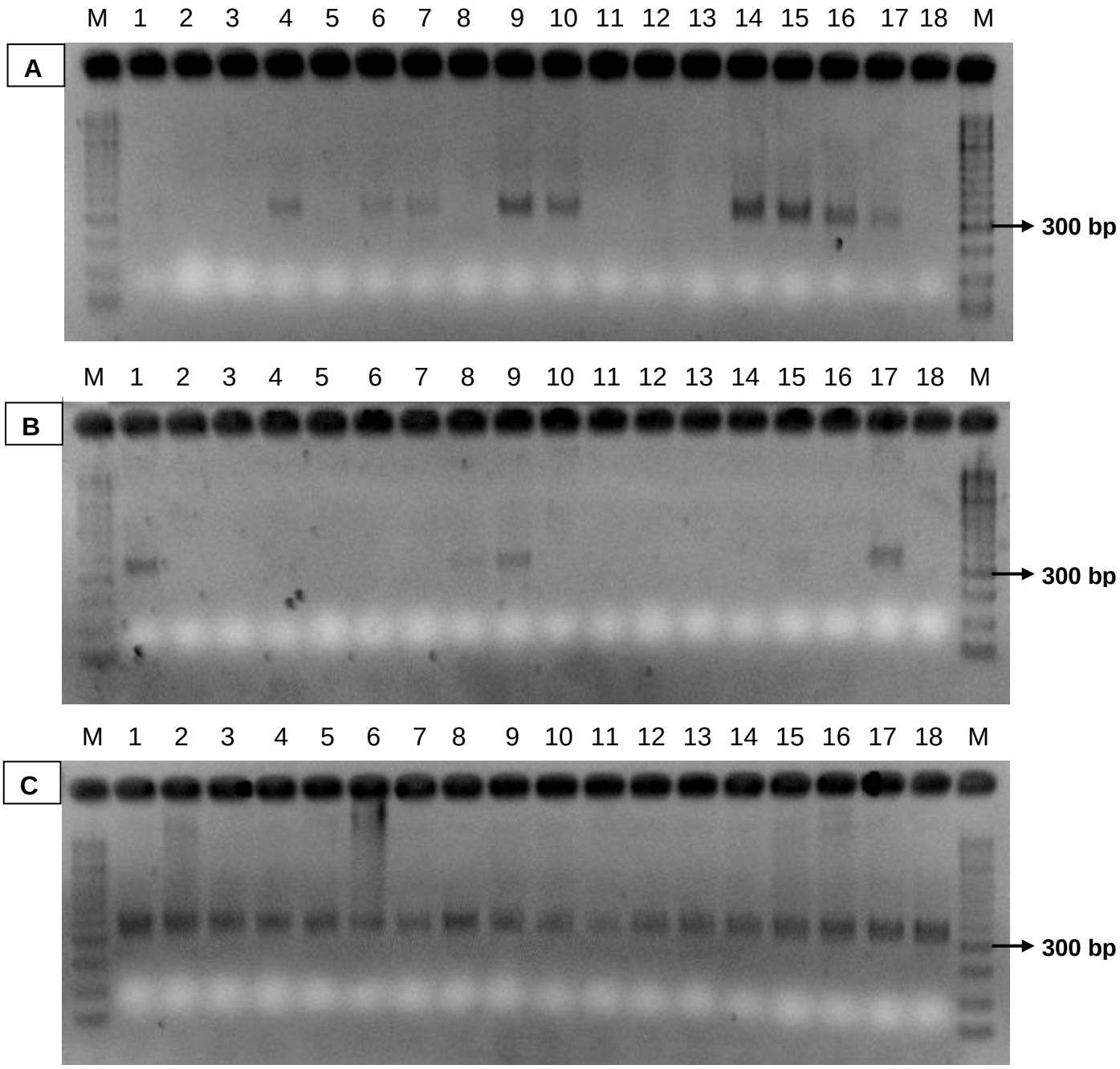

Figure 3.1: Negative inverted 2% gel images of the PCR product of a specific gene segment, *SCNN1B* exon 13 (346 bp), for 18 samples for A) using DNA obtained from purified FTA® discs used directly in the PCR reaction, B) DNA extracted from blood on FTA® discs with a modified methanol method, and C) DNA extracted from blood on FTA® paper with a modified CTAB protocol. For each DNA extraction method the same 18 samples were used and loaded in lanes 1 to 18. A 100 bp marker (lanes M), was loaded in lanes indicated as M.

3.2. Optimization of the long range PCR assay

The Expand High Fidelity PCR system kit (Roche) was initially used in long range PCR. However, for eight sample tested, the endogenous amplification control target region (exons one to five of *CYP11B2*) failed to amplify using this kit (Figure 3.2 A). As a result, the Q5® High-Fidelity PCR kit (New England Biolabs), which did allow amplification of the control target region (Figure 3.2 B), was subsequently used in long range PCR to screen for the presence of the chimeric *CYP11B1/CYP11B2*. Non-specific amplification was observed for the long range PCR assay (Figure 3.2 A, B). However, increasing the annealing temperature from 68°C, which was used previously (Johnsson *et al.* 1995), to 70°C did not appear to improve the specificity of the PCR assay (data not shown). As a result, an annealing temperature of 68°C was used in the long range PCR assay. Since the amplification of the target control using the Q5® High-Fidelity PCR kit was successful (Figure 3.2 B), further optimization was not performed.

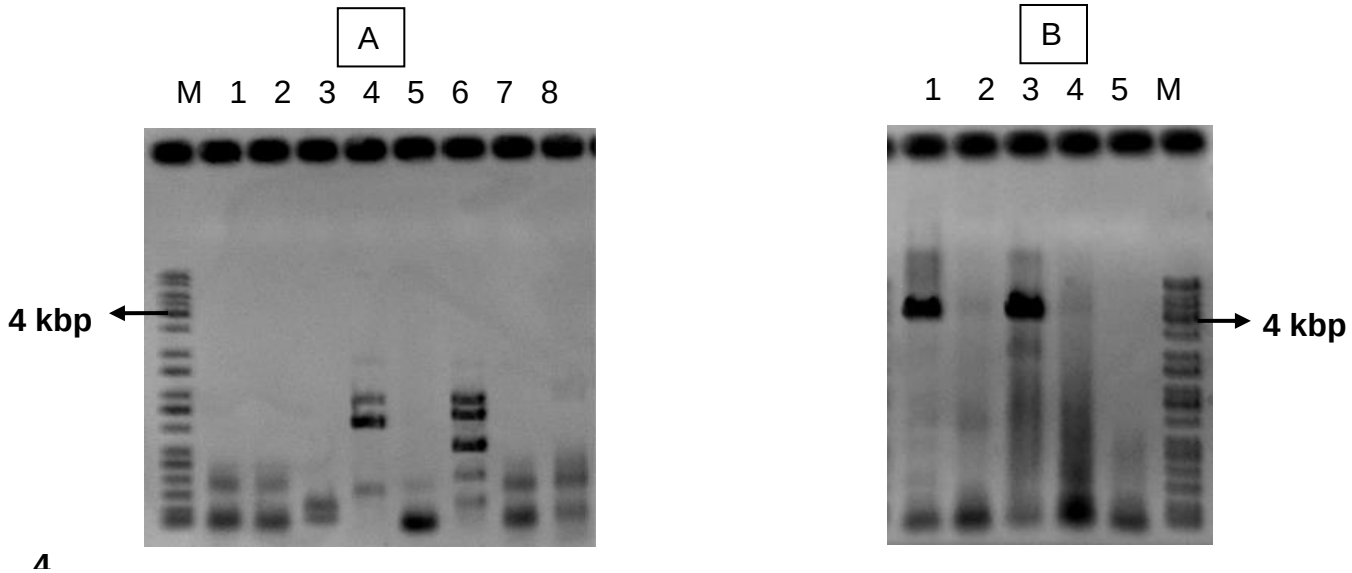

Figure 3.2: Negative inverted 0.7% gel images of long range PCR amplicon using the Expand High Fidelity PCR system kit (Roche) (A) and the Q5® High-Fidelity PCR kit (New England Biolabs) (B). A) Amplicon of the control gene (*CYP11B2*) of eight samples using the Expand High Fidelity PCR system was loaded in lanes 1 to 8. B) Amplicon of the chimeric *CYP11B1/CYP11B2* (lanes 1 and 3) and the control target (lanes 2 and 4) of two samples using the Q5® High-Fidelity PCR kit, with a no-template control loaded in lane 5. A molecular weight marker (100 bp to 10,000 bp) (KAPA Biosystems) was loaded in lanes M. The arrows show the position of the 4 kbp marker.

3.3. Optimization of conventional PCR

The first objective for PCR optimization was to determine the optimal annealing temperature for primer pairs *NR3C1* exons 6, 7, 9, 10 and 11, *HSD11B2* exons 3, 4, 5-1 and 5-2, *SCNN1B* exon 13, *SCNN1G* exon 13, and *WNK4* exons 7 and 17. As mentioned previously, due to the size of *HSD11B2* exon 5, two primer pairs (referred to as 5-1 and 5-2) were used to amplify this target region. Thereafter, annealing and extension times were optimized for each respective primer pair. All of the PCR assays were successfully optimized using this approach, with the exception of the assay for *HSD11B2* exon 4.

The optimal annealing temperature each of the primer pairs was determined by using an annealing temperature gradient ranging from 50^{o}C to 70^{o}C. Except for primer pair *HSD11B2* exon 4 which yielded non-specific products (Figure 3.3 L, M), the optimal annealing temperature for each of the primer pairs varied between 55^{o}C and 65^{o}C (Figure 3.3 A to K; Table 3.3). As far as possible the annealing temperature of the different primer pairs was selected at 55^{o}C, 58^{o}C, 60^{o}C, or 65^{o}C to allow more than one assay to be run simultaneously. Thereafter, the optimal annealing time and extension time were determined for each of the primer pairs (excluding *HSD11B2* exon 4). The annealing time evaluated was 30 seconds and 1 minute, while the extension time evaluated was 30 seconds, 1 minute, 1.5 minutes, and 2 minutes. The cycling conditions that resulted in the highest yield and minimal non-specific amplification product as visualized on a 2% agarose gel, was selected for each of the primer pairs (Table 3.3). As far as possible, the annealing time was selected at 30 seconds or 1 minute, and the extension time was selected at 1 minute or 2 minutes to allow more than one assay to be run simultaneously.

PCR amplification for primer pair *HSD11B2* exon 4 (Coeli *et al.* 2008) yielded non-specific products (Figure 3.3 L, M). A second primer pair was subsequently designed with Primer3Plus (Untergasser and Nijveen 2007), using NG_016549.1 as reference sequence. PCR amplification using the second primer pair also yielded non-specific products (data not shown). Thereafter, a combination of the two primer pairs was used in semi-nested PCR (Table 3.4), which yielded a specific PCR product of the correct size (Figure 3.4). The expected fragment size of the first PCR product (using forward primer F1) was 335 bp while the fragment size of the second PCR product (using forward primer F2) was 300 bp (Table 3.4). The amplicon of the first PCR reaction was diluted (1:100,000) and purified using FastAP in conjunction with Exo 1 (Thermo Scientific) according to the manufacturer's instructions, where after the FastAP treated PCR product was used as template in the semi-nested PCR reaction. Sequencing of the semi-nested PCR product confirmed that the correct target fragment was amplified (data not shown). The semi-nested PCR was optimized using an annealing temperature of 60^{o}C and annealing and extension times of 1 minute and 2 minutes, respectively.

Table 3.3: The optimized annealing temperature (T_a), annealing time and extension time for *NR3C1* (exons 6, 7, 9, 10 and 11), *HSD11B2* (exons 3 and 5), *SCNN1B* (exon 13), *SCNN1G* (exon 13), and *WNK4* (exons 7 and 17).

Primer pair	Size (bp)	T_a (^oC)	Annealing time	Extension time
NR3C1 exon 6	232	58	1 minute	1 minute
NR3C1 exon 7	204	58	1 minute	1 minute
NR3C1 exon 9	226	55	1 minute	1 minute
NR3C1 exon 10	241	58	1 minute	1 minute
NR3C1 exon 11	281	60	30 seconds	1 minute
HSD11B2 exon 3	292	65	30 seconds	2 minutes
HSD11B2 exon 5-1	182	58	1 minute	1 minute
HSD11B2 exon 5-2	401	65	1 minute	1 minute
SCNN1B exon 13	346	65	1 minute	1 minute
SCNN1G exon 13	432	65	30 seconds	2 minutes
WNK4 exon 7	198	65	30 seconds	1 minute
WNK4 exon 17	323	65	30 seconds	1 minute

Table 3.4: Primer sequences for semi-nested PCR for *HSD11B2* exon 4.

Target region	Primer sequence (5' to 3')[a]	Fragment size (bp)	Reference
HSD11B2 exon 4	F1: ACTGGAGCAAAAAGGAGCC	F1 + R: 335; F2 + R: 300	Coeli *et al.* (2008)
	F2: GGCTTAGGGAGCCCCTTG		Smith (2016)[b]
	R: ACACCCCCAACCAAAACCAT		Smith (2016)[b]

[a] F1 and F2 refers to forward primer 1 and forward primer 2, with R referring to the reverse primer;

[b] Primers were designed for this study with Primer3Plus using reference sequence NG_016549.1.

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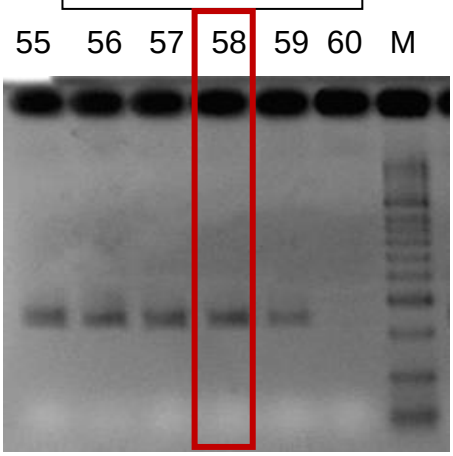
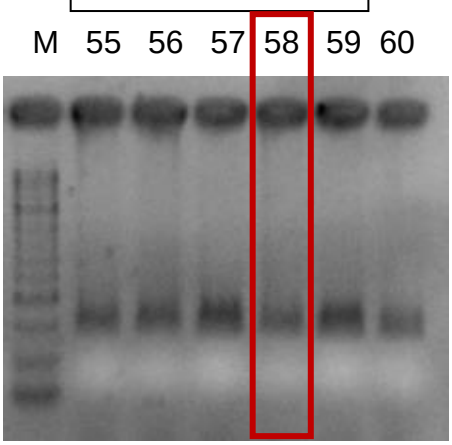
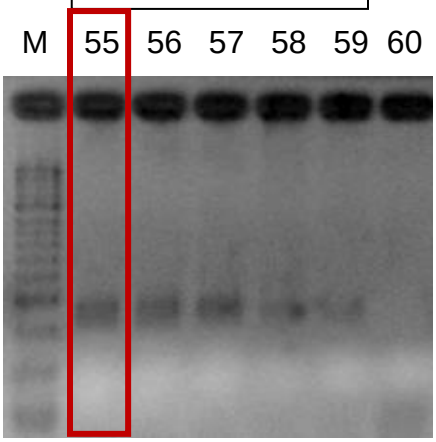
A: *NR3C1* exon 6



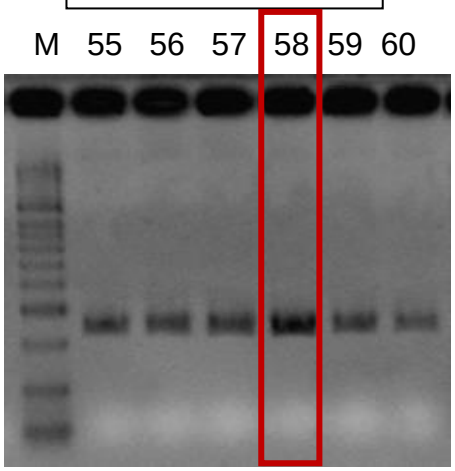
B: *NR3C1* exon 7



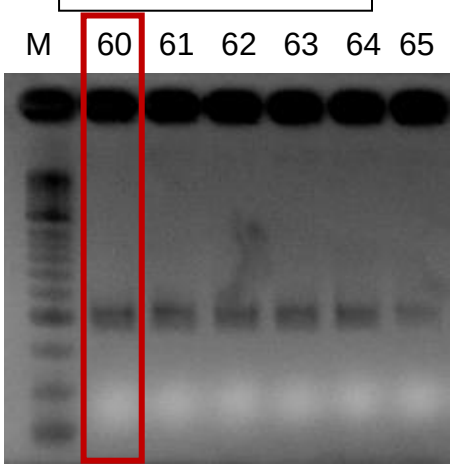
C: *NR3C1* exon 9



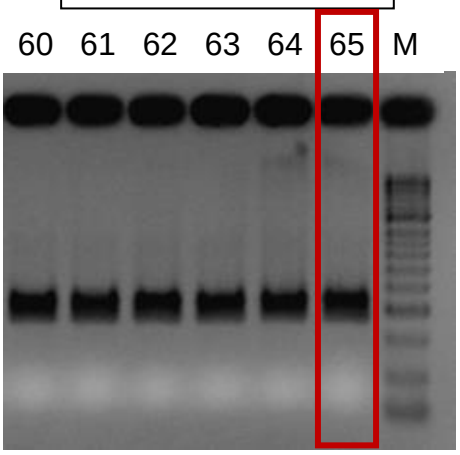
D: *NR3C1* exon 10



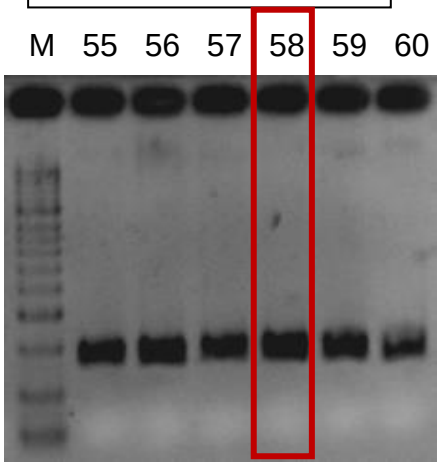
E: *NR3C1* exon 11



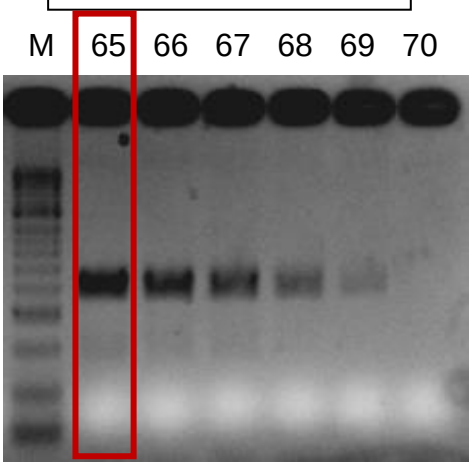
F: *HSD11B2* exon 3



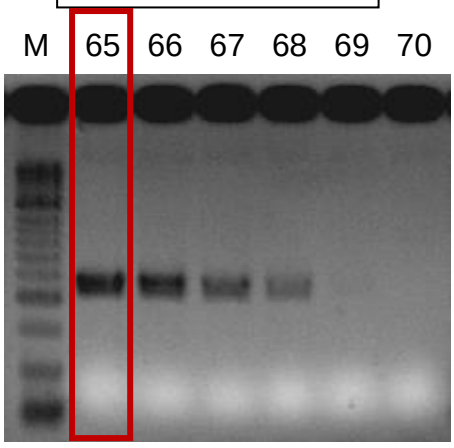
G: *HSD11B2* exon 5-1



H: *HSD11B2* exon 5-2



I: *SCNN1B* exon 13



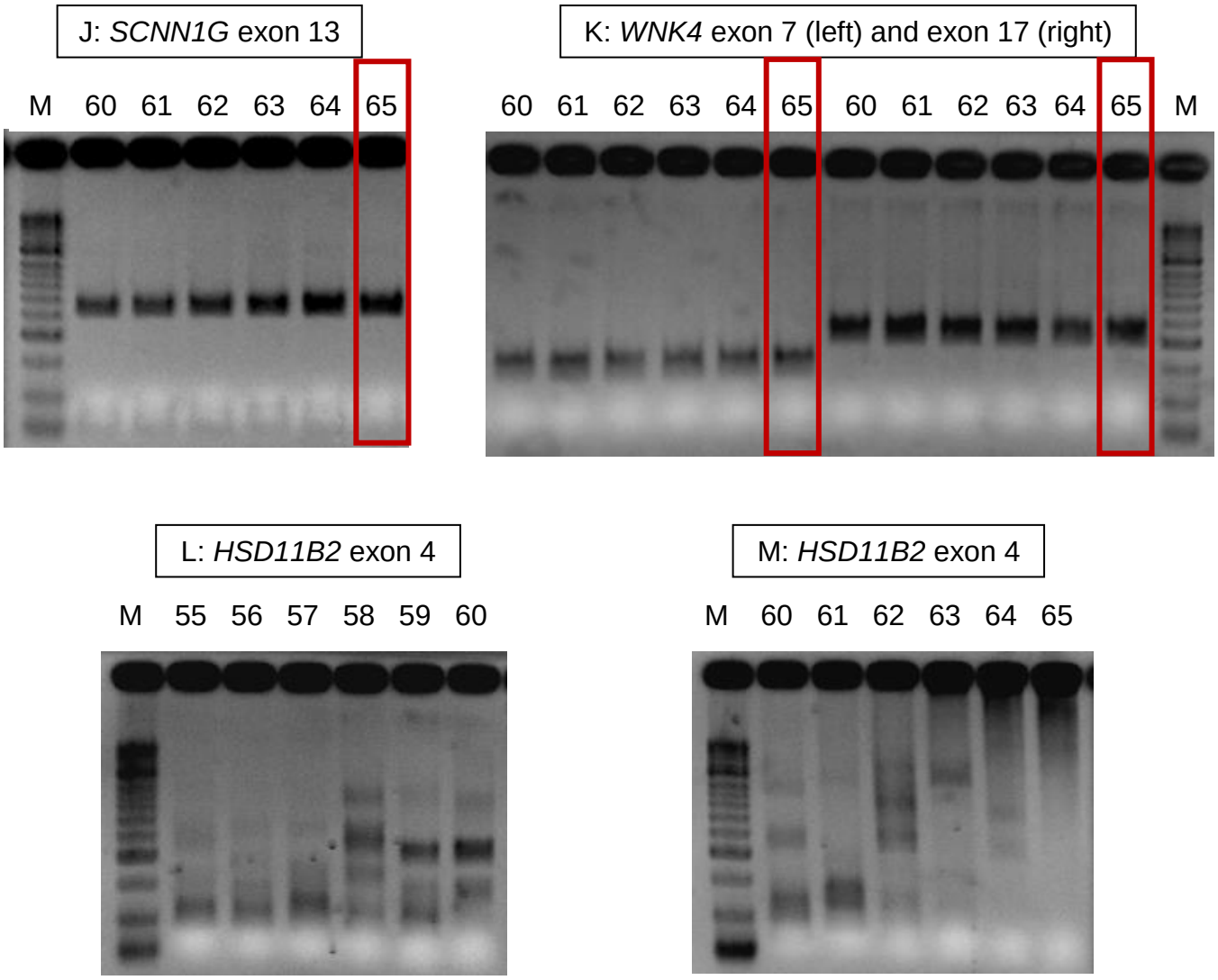


Figure 3.3: Negative inverted 2% gel images of the PCR product of an annealing temperature gradient for 13 primer pairs. A) *NR3C1* exon 6; B) *NR3C1* exon 7; C) *NR3C1* exon 9; D) *NR3C1* exon 10; E) *NR3C1* exon 11; F) *HSD11B2* exon 3; G) *HSD11B2* exon 5-1; H) *HSD11B2* exon 5-2; I) *SCNN1B* exon 13; J) *SCNN1G* exon 13; K) *WNK4* exons 7 (left) and 17 (right); L, M) *HSD11B2* exon 4. For *HSD11B2* exon 4, annealing temperature gradients ranging from 55 to 60^{o}C (L) and 60 to 65^{o}C (M) both yielded non-specific products. Except for *HSD11B2* exon 4, the red block indicates the selected annealing temperature for each of the primer pairs. A 100 bp molecular weight marker (Bioline) was also loaded in lanes M.

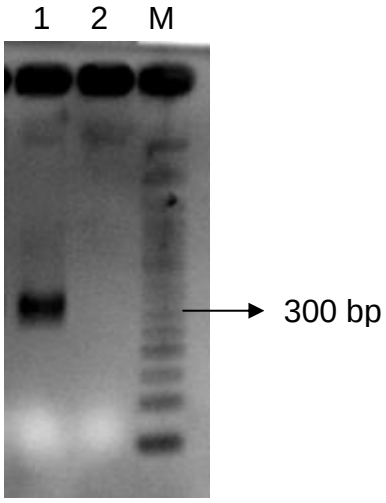


Figure 3.4: A negative inverted 2% gel image of the PCR product of the 2nd semi-nested PCR reaction. PCR product of one sample was loaded in lane 1 and a no-template control was loaded in lane 2. A 50 bp marker (New England Biolabs) was also loaded in lane M. The expected fragment size is 300 bp. The arrow shows the position of the 300 bp marker.

3.4. Optimization of HRM analysis

One of the objectives of the current study was to use HRM analysis to identify potential sequence variants in selected target regions in *NR3C1, HSD11B2*, *SCNN1B, SCNN1G* and *WNK,* in order to reduce the number of samples required to be sequenced. HRM analysis was used to screen for sequence variants in all the assays, except for *HSD11B2* exon 4 due to the problems initially to obtain a single fragment of the expected size.

The HRM PCR reaction for the assays for *NR3C1* (exons 6, 7, 9, 10 and 11)*, HSD11B2* (exons 3, 5-1 and 5-2), *SCNN1B* (exon 13)*, SCNN1G* (exon 13), and *WNK* (exons 7 and 17) was optimized in terms of the dilution factor of the PCR product used as template in the HRM PCR reaction, as well as the annealing and extension times. Three dilutions (1:10,000, 1:100,000 and 1:1,000,000) of the PCR product were

compared for use as template in HRM analysis in order to obtain threshold cycle (Ct) values ranging from 15 to 25. Based on the Ct value and level of fluorescence, a 1:100,000 dilution of the PCR amplicon was used in subsequent HRM reactions for each of the assays (data not shown). PCR amplicon from a random sample was selected and sequenced to obtain a wild type (WT) control. If a polymorphism was identified in a particular sample, another sample was then randomly selected for sequencing.

To determine the optimal annealing and extensions times for each of the assays, five sets of cycling conditions were compared: 1) 1 minute combined annealing and extension time; 2) 1 minute annealing with 1 minute extension; 3) 30 seconds annealing with 30 seconds extension; 4) 10 seconds annealing with 30 seconds extension and 5) 10 seconds annealing with 10 seconds extension. It was determined that 10 seconds annealing with 30 seconds extension was optimal for all of the primer pairs (data not shown). However, when HRM for each assay was tested on four patient samples, the level of amplification achieved with the HRM PCR reaction using 10 seconds annealing with 30 seconds extension was not always optimal (data not shown). It was found the amplification efficiency was not consistent across all the samples. As a result, a second PCR reaction was introduced prior to the HRM PCR reaction to achieve consistent amplification. The cycling conditions for the HRM PCR reaction were once again optimized for each assay in order to obtain maximum amplification for the 12 primer pairs. The cycling conditions that were compared were as follows: 1) 30 seconds annealing with 30 seconds extension; 2) 1 minute annealing with 1 minute extension; 3) 30 seconds annealing with 2 minutes extension; 4) 1 minute combined annealing and extension, and 5) 1.5 minutes combined annealing and extension. As far as possible, a combined annealing and extension time of 1 minute or 1.5 minutes, or an annealing time of 30 seconds with an extension time of 2 minutes was selected to allow more than one assay to be run simultaneously (Table 3.5).

Table 3.5: The optimized HRM PCR cycling conditions for 12 primer pairs. For each assay, amplicon (1:100,000) of the second PCR reaction was used as template in the HRM PCR reaction.

Primer pair	Optimized cycling conditions for the HRM PCR reaction
NR3C1 exon 6	2 step reaction: 1.5 minutes combined annealing and extension
NR3C1 exon 7	2 step reaction: 1 minute combined annealing and extension
NR3C1 exon 9	2 step reaction: 1.5 minutes combined annealing and extension
NR3C1 exon 10	2 step reaction: 1 min combined annealing and extension
NR3C1 exon 11	3 step reaction: 30 seconds annealing, 2 minutes extension
HSD11B2 exon 3	3 step reaction: 30 seconds annealing, 2 minutes extension
HSD11B2 exon 5-1	2 step reaction: 1 minute combined annealing and extension
HSD11B2 exon 5-2	3 step reaction: 30 seconds annealing, 2 minutes extension
SCNN1B exon 13	2 step reaction: 1 minute combined annealing and extension
SCNN1G exon 13	3 step reaction: 30 seconds annealing, 2 minutes extension
WNK4 exon 7	3 step reaction: 30 seconds annealing, 2 minutes extension
WNK4 exon 17	3 step reaction: 30 seconds annealing, 2 minutes extension

The optimized cycling conditions (Table 3.5) were subsequently used to perform HRM analysis on four samples, using 1:100,000 diluted amplicon of the second PCR reaction as template. For each primer pair, a WT control was included in the HRM analysis. Samples identified as variants to the WT in the difference plots obtained from the HRM analysis were sequenced, with the exception of the *WNK4* exon 7 and *NR3C1* exon 9 assays.

- *NR3C1* exon 10: The difference plot obtained from the HRM analysis for *NR3C1* exon 10 identified one variant group in addition to the WT group (Figure 3.5 A). Sequencing of the sample in the variant group (sample 16.1) confirmed the presence of a heterozygous sequence variant (Asp678Glu, rs258751) (Figure 3.6 A;

Table 3.6). The Asp678Glu variant has previously been described (Feng *et al*. 2000; Chung *et al.* 2009; Souza *et al*. 2014).

- *NR3C1* exon 11: The difference plot obtained from the HRM analysis for *NR3C1* exon 11 identified one variant group in addition to the WT group (Figure 3.5 B). Sequencing of the samples in the variant group (samples 14.1 and 16.1) confirmed the presence of heterozygous genetic variant (Asn767Asn; rs6196) in both samples (Figure 3.6 B; Table 3.6). The sequence variant has been described previously (Koper *et al.* 1997; Feng *et al*. 2000; Chung *et al.* 2009; Krupoves *et al.* 2011; Souza *et al*. 2014).
- *SCNN1B* exon 13: The difference plot obtained from the HRM analysis for *SCNN1B* exon 13 identified one variant group in addition to the WT group (Figure 3.5 C). Sequencing of the sample in the variant group (sample 10.2) confirmed the presence of a heterozygous genetic variant (Pro617Leu, CM081432), which has previously been described (Rossi *et al.* 2008; Rossi *et al*. 2011) (Figure 3.6 C; Table 3.6).
- *WNK4* exon 7: The difference plot obtained from HRM analysis for *WNK4* exon 7 identified one variant group, which consisted of all four patient samples, in addition to the WT group (Figure 3.7 A). Since all four samples grouped separately from the WT control, it was thought that it was unlikely that each of the four contained a sequence variant and these four samples were, therefore, not sequenced. However, in hindsight, after having obtained the sequencing data for *WNK4* exon 7, which showed that 45 out of 90 individuals contained a sequence variant (Ala547Ala) (Chapter 4, Table 4.5), it is possible that a variant could have been present in all four of these samples. However, Mendes *et al.* (2011) reported the presence of genetic variants in *WNK4* in only 4.3% of the study participants. In addition, Han *et al.* (2011) reported that the Ala547Ala variant was present in 14.2% and 14.8% of study participants in two independent populations, respectively. The four samples that were included in HRM analysis did not form part of the cohort of 90 individuals that were screened for sequence variants and whether a variant was in fact present is,

therefore, unknown. PCR amplicon of the four samples was resolved on a 2% agarose gel and non-specific products were not visible, although a faint smear was present above the band (Figure 3.8 A).

- *NR3C1* exon 9: Similar to *WNK4* exon 7, the difference plot obtained from HRM analysis for *NR3C1* exon 9 identified one variant group, which consisted of all four patient samples, in addition to the WT group (data not shown). Since all four samples grouped separately from the WT control, it was thought that it was unlikely that each of the four contained a sequence variant and these four samples were, therefore, not sequenced. Sequence data of 90 individuals revealed that only one individual had a sequence variant, and it is therefore unlikely that all four samples could have contained a variant (Chapter 4, Table 4.1). PCR amplicon of the four samples was resolved on a 2% agarose gel and non-specific products were not visible (Figure 3.8 B).
- *NR3C1* exon 6: The difference plot obtained from the HRM analysis for *NR3C1* exon 6 identified one variant group, which included samples 10.2 and 11.1, in addition to the WT group (Figure 3.7 B). However, sequencing the conventional PCR product of samples 10.2 and 11.1 did not identify a sequence variant (data not shown). PCR amplicon of the four samples was resolved on a 2% agarose gel and a single band of the correct size was observed (data not shown).
- *NR3C1* exon 7: The difference plot obtained from the HRM analysis for *NR3C1* exon 7 identified one variant group, which included sample 14.1, in addition to the WT group (data not shown). However, sequencing the conventional PCR product of sample 14.1 did not identify a sequence variant (data not shown). PCR amplicon of the four samples was resolved on a 2% agarose gel and a single band of the correct size was observed (data not shown).
- *WNK4* exon 17: The difference plot obtained from the HRM analysis for *WNK4* exon 17 identified one variant group, which included samples 10.2 and 11.1, in addition to the WT group (data not shown). However, sequencing the conventional PCR product of samples 10.2 and 11.1 did not identify a sequence variant (data not

shown). PCR amplicon of the four samples was resolved on a 2% agarose gel and a single band of the correct size was observed (data not shown).

- *HSD11B2* exon 5-1: The difference plot obtained from the HRM analysis for *HSD11B2* exon 5-1 identified one variant group, which included sample 10.2, in addition to the WT group (data not shown). However, sequencing the conventional PCR product of sample 10.2 did not identify a sequence variant (data not shown). PCR amplicon of the four samples was resolved on a 2% agarose gel and a single band of the correct size was observed (data not shown).
- *SCNN1G* exon 13: The difference plot obtained from the HRM analysis for *SCNN1G* exon 13 identified one variant group, which included sample 10.2, in addition to the WT group (data not shown). However, sequencing the conventional PCR product of sample 10.2 did not identify a sequence variant (data not shown). PCR amplicon of the four samples was resolved on a 2% agarose gel and a very faint second band was visible below the expected band (Figure 3.8 C)
- *HSD11B2* exon 3: The difference plot obtained from the HRM analysis for *HSD11B2* exon 3 identified one variant group, which included samples 11.1 and 16.1, in addition to the WT group (Figure 3.7 C). However, sequencing the conventional PCR product of samples 11.1 and 16.1 only identified a sequence variant (Ala196Ala, rs5480) in sample 11.1 (Figure 3.9 A, B). The Ala196Ala variant has previously been reported (dbSNP, rs5480). PCR amplicon of the four samples was resolved on a 2% agarose gel and a very faint second band was visible below the expected band (Figure 3.8 D).
- *HSD11B2* exon 5-2: The difference plot obtained from the HRM analysis for *HSD11B2* exon 5-2 identified one variant group, which included sample 10.2, in addition to the WT group (data not shown). However, sequencing the conventional PCR product of sample 10.2 did not identify a sequence variant (data not shown). PCR amplicon of the four samples was resolved on a 2% agarose gel and a second band below the expected band was clearly visible for all four samples (Figure 3.8 E).

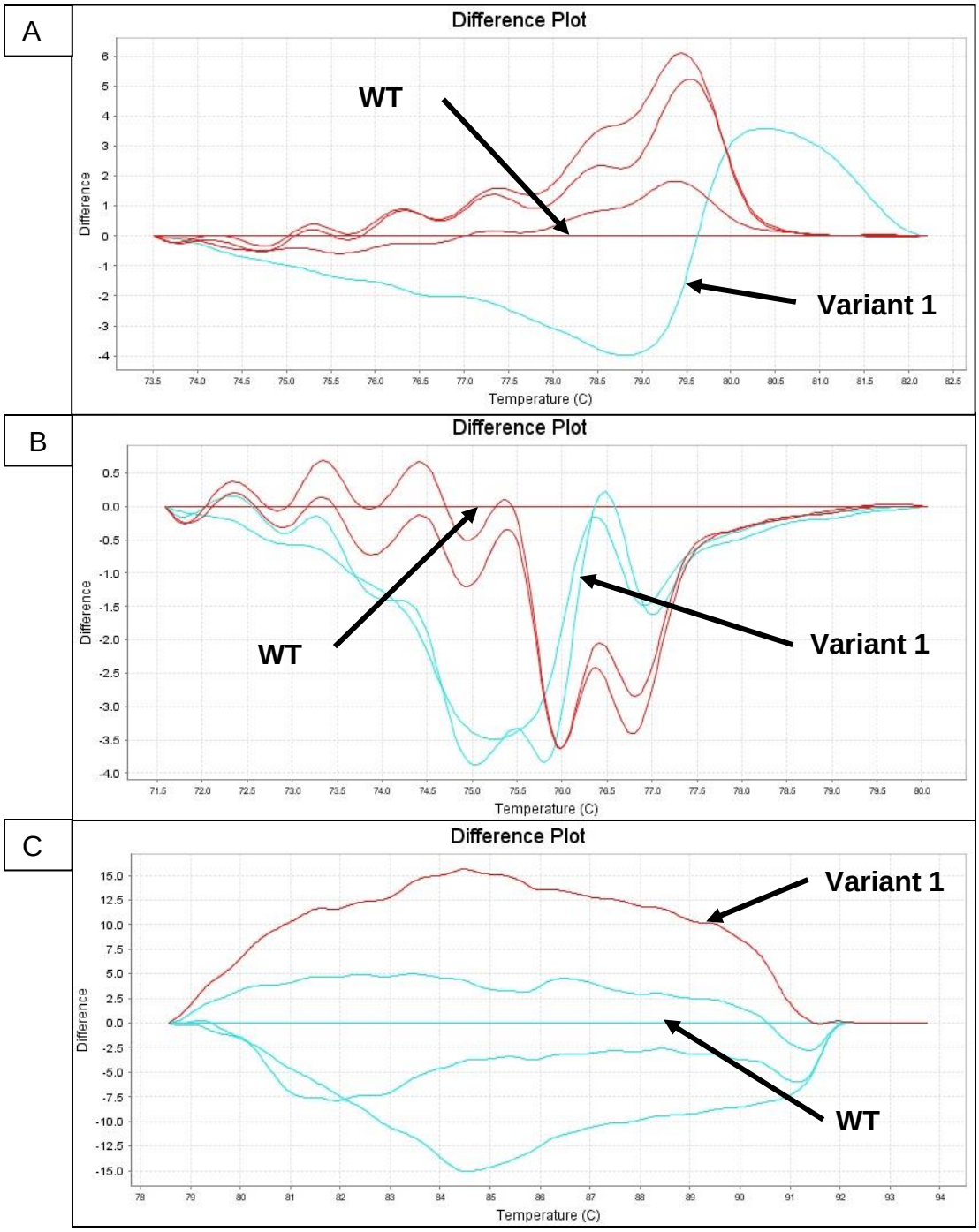


Figure 3.5: Difference plots obtained from HRM analysis for primer pairs *NR3C1* exon 10 (A), *NR3C1* exon 11 (B), and *SCNN1B* exon 13 (C). For each of the assays, one variant group was detected in addition to the wild type (WT) group.

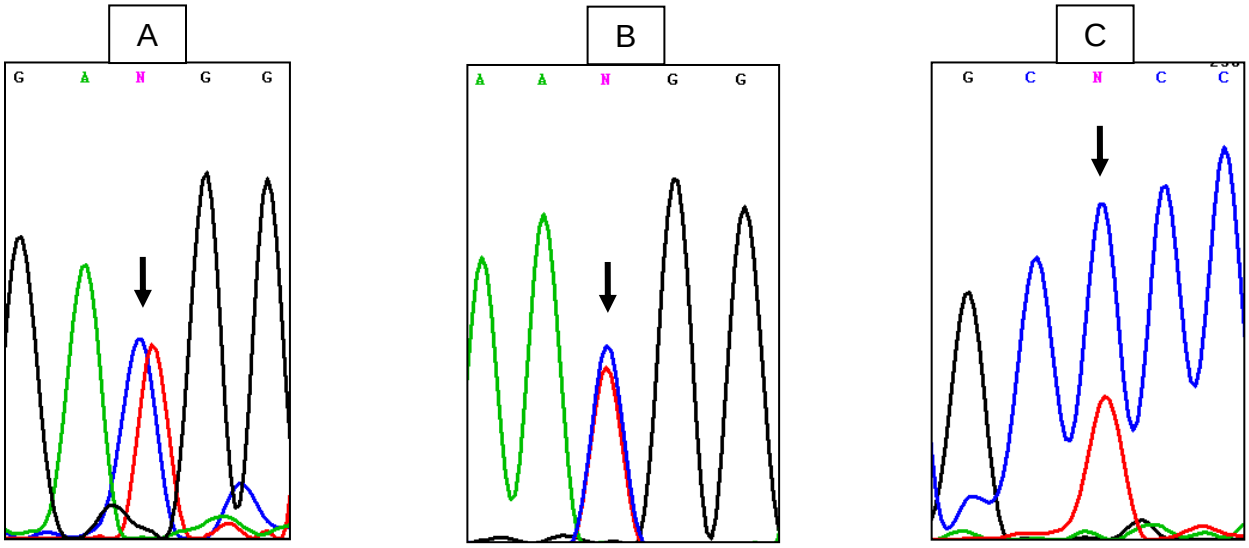


Figure 3.6: Sequence chromatographs of the genetic variants detected in *NR3C1* exon 10 (A), *NR3C1* exon 11 (B) and *SCNN1B* exon 13 (C). A) Heterozygous Asp678Glu (C>T) identified in *NR3C1* exon 10 in sample 16.1; B) Heterozygous Asn767Asn (T>C) identified in *NR3C1* exon 11 sample 14.1; C) Heterozygous Pro617Leu (C>T) identified in *SCNN1B* exon 13 in sample 10.2. The arrows indicate the position of the genetic variants in the chromatographs.

Table 3.6: Genetic variants identified in *NR3C1* exon 10, *NR3C1* exon 11 and *SCNN1B* exon 13.

Primer pair	Sample	Variant identified with sequencing	Reference
NR3C1 exon 10	16.1	Asp678Glu (rs258751)	Feng *et al*. (2000); Chung *et al.* (2009)
NR3C1 exon 11	14.1 16.1	Asn767Asn (rs6196)	Koper *et al.* (1997); Chung *et al.* (2009); Krupoves *et al.* (2011)
SCNN1B exon 13	10.2	Pro617Leu (CM081432)	Rossi *et al*. (2008); Rossi *et al*. (2011)

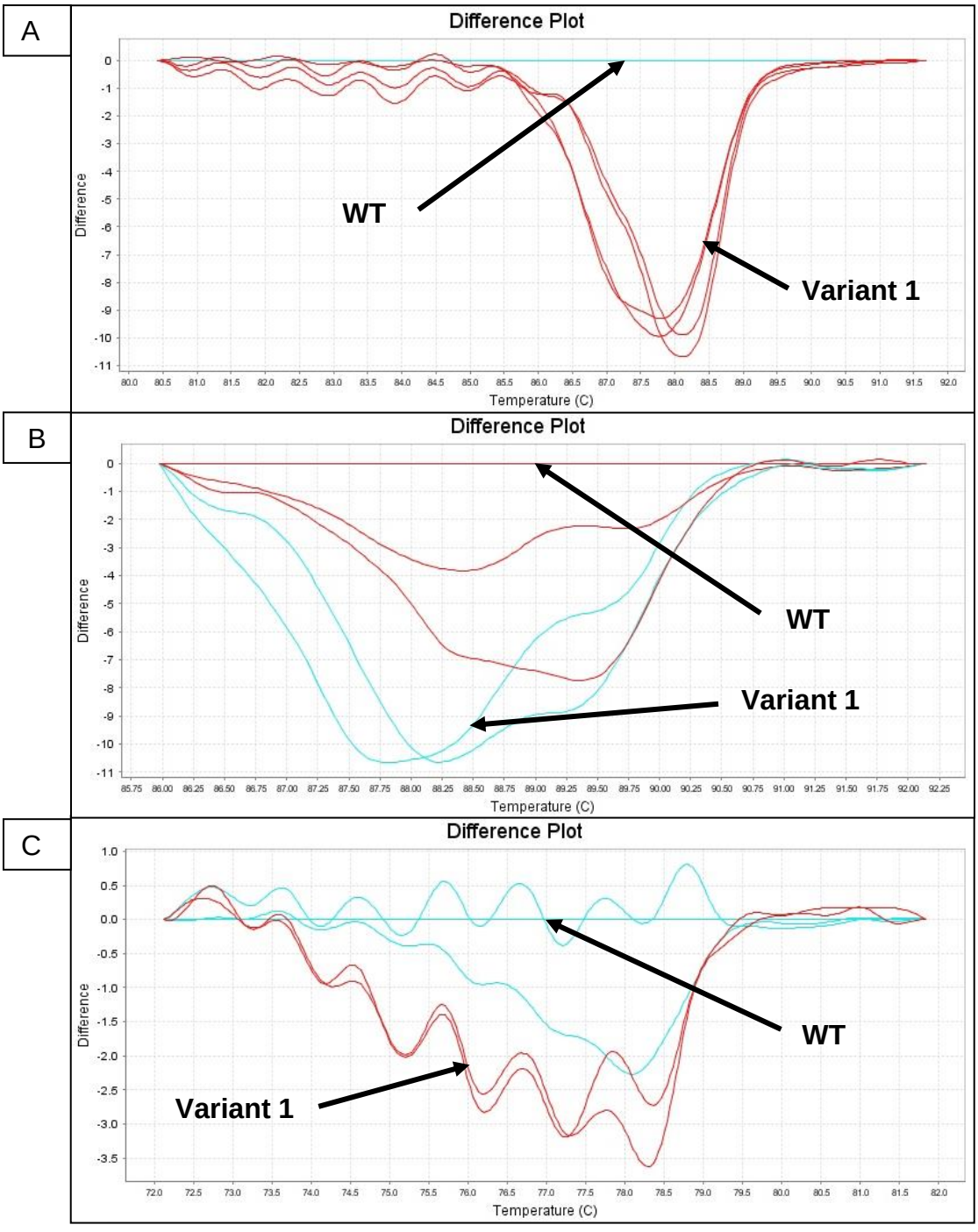


Figure 3.7: Difference plots obtained from HRM analysis for primer pairs *WNK4* exon 7 (A), *NR3C1* exon 6 (B), and *HSD11B2* exon 3 (C). For each of the assays, one variant group was detected in addition to the wild type (WT) group.

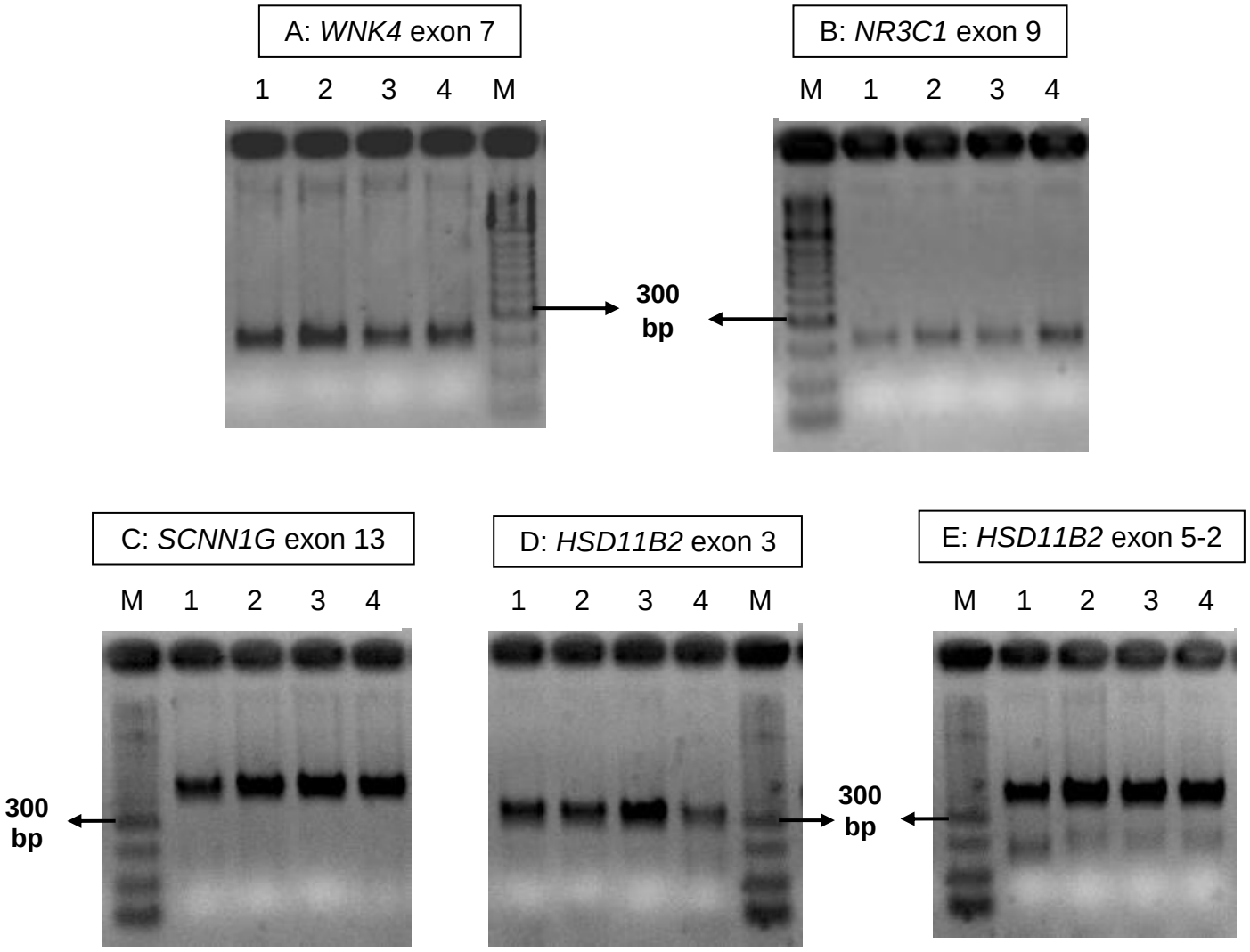


Figure 3.8: Negative inverted 2% gel images of the PCR products for primer pairs *WNK4* exon 7 (A), *NR3C1* exon 9 (B), *SCNN1G* exon 13 (C), *HSD11B2* exon 3 (D), and *HSD11B2* exon 5-2 (E). Amplicon was loaded in lanes 1(10.2), 2 (11.1), 3 (14.1), and 4 (16.1). A100 bp molecular weight marker (Bioline) was also loaded in lanes M. The expected fragment sizes are 198 bp for *WNK4* exon 7, 226 bp for *NR3C1* exon 9, 432 bp for *SCNN1G* exon 13; 292 bp for *HSD11B2* exon 3, and 401 bp for *HSD11B2* exon 5-2, respectively. The arrow shows the position of the 300 bp marker.

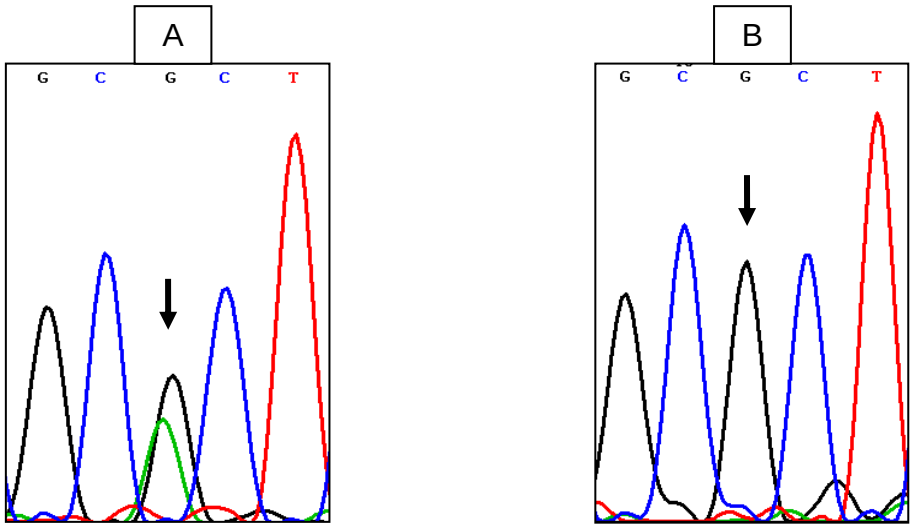


Figure 3.9: Sequence chromatographs of *HSD11B2* exon 3 in two samples. The Ala196Ala variant (G>A) was present in heterozygous form in sample 11.1 (A), but no sequence variant was identified in sample 16.1 (B). The arrow indicates the position of the sequence variant in chromatograph A and the wild type nucleotide in chromatograph B.

To summarize, only HRM assays for *NR3C1* exon 10, *NR3C1* exon 11 and *SCNN1B* exon 13 was shown to successfully identify samples with sequence variants. The conventional PCR product of these samples was sequenced and the presence of a genetic variant confirmed (Table 3.6). However, for *NR3C1* exon 10, *NR3C1* exon 11 and *SCNN1B* exon 13, the distance between the different samples in a particular grouping, WT or variant, in the difference plot made the interpretation of the HRM results difficult. HRM analysis for the assays for *WNK4* exon 7 and *NR3C1* exon 9 identified all four samples as variants to the WT. Since it was thought unlikely that all four samples contain sequence variants in *WNK4* exon 7 and *NR3C1* exon 9, these samples were not sequenced. However, in hindsight, sequencing data revealed that 45 out of 90 participants harboured a sequent variant in *WNK4* exon 7 (Chapter 4, Table 4.5), and it is therefore possible that a sequence variant could have been present in these four samples. This was not the case for *NR3C1* exon 9, however, since

sequencing data from 90 participants only revealed a variant in one individual (Chapter 4, Table 4.1). Therefore, it is unlikely that these four samples all contained a sequence variant, which suggests that HRM analysis for *NR3C1* exon 9 failed to successfully identify samples with sequence variants. HRM analysis for the assays for *NR3C1* exons 6 and 7, *HSD11B2* exons 3, 5-1 and 5-2, *SCNN1G* exon 13, and *WNK4* exon 17 also failed to successfully identify samples with sequence variants. For these seven assays, sequencing either did not identify a sequence variant, or did not identify a sequence variant in all of the samples that were identified as variants to the WT. As a result of the difficulty in identifying potential sequence variants, it was decided not to spend further laboratory resources on HRM analysis. Instead, it was decided to use DNA sequencing to identify sequence variants in genes implicated in monogenic forms of hypertension.

3.5 Conclusion

In the current study, PCR amplification using FTA® discs prepared according to the Whatman® FTA® protocol (GE Healthcare Life Sciences) did not result in successful amplification of all samples. PCR amplification using DNA extracted from FTA® discs with a modified methanol extraction method also did not result in successful amplification of all samples. It is suspected that, since the FTA® paper was saturated with buffy coat and red blood cells, inhibitory factors were present and resulted in PCR inhibition for both of these methods. Finally, PCR amplification using a modified CTAB extraction method resulted in a 100% PCR success rate for 18 samples. Thus, the CTAB extraction method was successfully used to extract DNA from FTA® paper, without the presence of PCR inhibitory factors. The CTAB method was subsequently used for all further DNA extractions.

In the current study, long range PCR and conventional PCR were used to amplify selected target regions in genes implicated in monogenic forms of hypertension. Long

range PCR was used to amplify the target regions of the chimeric *CYP11B1/CYP11B2* and *CYP11B2* that served as an endogenous amplification control. Long range PCR using the Expand High Fidelity PCR system (Roche) did not yield the control target for eight samples. As a result, it was decided to use the Q5® High-Fidelity PCR kit (New England Biolabs), which successfully amplified the control target. The Q5® High-Fidelity PCR kit (New England Biolabs) was subsequently used in the long range PCR reactions. In addition, the optimal annealing temperature and the optimal annealing and extension times were determined for conventional PCR for assays *NR3C1* exons 6, 7, 9, 10 and 11, *HSD11B2* exons 3, 5-1 and 5-2, *SCNN1B* exon 13, *SCNN1G* exon 13, and *WNK4* exons 7 and 17. For the conventional PCR assay for *HSD11B2* exon 4, semi-nested PCR was performed due to non-specific amplification that was initially encountered. Thus, in the current study, PCR reactions to amplify the chimeric *CYP11B1/CYP11B2* gene, as well as selected target regions in *NR3C1* (exons 6, 7, 9, 10 and 11), *HSD11B2* (exons 3, 4, and 5), *SCNN1B* (exon 13), *SCNN1G* (exon 13) and *WNK4* (exons 7 and 17), were successfully optimized.

HRM analysis was attempted in this study to screen for sequence variants in genes implicated in monogenic forms of hypertension, in order to reduce the number of samples required to be sequenced. HRM analysis was used to screen for sequence variants in all the assays, except for *HSD11B2* exon 4 and the long range PCR assay. The HRM PCR reaction for the assays for *NR3C1* (exons 6, 7, 9, 10, and 11)*, HSD11B2* (exons 3, 5-1, and 5-2), *SCNN1B* (exon 13)*, SCNN1G* (exon 13), and *WNK* (exons 7 and 17) was optimized in terms of the dilution factor of the PCR product used as template in the HRM PCR reaction, as well as the annealing and extension times. Only assays for *NR3C1* exon 10, *NR3C1* exon 11, and *SCNN1B* exon 13 were shown to successfully identify samples with sequence variants. The conventional PCR product of these samples was sequenced and the presence of a genetic variant confirmed. However, for *NR3C1* exon 10, *NR3C1* exon 11 and *SCNN1B* exon 13, the distance between the difference plot of the WT and the difference plot of the sample(s) made

interpretation of the HRM results difficult. Thus, identifying sequencing variants using HRM analysis proved to be difficult in the current study and as a result, it was decided not to spend further laboratory resources on HRM analysis. Instead, Sanger DNA sequencing was used to identify sequence variants in selected target regions in genes implicated in monogenic forms of hypertension.

Chapter 4

Results and discussion: Long Range PCR and DNA Sequencing

4.1. Long range PCR for the chimeric *CYP11B1/CYP11B2*

Long range PCR was used to screen for the presence of the chimeric *CYP11B1/CYP11B2* gene in the study cohort. The *CYP11B1/CYP11B2* gene fragment is approximately 3.9 kbp in size (Johnsson *et al.* 1995). A region of *CYP11B2* (exons one to five) was amplified in conjunction with the chimeric gene and served as an endogenous amplification control. The size of the *CYP11B2* target region is approximately 4.1 kbp, which was estimated using the reference sequence (NG_008374.1) from NCBI (http://www.ncbi.nlm.nih.gov/). In the current study, the Q5® High-Fidelity PCR kit (New England Biolabs) was used to amplify the *CYP11B2* and the chimeric *CYP11B1/CYP11B2* target regions of 90 hypertensive individuals. According to literature the *CYP11B1/CYP11B2* gene results in the constitutive secretion of aldosterone, with the resulting increase in mineralocorticoid receptor (MR) activity leading to elevated blood pressure (BP) through plasma volume expansion (Lifton *et al.* 1992a).

In the current study long range PCR allowed the identification of the chimeric *CYP11B1/CYP11B2* gene in at least one hypertensive individual (Figure 4.1, sample 8), and could potentially explain the elevated BP in this individual. In addition, a second hypertensive individual (Figure 4.1, sample 5) was identified that appeared to be positive for the chimeric *CYP11B1/CYP11B2* gene. The PCR reaction produced non-specific amplification despite using an annealing temperature of 68^{o}C (Figure 4.1). The presence of multiple bands made interpretation of the results difficult and it is possible that the long range PCR assay could have produced false negative results due to

competition with non-target primer binding. It is suspected that specific binding of the primers was problematic due to the high degree of similarity in the coding (95%) and non-coding (90%) regions between *CYP11B1* and *CYP11B2* (Mornet *et al*. 1989), resulting in non-specific amplification. As a result, the chimeric *CYP11B1/CYP11B2* gene could be more prevalent in the current study cohort. In addition, the endogenous control PCR fragment failed to amplify in 13 samples despite repeating the respective PCR reactions (data not shown). For future studies involving the detection of the chimeric *CYP11B1/CYP11B2* gene, new primers that bind more specifically to the target region could be designed. Since the chimeric *CYP11B1/CYP11B2* gene was identified in at least one in 77 hypertensive individuals (1%) in the current study, it could be a good candidate for future genetic studies relating to hypertension involving black individuals from Mangaung.

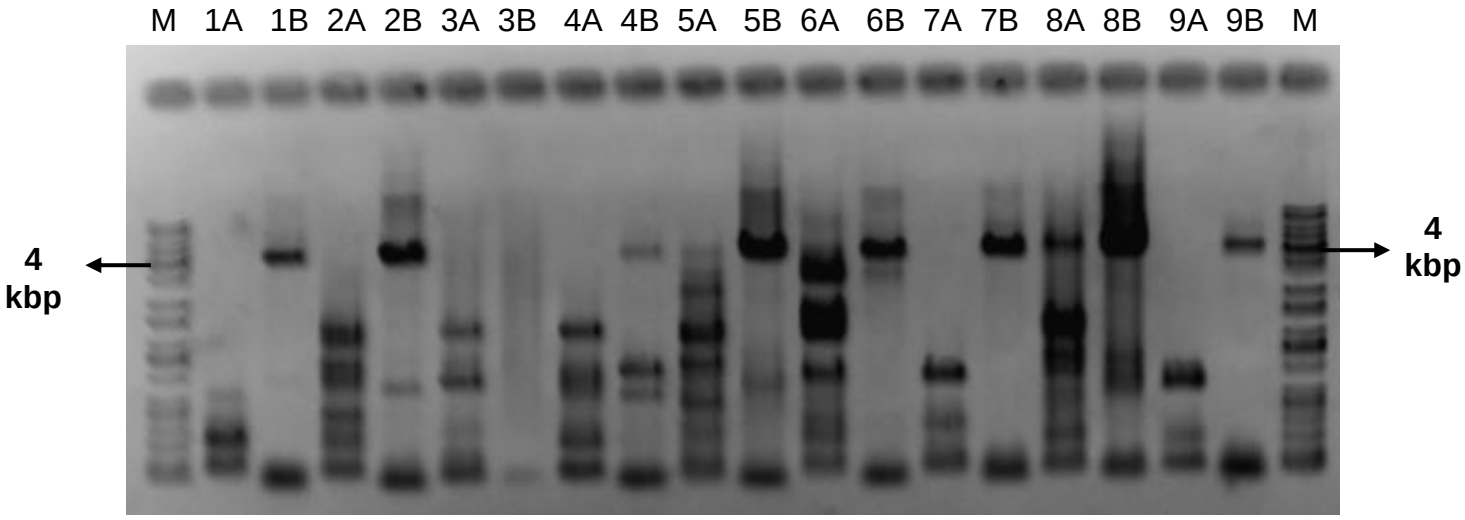


Figure 4.1: A negative inverted 0.7% gel image of the long range PCR products for nine samples, with amplicon of the chimeric *CYP11B1/CYP11B2* loaded in lanes 1A to 9A and amplicon of *CYP11B2* loaded in lanes 1B to 9B. Sample number eight (8A) tested positive for the presence of the chimeric *CYP11B1/CYP11B2*, while sample number five (5A) is potentially positive for the chimeric *CYP11B1/CYP11B2* gene. A molecular weight marker ranging from 100 bp to 10,000 bp (KAPA Biosystems) was loaded in lanes M. The expected fragment sizes for the chimeric *CYP11B1/CYP11B2* and *CYP11B2* target regions are 3.9 kbp and 4.1 kbp, respectively. The arrows indicate the position of the 4 kbp marker.

4.2. DNA sequencing

DNA sequencing is considered to be the gold standard for detecting genetic variants. It is however, time-consuming and expensive. In the current study we attempted to reduce the cost of sequencing by first using HRM analysis to screen for the presence of genetic variants. Unfortunately HRM analysis proved to be difficult in the current study despite extensive attempts to optimize the assays. As a result, it was decided not to spend further laboratory resources on HRM analysis. Instead, Sanger DNA sequencing was used to investigate the presence of genetic variants in *NR3C1* (exons 6, 7, 9, 10

and 11), *HSD11B2* (exons 3, 4, and 5), *SCNN1B* (exon 13), *SCNN1G* (exon 13), and *WNK4* (exons 7 and 17) in 90 black hypertensive individuals from Mangaung.

4.2.1. DNA sequencing of the *NR3C1* gene

DNA sequencing identified four genetic variants in the gene that encodes the glucocorticoid receptor *(NR3C1)* (Table 4.1). Interestingly, all four genetic variants identified occur in the hormone binding domain of the glucocorticoid receptor. The hormone binding region consists of residues 496 to 777 and controls the activity of the glucocorticoid receptor through interactions with proteins, hormones and co-activators (Feng *et al.* 2000). All four of the genetic variants were found to be in Hardy-Weinberg equilibrium (HWE) (Table 4.1).

- G145061A in intron 9: A single base change (G>A; rs763457533) in intron 9 was present in heterozygous form in one study participant (Figure 4.2 A). This intronic variant has previously been described (dbSNP, rs763457533), but its effect on BP is not currently known. However, this intronic variant is located in a splice region (Ensembl 2015) and it is therefore possible that this genetic variant could affect the splicing process. Aberrant RNA splicing can have a number of effects, including differential expression levels, skipping of an exon, the generation of a truncated protein, or the intron not being excised and therefore forms part of the expressed protein (Kuivenhoven *et al.* 1996; Webb *et al.* 1996; Nunez *et al.* 1999; Kuehl *et al.* 2001; Busst *et al.* 2011).
- Asp678Asp: A single base change (C>T; rs258751) in exon 10 was identified in 13 study participants (Figure 4.2 B). Eight of the individuals were heterozygous for the Asp678Asp synonymous variant and five individuals were homozygous. The Asp678Asp variant has previously been reported in Asian, African, African American, and Caucasian populations (Feng *et al*. 2000; Chung *et al.* 2009; Souza *et al*. 2014). Despite the lack of an amino acid change, synonymous variants have previously shown to affect gene expression (Duan *et al*. 2003). In a study by Duan *et al.* (2003), a synonymous variant in the dopamine receptor gene has been shown

to affect the production of the dopamine receptor through its effect on the folding and stability of mRNA. However, according to Chung *et al.* (2009), the Asp678Asp synonymous variant is not significantly associated with elevated BP in an African American population.

- Ser766Leu: A single base change (C>T) in exon 11 was present in one participant in heterozygous form (Figure 4.2 C). This sequence variant results in an amino acid change from serine (Ser) to leucine (Leu) at codon 766. The Ser766Leu variant has not previously been described, and its effect on BP is currently unknown.
- Asn767Asn: A single base change (T>C; rs6196) in exon 11 was found in 21 study participants (Figure 4.2 D). Of the 21 individuals, the Asn767Asn variant was present in heterozygous form in 18 individuals, and in homozygous form in three. This synonymous genetic variant has previously been described in several populations, including Asian, African, African American, Mexican American, Canadian and Caucasian populations (Koper *et al.* 1997; Feng *et al*. 2000; Chung *et al.* 2009; Krupoves *et al.* 2011; Souza *et al*. 2014). Chung *et al.* (2009) did not find any significant association between the Asn767Asn variant and elevated BP in a study of African American, Mexican American and European American individuals. However, the Asn767Asn has been associated with glucocorticoid resistance. Krupoves *et al.* (2011) found an association between the Asn767Asn variant and corticosteroid resistance in patients from two Canadian clinics ($p=0.042$). Compared to this, Koper *et al.* (1997) conducted a study in the Netherlands and found that the Asn767Asn variant was not associated with glucocorticoid response. The differences in populations could contribute to the variable association of the Asn767Asn polymorphism and glucocorticoid response. If the binding affinity of the glucocorticoid receptor for cortisol is reduced, cortisol levels are underestimated by the receptor and continuously perceived to be low. The resulting increase in the secretion of cortisol and deoxycorticosterone (DOC)

could result in enhanced activity of the mineralocorticoid receptor (MR) and ultimately elevated BP through plasma volume expansion (Hurley *et al.* 1991).

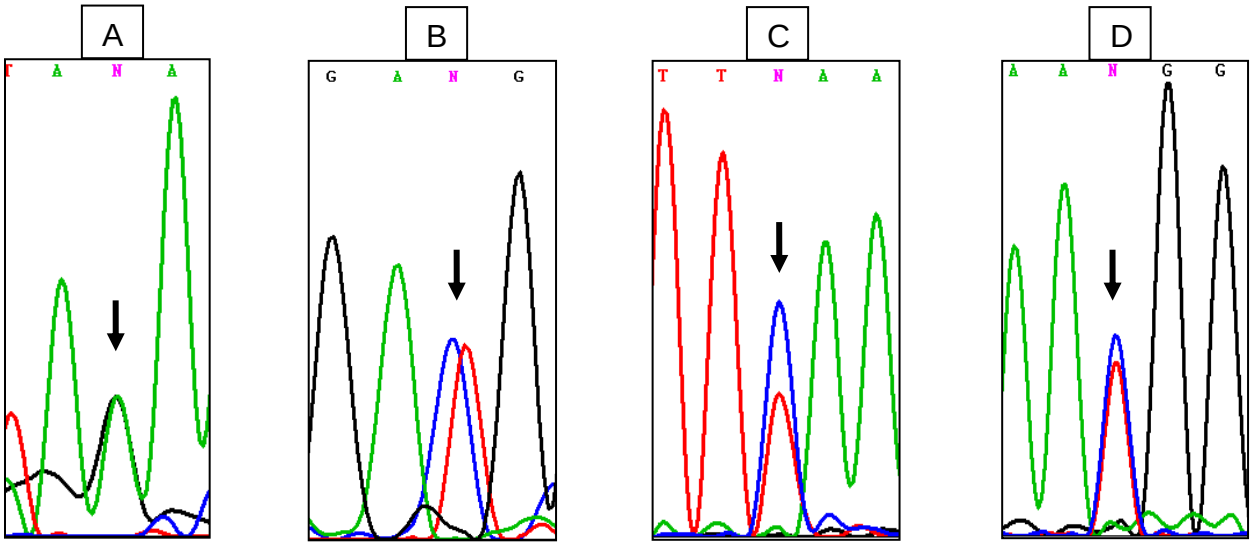


Figure 4.2: Sequencing chromatographs of the genetic variants detected in *NR3C1.* A) Heterozygous G145061A variant detected in intron 9 in sample 181.1; B) Heterozygous Asp678Asp (C>T) variant detected in exon 10 in sample 46.1; C) The novel heterozygous Ser766Leu (C>T) variant detected in exon 11 in sample 80.1; D) Heterozygous Asn767Asn (T>C) variant detected in exon 11 in sample 66.1. The arrows indicate the position of the sequence variants in the chromatographs.

4.2.2. DNA sequencing of the *HSD11B2* gene

Several genetic variants in *HSD11B2* have been identified in the current study (Table 4.2). A total of 35 single nucleotide variants and two deletions in intron 4 were identified in the study cohort. All of the identified genetic variants were in HWE (Table 4.2).

- Trp162Arg: A single base change (T>A) in exon 3 was present in one individual in heterozygous form (Figure 4.3 A). This sequence variant results in an amino acid change from tryptophan (Trp) to arginine (Arg) at codon 162. The Trp162Arg variant has not previously been described, and its effect on BP is currently not known.

- Val173Ala: A single base change (T>C; rs778006489) in exon 3 was present in one individual in heterozygous form (Figure 4.3 B). This sequence variant results in an amino acid change from valine (Val) to alanine (Ala) at codon 173. The Val173Ala variant has previously been described (dbSNP, rs778006489), but the effect of the genetic variant on BP is currently not known.
- Glu178Glu: A single base change (G>A; rs45483293) in exon 3 was present in two individuals in heterozygous form (Figure 4.3 C). Although a positive association between the Glu178Glu variant and hypertension was not found in a French population (Brand *et al.* 1998). Melander *et al.* (2000) suggested that Glu178Glu could increase the susceptibility to primary hypertension in a Swedish population.
- Val191Glu: A single base change (T>A) in exon 3 was present in individual in heterozygous form (Figure 4.3 D). This sequence variant results in an amino acid change from valine (Val) to glutamic acid (Glu) at codon 191. TheVal191Glu variant has not previously been described, and its effect on BP is currently unknown.
- Ala196Ala: A single base change (G>A; rs5480) in exon 3 was identified in 35 individuals (Figure 4.3 E). Of these, 31 were heterozygous and four were homozygous for this synonymous polymorphism. The Ala196Ala variant has been reported previously (dbSNP, rs5480), but is not considered to be clinically significant for hypertension.
- C10021G in intron 3: A single base change (C>G; rs770523823) in intron 3 was present in one individual in heterozygous form (Figure 4.3 F). This variant has previously been reported (dbSNP, rs770523823), but its effect on BP is currently not known. However, this intronic variant (C10021G) lies three nucleotides upstream to another variant in intron 3 (CS951448; C10024T) that has been associated with elevated BP due to aberrant splicing (Nunez *et al.* 1999).
- Val239Met: A single base change (G>A; rs76897343) in exon 4 was present in one individual in heterozygous form (Figure 4.4 A). This sequence variant results in an amino acid change from valine (Val) to methionine (Met) at codon 239. The

Val239Met variant has previously been reported (dbSNP, rs76897343), but its effect on BP is currently not known.

- Glu268X: A single base change (G>T) in exon 4 was present in one individual in heterozygous form (Figure 4.4 B). This novel sequence variant occurs at the last nucleotide in exon 4 and results in the substitution of glutamic acid (Glu) for a stop codon (GAG>UAG). Previous studies have shown that genetic variants in *HSD11B2* that result in a truncated peptide, leads to a decrease in the activity of the HSD11B2 enzyme due to reduced protein stability (Obeyesekere *et al.* 1997; Lavery *et al.* 2003; Quinkler *et al.* 2004). Therefore, it is possible that the Glu268X variant could affect HSD11B2 activity and as a result, have an effect on BP.
- Glu268Lys: A single base change (G>A) in exon 4 was identified in one individual in heterozygous form (Figure 4.4 C). This sequence variant also occurs at the last nucleotide in exon 4, and results in an amino acid change from glutamic acid (Glu) to lysine (Lys) at codon 268. The Glu268Lys variant has not previously been reported and its effect on BP is currently not known.
- G10255A in intron 4: A single base change (G>A) at the beginning of intron 4 was present in two individuals. One of these individuals was heterozygous for this genetic variant, while the other was homozygous (Figure 4.4 D, E). This intronic variant has not previously been described and its effect on BP is currently unknown. However, since the first and last nucleotides of the intron are thought to be necessary for RNA splicing (Ruis *et al.* 1994), it is possible that the G>A transition could affect the splicing process. As mentioned previously, aberrant RNA splicing can have a number of effects on gene expression (Kuivenhoven *et al.* 1996; Webb *et al.* 1996; Nunez *et al.* 1999; Kuehl *et al.* 2001; Busst *et al.* 2011).
- G10281A in intron 4: A single base change (G>A; rs749968834) in intron 4 was present in two individuals, both of whom were heterozygous for this sequence variant. (Figure 4.4 F). This variant has previously been described (dbSNP, rs749968834), but its effect on BP is not currently known. However, this intronic variant (G10281A) occurs in a predicted functional region 21 to 30 nucleotides

downstream from the 5' splice site of intron 4 (Lomelin *et al.* 2010). This predicted functional splice region is thought to contain intronic splicing enhancers and consequently play a role during mRNA processing (Lomelin *et al.* 2010). Several studies have observed that genetic variation in this predicted functional splice region is associated with changes in gene expression through their effect on RNA processing (Matsushima *et al.* 1995; McCarthy and Phillips 1998; Lew *et al.* 2004; Seth *et al.* 2008).

- A10292G in intron 4: A single base change (A>G; rs45574737) in intron 4 was present in 27 individuals (Figure 4.4 G). Of these, two were heterozygous and 25 were homozygous for this sequence variant. Although this intronic variant has previously been described (dbSNP, rs45574737), its effect on BP is currently unknown.
- g.10273_10302del and g.10260_10302del in intron 4: A 30 nucleotide deletion (Figure 4.5) and a 43 nucleotide deletion (Figure 4.6) in intron 4 have been identified in the study cohort. Five of the study participants were homozygous for the 30 nucleotide deletion and one of the study participants was homozygous for the 43 nucleotide deletion. Neither of these intronic deletions has previously been described and their effect on BP is not currently known. However, several intronic deletions that affect the splicing process have been reported previously (Wilson *et al.* 2001a; Ichikawa *et al.* 2006). In addition, both the 30 and 43 nucleotide intronic deletions are estimated to span the functional splice region predicted by Lomelin *et al.* (2010) (Figure 4.5; Figure 4.6). Since the predicted functional splice region is thought to contain intronic splicing enhancers (Lomelin *et al.* 2010), it is possible that the absence of this functional splice region could affect RNA processing in individuals affected by either of the two deletions.
- An additional 17 single nucleotide variants were identified in intron 4 in the study cohort. These 17 variants have not previously been described, and their effect on BP is currently unknown. However, seven of these variants (G10277A, G10278A, G10282A, G10283A, G10283T, G10284A, and G10285A) occur in the predicted

functional splice region 21 to 30 nucleotides downstream from the 5' splice site (Lomelin *et al.* 2010).

- Leu283Leu: A single base change (G>A) in exon 5 was present in one individual in heterozygous form (Figure 4.7 A). The Leu283Leu synonymous variant has not previously been described, and its effect on BP is not currently known.
- Tyr295Tyr: A single base change (C>T) in exon 5 was present in one individual in homozygous form (Figure 4.7 B). The Tyr295Tyr variant has previously been described in a Japanese population (Kamide *et al.* 2006), but its effect on BP is currently unknown.
- Arg405Arg: A single base change (G>A; rs769638876) in exon 5 was present in one individual in heterozygous form (Figure 4.7 C). The Arg405Arg variant has previously been described (dbSNP, rs769638876), but its effect on BP is not currently known.
- Met315Ile: A single base change (G>A; COSM2994871) in exon 5 was present in three individuals, all of whom were heterozygous for the sequence variant (Figure 4.7 D). This variant results in an amino acid change from methionine (Met) to isoleucine (Ile) at codon 315. The Met315Ile variant has previously been described (Ensembl 2015), but its effect on BP is not currently known.
- Arg374Gln: A single base change (G>A; rs45442297) in exon 5 was present in one individual in heterozygous form (Figure 4.7 E). This sequence variant results in an amino acid change from arginine (Arg) to glutamine (Gln) at codon 374. The Arg374Gln variant has previously been described (dbSNP, rs45442297), but its effect on BP is currently unknown.
- Ser396Gly: A single base change (A>G) in exon 5 was identified in one individual in heterozygous form (Figure 4.7 F). This sequence variant results in an amino acid change from serine (Ser) to glycine (Gly) at codon 396. The Ser396Gly variant has not previously been reported, and its effect of BP is currently unknown.

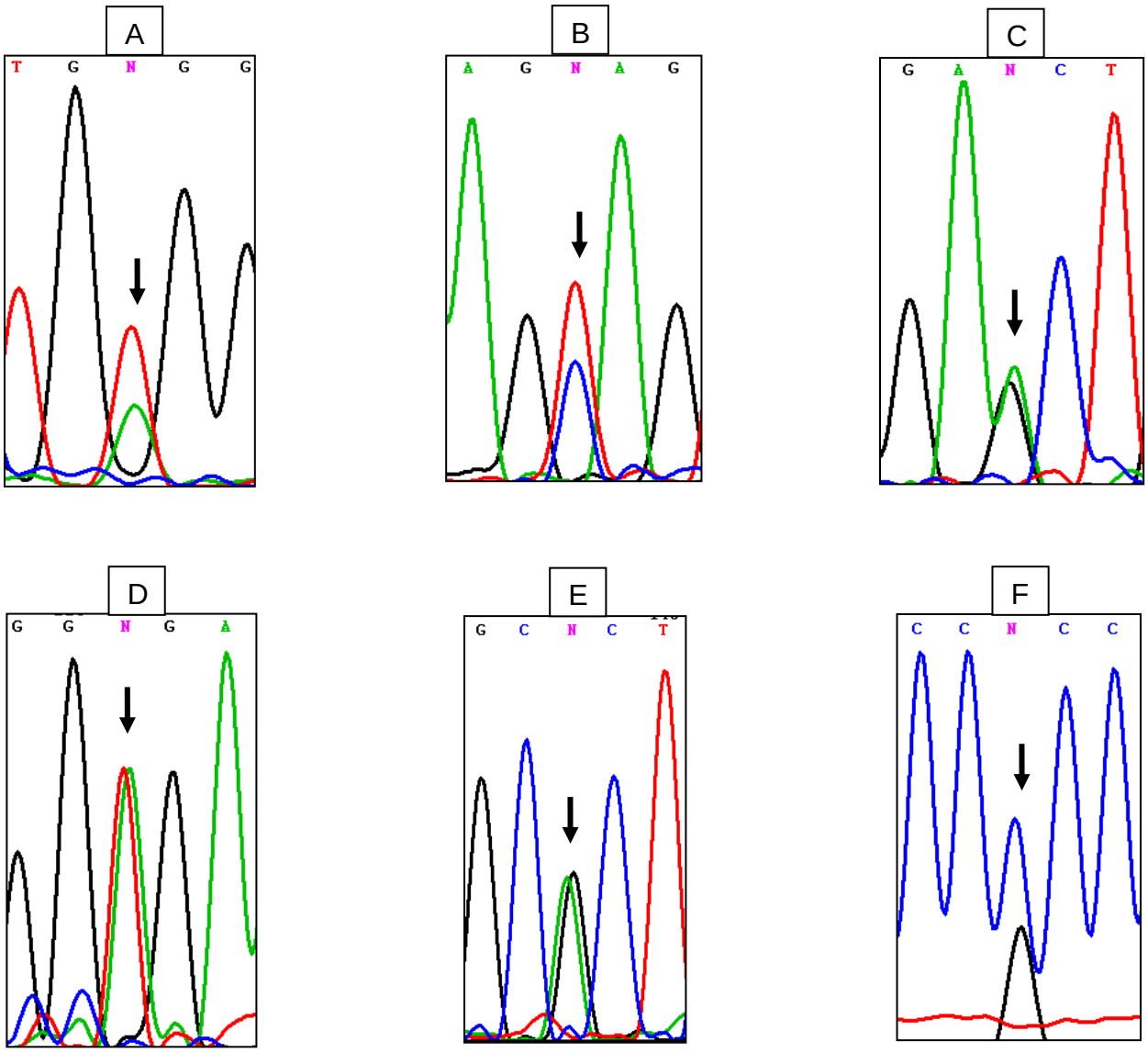


Figure 4.3: Sequencing chromatographs of the genetic variants detected in *HSD11B2* exon 3 (A to E) and intron 3 (F). A) Heterozygous Trp162Arg (T>A) detected in sample 257.1; B) Heterozygous Val173Ala (T>C) detected in sample 208.1; C) Heterozygous Glu178Glu (G>A) variant detected in sample 300.1, D) Heterozygous Val191Glu (T>A) detected in 267.1; E) The synonymous Ala196Ala variant identified in heterozygous form (G>A) in sample 352.1; F) Heterozygous C10021G (C>G) variant in intron 3 identified in sample 315.1. The arrows indicate the position of the sequence variants in the chromatographs.

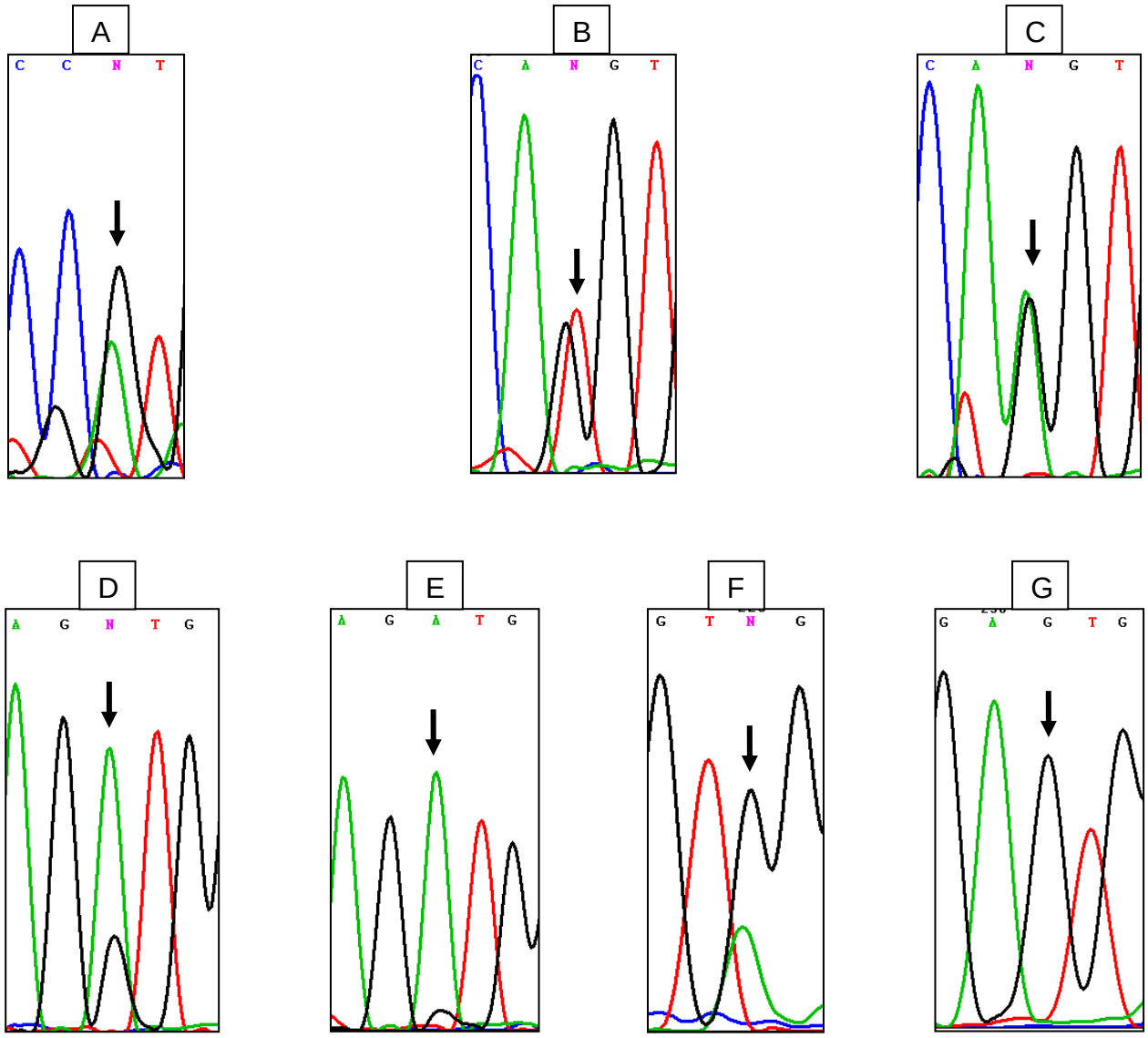


Figure 4.4: Sequencing chromatographs of the genetic variants detected in *HSD11B2* exon 4 (A to C) and three of the genetic variants identified in intron 4 (D to G). A) Heterozygous Val239Met (G>A) variant detected in sample 291.1; B) Heterozygous Glu268X (G>T) variant detected in sample 46.1; C) Heterozygous Glu268Lys (G>A) variant detected in sample 267.2; D to E) The G10255A variant (at the first intronic nucleotide) detected in heterozygous (D) and homozygous (E) form in samples 306.1 and 340.1, respectively; F) Heterozygous G10281A variant detected in sample 68.1; G) Homozygous A10292G variant detected in sample 66.1. The arrows indicate the position of the sequence variants in the chromatographs.

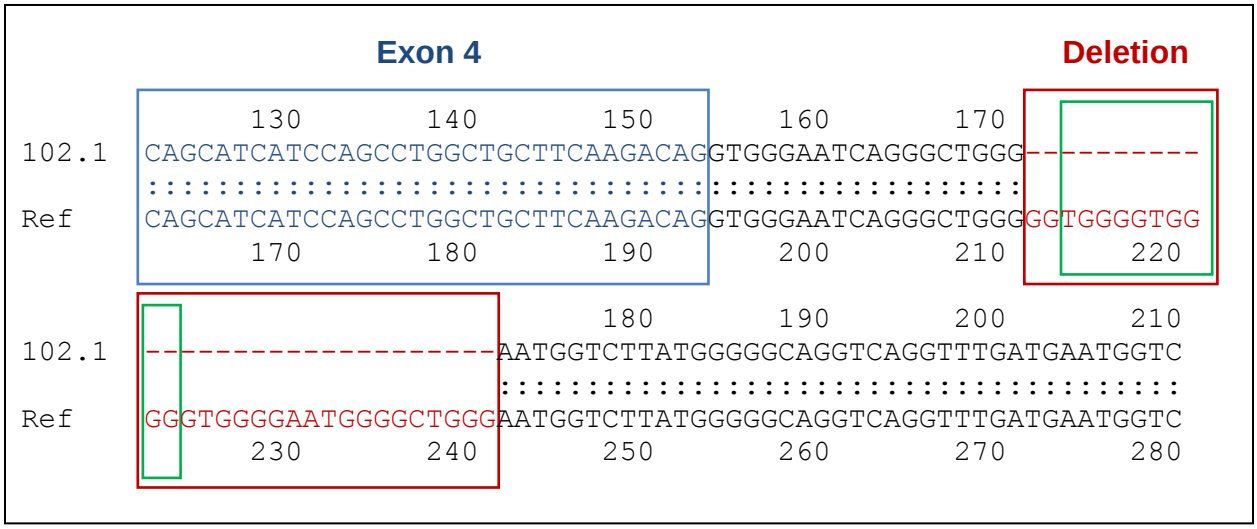


Figure 4.5: Alignment of the homozygous 30 nucleotide deletion variant in *HSD11B2* intron 4 in sample 102.1. The position of the deletion (shown in red) is 19 bases downstream of exon 4. The end of exon 4 is shown in blue. The predicted functional splice region is shown in green.

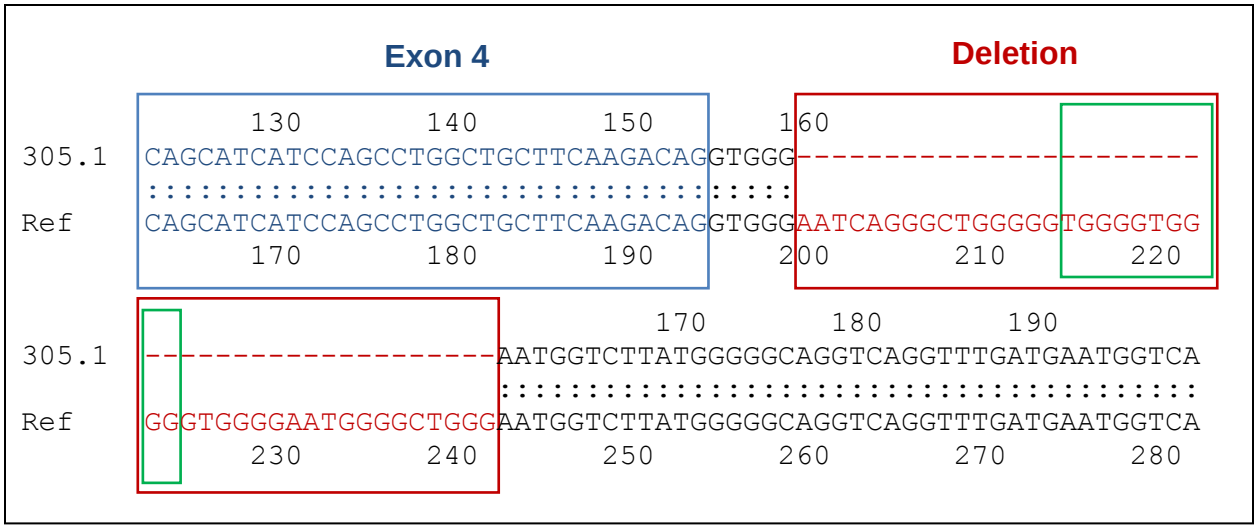


Figure 4.6 Alignment of the homozygous 43 nucleotide deletion variant in *HSD11B2* intron 4 in sample 305.1. The position of the deletion (shown in red) is 6 bases downstream of exon 4. The end of exon 4 is shown in blue. The predicted functional splice region is shown in green.

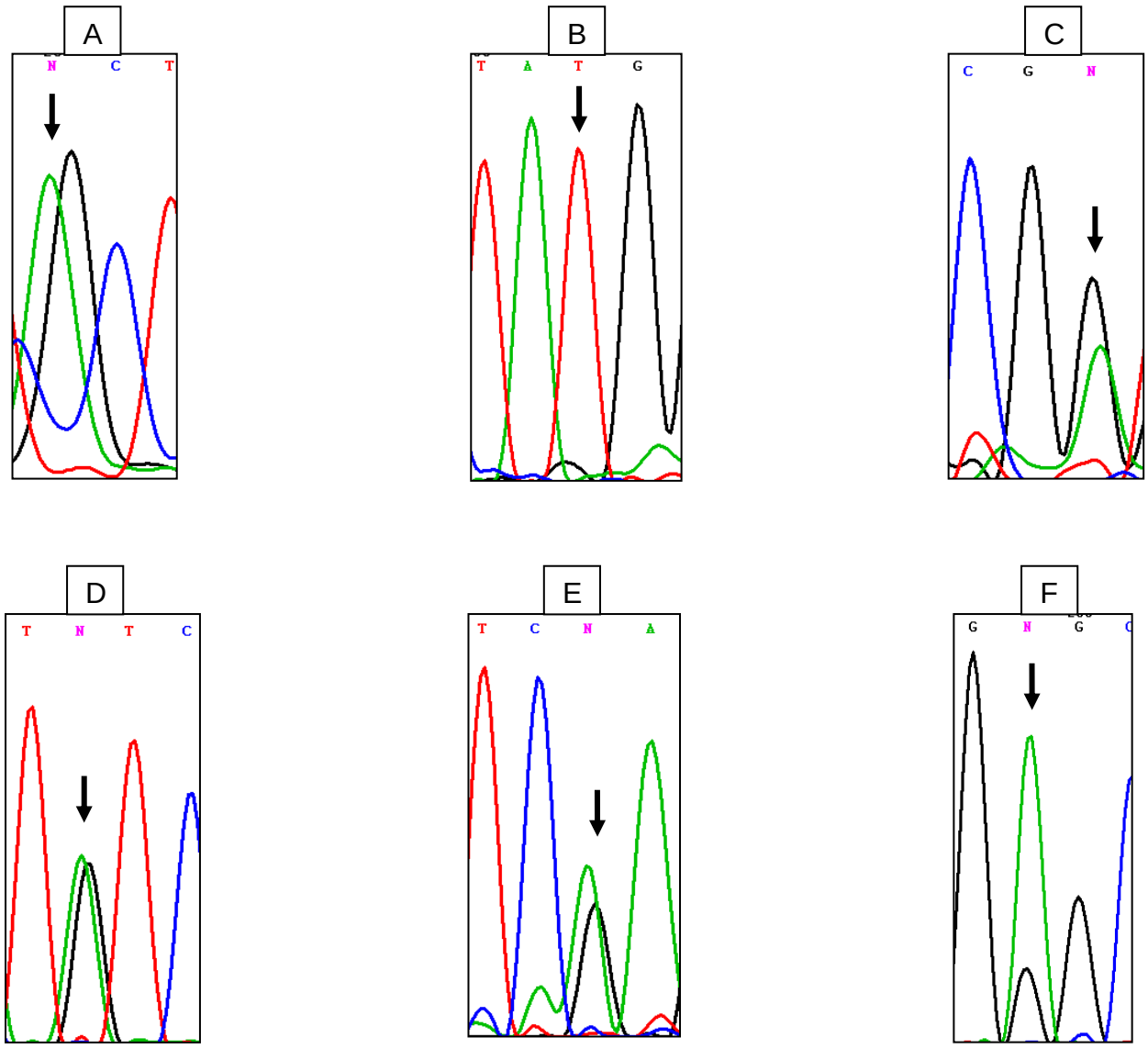


Figure 4.7: Sequencing chromatographs of the genetic variants detected in *HSD11B2* exon 5. A) Heterozygous Leu238Leu (G>A) variant detected in sample 23.1; B) Homozygous Tyr295Tyr (C>T) variant detected in sample 305.1; C) Heterozygous Arg405Arg (G>A) variant detected in sample 86.1; D) Heterozygous Met315Ile (G>A) variant detected in sample 67.1; E) Heterozygous Arg374Gln (G>A) variant detected in sample 345.1; F) Heterozygous Ser396Gly (A>G) variant detected in sample 55.1. The arrows indicate the position of the sequence variants in the chromatographs.

4.2.3. DNA sequencing of the *SCNN1B* gene

DNA sequencing identified six genetic variants in exon 13 of the gene that encodes the beta-subunit of the epithelial sodium channel (ENaC) (Table 4.3). All six of the sequence variants detected in *SCNN1B* were in HWE (Table 4.3).

- Ile545Ile: A single base change (C>T; rs139050851) in exon 13 was present in one individual in heterozygous form (Figure 4.8 A). Although the Ile545Ile variant has previously been described (dbSNP, rs139050851), its effect on BP is currently unknown.
- Arg563Arg: A single base change (G>A) in *SCNN1B* was present in one individual in heterozygous form (Figure 4.8 B). The Arg563Arg variant has not previously been described, and its effect on BP is currently not known.
- Thr577Thr: A single base change (C>T; rs61759923) in exon 13 was present in five individuals, all of whom were heterozygous for the sequence variant (Figure 4.8 C). The Thr577Thr variant has previously been described, and is not considered to be clinically significant to hypertension (dbSNP, rs61759923).
- Gly541Glu: A single base change (G>A) in exon 13 was present in 17 individuals, all of whom were heterozygous for the sequence variant (Figure 4.8 D). This variant results in an amino acid change from glycine (Gly) to glutamic acid (Glu) at codon 541. The Gly541Glu variant has not previously been described, and its effect on BP is not currently known.
- Arg563Gln: A single base change (G>A; rs149868979) in exon 13 was present in five individuals, all of whom were heterozygous for the sequence variant (Figure 4.8 E). This variant results in amino acid change from arginine (Arg) to glutamine (Gln) at codon 563. The Arg563Gln variant has previously been associated with hypertension in black and mixed-ancestry South Africans (Rayner *et al.* 2003; Jones *et al.* 2012). Rayner *et al.* (2003) proposed that the Arg563Gln results in the failure of the ENaC to be efficiently down-regulated and therefore contributes to the development of hypertension in affected individuals. In the study by Rayner *et al.* (2003), the Arg563Gln variant was present in two individuals with more severe

symptoms of low-renin hypertension and hypokalemia, which is indicative of the monogenic form of hypertension known as Liddle syndrome. Rayner *et al.* (2003) suggested that other genetic and environmental factors such as dietary sodium intake could account for the range in phenotype observed in individuals affected by the Arg563Gln variant. Jones *et al.* (2012) reported a positive association between hypertension and the Arg563Gln variant in individuals from urban areas, but not in individuals from rural areas. Jones *et al.* (2012) suggested that the lack of association between Arg563Gln and hypertension in individuals from rural areas could be due to the lower intake of sodium. Thus, the Arg563Gln appears to contribute to the development of hypertension, but it could be dependent on environmental factors such as dietary sodium intake. In addition, Jones *et al.* (2014) reported that the identification of the Arg563Gln in 22 individuals with resistant hypertension, allowed these individuals to be treated with medication aimed specifically at inhibiting the activity of the ENaC. This targeted treatment resulting in an average decrease of 36/17 mmHg ($p<0.0001$ for both systolic and diastolic BP).

- Thr594Met: A single base change (C>T; rs1799979) in exon 13 was present in three patients, all of whom were heterozygous for the sequence variant (Figure 4.8 F). This variant results in an amino acid change from threonine (Thr) to methionine (Met) at codon 594. The Thr594Met variant, which alters a binding site that is thought to play a role in inhibiting the activity of the ENaC, is considered to be a commonly identified mutation in the ENaC in black individuals (Su *et al.* 1996; Cui *et al.* 1997; Baker *et al.* 1998). The Thr594Met variant has been associated with hypertension in black individuals in England through an increase in sodium reabsorption (Baker *et al.* 1998). However, subsequent studies have failed to find an association between the Thr594Met variant and hypertension in individuals of African ancestry, including black South Africans (Persu *et al.* 1998; Nkeh *et al.* 2003). In addition, similar frequencies of the Thr594Met variant allele between normotensive and hypertensive African Americans and black South Africans have been reported (Su *et al.* 1996; Nkeh *et al.* 2003). However, Su *et al.* (1996)

suggested that other genetic or environmental factors could contribute to the development of hypertension in individuals with the Thr594Met variant, which could explain the discrepancy between studies. Thus, the Thr594Met variant does not appear to have a major effect on BP on its own, but could possibly contribute to the development of hypertension in affected individuals.

- Pro617Leu: During optimization of the HRM assay for *SCNN1B* exon 13, a single base change (C>T; CM081432) was identified in one normotensive individual (Chapter 3, Figure 3.6 C). The BP of this individual was 131/64 mmHg, which is classified as pre-hypertensive (NIH 2004). This variant was, however not identified in the cohort of 90 hypertensive individuals. This sequence variant results in an amino acid change from proline (Pro) to leucine (Leu) at codon 617. The Pro617Leu variant (CM081432) has previously been implicated in Liddle syndrome in two Sicilian families (Rossi *et al.* 2008, Rossi *et al.* 2011). Codon 617 forms part of the critical PY motif in the C-terminus that is necessary for degradation of the ENaC channel via Nedd-4 (Snyder *et al.* 1995; Staub *et al*. 1996). Several genetic variants that alter the PY motif have previously been associated with elevated BP (Appendix A, Table 1.6). An expression study has shown that the Pro617Leu variant result in a threefold increase in ENaC activity compared to the WT (Rossi *et al.* 2008). Increased ENaC activity result in more sodium being reabsorbed into the blood, leading to elevated BP through plasma volume expansion (Snyder *et al.* 1995; Uehara *et al*. 1998).

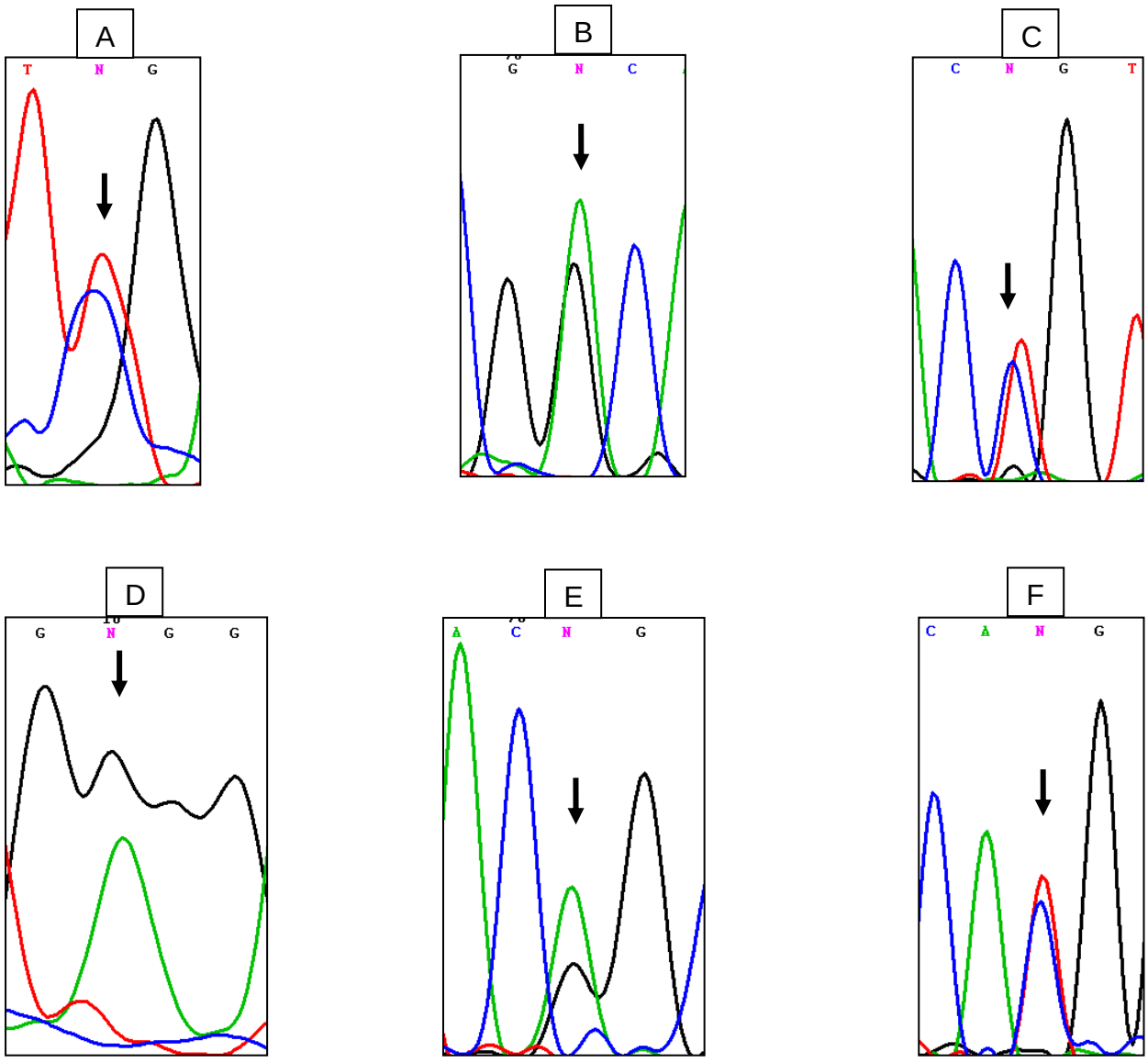


Figure 4.8: Sequencing chromatographs of the genetic variants detected in *SCNN1B* exon 13. A) Heterozygous Ile545Ile (C>T) variant detected in sample 149.1; B) Heterozygous Arg563Arg (G>A) variant detected in sample 153.5; C) Heterozygous Thr577Thr (C>T) variant detected in sample 197.1; D) Heterozygous Gly541Glu (G>A) variant detected in sample 48.1; E) Heterozygous Arg563Gln (G>A) variant detected in sample 267.1; F) Heterozygous Thr594Met (C>T) variant detected in sample 352.1. The arrows indicate the position of the sequence variants in the chromatographs.

4.2.4. DNA sequencing of the *SCNN1G* gene

DNA sequencing identified a genetic variant in exon 13 of the gene that encodes the gamma-subunit of the ENaC. This genetic variant in *SCNN1G* was in HWE (Table 4.4).

- Leu649Leu: A single base change (T>A; rs5723) in *SCNN1G* was identified in 43 individuals (Figure 4.9 A, B, C). Of these, 29 were heterozygous and 14 were homozygous for this synonymous variant. According to literature, the Leu649Leu variant is closely linked to another genetic variant (T>A; rs5729) in the 3' UTR of *SCNN1G* (Vormfelde *et al.* 2007; Barbosa *et al.* 2014). The GA haplotype, consisting of the rs5723 and rs5729 variants, has been associated with higher ENaC activity and it has been suggested that it could predispose individuals to hypertension (Vormfelde *et al.* 2007). Barbosa *et al.* (2014) reported an association between the GA haplotype and BP. However, it is not currently known which of the rs5723 and rs5729 is the causative variant, or perhaps whether another linked variant is involved (Vormfelde *et al.* 2007). In the current study, DNA sequencing did not extend to the 3' UTR and whether the rs5729 variant is present is unknown.

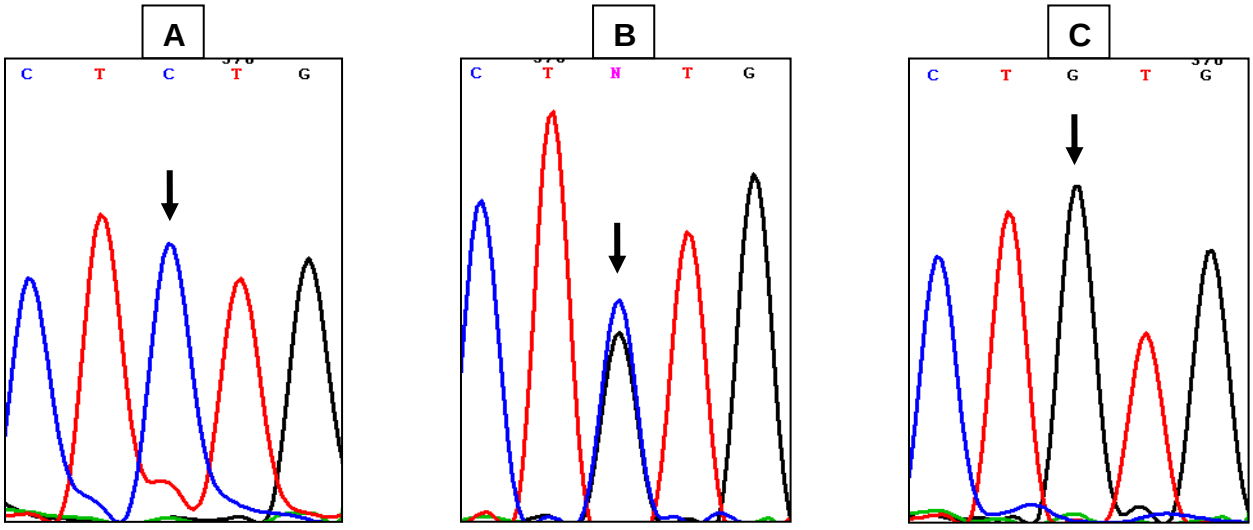


Figure 4.9: Sequencing chromatographs of the genetic variant detected in *SCNN1G* exon 13. A) Wild type; B) Heterozygous Leu649Leu variant detected in sample 47.1; C) Homozygous Leu649Leu variant detected in sample 48.1. The arrows indicate the position of the sequence variants (or wild type nucleotide) in the chromatographs.

4.2.5. DNA sequencing of the *WNK4* gene

DNA sequencing identified five genetic variants in *WNK4* in the study cohort (Table 4.5). All five genetic variants were found to be in HWE (Table 4.5).

- Ala547Ala: A single base change (C>T; rs9916754) in exon 7 was identified in 45 individuals (Figure 4.10 A). Of these, 29 were heterozygous and 16 were homozygous for this synonymous sequence variant. Han *et al.* (2011) reported a significant association between the Ala547Ala variant and hypertension in two Chinese populations. However, a study on a Portuguese population did not find an association between the Ala547Ala variant and hypertension (Mendes *et al.* 2011). According to Han *et al.* (2011), it is possible that another genetic variant that is in linkage disequilibrium with Ala547Ala could be the causative variant of the altered activity of the sodium chloride co-transporter (NCCT).
- His573His: A single base change (C>T; rs56243382) in exon 7 was present in six individuals (Figure 4.10 B). Of these, five were heterozygous and one was homozygous for this synonymous variant. Although the His573His variant has previously been reported, its effect on BP is currently unknown (Mendes *et al.* 2011).
- Pro555Arg: A single base change (C>G) in exon 7 was present in eight individuals (Figure 4.10 C). Of these, six were heterozygous and two were homozygous for this sequence variant. This variant results in an amino acid change from proline (Pro) to arginine (Arg) at codon 555. Although the Pro555Arg variant has been reported previously, its effect on BP is not currently known (Mendes *et al.* 2011).
- A11920C: A single base change (A>C) in intron 7 was present in one individual in heterozygous form (Figure 4.10 D). This intronic variant has not previously been described, and its effect on BP is not currently known.
- Arg1198His: A single base change (A>G; rs61755632) in exon 17 was identified in one individual in heterozygous form (Figure 4.10 E). This sequence variant results in an amino acid change from arginine (Arg) to histidine (His) at codon 1198. Arg1198His has previously been reported (dbSNP, rs61755632), but its effect on BP is currently unknown.

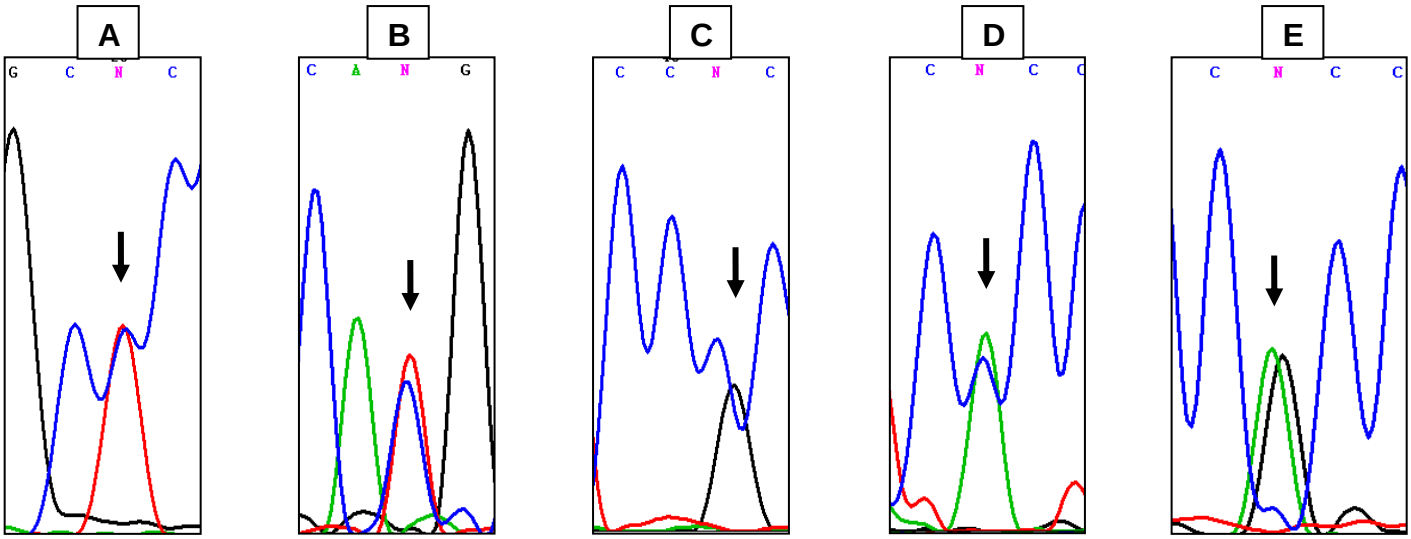


Figure 4.10: Sequencing chromatographs of the genetic variants detected in *WNK4*. A) Heterozygous Ala547Ala (C>T) variant detected in sample 47.2; B) Heterozygous His573His (C>T) variant detected in sample 46.1; C) Heterozygous Pro555Arg (C>G) variant detected in sample 159.1; D) Heterozygous A11920C variant detected in sample 315.1; E) Heterozygous Arg1198His (A>G) variant detected in sample 255.1. The arrows indicate the position of the sequence variants in the chromatographs.

4.3. Conclusion

In the current study, long range PCR allowed the conclusive identification of the chimeric *CYP11B1/CYP11B2* gene in at least one individual in the cohort (Figure 4.1). However, non-specific PCR amplification occurred despite using an annealing temperature of 68^{o}C, which may have lead to false negative results due to competitive primer binding. Thus, the chimeric *CYP11B1/CYP11B2* gene could be more prevalent in this study cohort. It is suspected that non-specific binding of the primers occurred due to the high degree of similarity reported between *CYP11B1* and *CYP11B2* (Mornet *et al*. 1989). For future genetic studies involving the chimeric *CYP11B1/CYP11B2*

gene, new primers that allow more specific amplification could be used in the long range PCR.

In addition, several variants in the genes implicated in monogenic forms of hypertension, namely *NR3C1, HSD11B2, SCNN1B, SCNN1G,* and *WNK4,* have been identified in the current hypertensive study cohort using DNA sequencing. Of these, one variant in *NR3C1* has been previously been associated with glucocorticoid response (Asn767Asn), while two variants in *SCNN1B* (Arg563Gln; Thr594Met), one variant in *SCNN1G* (Leu649Leu) and one variant in *WNK4* (Ala547Ala) have previously been associated with elevated BP. The Asn767Asn variant in *NR3C1* was present in 25% of this study cohort. This synonymous variant has been positively associated with glucocorticoid resistance in a Canadian population, but not in a population in Netherlands (Koper *et al.* 1997; Chung *et al.* 2009; Krupoves *et al.* 2011). Reduced binding affinity of the glucocorticoid receptor for cortisol leads to cortisol levels being underestimated by the receptor. The resulting increase in the secretion of cortisol and deoxycorticosterone (DOC) could result in enhanced activity of the mineralocorticoid receptor (MR) and ultimately elevated BP through plasma volume expansion (Hurley *et al.* 1991). The Arg563Gln variant in *SCNN1B* was present in 6% of this cohort, and has previously been associated with hypertension in black and mixed-ancestry South Africans from urban areas, but not rural areas (Rayner *et al.* 2003; Jones *et al.* 2012). Rayner *et al.* (2003) proposed that the Arg563Gln variant results in the failure of the ENaC to be efficiently down-regulated, which could lead elevated BP through plasma volume expansion (Snyder *et al.* 1995; Uehara *et al*. 1998). The Thr594Met variant in *SCNN1B* was present in 4% of this cohort. The Thr594Met variant has been associated with hypertension in black individuals in one study, but not in subsequent studies on black individuals (Baker *et al.* 1998; Persu *et al.* 1998; Nkeh *et al.* 2003). The Thr594Met variant is thought to alter a binding site that plays a role in inhibiting the activity of the ENaC (Cui *et al.* 1997; Baker *et al.* 1998). The resulting increase in sodium reabsorption could lead elevated BP through plasma volume expansion (Snyder

et al. 1995; Uehara *et al*. 1998). The Leu649Leu variant in *SCNN1G* was identified in 51% of this cohort and is reported to be closely linked to another variant in the 3' UTR of *SCNN1G* (Vormfelde *et al.* 2007; Barbosa *et al.* 2014). The haplotype, consisting of Leu649Leu and the linked variant, has been associated with increased activity of the ENaC and BP (Vormfelde *et al.* 2007; Barbosa *et al.* 2014). The Ala547Ala polymorphism in *WNK4* was identified in 43% of the study cohort. Ala547Ala has been associated with hypertension in a Chinese population, but not in a Portuguese population (Han *et al.* 2011; Mendes *et al.* 2011). In addition to these variants, the Pro617Leu variant in *SCNN1B* was identified in one individual during optimization of this assay for HRM analysis, but was not present in the cohort of 90 hypertensive individuals. The Pro617Leu variant has been implicated in Liddle syndrome, and an expression study has shown that the genetic variant results in a threefold increase in ENaC activity compared to the WT (Rossi *et al.* 2008). For 34 out of 53 sequence variants identified in the 90 hypertensive individuals, the effect on BP is currently unknown. Of these, 26 sequence variants were novel. Further investigation is therefore required to determine if any of these variants could contribute to the high prevalence of hypertension in an urban black population in Mangaung.

It has been suggested that the division between monogenic and polygenic hypertension might not be as definite as previously thought (Persu 2003). For the two variants in *SCNN1B* identified in the study cohort (Arg563Gln and Thr594Met), it has been suggested that other genetic and environmental factors (e.g. dietary sodium intake) contribute to the development of hypertension in affected individuals (Su *et al.* 1996; Rayner *et al.* 2003; Jones *et al.* 2012). However, in a previous study, the Arg563Gln variant was found in a minority of individuals with more severe symptoms indicative of the monogenic form of hypertension known as Liddle syndrome (Rayner *et al.* 2003). Thus, there appears to be an overlap between polygenic hypertension, which is considered to be multifactorial, and monogenic hypertension, which is thought to be primarily due to a genetic component (Lifton *et al.* 2001). In this study, several genetic

variants in genes implicated in monogenic forms of hypertension were detected in a hypertensive cohort. Of these, the chimeric *CYP11B1/CYP11B2* gene, Arg563Gln (*SCNN1B*), Thr594Met (*SCNN1B*), Leu649Leu (*SCNN1G*) and Ala547Ala (*WNK4*) have previously been associated with elevated BP. Thus, the current study has shown that genetic variants in genes implicated in monogenic forms of hypertension, could contribute to the prevalence of hypertension in the black population in Mangaung. However, since this cohort only comprised of hypertensive individuals, the presence of the variants that have been identified in this study needs to be established in normotensive black individuals in Mangaung as well. In addition, the effect of most of the sequence variants identified in the current study on BP is currently unknown. The effect of these genetic variants on BP, especially those that could affect RNA splicing, needs to be investigated in future.

Table 4.1: Summary of the *NR3C1* variants detected in the current study. For each genetic variant the number of participants it was found in, the genotype frequency, the allele frequency, whether the alleles are in Hardy-Weinberg equilibrium (HWE), and the significance to blood pressure (BP) is provided.

Variant description [a]	Location	No. of individuals with the variant	Genotype frequency	Allele frequency	HWE	Significance to BP	Reference
rs763457533; 145 061 G>A	Intron 9	1	G/G: 0.9884 G/A: 0.0116 A/A: 0	G: 0.9942 A: 0.0058	Yes	Splice region variant; significance to BP is unknown	dbSNP, rs763457533
rs258751; Asp678Asp; 157 798 C>T	Exon 10	13	C/C: 0.8523 C/T: 0.0909 T/T: 0.0568	C: 0.8977 T: 0.1023	Yes	Not significantly associated with BP	Chung *et al.* (2009)
Ser766Leu; 58 584 C>T	Exon 11	1	C/C: 0.9880 C/T: 0.0120 T/T: 0	C: 0.9940 T: 0.0060	Yes	Unknown	Not previously described
rs6196; Asn767Asn; 158 588 T>C	Exon 11	21	T/T: 0.7470 T/C: 0.2169 C/C: 0.0361	T: 0.8554 C: 0.1446	Yes	Has been associated with corticosteroid	Koper *et al.* (1997); Krupoves *et al.* (2011)

[a] The nucleotide positions of the genetic variants that were identified in the current study were determined using NG_009062.1 as reference sequence, while amino acid numbering were determined using transcript ENST00000231509 as reference.

Table 4.2: Summary of the *HSD11B2* variants detected in the current study. For each genetic variant the number of participants it was found in, the genotype frequency, the allele frequency, whether the alleles are in Hardy-Weinberg equilibrium (HWE), and the significance to blood pressure (BP) is provided.

Variant description [a]	Location	No. of individuals with the variant	Genotype frequency	Allele frequency	HWE	Significance to BP	Reference
Trp162Arg; 9 830 T>A	Exon 3	1	T/T: 0.9878 T/A: 0.0122 A/A: 0	T: 0.9939 A: 0.0061	Yes	Unknown	Not previously described
rs778006489; Val173Ala; 9 864 T>C	Exon 3	1	T/T: 0.9878 T/C: 0.0122 C/C: 0	T: 0.9939 C: 0.0061	Yes	Unknown	dbSNP, rs778006489
rs45483293; Glu178Glu; 9 880 G>A	Exon 3	2	G/G: 0.9756 G/A: 0.0244 A/A: 0	G: 0.9878 A: 0.0122	Yes	Could increase susceptibility to primary HT	Brand *et al.* (1998); Melander *et al.* (2000)
Val191Glu; 9 918 T>A	Exon 3	1	T/T: 0.9878 T/A: 0.0122 A/A: 0	T: 0.9939 A: 0.0061	Yes	Unknown	Not previously described
rs5480; Ala196Ala; 9 934 G>A	Exon 3	35	G/G: 0.5732 G/A: 0.3780 A/A: 0.0488	G: 0.7622 A: 0.2378	Yes	Not clinically significant	dbSNP, rs5480
rs770523823; 10 021 C>G	Intron 3	1	C/C: 0.9878 C/G: 0.0122 G/G: 0	C: 0.9939 G: 0.0061	Yes	Unknown	dbSNP, rs770523823
rs768979343; Val239Met; 10 167 G>A	Exon 4	1	G/G: 0.9873 G/A: 0.0127 AA: 0	G: 0.9937 A: 0.0063	Yes	Unknown	dbSNP, rs768979343

Variant description [a]	Location	No. of individuals with the variant	Genotype frequency	Allele frequency	HWE	Significance to BP	Reference
10 254 G>T; 10 254 G> A	Exon 4	1 1	G/G: 0.9747 G/T: 0.0127 T/T: 0 G/A: 0.0127 A/A: 0	G: 0.9873 T: 0.0063 A: 0.0063	Yes	Unknown	Not previously described
10 255 G>A	Intron 4	2	G/G: 0.9747 A/G: 0.0127 A/A: 0.0127	G: 0.9810 A: 0.0190	Yes	Unknown	Not previously described
30 nucleotide deletion (g.10 260_10 289)	Intron 4	5	WT: 0.9367 Del/WT: 0 Del/Del: 0.0633	WT: 0.9367 Del: 0.0633	Yes	Unknown	Not previously described
43 nucleotide deletion (g.10 260_10 302)	Intron 4	1	WT: 0.9873 Del/WT: 0 Del/Del: 0.0127	WT: 0.9873 del: 0.0127	Yes	Unknown	Not previously described
10 270 G>A	Intron 4	2	G/G: 0.9747 A/G: 0.0253 A/A: 0	G: 0.9873 A: 0.0127	Yes	Unknown	Not previously described
10 271 G>A	Intron 4	5	G/G: 0.9367 A/G: 0.0506 A/A: 0.0127	G: 0.9621 A: 0.0380	Yes	Unknown	Not previously described
10 273 G>A	Intron 4	2	G/G: 0.9747 A/G: 0.0253 A/A: 0	G: 0.9873 A: 0.0127	Yes	Unknown	Not previously described

Variant description [a]	Location	No. of individuals with the variant	Genotype frequency	Allele frequency	HWE	Significance to BP	Reference
10 278 G>A	Intron 4	1	G/G: 0.9873 G/A: 0.0127 AA: 0	G: 0.9937 A: 0.0063	Yes	Unknown	Not previously described
10 278 G>A	Intron 4	1	G/G: 0.9873 G/A: 0.0127 AA: 0	G: 0.9937 A: 0.0063	Yes	Unknown	Not previously described
10 281 G>A	Intron 4	2	G/G: 0.9747 A/G: 0.0253 A/A: 0	G: 0.9873 A: 0.0127	Yes	Unknown	Not previously described
10 282 G>A	Intron 4	2	G/G: 0.9747 A/G: 0.0127 A/A: 0.0127	G: 0.9810 A: 0.0190	Yes	Unknown	Not previously described
10 283 G>A, 10 283 G>T	Intron 4	1 1	G/G: 0.9747 G/A: 0 A/A: 0.0127 G/T: 0.0127 T/T: 0	G: 0.9810 A: 0.0127 T: 0.0063	Yes	Unknown	Not previously described
10 284 G>A	Intron 4	1	G/G: 0.9873 G/A: 0.0127 AA: 0	G: 0.9937 A: 0.0063	Yes	Unknown	Not previously described
10 284 G>A	Intron 4	1	G/G: 0.9873 G/A: 0.0127 AA: 0	G: 0.9937 A: 0.0063	Yes	Unknown	Not previously described
10 285 G>A	Intron 4	1	G/G: 0.9873 G/A: 0.0127 AA: 0	G: 0.9937 A: 0.0063	Yes	Unknown	Not previously described

Variant description [a]	Location	No. of individuals with the variant	Genotype frequency	Allele frequency	HWE	Significance to BP	Reference
10 287 G>A	Intron 4	2	G/G: 0.9747 A/G: 0.0127 A/A: 0.0127	G: 0.9810 A: 0.0190	Yes	Unknown	Not previously described
10 288 G>A	Intron 4	6	G/G: 0.9241 A/G: 0.0506 A/A: 0.0253	G: 0.9494 A: 0.0506	Yes	Unknown	Not previously described
10 289 G>A	Intron 4	2	G/G: 0.9747 A/G: 0.0253 A/A: 0	G: 0.9873 A: 0.0127	Yes	Unknown	Not previously described
rs45574737; 10 292 A>G	Intron 4	27	A/A: 0.6582 A/G: 0.0253 G/G: 0.3165	A: 0.6709 G: 0.3291	Yes	Unknown	dbSNP, rs45574737
10 294 G>A	Intron 4	1	G/G: 0.9873 G/A: 0 A/A: 0.0127	G: 0.9873 A: 0.0127	Yes	Unknown	Not previously described
10 295 G>A	Intron 4	2	G/G: 0.9747 G/A: 0 A/A: 0.0253	G: 0.9747 A: 0.0253	Yes	Unknown	Not previously described
10 300 G>A	Intron 4	1	G/G: 0.9873 G/A: 0.0127 AA: 0	G: 0.9937 A: 0.0063	Yes	Unknown	Not previously described
10 301 G>A	Intron 4	1	G/G: 0.9873 G/A: 0.0127 AA: 0	G: 0.9937 A: 0.0063	Yes	Unknown	Not previously described

Variant description [a]	Location	No. of individuals with the variant	Genotype frequency	Allele frequency	HWE	Significance to BP	Reference
Leu283Leu; 10 502 G>A	Exon 5	1	G/G: 0.9873 G/A: 0.0127 AA: 0	G: 0.9937 A: 0.0063	Yes	Unknown	Not previously described
Tyr295Tyr; 10 538 C>T	Exon 5	1	G/G: 0.9873 G/A: 0.0127 AA: 0	C: 0.9873 T: 0.0253	Yes	Unknown	Kamide *et al.* (2006)
COSM2994871; Met315Ile; 10 598 G>A	Exon 5	3	G/G: 0.9610 G/A: 0.0390 A/A: 0	G: 0.9805 A: 0.0195	Yes	Unknown	Ensembl, COSM2994871 (2015)
rs45442297; Arg374Gln; 10 774 G>A	Exon 5	1	G/G: 0.9870 A/G: 0.0130 A/A: 0	G: 0.9935 A: 0.0065	Yes	Unknown	dbSNP, rs769638876
Ser396Gly; 10 839 A>G	Exon 5	1	A/A: 0.9870 A/G: 0.0130 G/G: 0	A:0.9935 G: 0.0065	Yes	Unknown	Not previously described
rs769638876; Arg405Arg; 10 868 G>A	Exon 5	1	G/G: 0.9870 A/G: 0.0130 A/A: 0	G: 0.9935 A: 0.0065	Yes	Unknown	dbSNP, rs769638876

[a] The nucleotide positions of the genetic variants that were identified in the current study were determined using NG_016549.1 as reference sequence, while amino acid numbering were determined using transcript ENST00000326152 as reference.

Table 4.3 Summary of the *SCNN1B* variants detected in the current study. For each genetic variant the number of participants it was found in, the genotype frequency, the allele frequency, whether the alleles are in Hardy-Weinberg equilibrium (HWE), and the significance to blood pressure (BP) is provided.

Variant description [a]	Location	No. of individuals with the variant	Genotype frequency	Allele frequency	HWE	Significance to BP	Reference
Gly541Glu; 83 231 G>A	Exon 13	17	G/G: 0.8023 G/A: 0.1977 A/A: 0	G: 0.9012 A: 0.0988	Yes	Unknown	Not previously described
rs139050851; Ile545Ile; 83 244 C>T	Exon 13	1	C/C: 0.9884 C/T: 0.0116 T/T: 0	C: 0.9942 T: 0.0058	Yes	Unknown	dbSNP, rs139050851
rs149868979; Arg563Gln; 83 297 G>A	Exon 13	5	G/G: 0.9419 G/A: 0.0581 A/A: 0	G: 0.9709 A: 0.0291	Yes	Has been associated with hypertension	Rayner *et al.* (2003); Jones *et al.* (2012)
Arg563Arg; 83 298 G>A	Exon 13	1	G/G: 0.9884 A/G: 0.0116 A/A: 0	G: 0.9942 A: 0.0058	Yes	Unknown	Not previously described
rs61759923; Thr577Thr; 83 340 C>T	Exon 13	5	C/C: 0.9419 C/T: 0.0581 T/T: 0	C: 0.9709 T: 0.0291	Yes	Not clinically significant	dbSNP, rs61759923
rs1799979; Thr594Met; 83 390 C>T	Exon 13	3	C/C: 0.9651 C/T: 0.0349 T/T: 0	C: 0.9826 T: 0.0174	Yes	Has been associated with hypertension	Persu *et al*. (1998); Baker *et al*. (2002); Persu (2003)

[a] The nucleotide positions of the genetic variants that were identified in the current study were determined using NG_011908.1 as reference sequence, while amino acid numbering were determined using transcript ENST00000343070 as reference.

Table 4.4: The *SCNN1G* variant detected in the current study. The number of participants it was found in, the genotype frequency, the allele frequency, whether the alleles are in Hardy-Weinberg equilibrium (HWE), and the significance to blood pressure (BP) is provided.

Variant description [a]	Location	No. of individuals with the variant	Genotype frequency	Allele frequency	HWE	Significance to BP	Reference
rs5723; Leu649Leu; 37 748 C>G	Exon 13	43	C/C: 0.4881 C/G: 0.3452 G/G: 0.1667	C: 0.6607 G: 0.3393	Yes	Has been associated with increased channel activity and diastolic BP	Vormfelde *et al.* 2007; Barbosa *et al*. 2014

[a] The nucleotide position of the genetic variant that was identified in the current study was determined using NG_011909.1 as reference sequence, while amino acid numbering were determined using transcript ENST00000300061 as reference.

Table 4.5: Summary of the *WNK4* variants detected in the current study. For each genetic variant the number of participants it was found in, the genotype frequency, the allele frequency, whether the alleles are in Hardy-Weinberg equilibrium (HWE), and the significance to blood pressure (BP) is provided.

Variant description [a]	Location	No. of individuals with the variant	Genotype frequency	Allele frequency	HWE	Significance to BP	Reference
rs9916754; Ala547Ala; 11 821 C>T	Exon 7	45	C/C: 0.4512 C/T: 0.3537 T/T: 0.1951	C: 0.6280 T: 0.3720	Yes	Has been associated with elevated BP	Han *et al.* (2011); Mendes *et al.* (2011)
rs57737815; Pro555Arg; 11 835 C>G	Exon 7	8	C/C: 0.9024 C/G: 0.0732 G/G: 0.0244	C: 0.9390 G: 0.0610	Yes	Unknown	Mendes *et al*. (2011)
rs56243382; His573His; 11 890 C>T	Exon 7	6	C/C: 0.9268 C/T: 0.0610 T/T: 0.0122	C: 0.9573 T: 0.0427	Yes	Unknown	Mendes *et al*. (2011)
11 920 A>C	Intron 7	1	A/A: 0.9878 A/C: 0.0122 C/C: 0	A: 0.9939 C: 0.0061	Yes	Unknown	Not previously described
rs61755632; Arg1198His; 20 654 A>G	Exon 17	1	A/A: 0.9882 A/G: 0.0118 G/G: 0	A: 0.9941 G: 0.0059	Yes	Unknown	dbSNP, rs61755632

[a] The nucleotide positions of the genetic variants that were identified in the current study were determined using NG_007984.1 as reference sequence, while amino acid numbering were determined using transcript ENST00000246914 as reference.

Chapter 5

General Discussion and Conclusion

Previous studies on an urban black population in Mangaung in the Free State have revealed a much higher prevalence of hypertension compared to the average for South Africa (Lategan 2011; Van Zyl *et al.* 2012; WHO 2014a). In a study by Lategan (2011), a positive association was found between BP of the urban black population in Mangaung and adiposity, as well as with sodium intake. Lategan (2011) also investigated the association of BP and polymorphisms associated with primary hypertension. Of these, only one of the polymorphisms was identified as a possible independent risk factor for hypertension in 2% of the Mangaung population (Lategan 2011). Monogenic forms of hypertension include a group of syndromes where hypertension (BP ≥ 140/90 mmHg) is a characteristic, typically as a result of increased sodium reabsorption in the distal nephron. Several genes that play a role in the sodium reabsorption pathway have been implicated in monogenic forms of hypertension (Lifton *et al.* 2001), including the chimeric *CYP11B1/CYP11B2, NR3C1, HSD11B2, CYP11B1, CYP17A1, NR3C2, SCNN1B, SCNN1G, WNK1,* and *WNK4*. Studies have shown that physiological characteristics indicative of increased sodium reabsorption, which are typical of monogenic forms of hypertension, are more commonly found in black hypertensive individuals, including black South Africans (Luft *et al.* 1991; Weinberger 1996; Morris *et al.* 1999; Baker *et al.* 2001; Rayner *et al*. 2001; Sagnella 2001; Franco and Oparil 2006). In the current study, we investigated whether sequence variants in genes implicated in monogenic forms of hypertension could contribute to the high prevalence of hypertension in the Mangaung population. Selected target regions in candidate genes implicated in monogenic hypertension, including the chimeric *CYP11B1/CYP11B2, NR3C1, HSD11B2, SCNN1B, SCNN1G,* and *WNK4*, were screened for previously reported genetic variants in a cohort of 90 black hypertensive individuals from Mangaung.

In the current study, DNA extraction from blood spotted onto FTA® paper, a long range PCR assay, conventional PCR assays, as well as HRM analysis assays, were optimized. After PCR amplification was unsuccessful using FTA® discs prepared according to the Whatman® protocol, as well as DNA extracted from FTA® discs with a modified methanol method, a modified CTAB method was used to extract DNA from FTA® paper. PCR amplification using DNA extracted with a modified CTAB method resulted in a 100% PCR success rate for 18 samples, and was subsequently employed for all further DNA extractions. Long range PCR was used to amplify the chimeric *CYP11B1/CYP11B2* target, along with an endogenous control target. The long range PCR assay resulted in non-specific amplification, possibly due to non-specific primer binding. However, increasing the annealing temperature from 68^{o}C to 70^{o}C did not appear to improve the specificity of the PCR reaction, and 68^{o}C was used in subsequent long range PCR reactions. Conventional PCR was used to amplify selected target regions in *NR3C1* (exons 6, 7, 9, 10 and 11)*, HSD11B2* (exons 3, 4, and 5)*, SCNN1B* (exon 13)*, SCNN1G* (exon 13)*,* and *WNK4* (exons 7 and 17)*.* Except for *HSD11B2* exon 4, the optimal annealing temperature and the optimal annealing and extension times were determined for each primer pair. For the conventional PCR assay for *HSD11B2* exon 4, non-specific amplification was initially encountered. A semi-nested PCR was subsequently performed, which resulted in a single fragment of the correct size. Thereafter, the HRM PCR reaction for all of the assays except for *HSD11B2* exon 4 and the chimeric *CYP11B1/CYP11B,2* was optimized in an attempt to identify samples with potential sequence variants. The HRM PCR reaction was optimized in terms of the dilution factor of the PCR product used as template, as well as the annealing and extension times for each of the primer pairs. However, HRM analysis was only successful in identifying samples with sequence variants for *NR3C1* exon 10, *NR3C1* exon 11, and *SCNN1B* exon 13. In addition, for *NR3C1* exon 10, *NR3C1* exon 11, and *SCNN1B* exon 13, the distance between the difference plot of the WT and the difference plot of the sample(s) made interpretation of the HRM results difficult. As a result of the difficulty experienced with HRM analysis in this study, it was decided not to spend further laboratory resources using this technique. Instead, Sanger sequencing

was used to screen for sequence variants in selected target regions in genes implicated in monogenic forms of hypertension.

Long range PCR using the Q5® High-Fidelity PCR kit (New England Biolabs) was employed to screen for the presence of the chimeric *CYP11B1/CYP11B2* gene in the study cohort. The chimeric *CYP11B1/CYP11B2* gene was present in one individual, and a second individual was identified that could possibly be positive for the chimeric *CYP11B1/CYP11B2*. However, non-specific primer binding occurred with the long range PCR assay despite using an annealing temperature of 68^{o}C, possibly as a result of the high degree of similarity between *CYP11B1* and *CYP11B2* (Mornet *et al*. 1989). As a result, this assay could have produced false negative results and the chimeric *CYP11B1/CYP11B2* could, therefore, be more prevalent in this study cohort. For future genetic studies involving the chimeric *CYP11B1/CYP11B2* gene, new primers that bind more specifically to the target regions could be designed.

Sanger sequencing identified several variants in genes implicated in monogenic forms of hypertension (*NR3C1, HSD11B2, SCNN1B, SCNN1G,* and *WNK*) in a hypertensive cohort from Mangaung. Of these, one variant in *NR3C1* has been previously been associated with glucocorticoid resistance (Asn767Asn). The Asn767Asn variant has previously been associated with glucocorticoid resistance in a Canadian population, but not in a population in the Netherlands (Koper *et al.* 1997; Chung *et al.* 2009; Krupoves *et al.* 2011). Reduced binding affinity of the glucocorticoid receptor for cortisol leads to cortisol levels being underestimated by the receptor. The resulting increase in the secretion of cortisol and deoxycorticosterone (DOC) could result in enhanced activity of the mineralocorticoid receptor (MR) and ultimately elevated BP through plasma volume expansion (Hurley *et al.* 1991; Van Rossum2006). In addition, DNA sequencing identified two variants in *SCNN1B* (Arg563Gln; Thr594Met), one variant in *SCNN1G* (Leu649Leu) and one variant in *WNK4* (Ala547Ala) that have previously been associated with elevated BP (Cui *et al.* 1997; Koper *et al.* 1997; Baker *et al.* 1998;

Persu *et al.* 1998; Nkeh *et al.* 2003; Rayner *et al.* 2003; Vormfelde *et al.* 2007; Chung *et al.* 2009; Krupoves *et al.* 2011; Jones *et al.* 2012; Barbosa *et al.* 2014;). These four variants identified (Arg563Gln, Thr594Met, Leu649Leu, and Ala547Ala) are thought to result in increased activity of the ENaC or the NCCT (Cui *et al.* 1997; Baker *et al.* 1998; Rayner *et al.* 2003; Vormfelde *et al.* 2007; Han *et al.*2011; Barbosa *et al.* 2014). The resulting increase in sodium reabsorption could lead to elevated BP through plasma volume expansion (Snyder *et al.* 1995; Uehara *et al*. 1998). Interestingly, the Arg563Gln variant in *SCNN1B* has been implicated in primary hypertension, as well as the monogenic form of hypertension known as Liddle syndrome (Rayner *et al.* 2003). During optimization of the HRM assay for *SCNN1B* exon 13, another sequence variant (Pro617Leu) that has been implicated in monogenic forms of hypertension, was identified in one individual. The question then is how much of a role does genetic variants implicated in monogenic forms of hypertension play in primary hypertension?

Several authors have suggested that monogenic forms of hypertension could be more common in the general population (Gates *et al.* 1996; Takeda *et al.* 1996; Findling *et al.* 1997; Li *et al.* 1998; O'Shaughnessy *et al.* 1998; Ferrari and Krozowski 2000; Huizenga *et al.* 2000; Wilson *et al*. 2001a; Wilson *et al*. 2001b; Morineau *et al.* 2006; Rossi *et al*. 2008). It has been suggested that the wide range in phenotype in individuals affected by monogenic forms of hypertension could result in patients being misdiagnosed as having primary hypertension instead of the monogenic form (Gates *et al.* 1996; Li *et al.* 1997; Li *et al.* 1998; O'Shaughnessy *et al.* 1998; Gates *et al.* 2001; Hassan-Smith and Stewart 2011). In a previous study, the Arg563Gln variant was found in a minority of individuals with more severe symptoms indicative of the monogenic form of hypertension known as Liddle syndrome (Rayner *et al.* 2003). It was suggested that the range in phenotype in individuals affected by the Arg563Gln could be due to other genetic and/or environmental factors (such as dietary sodium intake) (Rayner *et al.* 2003; Jones *et al.* 2012). Thus, there appears to be an overlap between polygenic hypertension, which is considered to be multifactorial, and monogenic hypertension, which is thought to be primarily due to a genetic component (Lifton *et al.* 2001).

According to Persu (2003), the division between monogenic and polygenic hypertension might not be as definite as previously thought.

To conclude, several sequence variants in genes implicated in monogenic forms of hypertension were identified in 90 hypertensive individuals from Mangaung. Of these, seven of the genetic variants were present in more than 10 individuals (Asp678Asp; Asn767Asn; Ala196Ala; A10292G; Gly541Glu; Leu649Leu; Ala547Ala). A further nine genetic variants were present in more than three individuals in the cohort (30 nucleotide deletion; G10271A; G10288A; Met315Ile; Arg563Gln; Thr577Thr; Thr594Met; Pro555Arg; His573His). The remaining 37 genetic variants that were identified in this study were present in one or two study participants. For most of the sequence variants (34 out of 53) identified in this study, 26 of which were novel, the effect on BP is currently unknown. The combination of these sequence variants identified in genes implicated in monogenic forms of hypertension, is surprisingly high. In this study, it was found that 84 out of 90 hypertensive individuals had one or more sequence variant in a gene implicated in monogenic forms of hypertension. Although the function of many of the sequence variants identified in the current study is unknown, it does suggest that monogenic forms of hypertension may play a greater role in the development of hypertension in the Mangaung population than previously thought.

Summary

Non-communicable diseases (NCDs), also known as chronic diseases of lifestyle, are the leading cause of death worldwide. Amongst the risk factors for NCDs, hypertension (blood pressure (BP) ≥140/90 mmHg) is one of the leading causes of death in South Africa. The prevalence of hypertension in an urban black population in Mangaung in the Free State is reported to be much higher than the average for South Africa. Monogenic forms of hypertension are a group of physiological disorders where the elevated BP is thought to be primarily due to a genetic component. Several genes that play a role in the sodium reabsorption pathway have been implicated in the syndromes associated with monogenic hypertension, including the chimeric *CYP11B1/CYP11B2, NR3C1, HSD11B2, SCNN1B, SCNN1G,* and *WNK4*. In a previous study involving the Mangaung population, it was found that BP correlated positively with adiposity, as well as with sodium intake. In addition, genetic analysis indicated that a genetic variant (A6986G in *CYP3A5*) implicated in primary hypertension, could be an independent risk factor for hypertension in 2% of the Mangaung population. It is not known, however, if genetic variants implicated in monogenic forms of hypertension could play a role in the Mangaung population. The aim of this study was to screen for genetic variants implicated in monogenic forms of hypertension in a black hypertensive cohort from Mangaung.

In this study, a generic CTAB method was successfully used to extract DNA from blood spotted onto FTA® paper, which resulted in successful PCR amplification. Thereafter, a long range PCR assay was successfully optimized in order to amplify the chimeric *CYP11B1/CYP11B2*. Conventional PCR assays to amplify selected target regions in *NR3C1* (exons 6, 7, 9, 10 and 11), *HSD11B2* (exons 3, 4, and 5), *SCNN1B* (exon 13), *SCNN1G* (exon 13) and *WNK4* (exons 7 and 17), were also successfully optimized.

High resolution melting (HRM) analysis was optimized in an attempt to identify samples with potential sequence variants, thereby reducing the cost of sequencing. However, HRM analysis was only successful in identifying samples with sequence variants for *NR3C1* exon 10, *NR3C1* exon 11, and *SCNN1B* exon 13. As a result of the difficulty experienced identifying sequence variants using HRM analysis, it was decided to use DNA sequencing instead to screen for sequence variants in this study cohort.

Long range PCR was used to screen for the presence of the chimeric *CYP11B1/CYP11B2* in this hypertensive cohort. The long range PCR assay allowed the conclusive identification of the chimeric *CYP11B1/CYP11B2* in at least one hypertensive individual and could potentially explain the elevated BP in this individual. However, multiple fragments were produced using the long range PCR assay. It is suspected that the high degree of similarity reported between *CYP11B1* and *CYP11B2* could have resulted in non-specific amplification.

Using DNA sequencing, 53 sequence variants in genes implicated in monogenic forms of hypertension were identified in this hypertensive cohort. Of these, one variant (Asn767Asn) has previously been associated with glucocorticoid resistance and four variants (Arg563Gln, Thr594Met, Leu649Leu, and Ala547Ala) have previously been associated with elevated BP. Out of the 53 sequence variants identified in this study, 26 were novel. The number of sequence variants identified in genes implicated in monogenic forms of hypertension is surprisingly high. Several authors have suggested that monogenic forms of hypertension could be more common in the general population.

To conclude, several sequence variants in genes implicated in monogenic forms of hypertension were identified in a hypertensive cohort from Mangaung. It was found that 84 out of 90 hypertensive individuals had one or more sequence variants in genes implicated in monogenic forms of hypertension. The data from this study suggests that

monogenic forms of hypertension may play an important role in the development of hypertension in the Mangaung population.

Keywords: Monogenic hypertension; genetic variants; hypertension; Mangaung population; conventional polymerase chain reaction (PCR); long range PCR; DNA sequencing.

Opsomming

Nie-oordraagbare siektes (NOS) is die hoof oorsaak van sterftes wêreldwyd. Hipertensie (bloeddruk ≥ 140/90 mmHg) is een van die risikofaktore vir die ontwikkeling van NOS en is een van die hoof oorsake van sterftes in Suid-Afrika. Volgens studies is die voorkoms van hipertensie in ‘n swart bevolking in Mangaung baie hoër as die gemiddeld vir Suid-Afrika. Monogeniese vorme van hipertensie is ‘n groep fisiologiese siektes waarin die verhoogde bloeddruk hoofsaaklik aan ‘n genetiese komponent toegeskryf word. Verskeie gene wat ‘n rol speel in die herabsorpsie van natrium is geassosieer met monogeniese vorme van hipertensie, insluitend die chimeriese *CYP11B1/CYP11B2*, *NR3C1, HSD11B2, SCNN1B, SCNN1G* en *WNK4*. ‘n Vorige studie in die Mangaung bevolking het ‘n positiewe korrelasie tussen bloeddruk en natrium inname gevind, sowel as indikatore van abdominale vetsug en liggaamsvet. Genetiese analise het getoon dat ‘n variant (A6986G in CYP3A5) wat voorheen met primêre hipertensie geassosieer is, moontlik ‘n onafhanklike risikofaktor vir verhoogde bloeddruk in 2% van die Mangaung populasie kan wees. Dit is onbekend of genetiese variante in gene, wat met monogeniese vorme van hipertensie geassosieer is, ‘n rol speel in die Mangaung bevolking. Die doel van hierdie studie was om genetiese variante, wat voorheen met monogeniese vorme van hipertensie geassosieer is, te ondersoek in ‘n swart bevolking van Mangaung.

In die huidige studie is ‘n gemodifiseerde CTAB metode gebruik om DNA te isoleer uit bloed wat op FTA® papier versamel is en dit het tot suksesvolle PKR gelei. Verlengde PKR is geoptimiseer om die chimeriese *CYP11B1/CYP11B2* teiken gebied te amplifiseer. Konvensionele PKR is suksesvol geoptimiseer om teiken gebiede in *NR3C1* (eksons 6, 7, 9, 10 en 11), *HSD11B2* (eksons 3, 4 en 5), *SCNN1B* (ekson 13), *SCNN1G* (ekson 13) en *WNK4* (eksons 7 en 17) te amplifiseer.

Hoë-resolusie-smeltkromme (HRS) analise is ook geoptimiseer om monsters waarin ‘n variant moontlik teenwoordig kan wees te identifiseer en sodoende die koste van DNS volgordebepaling te verminder. HRS kon slegs vir *NR3C1* ekson 10, *NR3C1* ekson 11 en *SCNN1B* ekson 13 suksesvol gebruik word om monsters met potensiële variante te identifiseer. Gevolglik is daar besluit om DNS volgordebepaling te gebruik om variante, wat voorheen met monogeniese vorme van hipertensie geassosieer is, in hierdie studiegroep te ondersoek.

Verlengde PKR is gebruik om die teenwoordigheid van die chimeriese *CYP11B1/CYP11B2,* wat voorheen met ‘n monogeniese vorm van hipertensie geassosieer is, te ondersoek. Verlengde PKR het daarop gedui dat die chimeriese *CYP11B1/CYP11B2* in ten minste een individu in die hipertensiewe groep teenwoordig is, wat moontlik die verhoogde bloeddruk in hierdie individu kan verduidelik. Meervoudige fragmente is egter geproduseer met verlengde PKR. Daar word vermoed dat die hoë graad van ooreenstemming tussen *CYP11B1* en *CYP11B2,* tot nie-spesifieke amplifisering gelei het.

Daar is 53 variante in gene, voorheen met monogeniese vorme van hipertensie geassosieer, deur middel van DNS volgordebepaling in hierdie hipertensiewe groep geïdentifiseer. Een van hierdie variante (Asn767Asn) is voorheen met glukokortikoïed weerstandigheid geassosieer, terwyl vier variante (Arg563Gln, Thr594Met, Leu649Leu en Ala547Ala) voorheen met verhoogde bloeddruk geassosieer is. Van die 53 variante wat geïdentifiseer is, is 26 nie voorheen beskryf nie. Die aantal variante wat in hierdie studie geïdentifiseer is, is verbasend hoog. Verskeie navorsers het voorgestel dat monogeniese vorme van hipertensie meer in die algemene bevolking kan voorkom.

Ten slotte, verskeie variante in gene wat voorheen met monogeniese vorme van hipertensie geassosieer is, is in ‘n hipertensiewe groep van Mangaung geïdentifiseer. Daar is bevind dat een of meer variante in ‘n geen, wat voorheen met monogeniese

vorme van hipertensie geassosieer is, in 84 uit 90 hipertensiewe individue teenwoordig is. Die data van hierdie studie dui daarop dat monogeniese vorme van hipertensie ‘n belangrike rol kan speel in die ontwikkeling van hipertensie in die Mangaung bevolking.

Sleutelterme: Monogeniese hipertensie; genetiese variante; hipertensie; Mangaung bevolking; konvensionele polimerase ketting reaksie (PKR); verlengde PKR; DNS volgordebepaling.

References

Abegunde DO, Mathers CD, Adam T, Ortegon M, Strong K (2007). The burden and cost of chronic diseases in low-income and middle-income countries. *Lancet* 370:1929-1938.

Achard JM, Disse-Nicodeme S, Fiquet-Kempf B, Jeunemaitre X (2001). Phenotypic and genetic heterogeneity of familial hyperkalaemic hypertension (Gordon syndrome). *Clinical and Experimental Pharmacology and Physiology* 28(12): 1048-1052.

Ahlgren R, Yanase T, Simpson ER, Winter JS, Waterman MR (1992). Compound heterozygous mutations (Arg 239→Stop, Pro 342→Thr) in the CYP17 (P450c17 alpha) gene lead to ambiguous external genitalia in a male patient with partial combined 17 alpha-hydroxylase/17,20-lyase deficiency. *The Journal of Clinical Endocrinology and Metabolism* 74(3): 667-672.

Assadi FK, Kimura RE, Subramanian U, Patel S (2002). Liddle syndrome in a newborn infant. *Pediatric Nephrology* 17(8): 609-611.

Atanosov AG, Ignatova ID, Nashev LG, Dick B, Ferrari P, Frey FJ, Odermatt A (2007). Impaired protein stability of 11 beta-hydroxysteroid dehydrogenase type 2: A novel mechanism of apparent mineralocorticoid excess. *Journal of the American Society of Nephrology* 18: 1262-1270.

Audrezet MP, Dabricot A, Le Marechal C, Ferec C (2008). Validation of high-resolution DNA melting analysis for mutation scanning of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene. *Journal of Molecular Diagnostics* 10(5): 424-434.

Baima J, Nicolaou M, Schwartz F, DeStefano AL, Manolis A, Gavras I, Laffer C, Elijovich F, Farrer L, Baldwin CT, Gavras H (1999). Evidence for linkage between essential hypertension and a putative locus on human chromosome 17. *Hypertension* 34(1): 4-7.

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

Baker EH, Ireson NJ, Carney C, Markandu ND, MacGregor GA (2001). Transepithelial sodium absorption is increased in people of African origin. *Hypertension* 38(1): 76-80.

Barbosa EJL, Glad CAM, Nilsson AG, Bosaeus N, Nystrom HF, Svensson PA, Bengtsson BA, Nilsson S, Bosaeus I, Boguszewski CL, Johansson G (2014). Extracellular water and blood pressure in adults with growth hormone (GH) deficiency: A genotype-phenotype association study. *PLoS One* 9(8): e105754.

Barr M, MacKenzie SM, Wilkinson DM, Holloway CD, Friel EC, Miller S, MacDonald T, Fraser R, Connell JMC, Davies E (2006). Functional effects of genetic variants in the 11β-hydoxylase (*CYP11B1*) gene. *Clinical Endocrinology* 65: 816-825.

Beeks E, Kessels AGH, Kroon AA, van der Klauw MM, de Leeuw PW (2004). Genetic predisposition to salt-sensitivity: a systematic review. *Journal of Hypertension* 22(7): 1243-1249.

Benetti-Pinto CL, Vale D, Garmes H, Bedone A (2007). 17-hydroxyprogesterone deficiency as a cause of sexual infantilism and arterial hypertension: Laboratory and molecular diagnosis – a case report. *Gynecological Endocrinology* 23(2): 94-98.

Bhangoo A, Aisenberg J, Chartoffe A, Ten S, Wallerstein RJ, Wolf R, Auchus RJ (2008). Novel mutation in cytochrome P450c17 causes complete combined 17alpha-hydroxylase-17,20-lyase deficiency. *Journal of Pediatric Endocrinology and Metabolism* 21(2): 185-190.

Biason-Lauber A, Kempken B, Werder E, Forest MG, Einaudi S, Ranke MB, Matsuo N, Brunelli V, Schönle EJ, Zachmann M (2000). 17α-Hydroxylase/17,20-Lyase Deficiency as a Model to study enzymatic activity regulation: role of phosphorylation. *The Journal of Clinical Endocrinology and Metabolism* 85(3): 1126-1231.

Biglieri EG, Herron MA, Brust N (1966). 17-hydroxylation deficiency in man. *Journal of Clinical Investigation* 45: 1946-1954.

Blanchard A, Gérard F, Paillard M (2002). Hyperaldostéronisme primaire: physiopathologie, investigations et traitements. *Sang Thrombose Vaisseaux* 14(8), 455-465.

Bogdanović R, Kuburović V, Stajić N, Mughal SS, Hilger A, Ninić S, Prijić S, Ludwig M (2012). Liddle syndrome in a Serbian family and literature review of underlying mutations. *European Journal of Pediatrics* 171: 471-478.

Botero-Velez M, Curtis JJ, Warnock DG (1994). Brief report: Liddle's syndrome revisited – a disorder of sodium reabsorption in the distal tubule. *The New England Journal of Medicine* 330(3): 178-181.

Bouligand J, Delemer B, Hecart AC, Meduri G, Viengchareun S, Amazit L, Trabado S, Feve B, Guiochon-Mantel A, Young J, Lombes M (2010). Familial glucocorticoid receptor haploinsufficiency by non-sense mediated mRNA decay, adrenal hyperplasia and apparent mineralocorticoid excess. *PLoS One* 5(10): e13563.

Boyden LM, Choi M, Choate KA, Nelson-Williams CJ, Farhi A, Toka HR, Tikhonova IR, Bjornson R, Mane SM, Colussi G, Lebel M, Gordon RD, Semmekrot BA, Poujol A, Välimäki MJ, De Ferrari ME, Sanjad SA, Gutkin M, Karet FE, Tucci JR, Stockigt

JR, Keppler-Noreuil KM, Porter CC, Anand SK, Whiteford ML, Davis ID, Dewar SB, Bettinelli A, Fadrowski JJ, Belsha CW, Hunley TE, Nelson RD, Trachtman H, Cole TR, Pinsk M, Bockenhauer D, Shenoy M, Vaidyanathan P, Foreman JW, Rasoulpour M, Thameem F, Al-Shahrouri HZ, Radhakrishnan J, Gharavi AG, Goilav B, Lifton RP (2012). Mutations in kelch-like 3 and cullin 3 cause hypertension and electrolyte abnormalities. *Nature* 482(7383): 98-102.

Bradshaw D, Steyn K, Levitt N, Nojilana B (2011). Non-communicable diseases - A race against time. *MRC Policy Brief September 2011*. [Online]. Available at: www.mrc.ac.za/policybriefs/raceagainst.pdf; accessed 28 June 2013.

Brand E, Kato N, Chatelain N, Krozowski ZS, Jeunemaitre X, Corvol P, Plouin PF, Cambien F, Pascoe L, Soubrier F (1998). Structural analysis and evaluation of the 11 beta-hydroxysteroid dehydrogenase type 2 (*11beta-HSD2*) gene in human essential hypertension. *Journal of Hypertension* 16(11): 1627-1633.

Brautbar N, Levi J, Rosler A, Leitesdorf E, Kjaldeti M, Epstein M, Kleeman CR (1978). Familial hyperkalemia, hypertension and hyporeninemia with normal aldosterone levels. A tubular defect in potassium handling. *Archives of Internal Medicine* 138(4): 607-610.

Brooke AM, Taylor NF, Shepherd JH, Gore ME, Ahmad T, Lin L, Rumsby G, Papari-Zareei M, Auchus RJ, Achermann JC, Monson JP (2006). A Novel Point Mutation in P450c17 (CYP17) Causing combined 17a-hydroxylase/17,20-lyase deficiency. *The Journal of Clinical Endocrinology and Metabolism* 91(6): 2428-2431.

Brooks AM, Owens M, Sayer JA, Salzmann M, Ellard S, Vaidya B (2011). Pseudohypoaldosteronism type 2 presenting with hypertension and hyperkalaemia due to a novel mutation in the *WNK4* gene. *Quarterly Journal of Medicine (Association of Physicians)* 105(8): 791-794.

Busst CJ, Bloomer LDS, Scurrah KJ, Ellis JA, Barnes TA, Charchar FJ, Braund P, Hopkins PN, Samani NJ, Hunt SC, Tomaszewski M, Harrap SB (2011). The epithelial sodium channel γ-subunit gene and blood pressure: Family based association, renal gene expression, and physiological analysis. *Hypertension* 58: 1073-1078.

Carvajal CA, Gonzalez AA, Romero DG, Gonzalez A, Mosso LM, Lagos ET, Hevia MDP, Rosati MP, Perez-Acle TO, Gomez-Sanchez CE, Montero JA, Fardella CE (2003). Two homozygous mutations in the 11β-hydroxysteroid dehydrogenase type 2 gene in a case of apparent mineralocorticoid excess. *The Journal of Clinical Endocrinology and Metabolism* 88(6): 2501-2507.

Chang SH, Lee HH, Wang PJ, Chen JH, Chu SY (2004). Congenital adrenal hyperplasia with 11 beta-hydroxylase deficiency. *Journal of the Formosan Medical Association* 103(11): 860-864.

Charmandari E, Raji A, Kino T, Ichijo T, Tiulpakov A, Zachman K, Chrousos GP (2005). A novel point mutation in the ligand-binding domain (LBD) of the human glucocorticoid receptor (hGR) causing generalized glucocorticoid resistance: the importance of the C terminus of hGR LBD in conferring transcactivational activity. *The Journal of Clinical Endocrinology and Metabolism* 90(6): 3696-3705.

Charmandari E, Kino T, Ichijo T, Jubiz W, Meijia L, Zachman K, Chrousos GP (2007). A novel point mutation in helix 11 of the ligand-binding domain of the human glucocorticoid receptor gene causing generalized glucocorticoid resistance. *The Journal of Clinical Endocrinology and Metabolism* 92(10): 3986-3990.

Chrousos GP, Vingerhoeds A, Brandon D, Eil C, Pugeat M, DeVroede M, Loriaux DL, MB Lipsett (1982). Primary cortisol resistance in man. *The Journal of Clinical Investigation* 69: 1261-1269.

Chrousos GP, Detera-Wadleigh SD, Karl M (1993). Syndromes of glucocorticoid resistance. *Annuals of Internal Medicine* 119(11): 1113-1124.

Chua SC, Szabo P, Vitek A, Grzeschik KH, John M, White PC (1987). Cloning of cDNA encoding steroid 11β-hydroxylase (P450c11).

Chung BC, Picado-Leonard J, Haniu M, Bienkowski M, Hall PF, Shively JE, Miller WL (1987). Cytochrome P450c17 (steroid 17α-hydroxylase/17,20 lyase): Cloning of human adrenal and testis cDNAs indicates the same gene is expressed in both tissues. *Proceedings of the National Academy of Sciences of the USA* 84: 407-411.

Chung CC, Shimmin L, Nataraian S, Hanis CL, Boerwinkle E, Hixson JE (2009). Glucocorticoid receptor gene variant in the 3' untranslated region is associated with multiple measures of blood pressure. *The Journal of Clinical Endocrinology and Metabolism* 94(1): 268-276.

Ciechanowicz A, Dolezel Z, Placha G, Starha J, Góra J, Gaciong Z, Brodkiewicz A, Adler G (2005). Liddle syndrome caused by P616R mutation of the epithelial sodium channel β subunit. *Pediatric Nephrology* 20: 837-838.

Coeli FB, Ferraz LFC, De Lemos-Marini SHV, Rigatto SZP, Belangero VMS, De-Mello MP (2008). Apparent mineralocorticoid excess syndrome in a Brazilian boy caused by the homozygous missense mutation p.R186C in the *HSD11B2* gene. *Arquivos Brasileiros de Endocrinologia & Metabologia* 52(8): 1277-1281.

Coffman TM (2006). A WNK in the kidney controls blood pressure. *Nature Genetics* 38: 1105-1106. [Online]. Available at: http://www.nature.com/ng/journal/v38/n10/fig_tab/ng1006-1105_F1.html; accessed 8 January 2015.

Connell JMC, Fraser R, Davies E (2001). Disorders of mineralocorticoid synthesis. *Best practice & Research Clinical Endocrinology and Metabolism* 15(1): 43-60.

Connor M, Rheeder P, Bryer A, Meredith M, Beukes M, Dubb A, Fritz V (2005). The South African stroke risk in general practice study. *South African Medical Journal* 95(5): 334-339.

Costa-Santos M, Kater CE, Auchus RJ, Brazilian Congenital Adrenal Hyperplasia Multicenter Study Group (2004). Two prevalent *CYP17* mutations and genotype-phenotype correlations in 24 Brazilian patients with 17α-hydroxylase deficiency. *The Journal of Clinical Endocrinology and Metabolism* 89(1): 49-60.

Costenaro F, Rodrigues TC, Kater CE, Auchus RJ, Papari-Zareei M, Czepielewski MA (2010). Combined 17α-hydroxylase/17,20-lyase deficiency due to p.R96W mutation in the *CYP17* gene in a Brazilian patient. *Arquivos Brasileiros de Endocrinologia & Metabologia* 54(8): 744-748.

Curnow KM, Slutsker L, Vitek J, Cole T, Speiser PW, New MI, White PC, Pascoe L (1993). Mutations in the *CYP11B1* gene causing congenital adrenal hyperplasia and hypertension cluster in exons 6, 7 and 8. *Proceedings of the National Academy of Sciences of the USA* 90: 4552-4556.

Cui Y, Su YR, Rutkowski M, Reif M, Menon AG, Pun RYK (1997). Loss of protein kinase C inhibition in the β-T594M variant of the amiloride-sensitive Na^+ channel. *Proceedings of the National Academy of Sciences of the USA* 94: 9962-9966.

Cutler JA, Sorlie PD, Wolz M, Thom T, Fields LE, Rocella EJ (2008). Trends in hypertension prevalence, awareness, treatment, and control rates in United States adults between 1988-1994 and 1999-2004. *Hypertension* 52(5): 818-827.

Dahl LK (1972). Salt and hypertension. *The American Journal of Clinical Nutrition* 25: 231-244.

Database of Single Nucleotide Polymorphisms (dbSNP). Bethesda (MD): National Center for Biotechnology Information (NCBI), National Library of Medicine. dbSNP accession: rs763457533; rs778006489; rs5480; rs770523823; rs768979343; rs45574737; rs769638876; rs769638876; rs139050851; rs61759923; rs61755632.

[Online]. Available at: http://www.ncbi.nlm.nih.gov/SNP/; accessed 31 January 2016.

Dave-Sharma S, Wilson RC, Harbison MD, Newfield R, Azar MR, Krozowski ZS, Funder JW. Shackleton CHL, Bradlow HL, Wei JQ, Hertecant J, Moran A, Neiberger RE, Balfe JW, Fattah A, Daneman D, Akkurt HI, de Santis C, New MI (1998). Examination of genotype and phenotype relationships in 14 patients with apparent mineralocorticoid excess. *The Journal of Clinical Endocrinology and Metabolism* 83(7): 2244-2254.

Delaloy C, Elvira-Matelot E, Clemessy M, Zhou XO, Imbert-Teboul M, Houot AM, Jeunemaitre X, Hadchouel J (2008). Deletion of WNK1 first intron results in misregulation of both isoforms in renal and extra-renal tissues. *Hypertension* 52(6): 1149-1154.

Dhir V, Reisch N, Bleicken CM, Lebl J, Kamrath C, Schwarz HP, Grotzinger J, Sippell WG, Riepe FG, Arit W, Krone N (2009). Steroid 17α-hydroxylase deficiency: functional characterization of four mutations (A174E, V178D, R440C, L465P) in the *CYP17A1* gene. *The Journal of Clinical Endocrinology and Metabolism* 94(8): 3058-3064.

Di Cerbo A, Biason-Lauber A, Savino M, Piemontese MR, Di Giorgio A, Perona M, Savoia A (2002). Combined 17alpha-hydroxylase/17,20-lyase deficiency caused by Phe93Cys mutation in the *CYP17* gene. *The Journal of Clinical Endocrinology and Metabolism* 87(2): 898-905.

Dluhy RG, Lifton RP (1995). Glucocorticoid-remediable aldosteronism (GRA): Diagnosis, variability of phenotype and regulation of potassium homeostasis. *Steroids* 60(1): 48-51.

Du Toit E (2014). Development and application of a real-time PCR method to detect selected single nucleotide polymorphisms associated with hypertension in a black South African population. Unpublished Master's thesis. University of the Free

State, Bloemfontein, South Africa. Unpublished Master's dissertation. University of the Free State. [Online]. Available at: http://scholar.ufs.ac.za:8080/xmlui/handle/11660/2125; accessed 19 February 2016.

Duan J, Wainwright MS, Comeron JM, Saitou N, Sanders AR, Gelernter J, Gejman PV (2003). Synonymous mutations in the human *dopamine receptor D2 (DRD2)* affect mRNA stability and synthesis of the receptor. *Human Molecular Genetics* 12(3): 205-216.

Dumic K, Wilson R, Thanasawat P, Grubic Z, Kusec V, Stingl K, New MI (2010). Steroid 11-beta hydroxylase deficiency caused by compound heterozygosity for a novel mutation in intron 7 (IVS 7 DS+4A to G) in one *CYP11B1* allele and R448H in exon 8 in the other. *European Journal of Pediatrics* 169: 891-894.

Edelheit O, Hanukoglu I, Gizewska M, Kandemir N, Tenenbaum-Rakover Y, Yurdakok M, Zajaczek S, Hanukoglu A (2005). Novel mutations in epithelial sodium channel (ENaC) subunit genes and phenotypic expression of multisystem pseudohypoaldosteronism. *Clinical Endocrinology (Oxford)* 62(5): 547-553.

Edwards CRW, Burt D, McIntyre MA, de Kloet ER, Stewart PM, Brett L, Sutanto WS, Monder C (1988). Localisation of 11β-hydroxysteroid dehydrogenase-tissue specific protector of the mineralocorticoid receptor. *Lancet* ii: 986-989.

Ensembl (genome browser 83) (2015), variant ID: COSM2994871. [Online]. Available at: http://www.ensembl.org/Homo_sapiens/Variation/Explore?r=16:67436230-67437230;v=COSM2994871;vdb=variation;vf=154339646; accessed 30 January 2016.

Ergun-Longmire B, Auchus R, Papari-Zareei M, Tansil S, Wilson RC, New MI (2006). Two novel mutations found in a patient with 17α-hydroxylase enzyme deficiency. *The Journal of Clinical Endocrinology and Metabolism* 91(10): 4179-4182.

Escher G, Cristiano M, Causevic M, Baumann M, Frey FJ, Surbek D, Mohaupt MG (2009). High aldosterone-to-renin variants of *CYP11B2* and pregnancy outcome. *Nephrology, Dialysis, Transplantation* 24(6): 1870-1875.

Fallo F, Pilon C, Williams TA, Sonino N, Morra Di Cella S, Veglio F, De Lasio R, Montanari P, Mulatero P (2004). Coexistence of different phenotypes in a family with glucocorticoid-remediable aldosteronism. *Journal of Human Hypertension* 18(1): 47-51.

Fardella CE, Hum DW, Homoki J, Miller WL (1994). Point mutation of Arg440 to His in cytochrome P450c17 causes severe 17α-hydroxylase deficiency. *The Journal of Clinical Endocrinology and Metabolism* 79(1): 160-164.

Fardella CE, Mosso L, Gomez-Sanchez C, Cortes P, Soto J, Gomez L, Pinto M, Huete A, Oestreicher E, Foradori A, Montero J (2000). Primary hyperaldosteronism in essential hypertensives: Prevalence, biochemical profile, and Molecular Biology. *The Journal of Clinical Endocrinology and Metabolism* 85(5): 1863-1867.

Feng J, Zheng J, Bennett WP, Heston LL, Jones IR, Craddock N, Sommer SS (2000). Five missense variants in the amino-terminal domain of the glucocorticoid receptor: no association with puerperal psychosis or schizophrenia. *American Journal of Medical Genetics (Neuropsychiatric Genetics)* 96(3): 412-417.

Ferrari P, Krozowski Z (2000). The role of the 11B-hydroxysteroid dehydrogenase type 2 in blood pressure regulation. *Kidney International* 57: 1374-1381.

Ferrari P (2010). The role of 11β-hydroxysteroid dehydrogenase type 2 in human hypertension. *Biochimica et Biophysica Acta* 1802: 1178-1187.

Findling JW, Raff H, Hansson JH, Lifton RP (1997). Liddle's syndrome: Prospective genetic screening and suppressed aldosterone secretion in an extended kindred. *The Journal of Clinical Endocrinology and Metabolism* 82(4): 1071-1074.

Flack JM, Neaton J, Grimm R Jr, Shih J, Cutler J, Ensrud K, MacMahon S (1995). Blood pressure and mortality among men with prior myocardial infarction. Multiple Risk Factor Intervention Trial Research Group. *Circulation* 92: 2437-2445.

Flack JM, Sica DA, Bakris G, Brown AL, Ferdinand KC, Grimm RH Jr, Hall WD, Jones WE, Kountz DS, Lea JP, Nasser S, Nesbitt SD, Saunders E, Scisney-Matlock M, Jamerson KA (2010). Management of high blood pressure in blacks: An update of the International society on hypertension in blacks consensus statement. *Hypertension* 56(5): 780-800.

Franco V, Oparil S (2006). Salt sensitivity, a determinant of blood pressure, cardiovascular disease and survival. *Journal of the American College of Nutrition* 25(33): 247S-255S.

Freundlich M, Ludwig M (2005). A novel epithelial sodium channel β-subunit mutation associated with hypertensive Liddle syndrome. *Pediatric Nephrology* 20:512-515.

Funder JW, Pearce PT, Smith R, Smith AL (1988). Mineralocorticoid action: Target tissue specificity in enzyme, not receptor, mediated. *Science* 242: 583-585.

Furuhashi M, Kitamura K, Adachi M, Miyoshi T, Wakida N, Ura N, Shikano Y, Shinshi Y, Sakamoto K, Hayashi M, Satoh N, Nishitani T, Tomita K, Shimamoto K (2005). Liddle's syndrome caused by a novel mutation in the proline-rich PY motif of the epithelial sodium channel β-subunit. *The Journal of Clinical Endocrinology & Metabolism* 90(1): 340-344.

Gadallah MF, Abreo K, Work J (1995). Liddle's syndrome, an unrecognized entity: A report of four cases, including the first report in black individuals. *American Journal of Kidney Diseases* 25(6): 829-835.

Gao PJ, Zhang KX, Zhu DL, He X, Han ZY, Zhan YM, Yang LW (2001). Diagnosis of Liddle syndrome by genetic analysis of β and γ subunits of epithelial sodium channel – a report of five affected family members. *Journal of Hypertension* 19: 885-889.

Garovic VD, Hilliard AA, Turner ST (2006). Monogenic forms of low-renin hypertension. *Nature Clinical Practice Nephrology* 2(11): 624-630.

Gates LJ, MacConnachie AA, Lifton RP, Haites NE, Benjamin N (1996). Variation in phenotype in patients with glucocorticoid remediable aldosteronism. *Journal of Medical Genetics* 33: 25-28.

Gates LJ, Benjamin N, Haites NE, MacConnachie AA, McLay JS (2001). Is random screening of value in detecting glucocorticoid-remediable aldosteronism within a hypertensive population? *Journal of Human Hypertension* 15: 173-176.

Geleijnse JM, Kok FJ, Grobbee DE (2003). Blood response to changes in sodium and potassium intake: A metaregression analysis of randomized trials. *Journal of Human Hypertension* 17(7): 471-480.

Geley S, Kapelari K, Johrer K, Peter M, Glatzl J, Vierhapper H, Schwarz S, Helmberg A, Sippell WG, White PC, Kofler R (1996). *CYP11B1* mutations causing congenital adrenal hyperplasia due to 11β-hydoxylase deficiency. *The Journal of Clinical Endocrinology and metabolism* 81(8): 2896-2901.

Geller DH, Auchus RJ, Mendonca BB, Miller WL (1997). The genetic and functional basis of isolated 17,20-lyase deficiency. *Nature Genetics* 17(2): 201-205.

Geller DS, Farhi A, Pinkerton N, Fradley M, Moritz M, Spitzer A, Meinke G, Tsai FT, Sigler PB, Lifton RP (2000). Activating mineralocorticoid receptor mutation in hypertension exacerbated by pregnancy. *Science* 289: 119-123.

Gereda JE. Bonilla-Felix M, Kalil B, Dewitt SJ (1996). Neonatal presentation of Gordon syndrome. *The Journal of Pediatrics* 129(4): 615-617.

Go AS, Mozaffarian D, Roger VL, Benjamin EJ, Berry JD, Borden WB, Bravata DM, Dai S, Ford ES, Fox CS, Franco S, Fullerton HJ, Gillespie C, Hailpern SM, Heit JA, Howard VJ, Huffman MD, Kissela BM, Kittner SJ, Lackland DT, Lichtman JH, Lisabeth LD, Magid D, Marcus GM, Marelli A, Matchar DB, McGuire DK, Mohler

ER, Moy CS, Mussolino ME, Nichol G, Paynter NP, Schreiner PJ, Sorlie PD, Stein J, Turan TN, Virani SS, Wong ND, Woo D, Turner MB, American Heart Association Statistics Committee and Stroke Statistics Subcommittee (2013). Heart disease and stroke statistics – 2013 Update: A report from the American Heart Association. *Circulation* 127(1): e6-e245. [Online]. Available at http://circ.ahajournals.org/content/early/2012/12/12/CIR.0b013e31828124ad.citation; accessed 14 May 2013.

Golbang AP, Murthy M, Hamad A, Liu CH, Cope G, Van't Hoff W, Cuthbert A, O'Shaughnessy KM (2005). A new kindred with pseudohypoaldosteronism type II and a novel mutation (564D>H) in the acidic motif of the *WNK4* gene. *Hypertension* 46: 295-300.

Gong H, Tang Z, Yang Y, Sun L, Zhang W, Wang W, Cui B, Ning G (2008). A patient with pseudohypoaldosteronism type II caused by a novel mutation in *WNK4* gene. *Endocrine* 33(3): 230-234.

Gordon RD, Ziesak MD, Tunny TJ, Stowasser M, Klemm SA (1993). Evidence for primary hyperaldosteronism may not be uncommon – twelve percent incidence among antihypertensive drug trial volunteers. *Clinical and Experimental Pharmacology and Physiology* 20(5): 296-298.

Gordon RD, Stowasser M, Tunny TJ, Klemm SA, Rutherford JC (1994). High incidence of primary aldosteronism in 199 patients referred with hypertension. *Clinical and Experimental Pharmacology & Physiology* 21(4): 315-318.

Gordon RD, Stowasser M (1998). Familial forms broaden the horizons for primary aldosteronism. *Trends in Endocrinology and Metabolism* 9(6): 220-227.

Grigorescu-Sido A, Weber MM, Grigorescu-Side P, Clausmeyer S, Heinrich U, Schulze E (2005). 21-hydroxylase and 11β-hydroxylase mutations in Romanian patients with classic congenital adrenal hyperplasia. *The Journal of Clinical Endocrinology and Metabolism* 90(10): 5769-5773.

Guyton AC, Hall JE (2006). Urine formation by the kidneys: I. Glomerular filtration, renal blood flow, and their control. In *Guyton and Hall Textbook of Medical Physiology* (11th ed), Chapter 26. Philadelphia: Elsevier Saunders.

Han Y, Fan XH, Sun K, Wang X, Wang Y, Chen J, Zhen Y, Zhang W, Hui R (2011). Hypertension associated polymorphisms in *WNK1/WNK4* are not associated with hydrochlorothiazide response. *Clinical Biochemistry* 44: 1045-1049.

Hansson JH, Nelson-Williams C, Suzuki H, Schild L, Shimkets R, Lu Y, Canessa C, Iwasaki T, Rossier B, Lifton RP (1995a). Hypertension caused by a truncated epithelial sodium channel γ subunit: Genetic heterogeneity of Liddle syndrome. *Nature Genetics* 11: 76-82.

Hansson JH, Schild L, Lu Y, Wilson TA, Gautschi I, Shimkets R, Nelson-Williams C, Rossier BC, Lifton RP (1995b). A *de novo* missense mutation of the β subunit of the epithelial sodium channel causes hypertension and Liddle syndrome, identifying a proline-rich segment critical for regulation of channel activity. *Proceedings of the National Academy of Sciences of the USA* 92: 11495-11499.

Hassan-Smith Z, Stewart PM (2011). Inherited forms of mineralocorticoid hypertension. *Current Opinion in Endocrinology, Diabetes and Obesity* 18: 177-185.

He FJ, Li J, MacGregor GA (2013). Effect of longer term modest salt reduction on blood pressure: Cochrane systematic review and meta-analysis of randomised trials. *British Medical Journal* 346: f1325.

He FJ, MacGregor GA (2002). Effect of modest salt reduction on blood pressure: A meta-analysis of randomized trials. Implications for public health. *Journal of Human Hypertension* 16(11): 761-770.

He FJ, Marciniak M, Visagie E, Markandu ND, Anand V, Dalton RN, MacGregor GA (2009). Effect of modest salt reduction on blood pressure, urinary albumin, and pulse wave velocity in white, black, and Asian mild hypertensives. *Hypertension* 54(3): 482-288.

Hertz RP, Unger AN, Cornell JA, Saunders E (2005). Racial disparities in hypertension prevalence, awareness, and management. *Archives of Internal Medicine* 165: 2098-2104.

Hiltunen TP, Hannila-Handelberg T, Petajaniemi N, Kantola II, Tikkanen I, Virtamo J, Gautschi I, Schild L, Kontula K (2002). Liddle's syndrome associated with a point mutation in the extracellular domain of the epithelial sodium channel γ subunit. *Journal of Hypertension* 20: 2383-2390.

Huang X, Muller W (1991). LALIGN (online). [Online]. Available at: www.h.embnet.org/software/LALIGN_form.html; accessed 28 December 2015.

Huang CL, Kuo E, Toto RD (2008). WNK kinases and essential hypertension. *Current Opinion in Nephrology and Hypertension* 17(2): 133-137.

Huizenga NA, de Lange P, Koper JW, de Herder WW, Abs R, Kasteren JH, de Jong FH, Lamberts SW (2000). Five patients with biochemical and/or clinical generalized glucocorticoid resistance without alterations in the glucocorticoid receptor genes. *The Journal of Clinical Endocrinology and Metabolism* 85(5): 2076-2081.

Humphries SV (1957). A study of hypertension in the Bahamas. *South African Medical Journal* 31(28): 694-699.

Hurley DM, Accili D, Stratakis CA, Karl M, Vamvakapoulos N, Rorer E, Constantine K, Taylor SI, Chrousos GP (1991). Point mutation causing a single amino acid substitution in the hormone binding domain of the glucocorticoid receptor in familial glucocorticoid resistance. *Journal of Clinical Investigation* 87: 680-686.

Ichikawa S, Sorenson AH, Imel EA, Friedman NE, Gertner JM, Econs MJ (2006). Intronic deletions in the *SLC34A3* gene cause hereditary hypophosphatemic rickets with hypercalciuria. *The Journal of Clinical Endocrinology and Metabolism* 91(10): 4022-4027.

Imai T, Globerman H, Gertner JM, Kagawa N, Waterman MR (1993). Expression and purification of functional human 17α-hydroxylase/17,20-lyase (P450c17) in *Escherichia coli*. *The Journal of Biological Chemistry* 268(26): 19681-19689.

Inoue J, Iwaoka T, Tokunaga H, Takamune K, Naomi S, Araki M, Takahama K, Yamaguchi K, Tomita K (1998). A family with Liddle's syndrome caused by a new missense mutation in the β subunit of the epithelial sodium channel. *The Journal of Clinical Endocrinology and Metabolism* 83(6): 2210-2213.

Jamieson A, Slutsker L, Inglis GC, Fraser R, White PC, Connell JM (1995). Glucocorticoid-suppressible hyperaldosteronism: effects of crossover sites and parental origin of chimaeric gene on phenotypic expression. *Clinical Science (London)* 88(5): 563-570.

Joehrer K, Geley S, Strasser-Wozak EM, Azziz R, Wollmann HA, Schmitt K, Kofler R, White PC (1997). *CYP11B1* mutations causing non-classic adrenal hyperplasia due to 11 beta-hydroxylase deficiency. *Human Molecular Genetics* 6(11): 1829-1834.

Johnson AL, Criddle LM (2004). Pass the salt: Indications for and implications of using hypertonic saline. *Critical Care Nurse* 24(5): 36-48.

Jonsson JR, Shelley AK, Tunny TJ, Stowasser M, Gordon RD (1995). A new genetic test for familial hyperaldosteronism type I aids in the detection of curable hypertension. *Biochemical and Biophysical Research Communications* 207(2): 565-571.

Jones ESW, Owen EP, Rayner BL (2012). The association of the R563Q genotype of the ENaC with phenotypic variation in Southern Africa. *American Journal of Hypertension* 25(12): 1286-1291.

Julier C, Delepine M, Keavney B, Terwilliger J, Davis S, Weeks DE, Bui T, Jeunemaitre X, Velho G, Froguel P, Ratcliffe P, Corvol P, Soubrier F, Lathrop GM (1997). Genetic susceptibility for human familial essential hypertension in a region of

homology with blood pressure linkage on rat chromosome 10. *Human Molecular Genetics* 6(12): 2077-2085.

Kahle KT, Wilson FH, Leng Q, Lalioti MD, O'Connell AD, Dong K, Rapson AK, MacGregor GG, Giebisch G, Hebert SC, Lifton RP (2003). *WNK4* regulates the balance between renal NaCl reabsorption and K^+ secretion. *Nature Genetics* 35: 372-376.

Kamide K, Kokubo Y, Hanada H, Nagura, Yang J, Takiuchi S, Tanaka C, Banno M, Miwa Y, Yoshii M, Matayoshi T, Yasuda H, Horio T, Okayama A, Tomoike H, Kawano Y, Miyata T (2006). Genetic variation in *HSD11B2* in hypertensive patients and in the general population, six rare missense/frameshift mutations. *Hypertension Research* 29(4): 243-252.

Kamide K, Yang J, Kokubo Y, Takiuchi S, Miwa Y, Horio T, Banno M, Nagura J, Okayama A, Tomoike H, Kawano Y, Miyata T (2005). A novel missense mutation, F826Y, in the mineralocorticoid receptor gene in Japanese hypertensives: It's implications for clinical phenotypes. *Hypertension Research* 28(9): 703-709.

Kannel WB, Wolf PA, Verter J, McNamara PM (1996). Epidemiologic assessment of the role of blood pressure in stroke: The Framingham Study 1970. *Journal of the American Medical Association* 276: 1269-1278.

Karl M, Lamberts SW, Koper JW, Katz DA, Huizenga NE, Kino T, Haddad BR, Hughes MR, Chrousos GP (1996). Cushing's disease preceded by generalized glucocorticoid resistance: Clinical consequences of a novel, dominant-negative glucocorticoid receptor mutation (Abstract). *Proceedings of the Association of American Physicians* 108: 296-307.

Katsumata N, Satoh M, Mikami A, Mikami S, Nagashima-Miyokawa A, Sato N, Yokoya S, Tanaka T (2001). New compound heterozygous mutation in the *CYP17* gene in a 46,XY girl with 17 alpha-hydroxylase/17,20-lyase deficiency. *Hormone Research* 55(3): 141-146.

Kawamoto T, Mitsuuchi Y, Toda K, Yokoyama Y, Miyahara K, Miura S, Ohnishi T, Ichikawa Y, Nakao K, Imura H, Ulick S, Shizuta Y (1992). Role of steroid 11β-hydroxylase and steroid 18-hydroxylase in the biosynthesis of glucocorticoids and mineralocorticoids in humans. *Proceedings of the National Academy of Sciences of the USA* 89(4): 1458-1462.

Kearney PM, Whelton M, Reynolds K, Whelton PK, He J (2004). Worldwide prevalence of hypertension: A systematic review. *Journal of Hypertension* 22: 11-19.

Kharrat M, Trabelsi S, Chaabouni M, Maazoul F, Kraoua L, Jemaa LB, Gandoura N, Barsaoui S, Morel Y, M'rad R, Chaabouni H (2010). Only two mutations detected in 15 Tunisian patients with 11β-hydroxylase deficiency: The p.Q356X and the novel p. G379V. *Clinical Genetics* 78: 398-401.

Kim GH, Masilamani S, Turner R, Mitchell C, Wade JB, Knepper MA (1998). The thiazide-sensitive Na-Cl cotransporter is an aldosterone-induced protein. *Proceedings of the National Academy Sciences of the USA* 95: 14552-14557.

Kitanaka S, Katsumata N, Tanae A, Hibi I, Takeyama KI, Fuse H, Kato S, Tanaka T (1997). A new compound heterozygous mutation in the 11β-hydroxysteroid dehydrogenase type 2 gene in a case of apparent mineralocorticoid excess. *The Journal of Clinical Endocrinology and Metabolism* 82(12): 4054-4058.

Klag MJ, Whelton PK, Randall BL, Neaton JD, Brancati FL, Ford CE, Shulman NB, Stamler J (1996). Blood pressure and end-stage renal disease in men. *New England Journal of Medicine* 334:13-18.

Knops NBB, Monnens LA, Lenders JW, Levtchenko EN (2011). Apparent mineralocorticoid excess: Time of manifestation and complications despite treatment. *Pediatrics* 127(6): 1610-1614.

Koper JW, Stolk RP, de Lange P, Huizenga NATM, Molijn GJ, Pols HAP, Grobbee DE, Karl M, de Jong FH, Brinkmann AO, Lamberts SWJ (1997). Lack of an association between five polymorphisms in the human glucocorticoid receptor gene and glucocorticoid resistance. *Human Genetics* 99: 663-668.

Kramer H, Han C, Post W, Goff D, Diez-Roux A, Cooper R, Jinagouda S, Shea S (2004). Racial/ethnic differences in hypertension and hypertension treatment and control in the multi-ethnic study of atherosclerosis (MESA). *American Journal of Hypertension* 17(10): 963-970.

Krone N, Riepe FG, Gotze D, Korsch E, Rister M, Commentz J, Partsch CJ, Grotzinger J, Peter M, Sippell WG (2005). Congenital adrenal hyperplasia due to 11-hydroxylase deficiency: functional characterization of two novel point mutations and a three-base pair deletion in the *CYP11B1* gene. *The Journal of Clinical Endocrinology and Metabolism* 90(6): 3724-3730.

Krone N, Grischuk Y, Muller M, Volk RE, Grotzinger J, Holterhus PM, Sippell WG, Riepe FG (2006). Analyzing the functional and structural consequences of two point mutations (P94L and A368D) in the *CYP11B1* gene causing congenital adrenal hyperplasia resulting from 11-hydroxylase deficiency. *The Journal of Clinical Endocrinology and Metabolism* 91: 2682-2688.

Krone N, Grotzinger J, Holterhus PM, Sippell WG, Schwarz HP, Riepe FG (2009). Congenital adrenal hyperplasia due to 11-hydroxylase deficiency – insights from two novel *CYP11B1* mutations (p.M92X, p.R453Q). *Hormone Research* 72(5): 281-286.

Krupoves A, Mack D, Deslandres C, Seidman E, Amre DK (2011). Variation in the glucocorticoid receptor gene (*NR3C1*) may be associated with corticosteroid dependency and resistance in children with Crohn's disease. *Pharmacogenetics and Genomics* 21(8): 454-460.

Kuehl P, Zhang J, Lin Y, Lamba J, Assem M, Schuetz J, Watkins PB, Daly A, Wrighton SA, Hall SD, Maurel P, Relling M, Brimer C, Yasuda K, Venkataramanan R, Strom S, Thummel K, Boguski MS, Schuetz E (2001). Sequence diversity in *CYP3A* promoters and characterization of the genetic basis of polymorphic CYP3A5 expression. *Nature Genetics* 27: 383-391.

Kuivenhoven JA, Weibusch H, Pritchard PH, Funke H, Benne R, Assmann G, Kastelein JJP (1996). An intronic mutation in a lariat bransch point sequence is a direct cause of an inherited human disorder (Fish-Eye disease). *Journal of Clinical Investigation* 98(2): 358-364.

Kuribayashi I, Massa G, van den Tooren-de Groot HK, Oostdijk W, Wit JM, Shizuta Y (2003). A novel nonsense mutation in the *CYP11B1* gene from a subject with the steroid 11β-hydroxylase form of congenital adrenal hyperplasia. *Endocrine Research* 29(4): 377-381.

Kuribayashi I, Nomoto S, Massa G, Oostdijk W, Wit JM, Wolffenbuttel BHR, Shizuta Y, Honke K (2005). Steroid 11-beta-hydroxylase deficiency caused by compound heterozygosity for a novel mutation, p.G314R, in one *CYP11B1* allele, and a chimeric *CYP11B2/CYP11B1* in the other allele. *Hormone Research* 63(6): 284-293.

Kyuma M, Ura N, Torii T, Takeuchi H, Takizawa H, Kitamura K, Tomita K, Sasaki S, Shimamoto K (2001). A family with Liddle's syndrome caused by a mutation in the β-subunit of the epithelial sodium channel. *Clinical and Experimental Hypertension* 23(6): 471-478.

Lebea PJ, Pretorius PJ (2008). Newborn screening for classic galactosaemia and primary congenital hypothyroidism in the Nkangala district of Mpumalanga Province, South Africa. *South African Journal of Child Health,* 2(1): 20.

LaFlemme N, Leblanc JF, Mailloux J, Faure N, Labrie F, Simard J (1996). Mutation R96W in cytochrome P450c17 gene causes combined 17 alpha-

hydroxylase/17,20-lyase deficiency in two French Canadian patients. *The Journal of Clinical Endocrinology and Metabolism* 81(1): 264-268.

Lam CW, Arit W, Chan CK, Honour JW, Lin CJ, Tong SF, Choy KW, Miller WL (2001). Mutation of proline 409 to arginine in the Meander region of cytochrome P450c17 causes severe 17α-hydroxylase deficiency. *Molecular Genetics and Metabolism* 72: 254-259.

Lamberts SW, Koper JW, Biemond P, den Holder FH, de Jong FH (1992). Cortisol receptor resistance: The variability of its clinical presentation and response to treatment (Abstract). *The Journal of Clinical Endocrinology and Metabolism* 74(2): 313-321.

Lategan R (2011). The association of body weight, 25-hydroxy vitamin D, sodium intake, physical activity levels and genetic factors with the prevalence of hypertension in a low income, black urban community in Mangaung, Free State, South Africa. Unpublished PhD thesis. University of the Free State. [Online]. Available at: http://etd.uovs.ac.za/ETD-db//theses/available/etd-08232012145515/unrestricted/LateganR.pdf; accessed 28 June 2013.

Lavery GG, Ronconi V, Draper N, Rabbitt EH, Lyons V, Chapman KE, Walker EA, McTernan CL, Giacchetti G, Mantero F, Seckl JR, Edwards CRW, Connell JMC, Hewison M, Stewart PM (2003). Late-onset apparent mineralocorticoid excess caused by novel compound heterozygous mutations in the *HSD11B2* gene. *Hypertension* 42: 123-129.

Lee HH, Won GS, Chaos HT, Lee YJ, Chung BC (2005). Novel missense mutations, GCC [Ala306] → GTC [Val] and ACG [Thr318] → CCG [Pro], in the *CYP11B1* gene cause steroid 11β-hydroxylase deficiency in the Chinese. *Clinical Endocrinology* 62: 418-422.

Lee IS, Kim SY, Jang HW, Kim MK, Lee JH, Lee YH, Jo YS (2010). Genetic analysis of the chimeric *CYP11B1/CYP11B2* gene in a Korean family with glucocorticoid-remediable aldosteronism. *Journal of Korean Medical Science* 25(9): 1379-1383.

Lee LS, Shu WJ, Wu CM, Hsieh CH, Chen SM, Hu CJ, Chen WY, Chung BC (2006). A novel compound heterozygous mutation of K494_V495 deletion plus R496L and D487_F489 deletion in extreme c-terminus of cytochrome P450c17 causes 17α-hydroxylase deficiency. *Molecular and Cellular Endocrinology* 249: 16-20.

Levy D, Larson MG, Vasan RS, Kannel WB, Ho KKL (1996). The progression from hypertension to congestive heart failure. *Journal of the American Medical Association* 275: 1557-1562.

Levy D, De Stefano AL, Larson MG, O'Donnell CJ, Lifton RP, Gavras H, Cupples LA, Myers RH (2000). Evidence for a gene influencing blood pressure on chromosome 17. Genome scan linkage results for longitudinal blood pressure phenotypes in subjects from the Framingam Heart Study. *Hypertension* 36(4): 477-483.

Lew JM, Fei YL, Aleck K, Blencowe BJ, Weksberg R, Sadowski PD (2004). *CDKN1C* mutation in Wiedemann-Beckwith syndrome patients reduces RNA splicing efficiency and identifies a splicing enhancer. *American Journal of Medical Genetics* 127A: 268-276.

Li A, Li KXZ, Marui S, Krozowski ZS, Batista MC, Whorwood CB, Arnhold IJP, Shackleton CHL, Mendonca BB, Stewart PM (1997). Apparent mineralocorticoid excess in a Brazilian kindred - hypertension in the heterozygote state. *Journal of Hypertension* 15: 1397-1402.

Li A, Tedde R, Krozowski ZS, Pala A, Li KXZ, Shackleton CHL, Mantero F, Palermo M, Stewart PM (1998). Molecular basis for hypertension in the "type II variant" of apparent mineralocorticoid excess. *American Journal of Human Genetics* 63: 370-379.

Liddle GW, Bledsoe T, Coppage WS (1963). A familial renal disorder simulating primary aldosteronism but with negligible aldosterone secretion. *Transactions of the Association of American Physicians* 76: 199-213.

Lifton RP, Dluhy RG, Powers M, Rich GM, Cook S, Ulick S, Lalouel JM (1992a). A chimeric 11 beta-hydroxylase/aldosterone synthase gene causes glucocorticoid-remediable aldosteronism and human hypertension. *Nature* 355: 262-265.

Lifton RP, Dluhy RG, Powers M, Rich GM, Gutkin M, Fallo F, Gill JR Jr., Feld L. Ganguly A, Laidlaw JC (1992b). Hereditary hypertension caused by chimeric gene duplications and ectopic expression of aldosterone synthase. *Nature Genetics* 2(1): 66-74.

Lifton RP (1996). Molecular genetics of human blood pressure variation. *Science* 272(5262): 676-680.

Lifton RP, Gharavi AG, Geller DS (2001). Molecular mechanisms of human hypertension. *Cell* 104: 545-556.

Lim PO, Dow E, Brennan G, Jung RT, MacDonald TM (2000). High prevalence of primary aldosteronism in the Tayside hypertension clinic population. *Journal of Human Hypertension* 14(5): 311-315.

Lin D, Harikrishna JA, Moore CC, Jones KL, Miller WL (1991). Missense mutation serine^{106} → proline causes 17 alpha-hydroxylase deficiency. *The Journal of Biological Chemistry* 266(4): 15992-15998.

Loh KC, Koay ES, Khaw MC, Emmanuel SC, Young WF, Jr (2000). Prevalence of primary aldosteronism among Asian hypertensive patients in Singapore. *The Journal of Clinical Endocrinology and Metabolism* 85(8): 2854-2859.

Lomelin D, Jorgenson E, Risch N (2010). Human genetic variation recognizes functional elements in noncoding sequence. *Genome Research* 20: 311-319.

Lovati E, Ferrari P, Dick B, Jostarndt K, Frey BM, Frey FJ, Schorr U, Sharma AM (1999). Molecular basis of human salt sensitivity: the role of the 11 beta-hydroxysteroid dehydrogenase type 2. *The Journal of Clinical Endocrinology and Metabolism* 84(10): 3745-3749.

Luft FC, Miller JZ, Grim CE, Fineberg NS, Christian JC, Daugherty SA, Weinberger MH (1991). Salt sensitivity and resistance of blood pressure. Age and race as factors in physiological responses. *Hypertension* 17(1 Suppl): I102-I108.

Martin RM, Lin CJ, Costa EMF, Leocadia de Oliveira M, Carrilho A, Villar H, Longui CA, Mendonca BB (2003). P450c17 deficiency in Brazilian patients: Biochemical diagnosis through progesterone levels confirmed by *CYP17* genotyping. *The Journal of Clinical Endocrinology and Metabolism* 88(12): 5739-5746.

Martinez F, Mansego ML, Escudero JC, Redon J, Chaves FJ (2009). Association of a mineralocorticoid receptor gene polymorphism with hypertension in a Spanish population. *American Journal of Hypertension* 22(6): 649-655.

Masilamani S, Kim GH, Mitchell C, Wade JB, Knepper MA (1999). Aldosterone-mediated regulation of ENaC α, β and γ subunit proteins in rat kidney. *The Journal of Clinical Investigation* 104(7): R19-R23.

Mathers CD, Loncar D (2006). Projections of global mortality and burden of disease from 2002 to 2030. *PLOS Medicine* 3(11): e442. [Online]. Available at http://www.plosmedicine.org/article/fetchObject.action?representation=PDF&uri=info:doi/10.1371/journal.pmed.0030442; accessed 12 November 2015.

Matsushima M, Kobayashi K, Eml M, Saito H, Saito J, Suzumori K, Nakamura Y (1995). Mutation analysis of the *BRCA1* gene in 76 Japanese ovarian cancer patients: four germline mutations, but no evidence of somatic mutation. *Human Molecular Genetics* 4(10): 1953-1956.

Matsuzaki S, Yanase T, Murakami T, Uehara S, Nawata H, Yajima A (2000). Induction of endometrial cycles and ovulation in a woman with combined 17α-hydroxylase/17,20-lyase deficiency due to compound heterozygous mutations on the P45017α gene. *Fertility and Sterility* 73(6): 1183-1186.

Mayan H, Munter G, Shaharabany M, Mouallem M, Pauzner R, Holtzman EJ, Farfel Z (2004). Hypercalciuria in familial hyperkalemia and hypertension accompanies hyperkalemia and precedes hypertension: Description of a large family with the Q565E *WNK4* mutation. *The Journal of Clinical Endocrinology and Metabolism* 89: 4025-4030.

Mayosi BM, Flisher AJ, Lalloo UG, Sitas F, Tollman SM, Bradshaw D (2009). The burden of non-communicable diseases in South Africa. *Lancet* 374:934-947.

McCarthy EMS, Phillips JA III (1998). Characterization of an intron splice enhancer that regulates alternative splicing of human GH pre-mRNA. *Human Molecular Genetics* 7(9): 1491-1496.

McCormick JA, Ellison DH (2011). The WNKs: Atypical protein kinases with pleiotropic actions. *Physiological Reviews* 91(1): 177-219.

McMohan GT, Dluhy RG (2004). Glucocorticoid-remediable aldosteronism. *Arquivos Brasileiros de Endocrinoligia e Metabologia* 48(5): 682-686. [Online]. Available at http://www.scielo.br/pdf/abem/v48n5/a14v48n5.pdf; accessed 3 Julie 2013.

Melander O, Orho M, Fagerudd J, Bengtsson K, Groop PH, Mattiasson I, Groop L, Hulthen UL (1998). Mutations and variants of the epithelium sodium channel gene in Liddle's syndrome and primary hypertension. *Hypertension* 31: 1118-1124.

Melander O, Orho-Melander M, Bengtsson K, Lindblad U, Rastam L, Groop L, Hulthen UL (2000). Association between a variant in the 11 beta-hydroxysteroid dehydrogenase type 2 gene and primary hypertension. *Journal of Human Hypertension* 14(12): 819-823.

Mendes AI, Mascarenhas MR, Matos S, Sousa I, Ferreira J, Barbosa AP, Bicho M, Jordan P (2011). A *WNK4* gene variant relates to osteoporosis and not to hypertension in the Portuguese population. *Molecular Genetics and Metabolism* 102(4): 465-469.

Mendonca BB, Leite MV, De Castro M, Kino T, Elias LLK, Bachega TAS, Arnhold IJP, Chrousos GP, Latronico AC (2002). Female pseudohermaphroditism caused by a novel homozygous missense mutation of the GR gene. *The Journal of Clinical Endocrinology and Metabolism* 87(4): 1805-1809.

Merke DP, Tajima T, Chhabra A, Barnes K, Mancilla E, Baron J, Cutler GB Jr. (1998). Novel *CYP11B1* mutations in congenital adrenal hyperplasia due to steroid 11β-hydroxylase deficiency. *The Journal of Clinical Endocrinology and Metabolism* 83(1): 270-273.

Milford DV, Shackleton CHL, Stewart PM (1994). Mineralocorticoid hypertension and congenital deficiency of 11β-hydroxysteroid dehydrogenase in a family with the syndrome of 'apparent' mineralocorticoid excess. *Clinical Endocrinology* 43: 241-246.

Milford DV (1999). Investigation of hypertension and the recognition of monogenic hypertension. *Archives of Disease in Childhood* 81: 452-455.

Monder C, Shackleton CH, Bradlow HL, New MI, Stoner E, Iohan F, Lakshmi V. (1986). The syndrome of apparent mineralocorticoid excess: Its association with 11β-dehydrogenase and 5β-reductase deficiency and some consequences for corticosteroid metabolism (Abstract). *The Journal of Clinical Endocrinology and Metabolism* 63(3): 550-557.

Monno S, Ogawa H, Date T, Fujioka M, Miller WL, Kobayashi M (1993). Mutation of histidine 373 to leucine in cytochrome P450c17 causes 17 alpha-hydroxylase deficiency. *Journal of Biological Chemistry* 268(34): 25811-25817.

Morineau G, Marc JM, Boudi A, Galons H, Gourmelen M, Corvol P, Pascoe L, Fiet J (1999). Genetic, biochemical and clinical studies of patients with A328V or R213C mutations in *11βHSD2* causing apparent mineralocorticoid excess. *Hypertension* 34(3): 435-441.

Morineau G, Sulmont V, Salomon R, Fiquet-Kempf B, Jeunemaitre X, Nicod J, Ferrari P (2006). Apparent mineralocorticoid excess: report of six new cases and extensive personal experience. *Journal of the American Society of Nephrology* 17: 3176-3184.

Mornet E, Dupont J, Vitek A, White PC (1989). Characterization of two genes encoding human steroid 11β-hydroxylase (P-450(11)β). *The Journal of Biological Chemistry* 264(35): 20961-20967.

Morris RC Jr, Sebastian A, Forman A, Tanaka M, Schmidlin O (1999). Normotensive salt sensitivity. Effects of race and dietary potassium. *Hypertension* 33: 18-23.

Mosso L, Carvajal C, Gonzalez A, Barraza A, Avila F, Montero J, Huete A, Gederlini A, Fardella CE (2003). Primary aldosteronism and hypertensive disease. *Hypertension* 42(2): 161-165.

Motaghedi R, Betensky BP, Slowinska B, Cerame B, Cabrera M, New MI, Wilson RC (2005). Update on the prenatal diagnosis and treatment of congenital adrenal hyperplasia due to 11β-hydroxylase deficiency. *Journal of Pediatric Endocrinology and Metabolism* 18: 133-142.

Mulatero P, Tizzani D, Viola A, Bertello C, Monticone S, Mengozzi G, Schiavone D, Williams TA, Einaudi S, La Grotta A, Rabbia F, Veglio F (2011). Prevalence and characteristics of familial hyperaldosteronism: The PATOGEN study (primary aldosteronism in Torino-genetic forms). *Hypertension* 58(5): 797-803.

Mune T, Rogerson FM, Nikkilä H, Agarwal AK, White PC (1995). Human hypertension caused by mutations in the kidney isozyme of 11 beta-hydroxysteroid dehydrogenase. *Nature Genetics* 10(4): 394-399.

Mussig K, Kaltenbach S, Machicao F, Maser-Gluth C, Hartmann MF, Wudy SA, Schnauder G, Haring HU, Seif FJ, Gallwitz B (2005). 17α-hydroxylase/17,20-lyase deficiency caused by a novel homozygous mutation (Y27Stop) in the cytochrome *CYP17* gene. *The Journal of Clinical Endocrinology and Metabolism* 90(7): 4362-4365.

Nader N, Bachrach BE, Hurt DE, Gajula S, Pittman A, Lescher R, Kino T (2010). A novel point mutation in helix 10 of the human glucocorticoid receptor causes generalized glucocorticoid resistance by disrupting the structure of the ligand-binding domain. *The Journal of Clinical Endocrinology and Metabolism* 95(5): 2281-2285.

Naiki Y, Kawamoto T, Mitsuuchi Y, Miyahara K, Toda K, Orii T, Imura H, Shizuta Y (1993). A nonsense mutation (TGG [Trp116] → TAG [Stop]) in *CYP11B1* causes steroid 11 beta-hydroxylase deficiency. *The Journal of Clinical Endocrinology and Metabolism* 77(6): 1677-1682.

Nakagawa Y, Yamada M, Ogawa H, Igarashi Y (1995). Missense mutation in *CYP11B1* (CGA [Arg-384] → GGA [Gly]) causes steroid 11β-hydroxylase deficiency. *European Journal of Endocrinology* 132: 286-289.

NIH (2004). The Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure. [Online]. Available at: http://www.nhlbi.nih.gov/files/docs/guidelines/jnc7full.pdf; accessed 15 September 2014.

New MI, Wilson RC (1999). Steroid disorders in children: Congenital adrenal hyperplasia and apparent mineralocorticoid excess. *Proceedings of the National Academy of Sciences* 96(22): 12790-12797.

Nkeh B, Samani NJ, Badenhorst D, Libhaber E, Sareli P, Norton GR, Woodiwiss AJ (2003). T594M variant of the epithelial sodium channel beta-subunit gene and hypertension in individuals of African ancestry in South Africa. *American Journal of Hypertension* 16(10): 847-852.

Norman R, Bradshaw D, Schneider M, Joubert J, Groenewald P, Lewin S, Steyn K, Vos T, Laubscher R, Nannan N, Nojilana B, Pieterse D, the South African Comparative Risk Assessment Collaborating Group (2007). A comparative risk assessment for South Africa in 2000: Towards promoting health and preventing disease. *South African Medical Journal* 97(7): 637-641.

Nunez BS, Rogerson FM, Mune T, Igarashi Y, Nakagawa Y, Phillipov G, Moudgil A, Travis LB, Palermo M, Shackleton C, White PC (1999). Mutants of 11β-hydroxysteroid dehydrogenase (*11βHSD2*) with partial activity: Improved correlations between genotype and biochemical phenotype in apparent mineralocorticoid excess. *Hypertension* 34: 638-642.

Obeyesekere VR, Li KXZ, Ferrari P, Krozowski Z (1997). Truncation of the N- and C-terminal regions of the human 11β-hydroxysteroid dehydrogenase type 2 enzyme and effects on solubility and bidirectional enzyme activity. *Molecular and Cellular Endocrinology* 131: 173-182.

Ohta A, Schumacher FR, Mehellou Y, Johnson C, Knebel A, Macartney TJ, Wood NT, Alessi DR, Durz T (2013). The CUL3-KLHL3 E3 ligase complex mutated in Gordon's hypertension syndrome interacts with and ubiquitylates WNK isoforms: Disease-causing mutations in *KLHL3* and *WNK4* disrupt interaction. *The Biochemical Journal* 451(1): 111-122.

O'Rorke JE, Richardson WS (2001). Evidence based management of hypertension: what to do when blood pressure is difficult to control. *British Medical Journal* 322: 1229-1232.

O'Shaughnessy KM, Fu B, Johnson A, Gordon RD (1998). Linkage of Gordon's syndrome to the long arm of chromosome 17 in a region recently linked to familial essential hypertension. *Journal of Human Hypertension* 12(10): 675-678.

Palmer BF, Alpern RJ (1998). Liddle's syndrome. *The American Journal of Medicine* 104(3): 301-309.

Paperna T, Gershoni-Baruch R, Baderneh K, Kasinetz L, Hochberg Z (2005). Mutations in *CYP11B1* and congenital adrenal hyperplasia in Moroccan Jews. *The Journal of Clinical Endocrinology and Metabolism* 90(9): 5463-5465.

Parajes S, Loidi L, Reisch N, Dhir V, Rose IT, Hampel R, Quinkler M, Conway GS, Castro-Feijoo L, Araujo-Vilar D, Pombo M, Dominquez F, Williams EL, Cole TR, Kirk JM, Kaminsky E, Rumsby G, Arit W, Krone N (2010). Functional consequences of seven novel mutations in the *CYP11B1* gene: Four mutations associated with nonclassic and three mutations causing classic 11β-hydroxylase deficiency. *The Journal of Clinical Endocrinology and Cell Biology* 95(2): 779-788.

Patocs A, Liko I, Varga I, Gergics P, Boros A, Futo L, Kun I, Bertalan R, Toth S, Pazmany T, Toth M, Szucs N, Horanyi J, Glaz E, Racz K (2005). Novel mutation of the *CYP17* gene in two unrelated patients with combined 17α-hydroxylase/17,20-lyase deficiency: Demonstration of absent enzyme activity by expressing the mutant *CYP17* gene and by three-dimensional modelling. *The Journal of Steroid Biochemistry and Molecular Biology* 97: 257-265.

Pavlin VN, Hyman DJ, Vallbona C (1996). Hypertension control in multi-ethnic primary care clinics. *Journal of Human Hypertension* 10(Suppl 3): S19-23.

Persu A, Barbry P, Bassilana F, Houot AM, Mengual R, Lazdunski M, Corvol P, Jeunemaitre X (1998). Genetic analysis of the β subunit of the epithelial Na^+ channel in essential hypertension. *Hypertension* 32: 129-137.

Persu A (2003). The epithelial Na^+ channel: Progressing from Liddle's syndrome to essential hypertension. *Journal of Hypertension* 21: 855-857.

Pestana JA, Steyn K, Leiman A, Hartzenberg GM (1996). The direct and indirect costs of cardiovascular disease in South Africa in 1991. *South African Medical Journal* 86(6): 679-684.

Poch E, Gonzalez D, Giner V, Bragulat E, Coca A, de la Sierra A (2001). Molecular basis of salt sensitivity in human hypertension. Evaluation of renin-angiotensin-aldosterone system gene polymorphisms. *Hypertension* 38(5): 1204-1209.

Polakova KM, Lopotova T, Klamova H, Moravcova J (2008). High resolution melt curve analysis: Initial screening for mutations in BCR-ABL kinase domain. *Leukemia Research* 23: 1236-1243.

Pradervand S, Wang Q, Burnier M, Beermann F, Horisberger JD, Hummler E, Rossier BC (1999). A mouse model for Liddle's syndrome. *Journal of the American Society of Nephrology* 10(12): 2527-2533.

Qiao J, Hu RM, Peng YD, Song HD, Peng YW, Gao GF, Hao JH, Hu NY, Xu MY, Chen JL (2003). A complex heterozygous mutation of His373Leu and Asp487-Ser488-Phe489 deletion in human cytochrome P450c17 causes 17 [alpha]-hydroxylase, 17, 20-lyase deficiency in three Chinese sisters. *Molecular and Cellular Endocrinology* 201(1-2): 189-195.

Quinkler M, Bappal B, Draper N, Atterbury AJ, Lavery GG, Walker EA, DeSilva V, Taylor NF, Hala S, Rajendra N, Stewart PM (2004). Molecular basis for the apparent mineralocorticoid excess syndrome in the Oman population. *Molecular and Cellular Endocrinology* 217: 143-149.

Quinkler M, Stewart PM (2003). Hypertension and the cortisol-cortisone shuttle. *The Journal of Clinical Endocrinology and Metabolism* 88(6): 2384-2392.

Ramirez-Salazar M, Romero-Gutierrez G, Zaina S, Malacara JM, Kornhauser C, Perez-Luque E (2011). Relationship of aldosterone synthase gene (C-344T) and mineralocorticoid receptor (S810L) polymorphisms with gestational hypertension. *Journal of Human Hypertension* 25(5): 320-326.

Rayner BL, Opie LH, Davidson JS (2000). The aldosterone/renin ratio as a screening test for primary aldosteronism. *South African Medical Journal* 90(4): 394-400.

Rayner BL, Myers JE, Opie LH, Trinder YA, Davidson JS (2001). Screening for primary aldosteronism – normal ranges for aldosterone and renin in three South African population groups. *South African Medical Journal* 91(7): 594-599.

Rayner BL, Owen EP, King JA, Soule SG, Vreede H, Opie LH, Marais D, Davidson S (2003). A new mutation, R563Q, of the beta subunit of the epithelial sodium channel associated with low-renin, low-aldosterone hypertension. *Journal of Hypertension* 21(5): 921-925.

Reed GH, Wittwer CT (2004). Sensitivity and specificity of single-nucleotide polymorphism scanning by high-resolution melting analysis. *Clinical Chemistry* 50(1): 1748-1754.

Rich GM, Ulick S, Cook S, Wang JZ, RP Lifton, Dluhy RG (1992). Glucocorticoid-remediable aldosteronism in a large kindred: Clinical spectrum and diagnosis using a characteristic biochemical phenotype. *Annals of Internal Medicine* 116(10): 813-820.

Riedl S, Nguyen HH, Clausmeyer S, Schulze E, Waldhauser F, Bernhardt R (2008). A homozygous L299P mutation in the *CYP11B1* gene leads to complete virilisation in 46,XX individuals with 11-beta-hydroxylase deficiency. *Hormone Research* 70(3): 145-149.

Rodriguez JA (2000). Syndrome of apparent mineralocorticoid excess caused by a deficiency of 11 beta-hydroxysteroid dehydrogenase: Clinical and genetic study in a Chilean family followed for 19 years. *Revista Médica de Chile* 128(1): 17-26.

Rogoff D, Smolenicka Z, Bergada I, Vallejo G, Barontini M, Heinrich JJ, Ferrari P (1998). The codon 213 of the 11beta-hydroxysteroid dehydrogenase type 2 gene is a hot spot for mutations in apparent mineralocorticoid excess. *The Journal of Clinical Endocrinology and Metabolism* 83 (12): 4391-4393.

Rosa S, Duff C, Meyer M, Lang-Muritano M, Balercia G, Boscaro M, Topaloglu AK, Mioni R, Fallo F, Zuliani L, Mantero F, Schoenle EJ, Biason-Lauber A (2007). P450c17 deficiency: Clinical and molecular characterization of six patients. *The Journal of Clinical Endocrinology and Metabolism* 92(3): 1000-1007.

Rosa S, Steigert M, Lang-Muritano M, l'Allemand D, Schoenle EJ, Biason-Lauber A (2010). Clinical, genetic and functional characteristics of three novel *CYP17A1* mutation causing combined 17alpha-hydroxylase/17,20-lyase deficiency. *Hormone Research in Paediatrics* 73(3): 198-204.

Rosler A, Leiberman E, Sack J, Landau H, Benderly A, Moses SW, Cohen T (1982). Clinical variability of congenital adrenal hyperplasia due to 11 beta-hydroxylase deficiency. *Hormone Research* 16(3): 133-141.

Rossi E, Farnetti E, Debonneville A, Nicoli D, Grasseli Ch, Regolisti G, Negro A, Perazzoli F, Casali B, Mantero F, Staub O (2008), Liddle's syndrome caused by a novel missense mutation (P617L) of the epithelial sodium channel β subunit. *Journal of Hypertension* 26: 921-927.

Rossi (2011). A clinical phenotype mimicking essential hypertension in a newly discovered family with Liddle's syndrome. *American Journal of Hypertension* 24(8): 930-935.

Rumsby G, Skinner C, Lee HA, Honour JW (1993). Combined 17 alpha-hydroxylase/17,20-lyase deficiency caused by heterozygous stop codons in the cytochrome P450c17 alpha-hydroxylase gene. *Clinical Endocrinology (Oxford)* 39(4): 483-485.

Ruis BL, Kivens WJ, Siliciano PG (1994). The interaction between the first and last intron nucleotides in the second step of pre-mRNA splicing is independent of other conserved intron nucleotides. *Nucleic Acids Research* 22(24): 5190-5195.

Ruiz M, Lind U, Gåfvels M, Eggertsen G, Carlstedt-Duke J, Nilsson L, Holtmann M, Stierna P, Wikström AC, Werner S (2001). Characterization of 2 novel mutations in the GCR gene in patients with primary cortisol resistance. *Clinical Endocrinology* 55: 363-371.

Sagnella GA (2001). Why is plasma renin activity lower in populations of African origin? *Journal of Human Hypertension* 15(1): 17-25.

Sahakitrungruang T, Tee MK, Speiser PW, Miller WL (2009). Novel P450c17 mutation H373D causing combined 17alpha-hydroxylase/17,20-lyase deficiency. *The Journal of Clinical Endocrinology and Metabolism* 94(8): 3038-3092.

Sahay M, Sahay RK (2012). Low renin hypertension. *Indian Journal of Endocrinology and Metabolism* 16(5): 729-739.

Sanders PW (2009). Dietary salt intake, salt sensitivity, and cardiovascular health. *Hypertension* 53: 442-445.

Sawathiparnich (2009). A novel mutation in the β-subunit of the ENaC gene (*SCNN1B*) in a Thai family with Liddle's syndrome. *Journal of Pediatric Endocrinology and Metabolism* 22: 85-89.

Seth P, Miller HB, Lasda EL, Pearson JL, Garcia-Blanco MA (2008). Identification of an intronic splicing enhancer essential for the inclusion of *FGFR2* exon IIIc. *The Journal of Biological Chemistry* 283(15): 10 058-10 067.

Schmider-Ross A, Wirsing M, Buscher U, Neitzel H, Krause M, Henrich W, Reles A, Dudenhausen JW (2004). Analysis of the S810L point mutation of the mineralocorticoid receptor in patients with pregnancy-induced hypertension. *Hypertension in Pregnancy* 23(1): 113-119.

Schwab KO, Moisan AM, Homoki J, Peter M, Simard J (2005). 17alpha-hydroxylase/17,20-lyase deficiency due to novel compound heterozygote mutations: Treatment for tall stature in a female with male pseudohermaphroditism

and spontaneous puberty in her affected sister. *Journal of Pediatric Endocrinology and Metabolism* 18(4): 403-411.

Schwartz GL, Turner ST (2005). Screening for primary aldosteronism in essential hypertension: Diagnostic accuracy of the ration of plasma aldosterone concentration to plasma renin activity. *Clinical Chemistry* 51(2): 386-394.

Sheats N, Lin Y, Zhao W, Cheek DE, Lackland DT, Egan BM (2005). Prevalence, treatment and control of hypertension among African American and Caucasians at primary care sites for medically under-served patients. *Ethniticity & Disease* 15(1): 25-32.

Shi JY, Chen X, Ren Y, Long Y, Tian HM (2010). Liddle's syndrome caused by a novel mutation of the gamma-subunit of epithelial sodium channel gene *SCNN1G* in Chinese (Abstract). *Chinese Journal of Medical Genetics* 27(2): 132-135.

Shimkets RA, Warnock DG, Bositis CM, Nelson-Williams C, Hansson JH, Schambelan M, Gill JR Jr., Ulick S, Milora RV, Findling JW, Canessa CM, Rossier BC, Lifton RP (1994). Liddle's syndrome: heritable human hypertension caused by mutations in the β subunit of the epithelial sodium channel. *Cell* 79(3): 407-414.

Skinner CA, Rumsby G, Honour JW (1996). Single strand conformation polymorphism (SSCP) analysis for the detection of mutations in the *CYP11B1* gene. *The Journal of Clinical Endocrinology and Metabolism* 81(6): 2389-2393.

Snyder PM, Price MP, McDonald FJ, Adams CM, Volk KA, Zeiher BG, Stokes JB and Welsh MJ (1995). Mechanism by which Liddle's syndrome mutations increase activity of a human epithelial Na^+ channel. *Cell* 83: 969-978.

Solyom J, Racz K, Peter F, Homoki J, Sippell WG, Peter M (2001). Clinical, hormonal and molecular genetic characterization of Hungarian patients with 11β-hydroxylase deficiency. *The Journal of Endocrine Genetics* 2(1): 37-44.

Soro A, Ingram MC, Tonolo G, Glorioso N, Fraser R (1995). Evidence of coexisting changes in 11β-hydroxysteroid dehydrogenase and 5β-reductase activity in subjects with untreated essential hypertension. *Hypertension* 25(1): 67-70.

Souza MCLA, Martins CS, Silva-Junior IM, Chriguer RS, Bueno AC, Antonini SR, Silva WA Jr, Zago MA, Moreira AC, de Castro M (2014). *NR3C1* polymorphisms in Brazilians of Caucasian, African, and Asian ancestry: Glucocorticoid sensitivity and genotype association. *Arquivos Brasileiros de Endocrinologia & Metabologia* 58(1): 53-61.

Sowers JR, Ferdinand KC, Bakris GL, Douglas JG (2002). Hypertension-related disease in African Americans. Factors underlying disparities in illness and its outcome. *Postgraduate Medicine* 112(4):24-48.

Staub O, Dho S, Henry PC, Correa J, Ishikawa T, McGlade J, Rotin D (1996). WW domains of Nedd4 bind to the proline-rich PY motifs in the epithelial Na^+ channel deleted in Liddle's syndrome. *The EMBO Journal* 15(10): 2371-2380.

Stewart PM, Krozowski ZS, Gupta A, Milford DV, Howie AJ, Sheppard MC, Whorwood CB (1996). Hypertension in the syndrome of apparent mineralocorticoid excess due to mutation of the 11 beta-hydroxysteroid dehydrogenase type 2 gene. *Lancet* 347: 88-91.

Stowasser M, Bachmann AW, Huggard PR, Rosetti TR, Gordon RD (2000). Severity of hypertension in familial hyperaldosteronism type I: relationship to gender and degree of biochemical disturbances. *The Journal of Clinical Endocrinology and Metabolism* 85(6): 2160-2166.

Stowasser M, Gunasekera TG, Gordon RD (2001). Relationship of hypertension severity to hybrid gene crossover point in familial hyperaldosteronism type 1 (FH-1). *Journal of Hypertension* 19(Suppl 2): S164-S164.

Stowasser M, Huggard PR, Rosetti TR, Bachmann AW, Gordon RD (1999). Biochemical evidence of aldosterone overproduction and abnormal regulation in normotensive individuals with familial hyperaldosteronism type I. *The Journal of Clinical Endocrinology and Metabolism* 84(11): 4031-4036.

Stowasser M, Machmann AW, Jonsson JR, Tunny TJ, Klemm SA, Gordon RD (1995). Clinical, biochemical and genetic approaches to the detection of familial hyperaldosteronism type I. *Journal of Hypertension* 13(12 Pt 2): 1610-1613.

Stowasser M, Sharman J, Leano R, Gordon RD, Ward G, Cowley D, Marwick TH (2005). Evidence of abnormal left ventricular structure and function in normotensive individuals with familial hyperaldosteronism type I. *The Journal of Clinical Endocrinology and Metabolism* 90(9): 5070-5076.

Strazzulo P, Galletti F (2007). Genetics of salt-sensitive hypertension. *Current Hypertension Reports* 9(1): 25-32.

Su YR, Rutkowski MP, Klanke CA, Wu X, Cui Y, Pun RYK, Carter V, Reif M, Menon AG (1996). A novel variant of the β-subunit of the amiloride-sensitive sodium channel in African Americans. *Journal of the American Society of Nephrology* 7: 2543-2549.

Subramanya AR, Yang CL, McCormick JA, Ellison DH (2006). WNK kinases regulate sodium chloride and potassium transport by the aldosterone-sensitive distal nephron. *Kidney International* 70: 630-634.

Sutherland DJ, Ruse JL, Laidlaw JC (1966). Hypertension, increased aldosteronism secretion and low plasma renin activity relieved by dexamethasone. *Canadian Medical Association Journal* 95(22): 1109-1119.

Suzuki Y, Nagashima T, Nomura Y, Origata K, Nagashima K, Morikawa A (1998). A new compound heterozygous mutation (W17X, 436 + 5G → T) in the cytochrome P450c17 gene causes 17 alpha–hydroxylase/17,20-lyase deficiency. *The Journal of Clinical Endocrinology and Metabolism* 83(1): 199-202.

Takeda Y, Miyamori I, Iki K, Inaba S, Furukawa K, Hatakeyama H, Yoneda T, Takeda R (1996). Endogenous renal 11 beta-hydroxysteroid dehydrogenase inhibitory factors in patients with low-renin essential hypertension. *Hypertension* 27(2): 197-201.

Takeda Y, Yoneda T, Demura M, Furukawa K, Koshida H, Miyamori I, Mabuchi H (2001). Genetic analysis of the cytochrome P-450c17α (*CYP17*) and aldosterone synthase (*CYP11B2*) in Japanese patients with 17α-hydroxylase deficiency. *Clinical Endocrinology* 54:751-758.

Tamura H, Schild L, Enomoto N, Matsui N, Marumo F, Rossier BC, Sasaki S (1996). Liddle disease caused by a missense mutation of β subunit of the epithelial sodium channel gene. *Journal of Clinical Investigation* 97(7): 1780-1784.

Taniyama M, Tanabe M, Saito H, Ban Y, Nawata H, Yanase T (2005). Subtle 17α-hydroxylase/17,20-lyase deficiency with homozygous Y201N mutation in an infertile woman. *The Journal of Clinical Endocrinology and Metabolism* 90(5): 2508-2511.

Tanira MOM, Al Balushi KA (2005). Genetic variations related to hypertension: A review. *Journal of Human Hypertension* 19: 7-19.

ten Kate-Booij MJ, Cobbaert C, Koper JW, de Jong FH (2004). Deficiency of 17,20-lyase causing giant ovarian cysts in a girl and a female phenotype in her 46,XY sister: Case report. *Human Reproduction* 19(2): 456-459.

Tiffin N, Meintjies A, Ramesar R, Bajic VB, Rayner B (2010). Computational analysis of candidate disease genes and variants for salt-sensitive hypertension in indigenous Southern Africans. *PLoS One* 5(9): e12989.

Tindall EA, Peterson DC, Woodbridge P, Schipany K, Hayes VM (2009). Assessing high-resolution melting curve analysis for accurate detection of gene variants in complex DNA fragments. *Human Mutation* 30(6): 876-883.

Tobin MD, Tomaszewski M, Braund PS, Hajat C, Raleigh SM, Palmer TM, Caulfield M, Burton PR, Samani NJ (2008). Common variants in genes underlying monogenic hypertension and hypotension and blood pressure in the general population. *Hypertension* 51: 1658-1664.

Tozawa M, Iseki K, Iseki C, Kinjo K, Ikemiya Y, Takishita S (2003). Blood pressure predicts risk of developing end-stage renal disease in men and women. *Hypertension* 41: 1341-1345.

Turan S, Bereket A, Guran T, Akcay T, Papari-Zareei M, Auchus RJ (2009). Puberty in a case with novel 17-hydroxylase mutation and the putative role of estrogen in development of pubic hair. *European Journal of Endocrinology* 160: 325-330.

Uehara Y, Sasaguri M, Kinoshita A, Tsuji E, Kiyose H, Taniguchi H, Noda K, Ideishi M, Inoue J, Tomita K, Arakawa K (1998). Genetic analysis of the epithelial sodium channel in Liddle's syndrome. *Journal of Hypertension* 16: 1131-1135.

Uehara S, Sato J, Nishiyama Y, Matsuzaki S, Funato T, Murotsuki J, Yaegashi N, Okamura K, Yajima A (2000). Compound heterozygous mutations (PHE53/54DEL and HIS373LEU) of the P450c17 gene results in a 17α-hydroxylase/17,20-lyase deficient male pseudohermaphrodite with unambiguous external genitalia. *The Tohoku Journal of Experimental Medicine* 190(4): 279-287.

Ulick S, Levine LS, Gunczler P, Zanconato G, Ramirez LC, Rauh W, Rosler A, Bradlow HL, New MI (1979). A syndrome of apparent mineralocorticoid excess associated with defects in the peripheral metabolism of cortisol. *The Journal of Clinical Endocrinology and Metabolism* 49(5): 757-764.

Umscheid CA, Gross R, Weiner MG, Hollenbeak CS, Tang SSK, Turner BJ (2010). Racial disparities in hypertension control, but not treatment intensification (Abstract). *American Journal of Hypertension* 23(1): 54-61.

Untergasser A, Nijveen H (2007). Primer3Plus. [Online]. Available at: http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/; accessed 14 April 2014.

Van den Akker ELT, Koper JW, Boehmer ALM, Themmen APN, Verhoef-Post M, Timmerman MA, Otten BJ, Drop SLS, de Jong FH (2002). Differential Inhibition of 17 alpha-hydroxylase and 17,20-lyase activities by three novel missense *CYP17* mutations identified in patients with P450c17 deficiency. *The Journal of Clinical and Endocrinology and Metabolism 87(12): 5714-5721.*

Van der Hoogen PC, Feskens EJ, Nagelkerke NJ, Menotti A, Nissinen A, Kromhout D (2000). The relation between blood pressure and mortality due to coronary heart disease among men in different parts of the world. Seven Countries Study Research Group. *New England Journal of Medicine* 342: 1-8.

Van Rossum EFC, Lamberts SWJ (2006). Glucocorticoid resistance syndrome: a diagnostic and therapeutic approach. *Best Practice and Research Clinical Endocrinology and Metabolism* 20(4): 611-626.

Van Zyl S, van der Merwe LJ, Walsh CM, Groenewald AJ, van Rooyen FC (2012). Risk-factor profiles for chronic diseases of lifestyle and metabolic syndrome in an urban and rural setting in South Africa. *African journal of Primary Health Care & Family Medicine* 4(1) aricle # 346. [Online]. Available at http://www.phcfm.org/index.php/phcfm/ article/view/346; 2 February 2013.

Vania A, Tucciarone L, Mazzeo D, Capodaglio PF, Cugini P (1997). Liddle syndrome: A 14-year follow-up of the youngest diagnosed case. *Pediatric Nephrology* 11(1): 7-11.

Vehaskari VM (2009). Heritable forms of hypertension. *Pediatric Nephrology* 24: 1929-2937.

Vormvelde SV, Sehrt D, Toliat MR, Schirmer M, Meineke I, Tzvetkov M, Nurnberg P, Brockmoller J (2007). Genetic variation in the renal sodium transporters NKCC2, NCC and ENaC in relation to the effects of loop diuretic drugs. *Clinical Pharmacology & Therapeutics* 82(3): 300-309.

Wang LP, Gao LG, Zhou XL, Wu HY, Zhang L, Wen D, Li YH, Liu YX, Tian T, Fan XH, Jiang XJ, Zhang HM, Hui RT (2012). Genetic diagnosis of Liddle's syndrome by mutation analysis of *SCNN1B* and *SCNN1G* in a Chinese family. *Chinese Medical Journal* 125(8): 1401-1404.

Warnock DG (1998). Liddle syndrome: An autosomal dominant form of human hypertension. *Kidney International* 53(1): 18-24.

Webb JC, Patel DD, Shoulders CC, Knight BL, Soutar AK (1996). Genetic variation at a splicing branch point in intron 9 of the low density lipoprotein (LDL)-receptor gene: A rare mutation that disrupts mRNA splicing in a patient with familial hypercholesterolaemia and a common polymorphism. *Human Molecular Genetics* 5(9): 1325-1331.

Weinberger MH (1996). Salt sensitivity of blood pressure in humans. *Hypertension* 27(3): 481-490.

White PC, Dupont J, New MI, Leiberman E, Hochberg Z and Rosler A (1991). A mutation in CYP11B1 (Arg-448→His) associated with steroid 11 beta-hydroxylase deficiency in Jews of Moroccan origin. *Journal of Clinical Investigation* 87: 1664-1667.

White PC (1994a). Disorders of aldosterone biosynthesis and action. *The New England Journal of Medicine* 331(4): 250-258.

White PC, Curnow KM, Pascoe L (1994b). Disorders of steroid 11 β-hydroxylase isozymes. *Endocrine Reviews* 15(4): 421-438.

White PC, Mune T, Agarwal AK (1997). 11β-hydroxysteroid dehydrogenase and the syndrome of apparent mineralocorticoid excess. *Endocrine Reviews* 18(1): 135-156.

Whitworth JA, Williamson PM, Mangos G, Kelly JJ (2005). Cardiovascular consequences of cortisol excess. *Vascular Health and Risk Management* 1(4): 291-299.

WHO (2008). WHO STEPS Surveillance: Guide to physical measurements. [Online]. Available at: http://www.who.int/chp/steps/ Part3_Section3.pdf; accessed September 15, 2004.

WHO (2012). World Health Statistics Report 2012. [Online]. Available at http://www.who.int/gho/publications/world_health_statistics/EN_WHS2012_Full.pdf ; accessed 14 May 2013.

WHO (2013). Global action plan for the prevention and control of non-communicable diseases 2013-2020. [Online]. Available at: http://apps.who.int/iris/bitstream/ 10665/94384/1/9789241506236_eng.pdf?ua=1; accessed 3 November 2015.

WHO (2014a). Global status report on non-communicable diseases. [Online]. Available at: http://apps.who.int/iris/bitstream/10665/148114/1/9789241564854_ eng.pdf?ua=1; accessed 3 November 2015.

WHO (2014b). Non-communicable diseases (NCD) country profiles, South Africa. [Online]. Available at: http://www.who.int/nmh/countries/ zaf_en.pdf; accessed 3 November 2015.

Williams SS (2007). Advances in genetic hypertension. *Current Opinion in Pediatrics* 19(2): 192-198.

Wilson RC, Harbison MD, Krozowski ZS, Funder JW, Shackleton CH, Hanausku-Abel HM, Wei JQ, Hertecant J. Moran A, Neiberger RE, Balfe JW, Fattah A, Daneman D, Licholai T, New MI (1995a). Several homozygous mutations in the gene for

11β-hydroxysteroid dehydrogenase type 2 in patients with apparent mineralocorticoid excess. *The Journal of Clinical Endocrinology and Metabolism* 80(11): 3145-3150.

Wilson RC, Krozowski ZS, Li K, Obeyesekere VR, Razzaghy-Azar M, Harbison MD, Wei JQ, Shackleton CH, Funder JW, New MI (1995b). A mutation in the *HSD11B2* gene in a family with apparent mineralocorticoid excess. *The Journal of Clinical Endocrinology and Metabolism* 80: 2263-2266.

Wilson RC, Dave-Sharma S, Wei JQ, Obeyesekere VR, Li K, Ferrari P, Krozowski ZS, Shackleton CH, Bradlow L, Wiens T, New MI (1998). A genetic defect resulting in mild low-renin hypertension. *Proceedings of the National Academy of Sciences of the USA* 95: 10200-10205.

Wilson FH, Disse-Nicodème S. Choate KA, Ishikawa K, Nelson-Williams C, Desitter I, Gunel M, Milford DV, Lipkin GW, Achard JM, Feely MP, Dussol B, Berland Y, Unwin RJ, Mayan H, Simon DB, Farfel Z, Jeunemaitre X, Lifton RP (2001a). Human hypertension caused by mutations in *WNK* kinases. *Science* 293: 1107-1112.

Wilson RC, Nimkarn S, New MI (2001b). Apparent mineralocorticoid excess. *Trends in Endocrinology & Metabolism* 12(3): 104-111.

Wilson FH, Kahle KT, Sabath E, Lalioti MD, Rapson AK, Hoover RS, Hebert SC, Gamba G, Lifton RP (2003). Molecular pathogenesis of inherited hypertension with hyperkalemia: the Na-Cl cotransporter is inhibited by wild-type but not mutant *WNK4*. *Proceedings of the Natural Academy of Sciences of the USA* 100: 680-684.

Wittwer CT, Reed GH, Gundry CN, Vandersteen JG, Pryor RJ (2003). High-resolution genotyping by amplicon melting analysis using LC Green. *Clinical Chemistry* 49(6): 853-860.

World Economic Forum (2011). The global economic burden of non-communicable diseases. A report by the World Economic Forum and the Harvard School of Public Health. [Online]. Available at: http://www3.weforum.org/docs/WEF_ Harvard_HE_GlobalEconomicBurdenNonCommunicableDiseases_2011.pdf; accessed 3 November 2015.

Won GS, Chiu CY, Tso YC, Jeng SF, Cheng PS, Jap TS (2007). A compound heterozygous mutation in the *CYP17* (17alpha-hydroxylase/17,20-lyase) gene in a Chinese subject with congenital adrenal hyperplasia. *Metabolism: Clinical and Experimental* 56(4): 504-507.

Wu C. Zhou Q, Wan L, Ni L, Zheng C, Qian Y, Jin J (2011). Novel homozygous p.R454C mutation in the *CYP11B1* gene leads to 11β-hydroxylase deficiency in a Chinese patient. *Fertility and Sterility* 95(3): 1122.e3-1122.e6.

Yamaguchi H, Nakazato M, Miyazato M, Toshimori H, Oki S, Shimizu K, Suiko M, Kangawa K, Matsukura S (1998). Identification of a novel splicing mutation and 1-bp deletion in the 17α-hydroxylase gene of Japanese patients with 17α-hydroxylase deficiency. *Human Genetics* 102(6): 635-639.

Yamashita Y, Koga M, Takeda Y, Enomoto N, Uchida S, Hashimoto K, Yamano S, Dohi K, Marumo F, Sasaki S (2001). Two sporadic cases of Liddle's syndrome caused by *de novo* ENaC mutations. *American Journal of Kidney Diseases* 37(3): 499-504.

Yanase T, Kagimoto M, Matsui N, Simpson ER, Waterman MR (1988). Combined 17 alpha-hydroxylase/17,20-lyase deficiency due to a stop codon in the N-terminal region of 17 alpha-hydroxylase cytochrome P-450. *Molecular and Cellular Endocrinology* 59(3): 249-253.

Yanase T, Waterman MR, Zachmann M, Winter JS, Simpson ER, Kagimoto M (1992). Molecular basis of apparent isolated 17,20-lyase deficiency: compound heterozygous mutations in the C-terminal region (Arg(496) – Cys, Gln(461) - Stop)

actually cause combined 17alpha-hydroxylase/17,20-lyase deficiency. *Biochimica et Biophysica Acta - Molecular Basis of Disease* 1139(4): 275-279.

Yanase T (1995). 17α-hydroxylase/17,20-lyase defects. *The Journal of Steroid Biochemistry and Molecular Biology* 53(1-6): 153-157.

Yang CL, Angell J, Mitchell R, Ellison DH (2003). WNK kinases regulate thiazide-sensitive Na-Cl cotransport. *Journal of Clinical Investigation* 111(7): 1039-1045.

Yang J, Cui B, Sun S, Shi T, Zheng S, Bi Y, Liu J, Zhao Y, Chen J, Ning G, Li X (2006). Phenotype-genotype correlation in eight Chinese 17α-hydroxylase/17,20-lyase-deficiency patients with five novel mutations of *CYP17A1* gene. *The Journal of Clinical Endocrinology and Metabolism* 91(9): 3619-3625.

Ye Z, Zhang M, Zhang H, Jiang J, Li X, Zhang K (2010). A novel missense mutation, GGC (Arg454) → TGC(Cys), of *CYP11B1* gene identified in a Chinese family with steroid 11β-hydroxylase deficiency. *Chinese Medical Journal* 123(10): 1264-1268.

Young WF (1999). Primary aldosteronism: A common and curable form of hypertension. *Cardiology in Review* 7(4): 207-214.

Yusuf S, Reddy S, Ounpuu S, Anand S (2001). Global burden of cardiovascular diseases. Part II: Variations in cardiovascular disease by specific ethnic groups and geographic regions and prevention strategies. *Circulation* 104: 2855-2864.

Zachmann M, Tassinari D, Prader A (1983). Clinical and biochemical variability of Congenital Adrenal Hyperplasia due to 11β-hydroxylase deficiency, a study of 25 patients (Abstract). *The Journal of Clinical Endocrinology and Metabolism* 56(2): 222-229.

Zhang C, Wang Z, Xie J, Yan F, Wang W, Feng X, Zhang W, Chen N (2011). Identification of a novel *WNK4* mutation in Chinese patients with Pseudohypoaldosteronism type II. *Nephron Physiology* 118: 53-61.

Zhang K, Weder AB, Eskin E, O'Connor DT (2010). Genome-wide case/control studies in hypertension: only the "tip of the iceberg". *Journal of Hypertension* 28(6): 1115-1123.

Zhao Q, Gu D, Hixson JE, Liu DP, Rao DC, Jaquish CE, Kelly TN, Lu F, Ma J, Mu J, Shimmin LC, Chen J, Mei H, Hamm LL, He J, for the Genetic Epidemiology Network of Salt Sensitivity Collaborative Research Group (2011). Common variants in epithelial sodium channel genes contribute to salt sensitivity of blood pressure: The GenSalt study. *Circulation: Cardiovascular Genetics* 4: 375-380.

Zhu YS, Cordero JJ, Can S, Cai LQ, You X, Herrera C, DeFillo-Ricart M, Chackleton C, Imperato-McGinley (2003). Mutations in *CYP11B1* gene: Phenotype-genotype correlations. *American Journal of Medical Genetics* 122A: 193-200.

Appendix A: Genetic markers associated with elevated blood pressure

Table 1: Genetic variants in the 11β-hydroxysteroid dehydrogenase gene (*HSD11B2*) that result in reduced activity of the enzyme.

Variant	Exon	Nucleotide change [a]	Proposed effect	Population	Reference
Arg74Gly (CP044522)	1	5 336 C>G	Predicted to cause a truncated protein, resulting in loss of HSD11B2 activity	Oman	Quinkler *et al.* (2004)
Asp144Val (CM067681)	2	9 661 A>T	Reduced enzyme activity	French	Morineau *et al.* (2006)
Leu179Arg (CM993570)	3	9 882 T>G	Abolishes enzyme activity	Caucasian	Nunez *et al.* (1999)
Ser180Phe (CM993571)	3	9 885 C>T	Reduces enzyme activity	Japanese	Nunez *et al.* (1999)
Phe185Ser (CM067682)	3	9 900 T>C	Reduce enzyme activity to approximately 30% of wild type	Guyana	Morineau *et al.* (2006)
Arg186Cys (CM950649)	3	9 902 C>T	Reduce enzyme activity to approx 5% of wild type activity	African American	Wilson *et al.* (1995a)
				African American	Dave-Sharma *et al.* (1998)
				Brazilian	Coeli *et al.* (2008)
Arg208Cys (rs121917780)	3	9 968 C>T	Reduces enzyme activity by at least 95%	Middle Eastern	Wilson *et al.* (1995a)
				Native American	Mune *et al.* (1995)
				Oman	Dave-Sharma *et al.* (1998)

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Variant	Exon	Nucleotide change [a]	Proposed effect	Population	Reference
Arg208His (rs28934592)	3	9 969 G>A	Abolishes enzyme activity	Japanese	Kitanaka *et al.* (1997)
				Mexican American	Nunez *et al.* (1999)
Arg213Cys (rs28934591)	3	9 983 C>T	Reduces enzyme activity by ca. 90%	Caucasian/ Native South American	Mune *et al.* (1995)
				Argentinean	Rogoff *et al*. (1998)
				French	Morineau *et al.* (1999)
				Chilean	Rodriguez JA (2000)
				Algerian	Morineau *et al*. (2006)
Ala221Val (CM041371)	3	10 008 C>T	Suspect that the mutation compromises pre-mRNA splicing	Oman	Quinkler *et al.* (2004)
9 279 C>T (CS951448)	intron 3	10 024 C>T	Suspect that the mutation causes skipping of exon 4 (which contains the sequence of the catalytic domain) and thus result in an inactive enzyme; Postulated to change 2nd structure of pre-mRNA which may affect splicing process	Mexican American	Mune *et al.* (1995)
				Mexican American	Nunez *et al.* (1999)
				Chilean	Carvajal *et al.* (2003)
Asp223Asn (rs121917833)	4	10 119 G>A	Reduces enzyme activity to about 6% of wild type	Chilean	Carvajal *et al.* (2003)

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Variant	Exon	Nucleotide change [a]	Proposed effect	Population	Reference
Tyr226Asn (CM031995)	4	10 127 A>T	Reduce enzyme activity to about 3% of wild type	Caucasian	Lavery *et al.* (2003)
Pro227Leu (rs121917782)	4	10 132 C>T	Reduces enzyme activity, mild AME	Mennonite	Wilson *et al.* (1998)
Tyr232Cys (CM031995)	4	10 147 A>G	Reduce enzyme activity to about 3% of wild type	Caucasian	Lavery *et al.* (2003)
Ala237Val (CM993572)	4	10 152 C>T	Reduces enzyme activity	Caucasian	Nunez *et al.* (1999)
Asp244Asn (CM981000)	4	10182 G>A	Associated with Arg250; Heterozygote mutation result in loss of enzyme activity	Italian Moroccan	Dave-Sharma *et al.* (1998)
Leu250Arg (CM981001)	4	10 201 T>G	Associated with Asn244; Heterozygote mutation result in loss of enzyme activity		
Leu250Pro (CX952214)	4	10 201 T>C; 10 203,4 CT>TC	Heterozygous, associated with Leu251Ser; Reduce enzyme activity to almost 0% of wild type	Native American; European Native American	Mune *et al.* (1995); Wilson *et al.* (1995a)
Arg279Cys (rs28934594)	5	10 488 C>T	Decreases enzyme activity by approximately 33%	Sardinian	Li *et al.* (1998)
Ala328Val (CM972867)	5	10 636 C>T	Reduce enzyme activity to about 5% of wild type activity	Brazilian	Li *et al.* (1997)

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Variant	Exon	Nucleotide change [a]	Proposed effect	Population	Reference
Arg337Cys (rs121917781)	5	10 662 C>T	Reduce enzyme activity to about 60% of wild type; Decreased half-life from 21 to 4 hours compared to wild-type	Portuguese	Morineau *et al.* (1999)
				Caucasian	Nunez *et al.* (1999)
				Portuguese	Morineau *et al.* (2006)
				Persian	Wilson *et al.* (1995b)
Arg337His, ΔTyr338 (rs28934593)	5	10 663 G>A	Tyr338 is essential for enzyme activity and the deletion thereof results in an inactive enzyme	Iranian	Dave-Sharma *et al.* (1999)
				Iranian; East Indian	Wilson *et al.* (1995a)
Tyr338His (CM076236)	5	10 665 T>C	Reduce enzyme activity to about 5% of wild type; Abolishes enzyme activity; decreased half-life from 21 to 3 hours compared to wild-type, probably due to degradation via the proteosomal pathway	Iranian; Indian Iranian	Dave-Sharma *et al.* (1999)
				Japanese	Kitanaka *et al.* (1997)
				German	Atanasov *et al.* (2007)
Arg359Trp (CM031996)	5	10 728 C>T	Reduce enzyme activity to aproximately 5% of wild type	Italian	Lavery *et al.* (2003)
Arg374X (rs45442297)	5	10 744 G>A	Generate a stop codon, which result in a truncated protein; Reduces enzyme activity to almost 0% of wild type	Asian	Milford *et al.* (1994)

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Variant	Exon	Nucleotide change [a]	Proposed effect	Population	Reference
Leu376Pro (CM031997)	5	10 780 T>C	Reduce enzyme activity to about 10% of wild type	Asian Pakistani	Stewart *et al.* (1996)
				Northern European	Knops *et al.* (2011)
				Caucasian	Lavery *et al.* (2003)

[a] – using NG_016549.1 as reference sequence

Table 2: Genetic variants in the glucocorticoid receptor gene (*NR3C1*) that result in the receptor being partially insensitive to cortisol.

Variant	Exon	Nucleotide change [a]	Proposed effect	Population	Reference
Arg469X (CM107217)	6	130 353 C>T	Reduced ligand-dependant nuclear translocation and transcriptional activity	French (Caucasian)	Bouligand *et al.* (2010)
Ile559Asn (rs104893909)	7	139 957 T>A	Interferes with translocation to the nucleus and thereby strongly reduces transcription activation, decreased GR binding sites, acts as dominant negative mutant	Dutch	Karl *et al.* (1996)
Val571Ala (rs104893911)	7	139 993 T>C	Decreased transcription activation and reduced affinity for the ligand (x 6)	Brazilian	Mendonca *et al.* (2002)
Asp641Val (rs104893908)	9	144 952 A>T	Decreased transcription activation and reduced affinity for the ligand (x3)	Not mentioned	Hurley *et al.* (1991)
Arg714Gln (CM102670)	10	157 905 G>A	Reduced transcription activation; dominant negative mutant	Not mentioned	Nader *et al.* (2010)
Phe737Leu (rs121909727)	11	158 499 T>C	Reduced transcription activation and reduced affinity for the ligand (x2.6)	Not mentioned	Charmandari *et al.* (2007)

Variant	Exon	Nucleotide change [a]	Proposed effect	Population	Reference
Leu773Pro (rs104893912)	11	158 608 T>C	Reduced transcription activation, reduced affinity for ligand (x2.6), abnormal interaction with GRIP1, acts as dominant negative mutant	Not mentioned	Charmandari *et al.* (2005)

[a] – using NG_009062.1 as reference sequence

Table 3: Genetic variants in the *CYP11B1* gene that result in reduced activity of 11β-hydroxylase.

Variant	Exon	Nucleotide change [a]	Proposed effect	Population	Reference
Gln19X (CM980493)	1	5 062 C>T	Abolish enzyme activity	Caucasian	Merke *et al.* (1998)
				British Caucasian	Parajes *et al.* (2010)
Pro42Ser (CM970405)	1	5 161 C>T	Reduce enzyme activity to 17% of wild type activity	Not described	Joehrer *et al.* (1997)
Leu83Ser (CM066027)	2	5 642 T>G	Reduced enzyme activity to 3% of wild type	Scottish Caucasian	Barr *et al.* (2006)
Pro94Leu (rs104894070)	2	5 642 C>T	Almost completely abolish CYP11B1 activity (0 to 2% activity)	Romanian	Grigorescu-Sido *et al.* (2005)
				German	Krone *et al.* (2006)
Trp116Cys (CM051445)	2	5 742 G>C	Reduce enzyme activity to 3% of wild type	Turkish	Krone *et al.* (2005)
Trp116Gly (CM100845)	2	5 740 T>G	Complete loss of enzyme activity	Eastern German	Parajes *et al.* (2010)
Trp116X (rs104894066)	2	5 741 G>A	Complete loss of enzyme activity	Japanese	Naiki *et al.* (1993)
Val129Met (CM960464)	2	5 779 G>A	Complete loss of enzyme activity	Caucasian	Geley *et al.* (1996)
Asn133His (CM970406)	3	7 600 A>C	Reduce enzyme activity to 17% of wild type activity	Not described	Joehrer *et al.* (1997)
Arg141X (CM010807)	3	7 624 C>T	Reduce enzyme activity	Hungarian	Solyom *et al.* (2001)
Leu158Pro (CM066024)	3	7 676 T>C	Reduced enzyme activity to 3% of wild type	Scottish	Barr *et al.* (2006)

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Variant	Exon	Nucleotide change [a]	Proposed effect	Population	Reference
Ala165Asn (CM100846)	3	7 697 C>A	Reduced enzyme activity to 2% of wild type	British Caucasian	Parajes *et al.* (2010)
Lys174X (CM930174)	3	7 723 A>T	Associated with Gln384	Not described	Curnow *et al.* (1993)
Trp247X (CM960465)	4	8 080 G>A	Abolish enzyme activity	Caucasian	Geley *et al.* (1996)
Gln265X (CM034881)	4	8 133 C>T	Complete loss of enzyme activity	Not described	Kuribayashi *et al.* (2003)
Leu299Pro (CM051446)	5	8 522 T>C	Reduce enzyme activity to 1.2%	Iraq	Krone *et al.* (2005)
				Turkish	Riedl *et al.* (2008)
Ala306Val (CM50575)	5	8 543 C>T	May affect the protein structure and enzyme activity	Chinese	Lee *et al.* (2005)
Gly314Arg (CM053825)	5	8 566 G>C	Complete loss of enzyme activity	Caucasian	Kuribayashi *et al.* (2005)
Thr318Met (rs104894061)	5	8 579 C>T	Thr at codon 318 absolutely conserved across all P450 enzymes; Complete loss of enzyme activity	Yemenite	Curnow *et al.* (1993)
				Yemenite	Motaghedi *et al.* (2005)
Thr318Arg (CM980494)	5	8 579 C>G	Affects the same residue as Met 318; might affect protein transfer to the bound oxygen molecule	Yemenite	Merke *et al.* (1998)
				Hungarian	Solyom *et al.* (2001)
				Romanian	Grigorescu-Sido *et al.* (2005)
Thr318Pro (CM050576)	5	8 578 A>C	Completely prevent protein transfer to the bound oxygen molecule	Chinese	Lee *et al.* (2005)

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Variant	Exon	Nucleotide change [a]	Proposed effect	Population	Reference
Thr319Met (CM970407)	6	8 944 C>T	Reduce enzyme activity to 37% of wild type activity	Not described	Joehrer *et al.* (1997)
Ala331Val (CM960468)	6	8 980 C>T	Complete loss of enzyme activity	Caucasian	Geley *et al.* (1996)
				Syrian	Motaghedi *et al.* (2005)
Gln338X (CM930176)	6	9 000 C>T	Complete loss of enzyme activity	Indian Sikhs	Curnow *et al.* (1993)
Gln356X (CM930177)	6	9 054 C>T	Complete loss of enzyme activity	African American	Curnow *et al.* (1993)
				Nigerian	Skinner *et al.* (1996)
				African American	Merke *et al*. (1998)
				African American	Motaghedi *et al.* (2005)
				Tunisian	Kharrat *et al.* (2010)
Ala368Asp (rs104894071)	6	9 091 C>A	Reduce enzyme activity to 1% of wild type	German	Krone *et al.* (2006)
Glu371Gly (CM960469)	6	10 000 A>G	Complete loss of enzyme activity	Caucasian	Geley *et al.* (1996)
Arg374Gln (rs104894062)	6	9 109 C>T	Complete loss of enzyme activity	Lebanese Arab	Curnow *et al.* (1993)
Gly379Val (CM105508)	7	9 523 G>T	Should modify the active site, thereby preventing the synthesis of a functional enzyme	Tunisian	Kharrat *et al.* (2010)
Arg384Gly (CM950330)	7	9 537 C>G	Probably induces classical 11β-hydrox-ylase deficiency (i.e. almost complete loss of enzyme activity)	Japanese	Nakagawa *et al.* (1995)

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Variant	Exon	Nucleotide change [a]	Proposed effect	Population	Reference
Arg384Gln (CM930179)	7	9 538 G>A	Abolish enzyme activity	Not described	Curnow *et al.* (1993)
				Italian	Motaghedi *et al.* (2005)
				Spanish	Paperna *et al*. (2005)
Tyr423X (CM970408)	8	9 735 T>G	Result in a truncated enzyme lacking an essential C residue that interacts with the heme prosthetic group	Not described	Joehrer *et al.* (1997)
Val441Gly (CM930180)	8	9 788 T>G	Abolish enzyme activity	Not mentioned	Curnow *et al.* (1993)
Gly444Asp (CM910103)	8	9 797 G>A	Greatly reduced (almost complete loss) enzyme activity	African American	Motaghedi *et al.* (2005)
Arg448Cys (CM960471)	8	9 808 C>T	Complete loss of enzyme activity	Iranian	Geley *et al.* (1996)
				Hungarian	Solyom *et al.* (2001)
				Moroccan Jew/Iraqi	Paperna *et al.* (2005)
Arg448His (rs28934586)	8	9 809 C>T	Complete loss of enzyme activity	Moroccan Jew	White *et al.* (1991)
				Caucasian	Geley *et al.* (1996)
				Hungarian	Solyom *et al.* (2001)
				Romanian	Grigorescu-Sido *et al.* (2005)
				Eastern German	Paperna *et al.* (2005)
				Croatian	Dumic *et al*. (2010)

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Variant	Exon	Nucleotide change [a]	Proposed effect	Population	Reference
Arg454Cys (CM103705)	8	9 826 C>T	Could abolish or reduce enzyme activity	Chinese	Ye *et al.* (2010)
				Chinese	Wu *et al.* (2011)
Leu461Pro (CM045137)	8	9 848 T>C	Could abolish or reduce enzyme activity	Aborigine Taiwanese	Chang *et al.* (2004)

[a] – using NG_007954.1 as reference sequence

Table 4: Genetic variants in *CYP17A1* that result in reduced activity of CYP17.

Variant	Exon	Nucleotide change [a]	Proposed effect	Population	Reference
Trp17X (rs104894141)	1	5 223 G>A	Completely abolishes enzyme activity	Japanese	Yanase *et al.* (1988)
				Japanese	Suzuki *et al.* (1998)
Tyr27X (rs104894152)	1	5 253 C>A	Completely abolish both 17alpha-hydroxylase and 17,20-lyase activity	Turkish	Mussig *et al.* (2005)
Tyr64Ser (CM931231)	1	5 363 A>C	Reduce 17alpha-hydroxylase activity to 15% of wild type	Caucasian	Imai *et al.* (1993)
Gly90Asp (CM952253)	1	5 441 G>A	Less than 1% CYP17 activity	Caucasian	Yanase *et al.* (1995)
Phe93Cys (rs104894146)	1	5 450 T>G	10% CYP17 activity	Italian	Di Cero *et al.* (2002)
Arg96Trp (rs104894138)	1	5 458 C>T	Almost complete abolishment of 17α-hydroxylase and 17,20-lyase activity	French Canadian	Laflame *et al.* (1996)
				Italian	Biason-Lauber *et al.* (2000)
				Brazilian	Martin *et al.* (2003)
				Caucasian	Sahakitrung-ruang *et al.* (2009)
				Brazilian	Costenaro *et al.* (2010)
Arg96Gln (rs104894153)	1	5 459 G>A	Complete loss of 17α-hydroxylase and 17,20-lyase activity	United Arab Emirates	Brooke *et al.* (2006)
Ser106Pro (rs104894135)	2	7 160 T>C	Less than 1% activity for both 17α-hydroxylase and 17,20-lyase	Guamanian	Lin *et al.* (1991)

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Variant	Exon	Nucleotide change [a]	Proposed effect	Population	Reference
Gly111Ser (CM101463)	2	7 175 G>A	Complete abolishment of both enzymes	Not mentioned	Rosa *et al.* (2010)
Phe114Val (rs104894147)	2	7 184 T>G	Less than 2.2% and less than 1% activity of 17-alpha-hydroxylase and 17,20-lyase	Not mentioned	Van Den Akker *et al.* (2002)
Asp116Val (rs104894148)	2	7 191 A>T	37.7% 17-alpha-hydroxylase activity and 10.7% 17,20-lyase activity	Not mentioned	Van Den Akker *et al.* (2002)
Arg125Gln (rs104894154)	2	7 218 G>A	Complete loss of 17α-hydroxylase and 17,20-lyase activity	British Italian	Ergun-Longmire *et al.* (2006)
Glu194X (CM930181)	3	7 663 G>T	Completely abolish enzyme activity	English	Rumsby *et al.* (1993)
Tyr201Asn (rs104894150)	3	7 684 T>A	Less than 33% of WT 17-alpha-hydroxylase activity and less than 35% of WT 17,20-lyase activity	Japanese	Taniyama *et al.* (2005)
Arg239Gln (CM090462)	4	8 461 G>A	Non-functional enzyme	English	Rumsby *et al.* (1993)
				Canadian	Ahlgren *et al.* (1992)
				Not mentioned	Turan *et al.* (2009)
Ala302Pro (CM070886)	5	9 476 G>C	Complete loss of 17α-hydroxylase and 17,20-lyase activity	Not mentioned	Rosa *et al.* (2007)
Tyr329Asp (rs104894144)	6	9 869 T>G	Retain less than 5% of wild type 17- alpha hydroxylase activity	Brazilian	Martin *et al.* (2003)
				Brazilian	Costa-Santos *et al.* (2004)

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Variant	Exon	Nucleotide change [a]	Proposed effect	Population	Reference
Ile332Thr (CM101461)	6	9 879 T>C	Partial loss of enzyme activity	Not mentioned	Rosa *et al.* (2010)
Pro342Thr (rs104894137)	6	9 908 C>A	40 to 45% activity of wild type	Canadian	Ahlgren *et al.* (1992)
Arg347His (rs61754278)	6	9 924 G>A	44% of 17-alpha-hydroxylase activity and less than 1% of 17,20-lyase activity	Brazilian	Geller *et al.* (1997)
				Not mentioned	Van Den Akker *et al.* (2002).
Arg347Cys (rs104894149)	6	9 923 C>T	13.6% of 17-alpha-hydroxylase activity and less than 1% of and 17,20-lyase activity	Not mentioned	Van Den Akker *et al.* (2002)
				Not mentioned	ten Kate-Booij *et al.* (2004)
Ala355Thr (CM101462)	6	9 947 G>A	Complete abolishment of both enzymes	Not mentioned	Rosa *et al.* (2010)
Arg358X (CM081228)	6	9 956 C>T	Selectively ablates 17,20-lyase activity, while preserving most 17alpha-hydroxylase activity	Korean	Bhangoo *et al.* (2008)
Arg358Gln (rs104894139)	6	9 957 G>A	65% 17-alpha-hydroxylase activity and less than 5% 17,20-lyase activity	Brazilian	Geller *et al.* (1997)
Arg362Cys (rs104894142)	6	9 968 C>T	Loss of 17-alpha hydroxylase activity	Brazilian	Martin *et al.* (2003)
				Brazilian	Costa-Santos *et al.* (2004)
				Chinese	Won *et al.* (2007)
His373Asp (CM094685)	6	10 001 C>G	Abolishes both 17α-hydroxylase and 17,20-lyase activity	Caucasian	Sahakitrung-ruang *et al.* (2009)

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Variant	Exon	Nucleotide change [a]	Proposed effect	Population	Reference
His373Leu (CM930182)	6	10 002 A>T	Less than 1% activity for both 17α-hydroxylase and 17,20-lyase	Japanese	Monno *et al.* (1993)
				Japanese	Yamaguchi *et al.* (1998)
				Japanese	Matsuzaki *et al.* (2000)
				Japanese	Uehara *et al.* (2000)
				Japanese	Katsumata *et al.* (2001)
				Chinese	Qiao *et al.* (2003)
Lys388X (CM056320)	7	10 945 A>T	Loss of 17,20-lyase activity	German	Schwab *et al.* (2005)
				German	Dhir *et al*. (2009)
Trp406Arg (rs104894143)	7	10 999 T>C	Loss of 17-alpha hydroxylase activity	Brazilian	Martin *et al.* (2003)
				Brazilian	Costa-Santos *et al.* (2004)
				Brazilian	Benetti-Pinto *et al.* (2007)
Pro409Arg (CM012743)	7	11 009 C>G	Less than 1% enzyme activity for both 17α-hydroxylase and 17,20-lyase	Chinese	Won *et al.* (2007)
				Chinese	Lam *et al.* (2001)
Arg416Cys (CM012357)	8	11 551 C>T	Destruct the conformation of a binding loop	Japanese	Takeda *et al.* (2001)
				Chinese	Yang *et al.* (2006)
Arg416His (rs104894155)	8	11 552 G>A	Complete loss of 17α-hydroxylase and 17,20-lyase activity	British Italian	Ergun-Longmire *et al.* (2006)
				Not mentioned	Rosa *et al.* (2007)

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Variant	Exon	Nucleotide change [a]	Proposed effect	Population	Reference
Pro428Leu (rs104894145)	8	11 588 C>T	Retain less than 5% of wild type 17-alpha hydroxylase activity	Brazilian	Martin *et al.* (2003)
				Brazilian	Costa-Santos *et al.* (2004)
				German	Schwab *et al.* (2005)
Phe434Leu (CM063943)	8	11 606 C>T	Partial loss of 17alpha-hydroxylase and 17,20-lyase activity (6% 17 alpha-hydroxylase activity)	Chinese	Yang *et al.* (2006)
Arg440Cys (HM040114)	8	11 623 C>T	Negligible 17-alpha hydroxylase and 17,20-lyase activity	Not mentioned	Patocs *et al.* (2005)
				Czech	Dhir *et al.* (2009)
Arg440His (CM940326)	8	11 624 G>A	Less than 1% activity for both 17α-hydroxylase and 17,20-lyase	German	Fardella *et al.* (1994)
				Not mentioned	Rosa *et al.* (2010)
Phe453Ser (rs104894151)	8	11 663 T>C	Partial loss of 17alpha-hydroxylase and 17,20-lyase activity (29% 17 alpha-hydroxylase activity)	Chinese	Yang *et al.* (2006)
Gln461X (CM920225)	8	11 686 C>T	Combined 17alpha-hydroxylase and 17,20-lyase deficiency	Swiss	Yanase *et al.* (1992)
Leu465Pro (CM094745)	8	11 699 T>C	Almost complete loss of 17α-hydroxylase and 17,20-lyase activity	Turkish	Dhir *et al.* (2009)

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Variant	Exon	Nucleotide change [a]	Proposed effect	Population	Reference
Arg496Cys (CM920226)	8	11 791 C>T	Less than 10% activity for both 17α-hydroxylase and 17,20-lyase	Swiss	Yanase *et al.* (1992)
Arg496Leu	8	11 792 G>T	30% 17alpha-hydroxylase activity and 29% 17,20-lyase activity	Chinese	Lee *et al.* (2006)

[a] – using NG_007955.1 as reference sequence

Table 5: Genetic variants in *SCNN1B* and *SCNN1G* that result in increased activity of the epithelial sodium channel (ENaC).

Variant	Exon	Nucleotide change [a]	Proposed effect	Population	Reference
SCNN1B					
Arg563Gln (rs149868979)	13	83 297 G>A	Result in failure of the ENaC to be down-regulated	South African (Black & mixed ancestry)	Rayner *et al.* (2003)
				South African (Black & mixed ancestry)	Jones *et al.* (2012)
Arg566X (rs137852704)	13	83 305 C>T	Truncated protein does not contain the critical PY-motif, resulting in elevated channel numbers and activity	Not mentioned	Shimkets *et al.* (1994)
				Swedish	Melander *et al.* (1998)
				Japanese	Kyuma *et al.* (2001)
				Chinese	Shi *et al.* (2010)
Gln591X (CM941274)	13	83 380 C>T	Truncated protein does not contain the critical PY-motif, resulting in elevated channel numbers and activity	Not mentioned	Shimkets *et al.* (1994)
Pro617Ser (rs137852708)	13	83 458 C>T	Mutate critical PY motif, result in elevated channel numbers and activity	Japanese	Inoue *et al.* (1998)
Pro617Leu (CM081432)	13	83 459 C>T	Elevated channel numbers and an increase (3x) in channel activity	Italian	Rossi *et al.* (2008)
				Italian	Rossi *et al.* (2011)

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Variant	Exon	Nucleotide change [a]	Proposed effect	Population	Reference
Pro617His (CM094431)	13	83 459 C>A	Mutate critical PY motif, result in elevated channel numbers and activity	Thai	Sawathipar-nich *et al.* (2009)
Pro618Ser (CM983944)	13	83 461 C>T	Mutate critical PY motif, result in elevated channel numbers and activity	Japanese	Uehara *et al.* (1998)
				Serbian	Bogdanović *et al.* (2012)
				Chinese	Wang *et al.* (2012)
Pro618His (CM056053)	13	83 462 C>A	Mutate critical PY motif, result in elevated channel numbers and activity	Afro Haitian	Freundlich and Ludwig(2005)
Pro618Leu (rs137852705)	13	83 462 C>T	Eight-fold increase in channel activity	African American	Hansson *et al.* (1995a)
				Not mentioned	Uehara *et al.* (1998)
				Chinese	Gao *et al.* (2001)
				Japanese	Yamashita *et al.* (2001)
Pro618Arg (rs137852705)	13	83 462 C>G	Mutate critical PY motif, result in elevated channel numbers and activity	Czech	Ciechanowicz *et al.* (2005)
				Japanese	Furuhashi *et al.* (2005)
Tyr620His (rs137852707)	13	83 467 T>C	Constitutive channel activation	Japanese	Tamura *et al.* (1996)
SCNN1G					
Asn530Ser (rs148985177)	13	37 390 A>G	Twofold increase in ENaC activity	Finnish	Hiltunen *et al.* (2002)
Gln567X (CM102172)	13	37 500 C>T	Truncated protein	Chinese	Shi *et al.* (2010)

APPENDIX A: GENETIC MARKERS ASSOCIATED WITH ELEVATED BLOOD PRESSURE

Variant	Exon	Nucleotide change [a]	Proposed effect	Population	Reference
Trp573X (rs137853342)	13	37 519 G>A	Truncated protein, result in elevated channel numbers and activity	African American	Hansson *et al.* (1995b)
Trp575X (CM014826)	13	37 525 G>A	7,5 X increase in channel activity	Japanese	Yamashita *et al.* (2001)

[a] – using NG_011908.1 and NG_011909.1 as reference sequences for *SCNN1B* and *SCNN1G*, respectively

Table 6: Genetic variants in the *WNK4* gene that result in reduced inhibition of the sodium-chloride co-transporter (NCCT).

Variant	Exon	Nucleotide change [a]	Proposed effect	Population	Reference
Glu560Gly (rs193922734)	7	11 850 A>G	Located in a region thought to be important for protein structure and function; computer simulation predicted to be pathogenic	Not mentioned	Brooks *et al.* (2011)
Pro561Leu (rs193922735)	7	11 853 C>T	Located in a region thought to be important for protein structure and function	Chinese	Gong *et al.* (2008)
Glu562Lys (rs1378533093)	7	11 855 G>A	Loss of NCCT inhibition; increase K^+ inhibition	Caucasian	Wilson *et al.* (2001a)
Asp564Ala (rs137853094)	7	11 862 A>C	Increase paracellular Cl^- permeability and phosphorylation claudins	Caucasian	Wilson *et al.* (2001a)
Asp564His (rs193922736)	7	11 861 G>C	No significant inhibitory effect on NCCT; increased inhibition of renal outer medullary potassium channel	Not mentioned	Golbang *et al.* (2005)
Gln565Glu (rs137853092)	7	11 864 C>G	Loss of NCCT inhibition; increase K^+ inhibition	Caucasian (Israeli)	Wilson *et al.* (2001a)
Lys1169Glu (rs193922737)	17	20 566 A>G	Loss of NCCT expression inhibition	Chinese	Zhang *et al*. (2011)
Arg1185Cys (rs137853095)	17	20 614 C>T	Partial loss of NCCT inhibition	Caucasian	Wilson *et al.* (2001a)

[a] – using NG_007984.1 as reference sequence

Appendix B: Epidemiological data for the study cohort

Table 1: Epidemiological data collected by the Department of Nutrition and Dietetics (UFS) for the 90 hypertensive individuals included in the current study.

Sample no.	Gender	Age	Weight (kg)	Height (cm)	BMI (kg/m^2)	Waist (cm)	Hip (cm)	SBP (mmHg)	DBP (mmHg)	Medication for HT
17.1	F	57	56	160	21.9	88	91	131	105	Yes
20.1	F	59	108	145	51.4	131	145	198	188	No
23.1	F	55	45	152	19.5	68	82	203	145	Yes
25.1	F	34	75.1	165.5	27.4	87	113	150	109	Yes
44.1	M	32	60	174	19.8	74	89	141	101	No
46.1	F	37	78	159.5	30.7	89	108	129	91	No
47.1	F	37	93	158	37.3	100	115	162	94	Yes
47.2	F	57	113.7	158	45.5	125	139	139	99	Yes
48.1	M	40	135	164.5	49.9	134	145	147	83	No
48.3	F	35	72	162	27.4	80	114	141	88	Yes
50.1	F	43	103.2	165	37.9	114	124	150	110	No
54.1	M	39	51.5	168.5	18.1	71	88	148	93	No
55.1	F	46	56	151	24.6	77	97	132	84	Yes
59.1	F	50	69.4	159.3	27.3	88	104	122	106	Yes
65.1	F	51	61.8	165	22.7	90.5	105	189	102	No

APPENDIX B: EPIDEMIOLOGICAL DATA FOR THE STUDY COHORT

Sample no.	Gender	Age	Weight (kg)	Height (cm)	BMI (kg/m^2)	Waist (cm)	Hip (cm)	SBP (mmHg)	DBP (mmHg)	Medication for HT
66.1	F	46	93.8	156	38.5	104.5	133	161	117	Yes
67.1	M	44	110	166	39.9	118	125	147	92	Yes
68.1	F	31	74.2	160	29.0	101	109	160	100	No
79.1	F	48	65	143	31.8	87	109	147	108	Yes
80.1	F	63	104	157	42.2	117	140	146	85	Yes
84.1	M	47	62	168	22.0	80	90	136	90	Yes
85.1	F	39	80	151	35.1	87	100	160	110	Yes
86.1	F	62	85	148	38.8	95	122	131	98	Yes
93.1	F	56	60	151	26.3	79	104	157	95	Yes
95.1	F	51	55	162	21.0	71	89	155	93	No
96.5	M	26	49	162	18.7	66	83	136	94	No
102.1	F	54	60	157	24.3	88	95	158	100	Yes
103.2	F	52	35	141	17.6	64	78	120	90	No
108.2	M	38	52	161	20.1	70	88	145	70	Yes
112.2	F	39	45	157	18.3	68	89	172	117	Yes
118.2	F	52	55.7	152	24.1	87.5	104.5	170	130	No
118.3	F	25	65.4	155	27.2	91	107	172	118	No
121.1	F	44	86	158	34.4	95	118	155	98	Yes
122.1	F	45	79.8	147.5	36.7	102	127	163	84	Yes
129.1	F	62	95	165	34.9	98	122	125	75	Yes

APPENDIX B: EPIDEMIOLOGICAL DATA FOR THE STUDY COHORT

Sample no.	Gender	Age	Weight (kg)	Height (cm)	BMI (kg/m^2)	Waist (cm)	Hip (cm)	SBP (mmHg)	DBP (mmHg)	Medication for HT
141.1	F	34	58.9	156.5	24.0	86	98	129	108	No
143.1	F	56	67.5	156	27.7	100	114	140	80	Yes
144.2	F	56	82.3	158	33.0	101	120	188	92	Yes
149.1	F	36	61	148.3	27.7	74	99	161	125	No
153.5	F	56	84.5	159	33.4	100	133	193	113	No
159.1	F	47	75.5	151.1	33.1	97	118	169	109	Yes
164.2	F	47	65	172	22.0	80	100	147	98	Yes
171.1	F	36	67.6	154.5	28.3	96	108.5	150	100	Yes
181.1	F	62	45.3	150	20.1	80	93	154	72	Yes
193.1	F	37	85	174.5	27.9	91.5	109	172	126	Yes
197.1	M	38	70	165.9	25.4	76	101	140	94	No
208.1	F	35	82.9	165	30.4	103	122	140	94	No
209.1	F	34	79	162	30.1	86	110	125	100	No
211.1	F	54	77	148	35.2	99	104	150	77	Yes
214.1	F	50	42.9	156	17.6	77	84	150	119	No
222.1	F	63	61	151	26.8	83	99	169	98	Yes
225.2	M	63	72	171	24.6	90	97	162	97	Yes
230.2	F	36	76.6	153.5	32.5	97	108	167	121	No
235.1	F	46	78	153.4	33.1	91	108	138	99	Yes
249.1	F	47	117.4	161	45.3	129	140	155	97	Yes

APPENDIX B: EPIDEMIOLOGICAL DATA FOR THE STUDY COHORT

Sample no.	Gender	Age	Weight (kg)	Height (cm)	BMI (kg/m^2)	Waist (cm)	Hip (cm)	SBP (mmHg)	DBP (mmHg)	Medication for HT
254.1	F	29	64	154.5	26.8	81	104	137	91	No
255.1	F	59	56.1	147	26.0	89	99.5	185	114	Yes
257.1	F	47	68	153.4	28.9	98	98	138	99	Yes
260.1	F	51	98.8	158	39.6	114	132	121	96	Yes
265.1	F	53	100.3	162.5	38.0	127	114	146	92	Yes
267.1	F	46	90	159.1	35.6	99	119	151	82	Yes
267.2	M	54	83.9	171.8	28.4	88	105	114	95	Yes
268.1	M	32	52	167	18.6	74	84	145	119	No
269.1	M	52	52	170.3	17.9	74	88	116	99	No
271.1	M	49	56.5	170	19.6	80.5	95	167	109	Yes
272.1	F	36	72	156	29.6	85	109	155	121	No
276.1	F	54	69	160.6	26.8	94	103	147	97	Yes
279.1	F	56	91	155.7	37.5	95	134	145	81	Yes
280.1	F	42	102	160.3	39.7	111.5	130	169	108	Yes
287.1	F	27	62	151	27.2	85.5	108	137	100	No
291.1	F	59	59	153.7	25.0	82	97	162	92	Yes
300.1	F	34	54	147	25.0	77	102	137	92	No
303.1	M	31	54	170.5	18.6	68	85	157	100	No
305.1	F	56	103.9	164	38.6	122	123	126	103	Yes
306.1	F	59	98	157	39.8	112	132	158	82	Yes

APPENDIX B: EPIDEMIOLOGICAL DATA FOR THE STUDY COHORT

Sample no.	Gender	Age	Weight (kg)	Height (cm)	BMI (kg/m^2)	Waist (cm)	Hip (cm)	SBP (mmHg)	DBP (mmHg)	Medication for HT
311.1	F	48	69.8	161	26.9	103	108	144	91	Yes
313.1	F	51	96	159	38.0	117	120	149	94	Yes
314.1	F	57	140	161.4	53.7	115	165	160	115	Yes
315.1	F	39	80	159	31.6	95	110	154	95	Yes
320.1	M	46	66.3	170	22.9	84	97	146	96	Yes
321.1	M	55	74.1	161	28.6	92	105	145	80	Yes
325.1	F	49	80.2	158	32.1	100	113	142	83	Yes
331.1	F	49	100	159.4	39.4	99	120	144	85	Yes
336.1	F	36	64	161.3	24.6	68.5	101	153	100	No
340.1	M	49	61.7	177	19.7	81.5	93	158	115	Yes
345.1	F	28	61.2	156	25.1	86	106	128	98	No
346.1	F	42	88	151.4	38.4	102	118	147	82	Yes
348.1	F	57	121	160.8	46.8	112	144	146	111	Yes
352.1	F	57	94.6	160	37.0	109	124	200	140	Yes
379.1	M	47	45	161	17.4	63	79.5	169	110	Yes

BMI: body mass index; SBP: systolic blood pressure; DBP; diastolic blood pressure; HT: hypertension.