PHARMACOGENETICS OF RESISTANT HYPERTENSION:

Evaluating the Role of Genetic Variation in *ABCB1, ADBR1, CES1, CYP3A4, CYP3A5, NEDD4L, NOS3, NR3C2* and *SCNN1B* among South African Hypertensive Patients

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A thesis submitted in fulfilment of the requirements for the degree of Master of Science in Medicine, MSc. (Med.) in Human Genetics

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12 February 2024

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DECLARATION

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ABSTRACT

Introduction

Globally, it is estimated that over one billion individuals live with hypertension. The prevalence of resistant hypertension can be as high as 19% among African populations. Genetic variation in genes that affect the metabolism and transport of antihypertensive drugs has been reported to influence treatment outcome in patients with hypertension. However, there is substantial lack of data on which genetic variants are important in influencing how African patients with hypertension respond to treatment. We hypothesise that genetic variants previously reported to influence treatment outcome in patients with hypertension, may play a role in the development of resistant hypertension in Africans, as well as other novel variants. Therefore, this study set out to investigate the role of genetic variation in nine genes, namely, *ABCB1, ADBR1, CES1, CYP3A4, CYP3A5, NEDD4L, NOS3, NR3C2* and *SCNN1B,* on the development of resistant hypertension in South African patients. Knowledge of pharmacogenetic variants that play a role in resistant hypertension may potentially improve treatment and management of hypertension in our populations.

Materials and Method

This was a retrospective matched case-control study. The study was conducted at the Hypertension Clinic at Groote Schuur Hospital, Cape Town, South Africa. The study was approved by the Human Research Ethics Committee (HREC) of the University of Cape Town (HREC 141/2022) and all patients had consented to be included in pharmacogenetic studies. Demographic and clinical variables were extracted from the patients' medical records. Patients with BP ≥ 140/90 mmHg on 3 or more antihypertensive drugs or BP < 140/90 mmHg on more than 3 antihypertensive drugs, were classified into the resistant hypertension group (i.e., the case group). Patients on less than 3 antihypertensive drugs were classified into the non-resistant hypertension group (i.e., the control group). Twenty variants in the *ABCB1, ADBR1, CES1, CYP3A4, CYP3A5, NEDD4L, NOS3, NR3C2* and *SCNN1B* genes were genotyped using either polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) or quantitative PCR and subsequently validated using Sanger sequencing. Genetic models of inheritance were used to determine associations between genotypes and resistant hypertension. Multivariable logistic regression was performed to determine the

association between genotypes and resistant hypertension while adjusting for potential confounding variables.

Results

A total of 379 participants (29% Black African and 71% Mixed Ancestry) were successfully recruited and genetically characterised for 19 SNPs and 1 copy number variant (CNV). The participants comprised 190 cases (resistant to hypertension treatment) and 189 controls (non-resistant to hypertension treatment). In each group, 54% were female and the median age for cases and controls was 43.8 (20 – 54.2) and 42.2 (26 – 52.6) years, respectively. Most genotyping results were consistent with the Hardy-Weinberg equilibrium (P >0.05) except for *ABCB1* rs2032582 (P = 0.01), *CES1* copy number (P < 0.01) *CYP3A4* rs2740574 (P = 0.01) and *SCNN1B* rs149868979 (P = 0.008) polymorphisms. Homozygosity for *CYP3A5* rs776746C/C (P = 0.02; OR: 0.44; CI: 0.22 - 0.89) and *NOS3* rs3918188A/A (P = 0.0003; OR: 0.21; CI: 0.08 – 0.49) was associated with protection against resistant hypertension. On the other hand, homozygosity for *ADRB1* rs1801252G/G (P = 0.02; OR: 3.30; CI: 1.17 – 10.03) and *NEDD4L* rs4149601A/A (P = 0.001; OR: 3.82; CI: 1.67 – 9.07) was associated with increased risk of resistant hypertension. Carriers of the *ADRB1* rs1801252 – rs1801253G-C haplotype were more likely to have resistant hypertension (P = 0.04; OR: 2.83; CI: 1.05 -8.20). Carriers of the *NOS3* rs2070744 – rs1798883 – rs3918188G–T–A haplotype appeared to be protected against resistant hypertension (P = 0.006; OR: 0.47; CI: 0.27 – 0.80). There were no statistically significant associations with resistant hypertension for selected polymorphisms in the *ABCB1, CES1, CYP3A4, NR3C2* and *SCNN1B* genes (P > 0.05).

Discussion

The *CYP3A5* rs776746C allele is functionally associated with reduced CYP3A5 expression. This may lead to reduced clearance of antihypertensive drugs such as amlodipine, enalapril or spironolactone, increasing their exposure and efficacy. *NOS3* rs3918188A allele may affect *NOS3* gene expression, or its effect may be due to variants in linkage disequilibrium with it, leading to increased efficacy of antihypertensives drugs such as enalapril. *ADRB1 rs1801252G* allele or *ADRB1* rs1801252A>G – rs1801253G>C, G–C haplotype alters ADRB1 receptor function, leading to reduced sensitivity to beta-blockers such as atenolol and consequently reduced efficacy. The *NEDD4L* rs4149601A allele results in a non-functional NEDD4L, impacting antihypertensive drugs with renal mechanisms, such as hydrochlorothiazide and amiloride.

Conclusion

CYP3A5 rs776746T>C, *NOS3* rs3918188C>A, *ADRB1* rs1801252A>G and *NEDD4L* rs4149601G>A may be variants of pharmacogenomics importance in Africans with respect to response to hypertension treatment. They seem to play a role in either predisposition to resistant hypertension or the pharmacogenomics of antihypertensive drugs. Pre-emptive screening for these variants may be of potential clinical utility in prescribing antihypertensive drugs by influencing decisions on suitable drug types or optimal drug doses in patients with hypertension.

ACKNOWLEDGEMENTS

I would like to thank the **Almighty God** for taking me through this journey. Without Him, none of this would have been possible,

'***For in Him we live and move and have our being…***'
Acts 17:28.

It is my genuine pleasure to also acknowledge everyone that has been in this journey with me. I would like to express my deepest gratitude to:

My supervisor and mentor, Professor Collet Dandara, for his unwavering support, guidance, kindness and encouragement throughout this journey. I am beyond humbled and privileged to have had the opportunity to learn from you, both academic and life lessons. Thank you for the opportunity to be supervised by you, and opening up a door for me to study at one of the best leaning institutions in Africa.

My co-supervisor, Dr. Nyarai Soko, for believing in me and offering me a stepping stone to explore my potential. I would like to thank you and for your ferventness, expertise and rigour in the field of genetics, something that I have benefited immensely from.

My co-supervisors and experts in different fields. I had the opportunity to be co-supervised by **Professor Erika Jones, Professor Brian Rayner, Professor Dirk Blom and Professor Phumla Sinxadi**.

To all my supervisors,

"*If I have seen further it is by standing on the shoulders of Giants – Sir Isaac Newton*".

Thank you for your guidance, constructive feedback, expertise and holding my hand throughout this journey.

My fellow colleagues and researchers in the Pharmacogenomics and Drug Metabolism Research group, both current and former: Dr. Arinao Ndadza, Dr. Nonkululeko Mkwanazi, Ms. Marguerite Beningfield, Ms. Smangele Malema, Ms. Bianca Kruger, Ms. Zizo Lusiki, Ms. Nosipho Mabizela, Mr. Manase Kilonzi and Mr. Migael Mouton. You brighten(ed) my day and made my postgraduate journey bearable.

The whole Division of Human Genetics. I would like to appreciate Mr. Faghri February, Mr. Akshay Vanmali, Ms. Letillia Xhakaza for their unwavering support in the lab and for always being there to assist.

My family: My Parents (Chestin & Jonathan), for their prayers, support and encouragement; my Aunt Constance, my Sister Yvonne, siblings – Caroline, George, Anesu, Lovejoy, Tariro for their support always when the going gets tough.

My friends who became/are family: Mr. Barnabas Tinarwo, Mr. Silent Dzikiti, Ms. Siphathisiwe Moyo, Mr. Mfundo Dhuma, Mr. Richard Chikeya, Ms. Kelechi Francesca Njoku, Mr. Thatohatsi Nelson Rafube, Mr. Gerald Muguto, Mr. Ngoni Mujuru, Mr. Premodial Zhou, Mr. Courage Chiringa, for always being there for me and making my stay in Cape Town a memorable one.

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LIST OF SYMBOLS AND ABBREVIATIONS

$>$ = greater than

$<$ = less than

$\geq$ = greater than or equal to

A = Adenine

ABCB1 = ATP-Binding Cassette Binding Protein-1

ABPM = Ambulatory BP Monitoring

ADRB1 = Adrenoceptor Beta-1

ACEI = Angiotensin Converting Enzyme Inhibitors

ARB(s) = Angiotensin Receptor Blocker(s)

ATP = Adenosine Triphosphate

BMI = Body Mass Index

BP = Blood Pressure

C = Cytosine

cAMP = Cyclic Adenosine Monophosphate

CCB(s) = Calcium Channel Blocker(s)

CES1 = Carboxylesterase-1

CHD = Coronary Heart Disease

CHF = Chronic Heart Failure

CKD = Chronic Kidney Disease

Cl^- = Chloride ion

CNV = Copy Number Variation

CPIC = Clinical Pharmacogenetics Implementation Consortium

CVD(s) = Cardiovascular Disease(s)

CYP3A4 = Cytochrome P450 3A4

CYP3A5 = Cytochrome P450 3A5

DBP = Diastolic Blood Pressure

ddNTP = Di-deoxy Nucleotide Triphosphate

DM = Diabetes Miletus

DME = Drug Metabolizing Enzyme

DNA = Deoxyribonucleic Acid

ENaC = Epithelial Sodium Channel

FDA = Food and Drug Administration

G = Guanine

GDP = Gross Domestic Product

H_A	= Alternative Hypothesis
H_0	= Null hypothesis
HCTZ	= Hydrochlorothiazide
HHD	= Hypertensive Heart Disease
HPLC	= High Performance Liquid Chromatography
HWE	= Hardy-Weinberg Equilibrium
IHD	= Ischaemic Heart Disease
ISH	= International Society of Hypertension
LMIC	= Low-middle Income Country
LVH	= Left Ventricular Hypertrophy
mmHg	= Millimetres Mercury
$MgCl_2$	= Magnesium Chloride
Na+	= Sodium ion
NaCl	= Sodium chloride
NCDs	= Non-communicable Diseases
NEDD4L	= Neural Precursor Cell Expressed Developmentally Down-Regulated 4-Like
NOS3	= Nitric Oxide Synthase-3
NR3C2	= Nuclear Receptor Subfamily-3 Group C Member-2
PCR	= Polymerase Chain Reaction
PCR-RFLP	= Polymerase Chain Reaction Restriction Fragment Length Polymorphism
PASCAR	= Pan-African Society of Cardiology
PGX-ID	= Pharmacogenomics Identity
PRECODE	= Pharmacogenomics of Cardiovascular Diseases: Focusing on Dyslipidaemia and Hypertension
qPCR	= Quantitative Polymerase Chain Reaction
RNA	= Ribonucleic Acid
RT-PCR	= Real Time Polymerase Chain Reaction
SBP	= Systolic Blood Pressure
SCNN1B	= Sodium Channel Epithelial-1 Subunit Beta
SNP	= Single Nucleotide Polymorphism
T	= Thiamine
TBE	= Tris-Borate-EDTA buffer
TDM	= Therapeutic Drug Monitoring
UCT-HREC	= University of Cape Town Human Research Ethics Committee
WHO	= World Health Organization

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 Background

Non-communicable diseases (NCDs) have been on the increase globally, accounting for annual mortalities of up to 41 million. According to the World Health Organisation (WHO), (2020), NCDs are classified into four major groups: cardiovascular diseases (CVDs), cancer, chronic respiratory diseases and diabetes. Out of the four, CVDs are responsible for the majority of mortality, globally and an annual death toll of 17.9 million was recorded in 2019. CVDs are a group of disorders that affect the heart and blood vessels. Examples are coronary heart disease (CHD), rheumatic heart disease (RHD), peripheral arterial disease, aortic disease and stroke. Ischaemic heart disease (IHD) and stroke have been listed as the top 5 causes of death in Africa according to the WHO (**Table 1.1**) and accounted for approximately 2.8 million deaths in 2019 (WHO, 2020). Studies (Olsen et al., 2016; Zhou et al., 2021) have reported that **hypertension** is an important risk factor for CVDs and CVD related death. Thus, control of hypertension may play a significant role in reducing CVDs and CVD related deaths.

Table 1.1: An extract of the top five causes of death in Africa according to latest evidence by the World Health Organisation (WHO). Ischaemic heart disease and stroke which rank fourth (and are non-communicable), are potentiated by hypertension.

Cause of Death	Communicable/Non-communicable	Number of deaths	Percentage (%) of total deaths in 2019
Lower respiratory tract infections	Communicable	774 252	9.9
Diarrhoea	Communicable	496 278	6.4
HIV/AIDS	Communicable	434 543	5.6
Ischaemic heart disease and stroke	**Non-communicable**	**429 179**	**5.5*** **
Parasitic diseases	Communicable	388 229	5.0

***Hypertension is the leading risk factor for ischaemic heart disease and stroke.

1.2 Epidemiology and Burden of Hypertension

Globally, an estimated 1.13 billion individuals live with hypertension (**Figure 1.1**). Africa contributes significantly to this and has an estimated hypertension prevalence > 27% (WHO, 2021; Zhou et al., 2021). Without appropriate intervention strategies it has been reported that the number of people with hypertension in Africa will grow to up to 216.8 million by 2030

(Adeloye & Basquill, 2014; WHO, 2021). South Africa has the second highest prevalence of hypertension among African countries (**Figure 1.2**). Several studies have reported an estimated prevalence that is above 40% (Davids et al., 2019; Gómez-Olivé et al., 2017; Ibrahim & Damasceno, 2012; Peer et al., 2013; Peer et al., 2021). It has also been reported that nearly 50% of people with hypertension still struggle to have their blood pressure under control resulting in many cases of uncontrolled hypertension (Berry et al., 2017). According to several reports across South Africa, the prevalence of uncontrolled hypertension ranges from 13.5 – 75.5% (Adeniyi et al., 2016; Berry et al., 2017; Masilela et al., 2020) and has recently been reported to be on the rise in South Africa (Makukule et al., 2023). However, these studies used different methodologies which may not be comparable in terms of reflecting the true prevalence.

Similarly, hypertension related morbidity such as IHD, stroke, hypertensive heart disease (HHD) and chronic kidney disease (CKD) are also on the rise. A recent study has estimated total healthcare costs associated with hypertension to be ZAR 39.5 billion or US$2.79 billion, which is representative of 0.76% of South Africa's gross domestic product (GDP) in 2020 (Bank, 2019; Kohli-Lynch et al., 2022). For a developing country, also burdened by a plethora of communicable diseases, these are extremely high costs. Thus, concerted efforts are required to alleviate the economic burden due to hypertension, while also improving the quality of life in patients with hypertension.

1.3 Resistant Hypertension

Approximately 21% of patients with hypertension globally, have been estimated to progress to resistant hypertension (Dudenbostel et al., 2016). There is a marked variation in the prevalence of resistant hypertension by region. Studies from American and European countries have shown that the prevalence of resistant hypertension ranges between 8.4% and 17.4% among patients with hypertension (Achelrod et al., 2015; Judd & Calhoun, 2014; Lotufo et al., 2015). In Africa, there is limited epidemiological data accurately estimating the burden and prevalence of resistant hypertension for the whole continent (Katsukunya et al., 2023b). It has been reported that the prevalence of resistant hypertension across Africa ranges from 4-19%. In South Africa, the prevalence of resistant hypertension has been reported to be 12.6% among patients attending a tertiary-level Hypertension Clinic in Cape Town, South Africa (Cherif et al., 2015; Moosa et al., 2016; Salako & Ayodele, 2003; Thinyane et al., 2015; Yaméogo et al., 2014; Youmbissi et al., 1994).

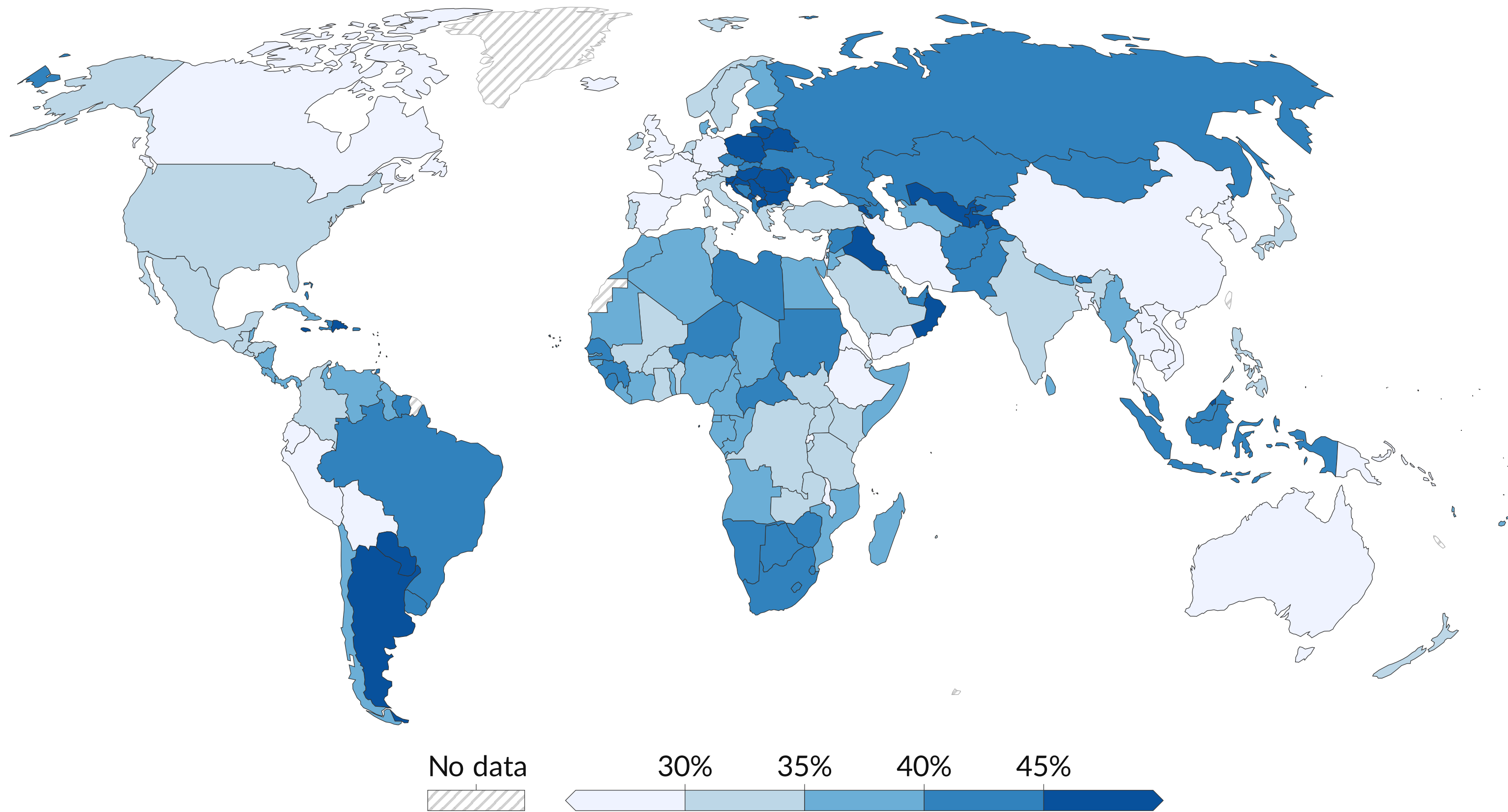

Figure 1.1: The global age-standardised prevalence of hypertension in individuals aged between 30-79 years based on population representative surveys and statistical modelling in 2019. Adapted from WHO, Global Health Observatory (2022) – processed by Our world in Data (*https://ourworldindata.org/grapher/hypertension-adults-30-79?time=2019, last accessed 14 December 2023*).

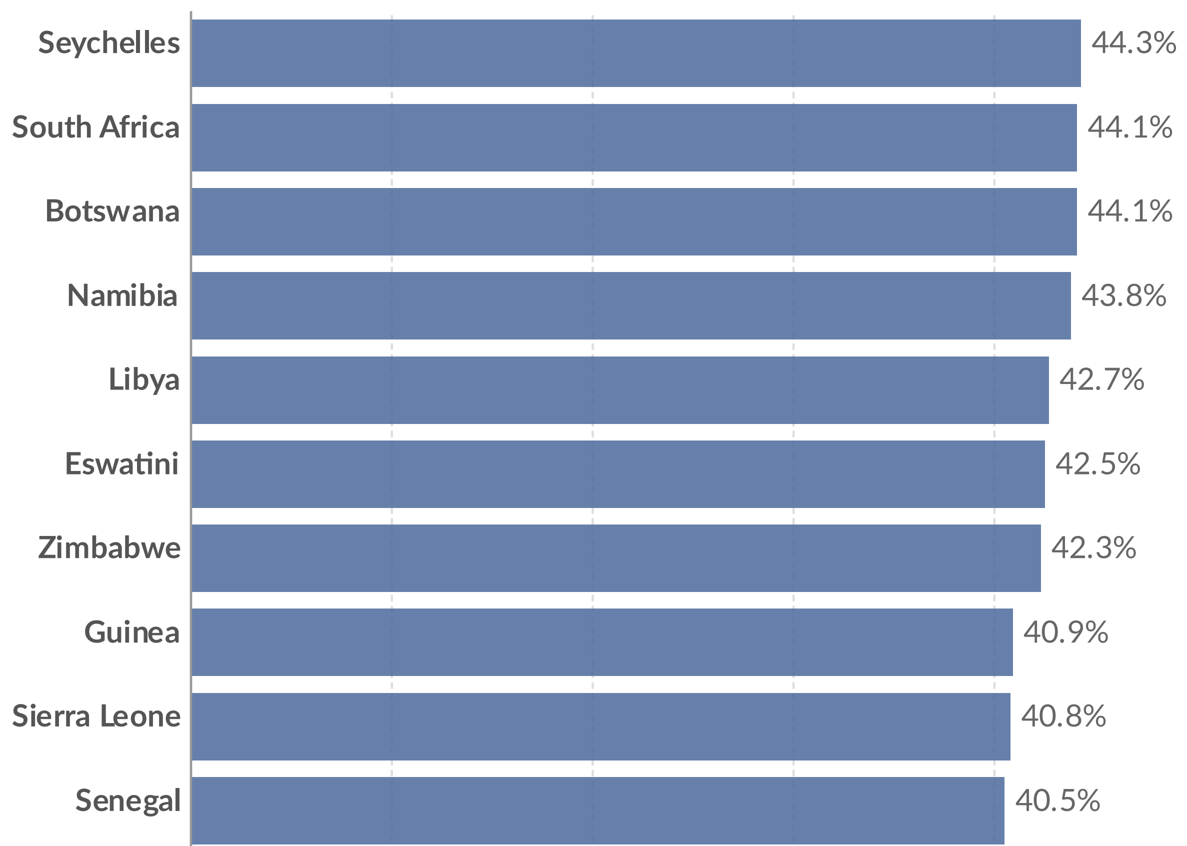

Figure 1.2: The top 10 African countries with the highest age-standardised prevalence of hypertension. South Africa has the 2nd highest prevalence of hypertension (44.1%) after Seychelles (44.3%). Adapted from WHO, Global Health Observatory (2022) – processed by Our world in Data (*https://ourworldindata.org/grapher/hypertension-adults-30-79?time=2019, last accessed 14 December 2023*).

1.3.1 Resistant Hypertension: A Historical Perspective and Evolution of the Definition

The challenge of managing high blood pressure has been a clinical concern since the introduction of hypotensive agents in the 1930s (**Figure 1.3**). As specific antihypertensive drugs started to be developed in the 1960s, the term "resistant hypertension" emerged, and referred to patients with hypertension that was difficult to control despite the use of antihypertensive drugs.

Over time, various definitions were proposed, in an attempt to classify patients with this phenotype, with each definition being a significant improvement over the previous one (Gifford Jr, 1988; Gifford & Tarazi, 1978; Van Dyne, 1960). However, it was only in 2008, when the definition of resistant hypertension was clearly established. According to the American Heart Association (AHA), resistant hypertension is,

"BP that remains elevated (≥140/90 mmHg) in a patient on three antihypertensive drugs in optimal doses and one of which must be a diuretic,"

or alternatively,

"If a patient requires four or more antihypertensive drugs for BP to be controlled (<140/90 mmHg), it is considered controlled resistant hypertension (Calhoun et al., 2008)."

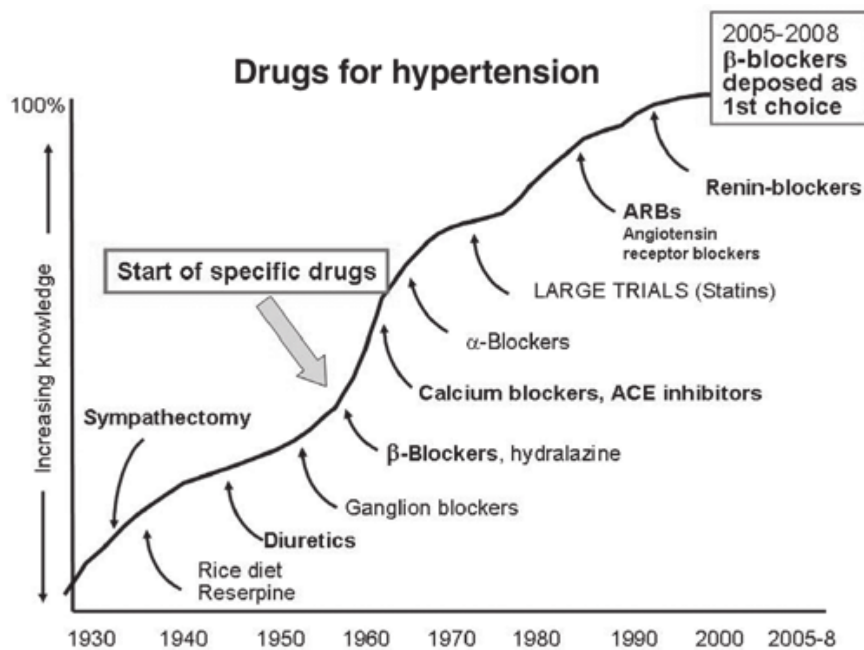

Figure 1.3: Timeline and evolution of the major classes of antihypertensive drugs between 1930 – 2008 (Opie, 2009). The start of the use of specific drugs began in the 1960s which is when the term resistant hypertension was coined.

This is the modern day basis of the definition of resistant hypertension and has been refined to distinguish phenotypes such as (1) controlled resistant hypertension from controlled hypertension, (2) uncontrolled resistant hypertension from uncontrolled hypertension and (3) refractory hypertension from uncontrolled resistant hypertension (**Figure 1.4**).

However, one of the major challenges in this definition is that it does not exclude patients with pseudo-resistant hypertension or apparent treatment resistant hypertension. Thus, it only

remains true, provided pseudo-resistance is excluded through accurate BP measurements and confirmation of antihypertensive drug adherence (Judd & Calhoun, 2014).

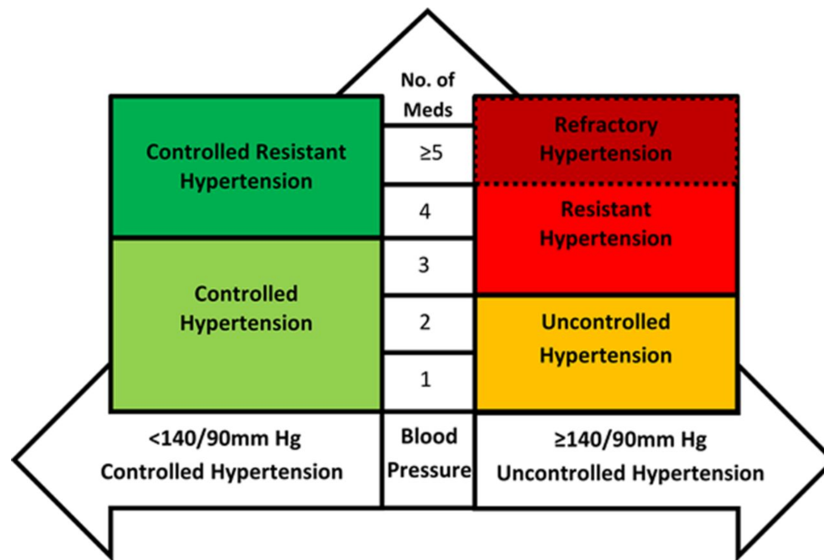

Figure 1.4: An algorithm summarising the modern-day criteria of classifying hypertension including resistant hypertension based on the number of antihypertensive drugs (No. of Meds) and BP levels. Resistant hypertension is classified as controlled resistant hypertension if it requires more than four antihypertensive drugs to control BP (<140/90 mmHg), or BP that remains elevated (≥140/90 mmHg) in a patient on three antihypertensive drugs. Refractory hypertension is classified as uncontrolled BP (≥140/90 mmHg) despite the use of more than five antihypertensive drugs (Dudenbostel et al., 2016).

1.3.2 Apparent Treatment Resistant Hypertension

Accurately measuring blood pressure and monitoring drug adherence are essential to avoid misdiagnosing true resistant hypertension as apparent treatment-resistant hypertension. Accurate and precise diagnosis of resistant hypertension heavily depends on accurate BP measurements. Therefore, when measuring BP, major hypertension guidelines (Mancia et al., 2023; Unger et al., 2020; Whelton et al., 2018) recommend that a patient be seated quietly in an upright position in a temperate environment, having their arm outstretched and supported (Cheung et al., 2023). During BP measurements, automated sphygmomanometers are used to measure several BP readings while the patient is sitting in a comfortable position. According to Seedat et al., (2014), six BP readings are usually taken at two-minute intervals. The initial BP reading is discarded while the remaining five BP readings are averaged to give the mean BP readings (Seedat et al., 2014). If white coating is suspected, ambulatory BP monitoring (ABPM) is recommended. White-coating is defined as BP that is elevated only in a clinical environment or when a physician is present but becomes normal when outside a clinical

environment (Nuredini et al., 2020). ABPM takes into account BP readings over 24 hours and assesses day and night-time BP changes. In this case, elevated BP is indicated by mean daytime and night-time BP ≥ 135/85 and 120/70 mmHg respectively (Kumanan, 2017).

Patient non-adherence has been reported to be very common among patients with hypertension and there have been reports that have estimated patient non-adherence to range between 7 – 66% (Ceral et al., 2011; Daugherty et al., 2012; Jung et al., 2013; Li et al., 2012; Raebel et al., 2012; Tomaszewski et al., 2014). Several strategies are used to detect non-adherence to antihypertensive therapy including direct questioning, pill counts, directly observed dosing and therapeutic drug monitoring (TDM). However, direct questioning and pill counts have been shown to provide unreliable estimates of detecting non-adherence and are less commonly used (James et al., 2017).

Directly observed dosing is the most used, even in resource limited settings. It involves measuring BP before and after a patient is observed taking the antihypertensive drugs by a healthcare professional in a clinical setting. In this case, when BP is found to be <140/90 mmHg after directly observed dosing, it is suggestive of non-adherence. However, if BP is >140/90 mmHg after directly observed dosing, a diagnosis of resistant hypertension may be likely. It is important to note that the method may only be reliable for antihypertensive drugs with a rapid onset of action and may have limited utility if drugs with very slow onset of action are given such as amlodipine or hydrochlorothiazide.

In cases where directly observed dosing may be unreliable, medication possession ratios (MPR) may be calculated. MPRs refer to the ratio of the total number of days of supply of a medication to the total number of days in the period of observation (Ágh, 2016). For example, the calculation of MPR may be:

$$\textbf{MPR} = \frac{\textbf{Days of supply of medication from last refill}}{\textbf{Last refill date – first refill date}}$$

Ratios suggestive of adherence differ by specific drugs, however, a ratio of 0.80 is conventionally used to denote adherence (Tang et al., 2017).

Therapeutic drug monitoring (TDM) remains the most preferred method of detecting non-adherence and has been referred to as the 'gold standard' of measure of drug adherence (**Figure 1.5**) (James et al., 2017; Tomaszewski et al., 2014). It involves measuring drug metabolites in either urine or serum using high-performance liquid chromatography-mass spectrometry (HPLC) and has been shown to be effective in detecting non-adherence to specific drug types (Ceral et al., 2011; Jung et al., 2013).

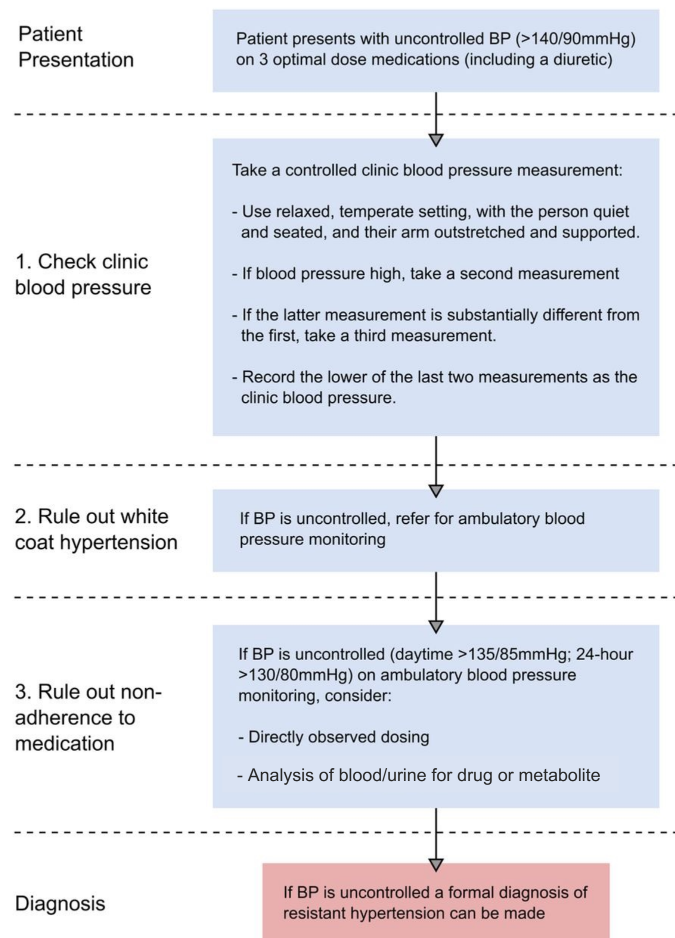

Figure 1.5: Overview of the stages involved in the diagnosis of resistant hypertension (adapted from James et al., 2017). The stages of diagnosis upon patient presentation until diagnosis include: (1) Checking clinic BP, (2) ruling out white-coat hypertension and (3) ruling out non-adherence (James et al., 2017).

This is particularly important for antihypertensive drugs with long half-lives such as amlodipine (Stangier & Su, 2000). TDM of amlodipine levels has become a routine part of clinical practise and is being implemented in clinics worldwide, even in South Africa, especially if non-adherence is suspected which could not be ruled out by other measures (Jones et al., 2017). In addition to the long half-life of amlodipine, the fact that amlodipine is one of the first line antihypertensive drugs, make it an attractive candidate for detecting non-adherence

even to other antihypertensives drugs that are prescribed in conjunction with it (Jones et al., 2017).

1.4 Pathogenesis of Resistant Hypertension

Several factors have been reported to contribute to the development of resistant hypertension (Sarwar et al., 2013). Broadly, factors that **contribute to elevated BP** and **poor response to antihypertensive drugs** underlie the pathogenesis of resistant hypertension. In both cases, the pathogenesis is both multifactorial, highly complex and involves the interaction of multiple organ systems (Oparil et al., 2018). The kidney is usually the contributing and major target organ for physiological processes contributing to elevated BP (Hall & Hall, 2017). As such, conditions that impair kidney function, such as chronic kidney disease (CKD) may contribute to elevated BP and resistant hypertension. In addition, diabetes mellitus (DM) has been reported to impair kidney function and may also be a secondary cause of resistant hypertension (Thomas & Rahman, 2021). For example, an increase in insulin levels has been reported to contribute to BP increase through increased renal sodium absorption. Insulin stimulates sodium uptake in multiple sections of the kidney nephron which in turn increases vascular volume and systemic BP (Zhou et al., 2014).

Many other pathways are at play and are interdependent, often intersect with each other and all work in unison to manage degrees of vasodilation or vasoconstriction within systemic circulation, facilitate the retention or excretion of sodium and water; and to maintain an adequate circulating blood volume (Bangash et al., 2020; Gallo et al., 2021; Grossman, 2008; Oparil et al., 2003; Sanchez-Lozada et al., 2020; Shariq & McKenzie, 2020; Zhou et al., 2014). These pathways act on a genetic background and thus, epigenetic and genetic factors have a major role to play in impairing these processes too (Oparil et al., 2018).

Environmental factors such as stress, physical inactivity, smoking, diet, excess salt intake, air and noise pollution have been reported to be associated with the development of hypertension or elevated BP (Brook et al., 2011). A recent cross-sectional study among South Africans by Masilela et al., (2020) showed that individuals who were physically inactive, consumed excess alcohol, were smokers and had a high salt intake diet had severely elevated BP (Masilela et al., 2020). It is also important to note that the interaction of environmental factors and the genome may lead to epigenetic changes that affect gene expression (Weinhold, 2006). Epigenetics

plays a crucial role in the development of hypertension (Liang et al., 2013; Wise & Charchar, 2016).

In contrast to epigenetic changes, heritable genetic changes do occur in the DNA sequence and over 60% of all hypertensive cases are reported to be of familial association (Saxena et al., 2018). Over 150 candidate genes have been reported to contribute to elevated BP and many of them are critical genes involved in renal sodium handling (Gaddam et al., 2010; Martinez et al., 2009). In **Table 1.2** examples of some of the genes contributing to elevated BP are listed and these genes are found on almost every chromosome (Butler, 2010). Thus, almost all chromosomes carry genomic determinants which contribute to elevated BP. According to Butler, (2010), an individual who develops hypertension usually inherits a set of genes associated with elevated BP, and this phenotype is usually expressed when one is exposed to environmental factors that favour raised BP (Butler, 2010).

Table 1.2: Genes contributing to the development of elevated BP

Genes reported to be associated with elevated BP or susceptibility to hypertension				
ADD1	*CLCNKB1*	*GPR83*	*LUC7L2*	*PR3*
AGT	*CNNM2*	***GRK4***	*MECOM*	*SH2B3*
APOA5	*CPS1*	*HFE*	*MOV10*	*SLC39A8*
ATP2B1	***CYP11B2***	*HSD3B1*	*MTHFR*	*SLC4A7*
B2	***CYP411***	*IGF2BP2*	***NOS3*****	*STK39*
BAG6	*EBF1*	*IGFBP3*	***NPPA***	*SUB1*
CDKAL1	*FGF5*	*JAG1*	***NEDD4L*****	*TBX5*
CEP83	*GNAS-EDN3*	*KCNJ1*	*PLCE1*	*TH*
CHIC2	***GNB3***	*LEP*	*PLEKHA7*	***WNK1/4***

Highlighted in bold are also genes with pharmacogenetic implications.
**Genes pertinent to this thesis.

Each of these factors contribute to the development of resistant hypertension in a unique way. However, they almost all exclusively result in all or each of the following: increased salt reabsorption, impaired renin-angiotensin-aldosterone system (RAAS) or sympathetic nervous system (SNS) over-activation.

1.4.1 Factors Contributing to Poor Drug Response

In addition to factors contributing to elevated BP, resistant hypertension is also caused by poor response to antihypertensive drugs and explains why resistant hypertensive patients are on multiple antihypertensive drugs for BP control (Hameed & Dasgupta, 2019). Numerous antihypertensive drugs classes are currently available and include calcium channel blockers

(CCB), angiotensin converting inhibitors (ACEI), angiotensin receptor blockers (ARB), diuretics, β-blockers and mineralocorticoid receptor antagonists (MRA). Major hypertension societies (Mancia et al., 2023; Unger et al., 2020; Whelton et al., 2018) have recommended different approaches to antihypertensive drug treatment for optimal BP control. However, Pan-African Society of Cardiology (PASCAR) supports the International Society of Hypertension (ISH) guidelines for the drug treatment of hypertension in Africa (Jones et al., 2020).

Briefly, a combination of an ACEI/ARB with a CCB or a combination of CCBs with thiazide or thiazide-like diuretics are recommended as first line therapy in African patients. β-blockers are recommended as second-line therapy and if BP is uncontrolled despite the use of 3 or more different classes of drug, which is usually the case in resistant hypertension, an MRA is added (Jones et al., 2020). However, with the multiple drugs available, some individuals still respond poorly to the antihypertensive drugs currently available (Pender & Omole, 2019). **Table 1.3** lists examples of the currently approved antihypertensive drugs in each class.

Table 1.3: FDA approved antihypertensive drugs and common antihypertensive drugs in South Africa including pharmacokinetic and pharmacodynamic genes associated with the drugs.

Class of antihypertensive drug	Names of drugs in the respective classes	Pharmacokinetic or Pharmacodynamic genes
Calcium channel blockers (CCBs)	**Amlodipine, nifedipine**, nilsodipine, **diltiazem**, felodipine, isradipine, nicardipine, **verapamil**, nimodipine	***ABCB1*****, *CACNA1C, CACNA1D*, ***CYP3A4**, CYP3A5*****, *NPPA, NUMA1, PICALM, POR, RYR3, TANC2*
Angiotensin converting inhibitors (ACEI)	Captopril, **enalapril**, **lisinopril**, perindopril, quinapril, ramipril, trandolapril, **benazepril**, fosinopril, moxipril	*ACE, BDKRB2*, ***CES1*****, *PRKCA, VEGFA*
Angiotensin receptor blockers (ARBs)	Eprosartan, **candesartan**, **losartan**, **valsartan**, irbesartan, telmisartan, azilsartan, olmesartan	*AGT, AGTR1, CAMK1D, CYP2C9, SCNN1G, GPR83, FUT4, Nphs1*
Diuretics	**Hydrochlorothiazide**, chorthalidone, chlorothiazide, metolazone, ethacrynic acid, indapamide, **furosemide**, bumetanide, torsemide, **amiloride**, triamterene	*ADD1 ALDH3, WNK1, WNK4*, ***NEDD4L*****, *CLIC5, CSDM1*, *CUB, STK39, TET2, YEATS4*
α and β-blockers	**Atenolol**, bisoprolol, metoprolol, propranolol, **carvedilol**, **doxazosin**, prazosin, terazosin	***ADRB1***,** *BST1, CYP2D6, SLC25A31, SLC4A1*
Mineralocorticoid receptor antagonists (MRA)	Eplerenone, **spironolactone**, finerenone	*AGT, CYP4A11, CYP11B2*, ***NR3C2*****, ***SCNN1B*****
Direct vasodilators	**Hydralazine, minoxidil**	*NAT2*
Peripherally acting adrenergic antagonists	Reserpine	*VMAT-2*

Highlighted in bold are commonly used antihypertensive drugs in South Africa, ** Genes pertinent to this thesis.

The reasons for poor response to antihypertensive drugs are numerous and diverse. For example, response to antihypertensive drugs is dependent on individual patient characteristics such as diet (Fravel & Ernst, 2021; Huggins et al., 2010), co-morbidities (Grossman & Grossman, 2017), body mass index (BMI) (Nedogoda et al., 2013), drug-drug interactions (Fravel & Ernst, 2021), drug-herb interactions (Thomford et al., 2015), age (Benetos et al., 2019; Freis, 1988; Mangoni & Jackson, 2004) and ancestral background (Franceschini et al., 2014), each contributing to some extent to response. However, age and ancestry have been reported to be the top clinical determinants of response to antihypertensive therapy and this has been validated in large clinical trials (Cooper-DeHoff & Johnson, 2016).

Age influences many physiological functions and induces changes in major organ systems such as the cardiac, renal and gastrointestinal systems. As such, age has an impact on the pharmacodynamics and pharmacokinetics of antihypertensive drugs including reduced drug absorption, reduced first pass metabolism or bioavailability, reduced drug distribution, reduced protein binding and reduced hepatic or renal clearance (Mangoni & Jackson, 2004). The influence of age on treatment response is augmented by ancestral background. Different ancestries also mean that the underlying genetics influencing antihypertensive drug response in different ancestries/populations are different. This implies that individuals from different ancestral backgrounds are mostly likely to respond differently to antihypertensives due to their genetics. For example, based on treatment response, young individuals of European Ancestry tend to respond better to ACE inhibitors/ARBs while in individuals of African Ancestry who are older, respond better to CCBs and diuretics (Johnson et al., 2009; Materson et al., 1993; Schwartz et al., 2013).

Rysz et al., (2020) in their review, highlight that up to 50% of variability in antihypertensive drug response emanates from genetic factors and from the extensive research that has been carried out across individuals from different ancestral backgrounds such as European, American and Asian populations, they show that there are quantifiable differences in antihypertensive drug response across these populations, with some populations being burdened with genetic variants associated with poor response more than others. Similar observations can also be drawn from earlier reviews by Oliviera-Paula et al., (2019), Johnson et al., (2019) and Cooper-DeHoff & Johnson, (2016). These findings from studies conducted in European, American and Asian populations, have shown that genetic polymorphisms in genes encoding antihypertensive drug metabolising enzymes (DMEs) or targets (**Table 1.3**),

hypertension susceptibility genes (**Table 1.2**) and novel genes or loci have implications on antihypertensive drug response (Cooper-DeHoff & Johnson, 2016; Johnson et al., 2019; Oliveira-Paula et al., 2019; Rysz et al., 2020).

Among the genetic variants previously reported, are variants in genes such as the *ABCB1, ADBR1, CES1, CYP3A4, CYP3A5, NOS3, NR3C2* and *NEDD4L* which influence the pharmacokinetics and pharmacodynamics of some of the commonly prescribed antihypertensive dugs in South Africa (**Table 1.3**). Specifically, antihypertensive drugs such as amlodipine, enalapril, atenolol, hydrochlorothiazide (HCTZ) and spironolactone were found to be among the most frequently prescribed antihypertensive drugs among patients attending a tertiary level Hypertension Clinic at Groote Schuur Hospital, South Africa (**Figure 1.6)**. Therefore, the *ABCB1, ADBR1, CES1, CYP3A4, CYP3A5, NOS3, NR3C2* and *NEDD4L* genes may be of pharmacogenomics importance in South African hypertensive patients and in the following section variation in these genes contributes to either poor or enhanced response to antihypertensive drugs commonly prescribed in South Africa. A standard literature search strategy was used to pool studies reporting on *ABCB1, ADBR1, CES1, CYP3A4, CYP3A5, NOS3, NR3C2* or *NEDD4L* and commonly prescribed antihypertensive drugs in South Africa from PubMed/Medline and Google Scholar databases.

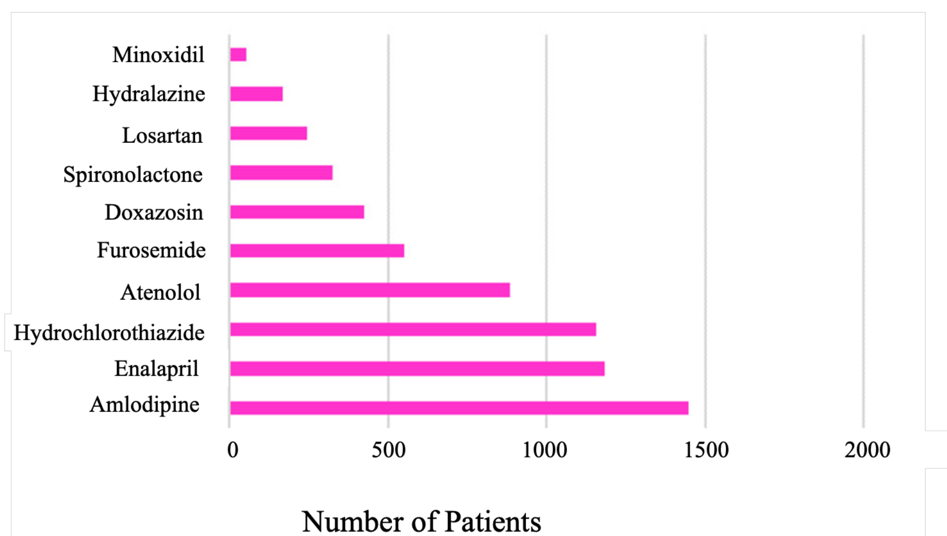

Figure 1.6: Commonly prescribed antihypertensive drugs among patients attending a Tertiary-level Hypertension Clinic at Groote Schuur Hospital (Soko et al., 2023).

1.5 Genes Involved in the Pharmacokinetics of Amlodipine

In a recently published review by Soko et al., (2023), amlodipine was the top prescribed antihypertensive drug (**Figure 1.6**). Amlodipine is a dihydropyridine CCB that lowers BP by

inhibiting the activity of calcium ion channels. This action stops calcium ion influx into the vascular smooth muscle cells, which would otherwise cause muscle contraction and vasoconstriction leading to elevated BP (Ferrari et al., 2019). Amlodipine is taken in orally, and its pharmacokinetics have been extensively reviewed by Shirley and McCormack, (2015). Amlodipine undergoes extensive first pass metabolism (Zisaki et al., 2015), is principally metabolised by CYP3A4, partially by CYP3A5 enzymes in the liver into its inactive pyridine derivatives (**Figure 1.7**) and is cleared by the ATP-binding cassette binding protein 1 (ABCB1) (Fares et al., 2016)

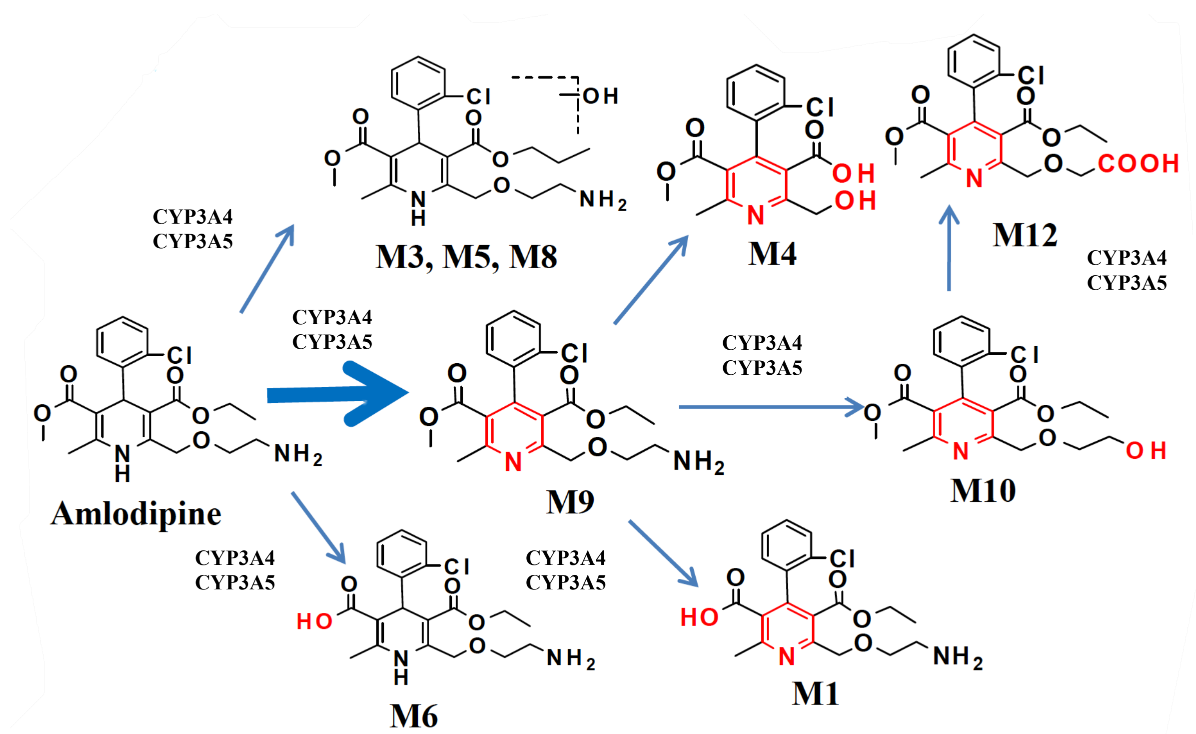

Figure 1.7: Role of CYP3A4 and CYP3A5 enzymes in the catalytic dehydrogenation of amlodipine to inactive pyridine derivatives. Amlodipine is predominantly inactivated to the M9 derivative, which is subsequently converted to M1, M4, M10 and M12 or it is directly converted to M3, M5, M6 and M8 (Zhu et al., 2014).

CYP3A4 and *CYP3A5* genes encoding the CYP3A4 and CYP3A5 enzymes respectively, share 85% sequence homology and have overlapping substrate specificities (Langman et al., 2016). *CYP3A4* is located on chromosome 7q22.1, is 27 kb long and has 13 exons, while *CYP3A5* is on chromosome 7q22.1, is ~33 kb long and has 14 exons. Variants in these genes have been previously shown to influence the pharmacokinetics of amlodipine and BP outcomes. For example, a single nucleotide polymorphism (SNP) in the *CYP3A4* promoter region, *CYP3A4* rs2740574C>T, was found to be associated with BP response to amlodipine in African American women who had early hypertensive nephrosclerosis but not in African American

men (Bhatnagar et al., 2010). Carriers of the *CYP3A4* rs2740574T allele had higher chances of reaching a mean arterial pressure (MAP) target of 107 mmHg compared to the *CYP3A4* rs2740574C allele carriers, indicating that *CYP3A4* rs2740574T allele carriers had a good response profile. Additionally, Bhatnagar et al., (2010), found *CYP3A4* rs2246709T>C to be associated with BP response to amlodipine in individuals randomised to a MAP of < 92 mmHg. Carriers of the *CYP3A4* rs2246709C allele, both males and females, were more likely to reach a MAP target of 107 mmHg.

According to Bhatnagar et al., (2010), no association was found between the *CYP3A5* rs776746T>C SNP and BP response to amlodipine among African Americans. This was similar to previous studies done in African and Asian populations (Guo et al., 2015; Masilela et al., 2022; Zhu et al., 2014). However, other studies have found significant associations of this SNP and amlodipine pharmacokinetics or efficacy. Kim et al., (2006), identified *CYP3A5* rs776746T>C SNP to be associated with plasma amlodipine concentrations in Korean males. Carriers of the rs776746T/T genotype were associated with higher plasma amlodipine concentration compared to rs776746C/C genotype carriers, although there was no significant correlation between the genotypes and BP or pulse rate (Kim et al., 2006).

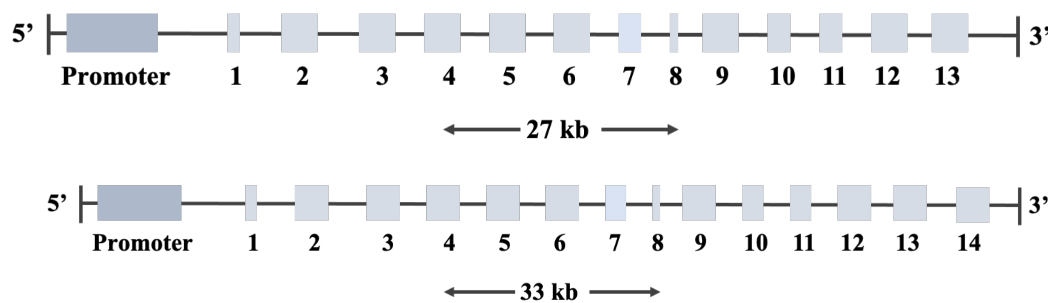

Figure 1.8: Structure of the *CYP3A4* and *CYP3A5* gene located on chromosome 7q22.1. CYP3A4 and CYP3A5 genes are 27 and 33 kb long and contain 13 and 14 exons respectively.

Not only is the *CYP3A5* rs776746T>C associated with altered pharmacokinetics of amlodipine, a recent study has also shown that it is associated with antihypertensive treatment switch and adverse outcomes (Türkmen et al., 2023). According to Türkmen et al., (2023), rs776746T/T genotype carriage was associated increased likelihood of switching antihypertensive therapy and chronic kidney disease (CKD), compared to rs776746C/C genotype carriage in European patients on amlodipine therapy (Türkmen et al., 2023).

The ABCB1, also critical in the metabolic clearance of amlodipine, is encoded by a the *ABCB1* gene located on chromosome 7q21.12. It is ~217 kb long and has 32 exons. Two missense SNPs, *ABCB1* rs1045642C>T and *ABCB1* rs2032582A>C, have been reported to affect amlodipine pharmacokinetics. According to Zuo et al., (2014), among the Chinese Han hypertensive patients, clearance of amlodipine was associated with *ABCB1* rs1045642C>T genotypes in a sex-specific manner. Carriers of the *ABCB1* rs1045642C/C or rs1045642C/T genotype who were males, had a higher amlodipine clearance rate than carriers of the same genotypes who were females (Zuo et al., 2014).

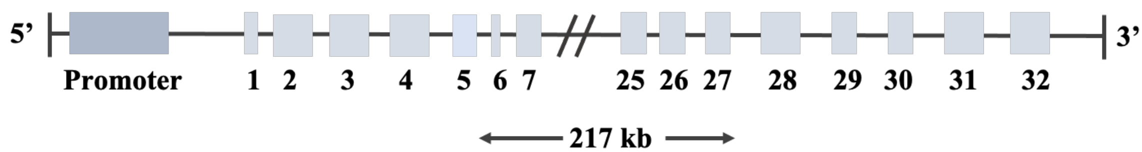

Figure 1.9: Structure of the ABCB1 gene located on chromosome 7q21.12. It is approximately 217 kb long and has 32 exons.

Expression of the ABCB1, has been previously shown to be affected by sex differences in several studies as reviewed by Mineiro and colleagues (Mineiro et al., 2023). In another study, individuals who were carriers of the *ABCB1* rs1045642T/T and rs2032582A/A genotypes were found to have higher clearance of amlodipine compared to carriers of the *ABCB1* rs1045642C and rs2032582C alleles (Kim et al., 2007).

1.6 Genes Involved in the Pharmacokinetics of Enalapril

Enalapril is the second most prescribed antihypertensive drug in South Africa (Soko et al., 2023). Enalapril lowers BP by inhibiting the activity of the angiotensin converting enzyme (ACE). ACE converts angiotensin I to angiotensin II, whose downstream activity is to upregulate pathways in the the kidneys, adrenal cortex, brain or arterioles to increase renal sodium reabsorption, fluid retention and vasoconstriction which would otherwise lead to elevated BP or hypertension (Davis, 2007). Enalapril is a pro-drug and conversion to enalaprilat is essential. Enalaprilat is up to 20 times more potent in lowering BP than enalapril and this conversion is needed for increased efficacy. Crucial for the activation step to occur, are serine esterases such as carboxylesterase 1 (CES1) (Davis, 2007; Ikonnikova et al., 2022).

CES1 is encoded for by the *CES1* (also referred to as *CES1A1*) gene. *CES1A1* is located on chromosome 16q12.2 has 14 exons and is ~33 kb long. *CES1A1* has a complicated structure and there are two main structural haplotypes. In one, *CES1A1* lies tail to tail with a non-functional pseudogene, also known as *CES1PI*. *CES1A1* is subject to segmental duplications which gives rise to another haplotype consisting of the original gene copy (*CES1A1*) and another copy with a pseudogene derived segment, commonly referred to as *CES1A2* (**Figure 1.10**). According to Yoshimura et al., (2008), the haplotype containing *CES1A2*, is associated with reduced transcriptional efficiency and this has implications on the pharmacokinetics or response to drugs metabolised by CES1 such as enalapril (Yoshimura et al., 2008).

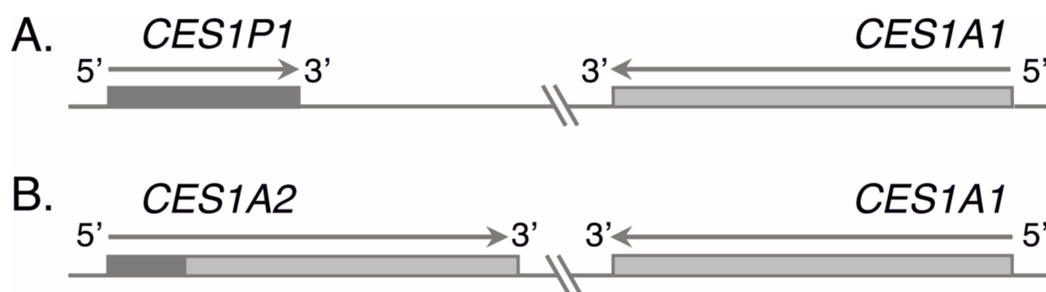

Figure 1.10: The main structural haplotypes of the *CES1* gene. Segmental duplications of the CES1A1 (A) results in a haplotype (B) containing a combination of *CES1A1* and *CES1A2*. *CES1A2* is identical to CES1A1 but contains 2.6 kb portion derived from the pseudogene, *CES1P1* (Ikonnikova et al., 2022)**.**

A limited number of studies have been reported on the pharmacogenetic implications of CES1 on enalapril and findings have been inconsistent. According to Nelveg-Kristensen et al., (2016), *CES1A2* did not appear to play a role on the pharmacokinetics of enalapril in European congestive heart failure (CHF) patients. There was no association either, between the number of copies of *CES1A2* or plasma angiotensin I/angiotensin II ratios (Nelveg-Kristensen et al., 2016). Moreover Stage et al., (2017) and Wang et al., (2016) also found no association between the number of *CES1* copies and enalapril pharmacokinetics (Stage et al., 2017; Wang et al., 2016). However, recently, a study by Ikonnikova et al., (2022), identified *CES1A2* to be associated with reduced enalaprilat concentrations in enalapril treated European hypertensive patients (Ikonnikova et al., 2022).

CES1 structural variation on enalapril response or outcomes is rarely investigated alone in most studies. However, most studies have reported on it in conjunction with other SNPs in the gene. The commonly reported SNPs in association with enalapril are the *CES1* rs71647871G>A and

CES1 rs2244613C>A. According to Her and Zhu et al., (2020), *CES1* rs71647871G>A is the first loss of function variant, located in exon 4 (**Figure 1.11**), that results in a change from glycine to glutamic acid in codon 143 (Her & Zhu, 2020). This causes a disruption of the hydrophobicity needed for the maintenance of the CES1 active site (Arena de Souza et al., 2015). Thus, Tarkiainen et al., (2015) and Wang et al., (2016) found *CES1* rs71647871G>A to be associated with impaired enalapril activation. Carriers of the rs71647871A/G genotype had lower enalaprilat concentrations as compared to rs71647871G/G genotype carriers (Tarkiainen et al., 2015; Wang et al., 2016). Recently, Ikonnikova et al., (2022) found this variant to have no effect on plasma enalaprilat concentration. However, they found the *CES1* rs2244613C>A SNP to be associated with enalapril pharmacokinetics (Ikonnikova et al., 2022).

According to Ikonnikova and colleagues, carriers of the *CES1* rs2244613C/C and rs2244613A/C genotypes were associated with reduced peak and trough concentrations of both enalapril and enalaprilat compared to rs2244613A/A genotype carriers in European arterial hypertensive patients. The *CES1* rs2244613C>A SNP is located in intron 11 (**Figure 1.11**), and its effect on the activity of CES1 is unknown. However, it has been hypothesised that the variant is in linkage disequilibrium with another variant in the *CES1* gene which is causal or may affect post-transcriptional processes introducing a splicing defect, which decrease enzyme activity (Ikonnikova et al., 2022). The influence of this SNP has also been confirmed for other drugs metabolised by CES1 such as dabigatran (Ji et al., 2021), capecitabine (Liu et al., 2021) and clopidogrel (Mirzaev et al., 2019) making it a plausible pharmacogenomic biomarker.

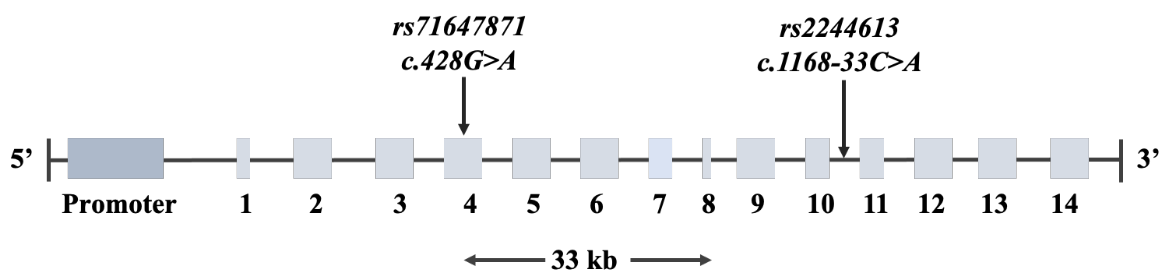

Figure 1.11: Structure of the *CES1* gene located on chromosome 16q12.2. It has 14 exons and is 33 kb long. SNPs rs7164787G>A and rs2244613C>A are located on exon 4 and intron 11 respectively.

1.7 Genes Involved in Response to Hydrochlorothiazide

The frequency of use of hydrochlorothiazide (HCTZ) is high in Africans due to its low cost, safety profile, and has been in use clinically for over 50 years (**Figure 1.2**) (Núñez-Acevedo et

al., 2018). Soko et al., (2023) reports that HCTZ is the third most frequent drug among South African hypertensive patients. HCTZ is orally administered, has an absolute bioavailability of 60-80% and is absorbed rapidly in the gut. Approximately 40% of HCTZ is distributed bound to plasma proteins and does not undergo first pass metabolism. Hence it is eliminated unchanged in urine (Beermann et al., 1976; Johnson et al., 2019; Patel et al., 1984; Zisaki et al., 2015). HCTZ functions by inhibiting the sodium-chloride co-transporter system in the distal convoluted tubules of the kidney. This reduces sodium ion influx or retention, resulting in decreases in the volume of the extracellular space, which in turn leads to a decrease in cardiac output and BP (Akbari & Khorasani-Zadeh, 2023).

Numerous genetic polymorphisms have been reported in association with HCTZ response or adverse effects and have been extensively reviewed elsewhere (Johnson et al., 2019). Findings from these studies have been conflicting. However, the strongest evidence was for the association of variants in the *NEDD4L* gene and BP or cardiovascular outcomes on individuals on HCTZ. The *NEDD4L* gene, located on chromosome 18q21.31, has 43 exons and is ~364 kb long (**Figure 1.12**), encodes the neural precursor cell expressed developmentally down-regulated 4-like (NEDD4L) E3 ubiquitin ligase which regulates the expression of sodium transporters in the kidney such as the SCNN1 (or epithelial sodium channel (ENaC)) and the SLC12A3, which are target sites for HCTZ and amiloride respectively.

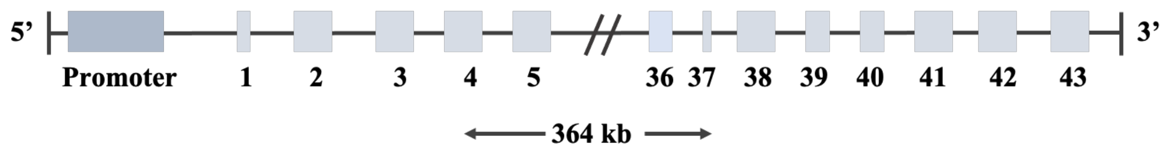

Figure 1.12: Structure of the *NEDD4L* gene located on chromosome 18q21.31. It has 43 exons and is 364 kb long.

For example, to regulate the expression of the ENaC, it has been reported that the intracellular carboxy terminal portion of the ENaC contains a PY motif which serves as a binding site for the *NEDD4* family of proteins. *NEDD4L* is composed of a *C2-WW(x4)-HECT* domain which facilitates binding to the *PY* motif of the ENaC leading to channel ubiquitination and endocytosis. This in turn suppresses ENaC function and thus inhibits epithelial sodium transport (Rotin & Schild, 2008).

Polymorphisms such as the *NEDD4L* rs4149601G>A, have been associated with upregulation or downregulation of the ENaC. According to Svensson-Färbom et al., (2011), individuals who carried the *NEDD4L* rs4149601G allele (G/G or A/G) derived the greatest benefit from HCTZ therapy and experienced greater BP reduction or reduced risk of myocardial infarction (Svensson-Färbom et al., 2011) as compared to those that were homozygous for the *NEDD4L* rs4149601A/A genotype. The G to A change causes alternative splicing and results in non-functional NEDD4L that is deficient of a C2 domain while the G allele results in functional NEDD4L with an intact C2 domain. As a result, G allele carriers express more of the ENaC due to a functional NEDD4L, and thus have more sodium reabsorption and elevated BP which explains why there were greater BP reductions in G allele carriers.

In another cohort, including individuals of European and African ancestry, McDonough et al., (2013), found G allele carriers for the *NEDD4L* rs4149601G>A polymorphism to be associated with greater BP decreases on HCTZ confirming previous findings by Svensson-Färbom and colleagues (Svensson-Färbom et al., 2011). Furthermore, they observed *NEDD4L* rs292449G>C, rs75982813A>G and rs1008899G>A to be associated with HCTZ response. Individuals who carried the two copies of the *NEDD4L* rs4149601 – rs292449G–C haplotype, had the strongest BP lowering effect of HCTZ. Such associations were observed in European and not African American participants. However, there is still need to perform more studies in African populations to pronounce on these effects seeing that genetic variation in *NEDD4L* appears to be predictive of HCTZ response.

1.8 Genes Involved in the Pharmacodynamics of Atenolol

Atenolol is a second-generation β-blocker indicated in the treatment of hypertension. Although current hypertension guidelines (Mancia et al., 2023; Unger et al., 2020; Whelton et al., 2018) recommend β-blockers as second line therapy, β-blockers such as atenolol are among the commonly prescribed antihypertensives worldwide due to their cost effectiveness and safety profile over other β-blockers such as metoprolol. As such, atenolol use ranks fourth in South Africa (Soko et al., 2023). Atenolol lowers BP by selectively blocking beta-1 adrenergic receptors (ADRB1) in the vascular smooth muscle and the heart. This action inhibits the binding of endogenous catecholamines such as norepinephrine and adrenaline, which would otherwise cause contraction or sympathetic stimulation resulting in increases in heart rate and BP (Ailani et al., 2021; Helfand et al., 2009). Atenolol is orally administered, and has a bioavailability of ~50%. It is independent of CYP2D6 metabolism, unlike other β-blockers.

Thus, it does not undergo extensive first pass metabolism (Zisaki et al., 2015). It is eliminated in its parent form in the kidney via organic cation transporters (Kirch & Görg, 1982; Ripley & Saseen, 2014; Yin et al., 2015).

The *ADRB1* gene is 9.8 kb long, has only one exon and is located on chromosome 10q25.3. It encodes the ADRB1 which are the primary target for β-blockers including atenolol. Thus, genetic variation in the *ADRB1* gene is of critical importance on the pharmacodynamics of atenolol. To date, the *ADRB1* gene is among the genes that have been proposed to the Clinical Pharmacogenetics Implementation Consortium (CPIC) for inclusion in prescription guidelines for β-blockers mainly due to a wide evidence base that has linked it to β-blocker response or adverse CVD outcomes (Thomas & Johnson, 2020).

Two missense polymorphisms, *ADRB1* rs1801252A>G and rs1801253C>G are the most commonly studied. *ADRB1* rs1801252A>G and rs1801253C>G result in decreased generation of cAMP by promoting agonist downregulation and decreasing adenylyl cyclase activity respectively. According to Pacanowski et al., (2008), the SNPs in ADRB1 are associated with adverse CVD outcomes (myocardial infarction (MI), stroke) and death in patients with coronary heart disease (CAD) (Pacanowski et al., 2008). It has been hypothesized that it is the combined effect of the two SNPs driving the observed associations rather than the individual SNPs (de Groote et al., 2005; Johnson et al., 2003; Karlsson et al., 2004; Liu et al., 2006; Thomas & Johnson, 2020; White et al., 2003). In most of these studies, Africans have been underrepresented. However, recently, Guerra et al., (2022) reported on the *ADRB1* rs1801253G>C polymorphism on survival in heart failure patients in a cohort consisting mainly of individuals of African ancestry. Individuals who carried the *ADRB1* rs1801253C allele derived the greatest benefit survival benefit from high β-blocker doses compared to non-carriers (Guerra et al., 2022).

1.9 Genes Involved in the Pharmacodynamics of Spironolactone

Individuals who have resistant hypertension have been found to have increased levels of aldosterone (Alvarez-Alvarez et al., 2010; Calhoun & White, 2008; Václavík et al., 2014). Therefore, prescription of spironolactone as a fourth agent, has been proven to be beneficial for the management of resistant hypertension (Williams et al., 2015). Spironolactone is one of the commonest MRAs globally and in South Africa, it is relatively common especially among

patients with resistant hypertension (Jones et al., 2020; Soko et al., 2023). Spironolactone directly blocks the action of aldosterone on mineralocorticoid receptors (MRs). Aldosterone is a component of the RAAS and an excess of it causes increased sodium reabsorption, vascular or cardiac stiffening and remodelling and fibrosis which all contribute to elevated BP. Spironolactone inhibits these processes and antagonises the downstream effects of aldosterone (Acelajado et al., 2019; Nishizaka et al., 2003).

The nuclear receptor nuclear receptor subfamily 3 group C member 2 (*NR3C2*) gene encodes the mineralocorticoid receptors where aldosterone/spironolactone binds. The *NR3C2* gene is located on chromosome 4q31.23, has 13 exons and is ~38 kb long (**Figure 1.13**). Polymorphisms such as the *NR3C2* rs5522T>C, rs2070950C>G and rs2070951C>T are commonly reported to be associated with BP response to spironolactone. According to Dumeny et al., (2021), Europeans previously diagnosed with heart failure, who were carriers of the *NR3C2* rs5522C allele and had poor diastolic function, appeared to benefit more from spironolactone therapy compared to carriers of rs5522T/T genotype since they experienced a greater reduction in BP and had significant improvement in diastolic function (Dumeny et al., 2021). Furthermore, Cavallari et al., (2010) had previously shown that carriers rs5522C allele to be associated with potassium level increases in individuals on spironolactone therapy (Cavallari et al., 2010).

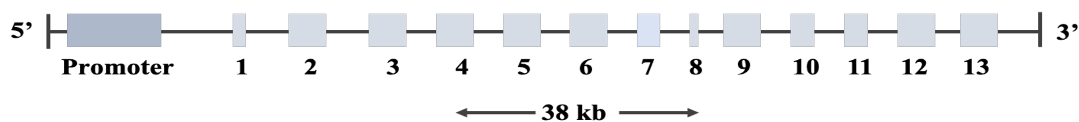

Figure 1.13: Structure of the NR3C2 gene located on chromosome 4q31.23. It has 13 exons and is 38 kb long.

In African populations, *NR3C2* rs2070950C>G polymorphism, was found to be associated with spironolactone response in Egyptians with heart failure. In this prospective cohort study, individuals who were carriers of the *NR3C2* rs2070950G/G and rs2070950G/C genotype were associated with improvements in echocardiographic parameters such as left ventricular end systolic volume (LVESV) and left ventricular end diastolic volume (LVEDV) but the genotypes could not explain changes in other outcomes such as diastolic dysfunction grade and quality of life (Sarhan et al., 2020).

1.10 Genes Associated with Susceptibility to Resistant Hypertension with Pharmacogenetic Implications

NOS3 and *SCNN1B* genes encode enzymes/proteins involved in critical BP regulating pathways. The *NOS3* gene located on chromosome 7q36.1, with 28 exons and is 31 kb long encodes a 1203 amino acid long nitric oxide synthase (NOS3). NOS3 is responsible for the production of nitric oxide (NO), a vasodilator, that plays an important role in BP regulation and the maintenance of vascular tone. Specifically, NO stimulates guanyl cyclase which converts GTP to cGMP whose downstream effects is to promote vascular smooth muscle relaxation and vasodilation. On the other hand, the *SCNN1B* gene on chromosome 16p12.2, encodes the β-subunit of the epithelial sodium channel (ENaC) which regulates sodium ion and water reabsorption in the kidney. According to Lifton et al., (2001) the ENaC accounts for 2% of the total sodium reabsorbed in the kidney and is the principal site where the net balance of sodium is determined (Lifton et al., 2001). As such, genetic polymorphisms in the NOS3 and SCNN1B genes have been reported to confer susceptibility to resistant hypertension (Cruz-González et al., 2009; Jáchymová et al., 2001) and to be of pharmacodynamics and pharmacogenetics importance.

1.10.1 Nitric Oxide Synthase (*NOS3*) Gene and Therapeutic Drug Response to ACE inhibitors

A number of SNPs in the *NOS3* gene have been reported to affect either NOS3 expression or activity leading to reduced levels of circulating NO. Among these *NOS3* polymorphisms, rs2070744C>T, rs3918188C>A and rs1799983T>G are examples of SNPs that have been implicated in both susceptibility and response to ACE inhibitors such as enalapril (**Figure 1.14**) (Oliveira-Paula et al., 2017). According to Oliviera-Paula and colleagues, ACE inhibitors also result in lowering of BP through another mechanism that involves the upregulation of NOS3 activity, stimulating it to produce more NO, a vasodilator (Oliveira-Paula et al., 2016).

NOS3 rs2070744C>T affects gene expression and is associated with increased levels of mRNA and the translated enzyme. It has been widely reported in pharmacogenetic studies in Brazilian populations in connection with response to angiotensin receptor blockers (ARBs) (Mason et al., 2012) and enalapril (Silva et al., 2013b). According to Silva and colleagues, *NOS3* rs2070744C/C and rs2070744C/T genotype carriers were frequent in good responders as compared to poor responders to enalapril. As a result, rs2070744C allele carriers were associated with favourable responses to enalapril (Silva et al., 2013b). In South African

participants, Masilela et al., (2021), reports on this SNP to be associated with BP response to enalapril. However, the influence of this SNP was only significant in the presence of the *VEGFA* rs69994A>T and *ABO* rs495828T>G SNPs, suggesting possible gene-gene interactions (or epistasis), implying that the effect of the *NOS3* rs2070744C>T SNP is dependent on *VEGFA* rs69994A>T and *ABO* rs495828T>G SNPs.

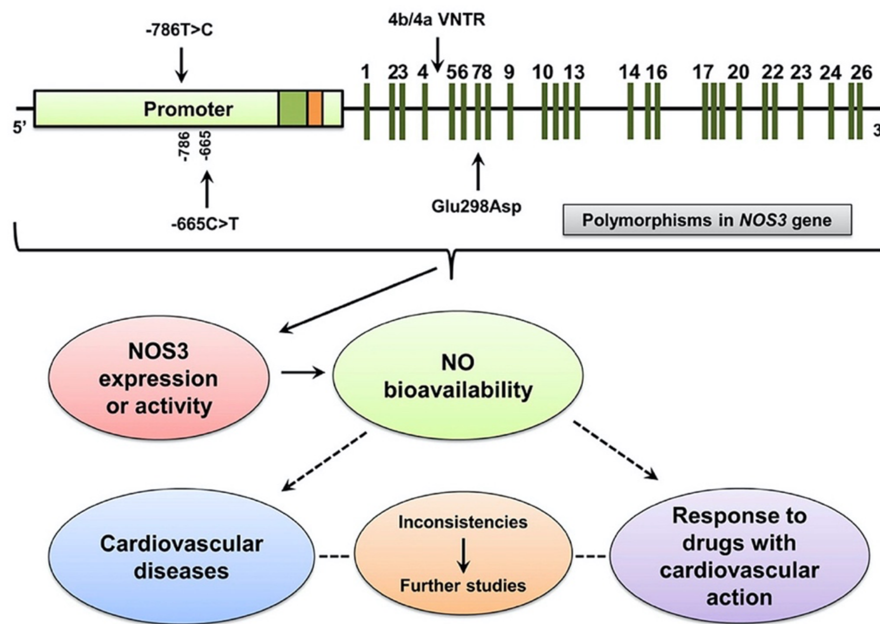

Figure 1.14: Structure and impact of SNPs in *NOS3* gene. The gene is located on chromosome 7q36.1, has 26 exons and is 31 kb long. Polymorphisms in this gene result in either reduced NOS3 expression or activity which reduces the bioavailability of NO. This results in increased susceptibility to CVDs or response to drugs acting on the cardiovascular system (Oliviera-Paula et al., 2017).

NOS3 rs3918188C>A has been reported to be associated with enalapril response in a cohort of Brazilian participants (Oliveira-Paula et al., 2016). It is in linkage disequilibrium with tagSNPs rs3918226C>T and rs743506G>A which have been shown to affect *NOS3* promoter activity (Luizon et al., 2012). According to Oliviera-Paula et al., (2016), carriers of the *NOS3* rs3918188A/A genotype, were found to have poor responses to enalapril which were indicated by lower decreases in BP. It was also shown that carriage of the *NOS3* rs3918188C allele was associated with better responses to enalapril upon haplotype analysis (Oliveira-Paula et al., 2016).

A missense SNP, *NOS3* rs1799983 (c.894T>G, p.Asp298Glu), has also been shown to affect the production of endogenous NO (Joshi et al., 2007). Despite evidence of the involvement of this SNP in susceptibility to hypertension and in modulation of drug response in preeclampsia patients (Sandrim et al., 2010), a study by Silva et al., (2013), did not find any association of this polymorphism with enalapril response. There were no significant differences in genotype and allele frequencies between good responders and poor responders (Silva et al., 2013b).

1.10.2 Sodium Channel Epithelial 1 Subunit Beta (SCNN1B) and Therapeutic Drug Response to Diuretics

Three missense SNPs in the *SCNN1B* gene, rs201279350G>A, rs1799980G>T and rs149868979G>A have been reported to occur in high frequency among Africans (Jones et., 2017). Among these SNPs, *SCNN1B* rs149868979G>A, has been reported to be associated with overactivity of the ENaC and resistant hypertension in African and Mixed Ancestry groups of South Africa. It has also been reported to be associated with BP responses to MRAs such as spironolactone, which are highly efficacious drugs used in the treatment of resistant hypertension (Seedat et al., 2014).

According to Jones et al., (2011), individuals harbouring the *SCNN1B* rs149868979A allele have poor response, needing the alternative use of amiloride as it directly blocks the ENaC. The study by Jones et al., (2011), among Xhosa and Mixed Ancestry patients who were carriers of the *SCNN1B* rs149868979A/G genotype and had a high mean BP of 172/99; administration of 5–10 mg of amiloride as an add-on drug, led to a mean decrease in BP of 36/17 mmHg (Jones et al., 2011).

1.11 Key Take-aways and Thesis Focus Area

Resistant hypertension represents a phenotype of hypertension which is both severe and is difficult to manage with conventional therapy. Its pathogenesis is complex and multifactorial, with genetic factors contributing up to 50% to its progression, especially in therapeutic drug responses. Thus, genetic factors governing therapeutic responses to antihypertensive medications currently in use (**Table 1.3**) have been the subject matter of a number of pharmacogenomics studies as evident in reviews by (Johnson et al., 2019; Katsukunya et al., 2023a; Oliveira-Paula et al., 2019; Rysz et al., 2020) and as reviewed here, with the ultimate goal of potentially rationalising optimal antihypertensive drug/dose regimens. However, research on the pharmacogenomics of hypertension currently lags behind, lacks consistent

findings across populations and hence there is no consensus yet on rational treatment strategies based on an individual's genetics as no antihypertensive drugs have been annotated in CPIC or FDA guidelines.

African populations have not been fully represented in these pharmacogenomics studies as we have shown here, yet it is among the continents that are heavily burdened with hypertension including resistant hypertension. Thus, we have recently advocated for the need for more studies in African populations for the ultimate realisation of a pharmacogenetic based approach for the treatment of hypertension in Africans (Katsukunya et al., 2023a) as pharmacogenetics bears great potential in reducing associated treatment costs, reducing drug burden and improving the quality of life in African patients (Dandara et al., 2019). As a result, this thesis is a step towards that direction and attempts to investigate whether genomic variation in *ABCB1, ADBR1, CES1, CYP3A4, CYP3A5, NR3C2*, *NEDD4L*, *NOS3* and *SCNN1B* contributes to resistant hypertension among African populations, using South Africa as a proxy population.

1.12 Basic Approaches to Pharmacogenomics Investigations and Thesis Approach

According to Cooper-DeHoff and Johnson, (2016), pharmacogenomic studies may utilise the non-targeted approach such as genome wide association studies (GWAS), the targeted approach such as whole exome/genome sequencing or the candidate gene approach (Cooper-DeHoff & Johnson, 2016). The GWAS approach tests multiple (up to > 10 million) SNPs across the entire genome with wide coverage and most techniques are CHIP based. The whole exome/genome approach are sequence-based techniques and test targeted genes. These two approaches are mostly used for discovery of novel genes or variants. This thesis utilises the candidate gene approach, which is hypothesis driven and focuses on genes or SNPs selected on the basis of existing knowledge. The genes were selected based on: (1) their involvement in the pharmacokinetics (*ABCB1, CES1, CYP3A4/5*) or pharmacodynamics (*ADRB1, NEDD4L, NR3C2*) of commonly used antihypertensive drugs in South Africa and (2) susceptibility to resistant hypertension (*NOS3, SCNN1B*). The SNPs were selected based on : (1) functional effect, (2) minor allele frequency >5% in Africans and (3) annotation in PharmGKB.

1.13 Thesis Methodological Approach

Lee et al., (2020), have extensively reviewed current technologies for pharmacogenomics. The most commonly used techniques currently are single nucleotide variant (SNV) panels

containing pre-selected SNPs (van der Lee et al., 2020). They have been extensively applied both in research and in clinical practise. However, it has been reported that they are limited in detecting structural variants such as copy number variants (CNVs) (Ingelman-Sundberg & Sim, 2010). Thus, next generation sequencing (NGS) and long-read sequencing have been reported to be of importance in pharmacogenomics research as they are able to resolve structural and rare variants (Levy & Myers, 2016). These techniques are robust for large sample sizes and can target multiple variants. However, for small scale and inexpensive genotyping procedures, simple polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP), real-time quantitative PCR (RT-qPCR) and Sanger sequencing are widely used and were techniques of choice for this thesis.

1.13.1 Polymerase Chain Reaction Restriction Fragment Length Polymorphism (PCR-RFLP)

PCR-RFLP is a molecular genotyping technique developed by Botstein et al., (1980). Polymorphisms including SNPs, often result in the creation or abolishment of restriction enzyme recognition sites within the DNA sequence and this is the principle of the technique. PCR-RFLP begins within amplification of a specific fragment of DNA containing the polymorphism of interest, followed by digestion with specific restriction enzymes. The presence or absence of restriction enzyme recognition sites results in the formation of DNA or restriction fragments of different sizes and alleles can be identified upon electrophoretic resolution of the DNA fragments (Botstein et al., 1980). In addition to the cost effectiveness of the technique for small scale genotyping procedures, PCR-RFLP does not require specialised instruments and the set-up of analyses is generally easy. However, it is a relatively time-consuming technique and there are difficulties in identifying the exact variation in the event that several SNPs affect the same restriction enzyme recognition site (Henrik Berg, 2012).

1.13.2 Real-time PCR or Quantitative PCR (qPCR)

Quantitative PCR is a PCR based technique that involves amplification of DNA region of interest coupled to quantification of the DNA species in the reaction (Dymond, 2013). Quantitative PCR adds to the PCR reaction fluorescent dyes and a fluorometer which allow for the detection and calculation of the starting DNA template concentration. As the qPCR progresses, amplification cycles generate PCR products in an exponential manner. However, PCR product formation enters a linear phase and the amplification efficiency decreases with more cycles due to the consumption of reactants in the reaction mixture such as dNTPs, primers

or enzymes. Finally, as the reactants get depleted, the reaction progresses to the plateau phase (**Figure A1, Appendix**) where there is little to no formation of PCR product (Dymond, 2013; Livak & Schmittgen, 2001).

Quantitative PCR is extensively applied in evaluating DNA copy number, viral load determination, SNP detection and allelic discrimination assays (Kralik & Ricchi, 2017). For this thesis, qPCR was applied in SNP genotyping and in determining DNA copy number. Quantitative PCR is a high resolution technique with high sensitivity, specificity and allows real-time monitoring as the amplification reaction progresses. Unlike, PCR-RFLP, qPCR can be performed in high throughput formats and has a quicker turnaround time. However, in addition to careful optimisation that is required for qPCR, sample quality and quantity can affect the accuracy of qPCR results, especially if there are inhibitors in the sample at a detectable level, false negative results may be probable (Rodriguez-Lazaro et al., 2013). Thus, validation of qPCR results may be required.

1.13.3 Sanger Sequencing

Sanger sequencing also referred to as the chain termination technique, is the gold standard for validating DNA sequences and has a 99.99% base accuracy score (Sanger & Coulson, 1975). Thus, it is particularly useful for verification of sequencing or genotyping. During Sanger sequencing, amplified DNA fragments are annealed to oligonucleotide primers followed by extension by DNA polymerase that incorporates chain-terminating dideoxnucleotide triphosphates (ddNTPs). Incorporation of the ddNTPs stops the elongation process, which results in DNA fragments of different lengths which can be distinguishable by electrophoretic separation to determine the nucleotide sequence (Stranneheim & Lundeberg, 2012) (**Figure A2, Appendix**).

CHAPTER 2: RESEARCH QUESTIONS, HYPOTHESES, AIMS AND OBJECTIVES

2.1 Research Questions

This study was based on the following primary and secondary research questions:

2.1.1 Primary Research Questions

1. Are there any significant differences in pharmacogene variant profiles between patients with resistant hypertension compared to those who present with non-resistant hypertension among Africans, focussing on 9 priority genes: ***ABCB1, ADBR1, CES1, CYP3A4, CYP3A5***, ***NOS3, NR3C2***, ***NEDD4L*** and ***SCNNB1***?
2. Is genetic variation in ***ABCB1, ADBR1, CES1, CYP3A4, CYP3A5***, ***NOS3, NR3C2***, ***NEDD4L*** and ***SCNNB1*** associated with resistant hypertension among people with hypertension?
3. Is there utility in developing a pharmacogenomic test that includes relevant genetic variants for use in deciding on hypertension treatment among Africans?

2.1.2 Secondary Research Question(s)

1. Do frequencies of the pharmacogenes variants compare to other previously studied world populations?

2.2 Hypotheses

From the above primary and secondary research questions, the following null and alternative hypotheses were generated:

2.2.1 Null Hypotheses (H_o)

1. H_o: There is no genetic contribution underlying resistance to commonly used antihypertensive drugs in South Africa among South African patients with hypertension.
2. H_o: There are no differences in the profiles of variants between individuals who have resistant hypertension (cases) and those without resistant hypertension (controls).
3. H_o: There is no utility in developing a pharmacogenomic test that includes relevant genetic variants for use in deciding on hypertension treatment among Africans.

4. ***H_o***: There are no comparable differences in the frequencies of the pharmacogenes variants to other previously studied world populations.

2.2.2 Alternative Hypotheses (H_A)

1. ***H_A***: There is a genetic contribution underlying resistance to commonly used antihypertensive drugs in South Africa among South African patients with hypertension.
2. ***H_A***: There are differences in the profiles of variants between individuals who have resistant hypertension (cases) and those without resistant hypertension (controls).
3. ***H_A***: There is utility in developing a pharmacogenomic test that includes relevant genetic variants for use in deciding on hypertension treatment among Africans.
4. ***H_A***: There are comparable differences in the frequencies of the pharmacogenes variants to other previously studied world populations.

2.3 Aim of Study

The aim of this study was to evaluate the role of genetic variation in selected pharmacogenes on the way patients with hypertension respond to antihypertensive drugs, comparing the outcomes between patients with resistant or non-resistant hypertension.

2.4 Objectives of the Study

To be able to test the hypotheses generated from the study research questions and to achieve the aim, the objectives of the study were:

1. To recruit study participants, obtain blood samples and collect data on demographic and clinical parameters of the study participants.
2. To classify the study participants into resistant (cases) and non-resistant hypertension (controls) phenotypes.
3. To extract DNA from blood samples and to genotype variants in the selected genes.
4. To compare the distribution of allelic variants between participants with resistant hypertension and those without.
5. To determine the baseline frequencies of the pharmacogene variants in the selected genes.

CHAPTER 3: MATERIALS AND METHOD

3.1 Reagents and Online Tools

All primers, enzymes, assays, consumables and chemicals of analytical grade used in this study were obtained from Inqaba Biotechnical Industries (Muckleneuk, Pretoria, South Africa), Thermofischer Scientific (Waltham, Massachusetts, USA), New England Biolabs (Ipswich, UK), Perkin Elmer (Midrand, South Africa) and other local suppliers. Primers were designed using the Integrated DNA Technologies (IDT) PrimerQuest™ (*https://eu.idtdna.com/pages*) and Oligo Analyzer™ (*https://eu.idtdna.com/calc/analyzer*) online tools followed by validating the primer sequences using the National Centre for Biotechnology Information (NCBI) PrimerBlast tool (*https://www.ncbi.nlm.nih.gov/tools/primer-blast/*) and the University of California Santa Cruz (UCSC) genome browser (*https://genome.ucsc.edu/index.html*). Restriction enzymes/cut sites were selected/mapped out using NebCutter (*https://nc2.neb.com/NEBcutter2/?noredir*) or Restriction Mapper (*https://restrictionmapper.org/*) and restriction enzyme optimum conditions (reaction/inactivation temperatures and buffer conditions) were obtained from NebCloner (*https://nebcloner.neb.com/#!/*). The Pharmacogenomics and Drug Metabolism Research Group is funded by (i) the South African Medical Research Council (SAMRC), (ii) the National Research Foundation (NRF) of South Africa and (iii) the University of Cape Town.

3.2 Ethical Considerations

This study is a sub-study to a larger project titled, "**Pharmacogenomics of Cardiovascular Diseases: Focusing on Dyslipidaemia and Hypertension (PRECODE)**", whose protocol was approved by the University of Cape Town Human Research Ethics Committee (UCT-HREC) with ethics clearance, "**HREC 694/2020**", and was granted permission to recruit consenting participants for the study from the Hypertension Clinic, Groote Schuur, Cape Town, South Africa (33°56′29″S 18°27′46″E). This sub-study also has its own ethical approval which was granted by the UCT-HREC with ethics clearance, "**HREC 141/2022**". The rights to privacy were respected and all medical information of the participants was kept confidential during the study. All patient samples were de-identified and labelled with a unique laboratory generated pharmacogenomics identity (**PGX-ID**).

3.3 Study Design

This is a retrospective matched case-control study. Participants were matched according to age, sex, and ethnicity in a preselected ratio of 1:1 (case : control) at the time of recruitment to eliminate confounding by these variables. All participants had a confirmed diagnosis of hypertension and participants with the following inclusion and exclusion criteria were included and excluded as appropriate.

3.3.1 Inclusion and Exclusion Criteria

The study included participants (i) with a confirmed diagnosis of primary or essential hypertension (defined as hypertension without any known underlying cause), (ii) of African ancestry (defined as individuals of Black African or Mixed ancestry descent), (iii) on at least one antihypertensive drug for at least a year prior, and (iv) >18 years at the time of recruitment. The study excluded participants (i) with a confirmed diagnosis of secondary hypertension (defined as hypertension with a known underlying cause), (ii) who were pregnant at the time of recruitment, (iii) with confirmed white coat hypertension or non-adherence, and (iv) not on treatment with any antihypertensive drugs.

3.4 Study Sample Size Determination

The sample size was calculated using the following formula,

$$\boldsymbol{N} = \frac{(\boldsymbol{Z} - \boldsymbol{\alpha})^2 \boldsymbol{P}(\mathbf{1} - \boldsymbol{P})}{\boldsymbol{D}^2}$$

where $\boldsymbol{N}$ = sample size, $\boldsymbol{Z\text{-}\alpha}$ = 1.96 (95% confidence level), $\boldsymbol{D}$ = 0.05 (absolute precision),

as previously described by Naing and co-workers (Naing et al., 2006). Given that the prevalence of resistant hypertension in South Africa is 12.6 % (Moosa et al., 2016), ***P*** **(*P*** = proportion with treatment resistant hypertension) of 12.6 % was used. So, we substituted **P** for 0.126 in the formula. Therefore,

$$\boldsymbol{N} = 163.$$

According to the calculation, samples will be required in each of the two groups, cases and controls. However, in order to take into account samples that may fail to work due to poor

quality DNA or other factors, a total sample size of 400 (200 controls and 200 cases) was used, enabling the study to remain within the required power of at least 80%.

3.5 Study Participant Recruitment

Participant recruitment for the study was retrospective and included patients attending the Hypertension Clinic at Groote Schuur Hospital (Cape Town, South Africa) between August 2021 and July 2022. Additional participants were recruited from patients who attended the Hypertension Clinic and had provided informed consent from January 2006 to December 2016. Briefly, at recruitment, all patients were assessed by consultants specialising in Nephrology and Hypertension (B.R and E.J) and underwent a full clinical assessment and the following laboratory examinations - assessing sodium, potassium, creatinine and eGFR, fasting lipogram, fasting glucose, uric acid, aldosterone, renin, amlodipine levels and the urine albumin-creatinine ratio. A trained nurse, measured all anthropometric variables such as height, weight, body mass index (BMI) and waist circumference. BP was measured using an automated office BP monitor (AOBP). Where white coating was suspected, 24-hour ambulatory BP monitoring was done to assess for presence of white coating. Furthermore, DNA samples from whole blood were collected and stored at -20°C until they were extracted.

3.6 Operational Classification of Participants into Cases and Controls

Cases were defined as: BP ≥140/90 mmHg on 3 or more antihypertensive drugs including a diuretic in optimal doses or, BP <140/90 mmHg on 4 or more antihypertensive drugs provided pseudo-resistance was excluded and adherence to antihypertensive therapy confirmed by clinician or through TDM of amlodipine levels where possible (i.e., plasma amlodipine levels >7.0 ng/µl). **Controls were defined as:** BP <140/90 mmHg or BP ≥140/90 mmHg on less than 3 antihypertensive drugs.

Operationally, classification was done following the workflow outlined in **Figure 3.1**. Briefly, stage 1 provides a checklist of the steps taken before a formal diagnosis of resistant hypertension. Stage 2 gives an overview of steps that were taken to classify participants into cases and controls mainly through counting the number of antihypertensive drugs and noting BP readings at each visit. Finally, stage 3 was a review and validation of the cases and controls by a clinician.

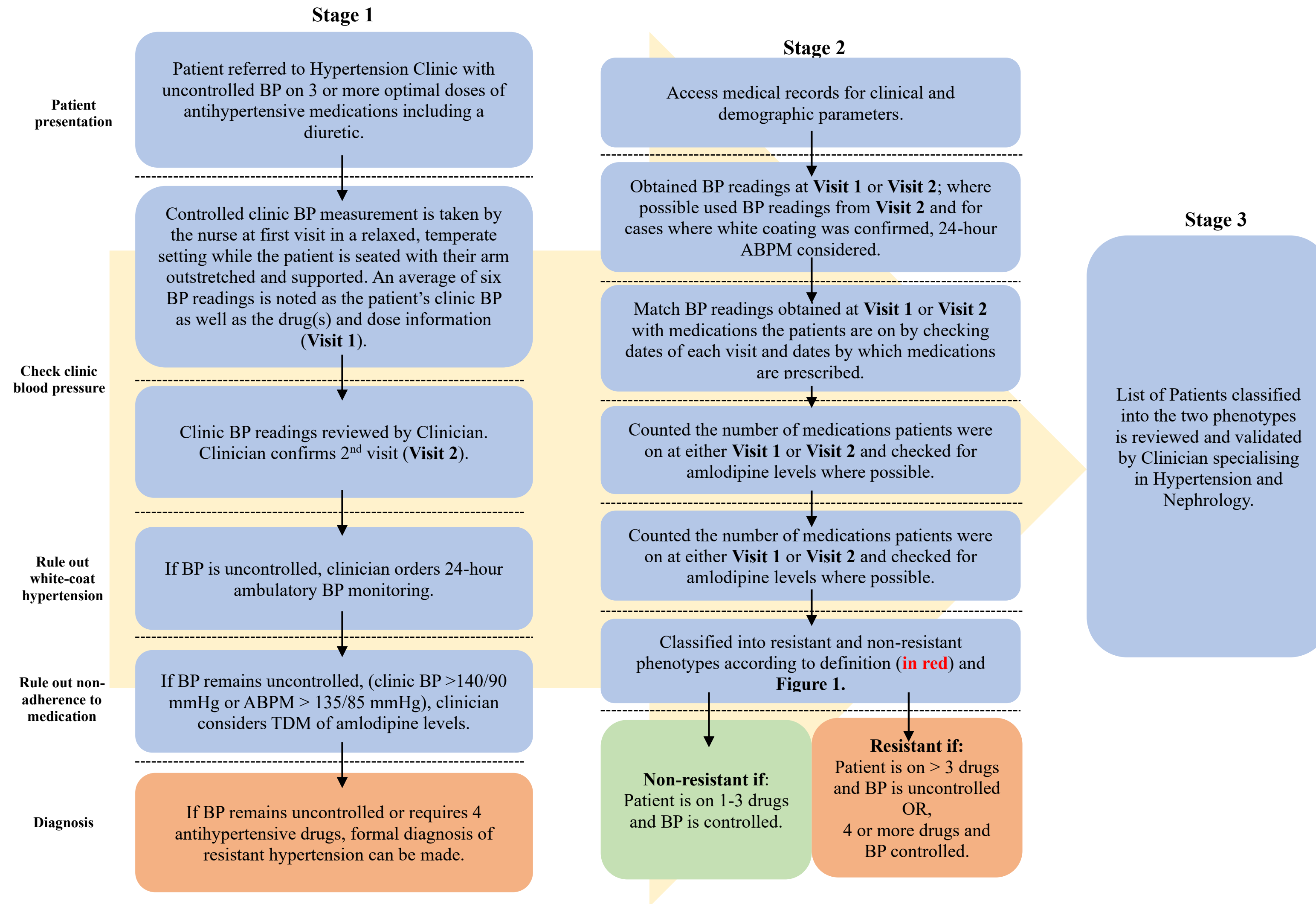

Figure 3.1: Operational Classification of Hypertensive Patients into the Resistant Hypertension and Non-resistant Hypertension Phenotypes in this study. Schema highlights the steps involved during diagnosis of resistant hypertension (Stage 1), steps taken to classify patients into the two phenotypes (Stage 2) and validation or review by Clinician (Stage 3).

3.7 Details on Matching of Cases to Controls and Assumptions

Participants classified into cases or controls were matched individually in a 1:1 ratio. A participant who was a case (classified as having resistant hypertension), was matched to a control (classified as not having resistant hypertension) of the same sex (male or female), same ethnicity (Black African or Mixed Ancestry) and same/exact age where possible. In the event where participants were not of the same/exact age, interval matching of age was used whereby matching on age was acceptable if it was within 5 years (age ± 5 years) between a case and a control.

3.8 Genomic DNA Isolation

Genomic DNA was extracted from whole blood using the automated nucleic acid extraction protocol for human blood developed by Chemagen Technologies GMBH/Perkin Elmer Incorporated (UK) on the Chemagic 360® instrument with integrated Chemagic dispenser. Briefly, five 24-deep well plates were prepared. To the first 24-deep well plate, a volume of 4 ml of whole blood was added into each well. This was followed by the addition of 15 µl of protease into each well containing 4 mL of whole blood. To a second 24-deep well plate, a volume of 400 µL of thoroughly mixed magnetic bead solution was added into each well. The remaining three 24-deep well plates were left empty for automatic addition of wash buffers 3, 4, 5 and 6. Furthermore, a total of 24 13 mL tubes were prepared on a special rack, followed by the addition of 300 µL of elution buffer 7 to each tube. On another special rack, 24 disposable tips were placed. The prepared plates, 13 mL tubes and disposable tips were then placed onto respective positions on special adapters in the Chemagic 360® instrument. Using the extraction protocol for human blood on the instrument software, the samples were mixed thoroughly, homogenized, and washed in multiple steps to increase the yield and purity of the DNA. The automatic transfer of magnetic beads between the tubes and racks, allowed for binding and separation of the DNA from cell debris. In the final step, the DNA was then released from the magnetic beads using elution buffer 7 from the prepared 13 mL prefilled tubes. The DNA was collected in these 13 mL tubes and transferred to 2 mL Eppendorf tubes for quantification and further downstream processing.

3.9 Genomic DNA Quantification and Integrity

The quantity of the extracted genomic DNA was determined using spectrophotometry on a NanoDrop c1000 UV spectrophotometer (Thermofischer Scientific Corporation, Waltham,

Massachusetts, USA). Briefly, the instrument was blanked by placing 1.5 μL of elution buffer 7 onto the instrument sample pedestal. This was followed by placing a volume of 1.5 μL of extracted DNA onto the sample pedestal. The instrument measured the quantity of the DNA (in ng/μL), absorbance at 230 nm, 260nm and 280 nm, and computed the 230/260 and 260/280 ratios.

This was followed by determination of DNA integrity using agarose gel electrophoresis. Briefly, a 1% (w/v) agarose gel was prepared by dissolving agarose powder in 1X TBE (1L: 1.08 g Tris-Ultra Pure; 5.5 g Boric Acid; 0.72 g EDTA-Disodium Salt) followed by addition of 5 μL of SafeView™ Classic (Applied Biological Materials Inc., Richmond, Canada) nucleic acid stain before casting the gel. The SafeView™ Classic nucleic acid stain intercalates the DNA molecules and allows for visualization of DNA under ultraviolet (UV) light. The gel was allowed to set and placed into an electrophoresis tank filled with 1X TBE (1L: 1.08 g Tris-Ultra Pure; 5.5 g Boric Acid; 0.72 g EDTA-Disodium Salt). DNA samples including a 1 kb molecular weight marker was loaded onto the wells and this was followed by electrophoresis at 100V for 1 hour. Visualization was done on a UV transilluminator (UVITEC, Avebury House, Cambridge, UK) using the Fire Reader software for Windows (D-56-26.MX, France).

3.10 Selection of SNPs for Genotyping

SNPs in the selected genes that have been previously reported to be associated with BP response to amlodipine, enalapril, hydrochlorothiazide, atenolol, or spironolactone among hypertensive patients were selected by accessing the Clinical Pharmacogenetics Implementation Consortium (CPIC) database, Food and Drug Administration (FDA) Table of Pharmacogenomic Biomarkers in Drug Labelling, Pharmacogenomics Knowledge Base (PharmGKB) database and surveying available literature. The selected SNPs were at least annotated in PharmGKB with a level of evidence of at least 3 which implied variant-drug combinations from multiple studies but with limited evidence of association. Furthermore, SNPs were selected if they had minor allele frequencies exceeding 0.05 in Africans and this was done by accessing the Ensembl Release 109 database (Cunningham et al., 2021) and the Allele Frequency Database (ALFRED) (Osier et al., 2002).

3.11 Genotyping

SNPs in the selected genes were characterized through polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP), qPCR either using TaqMan Allelic Discrimination assays or TaqMan copy number variation (CNV) assays and Sanger sequencing.

3.11.1 Polymerase Chain Reaction (PCR)

All primers used for PCR were either designed or obtained from previous studies (**Table 3.1**). Forward and reverse primers, covering the region of interest containing the SNP, were first optimized using temperature gradient PCR to determine the optimum annealing temperature. The range of the temperatures used for each individual gradient PCR were chosen depending on the melting temperatures (T_m, °C) specific to the primers which was determined by the formula,

$$\mathbf{T_m = 4\ °C\ (number\ of\ G/C\ nucleotides) + 2\ °C\ (number\ of\ A/T\ nucleotides)}.$$

Each gradient PCR temperature range was specific to each SNP. However, all the temperature gradient PCRs were carried out using temperature ranges that were between 50 °C - 70 °C on a T100^{TM} Thermal Cycler (Bio-Rad Laboratories, Inc., Singapore). Annealing temperatures which were specific for amplification of the region of interest covering either of the selected 20 SNPs, which gave the optimum yield of PCR product while achieving the good specificity (**Table 3.1**), were used for PCR amplification of the region containing the SNP of interest in all DNA samples.

For each PCR reaction, 2 µL of 50 ng/µl genomic DNA; 5 µL of 5X Green GoTaq Flexi Reaction Buffer (Promega Corporation, Madison, WI, USA); 1 µL of 10 µM of deoxynucleotide triphosphates or dNTPs (Promega Corporation, Madison, WI, USA); 1.5 µl of 25 mM magnesium chloride (Promega Corporation, Madison, WI, USA); 1 µL each of 10 µM forward and reverse primers (Inqaba Biotechnical industries (Pty) Ltd., South Africa) and 0.13 µl of 5 U/µL of GoTaq Flexi DNA Polymerase (Promega Corporation, Madison, WI, USA) were added to a 0.2 mL PCR reaction tube (NEST Biotechnology, Wuxi, China). A volume of 13.37 µL of sterile distilled water was added to make a total PCR reaction volume of 25 µL followed by gentle vortexing on a vortex mixer (Labnet International Inc., Edison,

New Jersey, USA) and spinning on a microcentrifuge (Whitehead Scientific, (Pty) Ltd., Cape Town, South Africa). Therefore, for a 25 µL PCR reaction, the final concentrations of genomic DNA, Green GoTaq Flexi Reaction Buffer, deoxynucleotide triphosphates, magnesium chloride, each primer and GoTaq Flexi DNA Polymerase were 100 ng/µL, 1X, 0.4 µM, 1.5 mM, 0.4 µM each and 0.03 U/µL respectively.

All the PCRs, amplifying the regions of interest containing the selected SNPs were carried out using a SimpliAmp™ Thermal Cycler (Applied Biosystems, ThermoFisher Scientific, Life Technologies, Singapore) in three basic stages namely initial denaturation stage, a cycling stage which consisted of a further denaturation step, annealing and initial extension steps, and lastly the final extension stage which was longer in duration. The reaction conditions were initial denaturation at 94 °C for 3 minutes, followed by 35 cycles of further denaturation at 94 °C for 30 seconds, annealing at temperatures specific for each SNP for 30 seconds (**Table 3.1**), (iv) initial extension at 72 °C for 30 seconds, and (v) final extension at 72 °C for 10 minutes. The PCR products were resolved on a 1.5% (w/v) agarose gel for 1 hour at 120V using a 100 bp molecular weight marker.

3.11.2 Restriction Fragment Length Polymorphism (RFLP)

The PCR products obtained from amplification of the regions of interest containing the *ABCB1* rs1045642C>T, rs2032582C>A, *ADRB1* rs1801252A>G, rs1801253G>C, *CES1* rs2244613C>A, *CYP3A5* rs776746T>C, rs10264272C>T, rs41303343insT, *NOS3* rs1799983G>T and *SCNN1B* rs149868979G>A SNPs were digested using *MboI, BSeYI, Sau96I, BstNI, AlwN1, SspI, DdeI, BanI* and *Scf1* restriction enzymes from New England BioLabs® (Ipswich, UK), which recognized specific cut sites within the DNA sequence which are shown in **Table 3.2.**

For each digest reaction, 10 µL of PCR product, 2 µL of 10X CutSmart™ Buffer (New England BioLabs®, Ipswich, UK), and 0.3 µL of 10 U/µL restriction enzyme specific for each SNP were added to a 0.2 mL PCR reaction tube (NEST Biotechnology, Wuxi, China). This was followed by addition of 17.7 µL of sterile distilled water to make up to 30 µL bringing the final concentration of the CutSmart™ Buffer and restriction enzyme to 0.67X and 3 U/µL respectively in a 30 µL digest reaction. The reaction mixture was vortexed gently on a vortex mixer (Labnet International Inc., Edison, New Jersey, USA), spun briefly using a microcentrifuge (Whitehead Scientific, (Pty) Ltd., Cape Town, South Africa) followed by

Table 3.1: Polymerase Chain Reaction (PCR) primers, optimum annealing temperatures and expected PCR product sizes for amplification of region of interest containing selected SNPs for this study

SNP	Primer Sequences (5'-3')		Ta (°C)*	PCR*	Reference
	Forward Primer	Reverse Primer			
ABCB1 rs1045642 (c.3435C>T)	TGCTGGTCCTGAAGTTGATCTGTGAAC	ACATTAGGCAGTGACTCGATGAAGGCA	60	248	(Turgut et al., 2007)
ABCB1 rs2032582 (c.2677C>A)	ATGGTTGGCAACTAACACTGTTA	AGCAGTAGGGAGTAACAAAATAACA	54	206	(Rhodes et al., 2007)
ADRB1 rs1801252 (c.145A>G)	GACCTCCCTCTGCGCACCAC	CTGAGGTCCACAGCTCGCAGA	61	508	(Moriyama et al., 2013)
ADRB1 rs1801253 (c.1165G>C)	ACGCTGGGCATCATCATGGGC	CTGAGGTCCACAGCTCGCAGA	57	332	(Moriyama et al., 2013)
CES1 rs2244613 (1168-33C>A)	TGTCGTCTGTTCCTCCTAAG	GTTGGTTGGTCAGTTTGTTT	58.4	480	designed
CYP3A4 rs2740574 (c.-392C>T)	GGACAGCCATAGARACAAGGGCT	AGGTTTCCATGGCCAAGTCT	64	334	(Tavira et al., 2013)
CYP3A5 rs776746 (c.219 237A>G)	CATCAGTTAGTAGACAGATGA	GGTCCAAACAGGGAAGAAATA	51	293	(van Schaik et al., 2002)
CYP3A5 rs10264272 (c.624G>A)	TGGAAGATGATTCAGCAGATA	GTGGGGTGTTGACAGCTAAAG	58	495	(van Schaik et al., 2002)
CYP3A5 rs41303343 (27131 27132insT)	CTTCAATAGTACTGCATGGAC	CTGTACCACGGCATCATAGCT	53	108	(van Schaik et al., 2002)
NEDD4L rs4149601 (c.49-16229G>A)	CGACTTCCGCATACTCTTCAG	CTGTCACGGTGTTCCTACATT	60	417	designed
NEDD4L rs292449 (c.-300G>C)	CCTCTTGTTCAAACTCCCTAAGA	TCTGTCCATCGTGAAGCATAC	60	239	designed
NEDD4L rs75982813 (g.5804376A>G)	TTGCTAGAGAAACGGTAGCTG	CTGAGCGTTTCTTCCTCC	60	450	designed
NOS3 rs1799983 (c.894G>T)	CATCAGTTAGTAGACAGATGA	CTGAGGTCCACAGCTCGCAGA	61.1	561	designed
NOS3 rs3918188 (c.1753-734C>A)	TAGCCAGGAGTGAGGAAAGA	AGTCTCGCTGTGTTGTTCAG	60	309	designed
NR3C2 rs5522 (c.538C>T)	TAACGGACTTGAGAGAGGAGAG	CCTATGAGCAGCAGAACCAA	60	443	designed
NR3C2 rs2070950 (c.-2-358C>G)	CTTTGGTCTCCATCGCTAACA	CAAGCCACCCACTTCACTAA	60	398	designed
SCNN1B rs149868979 (c.1688G>A)	AAGATCCCTAAGACAGTCCCA	AGACGCAGGGAGTCATAGT	60	485	designed

*Ta, Annealing temperature of the primer; **PCR product size in base pairs (bp).

incubation and inactivation on a SimpliAmp™ Thermal Cycler (Applied Biosystems, ThermoFisher Scientific, Life Technologies, Singapore) at temperatures and periods specific for each enzyme (**Table 3.2**).

Table 3.2: Restriction enzymes, cut sites and conditions used for genotyping the selected SNPs.

SNP	Restriction Enzyme	Restriction Enzyme Cut Sites	Restriction Enzyme Conditions; Period	
			Incubation	**Inactivation**
ABCB1 rs1045642 (c.3435C>T)	*MboI*	xGATC	37 °C; 2 h	65 °C; 20 min
ABCB1 rs2032582 (c.2677C>A)	*BSeYI*	xCCCAGC	37 °C; 16 h	80 °C; 30 min
ADRB1 rs1801252 (c.145A>G)	*Sau96I*	GxGNCC	37 °C; 2 h	65 °C; 20 min
ADRB1 rs1801253 (c.1165G>C)	*BstNI*	CCxGG	60 °C; 2 h	No
CES1 rs2244613 (1168-33C>A)	*AlwN1*	CAGNNNCxTG	37 °C; 2 h	80 °C; 20 min
CYP3A5 rs776746 (c.219-237A>G)	*SspI*	AATxAAT	37 °C; 4 h	65 °C; 20 min
CYP3A5 rs10264272 (c.624G>A)	*DdeI*	CxTNAG	37 °C; 2 h	65 °C; 20 min
CYP3A5 rs41303343 (insT)	*DdeI*	CxTNAG	37 °C; 2 h	65 °C; 20 min
NOS3 rs1799983 (c.894T>A)	*BanI*	GxGNNCC	37 °C; 16 h	65 °C; 20 min
SCNN1B rs149868979 (c.1688G>A)	*Scf1*	CTNNxAG	37 °C; 2 h	65 °C; 20 min

x: restriction enzyme cut site; h: hours; min: minutes; N: any random nucleotide; *ABCB1*: ATP-Binding cassette protein-1; *ADRB1*: Beta-1 adrenergic receptor; *CES1*: Carboxylesterase-1; *CYP3A5*: Cytochrome P450 family 3 subfamily A member 5; *NOS3*: Nitric oxide synthase 3, *SCNN1B*: Sodium channel epithelial 1 subunit beta.

The reaction digest products were resolved on a 3% (w/v) agarose gel stained with gel red in 1X TBE (1L: 1.08 g Tris-Ultra Pure; 5.5 g Boric Acid; 0.72 g EDTA-Disodium Salt) at 120 V for 20 minutes then at 80 V for 2 hours alongside a DNA molecular weight maker (50 bp, 100 bp or 1 kb). The agarose gels were visualized on a UV transilluminator (UVITEC, Avebury House, Cambridge, UK) using the Fire Reader software for Windows (D-56-26.MX, France). The appearance of different sized DNA fragments on the agarose gel corresponded to the genotype of each sample (**Table 3.3**).

Table 3.3: Genotypes corresponding to DNA fragment sizes (in bp). These are obtained for each SNP after a restriction enzyme digest and visualization on a 3% (w/v) gel red stained agarose gel.

SNP	Genotypes according to fragment sizes for:		
	Wild Type	**Homozygous Variant**	**Heterozygote**
ABCB1 rs1045642 (c.3435C>T)	C/C: 60 bp, 172 bp	T/T: 232 bp	C/T: 60 bp, 172 bp, 232 bp
ABCB1 rs2032582 (c.2677C>A)	G/G: 208 bp	C/C: 84 bp, 124 bp	G/C: 84 bp, 124 bp, 208 bp
ADRB1 rs1801252 (c.145A>G)	A/A: 54 bp, 389 bp	G/G: 54 bp, 190/199 bp	A/G: 54 bp, 190/199 bp, 389 bp
ADRB1 rs1801253 (c.1165G>C)	G/G: 52 bp, 138/142 bp	C/C: 52 bp, 280 bp	C/G: 52 bp, 138/142 bp, 280 bp
CES1 rs2244613 (1168-33G>T)	G/G: 480 bp	T/T: 92 bp, 388 bp	G/T: 92 bp, 388 bp, 480 bp
CYP3A5 rs776746 (c.219-237T>C)	T/T: 125 bp, 148 bp	C/C: 125 bp, 168 bp	C/T: 125 bp, 148 bp, 168 bp
CYP3A5 rs10264272 (c.624C>T)	C/C: 103 bp, 137 bp, 230 bp	T/T: 128 bp, 137 bp, 230 bp	C/T: 103 bp, 128 bp, 137 bp, 230 bp
CYP3A5 rs41303343 (insT)	A/A: 22/24 bp, 62 bp	T/T: 22/24 bp, 41 bp	A/T: 22/24 bp, 41 bp, 62 bp
NOS3 rs1799983 (c.894T>G)	G/G: 86 bp, 443 bp	T/T: 529 bp	G/T: 86 bp, 443 bp, 529 bp
SCNN1B rs149868979 (c.1688G>A)	G/G: 485 bp	A/A: 190 bp, 295 bp	A/G: 190 bp, 295 bp, 485 bp

ABCB1: ATP-Binding cassette protein-1; *ADRB1*: Beta-1 adrenergic receptor; *CES1*: Carboxylesterase 1; *CYP3A5*: Cytochrome P450 family 3 subfamily A member 5; *NOS3*: Nitric oxide synthase 3, *SCNN1B*: Sodium channel epithelial 1 subunit beta.

3.11.3 TaqMan Allelic Discrimination Assays

Validated TaqMan allelic discrimination genotyping assay kits from ThermoFisher Scientific Corporation (Waltham, Massachusetts, USA) were used for genotyping the *CYP3A4* rs2740574C>T, rs2246709A>G*,* *NR3C2* rs5522G>A, rs2070950C>G, *NEDD4L* rs4149601G>A, rs292449G>C, rs75982813A>G and *NOS3* rs2070744T>C, rs3918188C>A SNPs. Each assay contained FAM or VIC fluorescent dye labelled probes which were specific to either the wild type or variant allele for each SNP (**Table 3.4**) and sequence specific forward and reverse primers designed and synthesized by ThermoFisher Scientific Corporation.

Table 3.4: TaqMan Allelic Discrimination Assays. Each assay has a context sequence where alleles (wild type or variant) at the position of the SNP [**highlighted in bold**] targeted by FAM/VIC fluorescent dye labelled probes.

SNP	Assay ID	Context Sequence	Alleles targeted by fluorescent probe	
			FAM dye	VIC dye
CYP3A4 rs2740574 (c.-392C>T)	C___1837671_50	CTATTAAATCGCCTCTCTC[**C/T**]TGCCCTTGTCTCTATGG	Variant: T	Wild type: C
CYP3A4 rs2246709 (c.16090A>G)	C___1845287_10	AATAGGCAAATCCATAGA[**A/G**]GCAGAAAGTTGATTA	Variant: G	Wild type: A
NR3C2 rs5522 (c.538C>T)	C__12007869_20	CATGATAGGGCTTTTAACAA**[C/T]**GGCGCGCATGACGC	Variant: T	Wild type: C
NR3C2 rs2070950 (c.-2-358C>G)	C___1594391_1_	AGGCAAAAAAAGGTAACT**[C/G]**AGGACTTAGTGAAGTG	Variant: G	Wild type: C
NEDD4L rs4149601 (c.49-16229G>A)	C___1424558_10	AACGTCTCGCATTTGAGCA**[A/G]**GTAACACTCGGTAAG	Wild type: G	Variant: A
NEDD4L rs292449 (c.-300G>C)	C___1132327_10	TGCTTCTGCTCCATTGCTGTT**[C/G]**AACTTTTTTTTGGT	Wild type: G	Variant: C
NEDD4L rs75982813 (g.58043776A>G)	C_103971334_10	CTGCTCGTCGGGGCCGCA**[A/G]**AGAATTTAAAGGGGTC	Variant: G	Wild type: A
NOS3 rs2070744 (c.-51-762T>C)	C__15903863_10	AAGCTCTTCCCTGGC**[C/T]**GGCTGACCCTGCCTCAGCCC	Wild type: T	Variant: C
NOS3 rs3918188 (c.1753-734C>A)	C__29193459_10	CAAGGCACACGTACAAGGG**[A/C]**GTTTGAGAGAGCACC	Wild type: C	Variant: A

CYP3A4: Cytochrome P450 family 3 subfamily A member 4, *NR3C2*: Nuclear receptor subfamily 3 group C member 2, *NEDD4L*: Neural precursor cell expressed developmentally down-regulated 4 like;, *NOS3*: Nitric oxide synthase 3

The reactions were set up in a total reaction volume of 10 µL in 0.2 mL white qPCR tubes (Bio-Rad Laboratories, Inc., California, USA). Each 10 µL reaction mixture was made up of 5 µL of 2X TaqPath™ ProAmp™ Master Mix (Taq DNA polymerase, dNTPs, Mg^{2+}, K^{+}, pH 7.85 - 8.15 at 25 °C) from ThermoFisher Scientific Corporation (Waltham, Massachusetts, USA), 0.5 µL of 20X TaqMan® genotyping assay specific for each SNP (**Table 3.4**) and 4.5 µL of 10 ng/µL of genomic DNA bringing final concentrations of each component in the reaction mixture to 1X, 1X and 4.5 ng/µL respectively. This was followed by gentle vortexing on a vortex mixer (Labnet International Inc., Edison, New Jersey, USA) and spinning on a microcentrifuge (Whitehead Scientific, (Pty) Ltd., Cape Town, South Africa).

All qPCR reactions were carried out on a CFX96 Touch Real Time PCR Thermal Cycler (Bio-Rad Laboratories, Inc., California, USA) and the reaction conditions were initial denaturation at 95 °C for 5 minutes followed by 50 cycles of further denaturation and annealing at 95 °C for 15 seconds and 60 °C for 1 minute respectively, then final elongation at 60 °C for 30 seconds. Genotypes were displayed in the form of clusters of three possible allelic combinations which were wildtype/wildtype allele, wildtype/variant allele, and variant/variant allele on an allelic discrimination plot.

3.11.4 TaqMan Copy Number Variation (CNV) Assays

A validated FAM dye labelled TaqMan CNV assay (Assay ID: Hs00139541_cn) from Thermofischer Scientific Corporation (Waltham, Massachusetts, USA) for determination of structural variation in the carboxylesterase 1 (*CES1*) gene was used to determine the copy number of *CES1A2* by quantitative real time PCR. The assay targeted intron 11 of the *CES1* gene on chromosome 16q12.2 (assay reference genome location: chr.16:55810581 on build GRCh38). The reference gene was the gene encoding ribonuclease P located on chromosome 14q11.2 and the VIC dye labelled TaqMan™ CNV RNase P assay (catalogue number: 4403326) served as the reference assay (assay reference genome location: chr.14:20343370 on build GRCh38).

Each qPCR reaction was carried out in a total reaction volume of 10 µL containing 1 µL of 5 ng/µL of genomic DNA, 0.5 µL of RNase P assay, 0.5 µL of TaqMan CNV assay (Hs00139541_cn), 5 µL of 2X TaqPath™ ProAmp™ Master Mix and 3 µL of sterile distilled water in 0.2 mL qPCR tubes. All qPCR reactions were carried out on the QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems, Life Technologies, Waltham,

Massachusetts, USA) and the reaction conditions were initial denaturation at 95 °C for 10 minutes followed by 40 cycles of further denaturation and annealing at 95 °C for 15 seconds and 60 °C for 1 minute respectively.

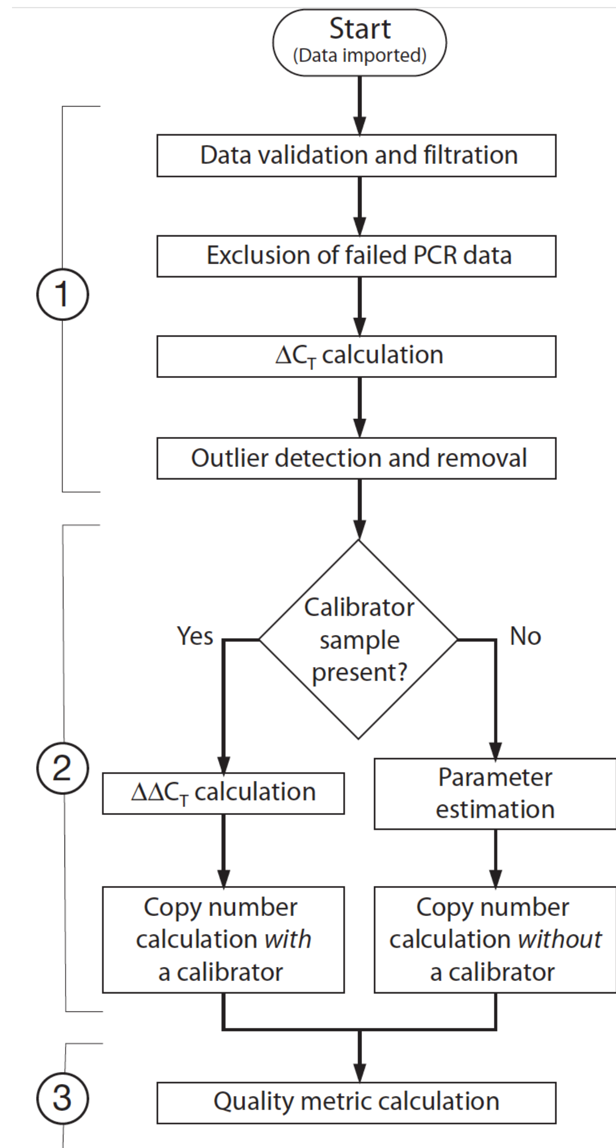

Figure 3.2: CopyCaller analysis workflow. Analysis includes three major stages namely, (1) preprocessing involving data validation, filtration, exclusion of failed PCR data, outlier detection and removal , (2) sample copy number assignment based on the presence or absence of a calibrator sample and (3) quality metric assignment based on Z-scores and confidence scores (adapted from Applied Biosystems by Life Technologies User Guide).

Calculations of gene copy numbers was done using CopyCaller software for Windows (Version 2.0, Applied Biosystems, Life Technologies, Waltham, Massachusetts, USA), according to **Figure 3.2**. Briefly, data from qPCR amplification imported onto the software underwent preprocessing which included data validation, filtration, exclusion of failed PCR data, ΔC_T calculation and outlier detection and removal. A calibrator sample was absent; thus, the copy

number was estimated by the CopyCaller software algorithm based on the most frequent copy number which we assigned as 2. Finally, a quality metric was assigned to the copy numbers generated for each sample, which incorporated both absolute *Z*-scores (±1) and confidence scores (0 – 100%).

3.11.4.1 Main Assumptions in the Calculation of *CES1* Copy Numbers

CES1 gene deletions have not been reported elsewhere, thus, we assumed the most frequent copy number to be 2, based on literature for parameter estimation in CopyCaller as there was no calibrator sample. Additionally, copy number calls with a confidence metric >50% were accepted with certainty.

3.11.5 Verification and Validation of Genotypes

Sanger sequencing was used as a verification method to confirm genotype calls obtained from primary genotyping methods - PCR-RFLP and TaqMan allelic discrimination assays. All sequencing reactions were carried out on the SeqStudio® Genetic Analyzer (Applied Biosystems Corporation, Waltham, Massachusetts, USA). Prior to capillary electrophoresis on the on the SeqStudio® Genetic Analyzer, post-PCR clean-up, cycle sequencing and post-sequencing clean up were done.

3.11.5.1 Post-PCR Clean-up

Post-PCR clean-up was employed to purify all PCR products by breaking down unincorporated PCR primers and nucleotides left over from PCR reactions. This was achieved by using exonuclease I (*ExoI*) and thermosensitive alkaline phosphatase (*FastAP*™) enzymes from ThermoFisher Scientific (Waltham, Massachusetts, USA). Each clean-up reaction was prepared by adding 1 µL of 1 U of *FastAP*™ and 0.2 µL of 4 U *ExoI* enzymes in a 0.2 mL PCR tube (NEST Biotechnology, Wuxi, China). This was followed by adding 3.8 µL of sterile distilled water to make up to 5 µL of the total reaction volume bringing the final concentration of *FastAP*™ and *ExoI* to 0.2 U and 0.16 U respectively. The reaction mixture was vortexed on a vortex mixer (Labnet International Inc., Edison, New Jersey, USA) and spun in a microcentrifuge (Whitehead Scientific, (Pty) Ltd., Cape Town, South Africa) for 10 seconds. The reaction mixture was incubated at 37 °C for 1 hour, followed by enzyme inactivation at 75 °C for 15 minutes in a SimpliAmp™ Thermal Cycler (Applied Biosystems, ThermoFisher Scientific, Life Technologies, Singapore).

3.11.5.2 Cycle Sequencing

Cycle sequencing is based on the addition of chain terminating dideoxy-nucleotide phosphates (ddNTPs) to the growing DNA strand during DNA synthesis. In each cycle of the sequencing reaction, PCR primers were selected—either forward or reverse—that maintained a minimum distance of 100 base pairs from the targeted SNP. The sizes of the cleaned-up PCR products ranged between 100 bp – 500 bp (**Table 3.1**). Thus, to each cycle sequencing reaction, 5 µL of the cleaned-up PCR product were added to 0.2 ml PCR reaction tubes (NEST Biotechnology, Wuxi, China). This was followed by adding 2 µL of BigDye™ Terminator v3.1 5X sequencing buffer, 2 µL of BigDye™ Terminator 3.1 Ready Reaction Mix from ThermoFisher Scientific (Waltham, Massachusetts, USA) and finally 1 µL of sequencing primer to make a total reaction volume of 10 µL. The reaction mixtures were vortexed on a vortex mixer (Labnet International Inc., Edison, New Jersey, USA) and spun briefly on a microcentrifuge (Whitehead Scientific, (Pty) Ltd., Cape Town, South Africa). The sequencing reaction was carried out on a SimpliAmp™ Thermal Cycler (Applied Biosystems, ThermoFisher Scientific, Life Technologies, Singapore). The sequencing reaction proceeded in two stages which were initial denaturation at 96 °C for 1 minute followed by 25 cycles of further denaturation at 96 °C for 10 seconds, annealing at 50 °C for 5 seconds and elongation at 60 °C for 4 minutes.

3.11.5.3 Post-Sequencing Clean-up and Capillary Electrophoresis

Sequencing products were cleaned up using the EDTA/ethanol precipitation method to remove unincorporated dye labelled terminators so as to obtain a consistent signal during capillary electrophoresis. A volume of 5 µL of 125 mM EDTA was mixed with 20 µL of sequencing products in a 0.2 mL 96-well plate. This was followed by addition of 60 µL of absolute ethanol and vortexing for 5 seconds on a vortex mixer (Labnet International Inc., Edison, New Jersey, USA). The mixture was left on ice in a freezer for 15 minutes, then centrifuged at 1 870 rcf for 45 minutes. The supernatant was discarded, and the plate was allowed to dry by tapping on a paper towel and centrifuging at 180 rcf for 1 minute. A volume of 60 µL of 70% ethanol was added, followed by a further centrifugation step at 1 870 rcf for 15 minutes and thereafter, the supernatant was discarded while tapping dry on a paper towel and centrifuging at 180 rcf as before. The plate was then allowed to airdry in the dark for 15 minutes. A volume of 10 µL of HiDi formamide was added followed by a denaturation step at 95 °C for 5 minutes in a thermal cycler. The plate was allowed to cool for at least 5 minutes before loading on the

SeqStudio® Genetic Analyzer for capillary electrophoresis. For all samples the Z_BigDye™ Terminator v3.1 dye set was selected on the instrument software. Furthermore, depending on the sizes of amplicons, ShortSeq_BDX, MediumSeq_BDX and LongSeq_BDX modules with expected read lengths of up to 350 bp, 500 bp and 800 bp respectively were selected depending on the size of the DNA fragment.

3.12 Statistical Analyses

Before proceeding with any statistical analyses, data quality control was done to ensure that variables with missing data, obvious errors or outliers are identified and dealt with according to appropriate statistical approaches. Data quality control also ensured that participants with the stated inclusion and exclusion criteria are included and excluded as appropriate. All Statistical analyses were performed using various packages in R statistical software (version 4.2.1, 2022-06-23, Vienna, Austria), while SHEsis online software (https://analysis.bio-x.cn/) was used to compute genotype and allele frequencies (Shen et al., 2016). For all analyses, categorical data were presented as N (%) [where N = the number of individuals in that category and % = the frequency of individuals within that category]. Continuous data were presented as mean ± standard deviation (SD) or median ± interquartile range (IQR) [25th – 75th percentile] depending on the distribution of the data.

The Shapiro-Wilks test or Q-Q plots (**Figure A3, Appendix**) were used to assess the data for normality and the Hardy-Weinberg Equilibrium (HWE) was determined for each SNP for Black African and Mixed Ancestry population groups using the chi-square test with one degree of freedom. This was followed by comparing the data between cases and controls. Continuous variables were compared between cases and controls either using the T-test or the Mann-Whitney U-test (Wilcoxon rank-sum test) for normally and non-normally distributed data respectively. Categorical variables were compared between cases and controls using Pearson's chi-square test or Fisher's exact test. The main outcome of interest was resistant hypertension. Therefore, to estimate associations of clinical and genetic variables with resistant hypertension, odds ratios (ORs) and 95% confidence intervals (CI) were used. ORs were calculated according to the formula:

$$\frac{\textbf{Odds of exposure in cases } (\boldsymbol{A/B})}{\textbf{Odds of exposure in controls } (\boldsymbol{C/D})}$$

where, $\boldsymbol{A}$ = exposure in cases, $\boldsymbol{B}$ = non-exposure in case, $\boldsymbol{C}$ = exposure in controls, $\boldsymbol{D}$ = exposure in controls.

Exposure was defined as exposure to any variable (i.e., genetics) potentially influencing the outcome of interest (i.e., resistant hypertension). Thus, OR = 1 indicated no association and that exposure was the same for cases and controls; OR > 1 indicated a positive association and that exposure was higher in cases than controls and OR < 1 indicated a negative association and that exposure is lower in cases than controls. To obtain these estimates, simple logistic regression analysis was used to associate study participant characteristics with resistant hypertension while genetic models of inheritance were used to test for associations between genotypes and resistant hypertension. Further analyses were streamlined to variants presenting with significant ($P<0.05$) or borderline/trending associations with resistant hypertension. Thus, construction of possible haplotypes for SNPs in genes with significant or borderline associations with resistant hypertension was done using SHEsis online software (https://analysis.bio-x.cn/). The frequencies of the resultant haplotypes were also compared between cases and controls using the Pearson's chi-square or Fisher's exact test and associations estimated using ORs and 95% CI as above.

This was followed by multivariable logistic regression including genetic (genotype and haplotypes) and non-genetic variables (clinical or demographic) identified to adjust for any confounding effect. Confounding variables were defined as predictor variables that significantly contributed to the main outcome of interest – resistant hypertension and have been previously reported to play as major risk factors for BP increases or antihypertensive drug response. **Table A1** (**Appendix**) highlights all clinical/demographic variables classified as predictor or outcome variables. No outcome variables were included in the model except the main outcome of interest - resistant hypertension. For all analyses, P-values less than 0.05 ($P < 0.05$) were considered significant.

CHAPTER 4: RESULTS

4.1 Observations from demographic and clinical characteristics of the study participants

The characteristics of the study participants are presented in **Table 4.1**. There were no significant differences in the distribution of age, sex, ethnicity, family history of hypertension, smoking, or alcohol use between cases or controls (P > 0.05) because of the matched case-control design. The median age for cases and controls was 43.8 (20 – 54.2) and 42.2 (26 – 52.6) years respectively, while 54% of cases or controls were female. The predominant ethnicity was Mixed Ancestry making up 69.5% and 72.5% among the cases and controls respectively. Black African participants comprised 30.5% and 27.5% of the cases and controls respectively. There were statistically significantly fewer cases (21.6%) with controlled BP (<140/90 mmHg), versus 64% among controls (P < 0.001; OR: 6.47; CI: 4.10 – 10.20). Left ventricular hypertrophy (LVH) was significantly more common among the cases (resistant hypertension, 47.4%) compared to 27.5% among the controls (P < 0.001; OR: 2.69; CI: 1.64 – 4.41). Comorbidities were more frequent in cases than controls. There were statistically significant differences between cases and controls for comorbidities such as chronic kidney disease (CKD) (P < 0.001; OR: 4.22; CI: 2.02 - 8.78), diabetes mellitus (DM) (P = 0.03; OR: 1.88; CI: 0.90 - 3.78) and dyslipidaemia (P = 0.03; OR: 2.19; CI: 1.14 - 4.22). There was no statistically significant difference for ischemic heart disease (IHD) (P = 0.11; OR: 1.99; CI: 0.86, 4.59) between cases and controls. Furthermore, there was borderline significance between cases and controls who had experienced previous stroke (P = 0.09; OR: 2.70; CI: 0.94 - 7.74). Similarly, there was a higher frequency of use of concomitant drugs. There was statistically significant difference between cases and controls on statins or lipid-lowering therapy (P < 0.0001; OR: 2.45; CI: 1.56 - 3.84). There was borderline significance between cases and controls on diabetic treatment (P = 0.08; OR: 1.88 CI: 0.93 - 3.78). As expected, use of a higher number of antihypertensive drugs was observed in cases, and this was significantly higher than in controls (P < 0.001).

4.2 Genetic characterisation

After successful DNA extraction and quality check on the DNA, genetic characterisation was done. **Figure 4.1** shows the quality of DNA extracted after electrophoresis on a 1% (w/v) agarose gel.

Table 4.1: Distribution of demographic and clinical characteristics between cases and controls and associations with resistant hypertension.

Variable(s)	Cases (N = 190)	Controls (N = 189)	P-Value	OR [95% CI]
Age, years	43.8 (20 - 54.2)	42.2 (26 - 52.6)	0.05	1.01 [0.99 – 1.03]
Sex,				
Male	87 (45.8%)	87 (46.0%)	1	1.00 [0.67 - 1.50]
Female	103 (54.2%)	102 (54.0%)		
Ethnicity,				
Black African	58 (30.5%)	52 (27.5%)	0.57	0.86 [0.55 - 1.35]
Mixed Ancestry	132 (69.5%)	137 (72.5%)		
Smoking (Active/Past),				
Yes	63 (33.2%)	64 (33.9%)	0.99	0.98 [0.65 - 1.52]
No/Unknown	127 (66.8%)	125 (66.1%)		
Alcohol (Active/Past),				
Yes	45 (23.7%)	47 (24.9%)	0.90	0.94 [0.59 - 1.52]
No/Unknown	145 (76.3%)	142 (75.1%)		
Family History of Hypertension,				
Yes	123 (64.7%)	121 (64.0%)	0.91	1.03 [0.68 - 1.57]
No/Unknown	63 (35.3%)	68 (36.0%)		
Blood Pressure (mmHg),				
Uncontrolled (>140/90)	149 (78.4%)	68 (36.0%)	<0.001	6.47 [4.10 - 10.20]
Controlled (<140/90)	41 (21.6%)	121 (64.0%)		
Left Ventricular Hypertrophy,*				
Yes	90 (47.4%)	52 (27.5%)	<0.001	2.69 [1.64 - 4.41]
No/Unknown	100 (52.6%)	189 (72.5%)		
Aldosterone, pmol/l	276 (31 - 435)	200 (2.3 - 313)	0.002	1.001 [1.00 - 1.003]
Comorbidities,				
Chronic Kidney Disease (CKD)*	36 (19.1%)	10 (5.3%)	<0.001	4.22 [2.02 - 8.78]
Diabetes Mellitus (DM)	33 (17.6%)	18 (9.6%)	0.03	1.88 [0.90 - 3.78]
Dyslipidaemia	30 (15.8%)	15 (7.9%)	0.03	2.19 [1.14 - 4.22]
Ischaemic Heart Disease (IHD)	17 (8.9%)	9 (4.8%)	0.11	1.99 [0.86 - 4.59]
Previous Stroke	13 (6.8%)	5 (2.6%)	0.09	2.70 [0.94 - 7.74]
Concomitant Drugs,				
Statin or Lipid-lowering Therapy	76 (40.0%)	45 (23.8%)	<0.001	2.45 [1.56 - 3.84]
Diabetic Treatment*	23 (12.1%)	15 (7.9%)	0.08	1.88 [0.93 - 3.78]
Analgesics	12 (6.0%)	10 (5.2%)	0.69	1.31 [0.55 - 3.11]
Antihypertensives Drugs,				
Amlodipine	172 (90.5%)	116 (61.3%)	<0.001	6.01 [3.41 – 10.6]
Hydrochlorothiazide	148 (77.9%)	108 (57.1%)	<0.001	2.64 [1.69 – 4.14]
Atenolol	123 (64.7%)	27 (14.2%)	<0.001	11.01 [6.65 - 18.24]
Enalapril	150 (78.9%)	88 (46.6%)	<0.001	4.30 [2.74 - 6.76]
Spironolactone	52 (27.3%)	7 (3.7%)	<0.001	9.79 [4.32 - 22.23]
Losartan	39 (20.5%)	14 (7.4%)	<0.001	3.23 [1.69 - 6.17]

OR: Odds Ratio; CI: Confidence Interval, P-value: Significance Level
*CKD indicated by eGFR MDRD > 60 ml/min; Abnormal LVH: females > 95 g/m^2, males > 115 g/m^2
*Diabetic treatment included metformin, insulin or gliclazide

4.3 Genotyping

Twenty variants in 9 genes were successfully genotyped using PCR-RFLP, TaqMan allelic discrimination/copy number assays and successfully validated using Sanger sequencing,

demonstrating the robustness, precision and accuracy of the genotyping methods used in this study for small scale genotyping.

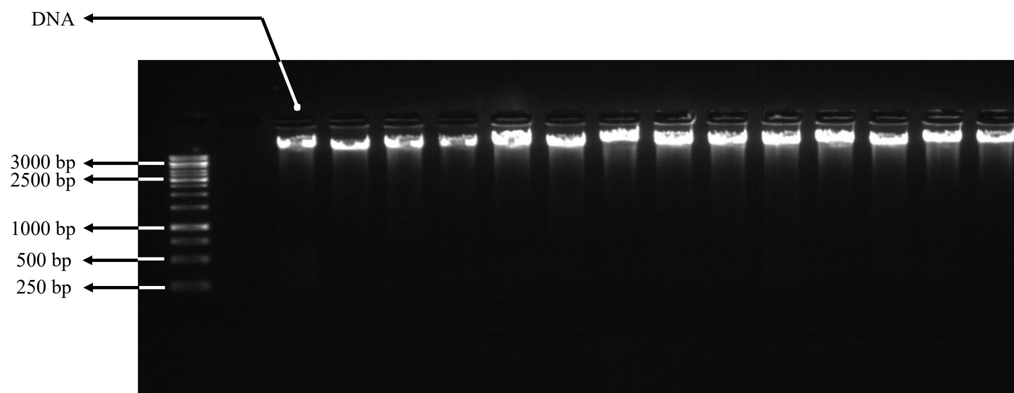

Figure 4.1: Appearance of extracted genomic DNA on 1% (w/v) agarose gel. The DNA appears intact and is of good integrity.

PCR-RFLP yielded DNA fragments corresponding to the genotype of the sample **(Figure 4.2)** while TaqMan allelic discrimination assays yielded plots with at least two or three possible clusters corresponding to the genotype groups of the samples **(Figure 4.3)**. The distributions of most variants across the study were consistent with the Hardy-Weinberg Equilibrium (HWE) for both Black African and Mixed Ancestry population groups (P > 0.05). However, the distributions some variants deviated significantly from the HWE, presenting with P < 0.05 for either Mixed Ancestry for *CYP3A4* rs2740574C>T or both population groups for *ABCB1* rs2032582C>A*, CES1* copy number and *SCNN1B* rs149868979G>A (**Table 4.2**).

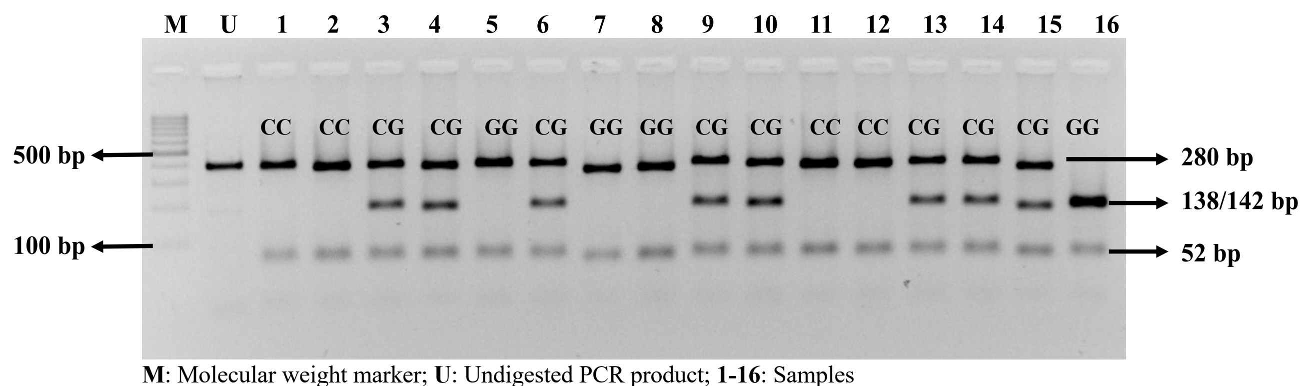

Figure 4.2: An example of PCR-RFLP used to genotype the *ADRB1* rs1801253G>C SNP. PCR-RFLP indicates different DNA band sizes corresponding to the G/G (52bp 138/142 bp), G/C (52bp, 138/142 bp, 280 bp) or C/C (52 bp, 280 bp) genotypes for *ADRB1* rs1801253G>C SNP.

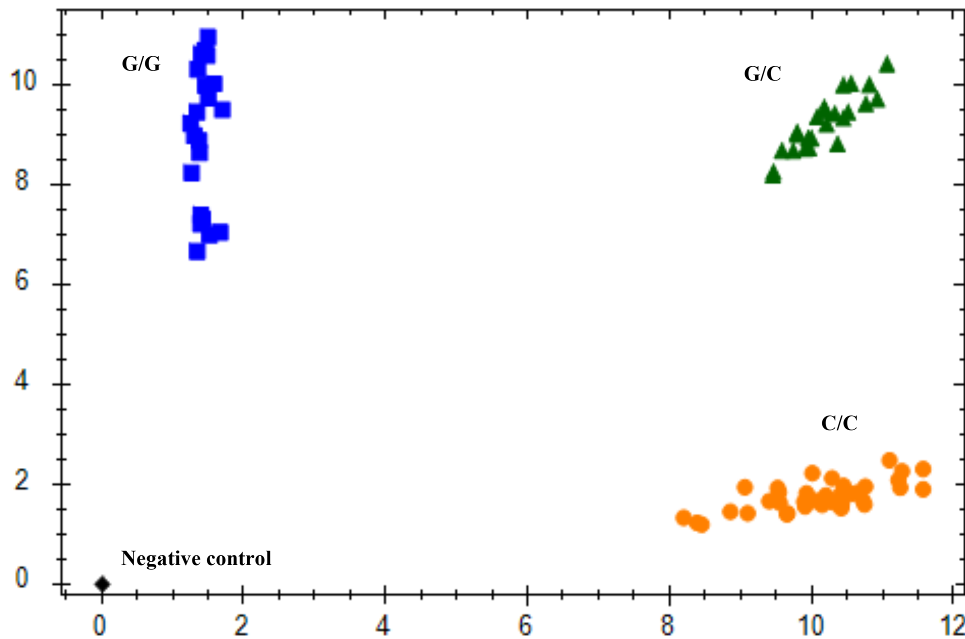

Figure 4.3: An example of a TaqMan allelic discrimination assay used to genotype *NR3C2* rs2070950C>G SNP. The assay results in a plot with 3 different clusters corresponding to three possible genotypes, in this case, G/G (blue), G/C (green) or C/C (orange) for the *NR3C2* rs2070950C>G SNP.

4.4 Observations from genotype and allele frequency distributions of the studied polymorphisms among cases and controls and association with resistant hypertension

The distribution of genotype and allele frequencies among cases or controls are presented in **Tables 4.2**, **4.3** and **4.4**. *CYP3A5* rs776746C allele carriers were statistically significantly more frequent in controls than cases (C/C: 29% controls vs. 18% cases, P = 0.03 and C allele: 49% controls vs. 37% cases, P = 0.02). *CYP3A5* rs776746C allele was significantly associated with reduced odds of resistant hypertension [C/C: OR: 0.54; CI: 0.31 - 0.97 and C allele: OR: 0.62; CI: 0.47 – 0.84).

There was borderline significant difference in the distribution of *NOS3* rs3918188A/C genotypes between cases and controls (P = 0.05). *NOS3* rs3918188A/C genotype was borderline associated with significantly reduced odds of resistant hypertension (OR: 0.65; CI: 0.41 – 1.03). *NOS3* rs3918188A allele carriers were significantly more frequent in the controls than cases (A/A: 17% controls vs. 11% cases, P = 0.02, and A allele: 39% controls vs. 29% cases, P = 0.005). *NOS3* rs3918188A allele was significantly associated with reduced odds of resistant hypertension [A/A: OR: 0.47; CI: 0.23 – 0.92 and A allele: OR: 0.65; CI: 0.48 – 0.88] (**Tables 4.3** and **4.4**).

Table 4.2: Genotype (or copy number) frequency distributions, comparisons between cases (resistant hypertension) or controls (no resistant hypertension) and associations with resistant hypertension for genes involved in the metabolism or pharmacokinetics of antihypertensive drugs

Variant	Genotype/ CNV	Genotype Frequencies		P-Value	OR [95% CI]	Hardy-Weinberg Equilibrium	
		Cases (freq)	Controls (freq)			Black African	Mixed Ancestry
ABCB1 rs1045642 (c.3435C>T)	C/C	99 (0.52)	92 (0.49)	1			
	C/T	70 (0.37)	72 (0.38)	0.66	0.90 [0.57 - 1.42]		
	T/T	21 (0.11)	25 (0.13)	0.51	0.78 [0.39 - 1.57]	0.61	0.19
ABCB1 rs2032582 (c.2677C>A)	C/C	112 (0.60)	107 (0.58)	1			
	A/C	54 (0.29)	55 (0.30)	0.82	0.93 [0.58 - 1.52]		
	A/A	22 (0.11)	24 (0.13)	0.74	0.88 [0.44 - 1.74]	0.01	0.01
CES1 rs2244613 (1168-33G>T)	T/T	101 (0.53)	111 (0.59)	1			
	G/T	74 (0.39)	65 (0.34)	0.33	1.25 [0.80 – 1.97]		
	G/G	15 (0.08)	13 (0.07)	0.68	1.27 [0.53 – 3.04]	0.87	0.69
CES1 Copy Number	2	132 (0.69)	129 (0.68)	1			
	3	48 (0.25)	54 (0.29)	0.56	0.86 [0.53 – 1.41]		
	4	3 (0.02)	6 (0.03)	0.50	0.48 [0.07 – 2.35]	<0.01	<0.01
CYP3A4 rs2740574 (c.-392C>T)	T/T	59 (0.32)	77 (0.41)	1			
	C/T	74 (0.40)	58 (0.31)	0.05	1.30 [0.78 – 2.17]		
	C/C	52 (0.28)	53 (0.28)	0.36	0.78 [0.47 – 1.30]	0.15	0.01
CYP3A4 rs2246709 (c.16090A>G)	A/A	75 (0.40)	63 (0.34)	1			
	A/G	92 (0.48)	101 (0.54)	0.23	0.77 [0.49 – 1.19]		
	G/G	23 (0.12)	23 (0.12)	0.61	0.84 [0.43 – 1.64]	0.37	0.09
CYP3A5 rs776746 (c.219-237T>C)	T/T	78 (0.41)	66 (0.35)	1			
	C/T	77 (0.41)	69 (0.37)	0.82	0.94 [0.58 – 1.54]		
	C/C	35 (0.18)	54 (0.29)	**0.03**	**0.54 [0.31 – 0.97]**	0.13	0.05
CYP3A5 rs10264272 (c.624C>T)	C/C	141 (0.74)	144 (0.76)	1			
	C/T	45 (0.24)	41 (0.22)	0.71	1.12 [0.67 – 1.87]		
	T/T	4 (0.02)	4 (0.02)	0.99	1.02 [0.18 – 5.60]	0.77	0.43
CYP3A5 rs41303343 (insT)	A/A	158 (0.83)	164 (0.87)	1			
	A/T	30 (0.16)	22 (0.12)	0.29	1.41 [0.75 – 2.69]		
	T/T	1 (0.01)	2 (0.01)	0.99	0.51 [0.01 – 10.08]	0.79	0.44

freq: frequency; OR: Odds ratio; CI: Confidence interval; P-Value: Level of significance

Table 4.3: Genotype frequency distributions, comparisons between cases (resistant hypertension) or controls (no resistant hypertension) and associations with resistant hypertension for genes involved in the pharmacodynamics of antihypertensive drugs

Variant	Genotype	Frequencies		P-Value	OR [95% CI]	Hardy-Weinberg Equilibrium	
		Cases (freq)	Controls (freq)			Black African	Mixed Ancestry
ADRB1	A/A	92 (0.48)	107 (0.57)	1			
rs1801252 (c.145A>G)	A/G	79 (0.42)	70 (0.37)	0.23	1.31 [0.84 - 2.05]		
	G/G	19 (0.10)	12 (0.06)	0.13	1.83 [0.80 - 4.39]	0.81	0.80
ADRB1	C/C	100 (0.53)	95 (0.50)	1			
rs1801253 (c.1165G>C)	C/G	74 (0.39)	79 (0.42)	0.67	0.89 [0.57 – 1.39]		
	G/G	16 (0.08)	15 (0.08)	0.98	1.01 [0.44 – 2.34]	0.50	0.69
NOS3	G/G	135 (0.71)	141 (0.75)	1			
rs1799983 (c.894T>G)	G/T	46 (0.24)	45 (0.24)	0.81	1.07 [0.65 – 1.76]		
	T/T	9 (0.05)	3 (0.02)	0.14	3.12 [0.75 – 18.3]	0.37	0.54
NOS3	T/T	123 (0.65)	122 (0.65)	1			
rs2070744 (c.-51-762C>T)	C/T	56 (0.30)	60 (0.32)	0.74	0.92 [0.58 – 1.48]		
	C/C	10 (0.05)	7 (0.03)	0.61	1.42 [0.47 – 4.53]	0.14	0.84
NOS3	C/C	100 (0.53)	75 (0.40)	1			
rs3918188 (c.1753-734C>A)	A/C	70 (0.37)	81 (0.43)	0.05	0.65 [0.41 – 1.03]		
	A/A	20 (0.11)	32 (0.17)	**0.02**	**0.47 [0.23 – 0.92]**	0.11	0.21
NEDD4L	G/G	59 (0.31)	75 (0.40)	1			
rs4149601 (c.49-16229G>A)	A/G	95 (0.50)	84 (0.45)	0.14	1.43 [0.89 – 2.31]		
	A/A	36 (0.19)	28 (0.15)	0.13	1.63 [0.86 – 3.11]	0.45	0.55
NEDD4L	G/G	52 (0.28)	48 (0.25)	1			
rs292449 (c.-300G>C)	C/G	89 (0.47)	101 (0.53)	0.25	0.73 [0.42 – 1.26]		
	C/C	48 (0.25)	40 (0.21)	0.77	0.90 [0.49 – 1.67]	0.97	0.90
NEDD4L	A/A	162 (0.85)	164 (0.87)	1			
rs75982813 (g.58043776A>G)	A/G	25 (0.13)	28 (0.15)	0.77	1.13 [0.61 – 2.12]	0.18	0.37
NR3C2	T/T	145 (0.76)	141 (0.75)	1			
rs5522 (c.538G>A)	C/T	45 (0.24)	45 (0.24)	0.99	0.97 [0.59 – 1.61]		
	C/C	0 (0)	1 (0.01)	0.49	0 [0 – 38.2]	0.35	0.04
NR3C2	G/G	88 (0.47)	83 (0.44)	1			
rs2070950 (c.-2-358C>G)	C/G	71 (0.38)	74 (0.39)	0.73	0.91 [0.57 – 1.44]	0.72	0.03
	C/C	30 (0.16)	32 (0.17)	0.77	0.88 [0.47 – 1.65]		
SCNN1B	G/G	180 (0.95)	174 (0.92)	1			
rs149868979 (c.1688G>A)	A/G	8 (0.04)	12 (0.06)	0.37	0.64 [0.22 – 1.76]		
	A/A	2 (0.01)	3 (0.02)	0.68	0.65 [0.05 – 5.70]	0.008	0.01

Table 4.4: Variant Allele Frequency Distributions, Comparisons between Cases (RHTN) or Controls (No RHTN) and associations with resistant hypertension

Gene	SNP	Variant Allele	Frequencies		P-Value	OR [95% CI]
			Cases (freq)	Controls (freq)		
ABCB1	rs1045642 (c.3435C>T)	T	112 (0.30)	122 (0.32)	0.40	1.14 [0.83 – 1.55]
	rs2032582 (c.2677C>A)	A	98 (0.26)	103 (0.28)	0.61	0.92 [0.67 – 1.27]
ADRB1	rs1801252 (c.145A>G)	G	117 (0.31)	99 (0.25)	0.06	1.46 [0.97 – 2.19]
	rs1801253 (c.1165G>C)	G	106 (0.28)	109 (0.29)	0.77	1.04 [0.76 – 1.44]
CES1	rs2244613 (1168-33G>T)	G	104 (0.27)	91 (0.24)	0.29	1.18 [0.86 – 1.65]
CYP3A4	rs2740574 (c.-392C>T)	C	178 (0.48)	164 (0.44)	0.21	1.19 [0.89 - 1.59]
	rs2246709 (c.16090A>G)	G	147 (0.39)	138 (0.36)	0.39	1.14 [0.84 -1.52]
CYP3A5	rs776746 (c.219-237T>C)	C	142 (0.37)	184 (0.49)	**0.02**	**0.62 [0.47 – 0.84]**
	rs10264272 (c.624C>T)	T	48 (0.13)	54 (0.14)	0.50	1.15 [0.76 – 1.75]
	rs41303343 (insT)	T	32 (0.08)	25 (0.07)	0.37	0.78 [0.45 – 1.34]
NOS3	rs1799983 (c.894G>T)	T	64 (0.17)	51 (0.14)	0.19	0.77 [0.52 – 1.15]
	rs2070744 (c.-51-762C>T)	T	76 (0.20)	74 (0.20)	0.84	1.03 [0.73 – 1.48]
	rs3918188 (c.1753-734C>A)	A	110 (0.29)	145 (0.39)	**0.005**	**0.65 [0.48 – 0.88]**
NEDD4L	rs4149601 (c.49-16229G>A)	A	167 (0.44)	140 (0.37)	0.07	1.31 [0.98 – 1.75]
	rs292449 (c.-300G>C)	G	185 (0.48)	181 (0.49)	0.77	0.95 [0.72 – 1.28]
	rs75982813 (g.58043776A>G)	G	28 (0.07)	25 (0.06)	0.68	0.89 [0.51 – 1.56]
NR3C2	rs5522 (c.538C>T)	C	45 (0.12)	47 (0.13)	0.76	0.93 [0.60 – 1.45]
	rs2070950 (c.-2-358C>G)	C	131 (0.35)	138 (0.37)	0.48	0.92 [0.68 – 1.24]
SCNN1B	rs149868979 (c.1688G>A)	A	18 (0.05)	12 (0.03)	0.25	0.65 [0.31 – 1.37]

freq: frequency; OR: Odds ratio; CI: Confidence interval; P-Value: Level of significance

There was a borderline significant difference (P = 0.06) in the distribution of *ADRB1* rs1801252G allele carriers between cases (31%) and controls (25%). *ADRB1* rs1801252G allele was borderline associated with increased odds of resistant hypertension (OR: 1.46; CI: 0.97 – 2.19). Similarly, there was borderline significant difference (P = 0.07) in the distribution of *NEDD4L* rs4149601A allele carriers between cases (44%) and controls (37%). *NEDD4L* rs4149601A allele was borderline associated with increased odds of resistant hypertension (OR: 1.31; CI: 0.98 – 1.75) (**Table 4.4**).

4.5 Observations from *CES1* Copy Number Variation between cases and controls and associations with resistant hypertension

Figure 4.4 shows the output from analysis of *CES1* copy number on CopyCaller software for representative samples. The number of copies of the *CES1* gene ranged from 2 - 4. The distribution of *CES1* copy numbers between cases and controls is shown in **Figure 4.5.** There were no significant differences in the distributions of each copy number between cases and controls (P > 0.05). *CES1* copy numbers were further categorized into copy number neutral (=2) and copy number gain (>2) as according to Chen et al., (2021) (**Figure 4.6**). There were no significant differences in copy number neutral/gain between cases and controls. No significant associations were observed between copy number gain and resistant hypertension (OR: 0.83; CI: 0.53 – 1.30) (**Table A4; Appendix**).

4.6 Assessing contribution of haplotypes constructed from SNPs in *ADRB1, CYP3A5, NEDD4L* and *NOS3* genes on distinguishing cases from controls

The frequency distributions in cases or controls and associations with resistant hypertension of possible haplotypes constructed from the studied SNPs, namely *ADRB1* rs1801252–rs1801253*; CYP3A5* rs776746–rs10264272–rs4130334*; NEDD4L* rs4149601–rs292449 and *NOS3* rs1799983–rs2070744–rs3918188 are shown in **Table 4.5**. *CYP3A5* rs776746–rs10264272–rs4130334C–C–A haplotype carriers were significantly (P = 0.03) more frequent in controls (46%) than cases (38%) and were significantly associated with reduced odds of resistant hypertension (OR: 0.73; CI: 0.54 - 0.97). *NOS3* rs1799983–rs2070744–rs3918188G–T–A haplotype carriers were significantly (P = 0.004) more frequent in controls (37%) than cases (27%) and were significantly associated with reduced odds of resistant hypertension (OR: 0.63; CI: 0.47 - 0.86).

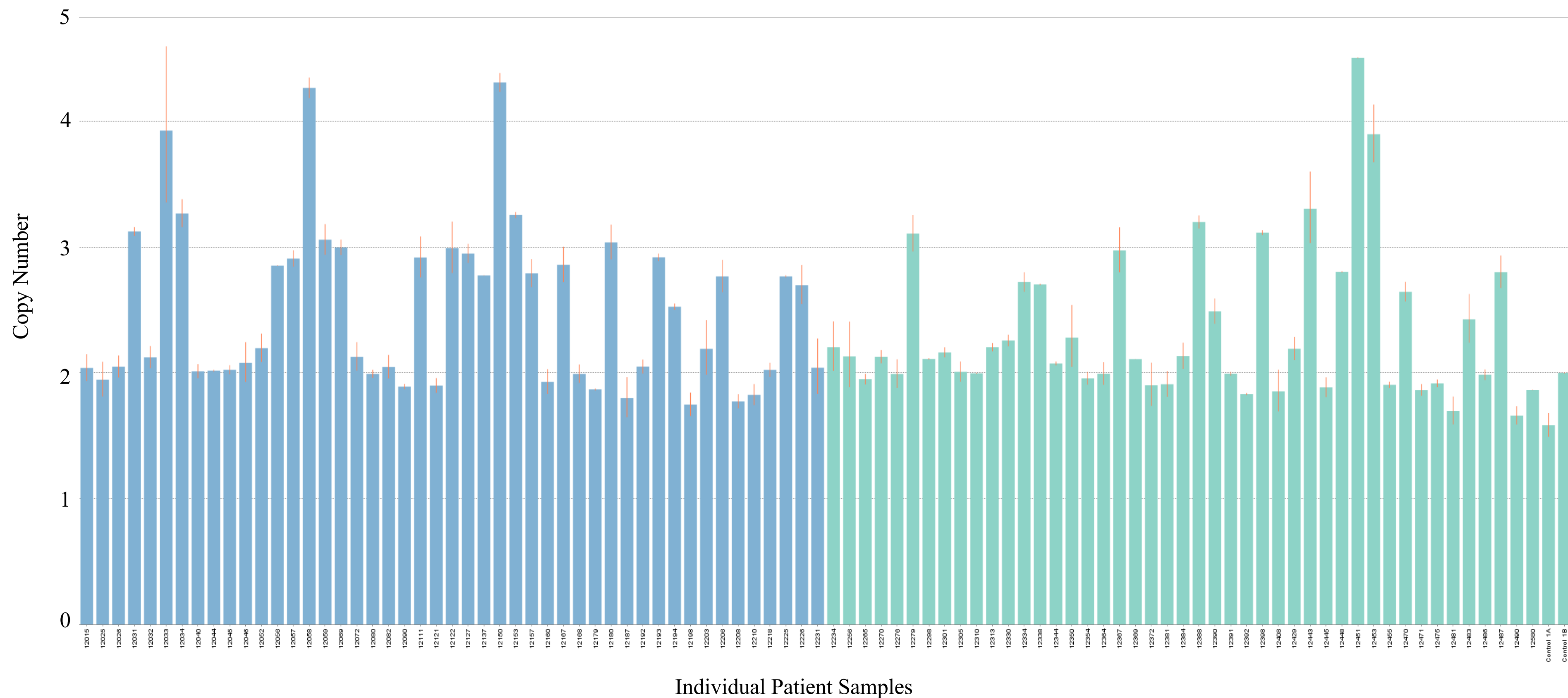

Figure 4.4: Representative output from CopyCaller software after calculation of copy number calls for each sample according to software algorithm. Copy number calls for each sample ranges from 2 - 4 with confidence scores >0.50.

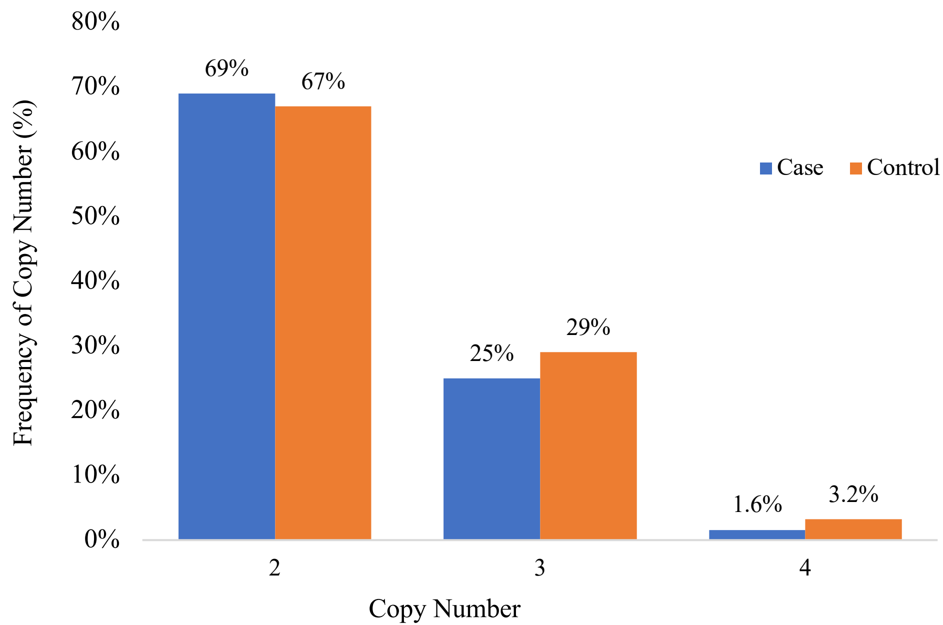

Figure 4.5: The distribution and comparison of *CES1* copy numbers between cases (resistant hypertension) and controls (no resistant hypertension). No significant differences were observed (Copy number 2: P = 0.99, copy number 3: P = 0.56, copy number 4: P = 0.50).

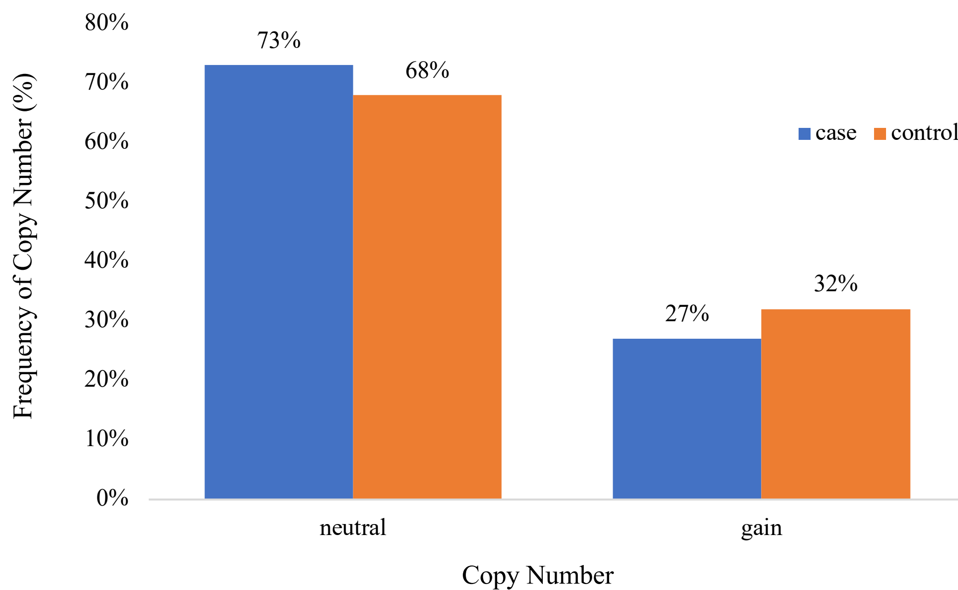

Figure 4.6: The distribution and comparison of *CES1* copy number neutral/gains between cases (resistant hypertension) and controls (no resistant hypertension). No significant differences between cases and controls or associations with resistant hypertension were observed.

There was borderline significance (P = 0.06) in the distribution of *ADRB1* rs1801252–rs1801253G–C haplotype carriers between cases (31%) and controls (25%). *ADRB1*

rs1801252–rs1801253G–C haplotype was borderline associated with increased odds of resistant hypertension (OR: 1.34; CI: 0.98 – 1.85). There was borderline significance (P = 0.07) in the distribution of *NEDD4L* rs4149601–rs292449A–C haplotype carriers between cases (25%) and controls (19%). *NEDD4L* rs4149601–rs292449A–C haplotype was borderline associated with increased odds of resistant hypertension (OR: 1.37; CI: 0.97 – 1.94) (**Table 4.5**).

Table 4.5: Haplotype frequencies and associations with resistant hypertension

Variants	Haplotype	Cases	Controls	P-Value	OR [95% CI]
ADRB1 rs1801252–rs1801253	A-C	157 (0.41)	175 (0.46)	0.17	0.82 [0.61 - 1.09]
	A-G	106 (0.28)	109 (0.29)	0.77	0.96 [0.69 - 1.31]
	G-C	117 (0.31)	94 (0.25)	0.06	1.34 [0.98 - 1.85]
CYP3A5 rs776746–rs10264272–rs41303343	C-C-A	144 (0.38)	172 (0.46)	**0.03**	**0.73 [0.54 - 0.97]**
	T-C-A	150 (0.40)	130 (0.35)	0.14	1.25 [0.93 - 1.68]
	T-C-T	30 (0.08)	25 (0.07)	0.43	1.25 [0.72~2.16]
	T-T-A	50 (0.13)	46 (0.12)	0.65	1.10 [0.72 - 1.70]
NEDD4L rs4149601–rs292449	A-C	93 (0.25)	72 (0.19)	0.07	1.37 [0.97 - 1.94]
	A-G	73 (0.19)	68 (0.18)	0.70	1.07 [0.75 - 1.55]
	G-C	100 (0.26)	123 (0.33)	0.05	0.73 [0.54 - 1.00]
	G-G	112 (0.30)	111 (0.30)	0.99	0.99 [0.73 - 1.37]
NOS3 rs1799983–rs2070744–rs3918188	G-C-C	46 (0.12)	45 (0.12)	0.970	1.01 [0.65 - 1.57]
	G-T-A	103 (0.27)	140 (0.37)	**0.004**	**0.63 [0.47 - 0.86]**
	G-T-C	159 (0.42)	135 (0.36)	0.060	1.32 [0.98 - 1.77]
	T-C-C	25 (0.07)	23 (0.06)	0.800	1.08 [0.60 - 1.92]
	T-T-C	38 (0.10)	27 (0.07)	0.180	1.42 [0.85 - 2.37]

OR: adjusted odds ratio; CI: confidence interval

4.6 Observations from association of genotypes or haplotypes with resistant hypertension after adjustment for confounding variables

Aldosterone levels, dyslipidaemia, diabetes mellitus and use of statins or lipid lowering therapy were identified as predictor variables potentially confounding the main outcome – resistant hypertension, based on their significant associations with resistant hypertension (P < 0.05) at univariate analysis (**Table 4.1**) and prior evidence from literature as major risk factors (Jones et al., 2020; Seedat et al., 2014). Associations of genotypes or haplotypes in *ADRB1, CYP3A5,*

NEDD4L and *NOS3* polymorphisms with resistant hypertension after adjusting for confounding variables are presented in **Table 4.6**.

It was observed that the *ADRB1* rs1801252G/G (P = 0.02; OR: 3.30; CI: 1.17 – 10.03) and *NEDD4L* rs4149601A/A (P = 0.001; OR: 3.82 CI: 1.67 - 9.07) genotypes were significantly associated with increased odds of resistant hypertension. Furthermore, the *ADRB1* rs1801252–rs1801253G–C haplotype was significantly associated with increased odds of resistant hypertension (P = 0.04; OR: 2.83; CI: 1.05 – 8.20). *NEDD4L* rs4149601–rs292449A–C haplotype was borderline associated with increased odds of resistant hypertension (P = 0.08; OR: 3.14; CI: 0.88 – 12.9).

Table 4.6: Multivariable logistic regression analysis adjusting for confounding variables

SNP/SNP Combination	Genotype/Haplotype	Coefficient	P-Value	OR [95% CI][a]
ADRB1 rs1801252	GG	1.19	**0.02**	**3.30 [1.17 – 10.03][b]**
ADRB1 rs1801252 – rs1801253	G-C	1.04	**0.04**	**2.83 [1.05 – 8.20][b]**
CYP3A5 rs776746	CC	-0.80	**0.02**	**0.44 [0.22 - 0.89][b]**
CYP3A5 rs776746 - rs1026427 - rs41303343	C-C-A	-0.14	0.59	0.86 [0.49 – 1.50]
NEDD4L rs4149601	AA	1.34	**0.001**	**3.82 [1.67 - 9.07][b]**
NEDD4L rs4149601 – rs292449	A-C	1.14	0.08	3.14 [0.88 – 12.9]
NOS3 rs3918188	AA	-1.53	**0.0003**	**0.21 [0.08 - 0.49][b]**
NOS3 rs1799983 - rs2070744 - rs3918188	G-T-A	-0.75	**0.006**	**0.47 [0.27 – 0.80][b]**

OR: adjusted odds ratio; CI: confidence interval; P-Value: significance level
[a] ORs for genotypes and haplotypes were adjusted for aldosterone levels, dyslipidaemia, diabetes mellitus and use of statins or lipid lowering therapy
[b] Statistically significant associations with resistant hypertension

CYP3A5 rs776746C/C genotype was significantly associated with increased odds of resistant hypertension [P = 0.02; OR: 0.44; CI: 0.22 – 0.89] while no significant association was observed for the *CYP3A5* rs776746–rs10264272–rs41303343C–C–A haplotype [P = 0.59; OR: 0.86; CI: 0.49 – 1.50]. *NOS3* rs3918188A/A genotype (P = 0.0003; OR: 0.21; CI: 0.08 - 0.49) and *NOS3* rs1799983–rs2070744–rs3918188G–T–A haplotype (P = 0.006; OR: 0.47 CI: 0.27 - 0.80) were associated with significantly reduced odds of resistant hypertension.

4.7 Variant Allele Frequency Distributions of the Studied Polymorphisms and Comparisons with other World Populations

The variant allele frequencies obtained for the studied polymorphisms are presented in **Table 4.7**. Briefly, variant allele frequencies obtained are comparable to other African populations and there are variable differences when compared to non-African populations. However, for the *CES1* rs2244613C>A*, CES1* copy number variation*, NOS3* rs1799983T>A*, NOS3* rs3918188C>A*, NOS3* rs2070744C>T, *NR3C2* rs5522G>A, rs2070950C>G*,* and *NEDD4L* rs75982813A>G*,* there are limited studies that have reported on the distribution of the polymorphisms among Africans in Southern Africa. Most notably, the frequency of *CES1* copy number gain (>2), has not been reported before for African populations and exists in frequencies of up to 17% in Black Africans and 34% among the Mixed Ancestry, respectively, in this Southern African study cohort. In Europeans and East Asians, studies have reported frequencies of 19% and 26%, respectively.

Table 4.7: Variant allele frequency distributions across different population groups

Gene	SNP	Variant Allele	Variant allele Frequencies (freq)							
			This Study		Bantu (Southern Africa) (N=503) **	Luhya (Kenya) (N=198) *	Yoruba (Nigeria) (N=216) *	European (N=1006) *	East Asian (N=1008) *	South Asian (N=978) *
			Black African (N=110)	Mixed Ancestry (N=269)						
ABCB1	rs1045642	T	0.15	0.38	0.12 [a] / 0.40 [b]	0.14	0.12	0.52	0.40	0.57
	rs2032582	A	0.05	0.36	-	0.24	0.30	0.16	0.60	0.39
ADRB1	rs1801252	G	0.33	0.26	-	0.30	0.21	0.13	0.14	0.13
	rs1801253	G	0.33	0.27	-	0.31	0.48	0.32	0.21	0.27
CES1	rs2244613	G	0.20	0.29	-	0.24	0.30	0.16	0.60	0.39
	Copy Number	*>2*	0.17	0.34	-	-	-	0.19 [f]	0.26 [f]	-
CYP3A5	rs776746	G	0.19	0.53	0.15 [a] / 0.58 [b]	0.12	0.17	0.94	0.71	0.67
	rs10264272	A	0.18	0.12	0.05 [a] / 0.24 [b]	0.24	0.17	0	0	0
	rs41303343	T	0.15	0.05	0.14 [a] / 0.04 [b]	0.12	0.12	0	0	0
NOS3	rs1799983	G	0.06	0.19	-	0.04	0.06	0.34	0.13	0.17
	rs2070744	C	0.12	0.23	0.15 [d]	0.14	0.12	0.44	0.12	0.25
	rs3918188	A	0.39	0.31	-	0.33	0.39	0.31	0.29	0.32
NR3C2	rs5522	C	0.14	0.11	-	0.10	0.08	0.08	0.15	0.10
	rs2070950	C	0.19	0.43	-	0.10	0.19	0.49	0.76	0.68
NEDD4L	rs4149601	A	0.51	0.36	0.52 [d]	0.50	0.38	0.35	0.21	0.20
	rs292449	G	0.52	0.49	0.49 [d]	0.47	0.45	0.66	0.17	0.59
	rs75982813	G	0.11	0.05	-	0.09	0.08	0.05	0	0.03
SCNN1B	rs149868979	A	0.04	0.04	0.06 [e]	-	-	-	-	-

*Variant allele frequencies obtained from the 1 000 genomes Project Phase 3 (*https://www.ensembl.org/index.html, last accessed 09/09/2023*). **Variant allele frequencies from previous published studies: [a] Black Africans from South Africa and Zimbabwe (Muyambo et al., 2022), [b] Mixed Ancestry from South Africa (Muyambo et al., 2022), [c] Black Africans from Zimbabwe (Mbavha et al., 2022), [d] Black Africans from South Africa (Masilela et al., 2020), [e] Black Africans from South Africa (Jones et al., 2012), [f] East Asians represented by Chinese Han (Chen et al., 2021) and Europeans by Danish (Nelveg-Kristensen et al., 2016).

CHAPTER 5: DISCUSSION

5.1 Characteristics of the Study Participants

Demographic variables including age, sex and ethnicity among the participants recruited into the study were found to be similar in both cases and controls. This is mostly likely as a result of matching as the study participants were matched according to these characteristics in a ratio of 1:1. However, overall, most participants in either group, cases or controls were female and were of Mixed Ancestry. This trend was similar to previous studies done in South Africa that show that a higher proportion of females are more likely to be recruited compared to males. Although more females have been shown to present with hypertension in several studies in some settings (Kohli-Lynch et al., 2022; Peer et al., 2021; Pender & Omole, 2019; Reddy et al., 2021), it can be argued that it has more to do with the frequency of clinic visits as other studies have shown that females attend clinic more regularly than males (Abera et al., 2017; Peer et al., 2021; Sikka et al., 2021). The higher proportion of Mixed Ancestry individuals is in keeping with Statistics South Africa reports, which show that the ethnic composition of Cape Town is 42.6% Mixed Ancestry and 38.6% Black African (StatsSA, 2022). The study participants were sampled at a Tertiary-level Hypertension clinic at Groote Schuur Hospital, Cape Town, where the surrounding neighboured is densely populated by individuals of Mixed Ancestry. This could be a possible explanation to this observation. We also speculate that there could be differences in health seeking behaviours in the two groups. However, there is no strong evidence supporting this (Christian et al., 2019; Edwards, 2016).

Clinical and biochemical variables for the study participants seemed to vary significantly between cases and controls (**Table 4.1**). More cases had elevated BP (>140/90 mmHg). This implies that a proportion of participants in this study with resistant hypertension had uncontrolled resistant hypertension that could not be controlled even on multiple BP lowering drugs (Dudenbostel et al., 2016). On the other hand, a proportion of the controls (36%) had uncontrolled BP (>140/90 mmHg) and were classified as having simple uncontrolled BP on less than 3 antihypertensive drugs since they had no other additional indications of resistant hypertension at the time of recruitment.

Resistant hypertension has been reported to be a major risk factor for adverse outcomes including several cardiovascular diseases, stroke, or hypertension mediated organ damage (Carey et al., 2018) and this underscores the importance of effectively managing BP. In this study, more cases (those with resistant hypertension) than controls were found to have developed more hypertension mediated organ damage as evidenced by the presence of chronic kidney disease (CKD) and left ventricular hypertrophy (LVH). Since patients with secondary hypertension were excluded at recruitment, it is likely that resistant hypertension caused hypertension mediated organ damage. CKD develops over time due to consistently high BP - which is the case in patients with resistant hypertension, particularly uncontrolled resistant hypertension (Song et al., 2021).

Similarly, when BP is consistently high, the heart consistently works harder to pump blood against the increased peripheral resistance in the blood vessels resulting in LVH (Delanaye et al., 2016). LVH is a common occurrence in patients with resistant hypertension and is usually a marker for the occurrence of other cardiovascular disease (CVD) complications such as ischaemic heart disease, coronary artery disease, heart failure and in some cases stroke. In line with previous findings, it was observed that more cases in this study, had been diagnosed with comorbidities such as ischaemic heart disease, and had previously experienced stroke, although the association with resistant hypertension was not significant.

On the other hand, other comorbidities including diabetes mellitus (DM) and dyslipidaemia have been previously shown to be risk factors for resistant hypertension (Romano et al., 2018). DM and dyslipidaemia were positively associated with resistant hypertension in this study. DM and dyslipidaemia often co-exist with resistant hypertension. Hypotheses for this include insulin resistance, which causes increased oxidative stress; impaired lipid metabolism or endothelial damage (Petrie et al., 2018). The underlying aetiology and physiological link of these conditions is not enough to account for the associations with resistance to antihypertensive therapy observed. However, it also includes the use of drugs managing each of these conditions, which were found to be more frequent in the cases in this instance. For example, statins - for dyslipidaemia and insulin or metformin – for diabetes. Hypertensive patients on statins or diabetic treatment, have been shown to require more rigorous treatment with antihypertensive drugs. It has been reported that in addition to diabetic treatment, a combination of ACEI/ARB plus CCB plus diuretic is normally required to achieve control (Ansari et al., 2023; Shaikh, 2017).

Similarly, aldosterone biochemical measurements were higher in cases than controls. According to Judd et al., (2014), aldosterone excess is a common feature in individuals with resistant hypertension and has been shown to increase expression of sodium chloride co-transporters in the kidney (Judd et al., 2014; Kim et al., 1998). Therefore, it may be one of the reasons why individuals with resistant hypertension are on multiple antihypertensive drugs. For example, when aldosterone is high, it has been shown that combination of thiazide diuretics and MRAs offers better prognostic outcomes while alleviating occurrence of adverse effects such as hypokalaemia (Guyton et al., 1967; Raheja et al., 2012).

The burden of antihypertensive drug use or combinations were found to be higher for cases than controls. By definition, cases were on multiple drugs or combinations thereof. This is reflective of the typical management of hypertension according to South African guidelines (Jones et al., 2020; Seedat et al., 2014). In addition to conventional treatment, spironolactone is mostly indicated in patients with resistant hypertension (Guerrero-García & Rubio-Guerra, 2018), and has been reported to be one of the most effective additional drugs for treating resistant hypertension (Acelajado et al., 2019). Hence, more cases in this study were on this drug than controls.

5.2 Genetic characterisation

Some of the observed deviations from HWE for some of the SNPs could have been due to non-random sampling as only patients with hypertension were preferentially recruited in the study. In addition, departure from HWE observed, especially for the Mixed Ancestry population group for some SNPs may be due to population admixture (Crow, 2017; Deng et al., 2001). According to Deng et al., (2001), population admixture results in new genetic combinations, and factors such as migration, selection, and differential mating patterns within the admixed population can lead to changes in allele frequencies over time. The Mixed Ancestry population group in this study consists of people resulting from intermarriages between/among African, European and Asian populations (Daya et al., 2013).

5.3 Associations of Genotypes and Haplotypes with Resistant Hypertension

CYP3A5 rs776746C allele was associated with reduced odds of resistant hypertension and appears to be protective. According to Lee et al., (2013), *CYP3A5* rs776746T>C nucleotide

change in intron 3 causes a disruption of the translational reading frame and skipping of exon 9 leading to a premature stop codon and production of a non-functional, truncated protein. At biochemical level, the enzyme produced lacks key functional domains essential for its structure and catalytic activity (Kuehl et al., 2001). Thus, *CYP3A5* rs776746C/C genotype carriers are non-expressors while CYP3A5 rs776746T/T genotype carriers express higher levels of CYP3A5 (Thervet et al., 2003). CYP3A5 partially metabolises and clears mainly calcium channel blockers such as amlodipine, some ACE inhibitors such as enalapril, some mineralocorticoid receptor antagonists such as spironolactone and thus, in non-expressors, exposure to the BP lowering effect of CYP3A5 substrate drugs is increased as there is reduced metabolic clearance. Therefore, it is possible that *CYP3A5* rs776746C allele carriers, although resulting in a non-functional enzyme, may derive the greatest benefit from antihypertensive drugs which are CYP3A5 substrates. Furthermore, carriage of the *CYP3A5* rs776746C allele has been shown to be associated with a reduced clearance of drugs and increased exposure to drugs metabolised by CYP3A5 in several pharmacokinetic studies (Fröhlich et al., 2004; Haufroid et al., 2004; Mouly et al., 2005; Thervet et al., 2003) further supporting the findings from this study. However, in a study by Kim et al., (2006), they reported an opposite effect. Clearance of amlodipine among carriers of the *CYP3A5* rs776746C allele was increased among Korean participants, while clearance was lower in *CYP3A5* rs776746T allele carriers (Kim et al., 2006).

CYP3A4 and CYP3A5 have broad overlapping substrate specificities and according to Zhu et al., (2014), CYP3A4 contributes the greatest in the metabolic clearance of amlodipine compared to CYP3A5 (Zhu et al., 2014). However, in this study, there were no associations between the studied *CYP3A4* polymorphisms and resistant hypertension to imply that the polymorphisms could affect the clearance of amlodipine. This is in contrast to a study by Bhatnagar reporting that *CYP3A4* rs2740574C>T and rs2246709A>G polymorphisms are associated with BP response to amlodipine in African Americans (Bhatnagar et al., 2010) and not *CYP3A5* rs776746T>C.

The *NOS3* rs3918188A allele was found to be protective and associated with reduced odds of resistant hypertension in this study. NOS3 plays a role in the production of nitric oxide (NO), a vasodilator and polymorphisms in *NOS3* have been widely reported to influence response drugs acting on the cardiovascular system such as enalapril (Silva et al., 2013a). While the impact of *NOS3* rs3918188C>A on NOS3 function is unknown, it has been reported to be in

high linkage disequilibrium (LD) with other tagSNPs, including rs3918226C>T and rs743506G>A (Oliveira-Paula et al., 2016). Previous studies (Gamil et al., 2017; Oliveira-Paula et al., 2016) have also reported *NOS3* rs3918188C>A to be in high LD with clinically relevant *NOS3* polymorphisms rs2070744C>T and rs1799983G>T, both studied here. The NOS3 rs1799983G>T–rs2070744C>T–rs3918188C>A*;* G–T–A haplotype, formed by these loci, showed a significant association with reduced odds of resistant hypertension in this study. Thus, it is possible that the allelic combination at these loci may be driving the observed association.

In this study, the *ADRB1* rs1801252G/G genotype and *ADRB1* rs1801252–1801253G–C haplotype was identified as associated with an increased likelihood of resistant hypertension, implying that carriers of these variants have poor response to antihypertensive therapy. *ADRB1* rs1801252A>G and *ADRB1* rs1801253G>C polymorphisms have been reported alter G-protein coupling and adenylyl cyclase activity, thereby influencing response to beta-blockers such as atenolol, metoprolol or bisoprolol (Sandilands & O'Shaughnessy, 2005). While there have been inconsistent findings across populations, previous studies indicate that *ADRB1* rs1801252A allele carriers respond better to beta-blocker treatment compared to *ADRB1* rs1801252G allele carriers, and individuals with the *ADRB1* rs1801252–1801253G–C haplotype exhibit a poor response (Johnson et al., 2003; Liu et al., 2006) - consistent with the outcomes of this study. Therefore, it is likely that associations with resistant hypertension observed in this study may be due to poor response to beta-blockers. Beta-blockers are typically prescribed as add-on drugs when other antihypertensive medications like diuretics, ACE inhibitors (ACEIs), or calcium channel blockers (CCBs) fail to achieve the target blood pressure (BP < 140/90 mmHg).

The *NEDD4L* rs4149601A/A genotype carriers were observed to have an increased risk of resistant hypertension in this study. Normally, functional NEDD4L serves as a principal regulator of sodium ion transporters in the kidney, which are targeted by antihypertensive medications such as hydrochlorothiazide (HCTZ). However, the presence of the *NEDD4L* rs4149601A allele causes alternative splicing, resulting in a non-functional NEDD4L (Svensson-Färbom et al., 2011). *NEDD4L* rs4149601A allele has been linked to adverse cardiovascular outcomes due to poor response in patients on HCTZ in several studies. For example, according to McDonough et al., (2016), carriers of the *NEDD4L* rs4149601A allele were found to not respond well to treatment with HCTZ, had less BP reduction and were at

increased risk of adverse CVD outcomes compared to *NEDD4L* rs4149601G allele carriers. Therefore, these prior associations with poor response to HCTZ resulting in adverse outcomes due to *NEDD4L* rs4149601G>A, are also consistent with findings from this study, although this study establishes a link with resistant hypertension.

5.4 Variant Allele Frequencies and Comparisons with World Population

The variant allele frequencies obtained were compared to other world populations especially for *ABCB1, CYP3A4* and *CYP3A5* variants (Dandara et al., 2011; Mbavha et al., 2022; Muyambo et al., 2022). For variants with few or limited studies in African populations including variants in *CES1, NEDD4L, NOS3* and *NR3C2* genes, this study revealed that variant allele frequencies are comparable to other African populations. However, there are qualitative and quantifiable differences in the frequencies of variant alleles when compared to non-African populations including European and Asian populations. The differences in allele frequencies across populations, especially those associated with resistant hypertension may imply that the susceptibility profile to resistant hypertension in different populations differ, and highlights how some populations may be burdened with resistant hypertension more than others. For the first time, this study reports on the frequency of the *CES1* copy number gain in African populations which arises from segmental duplications in the *CES1* gene resulting in reduced transcriptional activity. This variant has been reported widely in non-African populations to influence response to other drugs such as methylphenidate, dabigatran and clopidogrel which are also widely prescribed in Africans, and considering that this study has reported on *CES1* copy number variation in Africans, it may be an important variant to consider in future pharmacogenetic studies in African populations.

5.5 Conclusion

This study set out to evaluate the role of genetic variation in *ABCB1, ADBR1, CES1, CYP3A4/5, NEDD4L, NOS3, NR3C2* and *SCNN1B* among South African hypertensive patients, comparing the outcomes between resistant hypertensive patients (cases) and non-resistant hypertensive patients (controls). *CYP3A5* rs776746 (c.219-237T>C) and *NOS3* rs3918188 (c.1753-734C>A) polymorphisms were found to be associated with reduced risk of resistant hypertension while *ADRB1* rs1801252 (c.145A>G) and *NEDD4L* rs4149601 (c.49-16229G>A) were associated with increased risk of resistant hypertension. Thus, screening for these variants may help in predicting patients who are likely to have good or poor responses to antihypertensive drugs in African patients. In addition to aiding in diagnosis, variants may

potentially aid in the development of safe and efficacious alternative antihypertensive drugs other than the ones affected by these polymorphisms.

5.6 Limitations and Recommendations

Not all recruited participants of this study had therapeutic drug monitoring (TDM) data for amlodipine levels. Thus, it is possible that there could have been misclassification bias of participants into cases or controls as TDM is the gold standard of monitoring adherence. Additionally, this study only established associations between *CYP3A5* rs776746T>C, *NOS3* rs3918188C>A, *ADRB1* rs1801252A>G, *NEDD4L* rs4149601G>A and resistant hypertension. However, this may not imply causality. Thus, there is need for future studies to focus on establishing causality of the reported associations and this may include performing pharmacokinetic studies or larger prospective cohort studies so that there is an aspect of follow up over a long period. Furthermore, our study targeted specific variants in genes involved in the pharmacodynamics and pharmacokinetics of antihypertensive drugs. It is likely that targeted approaches may fail to detect variants with unknown biological links to hypertension. Therefore, it is crucial that future studies also utilize other approaches such as the GWAS approach, or whole exome sequencing for possible discovery of African specific variants associated with refractory response to antihypertensive therapy.

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7 APPENDICES

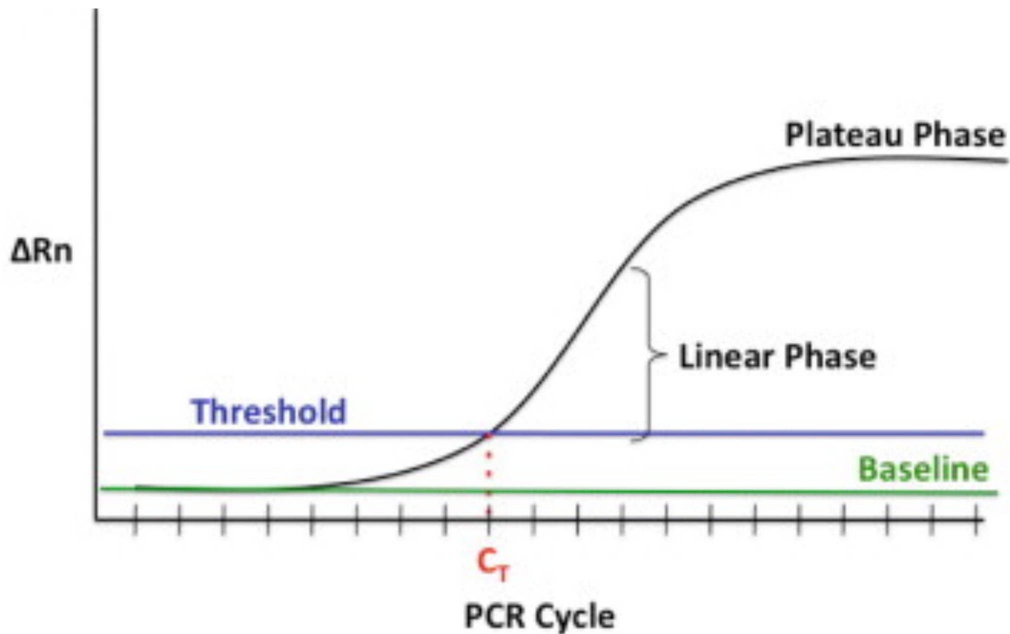


Figure A1: A sample quantitative PCR plot highlight the linear and plateau phases during amplification. Baseline (green line): represents the background fluorescence before accumulation of PCR product. Threshold (blue line): represents the standard deviation of the baseline fluorescence for the first 15 cycles. C_T: represents the amplification cycle at which the fluorescence exceeds the threshold (Dymond, 2013).

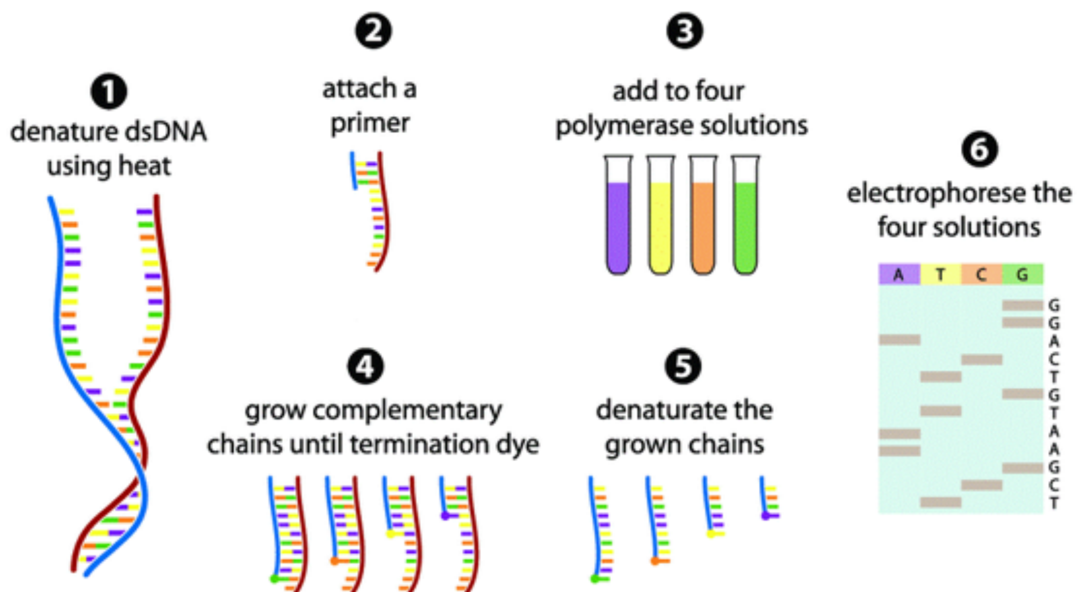
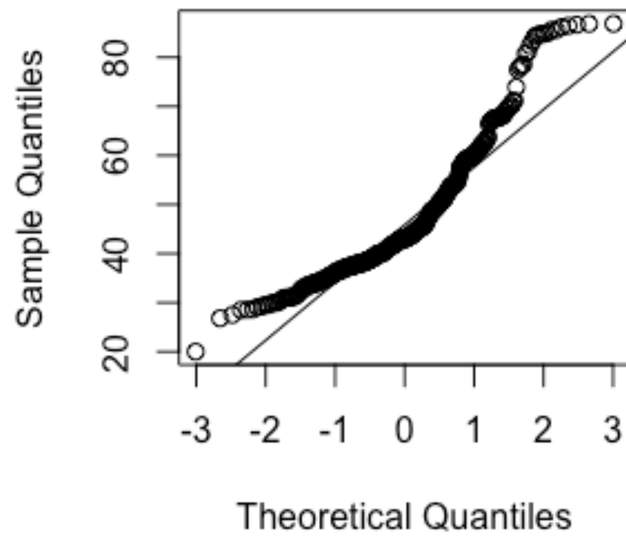
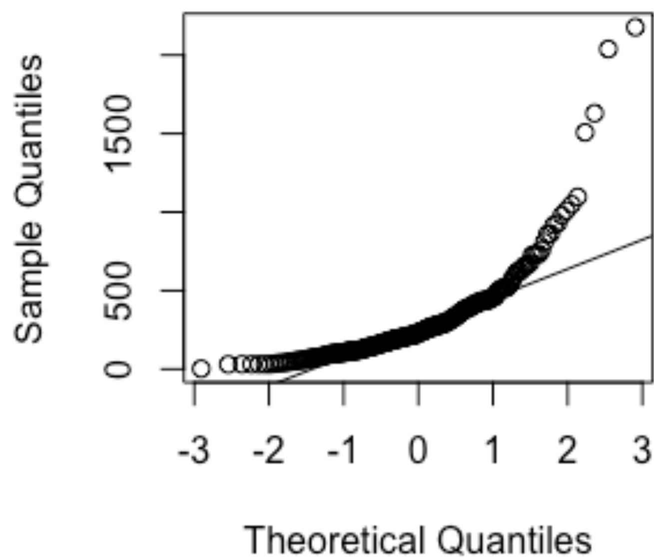


Figure A1: The basic steps involved in Sanger sequencing. Double-stranded DNA (dsDNA) is denatured followed by attachment of specific primers. Solutions containing ddNTPs are then added followed by elongation of the DNA strand until a ddNTP is incorporated. The resultant DNA fragments are denatured followed by electrophoresis to determine the DNA sequence (Gauthier, 2007).

Assessment of continuous data for normality for (a) age and aldosterone measurements (b) using Q-Q plots



(a)



(b)

Figure A3: Q-Q plots for age and aldosterone levels indicate that data are not normally distributed

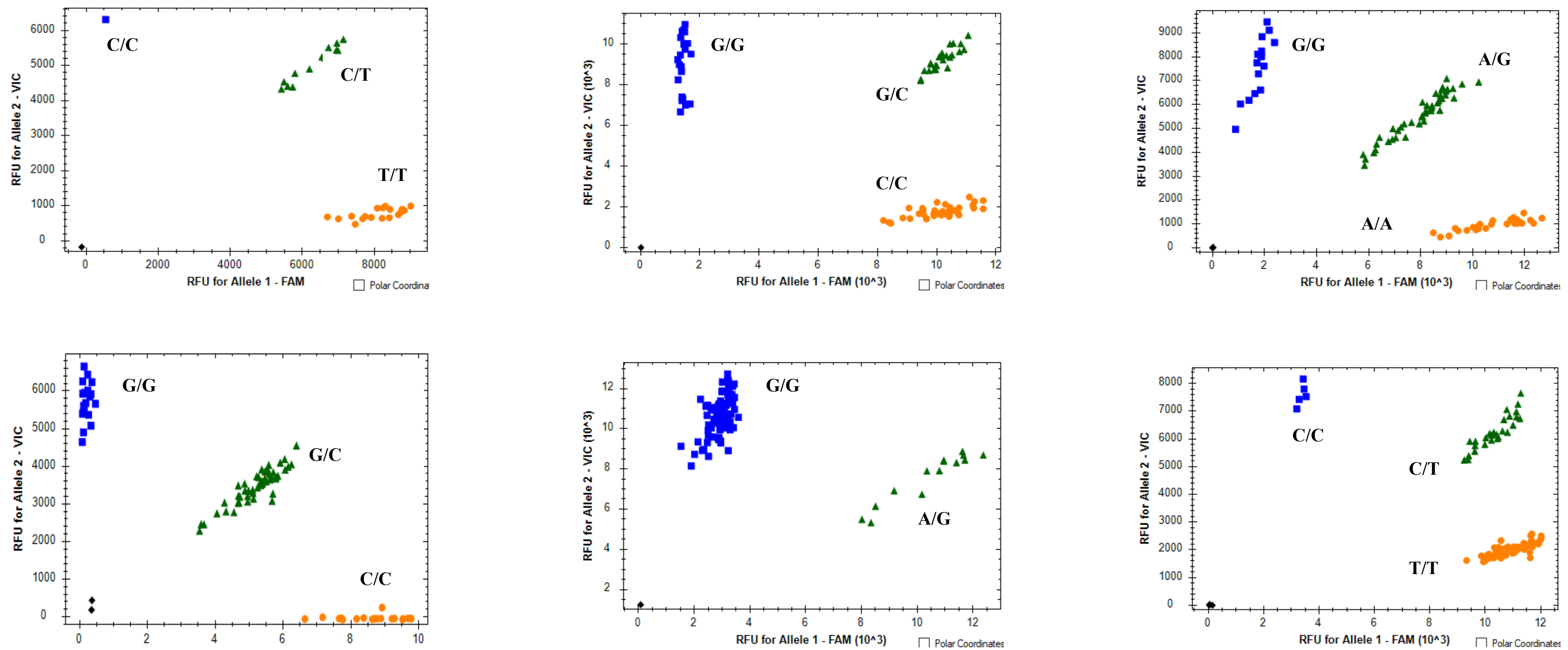


Figure A4: Representative TaqMan Assay Allelic Discrimination Plots. Each plot indicates the clustering of possible genotype combinations, i.e., (a) TT, CT, or CC for *NR3C2* rs5522, (b) GG, GC and CC for *NR3C2* rs2070950 (c) GG, AG and AA for *NEDD4L* rs4149601 (d) GG, GC and CC for *NEDD4L* rs292449 (e) AA and AG for *NEDD4L* rs75982813 (f) CC, CT, TT for *NOS3* rs2070744.

Table A1: Description of variable types and classification into whether they are predictor or outcome variables

Variable	Reason for Inclusion as a Study Variable	Level of Measurement (Nominal OR Ordinal OR Ratio)	Independent/Predictor Variable OR Dependent/Outcome Variable	Continuous Variable OR Categorical Variable
Systolic and Diastolic BP levels	Major Risk factor	Interval	Outcome	Continuous
Gender/Sex	Major Risk factor	Nominal	Predictor	Categorical
Age	Major Risk factor	Interval	Predictor	Continuous
Ethnicity	Major Risk factor	Nominal		
Height/Weight/BMI	Major Risk factor	Ratio	Predictor	Continuous
Waist Circumference	Major Risk factor	Interval	Predictor	Continuous
Family History of Hypertension	Major Risk factor	Nominal	Predictor	Categorical
Diabetes Mellitus	Major Risk factor/Side Effect of Diuretics and Calcium Channel Blockers	Ordinal	Outcome	Categorical
Dyslipidaemia	Major Risk factor	Ordinal	Predictor	Categorical
Smoking/Alcohol	Major Risk factor	Nominal	Predictor	Categorical
Coronary Heart Disease	Complication	Ordinal	Outcome	Categorical
Heart Failure	Complication	Ordinal	Outcome	Categorical
Chronic Kidney Disease	Complication	Ordinal	Outcome	Categorical
Stroke	Complication	Ordinal	Outcome	Categorical
Left Ventricular Hypertrophy (LVH)	Target Organ Damage (TOD)	Nominal	Outcome	Categorical
Electrolytes	Risk factor, Side Effect of Diuretics	Interval	Outcome	Continuous
Creatinine	Calculate eGFR, Target Organ Damage (TOD)	Interval	Outcome	Continuous
Cholesterol	Major Risk factor	Interval	Predictor	Continuous
Uric Acid	Side Effect of Diuretics	Nominal	Outcome	Continuous
Aldosterone/renin ratio	Major Risk factor	Ratio	Predictor	Continuous
Type/Dose of Antihypertensive Drug	Treatment	Ordinal	Outcome	Categorical (Type) and Continuous (Dose)

Table A2: Multivariable logistic regression analysis showing association of genotypes including the clinical variables adjusted for.

SNP/Variable	Genotype/Haplotype	Coefficient	P-Value	OR [95% CI][a]
ADRB1 rs1801252	GG	1.19	**0.02**	**3.30 [1.17 – 10.03]**
CYP3A5 rs776746	CC	-0.80	**0.02**	**0.44 [0.22 – 0.89]**
NEDD4L rs4149601	AA	1.34	**0.001**	**3.82 [1.67 – 9.07]**
NOS3 rs3918188	AA	-1.53	**0.0003**	**0.21 [0.08 – 0.49]**
Aldosterone	-	0.001	0.06	1.00 [0.99 – 1.02]
Dyslipidaemia	-	1.41	**0.007**	**4.10 [1.52 – 12.28]**
Diabetes Mellitus	-	0.41	0.35	1.51 [0.64 – 3.67]
Statin or Lipid Lowering Therapy	-	0.55	0.08	1.75 [0.95 – 3.27]

[a] Adjusted Odds Ratio

Table A3: Multivariable logistic regression analysis showing association of haplotypes including the clinical variables adjusted for.

SNP Combination/Variable	Genotype/Haplotype	Coefficient	P-Value	OR [95% CI][a]
ADRB1 rs1801252 – rs1801253	G-C	1.04	**0.04**	**2.83 [1.05 – 8.20]**[b]
CYP3A5 rs776746 - rs1026427 - rs41303343	C-C-A	-0.14	0.59	0.86 [0.49 – 1.50]
NEDD4L rs4149601 – rs292449	A-C	1.14	0.08	3.14 [0.88 – 12.9]
NOS3 rs1799983 - rs2070744 - rs3918188	G-T-A	-0.75	**0.006**	**0.47 [0.27 – 0.80]**[b]
Aldosterone	-	0.001	0.10	1.00 [0.99 – 1.02]
Dyslipidaemia	-	1.04	**0.04**	**2.82 [1.09 – 8.02]**
Diabetes Mellitus	-	0.24	0.55	1.28 [0.56 – 2.96]
Statin or Lipid Lowering Therapy	-	0.60	**0.04**	**1.83 [1.02 – 3.34]**

[a] Adjusted Odds Ratio

Table A4: Distribution, comparisons between cases and controls and association of CES1 copy numbers with resistant hypertension.

CES1 **Copy Number**	**Cases**	**Controls**	**P-Value**	**OR [95% CI]**
2	132 (69.4%)	129 (68.3%)	1	
3	48 (25.2%)	54 (28.6%)	0.56	0.86 [0.53 – 1.41]
4	3 (1.6%)	6 (3.2%)	0.50	0.48 [0.07 – 2.35]
Neutral	132 (73%)	129 (68%)	1	
Gain	51 (27%)	60 (32%)	0.43	0.83 [0.53 - 1.30]