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Renin genotype as a predictor of response to antihypertensive therapy: a personalized approach to management of high blood pressure

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RCSI

Renin genotype as a predictor of response to antihypertensive therapy: a personalized approach to management of high blood pressure

A thesis submitted to the School of Postgraduate Studies, Faculty of Medicine and Health Sciences, Royal College of Surgeons in Ireland, for the degree of
MD

By

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I declare that this thesis, which I submit to RCSI for examination in consideration of the award of a higher degree, MD, is my own personal effort. Where any of the content presented is the result of input or data from a related collaborative research programme this is duly acknowledged in the text such that it is possible to ascertain how much of the work is my own. I have not already obtained a degree in RCSI or elsewhere on the basis of this work. Furthermore, I took reasonable care to ensure that the work is original, and, to the best of my knowledge, does not breach copyright law, and has not been taken from other sources except where such work has been cited and acknowledged within the text.

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Table of Contents

Abbreviations

ABPM	Ambulatory Blood Pressure Monitor
ACE	Angiotensin Converting Enzyme
ACE2	Angiotensin Converting Enzyme 2
ACEi	Angiotensin Converting Enzyme Inhibitor
ACTH	Adrenocorticotrophic hormone
AGT	Angiotensinogen
Ang I	Angiotensin I
Ang II	Angiotensin II
ANOVA	Analysis of Variance
aPWV	Aortic Pulse Wave Velocity
AT1R	Angiotensin Type-1 Receptor
ARB	Angiotensin Receptor Blocker
BMI	Body Mass Index
BP	Blood Pressure
CAD	Coronary Artery Disease
CCB	Calcium Channel Blocker
cDNA	Complementary Deoxyribonucleic Acid
CG	Clinical Guidance
CHD	Coronary Heart Disease
CO	Cardiac Output
cwPWV	Carotid-femoral Pulse Wave Velocity
DBP	Diastolic Blood Pressure
DNA	Deoxyribonucleic Acid
DRI	Direct Renin Inhibitor
DZ	Dizygotic twins
ECG	Electrocardiogram
EDTA	Ethylenediaminetetraacetic Acid
ENaC	Epithelial Sodium Channel
ESC	European Society of Cardiology
ESH	European Society of Hypertension
GWAS	Genome Wide Association Studies
HCTZ	Hydrochlorthiazide diuretic
HBPM	Home Blood Pressure Monitoring

HR	Heart Rate
HRP	Handle Region Peptide
Hr	Hour
ICH GCP	International Conference on Harmonisation, Good Clinical Practice
IMB	Irish Medicines Board
I/D	Insertion/Deletion Polymorphism
kDa	Kilodalton
Kg	Kilograms
LD	Linkage disequilibrium
LVH	Left Ventricular Hypertrophy
mg	Milligrams
mL	Millilitres
mm Hg	Millimetres of Mercury (Mercury, Hg)
mmol/L	Millimoles per Litre
mRNA	Messenger Ribonucleic Acid
MR	Mineralocorticoid Receptor
MZ	Monozygotic twins
ng	Nanograms
NICE	National Institute for Clinical Excellence
PBS	Phosphate-buffered Saline
PCR	Polymerase Chain Reaction
(P)RR	Prorenin Receptor
PRA	Plasma Renin Activity
RAS	Renin Angiotensin System
RAAS	Renin Angiotensin Angiotensinogen System
RIA	Radioimmunoassay
rRNA	Ribosomal Ribonucleic Acid
rpm	Revolutions Per Minute
RNA	Ribonucleic Acid
SAEs	Serious Adverse Events
SBP	Systolic Blood Pressure
SEM	Standard Error of Mean
SLR	Specified Laboratory Reagent
SNS	Sympathetic Nervous System

SNP	Single Nucleotide Polymorphism
STEMI	ST Segment Elevation Myocardial Infarction
TPR	Total Peripheral Resistance
tSNP	Tagging Single Nucleotide Polymorphism
UK	United Kingdom
US	United States
WTCCC	Wellcome Trust Case Control Consortium
WHO	World Health Organisation
µL	Microlitre
µmol	Micromolar

List of Figures

List of Tables

Thesis Summary

A renin gene distal enhancer single nucleotide polymorphism, REN-5312C/T, has been reported to influence *in vitro* renin gene transcription in transfected human choriodecidual cells and in diseased kidney tissue in humans. The polymorphism has also been shown to have *in vivo* functional activity in humans, with carriage of the REN-5312T allele associated with elevated blood pressure.

This thesis consists of two studies with the common aim of further exploring the functionality of the REN-5312C/T variant in humans.

In a tissue-based study in normal adrenal glands, we observed greater renin mRNA expression levels in REN-5312T allele carriers. This finding confirmed functional activity of REN-5312C/T in normal adrenal gland tissue.

A randomized, cross-over clinical trial in 98 patients was completed in order to determine whether or not REN-5312C/T would predict response to each of three blockers of the renin-angiotensin system (RAS). A statistically significant difference was observed for supine plasma renin activity (PRA) and REN-5312C/T genotype, with baseline PRA higher amongst REN-5312T allele carriers. Blood pressure lowering responses were observed to be greater in REN-5312T allele carriers with high baseline BP and/or low baseline PRA levels. Additionally, blood pressure lowering responses were observed to be greater with candesartan treatment compared with aliskiren or perindopril treatments; in females compared to males; and in participants with high baseline blood pressure and high PRA levels.

The findings of this thesis further illustrate the functionality of the REN-5312C/T polymorphism in determining optimal antihypertensive treatment for individual patients. Greater blood pressure lowering was observed in the clinical trial amongst REN-5312T allele carriers. Greater tissue renin mRNA expression was observed in the tissue study amongst REN-5312T allele carriers. It is thus plausible that greater tissue RAS activity of REN-5312T allele carriers is at least

in part responsible for the greater antihypertensive effect of REN-5312 T-allele carriers with RAS blockade.

REN-5312C/T may yet become a genotype used in practice to identify patients who will gain maximal benefit from RAS blockade both in terms of blood pressure lowering and protection from end-organ damage.

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First and foremost, I must thank the patients who took part, without them this would not have been possible.

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Dedication

To family.
For my parents Michael and Frances Quinn and my little sister Adeline. For being my family, recognizing the power of education, and always supporting me in achieving my goals.

Chapter 1 – Thesis Introduction

1.1 Hypertension

1.1.1 Background

Hypertension is a leading cause of disease burden with complications of hypertension accounting for 9.4 million deaths worldwide per year (Lim et al. 2012). In 2008, an estimated 40% of the world's population aged 25 years and above had been diagnosed with hypertension (Lim et al. 2012; World Health Organization 2011). The World Health Organisation (WHO) identifies high blood pressure (BP) as one of the most important preventable causes of premature morbidity and mortality in developed and developing countries. It's prevalence is such that data from the Framingham Heart Study concluded the likelihood that those who are normotensive at age 55 have a 90 percent life-time risk for developing hypertension (Vasan et al. 2002).

The consequences of uncontrolled hypertension are heavily implicated in the pathogenesis of cerebrovascular disease and myocardial infarction. In patients presenting with acute cerebrovascular events and myocardial infarction a significant proportion of cases are attributable to uncontrolled hypertension. Hypertension is responsible for at least 45% of deaths from myocardial infarction and 51% of deaths from stroke (World Health Organization 2009) . It is also implicated as a causative factor in heart failure, renal disease, renal failure and retinal disease.

A Lancet publication in 2008 (Lawes et al. 2008) concluded that hypertension was the cause of approximately 7.6 million deaths worldwide (13.5% of total deaths) in the year 2001. It is now well recognised that cardiovascular disease is not limited to higher-income developed countries. In the Lancet publication, over 80% of attributable disease burden was seen in low-income and middle-income regions (Lawes et al. 2008).

A meta-analysis performed in 2002 provided data from observational studies whose cohorts were patients with no prior history of stroke or heart disease (Lewington et al. 2002). The study concluded that death from coronary artery disease (CAD) and stroke increases continously and linearly from blood

pressures as low as 115mmHg systolic and 75mmHg diastolic. Lewington (2002) demonstrated that an increment of 20mmHg in systolic blood pressure (SBP) or 10mmHg in diastolic blood pressure (DBP) in the elderly and middle-aged persons is associated with a two-fold increase in cardiovascular disease mortality (Lewington et al. 2002). Consequently, optimal management of hypertension is ever sought, to minimise the risk to the individual of adverse events. In clinical trials, antihypertensive therapy has been shown to reduce stroke incidence on average 35-40%; myocardial infarction 20-25%; and heart failure more than 50% (Neal et al. 2000).

In those who have established cerebrovascular disease, existing target-organ damage such as left ventricular hypertrophy (LVH), diabetes, especially type II diabetes and chronic renal disease, the effective management of hypertension becomes even more important. Further challenges to the management of hypertension have come from racial origin with well documented differing pharmacological responses to ACE inhibitors, for example, in black populations whose physiology favours the use of a calcium channel blocker (CCB) or diuretic-based antihypertensive regime.

It is now acknowledged that less than 35% of hypertensive patients are able to achieve their target systolic and diastolic blood pressure with the current available treatment classes (Thoenes et al. 2009). Up to 50% of patients gain little or no blood pressure lowering with any particular drug class. Since the 1950s it has been established that genetics have an influence on response to treatment (Lewington et al. 2002; Kalow 1962; Kalow 1992).

1.1.2 Definitions

Primary or essential hypertension is a term used for the majority of cases in which no cause for hypertension is identified (Calhoun et al. 2000; Carretero & Oparil 2000). Its pathogenesis is multifactorial, with genetics playing an important role along with environmental influence. The age of onset is usually between 25 and 55 years as hypertension is not a common entity prior to 20 years of age. It is much less common that a cause for a patient's hypertension is identified on investigation. If so, it is referred to as secondary hypertension for

which there are a variety of causes including renal and endocrine abnormalities (see Table 1.1).

Table 1. 1 – Causes of Secondary Hypertension

Secondary Hypertension
Renal parenchymal disease
Renovascular disease - renal artery stenosis
Phaeochromocytoma (adrenal gland tumour, associated with increased secretion of epinephrine and norepinephrine)
Cushing's syndrome (pituitary or adrenal gland tumour resulting in excess cortisol secretion), exogenous steroid therapy
Hyperaldosteronism
Thyroid or parathyroid disease
Oestrogen use, pregnancy-associated hypertension (Gestational Hypertension, Pre-Eclampsia)
Sleep apnoea, obesity
Coarctation of the aorta
Drug induced, including cocaine, methamphetamine, erythropoietin
Excess alcohol intake

1.1.3 Pathophysiology of Essential Hypertension

High blood pressure results from an imbalance between cardiac output and systemic vascular resistance and is represented by the following equation:

$$BP = CO \times TPR$$

(BP = blood pressure; CO = cardiac output; TPR = total peripheral resistance). Cardiac output is the amount of blood being pumped out per cardiac cycle, and equals stroke volume multiplied by the heart rate. Elevated vascular resistance, elevated stroke volume, elevated heart rate or a combination of all three can increase blood pressure.

$$CO = SV \times HR$$

$$BP = CO \times SVR$$

Hypertension commonly results from an increase in systemic vascular resistance (SVR) with normal cardiac output. There are exceptions to this however, for example in young people with an increase in cardiac output due to excess sympathetic nervous stimulation, or in those with fluid overload where an increase in cardiac output is often followed by an increase in SVR.

Essentially, many different physiologic factors are implicated in the pathogenesis of hypertension (Figure 1.1). The major mechanisms involve heightened sympathetic nervous system (SNS) activity and the renin-angiotensin aldosterone system (RAAS) with or without other factors involving vascular tone and remodelling amongst others. We have long been taught that the kidney probably plays the primary role and other systems such as the SNS augment this effect (Guyton 1991a). Complex interactions mean several systems often need to be targeted to achieve adequate blood pressure control.

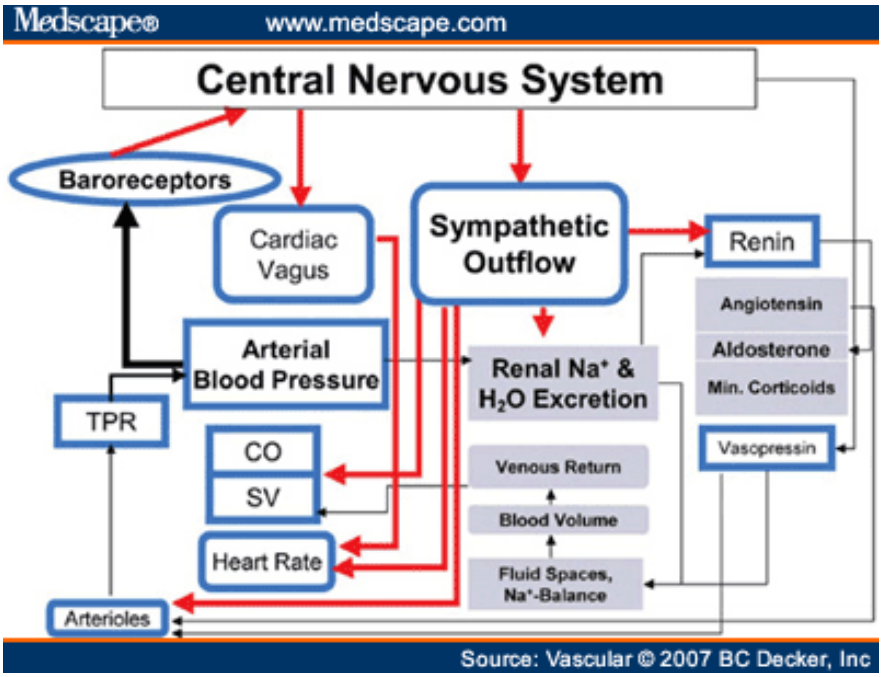


Figure 1. 1 - Blood pressure regulation is a complex interplay of many systems. Reproduced from (Schmidli et al. 2007).

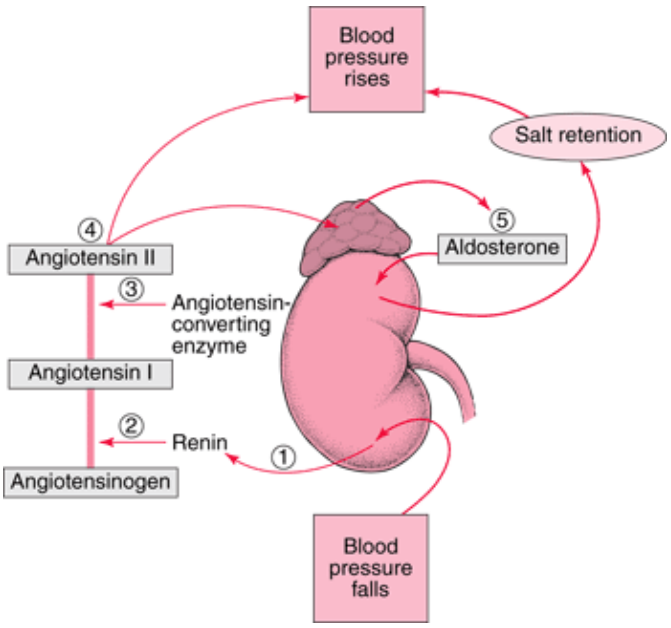


Figure 1.2 - **Role of the kidney in blood pressure regulation.** Renin release in response to hypovolaemia ultimately results in the production of Angiotensin II, vasoconstriction and aldosterone release with salt retention. Both processes contribute to an increase in blood pressure. Reproduced from (Merck 2011).

1.1.3.1 Sympathetic Nervous System

Heightened sympathetic nervous system (SNS) activity causes an increase in heart rate and vasoconstriction. An increase in sympathetic nerve release of noradrenaline causes a positive chronotropic effect with a resultant increase in heart rate at the sino-atrial node. An increase in release of noradrenaline from the adrenal medulla has the same effect. The opposite, parasympathetic release of acetylcholine causes a negative chronotropic effect (Neal et al. 2000; Boarder et al. 2010). Cardiac receptors for noradrenaline and adrenaline exist mainly as β_1-adrenoceptor subtype though other subtypes can be found. Voltage-dependent calcium channels and again β-adrenoceptors also play a role in cardiac myocyte contraction and in determining stroke volume. As such, β-receptor blockers are of significant interest as a therapeutic intervention in cardiovascular pharmacology.

Sympathetic activity affects both veins and arteries. It causes large vein constriction with a resultant increased venous return to the heart, increased end-diastolic volume, a subsequent increased force of atrial contraction and ultimately contributes to an increased stroke volume. This preload can be augmented by changes in fluid status of the individual as a result of the kidneys

regulation of circulating volume, and salt and fluid-retaining properties of aldosterone (Guyton 1977). As a consequence of increased sympathetic activity on the kidney, elevated renin or angiotensin levels also play a role as additional biochemical stimulants of blood pressure. Cardiac output increases, peripheral vascular resistance increases and fluid retention occur (Guyton 1991b). An increase in arterial pressure as a result of sympathetic activity means the heart has a greater pressure to overcome in order to pump blood outward, known as the afterload. The higher the afterload the greater the work the cardiac muscle has to do per cardiac cycle, resulting in hypertrophy of the ventricular muscle when longstanding, as in the case of poorly controlled arterial hypertension.

Peripheral resistance lies at the level of the precapillary bed or arterioles. The receptors for sympathetic stimulation here are predominantly α_1-adrenoceptors, activation of which cause arteriolar constriction with resultant increase in resistance. Vasoconstriction is further stimulated by intracellular calcium. B_2-adrenoceptors are responsible for arteriolar dilatation.

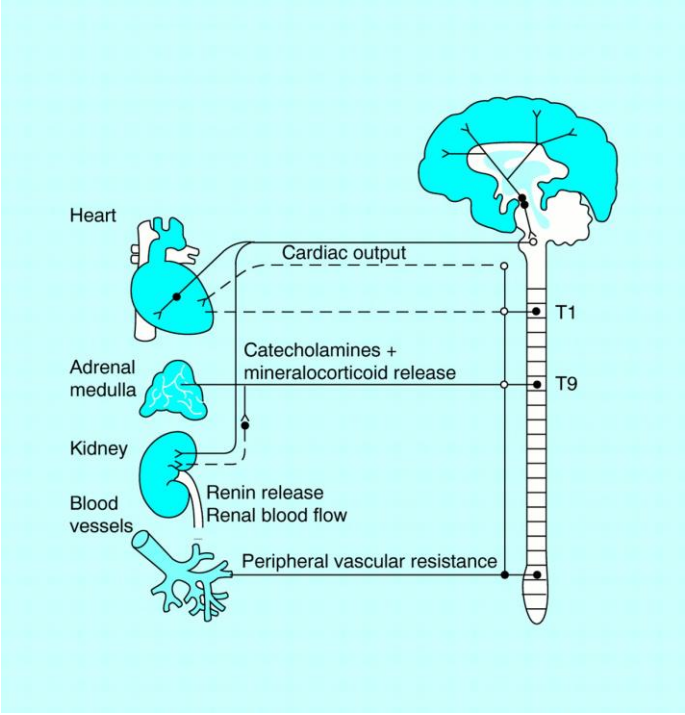


Figure 1.3 - **Autonomic nervous system targets in blood pressure regulation**. The sympathetic nervous system can stimulate vessel constriction, release of renin and catecholamines, and the heart rate affecting cardiac output. Reproduced from (Beevers et al. 2001).

The Coronary Artery Risk Development in Young Adults (CARDIA) study showed an association between elevated heart rate and the development of

elevated diastolic hypertension (Kim et al. 1999). With these young patients it is postulated that through chronic sympathetic stimulation of heart rate and peripheral vessels, vascular remodelling and smooth-muscle cell proliferation occurs, adding further to the sympathetic effect of vasoconstriction. As a result vascular resistance increases, and the diastolic pressure increases. It is also known that chronic sympathetic stimulation induces left ventricular hypertrophy (LVH), contributing to target-organ damage. Increases in circulating levels of norepinephrine are associated with reduced radial artery compliance (and therefore increased vascular hypertrophy).

Arterial baroreceptors are stretch receptors in the arterial wall and are stimulated when the wall of the artery changes as pressures within the lumen change. Reflex responses from the brainstem respond by triggering an increase or decrease in heart rate (Figure 1.4). Hormone secretions that target the heart rate and blood vessels are also stimulated by this baroreceptor response (Calhoun et al. 2000; Stanfield & Germann 2008; Carretero & Oparil 2000). Baroreceptors can make management of blood pressure difficult – if cardiac output falls, the baroreceptor reflex stimulates sympathetic activity with a resultant increase in heart rate, cardiac output, peripheral resistance and blood pressure (Guyton 1991; Boarder et al. 2010).

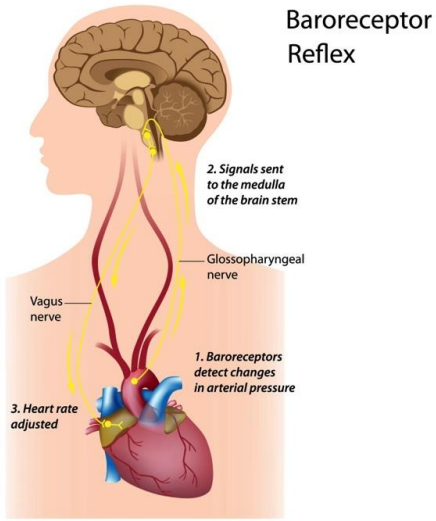


Figure 1.4 - **The Baroreceptor Reflex.** Reflex responses from the brainstem respond by triggering an increase or decrease in heart rate. Reproduced from http://www.interactive-biology.com/4008/blood-pressure-and-its-regulation/

Angiotensin II, the effector chemical of the renin-angiotensin system, amplifies the response to SNS stimulation by facilitating presynaptic noradrenaline release peripherally (Boarder et al. 2010) and may also have a role in resetting the aortic baroreflex resulting in a lack of inhibition once the aortic baroreceptor nerves have been activated (Boarder et al. 2010). Centrally acting α- and β-blockers are effective in helping lower blood pressure in patients with essential hypertension, confirming the importance of the role of central sympathetic tone in maintenance of hypertension.

1.1.3.2 The Kidney, Salt Homeostasis and Blood Pressure Control

Over sixty per cent of our filtered sodium load is reabsorbed in the proximal tubule (PT) of the nephron with a further thirty per cent reabsorbed in the thick ascending limb of the loop of Henle (TAL). By the time it has reached the cortical collecting tubule (CCT), a further 7 per cent has been reabsorbed by the distal convoluted tubule (DCT). Whilst the meagre-sounding 2 per cent yet to be reabsorbed at the CCT may appear to be very small in terms of what has been reabsorbed already, nothing could be further from true in terms of sodium homeostasis. In fact, salt reabsorption via the epithelial Na+ channel (ENaC) in the CCT is the principal site at which the individual's net sodium balance is determined. This is due to ENaC activity being heavily controlled by the renin-angiotensin system, with aldosterone having it's principal effects here (Lifton et al. 2001).

If the thick ascending limb of the loop of Henle detects a reduced salt load, renin is secreted from the juxtaglomerular apparatus. Ultimately angiotensin II (Ang II) is formed. Ang II binds to a specific G-protein coupled receptor in the adrenal glomerulosa and aldosterone is secreted. Aldosterone is the principal mineralocorticoid steroid hormone in the body, being responsible for salt and water retention with subsequent pressor effects. Aldosterone binds to the mineralocorticoid receptor (MR). The MR is found in the distal nephron, and is a nucelar hormone receptor, the binding of which by aldosterone ultimately results in increased activity of ENaC. As a result, increased salt absorption occurs and subsequent elevated blood pressure.

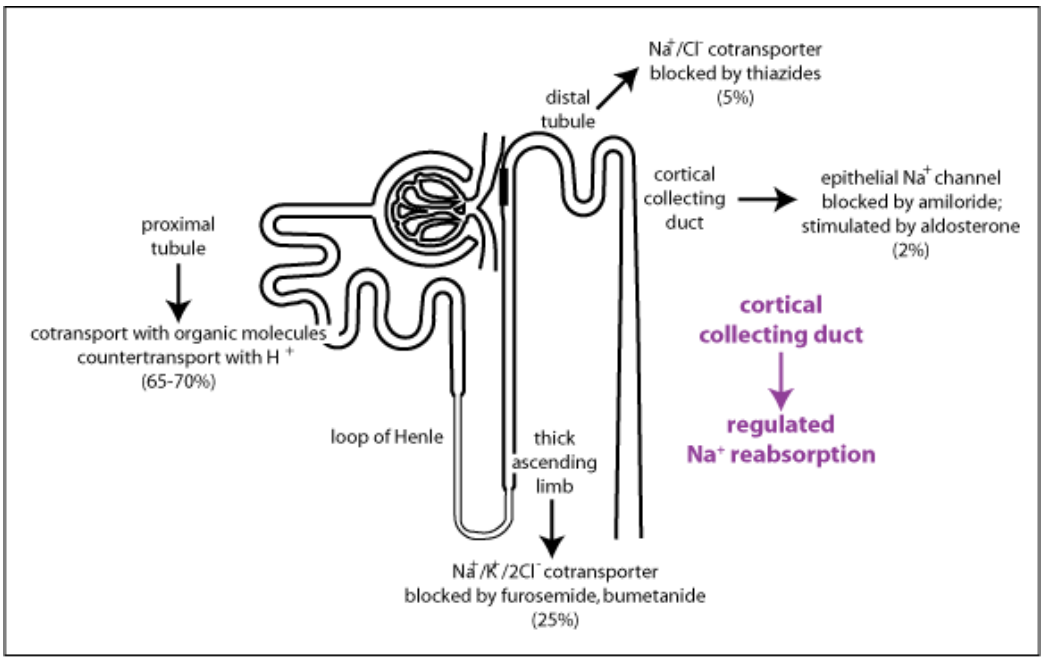


Figure 1.5 - Regulation of sodium (Na^+) balance and drug targets in its manipulation. Percentage of sodium reabsorbed at each section of the nephron is represented in brackets. Reproduced from http://courses.washington.edu/conj/bess/ sodium/sodium.htm

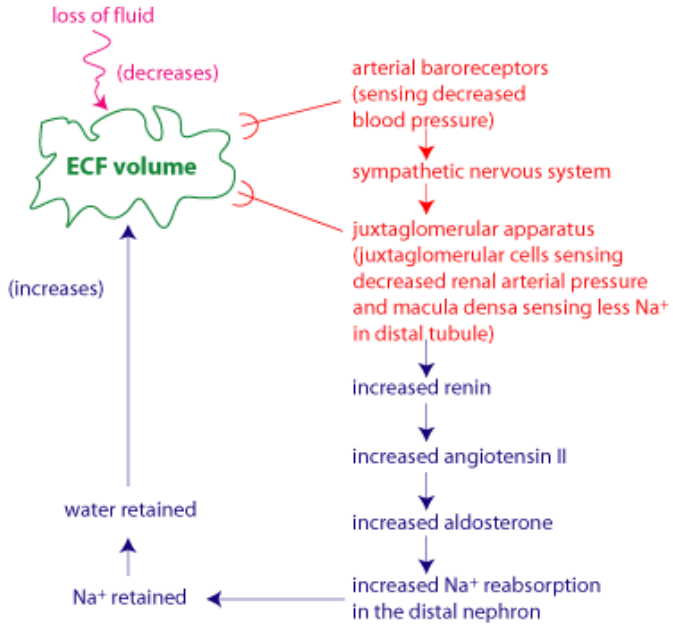


Figure 1.6 - **Summarising homeostasis of extracellular fluid volume and sodium homeostasis.** Reduced extracellular fluid volume stimulates arterial baroreceptors and the sympathetic nervous system together with the juxtaglomerular apparatus to increase renin, aldosterone secretion, sodium and water retention. Reproduced from http://courses.washington.edu/conj/bess/ sodium/sodium.htm

1.1.3.3 Renin-Angiotensin System

The renin-angiogtensin system (RAS) has a central role in the regulation of blood pressure control. It also influences regulation of electrolytes and atherosclerosis by a complex set of reactions of which renin is the first and rate-limiting catalytic enzyme. Renin (REN) maps to chromosome 1q32, spans 12.5 kb in length and encodes 10 exons. Individual REN polymorphisms have been associated with increased BP, plasma renin activity (PRA), and susceptibility to hypertension in a variety of ethnic groups, although often with inconsistent results.

Renin, synthesised by the juxtaglomerular cells of the renal cortex, sets off a cascade of chemical events commencing with the cleavage of the renin substrate angiotensinogen to produce the decapeptide angiotensin I. Ultimately the end product Angiotensin II, a potent vasoconstrictor and major stimulus of aldosterone production and release from the adrenal gland, can act on several receptors if it is not further processed. Each of these products of the action of renin on angiotensinogen have differing roles to play in the aetiology of hypertension and endothelial damage. Renin concentration itself can influence an individual's response to antihypertensive therapy and has also been implicated when present in high levels in an individual at greater risk of myocardial infaction. Indeed, pre-treatment plasma renin activity (PRA) is an independent risk factor for myocardial infarction in hypertensive patients and for microvascular complications in diabetes (Schmidli et al. 2007; Alderman et al. 1991; Alderman et al. 1997; Luetscher et al. 1985).

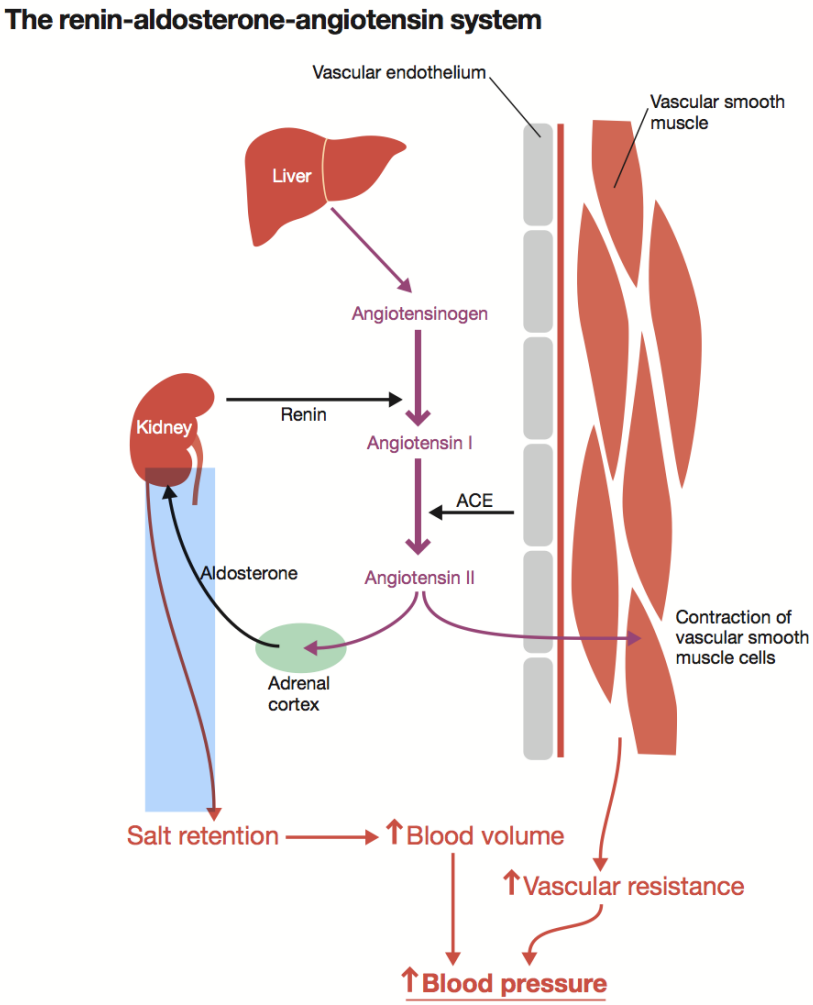


Figure 1.7 - Aspects of the renin-angiotensin system and control of blood pressure. Reproduced from (Merck 2011; Boarder et al. 2010), Chapter 5

Renin release is stimulated by reduced renal arterial blood pressure (BP), reduced extracellular fluid volume, or by reduced sodium concentrations in renal tubular fluid. The macula densa and renal sympathetic nervous system respond to these changes by stimulating the release of renin. Any physiological factor which is deemed to reduce renal blood flow will increase renin activity, for example upright posture, haemorrhage, renal artery narrowing, dehydration and salt restriction. Conversely, supine posture, high salt intake and peripheral vasoconstrictors will decrease renin levels (Wadei et al. 2012). Potassium concentration is also linked to renin release where hypokalaemia will increase renin release and hyperkalaemia will decrease renin release (Penton et al. 2015). In some forms of hypertension, a marked derangement of the renin system can be identified. For example, in those individuals with primary hyperaldosteronism, a cause of secondary hypertension, renin secretion is

suppressed with invariably undetectable PRA accompanied by aldosterone excess (Sahay et al. 2012). As well as sodium and water retention, marked hypokalaemia as a result of an increase in renal tubule potassium loss, can arise as a result of the hyperaldosteronism.

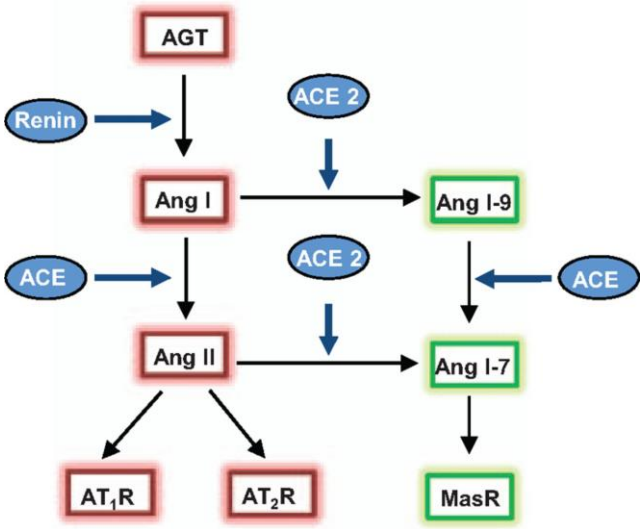


Figure 1.8 - **Overview of the renin-angiotensin system (RAS).** AGT indicates angiotensinogen; Ang, angiotensin; ACE, angiotensin-converting enzyme; ACE2, angiotensin-converting enzyme 2; AT_1R, angiotensin type 1 receptor; AT_2R, angiotensin type 2 receptor; MasR, Mas receptor. Reproduced from (Conti et al. 2012).

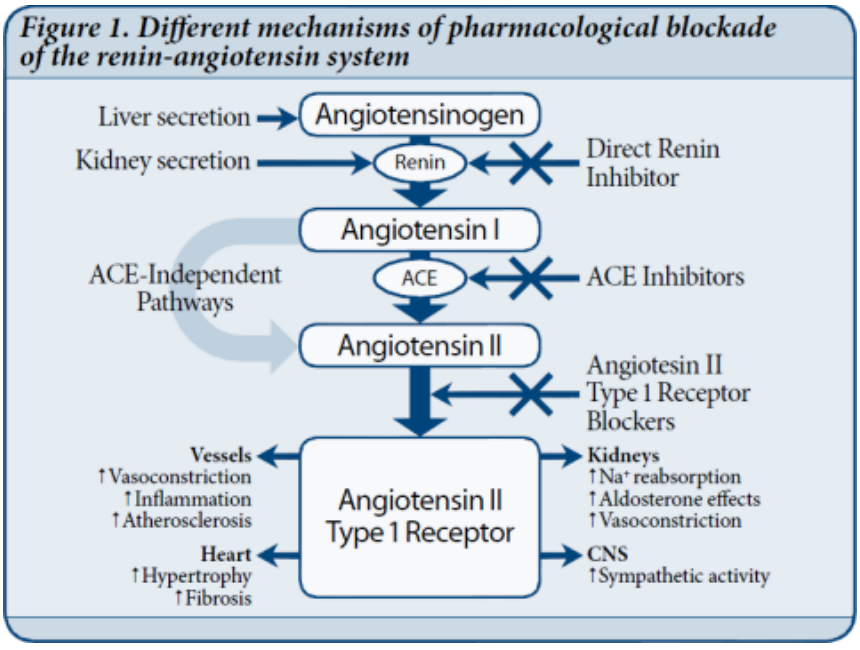


Figure 1.9 - Renin Angiotensin System as a Therapeutic Target. This diagram illustrates the various steps of the renin angiotensin system that are currently targeted by renin angiotensin system blockade. Reproduced from (Duke Evidence Based Practice Center 2011).

1.1.3.4 Prorenin and the (Pro)Renin Receptor, (P)RR

As early as 1971 it was determined that renin had an enzymatically inactive precursor. When amniotic fluid was left at low pH in the cold it was seen to acquire renin activity (Lumbers 1971). This was subsequently replicated in plasma (Skinner et al. 1975). With a molecular weight 5 kDa higher than that of renin it was determined that this “big” renin was the enzymatically inactive precursor of renin, later renamed prorenin. The cloning of the renin gene in 1984 allowed the formal identification of prorenin as the precursor of renin (Hobart et al. 1984).

Renin is an aspartic protease with two lobes. Residing in the cleft is the active site with its 2 catalytic aspartic residues. Prorenin is structurally different from renin in that the active site is covered by a prosegment of 43 amino acids, the propeptide. The propeptide covers the enzymatic cleft of the renin molecule and does not allow binding of angiotensinogen (Danser & Deinum 2005). Prorenin can be activated proteolytically through enzymatic removal of the propeptide and non-proteolytically through unfolding of the propeptide from the enzymatic cleft (Figure 1.10). Non-proteolytic activation occurs through exposure to low pH and low temperature – acid and cryoactivation (Pitarresi et al. 1992; Suzuki, Hayakawa, Nakagawa & Nasir 2003a). Proteolytic activation in vivo occurs in the kidney. The prosegment is proteolytically cleaved in the glomerular afferent artery, although the proconvertase responsible for the prosegment cleavage remains unidentified (Nguyen & Muller 2010).

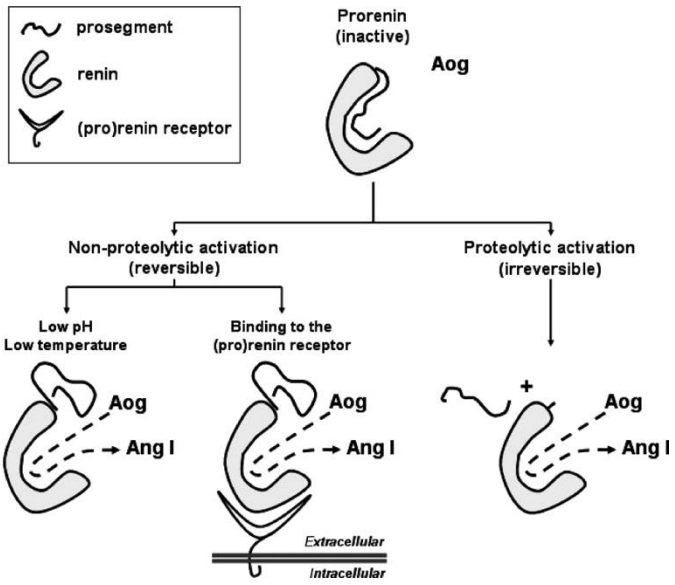


Figure 1.10 - **Proteolytic and non-proteolytic activation of prorenin.** Reproduced from (Danser & Batenburg 2007).

Prorenin itself is also enzymatically active in vivo (Methot et al. 1999). These authors demonstrated an increase in local pituitary Angiotensin I production in double transgenic animals when native prorenin or a mutated prorenin, that could not be cleaved into active renin, was coexpressed with angiotensinogen. A further observation that transgenic rats, with increased expression of liver prorenin and normal blood pressure, had severe liver fibrosis and nephroangiosclerosis, confirmed the activity of prorenin on processes not only related to blood pressure control.

Prorenin has also been found to be at higher concentrations in type II diabetic patients with nephropathy and retinopathy. Prorenin is increased out of proportion to renin in microalbuminuric diabetic subjects (Danser et al. 1989). Of greater interest is the fact that this rise in prorenin occurs before the microalbuminuria becomes apparent. It has been suggested that taking prorenin measurements may be utilized in conjunction with glycosylated haemoglobin measurements to determine those diabetic individuals more at risk of microalbuminuria later on (Guyton 1991; Deinum et al. 1999).

In 2002 a (pro)renin receptor, (P)RR was cloned. This (pro)renin receptor is a 350-amino acid protein with a single transmembrane domain binding both renin and prorenin (Nguyen et al. 2002). The receptor has been localized by immunohistochemistry and insitu hybridization studies to the vascular smooth

muscle cells in human heart and kidney. Kidney sites include the glomerular mesangial cells and distal and collecting tubular cells.

On binding to the receptor prorenin is non-proteolytically activated, with neither renin nor prorenin internalized but rather prorenin activation occurring through unfolding of the propeptide. Binding of prorenin to the (P)RR allows it to become enzymatically active on the cell surface (Danser et al. 2005). Activation of the receptor by binding of renin or prorenin activates different intracellular signalling pathways which upregulates profibrotic genes, including mitogen-activated protein (MAP) kinases p44/p42 or extracellular regulated kinases 1/2 (ERK 1/2) (Nguyen 2011). This occurs even in the presence of the angiotensin receptor antagonist losartan, demonstrating angiotensin-II independent effects of prorenin. Tissue fibrosis and vascular remodelling result from activation of these pathways.

Studies have reported a link between (P)RR and hypertension. A (P)RR gene named ATP6ap2/PRR located on the X chromosome was identified in early studies (Hirose et al. 2009). 1,112 subjects from a Japanese population (357 male subjects, 755 female subjects, mean age 58.9 ± 10.1 years) were recruited to investigate the association of (P)RR polymorphisms with ambulatory blood pressure. Hirose (2009) observed that the intervening sequence polymorphism IVS5+169C>T in the (P)RR gene was associated with significantly higher systolic and diastolic blood pressure in a cohort of Japanese men. Carriage of the T allele was associated with higher ambulatory blood pressures in male subjects (Hirose et al. 2009). Systolic BP tended to be higher in female TT-homozygote subjects when compared to CC-homozygote and CT-heterozygote females. This result did not achieve statistical significance, however this was likely due to the small size of the TT-homozygote female group (n=15) (Hirose et al. 2009). These findings are illustrated in Table 1.2.

Table1.2 - Blood pressure values according to the genotypes of the (P)RR gene IVS5+169C>T. Continuous values are expressed as mean ± standard deviation. * P < 0.05, ** P < 0.01, § P < 0.001 (Bonferroni correction). || P < 0.05, ¶ P < 0.001 (adjusted for age, body mass index, use of antihypertensive medications, prevalence of diabetes mellitus, history of previous cardiovascular disease, current smoking, and current drinking). Reproduced from Hirose et al. 2009.

	Males (n=357)		Females (n=755)		
	C (n = 310)	T (n = 47)	CC (n = 582)	CT (n = 158)	TT (n = 15)
24-h systolic, mmHg	125.0 ± 11.8	131.4 ± 14.6 §, ||	120.6 ± 12.7	119.7 ± 12.8	125.0 ± 14.2
24-h diastolic, mmHg	73.9 ± 6.9	77.7 ± 8.3 §, ||	70.5 ± 7.6	70.1 ± 7.1	71.0 ± 7.5
Daytime systolic, mmHg	130.5 ± 12.6	136.5 ± 16.2 **, ||	126.6 ± 14.0	125.3 ± 13.5	131.3 ± 17.3
Daytime diastolic, mmHg	78.0 ± 7.5	81.4 ± 9.2 **, ||	74.7 ± 8.5	74.1 ± 7.6	75.2 ± 9.6
Night-time systolic, mmHg	114.4 ± 12.8	121.5 ± 14.6 §, ¶	108.3 ± 13.0	108.2 ± 13.8	113.1 ± 12.2
Night-time diastolic, mmHg	66.1 ± 7.1	70.4 ± 7.7 §, ¶	61.9 ± 7.4	61.7 ± 7.5	63.1 ± 6.0

The association was replicated in a Caucasian male population for the same SNP (Ott et al. 2011). In this study, the SNP was genotyped in a population of 266 men with normal or mildly elevated BP, not taking any antihypertensive medication. No females were evaluated in this Caucasian population. Office and resting systolic BP was lower in male C-allele than T-allele carriers of the (P)RR gene, P<0.05 (Office systolic BP 145 ± 16 C-allele carriers vs. 153 ± 17 in T-allele carriers, p=0.026; resting systolic BP 136 ± 17 C-allele carriers vs. 144 ± 17 T-allele carriers, p=0.046). No significant difference between male C-allele and T-allele carriers was observed for office and resting diastolic BP. The authors found an association between carriage of the C-allele and lower serum aldosterone (P<0.05) and postulated that the (P)RR may influence BP regulation through altered aldosterone release (Ott et al. 2011).

In recent unpublished work by another colleague in our group, 8 (P)RR tagging SNPs were genotyped in a population of hypertensives treated with losartan or aliskiren. This analysis was performed in a population previously described (Vangjeli et al. 2010; Moore et al. 2007). No statistically significant associations

were observed between carriage of any of the (P)RR SNPs and response to treatment with the angiotensin II receptor antagonist losartan. In those individuals randomised to treatment with the direct renin inhibitor aliskiren, there was an overall pattern of increased daytime and clinic BP response to treatment associated with carriage of the minor alleles of the 8 genotyped SNPs. In the sex-specific analysis, specifically the male-only cohort, a number of associations were observed at P≤0.05 between SNPs in the (P)RR and increased clinic SBP and DBP response to treatment. There were no significant associations in the female-only cohort. The study did not show any association between 9 (P)RR SNPs and mRNA transcript levels of two primers of the (P)RR in adrenal tissue.

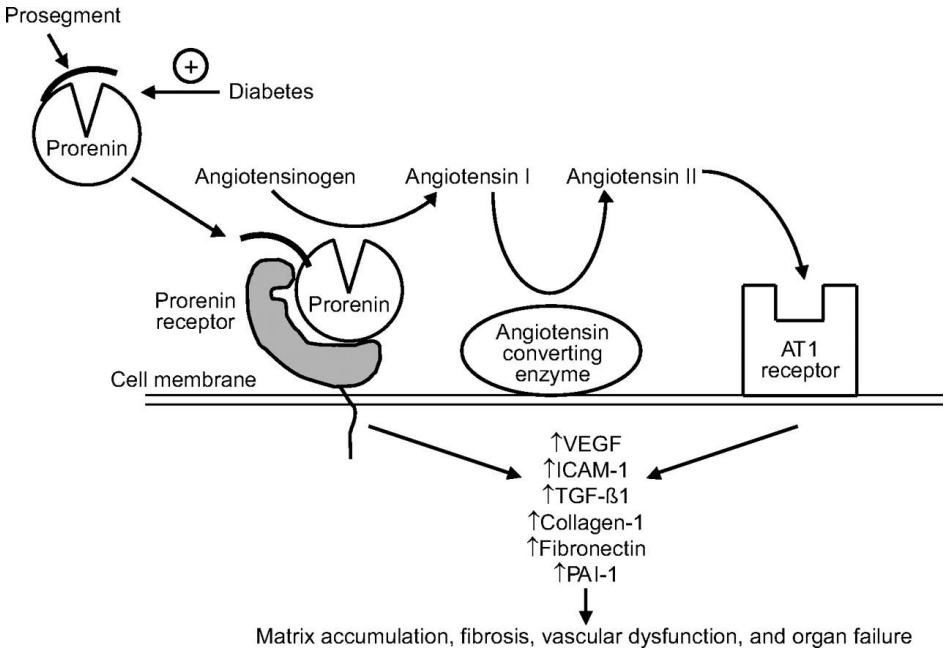


Figure 1.11 - Schematic outline of the potential consequences of prorenin binding to the (pro)renin receptor. Intracellular pathways are activated that can result in fibrotic change, vascular dysfunction and organ failure. Reproduced from (Wilkinson-Berka & Campbell 2009).

As a drug target prorenin has been of interest. Two groups described the prosegment as having a “handle” region that binds to the (P)RR which then allows the “gate” to open, or the prosegment to unfold thus activating the prorenin to renin (Vasan et al. 2002; Lim et al. 2012; Suzuki, Hayakawa, Nakagawa & Nasir 2003b). An antagonist has been developed that mimics the handle region, known as the “handle region peptide” or HRP. By binding the

handle region and not allowing the gate to open, prorenin can theoretically be kept in a “locked” state.

Unfortunately, experiments performed in two-kidney, one-clip Goldblatt rats has not supported a reduction in target organ damage with use of the HRP in renovascular hypertensive rats (Muller et al. 2008). In an experimental heart failure model in sheep however, the use of the HRP was shown to result in cardiovascular and renal benefits in association with inhibition of the renin-angiotensin aldosterone system (Rademaker et al. 2012). A nominally significant reduction in plasma sodium concentrations (P=0.024) in addition to a natriuresis (P= 0.046) was also seen in the heart failure model versus controls. In general however, the efficacy of the HRP remains doubtful with no firm conclusive evidence to support its use as routine (Krop et al. 2013). Further studies are needed to determine its possible use in future therapy.

1.1.4 Diagnosis of Hypertension

Traditionally, detection of hypertension has consisted of multiple readings taken by the auscultatory method over a period of visits to the clinician (Pickering et al. 2006). This is not without criticism, as factors such as variability of blood pressure and patient anxiety, or “white-coat” effect can skew findings considerably. Inter-observer differences also play a role, and NICE guidance recommends that those taking blood pressure measurements ought to be adequately trained and should have their performance reviewed regularly (NICE Clinical Guidance 127, 2011). The ideal and more accurate method of obtaining a true reading involves calculating a mean level over prolonged periods. The way in which this may be achieved is either with self-monitoring (usually home blood pressure monitoring, HBPM) or ambulatory monitoring.

Ambulatory blood pressure monitoring (ABPM) has been in existence for more than fifty years (Lifton et al. 2001; Kain et al. 1964). Patients wear the fully automatic unit for 24 hours or longer and continue about their daily business wearing them on a belt or in a pouch. NICE (2011) Guidance suggests that ABPM readings are taken twice per hour during the patients usual waking hours, for example between 08:00 and 22:00. Patients are recommended to

keep their arm still where possible and safe to do so while the cuff is inflating for greater accuracy of measurement. At the end of the period of recording, the information is downloaded to a computer for analysis. The NICE recommendation suggests using the average value of at least 14 measurements taken during the patient's usual waking hours to confirm the diagnosis. Approved devices are usually accurate to within 5mm Hg of readings taken with a traditional mercury sphygmomanometer. An updated list of monitors validated for use in ambulatory monitoring is available (www.dableducational.org).

ABPM can provide three distinct categories of information:

1. an estimate of the mean blood-pressure level, supposedly equivocal to "true" blood-pressure measurement
2. the diurnal rhythm of blood-pressure – subjects with normotension displaying more pronounced diurnal rhythm of blood pressure (Pickering et al 2006). Normally blood pressure is seen to fall to its lowest levels during the first few hours of sleep with a marked surge in the morning hours coinciding with awakening. A nocturnal dip (10 mm Hg or greater) is seen in most normal subjects, however this has been noted to be absent in some normotensives, in some african-american subjects and is only present in a minority of hypertensive individuals (Profant & Dimsdale 1999). These individuals are referred to as non-dippers. The absence of nocturnal dipping is thought to be due to multiple causes, such as high level of activity during the day, poor quality of sleep, highly active sympathetic nervous system, use of glucocorticoids and presence of renal disease. Interestingly, nondipping has been proposed in some studies as to why african-americans are at higher risk for cardiovascular disease than other racial or ethnic groups.
3. blood-pressure variability – as the blood pressure measurements are taken intermittently and not continuously, a rough estimate of blood pressure variability is obtained (Pickering et al. 2006).

ABPM has an important role in the diagnosis of white-coat hypertension. This is defined as a blood pressure of at least 140/90 on at least three occasions in a

clinic setting, with at least two sets of measurements in non-clinic settings less than the value. To meet criteria, the patient must be free of target-organ damage. It is an important diagnosis to make as it is widely purported that those with white-coat hypertension are generally at low risk. Thus, unnecessary medications are unlikely to benefit the patient. Conversely, hypertension by definition may develop in some of these white-coat patients, with risk of stroke increasing after six years of “clinically” elevated blood pressures (Verdecchia et al. 2005). Hence these are an important cohort of patients to identify. In some instances use of ABPM can avert the need for unnecessary antihypertensives in those without blood pressure control issues whilst identifying those who would warrant close follow up in the future, for example those in the category of prehypertension (Uallachain et al. 2006).

The day-time level of ambulatory blood pressure that is considered the upper limit of normal is 135/85, with both the UK NICE guidance and European Society of Hypertension/European Society of Cardiology (ESH/ESC) agreeing on this figure as a cut-off. This number has been chosen as it approximates to a clinic blood pressure of 140/90 (Conti et al. 2012; Pickering et al. 2005). 140/90 mmHg is the threshold above which an individual’s cardiovascular risk increases significantly (Verdecchia 2000). The general conclusion of several trials looking at efficacy of ABPM has shown that it predicts cardiovascular events better than clinic blood pressure does.

The most widely used predictor of cardiovascular risk is the mean 24-hour blood pressure. Some studies have compared daytime blood pressure with that of night time pressure in their respective predictive values, with some showing no difference (Björklund et al. 2004) and others reporting best indicator of future risk comes from the nocturnal pressure readings (Staessen et al. 1999). The ESH/ESC guidelines consider a mean 24-hour blood pressure greater than 130/80 mmHg a cut-off for the definition of hypertension.

ABPM also has roles in detection of labile hypertension, monitoring resistant hypertension and uncovering masked hypertension. Evaluating response to antihypertensive treatment particularly in clinical trials has proven beneficial in determining true drug-lowering effects in hypertensive cohorts of patients. It has

become established as an accurate method of determining those with “true” hypertension and in assessing efficacy of treatments, particularly during study trials. While it may be costly to run, there are significant long-term economic benefits particularly in terms of appropriate treatment of affected individuals.

1.1.5 Classification of Hypertension

The UK NICE clinical guideline 127 (August 2011) defines Stage 1, Stage 2 and Severe hypertension as follows:

- Stage 1 - clinic blood pressure 140/90 mmHg or higher and subsequent ambulatory blood pressure monitoring (ABPM) daytime average or home blood pressure monitoring (HBPM) average blood pressure 135/85 mmHg or higher
- Stage 2 – clinic blood pressure is 160/100 mmHg or higher and subsequent ABPM daytime average of HBPM average blood pressure is 150/95 mmHg or higher
- Severe – clinic systolic blood pressure is 180 mmHg or higher, or clinic blood diastolic blood pressure is 110 mmHg or higher.

If clinic blood pressure is 140/90 mmHg or higher, NICE guidance advocates offering ambulatory blood pressure monitoring to confirm the diagnosis. Home blood pressure monitoring (HBPM) may also be used to confirm a diagnosis of hypertension. Patients are instructed that for each blood pressure recording, two consecutive measurements are taken, at least one minute apart and with the person seated. BP should be recorded twice daily, morning and evening, and the series of readings should be taken for at least 4 days, ideally 7 days. The measurements obtained on the first day are discarded and the average of remaining values are used to confirm the diagnosis of hypertension.

1.2– Genetics of Hypertension

1.2.1. What is known?

Identifying genes responsible for particular disease pathways should ideally allow us determine the functionality of that gene and that gene's effect on the disease process. This in turn has the potential to identify targets and specific treatments for that disease entity. This is an area of great interest within the field of hypertension research currently as we seek to explain disease process and target existing and new treatments for better blood pressure control. Genetics is already well established as having a role in the aetiology of hypertension. Up to 50% of blood pressure variation has been shown to be explained by genetic factors across several populations (Arnett et al. 2006). Early twin studies have been very important as a means of quantitative genetic analyses in humans (Luft 2001).

Monozygotic twins (MZ) are a result of division of one zygote, therefore two identical copies or clones of genetic material are formed. Dizygotic twins (DZ) are more like siblings in the traditional sense, in that only half their genes are shared, they originate from two separately fertilised eggs and no "cloning" occurs. In theory therefore, any phenotypic or physically measured differences between the monozygotes will be caused by external or environmental factors. MZ twin pairs and DZ twin pairs are likely to be exposed to the same environmental factors. Genetically however, they are two distinct groups. Luft identifies DZ twins as ideal control subjects in the quest for causative genetic factors in disease processes (Luft 2001). The difference between MZ and DZ twins also allows us to evaluate interactions between genetic and environmental factors in both groups. Hypertension can be considered a complex genetic disease because of such interplay between genetics and environmental factors. The total number of genes related with hypertension remains unknown.

One of the earliest and well known twin study was performed by Stocks (Duke Evidence Based Practice Center 2011; Stocks 1930). This study compared the incidence of hypertension between monozygotic (MZ) and dizygotic (DZ) twins. Stocks (1930) studied 93 MZ English twin pairs, 101 DZ pairs opposite in gender, 85 same-sex DZ pairs, 248 pairs of brothers and sisters and 286 sets of

same-gender sibling relations. Their intra-class blood pressure correlation was 0.81 for MZ twins, 0.44 for same-sex DZ twins and 0.45 for same-sex sibling relationships. In 1977, twin studies demonstrated greater concordance of blood pressures of monozygotic twins than dizygotic twins, further confirming a genetic influence on blood pressure (Lumbers 1971; Feinleib et al. 1977).

In the Montreal Adoption Study both genetics and household environment and their effects on normal blood pressure variation were studied. This study was a cross-sectional epidemiologic study in French-Canadian families conducted from 1972 to 1974 (Mongeau et al. 1986). Readings were taken from some 756 adopted and 445 natural children. These were compared with readings of 1176 parents. Systolic blood pressure (SBP) correlation coefficients (r) between siblings were 0.38 and 0.16 for between biological and between adoptive siblings respectively. Similarly, diastolic BP (DBP) coefficients between siblings were 0.53 versus 0.29 for biological and adoptive siblings respectively, demonstrating the strong genetic component of blood pressure variance.

Correlations between mothers and their natural children for SBP were observed at 0.27 with a significantly less ($p=0.001$) correlation seen between mothers and adopted children at 0.08. Likewise for fathers, the correlation with their natural children was significantly different from that of their adopted children at 0.24 and 0.09 respectively ($p=0.001$). Within the study models, the study also demonstrated that for systolic blood pressure, 61% of the expected correlation was due to shared genes, and about 39% due to the environments within which both parents and children lived (Mongeau et al. 1986). The observed results demonstrated the importance of considering genetic factors in blood pressure control and the interplay between genes and the environment.

Whilst it is important to recognise genetic associations with blood pressure, we need to know what these associations are, and how they work. Aside from the 10 or so known Mendelian forms of hypertension, it has not been possible to ascribe one gene as the sole causative agent in other forms of hypertension. In other words, essential hypertension does not seem to follow a clear pattern of inheritance (Danziger 2001). Within essential hypertension a single gene's variants might account for only a small portion of the individual's susceptibility to

the disease. I will expand this point by describing some of the more commonly known monogenic forms of hypertension that result from a single gene defect. Such defects are inherited in simple Mendelian fashion.

1.2.1.1 Mendelian Blood Pressure Variation – Common Monogenic Traits

Monogenic variations responsible for blood pressure differences have been instrumental in allowing us to understand why hypertension and indeed hypotension develop (Lifton et al. 2001). Through them we have identified a particular physiologic pathway in the kidney, all variants being involved in pathways of renal salt absorption. Renal tubular electrolyte transport functions are the target for each of these genes (Padmanabhan et al. 2009). These Mendelian or monogenic genes are typically responsible for large effect changes in blood pressure.

It is mutations along this and other renal pathways that result in what we now recognise as monogenic or Mendelian forms of hypertension – single gene defects with loss of blood pressure control and hyper- or hypotension as the consequence. To date, molecular genetic studies have identified mutations in eight nuclear genes and one mitochondrial gene causing Mendelian forms of blood pressure (M. Gong & Hubner 2006). The most common of these are discussed briefly below and in Table 2.

The first to be recognised as a single-gene hypertensive disorder was glucocorticoid-remediable aldosteronism (GRA) or familial hyperaldosteronism type I (FH-I) (Lifton et al. 1992). Interestingly, an unexpectedly high prevalence of Irish ancestry of the GRA allele was observed, with a documented Irish allele in six pedigrees and a likely Irish origin in a further two. The genetic defect is the presence of a chimeric gene (miotic mismatch and unequal crossing over) on chromosome 8 consisting of the promoter-regulatory 5’ 11-hydroxylase gene, CYP11B1, coupled with the distal structural portion of aldosterone synthase gene, CYP11B2. A gene duplication occurs by this unequal crossing-over between two closely related genes involved in adrenal steroid biosynthesis. What results is an autosomal dominant transmission of hypertension, with variable levels of elevated aldosterone, suppressed plasma

renin levels and high levels of abnormal adrenal steroids 18-hydroxycortisol and 18-oxycortisol. As a result of the genetic change, aldosterone now responds to adrenocorticotrophic hormone (ACTH) instead of Angiotensin II (Ang II), and is consequently suppressible by exogenously administered glucocorticoids. Penetrance is high, often with hypertension manifesting from childhood (Lifton et al. 1992).

Liddle's syndrome is also an autosomal-dominant form of monogenic hypertension. To confirm that this hypertensive disorder resulted from a structural defect within the kidney, a renal transplant was performed on a patient with Liddle's syndrome which was curative (Botero-Velez et al. 1994). Further studies have shown that the problem is characterised by hyperactivity of the amiloride-sensitive sodium channel (ENaC) of the principal cell of the cortical collecting tubule (CCT) (Shimkets et al. 1994; Bubien 2010). The defective gene encodes for the ß subunit (SCNN1B) of the ENaC and remains inappropriately open or permeable. A mutation in the γ subunit of ENaC, SCNN1G was also found which can result in Liddle's syndrome (Hansson et al. 1995). These mutations result in constitutive expression of sodium channels at the apical surface of tubular cells (Schild et al. 1995; Lifton et al. 2001),they are never internalised or degraded and remain activated (Shimkets et al. 1994). As such, there are increased rates of sodium reabsorption, volume expansion and hypertension.

Pseudohypoaldosteronism type II or Gordon's syndrome manifests as familial hypertension with hyperkalaemia. Mutations within a family of serine-threonine kinases, WNK1 and WNK4, were found to be responsible for the biochemical changes (Wilson et al. 2001). Increased expression of WNK1 and increased activity of WNK4 as a result of genetic mutations (large intronic deletions and missense respectively) are associated with increased sodium (Na) chloride (Cl) Na-Cl cotransporter activity in the distal tubule, with excessive chloride and sodium reabsorption and volume expansion (Wilson et al. 2003). The resultant increase in WNK1 expression results in prevention of WNK4 suppression of the cotransporter.

An autosomal-recessive disorder, apparent mineralocorticoid excess, is also associated with a phenotype that includes hypertension. An individual ingesting large amounts of liquorice, “liquorice gluttony”, would manifest the same biochemical changes – low renin, low aldosterone and a salt-sensitive form of hypertension (Luft 2004). Antagonists of MR were found to lower blood pressure in these patients which was what led investigators to seek another circulating mineralocorticoid in the late seventies (New & Levine 1977). It results from mutations in the 11-beta hydroxysteroid dehydrogenase type II gene, HSD11B2. Normal rapid conversion of cortisol to cortisone is prevented. This usually has a protective function in that cortisol will bind the mineralocorticoid receptor at an equal affinity to aldosterone, and by converting from cortisol to cortisone it is no longer able to do so (Funder 1987; Stewart et al. 1987). This is important as the amound of circulating cortisol is some 1000-fold higher than aldosterone. So, in 11ßHSD deficiency, cortisol then acts on the mineralocorticoid receptor (MR) to increase renal sodium reabsorption via EnaC (White et al. 1997).

Severe hypertension during pregnancy is seen in women with a leucine for serine substitution at codon 810 in the MR at it’s hormone-binding domain. This substitution results in the MR being highly sensitive to steroid hormones lacking a 21-hydroxyl group (Geller et al. 2000). These normally antagonistic steroid hormones, including progesterone, become MR receptor agonists. The near-100 fold increase in progesterone during pregnancy enhances the agonist effect with affected females exhibiting severe hypertension during pregnancy as a result. Hypertension in pregnancy is a known major cause of maternal and foetal morbidity. 5% of stillbirths in infants without congenital abnormalities in the UK are attributable to maternal pre-eclampsia. In addition, hypertension remains one of the leading causes of maternal death in the UK (Redman 2011).

Autosomal dominant hypertension with brachydactyly (HTNB) is characterised by severe high blood pressure with short fingers, involving a defect in baroreflex blood pressure regulation. This does not affect heart rate, but results in high blood pressure that is salt-independent, age-dependent and at worst, uncontrolled. If left untreated, stroke may result before 50 years of age. In a cohort of 15 individuals with the syndrome, neurovascular anomalies (NVA)

were identified that were thought to contribute to the impaired baroreflex function by direct pressure on the ventrolateral medulla (Naraghi et al. 1997).

Luft et al., mapped the condition to the short arm of chromosome 12 in work that had been ongoing since 1994 to identify the gene involved (Luft 2008). In 2014, 6 missense mutations in phosphodiesterase 3A (PDE3A) gene were identified in 6 unrelated families with HTNB (Skinner et al. 1975; Maass et al. 2015). Phosphodiesterase (PDE) proteins have a role in early osteogenesis, and in mice PDE3A is expressed in the developing limbs. The PDE3A mutation also promotes vascular smooth muscle proliferation, resulting in vessel wall hyperplasia and increased peripheral vascular resistance. In addition, PDE3A mutants contribute to downregulation of expression of the parathyroid hormone-related protein gene (PTHLH) which encodes parathyroid hormone-related peptide (PTHrP). PTHLH downregulation was observed in chondrogenically induced fibroblasts from affected individuals, with dysregulated PTHrP levels. Two translocations within chromosome 12 of individuals with brachydactyly had previously been observed to downregulate the PTHLH gene (Maass et al. 2012), further supporting the findings of Maass et al (2012). It has been suggested that VSMC-expressed PDE3A ought to be a focus for drug development in the treatment of hypertension (Maass et al. 2015).

A Caucasian kindred (K129) with hypomagnesaemia was identified by Wilson et al. (Wilson et al. 2004). Through the evaluation of 142 blood relatives, the Study revealed a high prevalence of hypomagnesaemia, hypertension and hypercholesterolaemia. With an initial focus on hypomagnesaemia, mitochondrial inheritance was deemed likely. Affected fathers did not transmit the gene to their offspring, but a high proportion of affected mothers transmitted the gene to 16 of 21 offspring studied (Wilson et al 2004). A marked increase in the urinary fractional excretion of magnesium was observed in members of the maternal lineage, with the same members having lower serum magnesium levels when compared to non-maternal lineage (Wilson et al 2004).

32 members of the kindred had clinically significant hypomagnesaemia, 38 were hypertensive (blood pressure >140/90 mmHg or treated for hypertension) and 33 had hypercholesterolaemia (Wilson et al 2004). 30 of 38 adults on the

maternal lineage were hypertensive versus 8 of 53 on the nonmaternal lineage. Hypertension in the maternal lineage was markedly age dependent. The prevalence of hypertension on the maternal lineage increased from 5% in subjects under 30 years of age to 95% in those over 50 years of age (19 of 20 subjects).

All affected individuals were found to have a thymidine-to-cytidine transition associated with the mitochondrial tRNA isoleucine gene (Wilson et al 2004). This results in impaired mitochondrial function. Mitochondria have a vital role in energy production, converting nutrients into adenosine triphosphate through the process of oxidative phosphorylation. The distal convoluted tubule requires ATP-dependent sodium reabsorption to allow magnesium reabsorption, with the highest energy consumption of the entire nephron (Reilly & Ellison 2000; Simon et al. 1996). In addition, reduced mitochondrial ATP increases cholesterol biosynthesis and has also been reported in animal models of hypertension (Zager et al. 2003; Atlante et al. 1998). Hence, this syndrome with loss of mitochondrial function results in hypomagnesaemia, hypercholesterolaemia and hypertension.

Table 1. 3 - Mendelian forms of hypertension - causative mutations. MOI, mode of inheritance; AD, autosomal dominant; AR, autosomal recessive; Mit., mitochondrial; CYP-, cytochrome-P450 enzyme; SCNN-, Sodium-channel non-voltage gated; WNK, with-no-lysine serine threonine kinase channel; HSD11B2, hydroxylsteroid (11-beta) dehydrogenase isoenzyme 2; NR3C2, nuclear receptor subfamily 3, group C, member 2 - gene encoding mineralocorticoid receptor; PDE3A, phosphodiesterase 3A; MT-TI, mitochondrially encoded tRNA isoleucine

Monogenic Syndrome	Causative Gene	MOI	Chromosome Location
Glucocorticoid-remediable aldosteronism, GRA	CYP11B1 and CYP11B2	AD	8p
Pseudo-aldosteronism (Liddle's Syndrome)	SCNN1B and SCNN1G	AD	16p
Familial hyperkalaemic hypertension, FHH (Gordon's Syndrome)	WNK1 and WNK4	AD	12p, 17q
Apparent mineralocorticoid excess, AME	HSD11B2	AR	16q
Hypertension exacerbated in pregnancy	NR3C2	AD	4q
Hypertension with brachydactyly, HTNB	PDE3A	AD	12p
Hypertension, hypercholesterolaemia and hypomagnesaemia	MT-TI	Mit.	Mit.

Through our understanding of some of the disorders above, researchers in the field of hypertension have been able to search for new candidate-genes assuming similar disease pathways are responsible (Turner & Boerwinkle 2003). There are two ways in which to explore complex and quantitative genetic traits, either candidate gene studies or genome-wide association scans (Hobart et al. 1984; Zhu & Zhao 2007).

1.2.1.2 Hypertension – A Complex Disorder

Pickering was one of the first to identify hypertension as a complex genetic trait (G. Pickering 1965). As hypertension is not a single-gene disease for the majority of patients, ways had to be found to identify and single-out individual genes from such polygenic systems. Association studies, linkage analysis and genome scanning have all been employed to better understand the genetic mechanisms underlying hypertension (Delles et al. 2010).

3 to 3.5 billion bases make up the human genome. Out of this total, approximately 3% encode genes accompanied by their regulatory sequences (Doris 2002). Genetic variations responsible for hypertensive phenotypes lie within these coding and regulatory regions. It is estimated that common sequence variants exist in around 1 in every 1000 bases of coding/regulatory genetic sequences (Halushka et al. 1999). Therefore, there are approximately 100,000 genetic variations from which hypertensive phenotypes may arise. Given that hypertension is a complex genetic trait, more than one genetic variation is responsible, unlike the monogenic Mendelian forms of inherited hypertensive states.

DNA sequence changes contributing to the hypertension phenotype originate from di, tri, and tetra nucleotide repeats; deletions, insertions or duplications and nucleotide substitutions (Ku et al. 2010). Single-nucleotide polymorphisms (SNPs), frequently considered as point mutations, represent one form of nucleotide substitution in the genome. At least 90% of human genes contain at least one SNP with more than 14 million SNPs having been identified in the human genome, some with no identifiable function and some with significant effect on gene function (Ma & A. Y. H. Lu 2011). Those occurring in the coding region of the gene can ultimately result in amino acid sequence change with the protein. More than 60,000 SNPs are located in these coding regions (Sachidanandam et al. 2001).

SNPs with significant function however can occur at any region along the gene even when not associated with the transcription start site itself (Ma & A. Y. H. Lu 2011). For the SNP to be considered common, it must have a minor allele frequency, or the frequency at which the less common allele occurs in the population, of at least 1% in the general population (Doris 2002). It is however increasingly apparent that it is very rare for a single SNP to account for large changes in blood pressure levels. More than likely, higher blood pressure results from the cumulative effect of several SNPs functioning together.

1.2.2 Candidate Gene Studies

Candidate gene studies look specifically at a candidate locus or a region of interest within a gene involved in the pathophysiology of hypertension. The prevalence of hypertension among individuals with varying genotypes at the locus is compared. Level of blood pressure associated with the various genotypes can also be assessed (Lifton et al. 2001). A major limitation with candidate gene approach is that some functionality of the gene, either physiological or biochemical, must already be known to then attempt to identify an association between that process and the disease in question.

Prominent polymorphisms have been identified in several systems including the renin-angiotensin-aldosterone system (RAAS) and genes acting on pathways for blood pressure regulation outside the renin-angiotensin-aldosterone system. The latter includes functional polymorphisms of the genes encoding subunits of the epithelial sodium channel (ENaC), polymorphisms in genes encoding the ß-adrenergic receptor and SNPs in the gene encoding alpha-adducin (Padmanabhan et al. 2010; Turner et al. 2003; Turner & Boerwinkle 2003). Most candidate gene work has focused on renal tubular sodium transport mechanisms and the RAAS. The genetics of the renin-angiotensin system will be considered as a separate section within this chapter.

A functional polymorphism within ENaC was shown to be associated with hypertension, particularly in black patients of African descent (Baker et al. 1998). This Study identified a point mutation, threonine 594 methionine, T594M, of the sodium channel beta subunit. T594M was observed in 8.3% of London Black hypertensives compared to 2.1% normotensives of the same population (Baker at al. 1998). T594M is a commonly identified sodium-channel point mutation and is associated with increased sodium-channel activity (Su et al. 1996). It was postulated that via increased ENaC activity, renal tubular reabsorption of salt was also increased with a subsequent rise in blood pressure.

A further variant in the ENaC beta-subunit, out of five studied, was found to be more common in blacks than whites, G442V (Ambrosius et al. 1999). This variant was identified in 16% of blacks studied, and only one white (249 white

participants and 181 black young participants took part). However, Ambrosius (1999) was unable to replicate this finding in a study of an older population. This mirrors the problem with candidate gene studies over the years – in the majority of cases findings are not replicated in subsequent studies.

The β2-adrenergic receptor has also been a focus for candidate gene studies (Bray et al. 2000). These receptors respond to β-agonists resulting in vasodilation. It is likely that β2-adrenergic receptors are also present on both the proximal tubule and distal convoluted tubule (Gesek & K. E. White 1997). Given their location here, they may also have a role in tubular sodium reabsorption. Early studies demonstrated the Arg16Gly receptor variant to be associated with hypertension. The Glycine (Gly) variant was shown by Gratze et al to be associated with higher blood pressures (Gratze et al. 1999). The Study demonstrated that the vasodilatory response to salbutamol infusion was significantly decreased in those homozygous Gly16 subjects compared with Arg16Arg subjects.

Alpha-adducin is thought to regulate activity of the sodium-potassium ATPase pump, influencing sodium reabsorption, and therefore sodium and water retention (Ferrandi et al. 1999). SNPs in the gene that encodes alpha-adducin have been associated with essential hypertension, particularly the salt-retaining form (Cusi 1997; Cusi et al. 1997). Glorioso (1999) and colleagues studied an alpha-adducin Gly460Trp polymorphism in 490 hypertensive and 176 normotensive individuals from Sassari, Italy and in 468 hypertensive and 181 normotensive individuals from Milan (Glorioso et al. 1999). The Milan cohort demonstrated a positive association, whilst the Sassari cohort did not. Unfortunately, a meta-analysis performed last year failed to provide evidence for a genetic assocation with hypertension (K. Liu et al. 2010). In this meta-analysis, 22 out of a possible 152 PubMed citations were included in the analysis with 14303 cases and 15961 controls.

Other candidate genes of interest in hypertension have included polymorphisms within human atrial natriuretic peptide (hANP) with its role in salt excretion (Nannipieri et al. 2001), 11-beta-hydroxysteroid dehydrogenase, with a role in hypertension via cortisol stimulation of the mineralocorticoid receptor (MR)

(Lovati et al. 1999), endothelium-related factors and the nitric-oxide synthase gene (Kato et al. 1999) and dopamine, produced by the renal proximal tubules with its effect on sodium excretion and resultant effects on blood pressure (Felder et al. 2002).

The effects of any of these candidate gene variations alone on blood pressure levels are known to be small. This may well partially explain the failure to replicate consistently throughout different trial cohorts. A significant risk with candidate gene studies is the potential for selection of the wrong gene for the study, yielding little insight. This could occur through a lack of understanding of the biology underlying the development of the trait. The ability to detect an association depends upon the researcher's assumptions about the function of a gene being accurate. In addition, new genes are difficult to identify *de novo* using this approach given that some previous knowledge is required about disease mechanism to find the gene of interest. Candidate gene analysis has however proven fruitful in some cases in predicting responses to antihypertensive agents.

1.2.3 Association Studies – Linkage Analyses

Association studies are used to identify particular genes as strong candidates after a chromosomal region of interest has been linked to the trait. To do this, association studies rely on linkage disequilibrium (LD). Linkage disequilibrium occurs when certain allele or genetic variant combinations at closely linked genes are more frequent than might be expected randomly. In other words, it is the increased sharing of alleles above that expected by random sharing (Doris 2002).

Genetic linkage occurs when particular alleles or gene variants are inherited together. This linkage of genes and genetic markers occurs because of their close proximity on the same chromosome. The degree of LD measures non-random associations between genes. A logarithm of the odds score (LOD) of three or more suggests that two gene loci are close to each other, and is supportive of genetic linkage. A LOD of three means the odds are a thousand to one in favour of genetic linkage (Nyholt 2000).

Individuals are genotyped at the genetic locus of interest in a particular gene. Then it is tested to identify whether or not individual differences at this locus are statistically associated with a particular disease phenotype. This is done for both individuals with hypertension and controls with no hypertension. Chi-square tests are often used to determine distribution differences between the disease phenotype population and the control population. If an allele has a convincingly different frequency in cases compared with unaffected controls then this could be due to the allele having a causal involvement in disease susceptibility. It could also suggest linkage disequilibrium with an allele at a nearby site. Analyses of families are frequently used to attempt to link various loci associated with hypertension. This is achieved through the use of microsatellite markers consisting of variable number of repeats (Luft 2004). A microsatellite is sought out that is always inherited with the trait to give information regarding the location of that particular trait.

The first genome-wide linkage analysis was performed in 1999 in the Rochester Minnesota (MN) population (Krushkal et al. 1999). 3974 members of 583 multi-generation pedigrees underwent measurement of systolic blood pressure. Three readings were obtained two minutes apart, with the average of the readings used in the linkage analysis. Of the 583 pedigrees, 55 were chosen and genotyped for 359 polymorphic marker loci. The 55 pedigrees chosen were selected as pedigrees with 1 or more siblings above sex- and age-specific 80^{th} percentile and 1 or more siblings below the sex- and age-specific 20^{th} percentile of the systolic blood pressure distribution (Krushkal et al. 1999). A total of 427 individuals were included in the final analysis. Regions encoding genes influencing blood pressure were found on chromosomes 2, 5, 6 and 15. These regions showed significant linkage to genes that influence SBP variation ($P<0.01$) (Kruskhal et al. 1999). The results of this study provided a focus for future genetic studies. Since then, several chromosomal regions have been demonstrated that are linked to blood pressure and essential hypertension.

1.2.3.1 The Family Blood Pressure Program

The National Heart, Lung and Blood Institute in the United States (NHLBI) established the Family Blood Pressure Program (FBPP) in 1995 (FBPP Investigators 2002). The objective was to localise and identify genes responsible for inter-individual blood pressure variation across multiple racial or ethnic groups. The FBPP also sought to identify genes influencing the occurrence of hypertension in the general population. The programme recruited participants from the African American, Mexican American, Asian and non-Hispanic white populations. Four networks were set up to facilitate the collection of data. This resulted in a pooled data set with over 120 phenotypic variables gathered for 11,357 subjects (FBPP Investigators 2002). The population studied is summarised in Table 3, overleaf.

The FBPP focused the search for genes associated with hypertension on chromosome 2, where evidence of genetic linkage suggested that one or more loci were associated with hypertension status, contributing to blood pressure (Barkley et al. 2004). 8 genes were selected for genotyping. 82 SNPS within the 8 candidate genes were genotyped in 4595 individuals from the African American, white and Mexican American "Genetic Epidemiology Network of Atherosclerosis" (GENOA) population. Subsequently, *SLC4A5* was identified as a candidate hypertension susceptibility gene (Barkley et al. 2004). *SLC4A5* is a sodium bicarbonate cotransporter, highly expressed in the liver and spleen with moderate expression levels in the heart and kidney. The sodium bicarbonate cotransporter mediates electrogenic sodium bicarbonate cotransport and contributes to intracellular pH regulation. SNPs within the *SLC4A5* gene were also associated with blood pressure levels and hypertension status when analysed in the African Americans and white populations (Barkley et al. 2004). One particular *SLC4A5* SNP, hcv8941031, was significantly associated with hypertension in the African American population of the GenNet and HyperGen (Hypertension Genetic Epidemiology Network) populations, p=0.027. Whether this ethnic difference was as a result of differences in allele frequencies or environment-genotype interactions was unknown. Still, *SLC4A5* had been

identified as a gene on chromosome 2 with the potential to influence hypertension in the wider population (Barkley et al. 2004).

Table 1.4 – Descriptive statistics for pooled FBPP population. Statistics are represented by percentage, mean and standard deviation of mean, [mean(SD)], and count for categorical variables. %, percentage; n, number; FBPP, Family Blood Pressure Programme; GenNet, GenNet; GENOA, Genetic Epidemiology Network of Atherosclerosis; HyperGEN, Hypertension Genetic Epidemiology Network; SAPPHIRe, Stanford Asian Pacific Program

Item	GenNet (n=1253)	GENOA (n=4329)	HyperGEN (n=3262)	SAPPHIRe (n=2513)	POOLED (n=11 357)
Male, %	43.8	39.2	41.1	44.5	41.4
Age at clinic visit (years)	42.7(13.1)	56.4(11)	55.7(11.2)	53.5(12.1)	54(12.3)
Race					
White (non-Hispanic)	617 (50.0%)	1267 (29.3%)	1580 (48.6%)	0	3464 (30.6%)
Black (non-Hispanic)	618 (50.0%)	1621 (37.5%)	1671 (51.4%)	0	3910 (34.5%)
Hispanic	0	1440 (33.3%)	0	0	1440 (12.7%)
Asian	0	0	0	2510 (100%)	2510 (22.2%)

1.2.3.2 Framingham linkage analyses

Linkage analyses of the largest families from the Framingham cohort were performed for longitudinally measured systolic and diastolic blood pressure data in the 100K Project (Levy et al. 2007). The Framingham Heart Study began in 1948, characterising blood pressure and arterial stiffness whilst collecting additional phenotypic data in two generations of patients (Levy et al. 2000). For long-term systolic blood pressure (SBP), linkage resulted in a LOD score of 3 on chromosome 15 (Levy et al. 2007). In addition, a linkage peak that had previously been described by the same study team was reproduced at Chromosome 17. In earlier work, Levy at al. (2000) had identified two markers with LOD scores of >2 associated with hypertension on chromosome 17, one overlying the locus encoding angiotensin converting enzyme (ACE) (Levy et al. 2000). The 100K findings provided further evidence for the involvement of chromosome 17 in hypertension. In the 100K project however, only weak

evidence of association with blood pressure and arterial stiffness was observed for 69 SNPs in 6 renin-angiotensin system genes (Levy et al. 2007).

1.2.3.3 The Medical Research Council (MRC) funded BRIGHT study

The MRC British Genetics of Hypertension (BRIGHT) study recruited 1599 severely hypertensive families, with each family containing at least two affected siblings. A total of 3599 individuals were genotyped for analysis. One chromosomal region with an LOD score greater than 3.12 was identified, on long arm-chromosome 6 (6q). This result remained significant at the genome-wide level (Caulfield et al. 2003). 3 further areas were identified with LOD scores >1.57 on chromosome 2q, 5q and 9q. Of interest, the short-arm chromosome 2p (2p) locus identified in the African American individuals in the Family Blood Pressure Programme (FBPP) is located proximal to the 2q locus identified in the BRIGHT cohort (Barkley et al. 2004).

In a subsequent study, 2142 severely hypertensive white sibling pairs identified from a refined BRIGHT cohort were stratified into two groups based on response to antihypertensive therapy. Patients were assigned to receive either renin angiotensin system (RAS) blockade (angiotensin-converting enzyme inhibitor/angiotensin receptor blocker or beta-blocker), “AB”, or antihypertensives that do not block the RAS (calcium channel blockers or diuretics), “CD” (Padmanabhan et al. 2006). This was in line with National Institute for Health and Care Excellence (NICE) guidance regarding the management of hypertension (B. Williams & Taryn Krause 2011). Non-responders from both treatment groups were analysed for evidence of linkage. Significant linkage was observed for the AB group on chromosome 2p (Padmanabhan et al. 2006). The region identified in White European non-responders co-localised to the region described in the African American population of the FBPP. It was therefore postulated that this region may contain one or several genes for the salt-sensitive form of hypertension.

A limitation to linkage studies is that they require large numbers of families with several affected generations. In addition, complex traits, where multiple genes

are involved in a disease process, are difficult to define using linkage studies. They are also less useful for identifying areas of disease risk in late-onset disease where high mortality may mean finding families with more than one affected generation is difficult.

1.2.4 Genome-wide association studies

By contrast with linkage analysis, genome-wide association studies (GWAS) can be conducted in unrelated hypertensive individuals. They search the entire genome for associations rather than focusing on small candidate areas. However, large numbers of SNPs need to be genotyped in a large number of individuals. On the positive side, the association analysis has far greater power to detect variants of modest effect and lower frequency (Hastie et al. 2010). GWAS is also more powerful than linkage in quantifying trait loci with effects on drug response (Risch & Merikangas 1996). This will be further discussed in our section on pharmacogenetics.

1.2.4.1 Wellcome Trust Case Control Consortium

The Wellcome Trust Consortium undertook genome-wide association (GWA) studies looking at genetic variation at 500,000 different positions in some 17,000 individuals living in the UK. 7 complex human diseases including hypertension and coronary artery disease were studied. Also evaluated for genetic associations were bipolar disorder, Crohn's disease, rheumatoid arthritis, type I diabetes and type II diabetes (Burton et al. 2007). Approximately 2000 individuals were evaluated for genetic associations for each of the 7 major diseases. The 2000 hypertension cases in the Wellcome Trust Case Control Consortium were unrelated participants from the British Genetics of Hypertension (BRIGHT) Study (Caulfield et al. 2003).

For the 7 disease phenotypes studied, evidence for association of 24 SNPs was identified for 6 of the diseases, excluding hypertension. P-values for those associations were lower than the threshold 5×10^{-7}. There were no SNPs defined for hypertension with significance or p-value less than 5×10^{-7}. Of note, 58 loci across all disease areas including hypertension were identified with p-values between 10^{-5} and 5×10^{-7}. For hypertension, six loci were located at

chromosomes 1, 8, 12, 13 and 15. These were thought to have the potential to confer risk for each of the 7 diseases. However, the Wellcome Trust CCC was undertaken more as a validation to the approach of GWAS and several observations were made in terms of various methodological issues that could improve future GWA studies. Subsequent to the WTCCC, two large GWA studies published in Nature Genetics in 2009 did identify several regions with genome-wide significant loci influencing blood pressure (Newton-Cheh et al. 2009; Levy et al. 2009).

The Global Blood Pressure Genetics (Global BPgen) Consortium study identified eight loci associated with either diastolic blood pressure (DBP) or systolic blood pressure (SBP) genome-wide in a population of 34,433 (Newton-Cheh et al. 2009). During the first stage, genome-wide association studies were performed in a total of 34,443 individuals of European descent. 13 population-based cohorts were compared with controls from 4 case-control series (Caulfield et al. 2009). The 17 cohorts studied formed the Global BPgen Consortium. On meta-analysis of the results for stage 1, 11 signals with $p < 10^{-5}$ were observed for SBP, and 15 for DBP. Follow-up genotyping was then performed in up to 71,225 individuals from 13 cohorts of European descent, and up to 12,889 individuals of Indian Asian descent (Stage 2a). Computer-based analysis (*in silico* analysis) was then performed alongside the Cohorts for Heart and Aging Research in Genome Epidemiology (CHARGE) Consortium by "exchange of association" (Stage 2b). Meta-analysis of Stage 1, 2a and 3a resulted in the identification of 8 loci associated with hypertension that attained genome-wide significance. 3 of these overlapped with the CHARGE Consortium findings. Of note, no significant interaction was observed between each of the 8 SNPs identified and gender.

Each association identified only contributed to a small degree to the total variation in blood pressure observed. Translation of effect resulted in an approximate 1 mmHg increase in SBP and an approximate 0.5 mmHg increase in DBP per allele. However it is recognised that hypertension is polygenic and the inheritance is complex. Therefore, several alleles in combination would potentially result in greater increases in blood pressure with an associated increase in cardiovascular risk. Observational data has shown us that a

sustained rightward shift in diastolic blood pressure of 5mm Hg is associated with a 34% increase risk for stroke and a 21% increase in risk of coronary events (MacMahon 1990). The aggregate effect of alleles on blood pressure status must not be dismissed.

The Cohorts for Heart and Aging Research in Genome Epidemiology (CHARGE) Consortium study was comprised of six groups (Levy et al. 2009). The CHARGE Study was populated mainly by individuals from the Framingham Heart Study (FHS) and Atherosclerosis Risk in Communities (ARIC). 13 significant SNP associations were identified for SBP, 20 for DBP and ten for hypertension in a sample size of 29,136. The top ten loci for each of SBP, DBP and hypertension were checked for significance against the Global BPGen results. Five of six SNPs assessed for replication in Global BPGen attained p-values <0.008 (Levy et al. 2009).

When the same 30 SNPs were included in a joint meta-analysis with the Global BPGen Consortium, four CHARGE loci attained genome-wide significance for SBP, six for DBP and one for hypertension. SNPs for each risk allele attaining genome-wide significance in the combined analysis with Global BPGen inferred modest effect sizes of approximately 1 mmHg and 0.5 mmHg increases in SBP and DBP respectively. Interestingly, the CHARGE investigators went on to look at the relationship between number of alleles carried and increase in blood pressure. They observed a linear increase in BP of several mmHg as alleles accumulated (Levy et al. 2009). If this was to be replicated in another large population it would support the notion of hypertension as a polygenic trait.

The strongest signals for systolic blood pressure (SBP) for each study were common to both studies – loci for ATP2B1 and steroid 17-α hydroxylase, CYP17A1 (Levy et al. 2009; Newton Cheh et al. 2009). The strongest evidence for association with SBP in the CHARGE Consortium was ATP2B1. Located on chromosome 12q21, the gene encodes the plasma membrane calcium-transporting ATPase 1. This enzyme plays an important role in calcium homeostasis. The strongest evidence for association with SBP in the Global BPGen study with systolic blood pressure (SBP) was located at 10q24. This locus includes six genes, the most notable being the CYP17A1. This is a

Cytochrome P450 enzyme that is key in biosynthesis of mineralocorticoids and glucocorticoids and also sex-steroid biosynthesis. A missense mutation results from a single nucleotide change in a codon coding for a different amino acid. The resulting protein may be rendered non-functional. Missense mutations in CYP17A1 cause adrenal hyperplasia, hypertension being one of the consequences.

SH2B3 was identified as significantly associated with both SBP and diastolic blood pressure (DBP) in both studies, and as the most significant SNP for diastolic blood pressure (DBP) in the CHARGE Consortium (Levy et al. 2009). The minor allele has recently been associated with autoimmune diseases such as type I diabetes (J. A. Todd et al. 2007) and coeliac disease (Hunt et al. 2008). SH2B3 is expressed in haematopoietic precursor and endothelial cells and is involved in a range of signalling activities by growth factor and cytokine receptors (Fitau et al. 2006). Minor allele expression is thought to be associated with loss of SH2B3 function in humans. Studies of murine knockouts of the SH2B3 gene, also known as lymphocyte-specific adaptor protein or LNK, have shown LNK to be a negative regulator of inflammatory signaling pathways in the endothelial cell. Thus with minor allele carriage of the SNP one could postulate an increase in inflammation around endothelial cells, a process central in blood pressure and atherosclerosis.

The following tables describe the SNPs that achieved significance and were common to both CHARGE and Global BPgen. Each SNP is identified by it's "Reference SNP cluster ID" or accession number (rs), it's location on chromosome, and the nearest gene. Functionality for the nearest gene is briefly described.

Table 1.5 – Top systolic blood pressure loci identified in the meta-analysis of CHARGE & Global BPGen. Functionality of associated genes are identified. SNPs above attained P < 5 x 10^{-8} in meta-analysis. Adapted from **(Levy et al. 2009)**. rs, Reference SNP cluster ID SNP; single nucleotide polymorphism; Chr, chromosome; CYP, cytochrome P450 enzymes; ATPase, adenosine triphosphatase (catalyses hydrolysis of ATP to ADP and phosphate); SH2B, SH2 domain containing adaptor protein B; SBP, systolic blood pressure; PMCA1, plasma membrance calcium ATPase

SNP Identifier	Chr	Nearest Gene	Functionality
Systolic BP			
rs1004467	10	Steroid 17-alpha-hydroxylase, CYP17A1	SBP; adrenal hyperplasia with mineralocorticoid and glucocorticoid excess
rs381815	11	Pleckstrin homology domain-containing family A member 7, PLEKHA7	SBP; adherens tissue protein or adherence junction (AJ) protein, found at epithelial junctions
rs2681492	12	ATPase 2B1, ATPase2B1	SBP; encodes PMCA1, an ATPase expressed in vascular endothelium, involved in calcium pumping from cytosol to extracellular space
rs3184504	12	SH2B adapter protein 3, SH2B3	SBP; growth factor and cytokine receptors, T-cell activation; pro-inflammatory

Table 1.6 – Top diastolic and hypertension blood pressure loci identified in the meta-analysis of CHARGE & Global BPGen. Functionality of associated genes are identified. SNPs above attained $P < 5 \times 10^{-8}$ in meta-analysis. Adapted from **(Levy et al. 2009)**. rs, Reference SNP cluster ID SNP; single nucleotide polymorphism; Chr, chromosome; CYP, cytochrome P450 enzymes; ATPase, adenosine triphosphatase (catalyses hydrolysis of ATP to ADP and phosphate); SH2B, SH2 domain containing adaptor protein B; DBP, diastolic blood pressure; PMCA1, plasma membrance calcium ATPase

SNP Identifier	Chr	Nearest Gene	Functionality
Diastolic BP			
rs9815354	3	Unc-51 like kinase 4, ULK4	DBP; little known of functionality
rs11014166	10	Voltage-gated calcium channel beta-2 subunit, CACNB2	DBP; Beta-2 subunit of a voltage-gated calcium channel
rs2681472	12	ATPase 2B1, ATP2B1	DBP; encodes PMCA1, an ATPase expressed in vascular endothelium, involved in calcium pumping from cytosol to extracellular space
rs3184504	12	SH2B adapter protein 3, SH2B3	DBP; growth factor and cytokine receptors, T-cell activation; pro-inflamma
rs2384550	12	T-box transcription factor, TBX3 and TBX5	DBP; associated with structural cardiac malformations and regulation of atrial natriuretic peptide
rs6495122	15	Tyrosine-protein kinase, CSK and Unc-51 like kinase 3, ULK3	DBP; CSK encodes cytoplasmic tyrosine kinase, involved in angiotensin II dependent vascular smooth muscle cell proliferation
Hypertension			
rs2681472	12	ATPase 2B1, ATP2B1	Encodes PMCA1, an ATPase expressed in vascular endothelium, involved in calcium pumping from cytosol to extracellular space

With the modest effects seen in both GWAS, it is likely that there are many more common variants with similarly weak effects on blood pressure yet to be discovered and replicated. Discovery of such loci can in the future guide fine-mapping efforts to determine causal variants of hypertension. Identification of novel loci may also permit development of new and potentially targeted antihypertensive treatments. However, this would require an understanding of the relationship between the various loci and gene functions.

The following 2 tables summarise the strengths and weaknesses for both GWAS and candidate gene studies.

Table 1.7 - Strengths and weaknesses of GWAS. Genome-wide association studies (GWAS) examine the association of a large number of genetic variants across the whole genome with a given phenotype. BP, blood pressure; SNP, single nucleotide polymorphism, GWAS, genome-wide association studies. Adapted from **(Delles & Padmanabhan 2012)**.

GWAS: Strengths	GWAS: Weaknesses
GWAS are hypothesis-free and therefore unbiased.	Large sample sizes are required to detect associations at genome-wide significance level.
This enables the discovery of new susceptibility genes and pathogenetic principles based on genetic findings.	Genotyping costs are relatively high.
Population-based GWAS can study all meiotic recombination events in a population at high marker density.	Due to the required large sample sizes phenotypic quality is not in all GWAS optimal.
GWAS detect stratifications within populations for example due to different ancestry or evolutionary events.	GWAS focus on common genetic variants and are blind to rare (private) mutations.
	Analysis of GWAS data involves robust quality control pipelines and bioinformatic methods.

Table 1.8 - Strengths and weaknesses of candidate gene studies. Candidate gene studies examine the association of a limited number of genetic variants within a limited number of genes with a given phenotype. Adapted from **(Delles & Padmanabhan 2012)**.

Candidate Gene Studies: Strengths	Candidate Gene Studies: Weaknesses
Candidate genes can be chosen based on a wide range of evidence from pathophysiological considerations, data in other species, data from gene expression studies and others.	The individual contribution of genetic variants to the BP phenotype is small. Most of the study designs lack power to detect these small effects.
Genotyping costs are relatively low.	Inconsistency of phenotyping does not allow results to be compared or combined.
Due to low genotyping costs more weight can be put on high-fidelity phenotyping.	Selection of candidate genes depends on known pathways of BP regulation precluding undiscovered pathways.
	Most candidate gene studies tend to be single SNP studies that are not completely informative of the variability within a gene. Additional SNP identification requires sequencing resources that may not be readily accessible.

Post GWAS analysis refers to the process by which genes identified are studied in order to determine their biological function. Bioinformatics allow identification of genes of interest that can then be studied through *in vitro* and *in vivo* experiments. The following figure illustrates the process that follows GWAS in order to understand what the association of a gene with a disease process means in the context of why the disease occurs (Figure 1.12).

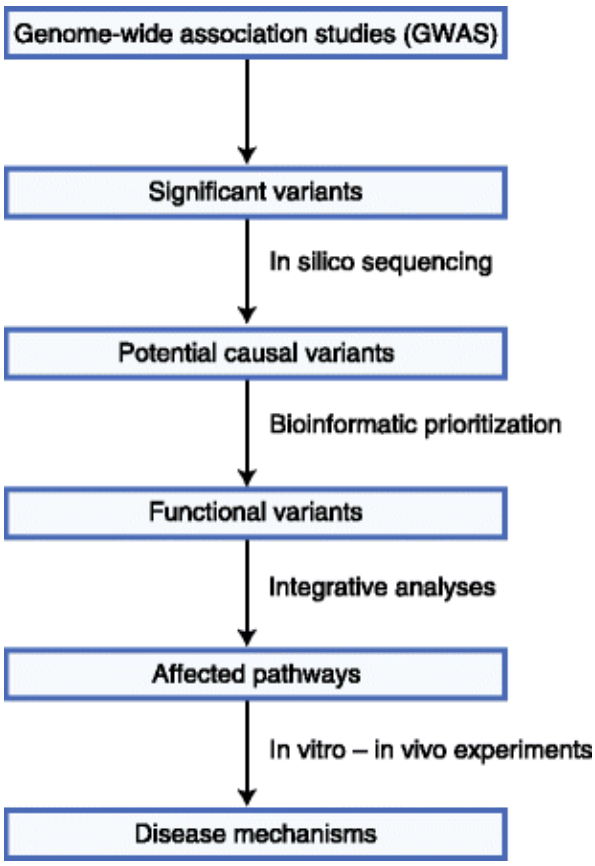


Figure 1.12 - From GWAS to disease mechanisms. This diagram illustrates how post-GWAS analysis aids the translation of significant GWAS signals into disease mechanisms. Reproduced from **(X. Wang et al. 2011)**.

1.2.4.2 Genetic Risk Scoring

GWAS have only identified genetic variations that individually account for a small proportion of variation in blood pressure, in the order of 1 mmHg per allele for systolic pressure and 0.5 mmHg for diastolic pressure (Levy et al. 2009; Newton-Cheh et al. 2009). The International Consortium for Blood Pressure GWAS performed a study examining the association between a genetic risk score and odds of hypertension. By looking at the effect that 29 single nucleotide polymorphisms (SNPs) had on systolic and diastolic blood pressure control they found that individuals who were one standard deviation (SD) above the mean genetic risk score had a 21% increase in the odds of hypertension (Ehret et al. 2011).

A second group attempted to replicate this finding in a population of over 17,000 Swedes participating in the Malmö Preventive Project (Berglund et al. 2000). In this Study, patients were examined at baseline and during a 23-year follow-up period. Using the same 29 SNPs, they sought to assess the utility of

genetic risk scoring in prediction of incidence of hypertension, and prediction of change in blood pressure over time (Fava et al. 2013). The genetic risk score was associated with higher baseline systolic and diastolic blood pressure at baseline and follow-up measurements of blood pressure. Fava (2013) confirmed a positive association between the genetic risk score with incidence and prevalence of hypertension. The genetic risk score however did not appear to predict incident hypertension significantly, when compared with traditional risk factors for hypertension.

There is always the possibility that if further SNPs of significance are identified in the future this could improve the utility of the genetic risk score. It is still possible that utilizing genetic information in combination with traditional risk scoring such as the Framingham Risk Score (D'Agostino et al. 2008) may help to improve their predictive abilities if the right genetic variants are identified.

1.2.3 Genetics of the Renin-Angiotensin Aldosterone System

The most studied candidate genes in terms of contribution to hypertension are those coding for the renin-angiotensin aldosterone system (Redon et al. 2004). Polymorphisms within the Renin-Angiotensin Aldosterone System (RAAS) such as the ACE I/D, angiotensinogen (AGT) M235T polymorphisms and angiotensin II type-1 receptor (AT_1R) polymorphism A1166C have been well characterised. These polymorphisms have been extensively tested in relation to their effect on hypertension however with conflicting results (Schunkert et al. 1997; Munroe 2000; Duncan et al. 2001). Still, genetic variations within the RAAS are popular in terms of determining treatments that may work with greater efficacy in their antihypertensive action.

1.2.3.1 Renin

The renin gene, REN, spans 12 kilobytes (kb) of DNA and contains 8 introns (Hobart et al. 1984). It is located at 1q32 (Cohen-Haguenauer et al. 1989). The Hypertension Pathotype cohort (HyperPATH) is an ongoing multi-site international project aiming to collect data investigating pathophysiological and genetic mechanisms of cardiovascular disease including hypertension (Pojoga et al. 2011). Data is collected under controlled conditions in that the protocol has been designed to minimise confounders of PRA such as salt intake, body posture and medications.

570 hypertensive and 222 normotensive individuals from the HyperPATH cohort were challenged with a high-salt diet of 200 mmol sodium intake per day (Sun et al. 2011). Phenotypic data was collected and the renin gene genotyped with the purpose of identifying renin genotypes associated with hypertension. Renin SNPs were selected from the HapMap CEU population (Utah residents with Northern and Western European Ancestry). 2 SNPs were significantly associated with higher hypertensive risk, rs6693954 and rs5705, with carriage of the major A allele demonstrating higher risk for hypertension (odd ratio, OR, 1.98 for rs6693954 and OR 2.04 for rs5705).

rs6693954 is in high LD with rs2368564 ($r^2 = 0.96$) located in intron 9, the latter having been found to be associated with hypertensive risk in a range of ethnic

populations (Frossard et al. 2001; Ahmad et al. 2005). rs2368564 (10607G>A) has been recognised as a *Mbo*I restriction fragment length polymorphism, and has been widely studied. Variation at this site in different populations is associated with hypertension (Okura et al. 1992; Frossard et al. 1998). Due to its location in Intron 9 it probably does not represent a functional genetic variation. Carriage of the A-allele is associated with hypertension. In a study of 86 West Mexican (Mestizo), 49 Huichol native Mexians and 39 Germans, frequency of carriage of the A-allele varied from 27-35%. In the USA European-derived population (CEU) a similar frequency of carriage of the A allele has been reported of 24% (Valdez-Velazquez et al. 2011). The frequency of this particular SNP has been shown to vary from 18% in American natives (Rupert et al. 2003) to 36.5% in Arab populations (Ahmad et al. 2005).

Another SNP in Intron 1, rs10900556 (1303G>A), has also been well studied and has been recognised as a Bgl-I restriction fragment length polymorphism. Variation at this site is thought to influence fluid homeostasis and thus blood pressure. Distribution of the SNP varies between populations, being frequent in Africans but not in Asians (Barley et al. 1991). In the Mexican and German population mentioned above, carriage of the G-allele ranged from 23-28% (Valdez-Velazquez et al. 2011), with a frequency not far off that reported in European populations (Frossard et al. 1999).

Interestingly, in Valdez-Velazquez's work (2011), a polymorphism was identified in the hypertensive Mexican (Mestizo and Huichol) population 98 kb upstream to the 1303G>A polymorphism, at position 1205 (1205C>T) (Valdez-Velazquez et al. 2011). Allele T was occurred more frequently in the German population than the Mexican group. A T allele frequency of 0.23 was observed in the German population, compared to a very low allele frequency of 0.03 in the Mestizo Mexican population ($P<0.00005$). The T allele was not identified in Huichol Mexicans. Carriage of 1205C>T was significantly associated with hypertension in this study and had not been reported previously (Valdez-Velazquez et al. 2011). Inter-population difference within the two Mexican populations was significant for the hypertensive population where carriage of the T allele was found in hypertensive individuals with respect to the Mestizo

group (11% vs 3%, p=0.02). These results support the potential for wide genetic diversity in disease-related genes when populations are compared.

1.2.3.2 Angiotensinogen (AGT)

As early as 1992 the relationship between angiotensinogen levels and higher blood pressure levels amongst family members was described (Watt et al. 1992). Higher angiotensinogen levels were observed in families in whom both parents and offspring had higher blood pressures. Jeunemaitre et al. (1992) demonstrated significant differences in plasma concentrations of angiotensinogen among hypertensive subjects with differing AGT genotypes (Jeunemaitre et al. 1992). The Study also demonstrated evidence of genetic linkage between the AGT gene and hypertension. 15 molecular variants were subsequently identified and used in case-control studies to test for an association with hypertension (Jeunemaitre et al. 1997).

Of the polymorphisms studied, an amino acid change from methionine to threonine at position 235 (M235T) and a further amino acid change encoding methionine instead of threonine at position 174 (T174M) were significant (Jeunemaitre et al. 1997). Carriage of the M235T AGT variant is associated with hypertension that is likely related to higher AGT levels in several populations (Corvol et al. 1999; Staessen et al. 1997). However, this is not seen to be consistent throughout populations and indeed a study in Slovenian adult Caucasians failed to find any link of M235T with hypertension (Glavnik & Petrovic 2007).

A recent publication in which 302 newly diagnosed stage I-II essential hypertensives were assessed (191 controls, age matched) for the M235T polymorphism found a significant difference in the prevalence of TT-homozygotes in the hypertensive population (15.9%) versus controls (6.3%, p<0.01) (Tousolis et al. 2012). The associated risk of essential hypertension with TT genotype carriership was significantly increased compared with all of the other genotypes with an odds ratio (OR) of 2.830 [1.051-5.480; p=0.002] (Tousoulis et al. 2012). Further replication studies in differing populations are necessary. Interestingly, Tousoulis (2012) demonstrated that an assessment of carotid-femoral pulse wave velocity (cfPWV) revealed higher cfPWV in TT

homozygotes compared with MM- and MT-genotypes in their population of hypertensive patients. Tousoulis (2012) postulated that the genotype may have effects on vascular biology resulting in potentiated angiotensin II mediated mechanisms influencing arterial stiffness (Tousolis et al. 2012).

1.2.3.3 ACE I/D

As discussed, angiotensin-converting enzyme functions as a kininase in the degradation of both the inactive decapeptide angiotensin I and bradykinin. A common variant in the angiotensin converting enzyme (ACE) gene is associated with variations in ACE levels. The gene that encodes ACE is located on the long arm of chromosome 17 (17q23). The gene is 21 kilobytes (kb) long and comprises 26 exons and 25 introns.

An insertion/deletion (I/D) polymorphism resulting in the presence or absence of a 287 base pair (bp) insert in the ACE gene has been associated with differences in ACE activity, the deletion allele strongly associated with an increased level of circulating ACE (Rigat et al. 1990). Carriage of the I/D polymorphism, rs4646994, has been thought related to 14-50% of the inter-individual variance in serum ACE activity (Rigat et al. 1990; Schunkert 1997). This was not replicated however for a Nigerian population studied in 2001 (Zhu et al. 2001).

In 2007 carriage of the D-allele was thought to be associated with diastolic hypertension (Jiménez et al. 2007). An association has also been made with carriage of the polymorphism and target-organ damage in hypertensive patients (Redon et al. 2000), progressive kidney disease, renal artery stenosis and atherosclerosis. Interestingly, a significant association between salt-sensitive hypertension and the I/D polymorphism of the ACE gene has been shown in a small study of 50 patients (Giner et al. 2000). Salt-sensitive hypertension was significantly higher (P=0.012) in II (67%) and DI patients (62%) compared with DD hypertensives (19%). A gene-environment interaction was also seen in another population with a similar pattern exacerbated by being overweight (Zhang et al. 2006). Knowing that salt-sensitive hypertensives respond well to diuretic antihypertensives this could possibly aid in guiding their treatment.

A meta-analysis from 2012 showed an association between the ACE I/D polymorphism and ACE inhibitor (ACEi) related cough in subjects >60 years old (Y.-F. Y. Li et al. 2012). In this meta-analysis, a greater I allele frequency gave rise to an increase in ACEi associated cough. The benefits of beta-blockers and high-dose ACE-inhibitors as antihypertensives have been shown to be maximal in ACE DD patients (McNamara et al. 2004). The location of the ACE I/D polymorphism in a non-coding region makes it unlikely to be a functional variant. Thus attempting to identify genes in linkage disequilibrium with it may help identify treatment targets (Sayed-Tabatabaei et al. 2006).

In 2001, Zhu et al. performed association studies in 1,343 Nigerians from 332 families (Zhu et al. 2001). The effect of 13 different ACE polymorphisms in the ACE gene on serum ACE and blood pressure were studied. They found another polymorphism, G2350A (Exon 17, ACE 8) had a greater effect on serum ACE level than the I/D polymorphism in their population, accounting for 19% of variation in ACE activity. G2350A alongside A-240T (ACE 4) was associated with systolic blood pressure ($P < 0.05$). With regards diastolic blood pressure only the latter polymorphism was significantly associated ($P < 0.05$). An association of G2350A with essential hypertension has also been shown in an Emirati hypertensive population (118 hypertensive individuals versus 136 normotensive controls, $P = 0.05$).

1.2.3.4 ACE2

ACE2 was first described in 2000 and is estimated to share 42% identity with the catalytic domain of ACE (Tipnis et al. 2000; Donoghue et al. 2000). With 18 exons, the gene maps to Xp22. ACE2 converts Angiotensin I to Angiotensin 1-9 (Ang 1-9) and Angiotensin II to Angiotensin 1-7 (Ang 1-7). Both these metabolites are thought to have opposing vasodilatory function versus Angiotensin II (Ferrario et al. 1997). The ACE2 gene has been studied not only in hypertension, but coronary artery disease. It is estimated that genetic factors account for 67% of variation in the amount of ACE2 present in the circulation (Rice et al. 2006). Multiple studies have been performed since 2004 investigating ACE2 SNPs and their associations with blood pressure and/or

hypertension. A greater number of studies have investigated two SNPs, rs1978124 and rs2285666.

A study in 2009 by Zhou et al., discounted a relationship between hypertension and an ACE2 polymorphism rs2285666, G8790A in Han Chinese population. No association was shown between the frequency of the A allele and genetic susceptibility to hypertension in male or female patients, sex being important as the ACE2 gene is carried on the X chromosome (Zhou & Yang 2009). In a separate study in Anglo-Celtic Australian subjects with hypertension, 4 SNPs evaluated in the ACE2 gene including the G8790A polymorphism have not revealed a statistically significant effect on incidence of hypertension (Benjafield et al. 2004).

A meta-analysis of case-control studies published in 2012 sought to comprehensively estimate the association between carriage of the G8790A polymorphism and essential hypertension in studies involving several ethnic populations with data for each sex analysed separately (N. Lu et al. 2012). Of the studies included in the meta-analysis, 8 were of Han-Chinese ethnicity. 2 studies were performed in Chinese minority ethnicity (Dong Xiang and Li) with 1 Anglo-Celtic Australian population. The total numbers analysed included 7,249 cases and 3,800 controls. An association was observed between carriage of 8790A and hypertension in females across all ethnicities studied, and in Han Chinese males (N. Lu et al. 2012).

For females, the frequency of the A allele was significantly higher in the essential hypertension group than in the control group, $P < 0.00001$. When 8790AA genotype was compared with 8790GG genotype, significant association was seen for AA-homozygotes with essential hypertension (OR 1.17, $P=0.049$). No difference in association with essential hypertension was seen between GA and GG genotypes. In males, no association was seen between carriage of the A allele and essential hypertension until the population was divided into sub-groups by ethnicity. Then, the association became significant with OR 1.21 in the Han Chinese subgroup, $P=0.006$.

In healthy subjects participating in the MONICA Augsburg echocardiographic substudy, 4 ACE2 SNPs including rs4240157 were not associated with hypertension. However, in a study of 503 Australian Caucasians with type 2 diabetes, the prevalence of hypertension was significantly higher in both men and women and was associated with the G allele of the *ACE2* SNP rs4240157 (Patel et al. 2012).

An interesting study in 555 adolescent males and females of French Canadian or European descent found that carriage of the ACE2 SNP rs233575 C allele was associated with higher baseline systolic and diastolic blood pressure in European males (Malard et al. 2013). In contrast, French Canadian females with the rs233575 T allele had a 4 mmHg higher baseline diastolic blood pressure compared to carriage of the C allele. In addition to evaluating SNPs for association with blood pressure, the study also evaluated SNPs for association with change in blood pressure over time. Data was evaluated from the baseline assessments, and compared with two follow-up assessments. The average age of individual participants at each of three assessments were 12.7 at baseline, 15.1 at the first follow-up and 17 years at the second follow-up.

SBP significantly increased over the five year follow-up period by an average of 10.1 mmHg in males and 2 mmHg in females. This result was statistically significant between males and females $P<0.001$. DBP increased by an average of 3.4 mmHg in males and 2.2 mmHg in females. This difference was not statistically significant by sex. French Canadian females carrying rs233575 or rs2158083 T allele were observed to have 4 mmHg higher baseline DBP than French Canadian C allele carriers of either SNP ($P<0.05$). For change from baseline, carriage of rs2074192 A allele in females of European descent was associated with a change in systolic blood pressure that was lower when compared to the alternative G allele in females of European descent. Carriage of rs233575 and rs2158083 C allele was associated with a change in systolic blood pressure that was higher compared to the alternative T allele in females of European descent. Conversely, no significant association between carriage of these SNPs and change in blood pressure from baseline was observed in either European or French Canadian males (Malard et al. 2013). This

demonstrated the association of the SNP with blood pressure was influenced by both gender and ethnicity.

Clearly further prospective studies are required to determine whether or not specific ACE2 polymorphisms have a role in determining genetic predisposition to hypertension.

1.2.3.5 Angiotensin II Type 1 receptor (AT1R)

The vasoconstrictor and growth promoting effects of Angiotensin II (Ang II) are mediated through the AT1R. Some fifty different polymorphisms have been described in the receptor whose gene spans >55 kb. The most studied of the approximate 50 SNPs in the AT1R is the A1166C AT1R receptor polymorphism, rs5186. This polymorphism in the AT1R has been shown to be linked with essential hypertension and hypertension in pregnancy in several different studies in Caucasians (Kobashi et al. 2004; Lapierre et al. 2006).

The presence of the A1166C AT_1R receptor polymorphism identified as C-allele carriers has been shown to predict greater likelihood of patients being in a prehypertension group and having higher systolic blood pressures, SBP (Fung et al. 2011). Carriage of the C-allele is associated with increased risk of hypertension in Caucasian population with an odds ratio of 7.3 (in C:C homozygotes compared with A:C and A:A) (Bonnardeaux et al. 1994).While the C-allele was associated again with increased risk of hypertension in a Chinese population (Jiang et al. 2001), this was not observed as a risk in a Japanese population (Ono et al. 2003). A recent meta-analysis including 56 studies with a total of 28,952 subjects suggested that the A1166C polymorphism was significantly associated with an increased hypertension risk in Asian and Caucasian populations (D.-X. Liu et al. 2015). The group did not identify any association for African populations.

Further work evaluating the relationship between A1166C and vessel hypertrophy and stiffness with the suggestion that the polymorphism is associated with Collagen type I synthesis and myocardial thickness in individuals with hypertensive heart disease (Díez et al. 2003). Early studies in

1999 and 2001 demonstrated opposing effects of C-allele carriage on response to the angiotensin receptor blocker – the former showing greater blood pressure lowering in the C-allele carrier (Miller et al. 1999) and the latter yielding no association between response and C-allele carriage (Kurland et al. 2001). The degree of influence of the SNP on severity of hypertension and indeed response to treatment remains unclear and warrants further evaluation.

1.2.4 Introducing the Renin Distal Enhancer Element Single Nucleotide Polymorphism (SNP), REN-5312

A common SNP in a renin distal enhancer element (REN-5312C/T) has been reported to influence *in vitro* gene transcription in transfected human choriodecidual cells (Germain et al. 1998). In 2002, Fuchs and colleagues noted 45% greater rates of renin gene transcription in the presence of a -5312T allele rather than a -5312C allele (Fuchs et al. 2002).

More recently, Stanton and colleagues provided the first evidence that this REN–5312C/T polymorphism has *in vivo* functional activity in humans (Moore et al. 2007). Carriage of the -5312T allele, a specific marker for a single renin haplotype, was found to be associated with both elevated ambulatory and elevated clinic blood pressure (BP) levels in healthy Allied Irish Bank employees. The magnitude of the effect associated with carriage of the -5312T allele ranged from 2.7 mm Hg to 1.5 mm Hg (Moore et al. 2007).

Stanton and colleagues have gone on to confirm the association of renin -5312C/T genotype with BP level in a second Irish population (Vangjeli et al. 2010) of 1024 current and retired white bank employees aged between 18 and 80 recruited to the Allied Irish Bank study between June 2003 and June 2004. Carriage of one REN-5312T allele was associated with age- and sex-adjusted increments in diastolic clinic pressures of 1.1 mm Hg (0.1 to 2.1 mmHg). These data support renin as an important susceptibility gene for arterial hypertension in whites and provide the stimulus for the further studies of this thesis (Vangjeli et al. 2010).

Furthermore, Vengjeli et al. (2010) also found evidence that the polymorphism predicts BP lowering responses to renin-angiotensin system (RAS) blockade in hypertensive patients, and that this prediction is additional to, and independent of plasma renin activity (PRA). Blood pressure lowering with an angiotensin receptor blocker (losartan 100 mg daily), among T-allele carriers with a baseline PRA greater than the median value, was more than twice that of CC homozygotes with a baseline PRA less than the median. While BP responses with a renin inhibitor (aliskiren 150 mg or 300 mg daily), were also positively correlated with baseline PRA, BP lowering, particularly at night, was

considerably greater amongst CC homozygotes compared to T-allele carriers, (-10.1(1.4)/-6.5(1.1) versus -5.4(2.0)/-4.1(1.3), $p<0.03$ for treatment*genotype interaction for night-time systolic and diastolic pressures) (Moore et al. 2007).

Interestingly, in the same group of hypertensive patients, no statistically significant difference was observed for PRA at baseline between renin -5312T allele carriers and CC homozygotes. Plasma renin activity of T-allele carriers was neither more resistant to suppression by aliskiren nor more elevated with losartan therapy than that of CC-homozygotes. The associations of genotype and baseline PRA, with nocturnal systolic and diastolic and clinic systolic BP lowering responses to renin inhibition and to angiotensin receptor blockade, were independent of each other(Moore et al. 2007). These three findings suggest that the renin -5312C/T polymorphism does not influence the highly regulated secretion of active renin from renal juxtaglomerular cells into the systemic circulation.

It is noteworthy that mice with two renin genes do demonstrate higher BP levels, but have lower levels of both plasma and renal renin, than mice with only one renin gene (Lum et al. 2004). Hence a plausible explanation is that functionality of the renin -5312C/T polymorphism is mediated by altered local tissue or even intracellular renin or prorenin levels (Bader et al. 2001; Re 2003). Possibly in keeping with this suggestion was an observation of a non-significant trend for aliskiren levels to be lower in T-allele carriers. This led to the speculation that high affinity binding of aliskiren by elevated tissue renin in -5312T allele carriers could have contributed to the lesser BP lowering achieved in these individuals after 4 weeks of aliskiren. Alternatively or additionally, a failure by aliskiren to inhibit the catalytic activity or the intracellular signalling that occurs when renin or prorenin binds to the above described (pro)renin receptor, could have contributed to the lesser BP lowering seen in renin -5312T allele carriers with aliskiren (Nguyen et al. 2002; Saris et al. 2006).

1.3 Blood Pressure Treatment and Control

1.3.1 Blood Pressure Treatment and Control

The physician's primary aim in treatment of hypertension is the prevention of cardiovascular disease and death. Lifestyle modification and pharmacologic treatments are the mainstay of effective reductions in blood pressure. The NICE guidance states that antihypertensive drug treatment ought to be offered to people aged under 80 years with stage 1 hypertension who have one or more of target organ damage, established cardiovascular disease, renal disease, diabetes or a ten year cardiovascular risk greater than or equal to 20%. People of any age with stage 2 hypertension also ought to be offered antihypertensive drug treatment. For those under the age of 40 years with stage 1 hypertension and evidence of target organ damage, cardiovascular disease, renal disease or diabetes, NICE advocates the consideration of specialist evaluation of secondary causes of hypertension and a more detailed assessment of potential target organ damage. The reason for this is that 10-year cardiovascular risk assessments can underestimate the lifetime risk of cardiovascular events in younger hypertensives.

The 2013 European Society of Hypertension and European Society of Cardiology (ESH/ESC) guidelines for the management of arterial hypertension makes several recommendations in terms of blood pressure goals in patients based on systolic and diastolic blood pressure, and also specific recommendations for elderly patients. These are referred to in Table 1.8 (Pitarresi et al. 1992; Mancia et al. 2014; Suzuki et al. 2003).

Table 1. 9 - Blood pressure goals in hypertensive patients. Adapted from **(Mancia et al. 2014)**. CHD – coronary heart disease; CKD – chronic kidney disease; CV = cardiovascular; DBP = diastolic blood pressure; SBP = systolic blood pressure; TIA = transient ischaemic attack. Class of recommendation and level of evidence are indicated.

Recommendations	Class	Level
A SBP goal <140 mmHg:		
a) is recommended in patients at low-moderate CV risk	I	B
b) is recommended in patients with diabetes	I	A
c) should be considered in patients with previous stroke or TIA	IIa	B
d) should be considered in patients with CHD	IIa	B
e) should be considered in patients with diabetic or non-diabetic CKD	IIa	B
In elderly hypertensives less than 80 years old with SBP≥160 mmHg, reduce to 150-140 mmHg	I	A
In fit elderly patients < 80 years old SBP <140 mmHg may be considered	IIb	C
In fragile elderly patients < 80 years old SBP adapted to tolerability	IIb	C
In individuals > 80 years with SBP ≥160 mmHg reduce to 150-140 mmHg once in good physical and mental condition	I	B
A DBP target of < 90 mmHg always recommended, except in diabetes: < 85 mmHg	I	A

“SLAN 2007 Survey of Lifestyle, Attitudes and Nutrition in Ireland” gave valuable insight into the lack of adequate blood pressure control experienced in Ireland (Morgan et al. 2008). SLAN was a survey involving face-to-face interviews with some 10,000 adults in addition to assessment of body size of approximately 1,000 younger adults (18-44) and a detailed physical examination of over 1,200 adults (45 years and over). It detailed the challenging state of our current population health, most notably with 94% of the population having one or more of three risk factors for CHD, namely hypertension, hypercholesterolaemia and/or obesity.

In the SLAN survey, 60% of 1,207 respondents over the age of 45 years had hypertension. 57% of these were not on medication and of those who were taking prescribed anti-hypertensives, 70% were not controlled to levels </=140/90 mmHg (Morgan et al. 2008). This is a problem which needs to be

tackled on a primary care basis, in the community, through adequate education and empowerment of patients to assume responsibilty for their blood pressure control. With an ageing population, the consequences of an increasing prevalence of hypertension and difficulties achieving control is worrisome, with an expected increase in the personal and economic burden of the consequences of stroke and myocardial infarction as a result.

Worldwide it is a similar story with less than 35% of hypertensive patients able to achieve their target systolic and diastolic blood pressure (Thoenes et al. 2009). Many factors contribute to the difficulty in obtaining control of blood pressure with antihypertensive treatment. Patient adherence and the influence of experienced side-effects on adherence is a significant factor, poor lifestyle is often a factor, or even prescription of a drug without optimal blood pressure lowering effect. Concomitant medications can influence the efficacy of, for example, the cytochrome P450 drug metabolising enzymes resulting in altered bioavailability of the antihypertensive agent, potentially with a reduced blood pressure lowering effect.

1.3.1.1 Lifestyle

Lifestyle modifications are recommended for all patients with hypertension. NICE Guidance 127 (2011) recommends “lifestyle advice should be offered initially and then periodically to people undergoing assessment or treatment for hypertension” (B. Williams & Taryn Krause 2011). Adoption of the Dietary Approaches to Stop Hypertension (DASH) eating plan showed that eight weeks of a diet of fruits, vegetables, low-fat dairy products, fish, nuts, poultry and whole grains while limiting intake of fats, red meats and sweets reduced blood pressure by 11.4 mm Hg systolic and diastolic 5.5 mm Hg (Sacks et al. 2001).

The restriction of sodium intake to less than 2g per day is also advocated and has itself been shown to reduce systolic pressure on average by 3.7 to 4.8 mm Hg and also lowers diastolic pressure by 0.9 to 2.5 mm Hg. Of importance, salt sensitivity is common in elderly hypertensives (August 2003). Weight loss is of significant importance in those who are overweight or obese, as well as an increase in physical activity and moderation of alcohol consumption (G. A. Kelley & K. S. Kelley 2000; Whelton et al. 2002; Xin et al. 2001). Lifestyle

modifications are inexpensive and ultimately safe to patients and should be encouraged therefore, as first-line or concomitant treatment with appropriate pharmacologic agents.

1.3.1.2 Pharmacologic Treatment

Pharmacologic agents either as monotherapy or as a treatment with other classes of agents have been shown to reduce complications of hypertension. Trials involving patients with stage 1 or 2 hypertension showed lowering systolic blood pressure by 10-12 mm Hg and diastolic pressure by 5 or 6 mm Hg reduced the risk of stroke by 40%, CHD by 16% and the risk of death from any cardiovascular cause by 20% (August 2003). It was found that the greater the number of risk factors and the higher the blood pressure, the greater the reduction in absolute risk with effective management.

A pharmacologic treatment model commonly followed is the National Institute for Clinical Excellence (NICE) clinical guideline on the management of hypertension (NICE, CG 127 2011) (McManus et al. 2012). Here, choice of antihypertensive agent is driven predominantly by patient age, recognising in particular the effect of age and race on the activity of an individual's renin-angiotensin system. We will now discuss the renin-angiotensin system as a target in treatment of hypertension and the main classes of antihypertensive drugs used in the following table.

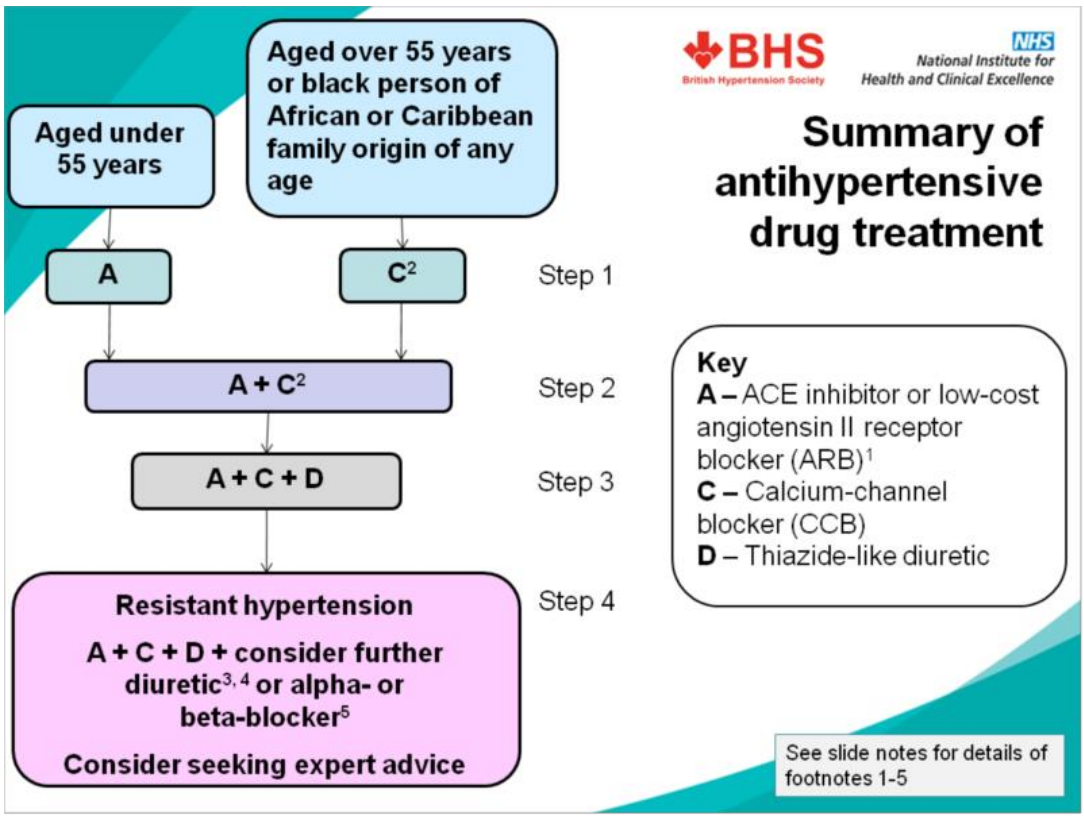


Figure 1.13 – BHS summary of antihypertensive treatment. Reproduced from NICE 2011 Clinical Guidance 127 **(National Institute for Health and Clinical Excellence 2011)**.

1.3.1.3 Renin-Angiotensin System (RAS) - a treatment target

International guidelines (US, European and UK) all advocate the use of agents which block varying steps of the RAS as first-line or second-line therapy for hypertension. Not only do they have a role in management of this condition, but have also been found to have indications in patients with established cardiovascular disease. For example, RAS-blockers have indications in patients who have suffered a stroke or myocardial infarction, and in those with diabetes mellitus and and associated nephropathy. Optimal management of blood pressure is mandatory for these patients in order to help minimise development and progression of macro- and microvascular complications of their disease.

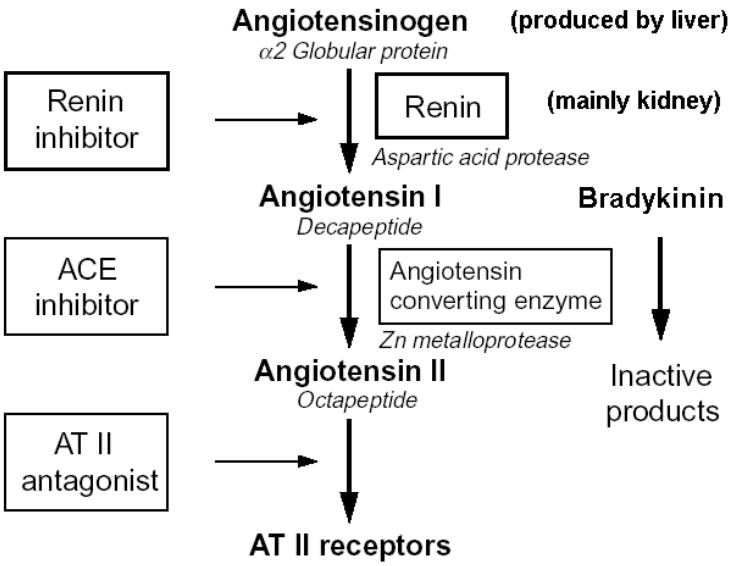


Figure 1.14 - **Drug targets of the Renin-Angiotensin System**. Reproduced from "Cardiovascular Diseases and Rational Drug Designs", Series of 3 Lectures, Dr Illingworth, http://www.bmb.leeds.ac.uk/illingworth/cardio

Angiotensin-converting enzyme or "ACE inhibitors" directly block the conversion of the inactive decapeptide Angiotensin I to the active Angiotensin II thus reducing the amount of active Angiotensin for angiotensin receptor type 1 stimulation. Bradykinin degradation relies on ACE activity for its degradation. This is also impaired through ACE inhibition and as a result nitric oxide release can be increased along with prostacyclin production (Dielis et al. 2005). Together these have vasodilatory effects. Whilst we know ACE inhibitors are effective antihypertensive agents they also have an important role in organ protection, in particular renal protection, with a further important role in cardiovascular protection (Tsukamoto & Kitakaze 2013). Unfortunately, ACE inhibitors do not entirely prevent the activation of Angiotensin I as alternative pathways exist via the chymase pathway to allow production of Angiotensin II. Thus ACE inhibitors do not completely block Angiotensin II production.

Angiotensin II receptor blockers (ARBs) have several advantages over the use of the traditional angiotensin converting enzyme inhibitor (ACEi) and were designed to overcome several deficiencies in the ACEi class. They directly block the Angiotensin II type 1 receptor. Losartan was the first commercially

licensed long-acting ARB in 1995 (Timmermans et al. 1990). ACE, being a relatively non-specific enzyme, has many substrates along with angiotensin I, including bradykinin and other tachykinins, with ACE inhibition allowing accumulation of these and several other substrates and their subsequent undesirable side-effects. Cough as a result of accumulation of bradykinin is the most commonly encountered troublesome side-effect. ARBs offer more effective angiotensin II inhibition by selective inhibition of the receptor site. Their design also allowed this class of drugs to overcome the compensatory rise in renin and angiotensin I levels seen with use of ACE inhibitors. ARBs displace angiotensin II from the angiotensin I receptor and produce their blood pressure lowering effects by antagonising angiotensin II vasoconstriction and subsequent hormonal and chemical events.

The Losartan Intervention for Endpoint Reduction in Hypertension (LIFE) study confirmed significant blood pressure reductions with atenolol and losartan by 30.2/16.6 mmHg: 29.1/16.8 mmHg respectively in a population of more than 9,000 Scandinavians aged 55 to 80 years (Dahlöf et al. 2002). 49% of patients in the losartan population achieved target blood pressure compated with 46% of those in the atenolol treatment group. Lower total primary cardiovascular events were seen in those receiving the ARB versus atenolol at 23.8 per 1000 patient years versus 27.9 per 1000 patient years respectively. The IRbesartan MicroAlbuminuria in Type 2 Diabetic Subjects (IRMA II) study again demonstrated an additional advantage in those hypertensive type II diabetics with persistent microalbuminuria, with significant reduction in rate of progression to overt diabetic nephropathy (Hellemons et al. 2011). Combination of ACE inhibitors with ARBs in the treatment of hypertension is not recommended due to higher incidence of serious side-effects including hyperkalaemia, hypotension and renal failure (Makani & Sripal 2013).

1.3.1.4 Calcium Channel Blockers

Calcium-channel blockers (CCBs) constitute non-Dihydropyridines (diltiazem, verapamil) and Dihydropyridines, DHP-CCB (such as amlodipine, nicardipine, felodipine). Most calcium channel blockers work via a negative inotropic effect and block the calcium channel during the plateau phase of the cardiac action

potential. Dihydropyridines are often used to reduce systemic vascular resistance and arterial pressure, with the non-Dihydropyridines, particularly verapamil, being relatively selective for myocardium. The latter reduce myocardial oxygen demand and reduce coronary vasospasm, but have minimal vasodilatory effects when compared with Dihydropyridines.
Over the past decade, major placebo-controlled trials have documented that CCBs reduce cardiovascular events in individuals with hypertension. In the Systolic Hypertension in the Elderly Program and the Systolic Hypertension in Europe trial, in which a thiazide-like diuretic (chlorthalidone) or a DHP-CCB was compared with placebo, major CHD events were reduced by 27% and 30%, and stroke by 37% and 42% respectively (Hulley et al. 1985). The Conduit Artery Functional Endpoint (CAFÉ) substudy of the Anglo-Scandinavian Cardiac Outcomes (ASCOT) trial, with amlodipine 5mg as the CCB of choice in combination with perindopril, demonstrated reduction in central systolic blood pressure and pulse pressure compared with a regimen of beta-blocker with or without diuretic (B. Williams et al. 2001). Patient compliance with this class of drugs is relatively preserved.

1.3.1.5 Thiazide-type diuretics

Thiazide-type diuretics have been the mainstay of antihypertensives employed for treatment of hypertension in a large majority of clinical trials, including The Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial (ALLHAT 2002). Thiazide and thiazide-like diuretics act on the nephron mainly at the proximal part of the distal tubule with a resulting increase in sodium excretion and urine volume. The long-term effect achieved with use of of diuretics is that of a reduction in peripheral resistance due to changes in the contractile responses of vascular smooth muscle. The main side-effects associated with use of thiazide and thiazide-like diuretics include hypokalaemia due to urinary potassium loss, hyperuricaemia with risk of acute gout and hypercalcaemia.

Despite being shown as highly effective and inexpensive drugs in an antihypertensive regime they remain quite underutilised (Kolasa 2003). The antihypertensive effect is long-lasting, and on chronic dosing, persists for 24

hours with once-daily dosing appropriate for most agents. When used in multidrug regimens, they have been shown to enhance efficacy of other agents, for example when used with an ACE inhibitor or angiotensin receptor blocker, antihypertensive effects are at least additive and incidence of hypokalaemia may be reduced. They can be used alone as a first-line agent in patients over the age of 55 intolerant to calcium channel blockers, with evidence of heart failure or in combination with other classes of drugs such as ACE inhibitors, ARBs, CCBs.

1.3.1.6 Combination Treatment

The majority of patients requiring antihypertensive treatment will require two or more antihypertensive medications to achieve BP goals (Cushman et al. 2002) and individual variation in responsiveness to drugs can be an issue (August 2003). It is recommended that when BP is more than 20/10 mm Hg above goal, two drugs should be considered for the initiation of therapy (Kolasa 2003). Caution is advised in utilising such treatment regimens particularly when the patient is diabetic, has autonomic dysfunction or is elderly as there is increased risk orthostatic hypotension with possible syncope and subsequent injury. The choice of therapy must also take into account patient age, race or ethnic group and response to previously used drugs, taking into consideration previous adverse reactions.

The Avoiding Cardiovascular Events in Combination Therapy in Patients Living with Systolic Hypertension (ACCOMPLISH) trial showed a single-tablet dual-mechanism therapy initiated in high-risk hypertensive patients significantly reduced the risk of morbidity and mortality by 20% compared with conventional therapy (Jamerson et al. 2004). The combination of the CCB amlodipine with ACEi benazepril was more effective than treatment with ACEi plus diuretic, to extent that the trial was discontinued early. At 36 months, 75% of patients in both treatment arms had blood-pressure levels less than 140/90. In patients with coronary artery disease and stage 2 hypertension, use of the combination of amlodipine and benazepril saw an 18% reduction in hazard ratio for cardiovascular events versus benazepril + hydrochlorthiazide (p=0.0016) (Bakris et al. 2013). In an era when it is now possible to combine

antihypertensive medications into a one tablet formulation, greater compliance and blood pressure control should theoretically be within easy reach.

1.3.1.7 Add-on Treatments

The NICE clinical guidance advocates the use of beta-blockers and alpha 1-adrenergic blockers in individuals who require additional antihypertensive treatment despite being on a combination of A+C+D or who have specific drug intolerances. An additional diuretic, in this case spironolactone may also be considered and is used predominantly in those with resistant hypertension, or those in whom a secondary cause of aldosterone producing adrenal adenoma is identified.

Where beta-blockers were once first-line in the treatment of hypertension a Cochrane review in 2007 demonstrated a “relatively weak effect of beta-blockers to reduce stroke, and the absence of effect on coronary heart disease when compared with placebo or no treatment” and a “trend toward worse outcomes in comparison with calcium channel blockers, renin-angiotensin system inhibitors, and thiazide diuretics.” This review was based on a meta-analysis of randomized trials comparing beta-blockers for hypertension to each of the other major classes of antihypertensives in adults 18 years of age and older (Wiysonge et al. 2007).

It is still held that in those with “compelling indications” such as a history of ischaemic heart disease that beta-blocker use is advocated (Kolasa 2003). However NICE clinical guidance 127 does recommend the use of beta-blockers be considered in young patients who are intolerant or in whom there is a contraindication to the use of ACE inhibitors, ARBs, or in whom there is evidence of sympathetic over-activity or in hypertensive women who are pregnant or post-partum. Blood pressure lowering achieved with alpha blockers is limited with only a “modest” reduction in blood pressure seen (Heran et al. 2012). Alpha-blockers are used as an add-on treatment once the NICE algorithm of A + C + D has been satisfied, or as a drug in individuals with multiple drug intolerances to the commonly used classes.

1.3.1.8 General Principles of Antihypertensive Drug Treatment

NICE clinical guidance (CG) 127 (www.nice.org.uk/guidance/CG127) recommends the use of drugs taken once daily where possible. Treatments used follow the British Hypertension Society (BHS) and NICE CG 127 recommendations illustrated above. Followup for patients commenced on an antihypertensive regimen should occur approximately every month in order to assess blood pressure control and any incidence of adverse or undesired side-effects of the medications. Serum electrolytes and creatinine should also be monitored though not as frequently, at least annually or bi-annually, though of course also taking into account co-existing disease (Bakris et al. 2003). Again, it is important to also address lifestyle modification and such frequent visits in the initial stages of blood pressure management provide opportunistic assessment of adherence to dietary and exercise interventions and success of smoking cessation programmes if applicable. Once BP is at goal and stable followup visits can usually be at 3 to 6 month intervals, again depending on any other comorbidities such as heart failure or diabetes.

1.3.2 Drug Metabolism and Genetic Variation

From a biological viewpoint the way in which we metabolise and physically respond to medications can differ significantly between individuals. Since the 1950s it has been established that genetics have an influence on response to treatment (Kalow 1962; Kalow 1992). Work performed by Kalow in the late nineties supported this further by looking at individual patient (intra-) and then population-based (inter-) responses to pharmacological treatments (Kalow et al. 1998). Their observation was that the variation in drug response was larger between individuals in a group (population variability) than the variation in drug response in one individual at different times (intrapatient variability).

Genetic polymorphisms that affect an individual's response to a particular drug can occur within genes that regulate drug metabolising enzymes, drug targets and drug transporters. In addition, genetic polymorphisms can also affect whether or not an individual experiences adverse drug reactions including drug toxicity and hypersensitivity, and to what extent (Ma & A. Y. H. Lu 2011). Individual variations as a result of genetic factors tend to be germ-line inherited

and not to undergo mutation or change over the course of a patient's life. The commonest genetic polymorphisms that contribute to this variation are those found in drug targeting, or pharmacodynamics, and drug metabolism, or pharmacokinetics, with subsequent changes in absorption and metabolism of drugs. Genetic variations are expressed either as a change in the coding region of the gene altering the structure of a target protein, or a change in the amount of protein expressed by modulating gene regulation (in our case, changes for example in messenger RNA levels of renin or angiotensinogen with subsequent changes in protein levels).

1.4 Personalised Medicine and Pharmacogenetics

1.4.1 What is "personalised medicine"? Clinical Applications

The following is the definition used by the International Conference on Harmonisation of technical requirements for registration of pharmaceuticals for human use, ICH (Guideline E15, November 2007):

" Pharmacogenomics (PGx) is defined as: The study of variations of DNA and RNA characteristics as related to drug response. Pharmacogenetics (PGt) is a subset of pharmacogenomics (PGx) and is defined as:The study of variations in DNA sequence as related to drug response" (Guideline 2007).

Through a more "personalised" or individual tailoring of drug treatment we could potentially reduce the incidence of unwanted side effects and also select medications with the maximum effect for many disease processes (Khullar & Sharma 2012).

1.4.2 Drug metabolism and pharmacogenetics

Within the drug metabolising cytochrome P450 enzymes (P450) *CYP2D6,* the gene encoding CYP2D6 isoenzyme, has the most variations of all genes for CYP isoenzymes. The variations result from point mutations, single base-pair deletions or additions, gene rearrangements, or deletion of the entire gene. The effect of this varies from some reduction in activity of the enzyme to total loss of activity (Ingelman-Sundberg & Evans 2001). This is important as CYP2D6 is responsible for the metabolism of approximately 20 to 25% of all marketed drugs (J. A. Williams et al. 2004).

Individuals known as extensive metabolisers have fully functional enzyme, and the opposite is so for those known as poor metabolisers with little or no functional enzyme (Siest et al. 2007). The degree of change in the activity of the enzyme can be measured by dosing the patient with a known CYP2D6 substrate, for example debrisoquine, and measuring the amount of excreted metabolised drug in the urine (compared to the amount of unmetabolised drug in the urine). Genotyping can also be used to determine metaboliser status and specific DNA chips are now on the market.

Non-P450 drug metabolising enzymes can also play a role. Another drug metabolising polymorphism resulting in loss of effect of thiopuine S-methyltransferase (TPMT) results in severe life-threatening haematopoietic toxicity and failure if patients receive the immunomodulating drug azathioprine (Eichelbaum & Ingelman-Sundberg 2006). TPMT converts drugs including azathioprine and 6-mercaptopurine to inactive compounds. More than 20 different alleles of the gene encoding TPMT have been discovered with 90% of white persons inheriting high enzyme activity, 10% intermediate activity (heterozygotes) and 0.3% inherit low or no activity (Ma & A. Y. H. Lu 2011).

An example of drug target genetic variation and effect is associated with statins, another drug in the cardiovascular primary and secondary prevention portfolio. Statin drugs work by inhibition of the HMG-CoA reductase (HMGCR gene) to lower cholesterol levels. Chasman and colleagues described two common SNPs within the HMGCR gene significantly associated with reduced efficacy of the statin pravastatin in achieving goal cholesterol levels (Chasman et al. 2004). A 22% smaller reduction in total cholesterol lowering achieved and a 19% less reduction in LDL cholesterol lowering achieved was seen in those individuals with a single copy of the SNP allele versus those who were homozygous for the major allele.

Again, in relation to statins, a transporter situated on the basolateral membrane of hepatocytes, involved in the uptake of statins into the liver, OATP1B1, is encoded by the gene *SLCO1B1*. Simvastatin is administered as an inactive medication and through various nonenzymatic and carboxylesterase-mediated processes, is activated to the simvastatin acid in the liver, plasma and intestinal mucosa. A common polymorphism of *SCLO1B1* (c.521T>C, rs4149056) has been shown to markedly affect individual variations in concentrations of circulating active statin (Pasanen et al. 2006). Reduced uptake of simvastatin acid into hepatocytes via the transporter in TC heterozygotes resulted in a 2- to 3-fold increase in simvastatin acid in the CC-homozygote individuals. With increased plasma concentration of the active metabolite comes an increased risk of adverse effects, namely myopathy or muscle pain and weakness associated with elevated creatine kinase (CK) levels.

One example of where pharmacogenetics has shown potential is in therapeutic anticoagulation using the coumarin-derived drug warfarin. Warfarin is a drug notorious for its narrow therapeutic index and dramatic and potentially fatal adverse events (intracerebral bleed or other haemorrhage when overly anticoagulated). The dose of warfarin required for adequate anticoagulation as measured by the "international normalised ratio" or INR can vary by up to ten-fold between individuals 1.5 – 14mg (Verhoef et al. 2013). The goal is to maintain the individual with an INR in the range of 2-3. This can take days or indeed several months to achieve with some patients never achieving consistently stable control. Whilst age, sex, weight and concomitant medications can all influence the dose of warfarin required, genetic factors without doubt have a role to play.

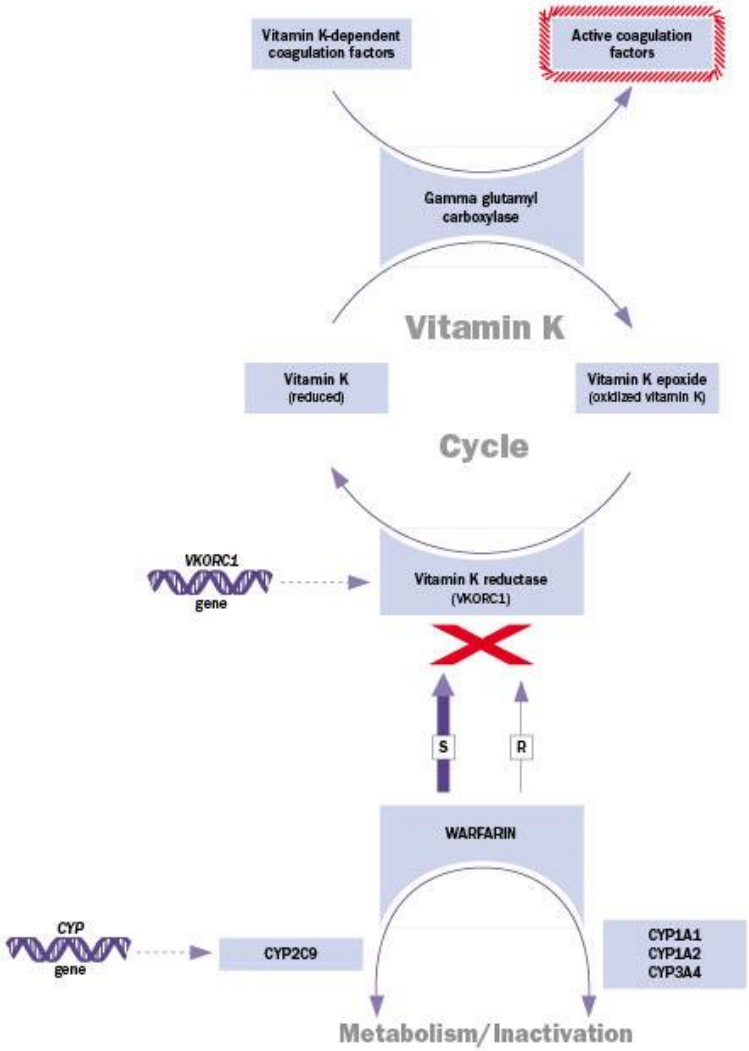


Figure 1.15 - Vitamin K cycle and warfarin metabolism. S, warfarin S-isomer. R, warfarin R-isomer. Of warfarin S- and R-isomers, S-warfarin is the most potent form. CYP2C9 metabolises S-warfarin to inactive products. Reproduced from Shindley L et al., Mayo Clinic Comminuque – Warfarin: Genotyping and Improving Dosing, August 2008.

CYP2C9 is the principle P450 isoenzyme to convert active S-enantiomer of warfarin to the inactive 6-hydroxywarfarin and 7-hydroxywarfarin (Schwarz & Stein 2006). Carriage of the CYP2C9*1 allele or wild-type is associated with normal warfarin metabolism. 2C9 is highly polymorphic with approximately 50 SNPs within the regulatory and coding parts of the gene identified (Lee et al. 2002). Some SNPs in 2C9 are associated with reduced enzyme activity compared with wild-type. Drug interactions with warfarin can exist because of changes in metabolism via the CYP2C9 pathway causing inhibition or induction

of the enzyme (Ufer 2005). As a result the amount of active warfarin available to the patient is altered.

CYP2C9*2 and *3 are the two most common allele variants known with largely reduced 2C9 activity as a result (Ma & A. Y. H. Lu 2011). In the region of 1% of white people are CYP2C9*2 homozygotes and 0.4% CYP2C9*3 homozygotes (Lee et al. 2002). Homozygous *2 and *3 carriers are very rare in Chinese and Japanese populations. Those carrying the CYP2C9*2 and CYP2C9*3 alleles have reduced warfarin metabolism and require a reduced dose to reduce the incidence of unwanted and potentially fatal side-effects. Such patients have an estimated two- to three-fold increased risk of having an adverse event (Ma & A. Y. H. Lu 2011).

Another gene of significance is VKORC1 encoding the Vitamin K epoxide reductase complex subunit 1 enzyme. The enzyme encoded by the gene is responsible for activating Vitamin K, essential for clotting. Fatal bleeding can result from endogenous Vitamin K deficiency due to a lack of Vitamin K-dependent clotting factors. Warfarin acts as a Vitamin K antagonist with the same effect – reduced clotting ability. It is the product of VKORC1 that is sensitive to warfarin. Those carrying a polymorphism 1173 TT of VKORC1 require around half the dose of warfarin daily than those wild-type 1173 CC-allele carriers. Polymorphisms can also occur in the coding region of VKORC1 resulting in varying degrees of warfarin resistance.

Given a lack of prospective, controlled studies the routine use of CYP2C9 and VKORC1 genotyping is not supported in those who are just beginning warfarin therapy (Limdi & Veenstra 2008). Indeed, the contribution of variable warfarin metabolism by CYP2C9 is estimated to be only around 10% of warfarin dose variation (Ma & A. Y. H. Lu 2011). VKORC1 is thought to contribute a little more at 25%. Clinical factors such as age, sex and additional medications contribute another 20% to the variation in metabolism of warfarin. The International Warfarin Pharmacogenetics Consortium assessed the contribution of CYP2C9 *1, -*2 and -*3, VKORC1 variants and clinical factors in 4043 patients from 9 countries to determine warfarin dose required for therapeutic anticoagulation (International Warfarin Pharmacogenetics Consortium et al. 2009). Using

genetic and clinical data the algorithm correctly identified that 50% of patient require ≤3mg/day and 25% require ≥7mg/day of warfarin to achieve target INR. Given that 50% of factors involved in variation in warfarin dose remain unknown there is still a significant piece of the puzzle yet to be discovered.

The next consideration is hypertension in focus – what we know to date regarding the genetics of hypertension, and how it can possibly influence and improve our treatment strategy going forward as we seek to improve control of a health concern whose incidence and prevalence continues to grow. The current approach to prescribing still has a "trial and error" component. Perhaps through enhancing our knowledge of the genetics of hypertension it is possible to identify optimal antihypertensive therapy.

1.4.3 Pharmacogenetic Studies related to Hypertension

The 8 Global BPGen SNPs associated with systolic or diastolic blood pressure were genotyped in the Nordic Diltiazem (NORDIL) antihypertensive study. In NORDIL, patients were treated with a combination of thiazide diuretic and β–blocker, or either drug alone, and this was compared with treatment with diltiazem. The magnitude of blood pressure lowering achieved with six months of either a beta-blocker, diuretic or diltiazem was assessed in relation to carriage of each of the SNPs of interest (Hamrefors et al. 2012). Two SNPs showed nominal evidence of an association with treatment response.

A phospholipase C delta-3 variant responsible for the generation of diacylglycerol and inositol 1,4,5-trisphosphate (IP3), *rs12946454,* was associated with a reduction of both systolic and diastolic blood pressure in the calcium channel blocker (diltiazem) group. No relationship between carriage of this SNP and blood pressure reduction in the beta-blocker or diuretic group was found (Hamrefors et al. 2012).

The second SNP of interest in the NORDIL study is within the gene encoding a member of the Cytochrome P450 superfamily of enzymes, CYP17A1. *rs11191548* carriage was associated with a reduction in the diastolic blood pressure lowering obtained with beta-blockers and diuretics. No treatment-

response association was observed in the diltiazem-treated group (Hamrefors et al. 2012).

Another SNP in NEDD4L, which encodes a regulatory protein that removes the epithelial sodium channel (ENaC) from the cell surface, has also been shown to have implications in terms of response to treatment. Knock-out mice have higher levels of ENaC expression and salt-sensitive hypertension. One particular SNP within NEDD4L, rs4149601 has been shown in previous work to be associated with hypertension (Svensson-Färbom et al. 2011). They found that G allele carriers had greater BP lowering than AA homozygote patients when treated with the thiazide or β–blocker, with no differences in response to diltiazem by genotype. The authors suggest that these SNPs could become significant in determining treatment of polygenic essential hypertension if further SNPs could be identified that also have a degree of effect in determining blood pressure lowering achieved with a particular class of antihypertensive drug(Svensson-Färbom et al. 2011).

Evaluation of Antihypertensive Responses (PEAR) Study was designed to test the association between 37 SNPs identified in Caucasians with their response to a beta-blocker, atenolol and a diuretic, hydrochlorthiazide (Y. Gong et al. 2012). Again, only one SNP achieved a nominal significance with a p-value <0.05, *rs1458038*. Carriage of this SNP was associated with a better response to both atenolol and the diuretic hydrochlorthiazide. A further 3 SNPs were associated with atenolol blood pressure response ($p<0.01$) (Y. Gong et al. 2012; Johnson et al. 2009). A SNP within NEDD4L, rs4149601 yielded significant association in the hydrochlorthiazide-treated patients where there was increasing lowering with each G allele carried, but no association was observed in the atenolol-treated patients (Johnson 2012). When taken into account alongside the NORDIL findings with the same SNP, it is likely that the NORDIL findings were driven by the diuretic.

Two candidate genes have been documented through GWAS as hypertension genes, CACNB2 and ADRB1. CACNB2 encodes the β2-regulatory subunit of the L-type calcium channel. Calcium channel genes can affect blood pressure regulation and act as targets of calcium channel blocker antihypertensives

(Levy et al. 2009). The β2-regulatory subunit regulates cell surface expression of the α1c subunit to which all CCBs bind. SNPs associated with systolic BP, diastolic BP and hypertension exist in both the 5' and 3' regions (Ehret et al. 2011; Levy et al. 2009). The INternational VErapamil SR-Trandolapril STudy GENEtic Substudy (INVEST-GENES) genotyped three common SNPs in CACNB2 (rs2357928, rs7069292, and rs61839258). In addition, GWAS identified an intronic SNP in CACNB2 (rs11014166) as part of a clinical association study in 5598 hypertensive patients. The INVEST-GENES patients had a diagnosis of coronary artery disease and randomized to either a β–blocker (BB) or CCB. A promoter SNP A>G , rs2357928, was found to have a significant interaction with treatment strategy for adverse cardiovascular outcomes. GG individuals had better outcomes if treated with a β–blocker than a CCB. By contrast, carriers of the A-allele did not experience the same differences in treatment outcomes. This was subsequently replicated in both Hispanic and black populations, suggesting that is may be a functional SNP. This study suggested that genetic variation within CACNB2 has the potential to influence treatment outcomes in high-risk patients with coronary artery disease and hypertension.

ADRB1 encodes the β1-adrenergic receptor, the target of β–blockers (BB). Two non-synonymous polymorphisms have been described Arg389Gly and Ser49Gly, the former determining contractile response to catecholamines (La Rosée et al. 2004). Arg389 form of the receptor leads to greater coupling to adenylyl cyclase with a resultant increase in downstream signaling in response to the agonist i.e. a more potent contractile response. This led to the hypothesis that the more responsive form of the receptor would also benefit most from BB therapy. Several studies have shown that Arg389Arg patients do indeed have the greatest BP lowering with BB therapy, while others have not observed this relationship between ADRB1 genotype and BP lowering with β-blockade (Johnson & Liggett 2011).

Of greater relevance to this work is the publication in Hypertension in 2007 by Moore and colleagues regarding the potential future use of the REN -5312 SNP in aiding selection of antihypertensive agent with the greatest blood pressure lowering effect (Moore et al. 2007). When compared with the direct renin

inhibitor aliskiren, blood pressure lowering with an angiotensin receptor blocker (losartan 100 mg daily) among T allele carriers was more than twice that of CC homozygotes. Blood pressure lowering particularly at night in those receiving aliskiren 150 mg or 300 mg daily was considerably greater amongst CC homozygotes compared to T allele carriers, (-10.1(1.4)/-6.5(1.1) versus -5.4(2.0)/-4.1(1.3), $p<0.03$ for treatment*genotype interaction for night-time systolic and diastolic pressures).

1.4.4 Hypothesis and Objectives

In Moore et al. (2007), findings of independent and disparate predictions of responses to renin inhibition and to angiotensin receptor blockade, by baseline PRA and by renin -5312C/T genotype, suggested that there might be clinical utility in measuring both PRA and renin genotype, before the prescription of antihypertensive therapy. Furthermore the divergent BP lowering responses with the two blockers of the RAS may be paralleled by differences in cardiovascular protection.

The primary hypotheses of this thesis are as follows:

1. REN-5312C/T genotype influences renin gene expression in the adrenal glands;
2. REN-5312C/T genotype influences levels of circulating plasma renin activity;
3. REN-5312C/T genotype influences antihypertensive responses to RAS blockade.

The primary objectives of this thesis are:

1. To determine whether the REN-5312C/T polymorphism influences renin gene expression in healthy adrenal glands;
2. To determine the relationship between REN-5312C/T genotype and PRA;
3. To determine whether genotyping of the REN-5312C/T polymorphism, alone or in combination with plasma renin activity (PRA), predicts BP lowering responses to renin-angiotensin system (RAS) blockade.

In Chapter 2 we will discuss the findings of a tissue-based study designed to confirm or refute the first primary hypothesis. Specifically, we will review the findings observed in relation to the role of REN-5312C/T in determining tissue expression of the renin gene and also the expression of other key renin angiotensin system genes.

In Chapter 3, we will discuss the findings of a clinical study, designed to confirm or refute the second and third primary hypotheses. Specifically, we will review

the findings observed in relation to the role of REN-5312C/T, alone or in combination with PRA, in determining response to RAS blockade in the management of hypertension.

Chapter 2 - Renin Genotype and Tissue Expression of Renin-Angiotensin Components

2.1 Introduction

The renin angiotensin system (RAS) plays important roles in the regulation of electrolytes, blood pressure (BP) and atherosclerosis (Oparil & Haber 1974a; Oparil & Haber 1974b; Dzau 2001). Renin catalyses the first and rate limiting step of this cascade, the conversion of angiotensinogen to angiotensin I. Hypertensive patients with high plasma renin levels are more likely than those with normal or low renin levels to experience myocardial infarctions (Brunner et al. 1972). Furthermore, BP lowering responses to antihypertensive drugs differ depending on the plasma renin status of the patient . This “circulating” RAS has been well described.

2.1.1 Tissue RAS

The concept of a local or “tissue” renin angiotensin system (RAS) has been around for some time and came about upon discovery of various RAS components in compartments such as the brain (Mukhopadhyay & Raizada 2013). It was not possible to explain the presence of renin, traditionally considered a “kidney enzyme” in the brain, particularly when the blood-brain barrier is known to exclude proteins as large as renin (Ganten et al. 1971). Angiotensin (Ang) II is also known to be produced in the tissue of organs such as the kidney, heart and adrenal gland (Bader & Ganten 2008).

In addition to the kidney, heart and adrenal gland, all components of the RAS have been identified in the vasculature, adipose tissue, placenta, pancreas and gonads in human and animal tissue (Paul et al. 2006). The skin, digestive organs (such as the salivary glands, stomach and intestine) and the lymphatic tissue (including the spleen and thymus) are also known to have local tissue RAS functionality (Paul et al. 2006).For example, human skin mast cells are a known source of renin synthesis (Silver et al. 2004), and it has also been observed that rat skin Ang II receptor expression is enhanced during experimental wound healing (Viswanathan et al. 1992). Such tissues with the capacity for local generation and action of Ang II can be described as tissues with local RAS functionality (Lavoie at al. 2003).

Early controversy surrounded the question of whether the identified components of the RAS were generated locally or were as a result of uptake from the peripheral circulation. Much of the information we have regarding this tissue-based RAS comes from transgenic and knockout mouse models. However, these animals are bred to either over-express or lack RAS components. This makes the ability to interpret experimental results difficult.

Elegant studies infusing ^{125}-I-labelled angiotensins I and II at levels known not to affect blood pressure have revealed substantial uptake of circulating radiolabelled Angiotensin II (Ang II) in preference to Angiotensin I (Ang I) at renal tissue sites (Van Kats et al. 2001). Renal tissue uptake of radiolabelled Angiotensin I did not occur, with higher levels of endogenous Angiotensin I in the tissue than in circulation. A significant majority of Ang I was therefore shown to have originated from renal tissue, not the circulating RAS. In addition, renal cortical tissue levels of radiolabelled Ang II were twice that of circulating levels, with endogenous levels of Ang II up to 60 times higher than circulating arterial concentration. In summary, uptake of both radiolabelled Ang I and II did not match endogenous tissue production, supporting a locally active tissue RAS.

Furthermore, the existence of a biologically active tissue RAS is supported by the fact that the clinical efficiency of RAS-blockers such as ACE inhibitors or AT1 receptor antagonists is in part related to their method of action. ACE inhibitors and AT1 receptor antagonists are known to inhibit not only the classical intravascular RAS, but also tissue-bound RAS (Mukhopadhyay & Raizada 2013; Bader & Ganten 2008). Thus, hypertensive patients with normal or even low levels of systemic RAS activity can effectively be treated with inhibitors of the RAS (Lavoie & Sigmund 2003; Dzau 2001).

The tissue RAS is thought to exert its effects locally, within the organs themselves. Whilst tissue RAS does not directly secrete its components into the circulating RAS, the tissue-mediated effects can in turn indirectly affect circulating RAS function. For example, adrenal gland tissue RAS is stimulated by Angiotensin II to release aldosterone from the glomerulosa cells into the circulation. As another example, inhibition of brain synthesis of angiotensinogen

results in significantly lower basal systolic blood pressure in transgenic rats when compared with controls (Paul et al. 2006). Thus the brain RAS is involved in control of auto regulation of blood pressure centrally, separate to the circulating RAS.

2.1.2 Kidney RAS

The kidney's juxtaglomerular (JG) apparatus as the source for circulating RAS activity made the kidney an early focus of determining whether or not a tissue-based RAS existed. Studies in the early nineties discovered renin and its messenger RNA (mRNA) existed outside the JG cells in the proximal tubules and the collecting duct (Moe et al. 1993). In a separate study of *in vitro* cultured rabbit proximal tubule cells, a time-dependent increase in renin and angiotensinogen activity was seen (Yanagawa et al. 1991). Such locally generated components of the RAS were thought to contribute to local proximal tubule tissue angiotensin generation.

In transgenic mice, overexpression of human angiotensinogen and renin cDNA in the proximal tubule results in hypertension that is independent of systemic RAS activity (Sigmund 2001; Davisson et al. 1999). Bader and colleagues demonstrated reduced hypertension-associated end-organ renal damage in mice lacking intra-renal angiotensinogen (AGT) over controls (Kang et al. 2002). Subsequent research observed that transgenic mice overexpressing rat AGT were found to develop high blood pressure and significant kidney injury, mediated by AGT-related overproduction of Ang II (Sachetelli et al. 2006).

To highlight the difference in activity between tissue and circulating RAS, a study was performed to evaluate the result of drug effects on components of the tissue and circulating systems. Administration of perindopril to Sprague-Dawley rats resulted in a reduction of intra-renal concentrations of the peptide Ang II to 14% of the control group with no effect on circulating levels (Campbell et al. 1991). Coupled with differential regulation of angiotensins between tissue and circulating RAS systems the results supported an independently active tissue RAS. It also provided significant evidence that perindopril was working at the level of the tissue RAS, and that the hypotensive effect of ACE inhibition was dependent more on tissue Ang II suppression than its effect on circulating RAS.

More recently, tissue work has demonstrated attenuated blood pressure and renal responses including sodium and water retention in mice lacking intra-renal ACE in response to infusion of Ang II (Gonzalez-Villalobos et al. 2013). This supports the opinion that tissue RAS has an additional effect on blood pressure from the circulating RAS, a topic of ongoing debate.

As discussed in Chapter 1, the prorenin receptor, (P)RR, has been implicated in Ang II-related and Ang II-independent organ damage through activation of bound prorenin, a renin precursor resulting in local Ang II generation (Nguyen et al. 2002). (P)RR activation also activates intracellular pathways, including mitogen-activated protein kinases (MAPKs). Ichihara went on to describe a reduction in renal damage when prorenin binding with the (P)RR was inhibited by a “decoy” protein (Ichihara et al. 2004).

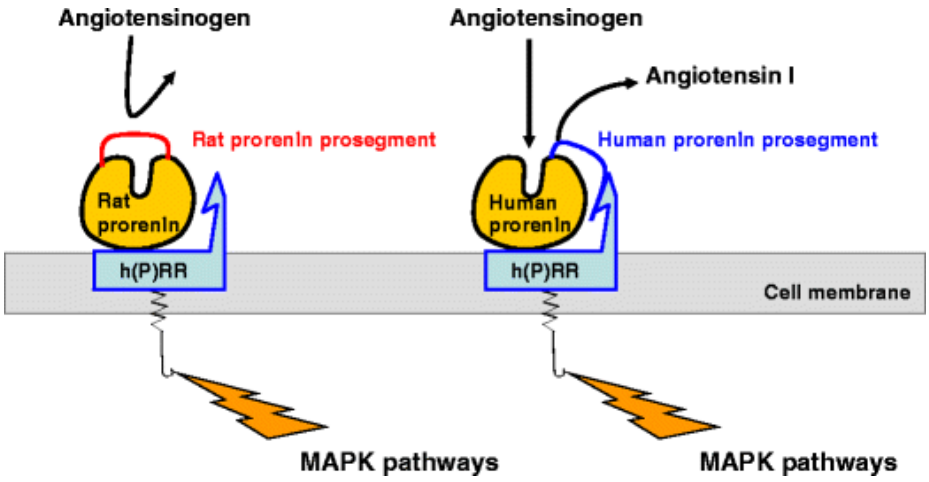


Figure 2.1 - Activation of intracellular pathways by prorenin binding to (pro)renin receptor. Studies in transgenic rats over expressing the human (pro)renin receptor demonstrated glomerulosclerosis with proteinuria. Recombinant rat prorenin stimulated mitogen-activated protein kinase (MAPK) activation in human (pro)renin receptor expressed cultured cells, but the human (pro)renin receptor was unable to enzymatically activate rat prorenin. Activation of the human (pro)renin receptor in these transgenic rats elicited nephropathy through intracellular pathways, including MAPK, and not through enzymatic activation of prorenin and angiotensin II generation. Reproduced from: “Involvement of (pro)renin receptor in the glomerular filtration barrier” **(Ichihara et al. 2008)**.

Most recently, a tubule-wide (P)RR receptor knock-out (KO) mouse has been developed in an attempt to understand the physiological significance of the

(P)RR in renal tubules (Ramkumar et al. 2016). (P)RR KO mice demonstrate lower epithelial sodium channel (ENaC-α), with absence of renal tubule (P)RR promoting sodium (Na^+) wasting with a reduction in hypertensive response to infused Ang II.

An association has also been described between (P)RR expression and repression of Wnt signaling. Wnt is an important cell signaling pathway involved in embryogenesis, cell differentiation, cell proliferation and cell migration with a role in tumorigenesis (Bernhard et al. 2012). With both (P)RR expression and Wnt involved in the pathogenesis of cardiac and renal end-organ damage, it means their interplay has an important role and makes them both potential drug targets in drug development.

2.1.3 Brain RAS

The discovery of a tissue RAS in the brain by Ganten et al. (1971), was the initial acknowledgement of the potential for organ-based tissue RAS separate to the circulating RAS (Bader & Ganten 2008). This was achieved through demonstration of local Ang II generation in the brain. Central to this was the fact that circulating Ang II cannot access most brain Ang II receptors due to the presence of the blood-brain barrier (Ganten et al. 1971).

In 1961, Bickerton and Buckley had shown evidence of response to angiotensin within the brain. Blood pressure increase was observed after direct administration of Ang II (Bickerton & Buckley 1961). Subsequent researchers demonstrated an interaction between Ang II and the autonomic nervous system, ANS (Ferrario et al. 1970). Renin activity is present in many regions of the rat brain and decreases with the age of the animal (Schelling et al. 1982). High levels of expression of the (P)RR in the brain have also been described (Nguyen et al. 2002; Nguyen et al. 2004).

Ang II receptors (AT1) have been shown to be present in most parts of the brain including the circumventricular organs (CVO), which can access circulating Ang II allowing activity on the high number of AT1 receptors present there. Activation of receptors there results in increased salt appetite, thirst and subsequently elevated blood pressure. In a series of experiments, transgenic mice with

increased Ang II generation localized to the brain developed increased salt appetite and hypertension (Morimoto & Sigmund 2002; Morimoto et al. 2001; Morimoto et al. 2002). In order for signals to be successfully translated from the CVO to physiological outputs such as activation of the sympathetic nervous system, a local RAS is required in areas of the brain inside the blood–brain barrier. Research performed by Donoghue and colleagues in their search for the ACE2 gene described the functionally active peptide Ang (1-7) in the brain (Donoghue et al. 2000).

Schinke et al., studied transgenic rats in whom an antisense RNA against astrocyte angiotensinogen (AGT) was expressed (Schinke et al. 1999). They demonstrated reduced blood pressure in the animals alongside reduced sympathetic nervous system activity and a blunted response to increased circulating Ang II. This further supported the functionality of a local brain RAS (Baltatu et al. 2001).

2.1.4 Adrenal Gland RAS

The adrenal gland has a central role in the regulation of blood pressure. In response to sodium depletion, the glomerulosa cells of the adrenal gland produce greater quantities of aldosterone in response to Angiotensin II resulting in sodium retention. In addition, circulating Angiotensin II increases local renin production within the adrenal cortex, further potentiating Angiotensin II levels with even greater release of aldosterone. Where sodium excess occurs, local renin release is suppressed. The effect of the local renin angiotensin system is thought to be localised to the gland itself, and does not directly influence PRA levels by secretion of renin into the circulation. However, the secretory tissue RAS in the adrenal gland does impact aldosterone release which has a direct effect on circulating renin levels and blood pressure.

As with the brain RAS, evidence for a tissue RAS in the adrenal gland has been present for around forty years. In 1967, Ryan described a “renin-like” enzyme in the rabbit adrenal gland (Ryan 1967) [abstract only, *Science Classic*] that appeared to generate a vasopressor polypeptide thought likely to be Ang I. During mouse embryogenesis, higher levels of renin transcripts have been

detected in the adrenal cortex of a murine two-renin gene strain over a one-renin gene model (Jones et al. 1990).
Later work demonstrated increased adrenal renin levels in spontaneously hypertensive rats compared with normotensive Wistar-Kyoto rats (Naruse & Inagami 1982). The spontaneously hypertensive animals had adrenal renin levels 6-8 fold higher than the normotensive controls. This difference was seen as early as 3 weeks of age, prior to the onset of hypertension in the spontaneously hypertensive rats, indicating an early role for adrenal renin levels in the development of hypertension. The adrenal renin level increased further with bilateral nephrectomy in both the spontaneously hypertensive and normotensive rats. This indicates that the adrenal RAS is independent of the circulating RAS. However, little difference was seen in the plasma renin levels between spontaneously hypertensive and normotensive rats either before or after nephrectomy.

It is thought that the possible mode of action of the adrenal gland RAS focuses on modulation of aldosterone secretion. Work in transgenic rats with normal circulating Ang II levels but an increased tissue adrenal RAS activity has demonstrated significantly altered steroidogenesis (Sander et al. 1992). Ang II has been shown to be a potent stimulus of aldosterone secretion *in vivo,* mediated by the AT1 receptor subtype of Ang II (Otis & Gallo-Payet 2007; Peters et al. 2008). Additionally it has been postulated that locally generated Ang II has an important role in adjusting the size of the adrenal glomerulosa to physiological needs, describing Ang II as a “potent growth factor for glomerulosa cells” (Otis & Gallo-Payet 2007).

2.1.5 REN -5312C/T genotype and tissue expression of RAS components

A common SNP in a renin distal enhancer element (REN-5312C/T) has been reported to influence *in vitro* gene transcription in transfected human choriodecidual cells (Germain et al. 1998). The same group subsequently demonstrated 45% greater rates of renin gene transcription in the presence of a -5312T allele rather than a -5312C allele in human choriodecidual cells (Fuchs et al. 2002). In diseased human kidneys, greater renin gene transcription was identified in the presence of the -5312T allele, for both TT-homozygotes and CT-heterozygotes when compared with CC-homozygotes (Makino et al. 2015).

The influence of REN-5312C/T on renin expression has not been studied in three important tissues involved in blood pressure control (kidney, adrenal gland and brain). It is difficult to access normal tissue in healthy humans. Access to normal adrenal glands is facilitated by kidney donation. During kidney donation, the adrenal gland and surrounding adipose tissue are removed alongside the donor organ. As a known site of renin-angiotensin system activity and with a central role in the regulation of blood pressure, adrenals are a suitable tissue to study the effect of this polymorphism.

2.2 Study Objective

This study was designed as a cross-sectional comparison study of levels of renin angiotensin system component mRNA in REN-5312T allele carriers and REN-5312CC homozygotes. The tissues studied were those of normal adrenal glands from healthy donors.

The primary objective of this tissue-based study was to test the hypothesis that the REN-5312C/T polymorphism influences renin mRNA levels in healthy adrenal glands.

The secondary objective was to test the hypothesis that the REN-5312C/T genotype influences mRNA levels of other key renin angiotensin system genes, namely, angiotensinogen, ACE and ACE2 in normal adrenal glands.

2.3 Methods

2.3.1 Study Participants

The study was reviewed and approved by the Beaumont Hospital Research Ethics Committee (Reference 07/64, Final Approval Date 7th August 2008). All tissue was obtained from cadaveric renal donors whose next-of-kin provided written consent for organ donation. All standard procedures for organ donation and transplantation were followed.

The process was coordinated through the Beaumont Hospital organ Transplant Coordinator. As per usual protocol, the next-of-kin was provided with written and verbal information to allow informed consent for organ donation and transplantation. The consent form included a list of the organs being donated, and also the following statement: “In the event that any of the above stated organs or tissues prove unsuitable for transplantation, they may be used for research.” This required the relative to either accept or decline.

All transplant coordinators were provided with information concerning the research project. In the event that the next of kin sought further information concerning the type of research that might make use of this tissue, the transplant coordinators were in a position to briefly describe this study. The next of kin were offered the opportunity to discuss it in more detail with a member of the study team. The consent forms for organ donation and tissue donation were kept in confidential storage with the transplant coordinators.

2.3.2 Study Procedures

When kidney donation and transplantation occurs, the kidney and adrenal gland are excised together from the donor. The adrenal gland, with surrounding fascia and fat, is separated from the kidney. The kidney is further prepared for transplantation. However the adrenal gland, fascia and fat tissues are not usually suitable for transplantation. Hence they are either used for research purposes or disposed of in a lawful and respectful way, in accordance with the precise instructions on the consent form signed by the next of kin.

Where consent for use of the adrenal glands for research purposes was given,

the tissue was collected at the time the kidney was prepared for transplant. The excised adrenals were divided and either snap frozen in liquid nitrogen (-80^{o}C) or immersed in RNAlater RNA Stabilization Reagent (Qiagen).

The following procedures were performed on the adrenal tissue donated by participants:

- DNA extraction for genotyping of REN-5312C/T
- Tissue mRNA extraction to quantify levels of the following renin angiotensin system components by real-time PCR:
    a. Renin;
    b. Angiotensinogen;
    c. ACE;
    d. ACE-2.

2.3.3 RNA extraction, cDNA generation and real-time (RT) PCR amplification

RNA in human tissue is not protected after harvesting until the sample is flash-frozen, treated with RNAlater RNA Stabilization Reagent (Qiagen), or disrupted and homogenized in the presence of RNase-inhibiting or denaturing reagents. Otherwise, unwanted changes in the gene expression profile will occur. It was therefore important that tissue samples were immediately frozen in liquid nitrogen and stored at –70°C, or immediately immersed in RNAlater RNA Stabilization Reagent (Qiagen). It was important that the procedures for tissue harvesting and RNA protection were carried out as quickly as possible after the tissue was removed from the donor to maximize preservation of RNA.

Arrangements were made with the Transplant Coordinators to contact the study team when they were en route to the organ donation procedure. A nominated member of the study team would attend at the time of adrenal and adipose tissue dissection from the donor kidney. Surrounding adipose tissue was dissected off the adrenal under sterile conditions. This was performed with minimal disruption to the adrenal tissue. The tissue was then cut into 7 to 10 transverse sections applying minimal pressure so as not to disrupt the RNA within the tissue.

All samples were labeled with a code indicating the study (“ADR”) and a sequential number (e.g. 001, 002, 003 etc.). Half of the tissue samples from each tissue donor were placed in labeled 2mL sterile Cryovials (Cruinn Diagnostics, Dublin, Ireland) and immediately flash-frozen in liquid nitrogen, then placed into the -80^{o}C freezer for storage. 1mL of RNALater (Qiagen) solution was dispensed into 3 to 5 sterile 2mL Cryovials (Cruinn Diagnostic) and the remainder of the tissue dispensed individually into these, placed in the +4^{o}C fridge for 24 hours and transferred to the -80^{o}C freezer the following day.

Materials needed:

14.3 M β-mercaptoethanol (β-ME) (Sigma-Aldrich)
RNeasy Mini Kit (Qiagen)
RNALater (Qiagen)
Sterile, RNase-free pipette tips
Microcentrifuge
70% ethanol (Sigma-Aldrich)
Disposable gloves
Blunt needle and syringe
Mortar and pestle

2.3.3.1 Preparation of Tissue for Disruption and RNA isolation

Adequate tissue disruption and homogenization is essential to the process of RNA extraction. It is also critical in order to ensure maximum tissue RNA yield that frozen tissue samples must not be allowed to thaw during handling. To achieve tissue disruption the frozen tissue was ground at -80^{o}C in a sterile mortar and pestle. To minimise RNA cross-contamination the pestle and mortar were cleansed with diethypyrocarbonate treated water (Sigma-Aldrich) and left under UV-light in the biohazard fumehood overnight.

Tissue disruption took place in the biological sample fumehood and liquid nitrogen was used at all times to ensure tissue thaw was not allowed. The tissue sample was ground into fine powder and transferred into chilled sterile labeled RNAse-free 2mL cryovials yielding approximately 20-30mg of tissue for processing. Total RNA was extracted from the ground tissue using QIAGEN

RNeasy Plus Mini kit, according to the protocol supplied.

Lysis buffer was added to the tissue powder having been activated by addition of 2-Mercaptoethanol (β-ME) (Sigma-Aldrich) to Buffer RLT in accordance with the RNEasy Mini Kit (Qiagen) protocol. The lysate was passed through a 1mL syringe with a 20-gauge needle to ensure adequate homogenization. The lysate was centrifuged at full speed for three minutes with the supernatant used in subsequent steps for the RNA extraction procedure as per the manufacturer's protocol.

2.3.3.2 RNA Extraction Procedure

1 volume of 70% ethanol (Sigma-Aldrich) was added to the cleared lysate, and mixed immediately by pipetting. Up to 700 µl of the sample was applied to an RNeasy (Qiagen) mini column placed in a 2 ml collection tube and centrifuged for 15 seconds at 10,000 rpm. Buffers RW1 and RPE were added in accordance with the RNeasy Plus Mini Kit (Qiagen)protocol and centrifuged.

To elute, the RNeasy (Qiagen) column was transferred to a new 1.5 ml collection tube and 30–50 µl RNase-free water (Qiagen) was pipetted directly onto the RNeasy silica-gel membrane and centrifuged for 1 minute at 10,000 rpm. The eluted RNA was kept on ice at all times and aliquoted into 2x20uL amounts in RNAse free tubing, to be ultimately frozen at -80 degrees Celsius.

2.3.3.3 RNA Concentration Determination using Nanodrop 8000

RNA was finally eluted in diethylpyrocarbonate-treated water (30µl) (Sigma-Aldrich) and stored at -80ºC. RNA concentrations in all samples prior to freezing were determined using the Nanodrop ® 8000 UV-Vis Spectrophotometer. The quantity and quality of the extracted RNA was confirmed by absorption measurements at 260 and 280 nm. Nucleic acids have a peak absorbance at 260nm wavelength and proteins at 280nm wavelength. The 260/280 ratio is designed to detect contamination of protein with nucleic acid, thus evaluating the quality of the isolated RNA. A ratio of approximately 2 is generally accepted as "pure" for RNA (Thermo Scientific, T009 Technical Bulletin).

The spectrophotometer was blanked using 100µL of H_2O and the samples loaded with 8 x 1µL samples loaded at a time. Nucleic acid concentration and purity were measured using the A_{260}/A_{280} ratio. The extracted RNA was aliquoted into 2 x 20uL samples and frozen. Extracted RNA was subsequently used for the purpose of cDNA generation and real-time PCR.

Table 2.1 – Determining purity of RNA obtained. Example of RNA concentrations obtained by Nanodrop 8000® utilizing the A260:280 ratio, important in evaluating the quantity and quality of extracted RNA. Contamination of protein with nucleic acid can be evaluated in each sample.

Sample ID	A260:280	Concentration ng/uL	Eluted Volume
17	2.05	265	40uL
18	2.07	233.8	40uL
19	2.06	1107	40uL
20	1.96	495.9	40uL
21	2.03	146.3	40uL
22	2.03	307.1	40uL
23	2.07	142.7	40uL

2.3.3.4 Complementary DNA generation

Materials Needed:

Multiply Pro 0.2mL Biospheres (Sarstedt)
ImProm II Reverse Transcriptase System (Promega, A3800)
Freezer Blocks (Eppendorf)
PCR Thermal Cycler - MJ Research Thermal Cycler Model PTC 220 DYAD
Mini-Vortex (Fisher-Scientific)
Polystyrene box filled with ice, Gilsen Pipettes and Pipette tips

Single-strand cDNA was synthesized using 1µg RNA using the ImProm II reverse transcriptase kit (Promega), in a total volume of 20µl. Calculations regarding various amounts of RNA, Oligo primer and nuclease free water were made using a pre-programmed reverse transcription Excel template. To a 1 µg concentration of RNA, 1 µL of Oligo deoxythymine (DT) and a calculated volume of nuclease free water were added to yield a total volume of 5 µL. Immediately prior to the RNA extraction the RNA was taken out and left on ice to thaw.

The agents for use in complementary DNA (cDNA) generation were prepared using the Improm II Reverse Transcriptase system (Promega) as per the kit protocol with the nuclease-free (NF) water, Oligo dT_{15} Primer, Improm II 5X Reaction Buffer, MgCl2, dNTP and RNasin Ribonuclease Inhibitor all taken out to thaw on ice. The thermal cycler was initialized and heating block prepared (MJ Research Thermal Cycler Model PTC 220 DYAD). The heating block was preheated to 70 degrees Celsius. Labeled Multiply Pro 0.2mL Biospheres (Sarstedt) were placed onto the Freezer Block (Eppendorf), identified with same identifier used in storage of RNA samples, e.g. ADR002.

Figure 2.2 - Thermal Cycler and heating block used in cDNA generation.
MJ Research Thermal Cycler Model PTC 220 DYAD

cDNA Generation Procedure

Using a P10 Gilsen pipette and 10uL tips, NF Water was pipetted into the chilled Biospheres first, followed by the thawed RNA sample and then 1uL of the Oligo DT Primer. The Biospheres were placed into the heating block for five minutes. The Master Mix was prepared as per a preprogrammed Excel template (4 µL Reaction Buffer, 5 µLMgCl2, 1 µL dNTP and 0.5 µLRNAsin Ribonuclease inhibitor combined for each sample) . Provision was made to allow for an additional sample amount to compensate for inadvertent loss during pipetting. The samples were returned back into a freezer block again for five minutes. To the cooled samples 10.5 µL of Master Mix, 3.5 µL NF water and 1 µLresulting in a final volume of 20 µL. Reverse Transcriptase (RT) were added in sequence. RT was left in the freezer until immediately before use to ensure minimal degradation of enzyme.

The tubes were vortexed to allow adequate mixing for approximately ten seconds, returned to the heat block and the reverse transcription reaction selected. The sample was then annealed at 25^{o}C for 5 minutes. First-strand synthesis reaction was carried out at 42°C for 60 minutes. When completed the samples were removed and stored in the minus 20^{o}C freezer for future quantitative real-time PCR.

2.3.3.5 Real-time PCR with TaqMan assays

Real-time PCR was used to quantify mRNA transcript levels, using the Taqman system. TaqMan real-time PCR is one of the two types of quantitative PCR methods. TaqMan uses a fluorogenic probe that is a single stranded oligonucleotide of 20-26 nucleotides and is designed to bind only the specific DNA sequence between the two PCR primers. Only a specific PCR product can generate fluorescent signal in TaqMan PCR. We used the TaqMan Real-time PCR system to quantify renin angiotensin system messenger RNA (mRNA) transcript levels. Primers were designed by Applied Biosystems for use with the Taqman reporter system.

The quantitative endpoint for real-time PCR is the threshold cycle (C_T). This is the point at which amplification of the PCR product is such that the fluorescent signal of the reporter dye crosses an arbitrarily placed threshold (Schefe et al. 2006). The larger the starting quantity of the target molecule, the earlier a significant increase in fluorescence is observed.

Determining the precise amount of total cDNA added to each reaction and its quality are both difficult to assess. To allow as accurate an estimation as possible, transcripts of the gene coding for 18S ribosomal RNA (rRNA) were also quantified as an endogenous or internal control. 18s rRNA represents the structural RNA for the small eukaryotic ribosomal subunit (40S). As 18s rRNA is ubiquitous to all eukaryotic cells each sample was normalized on the basis of its 18S rRNA content.

Gene expression was quantified using relative quantification, or the 'comparative C_T' method, where the data are presented relative to another gene, often referred to as an internal control. This method assumes that the efficiency of the PCR is close to 1 and that the PCR efficiency of the target gene is similar to the internal control gene. 18S rRNA represented the internal control for these samples (Schmittgen & Livak 2008).

For quantitative real-time PCR, 2µl (20ng/ µl) cDNA was loaded in an optical 96-well plate with 10µl TaqMan Gene Expression Master mix (Applied Biosystems), 1µl primer mix (Applied Biosystems, 20x stock concentration 18µM per primer) and 7µl PCR-grade water (Sigma-Aldrich). Amplification was carried out in a 7500 Fast Real-time PCR System (Applied Biosystems) as

follows: a preincubation step at 95°C for 10 min was followed by 40 cycles of denaturation at 95°C for 20 sec, annealing at 60°C for 30 sec, and elongation at 72°C for 30 sec. All samples were normalized for 18S rRNA expression levels.

Taqman gene expression assay primers were used (Applied Biosystems, Invitrogen). The targets of interest were:

- Renin (hs00982555_m1): Amplicon length 62 base pairs, amplifies exons 8-9;
- Angiotensinogen (hs01586213_m1): Amplicon length 121 base pairs, amplifies exons 4-5;
- ACE (hs00174179_m1) Amplicon length 74 base pairs, amplifies exons 19-20; and
- ACE2 (hs01085333_m1): Amplicon length 141 base pairs, amplifies exons 16-17.

All samples were assayed in triplicate. The mean C_T values of the 18S endogenous controls for each sample were within the range 10.0-13.7 for 21 of 25 samples assayed. The four samples for which this was not the case were excluded from further analysis.

Within-replicate standard deviation of C_T values was consistently <0.05 for each of the primers used. The reference sample was chosen as the sample for which ΔCT (C_T for each primer assay- C_T for the endogenous control) was closest to the population mean value. The following reference samples were selected for each target of interest on the basis of mean 18S ΔCT value observed: renin primer, ADR014b; angiotensinogen primer, ADR017; ACE primer, ADR019 and ACE2 primer, ADR013. ΔΔ C_T was calculated as the difference between Δ C_T of each sample and Δ C_T of the reference sample. Final fold changes for each sample relative to the reference sample were calculated as 2^(-ΔΔ C_T). A pre-programmed Excel sheet was used for the purpose of the calculations.

2.3.3.6 DNA Extraction from adrenal tissue

Materials needed

QIAamp DNA Mini kit (Qiagen)
Sterile pipette tips (Sigma-Aldrich)
Microcentrifuge
Water bath x2
Disposable gloves
Blunt needle and syringe
Mortar and pestle
Sterile 96-well plate (Greiner, Sigma-Aldrich)

Procedure

Samples were equilibrated to room temperature (15–25°C). Two water baths were heated to 56°C and 70°C. Buffers AW1 and AW2 were prepared according to the QIAamp DNA Mini kit protocol (Qiagen).

The frozen tissue was removed from storage, placed on dry ice, and mechanically disrupted with pestle and mortar as with the RNA extraction procedure previously described. 180 µl of Buffer ATL was added in accordance with the QIAamp DNA Mini kit protocol (Qiagen). 20 µl (50µg/mL) proteinase K was added to the sample and vortexed, then incubated at 56°C to allow for tissue lysis and briefly centrifuged. 200 µl Buffer AL was added to the sample, pulse-vortexed for 15 seconds, and incubated at 70°C for 10 min. 200 µl ethanol (96–100%) was added to the sample, and mixed by pulse-vortexing for 15 seconds (Fisher-Scientific).

The mixture and precipitate were applied to the QIAamp Mini spin column (Qiagen) and centrifuged at 8000 rpm for 1 minute. 500 µl of Buffer AW1, Buffer AW2 and Buffer AE were all added in sequence with the sample centrifuged in between each buffer addition. To allow for long-term storage of DNA, the sample was eluted in buffer AE and stored –20°C since DNA stored in water is subject to acid hydrolysis.

2.3.3.7 DNA plating

Adrenal DNA was pipetted carefully onto a sterile 96-well plate (Greiner, Sigma-Aldrich). Random duplicates were incorporated with new sample IDs along with two blank wells as controls. The plates were left to dry overnight. Adhesive film was placed on top prior to packaging and preparation for transport. The plates were sent by registered airfreight to K Biosciences, Herts. Genotyping was performed by Kbiosciences using modified TaqMan assays (http://www.kbioscience.co.uk).

2.3.4 Statistical Analyses

2.3.4.1 Data Handling

All data generated by the study remains confidential and no current or future report contains or will contain any information that would allow an individual participant in the study to be identified. Paper copies of data are stored in a locked filing cabinet. Electronically stored data, identified by a unique registration number, the participants' date of birth, and the participants' initials are stored on password-protected computers.

2.3.4.2 Statistical Analysis

Data were represented as mean ± SD. Unpaired Student's T-Tests were used to compare tissue levels of renin, angiotensinogen, ACE and ACE2 mRNA between REN-5312 CC-homozygotes and T allele carriers (CT-heterozygotes and TT-homozygotes). One-tailed significance values were used given the hypothesis that mRNA expression would be greater in T-allele carriers than CC-homozygotes.

2.4 Results

2.4.1 Donor Participants

In total, 27 adrenal tissue samples were obtained from cadaveric renal donors. Detailed data regarding cause of death is not available for this population. The first sample, ADR001, was excluded from further analysis. This was due to a deviation in initial storage conditions rendering it unsuitable for cDNA generation and real-time PCR (RT-PCR). Sample ADR010 was excluded from RT-PCR and further analysis due to replacement of normal adrenal tissue by multiple, large adrenal adenomas observed on preparation of the sample. 25 samples were analysed by RT-PCR.

The table below illustrates the renin SNP against which mRNA expression levels of renin, angiotensinogen, ACE and ACE2 were evaluated.

Table 2.2 - Renin distal enhancer element, REN-5312C/T SNP. SNP, single nucleotide polymorphism; rs, reference SNP cluster ID – an accession number used by researchers and databases to refer to specific SNPs. Adapted from **(Moore et al. 2007)**.

SNP Location	Alleles	rs Number
Distal enhancer -5312	C/T	rs12750834

For the 25 samples analysed, the range of mean C_T values of the 18S endogenous control was 6.98, with a minimum and maximum value of 6.94 and 13.91 respectively. The 18S mean C_T measured 11.78 (SD 1.9). After exclusion of outliers, 21 of 25 samples were chosen for analysis.

Figure 3.4 illustrates the number of donor participants by each REN -5312C/T genotype group. The population was represented by more CT-heterozygotes than CC-homozygotes, and only one TT-homozygote.

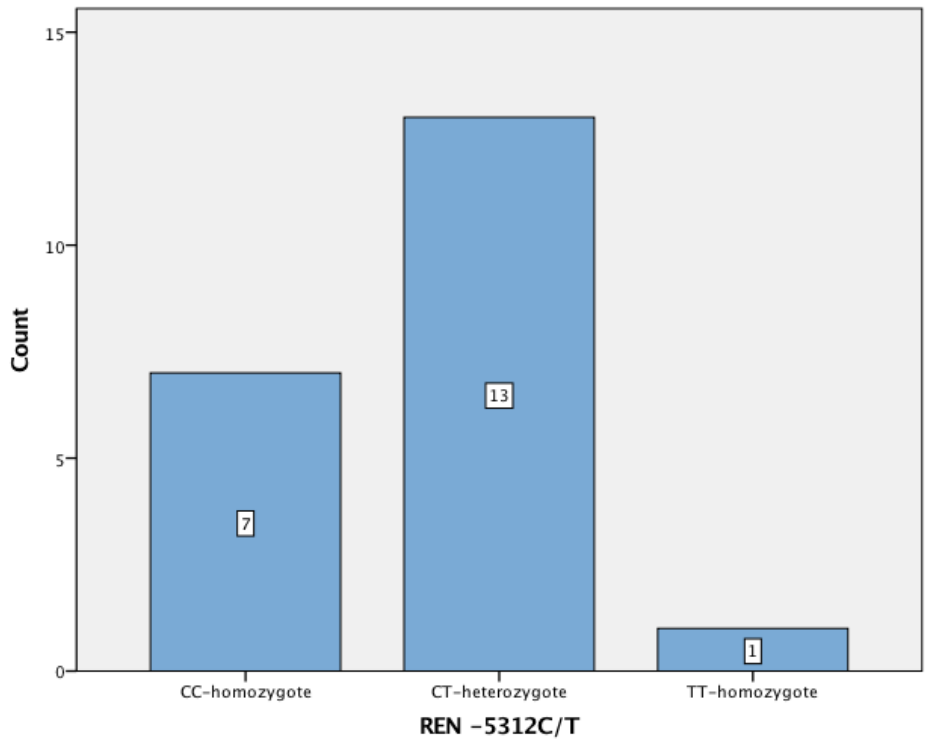


Figure 2.3 – Study Population. The population split for the purpose of analysis by genotype of the distal enhancer element SNP, REN -5312C/T, rs12750834. Data expressed as number of participants by REN -5312C/T genotype.

Table 2.3 illustrates the donor participant characteristics by REN -5312C/T genotype. Table 2.4 illustrates the donor participant characteristics where donors who expressed the T-allele as either hetero- or homozygotes were combined to form a single participant group. This was performed for the purpose of statistical analysis given that the TT-homozygote group contained only one female donor participant.

Table 2.3 - Donor participant characteristics by REN -5312C/T genotype. Data expressed as mean and standard deviation of mean, mean(SD), or as the count for categorical variables.

Variable	CC-homozygote (N=7)	CT-heterozygote (N=13)	TT-homozygote (N=1)
Age, years	45.71(16.11)	46.85(14.33)	19(0)
Male	3	7	0
Female	4	6	1

Table 2.4 - Donor participant characteristics categorised by genotype. CC vs. CT and TT. Data expressed as mean and standard deviation of mean, or as the count for categorical variables.

Variable	CC-homozygote (N=7)	CT-heterozygote and TT-homozygote (N=14)	p-value
Age, years	45.71(16.11)	44.86(15.65)	0.45
Male	3	7	0.38
Female	4	7	

The minor allele frequency for the donor participant population was 0.36. This minor allele frequency was considerably higher than previously described frequencies, which have measured up to 0.20 (Moore et al., 2007). Hardy Weinberg Equilibrium in the population is demonstrated by the following equation, where q represents the minor allele:

Hardy Weinberg Equation
$p^2 + 2pq + q^2 = 1$
$0.413 + 0.459 + 0.127 = 0.999$

2.4.2 REN-5312C/T genotype and adrenal tissue mRNA levels of renin

For mean fold change of renin mRNA, a significant difference was observed between CT-heterozygotes combined with the TT-homozygote by comparison with CC-homozygotes, p=0.036. (Figure 2.4 (a) and Table 2.5). On review of individual participant fold change values, a greater number of participants within the CT-heterozygote group were observed to have higher mRNA expression levels than individual CC-homozygotes (Figure 2.4 (b)).

The significant difference in mean fold change between the two participant groups and observation of a difference in individual fold change values between CT-heterozygotes and CC-homozygotes supports greater influence of the REN -5312C/T on tissue renin expression in CT-heterozygotes *versus* CC-homozygotes.

Table 2.5 – mRNA expression levels for renin, angiotensinogen, ACE and ACE2 by REN-5312C/T genotype. This table illustrates the results of mRNA transcript expression levels from 21 adrenal samples for each of renin, angiotensinogen, ACE and ACE2 by genotype REN -5312C/T, rs12750834. Fold change value obtained for each of renin, angiotensinogen, angiotensin-converting enzyme (ACE) and angiotensin-converting enzyme-2 (ACE2) messenger RNA, mRNA, are tabulated with mean and standard deviation (SD) values shown. One-tailed significance values obtained by independent T-test are shown for each mRNA transcript according to genotype with CC-homozygotes *versus* CT-heterozygotes and TT-homozygotes

Fold Change mRNA	REN -5312C/T	N=	Mean(SD)	p-value
Renin	CC-homozygote	7	1.07(1.07)	0.036*
	CT-heterozygote and TT-homozygote	14	3.07(3.61)	
Angiotensinogen	CC-homozygote	7	1.2(1.02)	0.097
	CT-heterozygote and TT-homozygote	14	2.27(2.59)	
ACE	CC-homozygote	7	0.99(0.64)	0.082
	CT-heterozygote and TT-homozygote	14	2.43(3.58)	
ACE2	CC-homozygote	7	1.49(1.33)	0.141
	CT-heterozygote and TT-homozygote	14	2.4(2.42)	

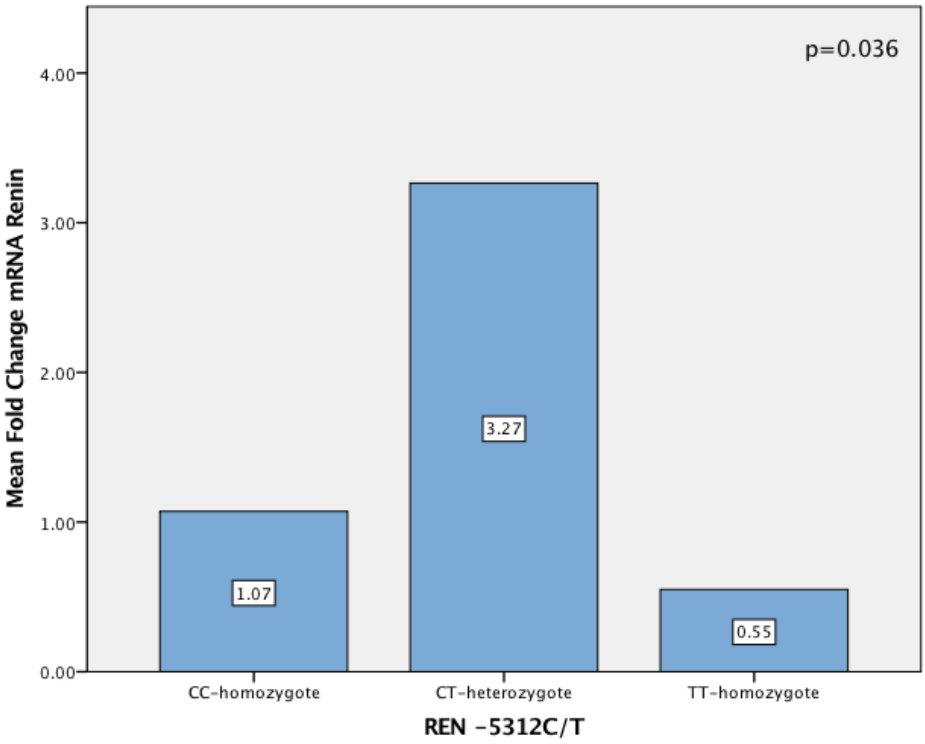


Figure 2.4 (a)

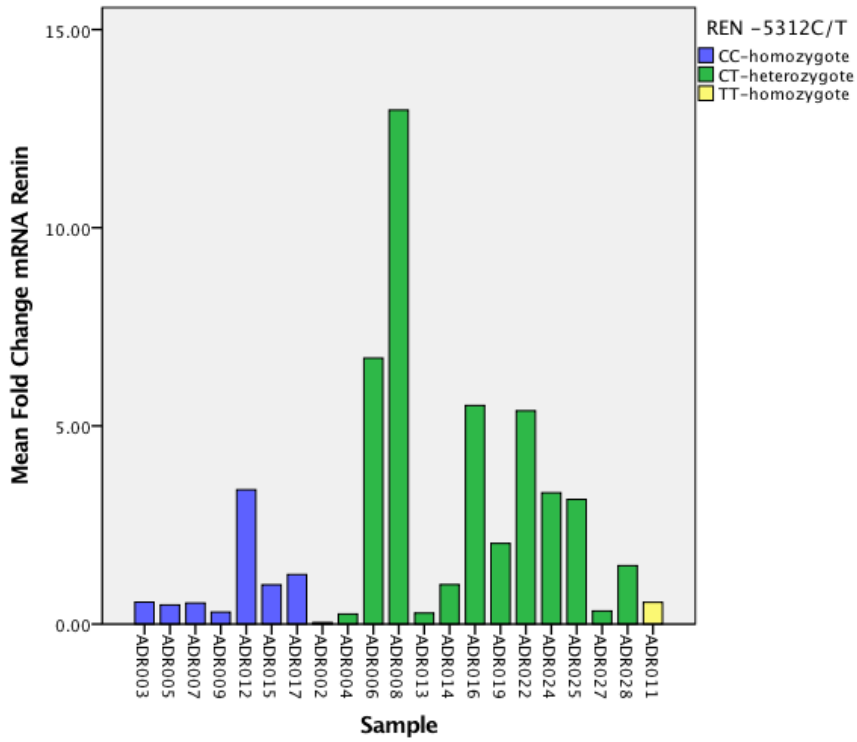


Figure 2.4 (b)

Figure 2.4 (a) and (b) - Fold changes of renin mRNA levels by genotype of the Renin SNP, rs12750834. Fold changes for primer 1, renin (hs00982555_m1) are shown. Sample identified by prefix ADR- and individual sample number. mRNA, messenger RNA.

2.4.3 REN-5312C/T genotype and adrenal tissue mRNA levels of angiotensinogen

For mean fold change of angiotensinogen mRNA, CT heterozygotes demonstrated higher mean values than either CC-homozygotes or the TT-homozygote (Figure 2.5 (a) and Table 2.5). These did not achieve statistical significance when analysed as two participant groups, p-value=0.097. On review of individual participant fold change values, a trend was observed of higher mRNA expression levels in CT-heterozygotes than individual CC-homozygotes (Figure 2.5 (b)).

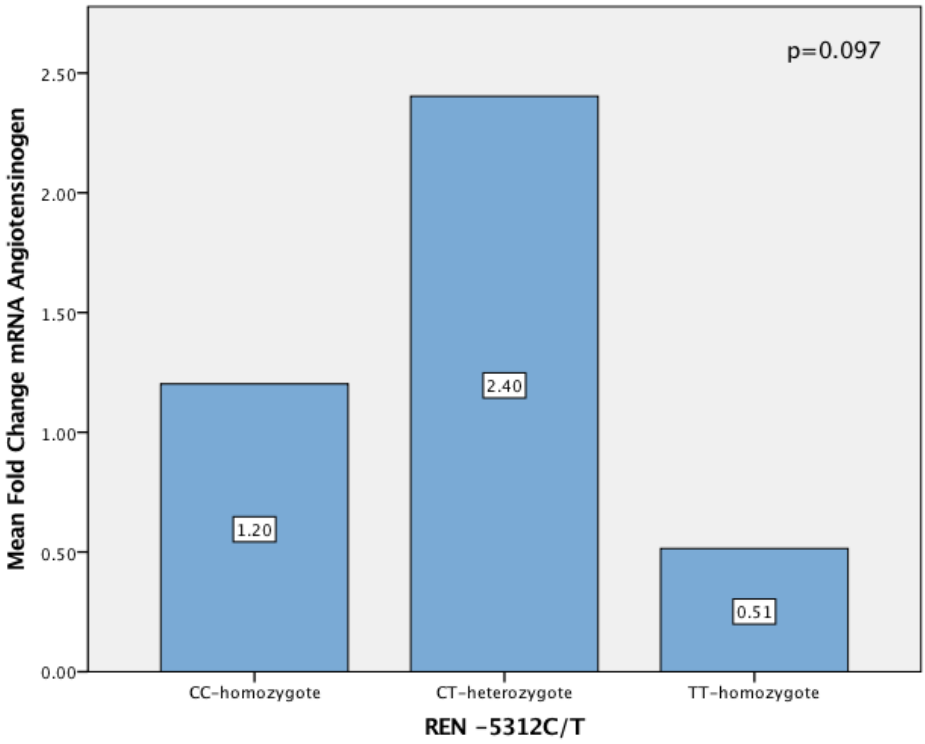


Figure 2.5 (a)

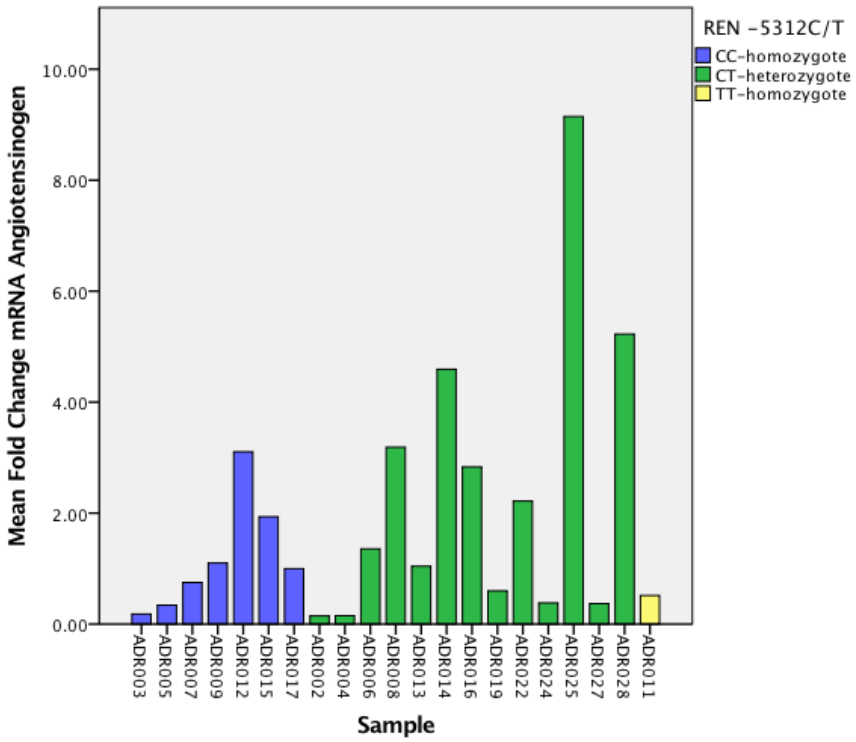


Figure 2.5 (b)

Figure 2.5 (a) and (b) - Fold changes of angiotensinogen mRNA levels by genotype of the Renin SNP, rs12750834. Fold changes for primer 2, angiotensinogen (hs01586213_m1), are shown. Sample identified by prefix ADR- and individual sample number. mRNA, messenger RNA.

2.4.4 REN-5312C/T genotype and adrenal tissue mRNA levels of ACE and ACE2

For mean fold change of ACE mRNA, CT-heterozygotes demonstrated higher mean values than either CC-homozygotes or the TT-homozygote (Figure 2.6 (a) and Table 2.5). These did not achieve statistical significance, p=0.082, and were observed only as a trend in this population.

On review of individual participant fold change values, a greater number of participants within the CT-heterozygote group appeared to have higher mRNA expression levels than individual CC-homozygotes (Figure 2.6 (b)). 5 participants out of 13 in the CT-heterozygote group had higher fold change values than the participant with the maximum fold change in the CC-homozygote group, n=7. The higher mean and individual mRNA expression levels in CT-heterozygotes would support a potential influence of REN -5312C/T on tissue ACE expression in CT-heterozygotes *versus* CC-homozygotes.

Similar observations were made for ACE2 mRNA expression, for both average values and individual participant trends (Figure 2.7 (a) and (b) and Table 2.5).

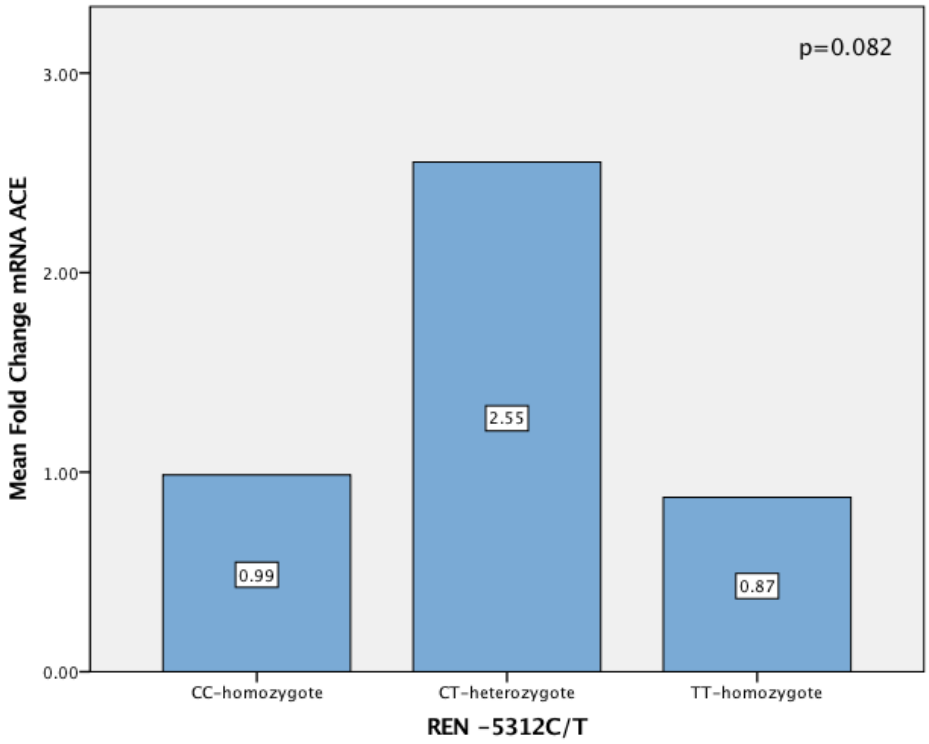


Figure 2.6 (a)

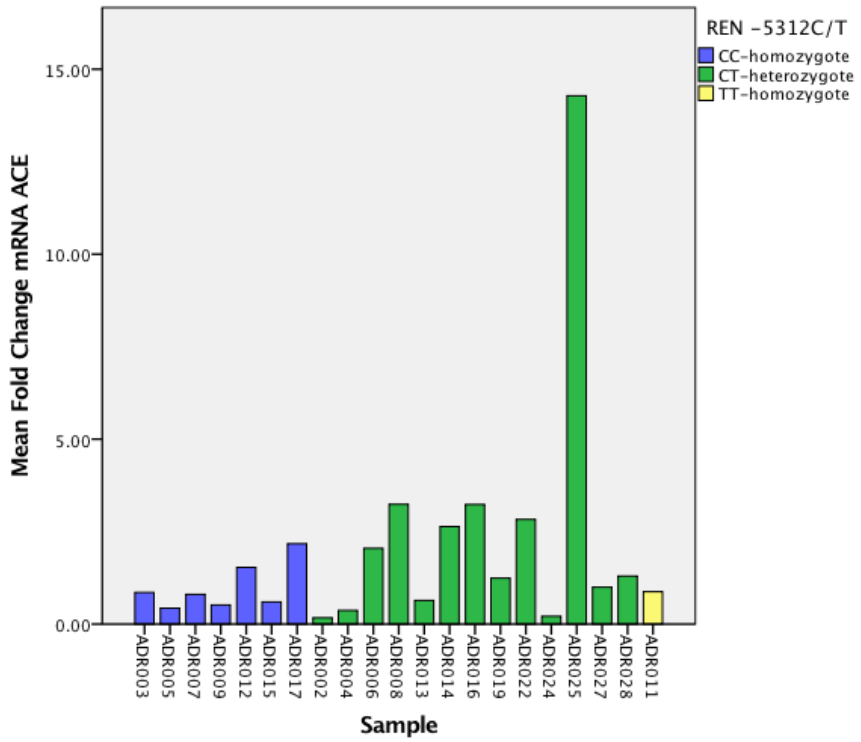


Figure 2.6 (b)

Figure 2. 6 (a) and (b) - Fold changes of ACE mRNA levels by genotype of the Renin SNP, rs12750834. Fold changes for primer 3, ACE (hs00174179_m1), are shown. Sample identified by prefix ADR- and individual sample number. mRNA, messenger RNA.

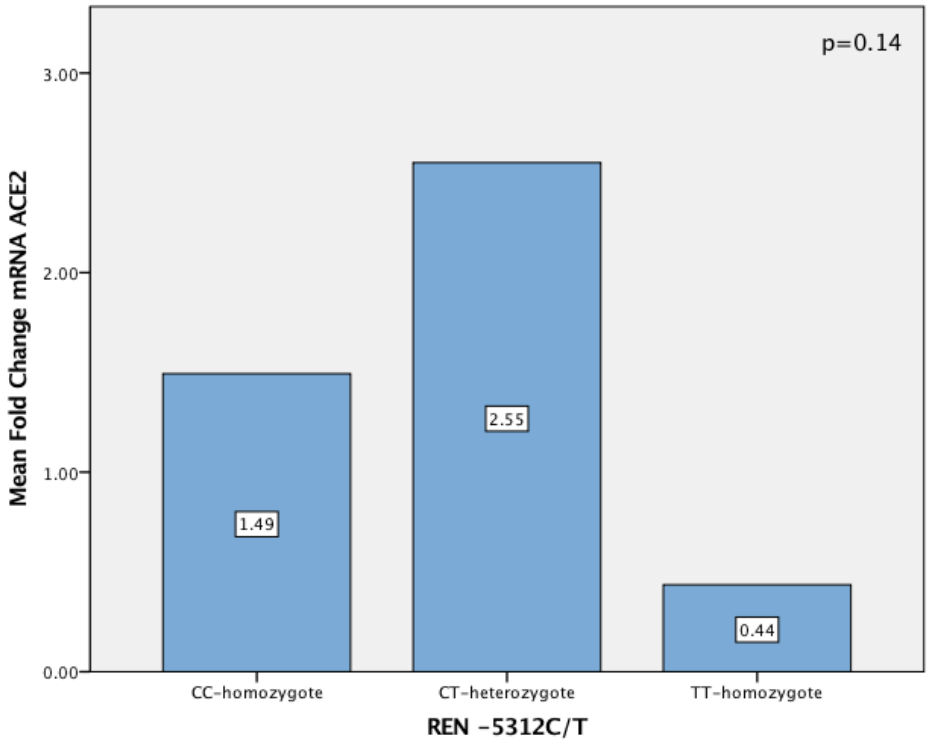


Figure 2.7 (a)

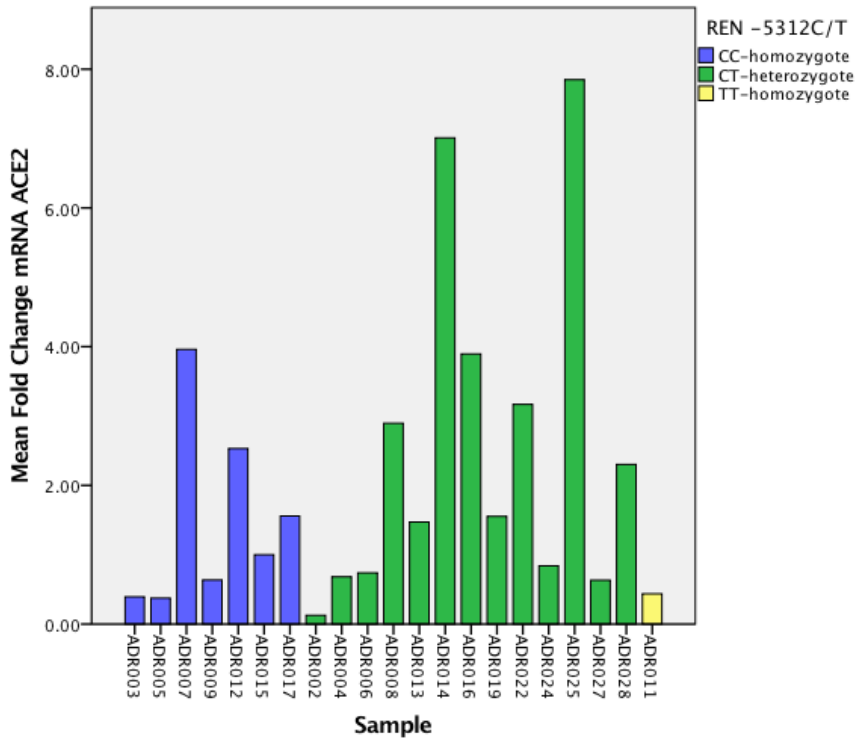


Figure 2.7 (b)

Figure 2.7 (a) and (b) - Fold changes of ACE2 mRNA levels by genotype of the Renin SNP, rs12750834. Fold changes for primer 4, ACE2 (hs01085333_m1) are shown. Sample identified by prefix ADR- and individual sample number. mRNA, messenger RNA.

2.5 Discussion

2.5.1 Summary of Results

REN -5312C/T significantly influenced adrenal tissue expression of renin mRNA. This confirmed the findings of previous work performed by Fuchs et al. (2002) evaluating the functionality of REN-5312C/T in choriodecidual tissue, and Makino et al. (2015) evaluating REN-5312C/T in diseased renal tissue. In this current work, significantly higher renin mRNA expression was observed in CT-heterozygotes, with an approximate three-fold higher renin mRNA expression seen in CT-heterozygotes compared to CC-homozygotes. Only one TT-homozygote was identified in the study population. A trend toward higher levels of mRNA expression of the remaining RAS components studied was observed in CT-heterozygotes.

2.5.2 Discussion

Transgenic studies in mice have suggested that the most important regulatory regions involved in renin expression reside within the renin 5' flanking region (Pan & Gross 2005). Both renin transcription and protein synthesis are known to occur in choriodecidual tissue from foetal membranes. Similar transcription factors exist within both the renal cortex and human choriodecidual cells, binding the human renin promoter. Consequently, primary cultures of choriodecidual cells have been used to determine activity of regions within the human renin promoter (Borensztein et al. 1994). A 225 base pair (bp) enhancer element located in the promoter region was previously described, 5777-5552 nucleotides upstream from the renin transcription start site (Germain et al. 1998). In human choriodecidual cells, this enhancer region has been observed to up regulate renin expression by a factor of 60 (Germain et al. 1998).

Fuchs et al., hypothesized that polymorphisms within or near the enhancer region could also affect renin expression (Fuchs et al. 2002). They subsequently identified two new single nucleotide polymorphisms, SNPs, at nucleotide positions -5434 and -5312. Only -5312 affected renin transcription, with the -5312T variant yielding 45% greater levels of renin transcription than the -5312C variant in choriodecidual cells (Fuchs et al. 2002). This was the first evidence of a direct effect *in vitro* of the -5312T polymorphism on the level of transcription of human renin.

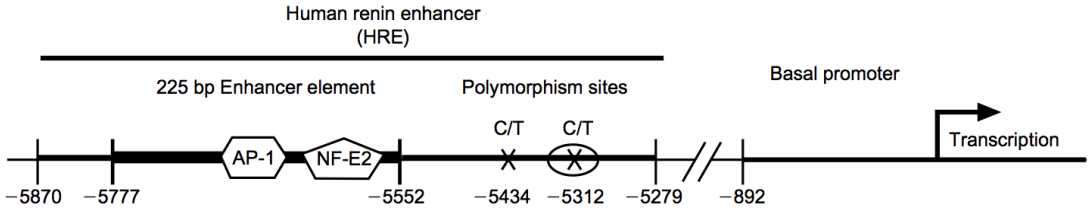


Figure 2.8 – Schematic representation of the regulatory regions of the human renin promoter. The previously described 225 bp enhancer element (positions -5777 to -5552) contains two major binding sites for two transcription factors, nuclear activation promoting-1 (AP-1) and nuclear-factor erythroid derived-2 (NF-E2), which are involved in its stimulatory activity **(Germain et al. 1998)**. The two new single nucleotide polymorphisms identified downstream from this enhancer element are located 5434 and 5312 bp upstream from the transcription initiation site. The human renin enhancer (HRE) comprises not only the 225 bp enhancer element, but also the -5312 SNP. Reproduced from **(Fuchs et al. 2002)**.

In a separate and more recent study in kidney tissue, 64 patients with biopsy-proven renal conditions were studied to evaluate whether REN-5312C/T genotype influenced kidney renin expression levels (Makino et al. 2015). Tissue was obtained by ultrasound-guided percutaneous renal biopsy. RNA was extracted from approximately 2mm of an 18G needle specimen of renal cortical tissue. The REN -5312C/T genotype was assayed with measurement of renin and endogenous control mRNA (glyceraldehyde 3-phosphate dehydrogenase, GAPDH) by real-time quantitative PCR. The authors observed a significant difference in renin gene expression between REN -5312C/T genotypes when analysed by analysis of variance (ANOVA). T-allele carriers were observed to have higher levels of renin gene expression when compared to CC-homozygotes. These findings support functionality of the SNP in a tissue other than choriodecidual tissue (Fuchs et al. 2002). The findings of Makino et al. (2015) are illustrated in Figure 2.9 (a) and (b).

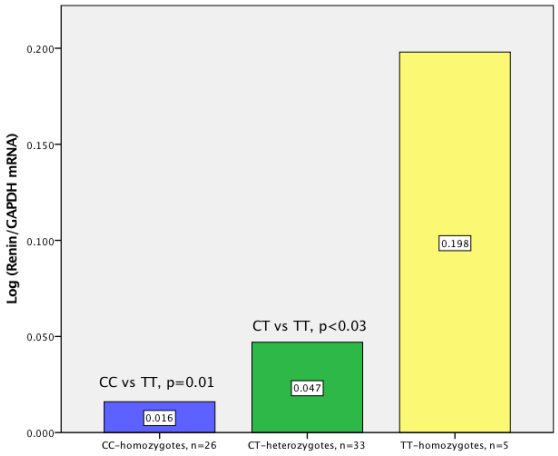


Figure 2.9 (a)

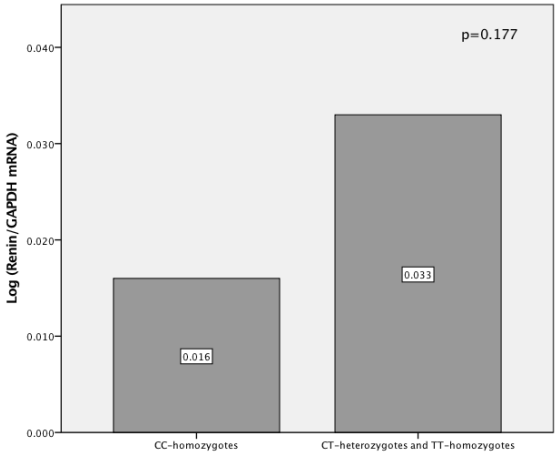


Figure 2.9 (b)

Figure 2.9 (a) and (b) – REN-5312C/T genotype and renin gene expression levels in diseased human kidney tissue. mRNA was measured and compared among REN-5312C/T genotypes. The values for genetic expression are expressed as logarithmically transformed values of their relative ratio to GAPDH (10^{-3}). Columns show the means. Adapted from **(Makino et al. 2015)**.

The current work described in this chapter confirms the findings of Fuchs et al. and those of Makino et al., with higher levels of renin expression seen in -5312T allele carriers (Fuchs et al. 2002; Makino et al. 2015). The groups assessed renin mRNA expression in two different tissue types, Fuchs et al. in human choriodecidual tissue from the uteroplacental unit, and Makino et al. in diseased human kidneys. Our study evaluated the effect of the REN-5312C/T genotype in normal human adrenal tissue, the first to replicate this finding in normal human tissue. The consistency of findings across all three studies and tissue types supports the functionality of REN-5312C/T in influencing tissue renin gene expression.

Adrenal tissue expression of angiotensinogen, ACE and ACE2 did not achieve statistical significance in relation to renin genotype. However, trends were observed toward higher individual expression of angiotensinogen, ACE and ACE2 mRNA amongst CT-heterozygotes compared to CC-homozygotes. Therefore, it may be surmised that in addition to renin, REN -5312C/T may directly or through association with other polymorphisms affect tissue mRNA expression levels of those components of the renin angiotensin system, RAS. One possible mechanism whereby this could occur is through prorenin to (pro)renin receptor interactions influencing intracellular pathways and gene expression (Nguyen & Muller 2010; W. Li et al. 2012).

In a population of 50 adolescents with insulin dependent diabetes mellitus, 25 of whom also had microalbuminuria, a familial disposition to higher plasma prorenin was suggested when they were compared to non-IDDM matched siblings with and without microalbuminuria (Daneman et al. 1994). Deinum et al. went on to identify a renin gene polymorphism associated with diabetic nephropathy(Deinum, Tarnow, et al. 1999). A *bgl*I restriction fragment length polymorphism located at base 1161 in Intron A of the renin gene represents a C-T mutation. A trend was identified in bb-homozygotes with diabetic nephropathy for higher prorenin levels, p=0.07. Their findings suggested that this renin gene polymorphism contributed to diabetic nephropathy through its effects on prorenin levels, and warrants further review.

A greater number of donor participants in this study population were T-allele carriers than expected, with a minor allele frequency of 0.36. By comparison, the minor allele frequency of our clinical trial cohort was 0.18 (see Chapter 3). This is consistent with the findings of Moore et al (2007) who observed a minor allele frequency in their hypertensive population of 0.18, and an additional confirmatory study by the same group who observed a minor allele frequency of 0.18 in two Irish study populations (Moore et al. 2007). In our study population, all participants were cadaveric organ donors. Detailed data regarding cause of death in this current study population was not available. An important limitation to note in this particular work is the fact that tissue was obtained from cadaveric tissue donors with limited phenotypic information available. In addition, the potential effect of ethnicity was not assessed. The sample size yielded only one TT-homozygote, which makes interpretation of data relating to that individual in

the context of the overall study difficult. Greater sample numbers would be necessary in the future in particular if a cadaveric donor population is studied again. These limitations would need to be taken into consideration in any future tissue work evaluating RAS polymorphisms and their influence on tissue mRNA expression of RAS components.

With greater renin expression seen in the t-allele group (CT/TT) in this study population, it would be fair to suggest that RAS blockade may play a role in limiting target organ damage in CT/TT individuals with cardiovascular disease. In current practice for example, it is recommended that RAS blockade is utilized as a drug therapy in diabetic patients to reduce incidence of diabetic nephropathy. The same can be said for those patients post myocardial infarction in an attempt to limit complications associated with cardiovascular disease such as heart failure or LVH. Carriage of a renin SNP indicating heightened tissue RAS activity would make a greater case for encouraging the use of RAS blockers as antihypertensives in individuals over the age of 55 who would otherwise traditionally receive a calcium channel blocker initially alone, by virtue of the evidence base available (McManus et al. 2012).

2.5.3 Conclusion

The key finding of this study is that the adrenal glands from healthy humans who are REN-5312 T-allele carriers exhibit greater renin mRNA levels than REN-5312 CC-homozygotes. This confirms that REN-5312C/T has definite functionality in agreement with Fuchs et al., and Makino et al.

Chapter 3 - Renin Genotype and Response to Renin Angiotensin System Blockade

3.1 Introduction

The renin-angiotensin system (RAS) has a central role in the regulation of blood pressure level. International guidelines (US, European and UK) all advocate the use of agents which block varying steps of the RAS as first-line or second-line therapy for hypertension. The most studied candidate genes in terms of contribution to hypertension are those coding for the renin-angiotensin aldosterone system (Redon et al. 2004). Polymorphisms within the Renin-Angiotensin Aldosterone System (RAAS) such as the ACE I/D, angiotensinogen (AGT) M235T polymorphisms and angiotensin II type-1 receptor (AT1R) polymorphism A1166C have all been well characterised.

Renin is an aspartic protease and is the rate-limiting step in the process that ultimately results in angiotensin II production. The renin gene, REN, spans 12kB of DNA and contains 8 introns (Hobart et al. 1984). It is located at 1q32 (Cohen-Haguenauer et al. 1989). A common single nucleotide polymorphism (SNP) in a renin distal enhancer element (REN-5312C/T, rs12750834) has been reported to influence *in vitro* gene transcription in transfected human choriodecidual cells (Germain et al. 1998). In 2002, Fuchs and colleagues noted 45% greater rates of renin gene transcription in the presence of a -5312T allele rather than a -5312C allele (Fuchs et al. 2002). A further study in diseased human kidney observed greater renin gene expression in T-allele carriers (Makino et al. 2015). In Chapter 2, we replicated the finding of increased renin gene expression associated with carriage of the -5312T allele.

The SNP has been shown to have *in vivo* functional activity in humans, with carriage of the -5312 T-allele associated with elevated ambulatory and clinic BP levels in a cohort of healthy bank employees (Moore et al. 2007). This was replicated in a second population (Vangjeli et al. 2010). It also appeared that presence of the polymorphism predicted BP lowering responses to RAS blockade in hypertensive patients, independent of plasma renin activity, (PRA) (Moore et al. 2007). Aliskiren, a direct renin inhibitor, when compared with losartan, an angiotensin receptor blocker, was found to have a significantly greater nocturnal BP lowering amongst -5312 CC homozygotes than T-allele

carriers. The reverse was true for losartan, with T-allele carriers achieving BP lowering twice that of CC homozygotes. Thus REN-5312 C/T carriership could have potential use as a pharmacogenetic assay (Moore et al. 2007).

3.2 Objectives

The main objectives of this study were:

1. To determine the relationship between REN-5312C/T genotype and plasma renin activity (PRA);
2. And, to confirm or refute whether genotyping of the REN-5312C/T polymorphism, alone or in combination with plasma renin activity (PRA), predicts BP lowering responses to renin-angiotensin system (RAS) blockade.

Drug class and potency, age, ethnicity and baseline BP are currently regarded as clinically useful predictors of BP lowering responses to antihypertensive therapy (Chapman et al. 2002). Hence the principal hypothesis to be tested in this trial was whether REN-5312C/T genotype would predict blood pressure lowering, and whether addition of PRA would yield additive predictive ability of BP lowering responses to renin-angiotensin system (RAS) blockade.

An exploratory analysis of whether REN-5312C/T genotype was predictive of BP lowering responses to the calcium channel blocker Amlodipine was also conducted, as a subset of patients received treatment with both a RAS-blocker and amlodipine.

Lastly, it was recognised that storage of patient DNA and clinical trial data would facilitate future related pharmacogenetic analyses (Beaumont Hospital Medical Research Ethics Committee Approval obtained for this purpose 7th August 2008).

3.3 Methods

3.3.1 Study Design

This was a phase IV single centre prospective randomized open cross-over pharmacogenetic clinical trial comparing BP lowering responses in hypertensive individuals to a renin inhibitor (Aliskiren), an angiotensin receptor blocker (Candesartan), and an angiotensin converting enzyme inhibitor (Perindopril) according to age, baseline BP, PRA level, and REN -5312 C/T genotype.

The primary endpoint was the change from baseline in mean 24-hour systolic blood pressure with each drug therapy. Secondary endpoints included the changes from baseline in daytime systolic, night-time systolic, 24-hour diastolic, daytime diastolic and night-time diastolic blood pressures with the three drug therapies.

3.3.2 Study Population and Selection

Patients with mild to moderate essential arterial hypertension attending Beaumont Hospital cardiovascular outpatient clinics were invited to participate in the study. There was no upper age limit set for the study as the prevalence of hypertension increases with age. Specific inclusion criteria were as follows:

- Male or female outpatients
- Age greater than 18 years
- Written informed consent provided
- Participants with essential hypertension who were either antihypertensive treatment naïve, or taking a maximum of two antihypertensive agents
- Baseline mean 24-hour systolic pressure > 130 mm Hg, and < 160 mm Hg, either treatment naïve, after a one-week washout period in those already on two or less antihypertensives, or on amlodipine 10 mg daily.

Specific exclusion criteria were as follows:

- Pregnancy, breastfeeding women, or women of childbearing years not using a medically accepted method of contraception
- Presence of any significant acute or chronic illness. In particular, participants must not have had a history of an acute cardiovascular morbid event within three months prior to study inclusion. Participants who had suffered with

malignant hypertension or congestive heart failure and who had a terminal illness were not eligible for randomisation

- Significant baseline electrolyte, serum creatinine or creatinine clearance abnormalities (potassium > 5.5 mmol/l, or serum creatinine > 150 micromol/l, or creatinine clearance less than 30mls/minute
- Known secondary hypertension
- Requirement for any specific antihypertensive drug therapy
- Contra-indications to any of the study drugs
- Participation in any other studies involving investigational or marketed products within one month prior to entry into our study or concomitantly with our study
- Participants deemed unlikely to comply well with study treatments or with the scheduled visits
- Participants with a history of alcohol or drug abuse, psychosis, antagonistic personality, or any emotional or intellectual problems that were deemed likely to invalidate informed consent, or limit the ability of the subject to comply with the protocol requirements.

3.3.3 Study Conduct

Approval was obtained from Beaumont Hospital Medical Research Ethics Committee (REC reference 08/60, date of approval 7th August 2008, Protocol Version 3 RGR001) and the Irish Medicines Board (Case number 2051960, date of approval 2nd June 2008) prior to study recruitment. Written informed consent was obtained from all participants before randomisation, having reviewed the patient information leaflet. At all times study procedures were carried out in compliance with International Conference on Harmonisation Good Clinical Practice (ICH GCP) guidelines. Recruitment took place between August 2009 and February 2011. The last participant completed the study June 2011.

Participants were reviewed at the Blood Pressure Unit, Beaumont Hospital and at the Clinical Research Centre, Beaumont Hospital. At the screening visit, a comprehensive medical history and physical examination was performed. Individuals not taking any antihypertensive medications were booked for their randomisation visit. Those individuals on antihypertensive medications were counselled regarding discontinuing their treatment for a one-week period. After

one week free of all vasoactive medications clinic BP and 24-hour ambulatory blood pressures were measured and phlebotomy performed.

Participants whose baseline mean 24-hour systolic pressure was greater than 130 mm Hg, and less than 160 mm Hg off all antihypertensive treatment were eligible for inclusion. Those, whose baseline mean 24-hour systolic pressure was greater than 160 mm Hg and less than 175 mm Hg, were prescribed amlodipine 10 mg daily, and 24-hour ambulatory BP monitoring was repeated 2 weeks later. Once their mean 24-hour systolic pressure was then greater than 130 mm Hg, and less than 160 mm Hg on amlodipine 10mg, these participants were eligible for inclusion. For these patients, treatment with amlodipine continued throughout the clinical trial alongside each of the renin-angiotensin system blockers ("dual-therapy").

Upon completion of this evaluation, participants were then allocated in random order (stratified according to amlodipine usage or not), to 4 weeks of treatment with a renin inhibitor (Aliskiren 300 mg daily), an angiotensin receptor blocker (Candesartan 32 mg daily) and an angiotensin converting enzyme inhibitor (Perindopril 10 mg daily) (Figure 3.2). The drugs used are licensed for use in treatment of hypertension, and the doses given were all at their individual maximum licensed doses. These doses were chosen to avoid a potentially incomplete blood pressure response due to inadequate dosing with any one drug. The assumption was made *a priori* that there was no carry-over effect between treatment periods. Randomisation was fixed, balanced and organised through use of sealed envelopes.

At the end of each 4-week treatment period clinic BP, 24-hour ambulatory BP and serum urea, electrolytes and creatinine were measured. Adverse events and concomitant disease activity were evaluated. Details of all concomitant treatments were recorded. Participants were instructed to notify the study team regarding new medications taken after the start of the clinical trial at each study visit.

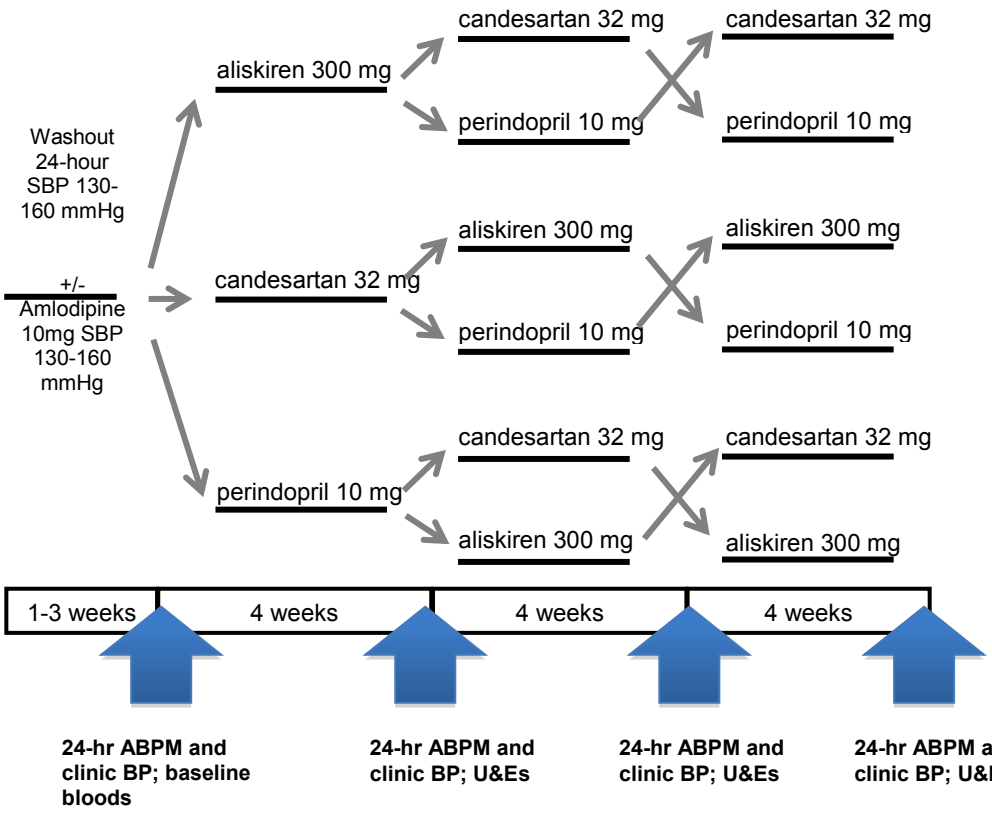


Figure 3.1 - **Study Schema – cross-over study design**. SBP, systolic blood pressure; mmHg, millimetres of Mercury, unit of measurement of blood pressure; mg, milligrammes; ABPM, 24-hour ambulatory blood pressure monitoring; U&Es, urea and electrolytes.

At study end, participants were prescribed the medication(s) resulting in the best BP control during the study, without resulting in any clinically relevant side effects or adverse events. A letter summarising their participation in the study was then sent to the participant's GP.

3.3.4 Investigational Medicinal Products

All study drugs were supplied as commercially available tablets (Perindopril, Coversyl®: Servier; Candesartan, Atacand®: Astra Zeneca; Aliskiren, Rasilez®: Novartis) and were stored in a secured area with restricted access at room temperature. Temperature monitoring logs were undertaken daily. The dosages of the three RAS blockers and the calcium channel antagonist were the maximum licensed maintenance dosages recommended for treatment of uncomplicated hypertension. Study treatments were dispensed to participants in accordance with the study protocol, and in accordance with the individual randomization schedule.

3.3.4.1 Identity and Labelling of Investigational Product(s)

The investigational product for each participant was supplied in a standard commercial medication box, comprising medication sufficient for 4 weeks of treatment and an additional one week supply. The medication box was labelled with a unique identifier, which corresponded to the patient's randomization number. The medication box required storage below 25^{o}C and the medication had to be protected from light and moisture. This was in keeping with the Ethics Committee and IMB-approved protocol.

3.3.5 Study Procedures

3.3.5.1 Clinical Measures

Medical history and Examination

On the initial screening visit a full medical history and examination was performed. Current diagnoses, past medical history, current medications, life-style assessment including diet, smoking history and family history were evaluated. Alpha-blockers already prescribed for indications other than hypertension (for example, in the treatment of benign prostatic hypertrophy, BPH), at a dose that would not change during the study were continued.

The participants' height, weight and waist circumference were measured with participants standing without shoes and in basic indoor clothing. Weight was measured to the nearest 0.1kg using Seca scales (Seca, Hamburg, Germany) that were calibrated regularly. Height was measured to the nearest 5mm on a Seca height gauge (Seca, Hamburg, Germany). A full cardiovascular examination was performed.

Electrocardiography (ECG)

A standard 12 lead ECG was performed at screening, and reviewed for abnormalities, and also on any occasion during the study where clinically indicated.

3.3.5.2 Phlebotomy

At baseline and off all antihypertensive medications (except alpha-blockers), erect and supine phlebotomy was performed with blood samples taken after 10 minutes in the erect position and after 20 minutes in the supine position (Sarstedt Monovette blood collection system).

At the baseline visit, phlebotomy was performed to allow the following:

- Measurement of baseline renal function - serum urea, electrolyte, creatinine and glomerular filtration rate
- Measurement of serum cholesterol and triglycerides, serum glucose
- Measurement of plasma renin activity, both upright and repeated after 20 minutes supine
- Immediate spinning down of blood samples and storage of plasma aliquots for future measurements
- DNA extraction for REN-5312C/T genotyping and storage of DNA for future genetic studies (Beaumont Hospital Medical Research Ethics Committee reference 08/60, date of approval 7th August 2008).

In total 60 mLs were drawn at the baseline visit, 15 mLs in the erect position, and 45 mLs in the supine position. On subsequent study visits, blood sampling occurred in the sitting position and was performed for renal function estimation only with a volume of up to 7.5mL taken.

3.3.5.3 Blood Pressure Measurement

Clinic Blood Pressure Measurement

Seated clinic BP measurement and heart rate (HR) were measured from the right arm using a regularly calibrated validated automated sphygmomanometer (Omron HEM-705CP). After at least 5 minutes in the sitting position, three measurements were taken at one-minute intervals. Seated BP and HR were taken as the average of the second and third readings.

Ambulatory Blood Pressure Measurement

Ambulatory Blood Pressure Measurements (ABPM) using the oscillometric method were obtained every half-hour throughout the 24-hour period using SpaceLabs 90207 monitors (SpaceLabs Medical Inc. Issaqua, West Virginia,

USA). Monitoring commenced between 07.00 and 14.00 hours on each occasion. Both study medication and concomitant medication were taken at the usual timing on the days where ABPM was performed. The ABPM reading was regarded as satisfactory if there were at least 14 daytime readings and 8 night-time readings. These were deemed the minimum acceptable number of readings to ensure the ABPM result was eligible for inclusion in study analysis.

The first and subsequent study medications were not commenced until the baseline and each ABPM thereafter was judged to have been satisfactory. In instances where the minimum number of ABPM measurements were not achieved, a second ABPM was placed within 24-72 hours. Where a second ABPM needed to be performed to satisfy the requirements of the analysis of data obtained, the medication period was extended by 24-72 hours. This was to ensure each end-of-treatment ABPM was a valid assessment of response to antihypertensive treatment. Mean 24-hour, daytime (mean of all readings obtained between 0900 and 2100), night-time systolic and diastolic blood pressures were calculated from the ABPMs.

3.3.5.4 Blood Sample Handling
Immediate Blood Assays

Serum urea, electrolytes and creatinine were quantified at baseline and at the end of each treatment period. Cholesterol, triglycerides and glucose were also quantified as they are known additional risk factors in cardiovascular disease. These biochemical blood assays were performed by Beaumont Hospital Clinical Laboratory.

Blood Sample Storage, DNA Extraction and Future Assays

At the baseline visit only, additional erect and supine blood samples were taken. These blood samples were immediately centrifuged at 3500 rpm for 15 minutes, the plasma was pipetted off, and up to four 1 ml plasma aliquots were stored at -80^{O}C in Cryovials (Cruinn Diagnostics).

One 1 ml aliquot was assayed to determine PRA within one month as considerable variations in PRA have been observed when samples were stored

at 2-8^{o}C or when stored frozen at -20 ^{O}C for prolonged periods when frozen (Locsei et al. 2009; Sealey et al, 1991). PRA was measured by radioimmunoassay (GammaCoat Plasma Renin Activity 125I RIA Kit, CA-1533; DiaSorin, Stillwater, MN) at St James' Hospital, Dublin 8. Prior to assay the frozen samples were rapidly defrosted to room temperature. Plasma renin activity determination involved an initial incubation of plasma to generate angiotensin I (up to 18 hours). Phenylmethylsulfonyl fluoride (PMSF) was added to the samples to prevent enzymatic conversion of angiotensin I to angiotensin II (GammaCoat Plasma Renin Activity 125I RIA Kit, Diasorin). Maleate generation buffer was also added to the samples to maximize the generation of angiotensin I from angiotensinogen. Angiotensin I generation was followed by quantitation of angiotensin I by a three-hour radioimmunoassay incubation (Rabbit anti-Angiotensin I coated tubes). PRA activity was expressed as ng/mL/hour of generated Angiotensin I.

The remainder of the plasma aliquots remain in storage so as to allow future measurements of bioactive molecules. The specific assays that will be performed are currently undetermined.

At the baseline visit only, whole blood samples were preserved in EDTA and immediately frozen at -80^{O}C to allow later DNA extraction for renin SNP genotyping, and storage of DNA for future genetic studies.

3.3.6 Blood DNA Extraction and Genotyping

3.3.6.1 Materials Needed

The following materials were used for the purpose of leukocyte and DNA extraction:

- Specified Laboratory Reagent (SLR) Solution (TRIS 2M pH 7.6, MgCl2 1M, NaCl 3M and distilled water)
- 50mL Sigma-Aldrich Greiner tubes
- Centrifuge, vortex and Gilsen pipette and pipette tips
- Gilsen "Macroman" pipette and 10mL, 20mL large plastic pipette tubes
- Phosphate-buffered saline (PBS)(Sigma-Aldrich), ethanol (96-100%) (Sigma-Aldrich), Proteinase K 50µg/mL (Sigma-Aldrich)

- Water bath heated to 37^{o}C
- Waste Container
- QIAamp DNA Blood Mini Kit (Qiagen), QIAcube machine (Qiagen)

Frozen blood was thawed in the heated water bath and lysed to obtain white cells. The QIAmp DNA Blood Mini Kit (Qiagen) and Qiagen QIACube were used to extract DNA from the white cells.

3.3.6.2 Extraction of Leukocytes

The 7.5mL EDTA bottles (Sarstedt Monovette) were removed from the -80^{o}C freezer and heated to 37^{o}C in a water-bath until thawed. SLR was freshly prepared in sterile graduate cylinders using sterile distilled water and stored at +4°C. The thawed samples were centrifuged in 50mL volume Sarstedt tubes, the plasma aspirated and discarded without disturbing the buffy coat. Mixing of the remaining red blood cells and leukocytes occurred and the volume was made up to 50mL with SLR, vortexed to resuspend and centrifuged at 3500 rpm for fifteen minutes. The remaining cells underwent lysis as a result of mixing with SLR to obtain a cellular pellet. The supernatant was removed, and further SLR was added to the Sarstedt tubes with two further repeat resuspensions and centrifugations at 3500 rpm for fifteen minutes. A cellular pellet was obtained which was then frozen at -80°C.

Figure 3.2 – **Extraction of DNA: QIACube (QIAGEN)** – the machine was used in combination with QIAmp DNA Blood Mini Kit to complete the extraction of DNA from leukocytes.

3.3.6.3 DNA Purification from Leukocytes

The cellular pellet was defrosted in a water bath at 50°C for one minute with lysis of the leuckocytes in 1x phosphate buffered saline (PBS) (137 mM NaCl, 10 mM Phosphate, 2.7 mM KCl, and pH of 7.4) and 50µg/mL proteinase K.

The Qiagen DNA Mini Kit buffers (Qiagen) and proteinase K (Sigma-Aldrich) were prepared in accordance with the kit protocol. Leukocytes were suspended in a mixture of PBS (Sigma-Aldrich) and 50µg/mL proteinase K (Sigma-Aldrich), with the kit Buffer AL (Qiagen) added to complete the lysis procedure. The samples were incubated, mixed with high concentration 96-100% ethanol, centrifuged and the resulting mixture transferred to the spin column. The kit buffers were added in sequence, samples centrifuged with each sequential buffer addition and the isolated DNA eluted. The procedure followed was exactly as per manufacturer's protocol (Qiagen). DNA was then stored at –20°C in Buffer AE (Qiagen) to avoid acid hydrolysis.

3.3.6.4 Determination of DNA concentration obtained

DNA concentrations in the samples prior to freezing were determined using the Nanodrop ® 8000 UV-Vis Spectrophotometer. The spectrophotometer was blanked using 100µL of H_2O and samples loaded with 8 x 1µL samples loaded at a time. Nucleic acid concentration and purity were measured. The A_{260}/A_{280} ratio was used to evaluate sample purity. Nucleic acids and proteins have absorbance maxima at 260 and 280 nm, respectively. The A_{260}/A_{280} ratio is a ratio of spectrophotometric absorbance of the sample at 260 nm to that of 280 nm, and is used to assess purity of nucleic acid samples in DNA extraction. A ratio of approximately 1.8 is generally accepted as "pure" for DNA (Glasel 1995; Murray & Rajeevan 2013).

Figure 3.3 – **DNA Concentration: Nanodrop 8000 Machine** – used to determine purity of DNA by measuring the A260/A280 ratio of 1 microlitre of DNA.

Table 3.1 - Example of output from Nanodrop ®. Used to determine purity of DNA obtained through measurement of A260/A280 ratio.

Sample ID	Concentration	Units	A260	A280	A260/A280	Volume
20	295.4	ng/ul	5.907	3.188	1.85	100 ul
21	158.8	ng/ul	3.177	1.697	1.87	100 ul
22	274	ng/ul	5.48	2.946	1.86	100 ul
23	338.6	ng/ul	6.773	3.653	1.85	100 ul
24	630	ng/ul	12.6	7.048	1.79	100 ul

3.3.6.5 DNA Plating and Genotyping

DNA plating

The DNA was pipetted carefully onto a sterile 96-well plate (10 µL at 10ng/ µLper SNP) (Greiner, Sigma-Aldrich). Random duplicates were incorporated with new sample IDs along with two blank wells as controls. The plates were left to dry overnight. Adhesive film was placed on top prior to packaging and preparation for transport. The plates were sent by registered airfreight to K Biosciences, Herts. Genotyping was performed by Kbiosciences using modified TaqMan assays (http://www.kbioscience.co.uk). Genotype calling was performed using Kluster software. Samples were excluded from the analysis with a genotyping call rate of <95%. All declared and hidden blanks were correctly called, and all hidden sample duplications were consistent.

3.3.7 Data Handling and Statistical Analyses

3.3.7.1 Data handling and Record Keeping

All data generated by the study remains confidential and no report or results contain any information that would allow an individual participant in the study to be identified. Paper clinical record forms were used in this study. Data was also stored electronically using EXCEL (Microsoft EXCEL 97, Microsoft Corporation, Redmond, WA, USA) database. Electronically stored data was identified by a unique registration number. All information relevant to the study is to be stored for at least 15 years after the end of the study.

3.3.7.2 Sample size and study power

Assuming a standard deviation of 6 mmHg for the change in baseline to end-of-treatment 24-hour systolic BP, a significance level of 0.05, 90 evaluable patients, and a renin -5312C/T minor allele frequency of 30%, this study had 80% power to detect 4 mm Hg differences in the primary end-point (change from baseline to end-of-treatment mean 24-hour systolic BP) with treatment with a renin inhibitor, an angiotensin receptor blocker, and an angiotensin converting enzyme inhibitor, between REN-5312 CC homozygotes *versus* CT heterozygotes *versus* TT homozygotes.

3.3.7.3 Statistical Analyses

Phenotypic data are expressed as mean ± standard deviation, as median [interquartile range], or as numbers (percentages). PRA followed a lognormal distribution and therefore was log-transformed before statistical analysis.

Departure from Hardy-Weinberg equilibrium was tested by Chi-squared tests. Genotypic analyses involved comparisons of phenotypic characteristics of REN-5312 CC homozygotes, CT heterozygotes and TT homozygotes.

The principal hypothesis tested within this clinical trial was whether REN-5312C/T genotype, alone or in combination with PRA, provides improved prediction of BP lowering responses to RAS blockade, additional to that provided by drug type, age, and baseline BP. The primary endpoint was the change from baseline in mean 24-hour systolic blood pressure with each drug therapy. Secondary endpoints included the changes from baseline in daytime systolic, night-time systolic, 24-hour diastolic, daytime diastolic and night-time

diastolic blood pressures with the three drug therapies. Generalised linear modelling (stepwise regression models with backward elimination) was used to test for independent predictors of BP lowering responses. The stepwise regression was performed by iteratively removing non-significant ($p<0.05$) variables from the model until only variables significantly associated with BP lowering responses remained. In order to test for additional prediction of BP lowering responses by REN-5312C/T genotype, alone or in combination with PRA, four linear regression models were tested for each endpoint;

- Model 1 included age, gender, drug treatment, and baseline BP as covariates.
- Model 2 included age, gender, drug treatment, baseline BP, and log supine PRA as covariates.
- Model 3 included age, gender, drug treatment, baseline BP, REN-5312C/T genotype, REN-5312C/T genotype*drug treatment interaction and REN-5312C/T genotype*baseline BP interaction as covariates.
- Model 4 included age, gender, drug treatment, baseline BP, log supine PRA, REN-5312C/T genotype, REN-5312C/T genotype*drug treatment interaction, REN-5312C/T genotype*baseline BP interaction and REN-5312C/T genotype*log supine PRA interaction as covariates.

Of note, repeated measurements were taken after four weeks of treatment on the assumption that there were no residual carryover effects from the previous treatment after that duration. If it is believed *a priori* that carryover does not exist, then it is thought appropriate to disregard the possibility of its existence in the statistical model employed {Senn:2003tn}- .

Analyses were performed using DataDesk® statistical software package version 7.0.2 (Data Description, Inc., Ithaca, NY 14852-4555, USA). All statistical tests were 2-sided. For all analyses $P<0.05$ was considered statistically significant.

3.4 Results

3.4.1 Study Population

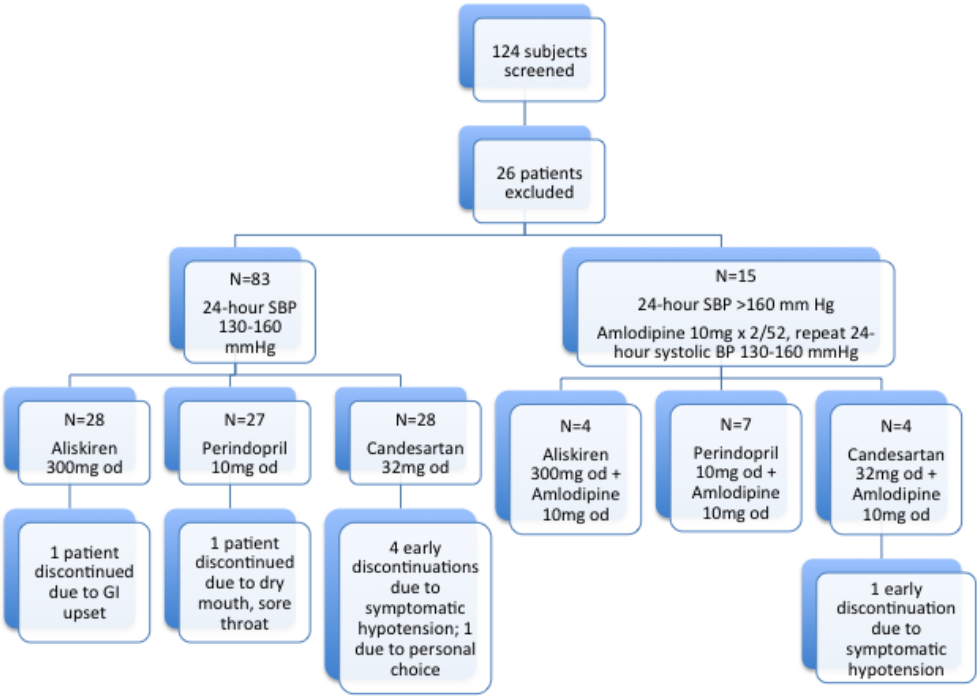


Figure 3.4 - Study flow diagram – this diagram details the number of patients screened, those subsequently excluded or randomised to active treatment. Each treatment group is further defined by first treatment received, number within each of the treatment groups, and number of withdrawals for each treatment group. N, number; mmHg, millimetres of Mercury; od, once daily; GI, gastrointestinal.

124 subjects were screened with 98 randomised to the study. Table 3.2 details the reasons for exclusion of the 26 participants who were not randomised.

Table 3.2 - Reasons for exclusion from participation in clinical study. 26 participants were excluded from clinical trial participation. A summary of exclusion reasons is presented in the table below. mmHg, millimetres of Mercury; µmol/L, micromoles per litre

Number	Exclusion Reason
12	Baseline 24-hour SBP after one-week washout period of <130 mmHg
4	24-hour systolic blood pressure <130 mmHg after treatment with amlodipine 10mg as per protocol
3	Patient change of mind
1	ECG revealed widespread ischaemic changes, urgent Cardiology referral with subsequent percutaneous intervention undertaken
1	Hypokalaemia on screening bloods, investigated for secondary hypertension
1	Deranged thyroid function with a palpable goitre
1	Left bundle branch block of unknown cause
1	Elevated creatinine of 150 µmol/L
1	Previous amlodipine intolerance in a patient in whom treatment with the drug was indicated (washout 24-hour SBP >160 mmHg)
1	Poor understanding of the trial procedures

3.4.2 Baseline Characteristics

98 participants were successfully enrolled in the study.

Expected variances were seen for gender between female and male participants. These included statistically significant differences in weight, waist, creatinine, and HDL-cholesterol levels (with respective p-values all <0.001). Fasting glucose was statistically different between females and males with a p-value of 0.02.

No statistically significant difference was seen in baseline 24-hour systolic and diastolic blood pressure and PRA between female and male participants. Alcohol intake was significantly different between female and male participants, but smoking history was not.

These data are illustrated overleaf.

Table 3.3 – Population characteristics by gender. Data expressed as mean and standard deviation, mean (SD), for continuous variables; count and percentage for categorical variables. Analysis of Variance and Chi-squared test used to determine significance values. mm Hg, millimetres of Mercury; PRA, plasma renin activity; kg, kilograms; ng/mL/hr, nanograms per milliliter per hour of Angiotensin I generation; HDL, high density lipoprotein cholesterol; LDL, low density lipoprotein cholesterol

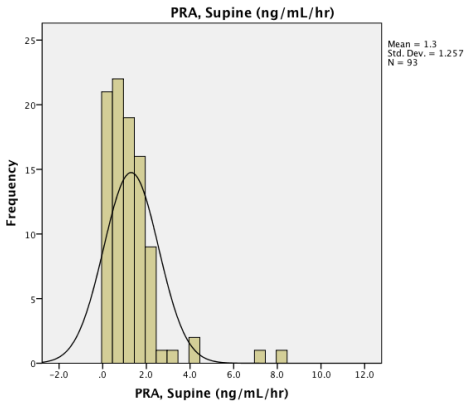
Variable		Male	Female	p-value
		n=63	n=35	
Age, years		55.1(9.6)	51.9(9.2)	0.106
Weight, kilograms (kg)		93.1(14.7)	75.6(15.3)	<0.001*
Body Mass Index (kg/m2)		29.9(4.4)	28.3(4.7)	0.09
Waist, centimetres (cm)		107.4(10.9)	95.3(11.6)	<0.001*
Smoking	Current smoker	13(21%)	3(8.5%)	0.904
	Ex-smoker	23(36.5%)	9(25.7%)	
	Never-smoker	27(42.8%)	22(62.8%)	
Alcohol intake, units per week		14.3(13.7)	6.6(6.9)	0.002*
Regular exercise	Yes	32(50.8%)	24(68.5%)	0.09
	No	31(49.2%)	11(31.4%)	
Baseline 24-hour Systolic Blood Pressure (mmHg)		142.5(9.0)	141.7(6.9)	0.32
Baseline 24-hour Diastolic Blood Pressure (mmHg)		86.3(6.4)	85.1(6.8)	0.35
Total cholesterol	(mmol/L)	4.9(1.1)	5.3(1.2)	0.11
Triglycerides	(mmol/L)	2.0(1.7)	1.5(1.0)	0.09
HDL cholesterol	(mmol/L)	1.3(0.3)	1.6(0.5)	<0.001*
LDL cholesterol	(mmol/L)	2.8(0.9)	3.0(1.0)	0.26
Fasting glucose	(mmol/L)	5.6(1.0)	5.1(1.2)	0.02*
Serum sodium	(mmol/L)	139.5(2.3)	139.7(2.1)	0.59
Serum potassium	(mmol/L)	4.0(0.3)	4.0(0.4)	0.89
Serum chloride	(mmol/L)	104.9(2.5)	105.5(1.5)	0.22
Creatinine	(micromol/L)	80.1(11.5)	63.8(10.8)	<0.001*
GFR	(mL/kg/hr)	88.3(15.4)	90.7(26.7)	0.57
Baseline PRA, upright	(ng/mL/hr)	1.6(1.4)	1.5(1.0)	0.54
Baseline PRA, supine	(ng/mL/hr)	1.3(1.3)	1.3(1.3)	0.99
Log PRA, upright		0.1(0.3)	0.1(0.3)	0.59
Log PRA, supine		0.0(0.4)	0.0(0.4)	0.8
REN-5312C/T	CC-homozygote	42(66.6%)	25(71.4%)	0.85
	CT-heterozygote	19(30.1%)	8(22.8%)	
	TT-homozygote	2(3.1%)	2(5.7%)	

3.4.2.1 Gender and age, baseline 24-hour systolic and diastolic blood pressure and smoking history

Female and male study participants were of a similar age, and had similar baseline 24-hour systolic and diastolic blood pressure and smoking habits.

3.4.2.2 Plasma Renin Activity

For the purpose of the analysis, both upright and supine plasma renin were treated as a continuous variable. Natural-logarithmic transformation was utilised to normalize the positively skewed distribution. In this study population, the expected change in plasma renin activity, PRA, was observed from upright to supine with a decrease in measured PRA. The observed mean upright PRA of 1.6 ng/mL/hr decreased to a mean PRA of 1.3 ng/mL/hr after 20 minutes of lying supine.



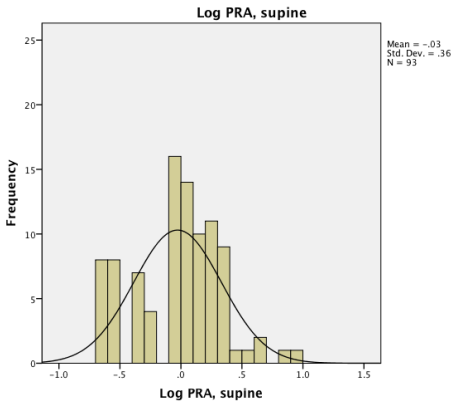
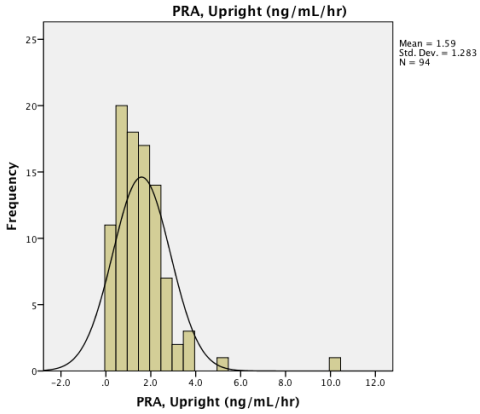


Figure 3.5 (a)



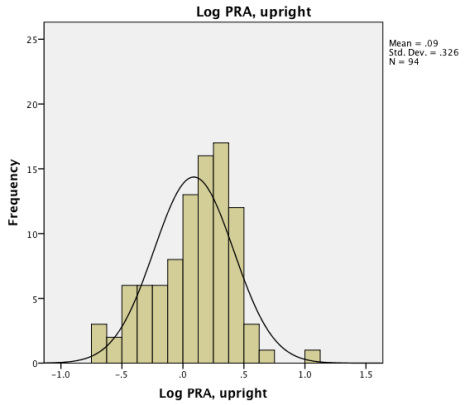


Figure 3.5 (b)

Figure 3.5 – Supine (a) and upright (b) plasma renin treated as a continuous variable (with natural-logarithmic transformation to normalize the positively skewed distribution on right). Log-renin used in statistical analysis of data. Ng/mL/hr, nanograms per millilitre per hour.

3.4.2.3 Plasma Renin Activity and participant age

As expected, increasing age was associated with a reduction in plasma renin activity, PRA. The association was seen for both upright (p=0.01) and supine PRA (p=0.01).

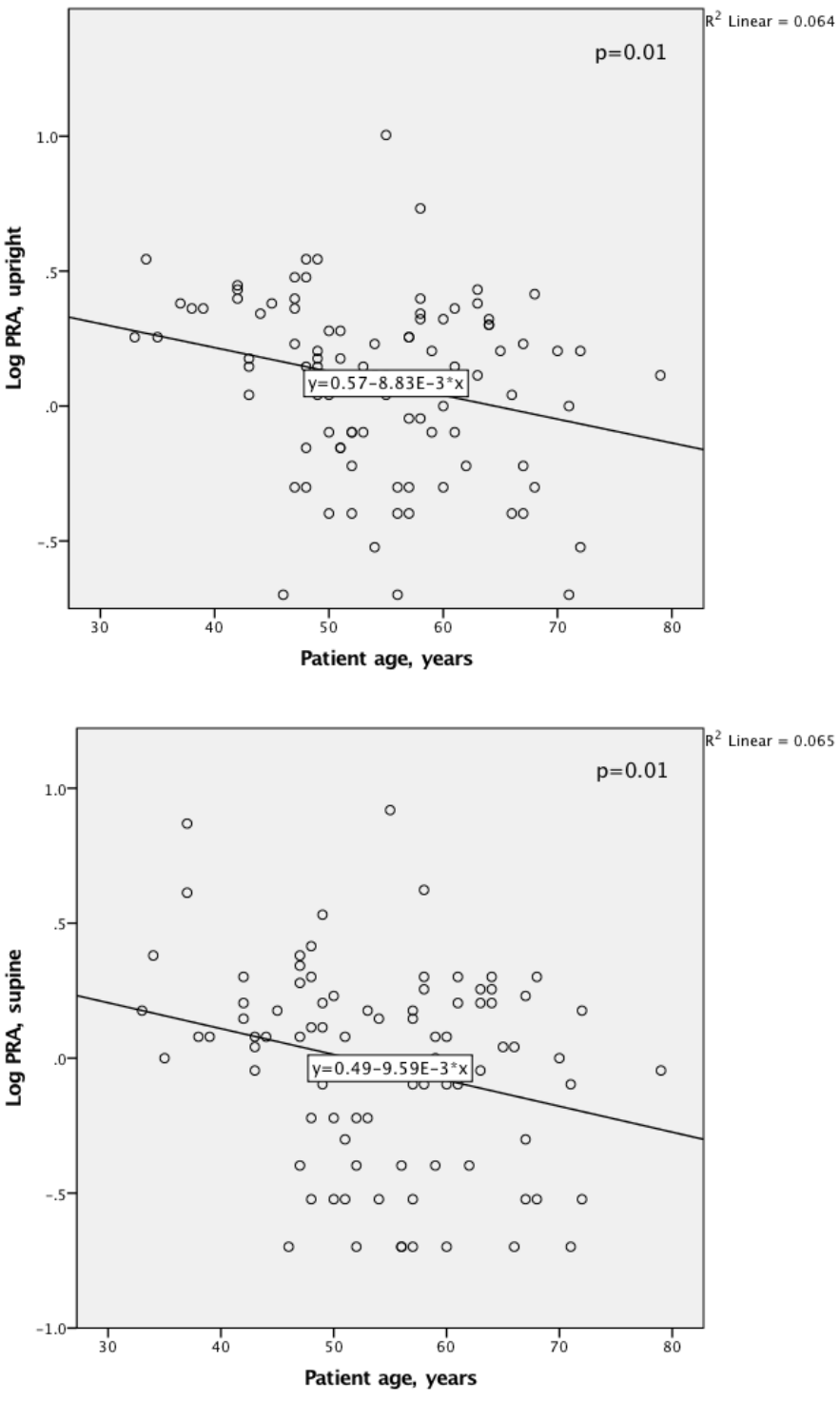


Figure 3.6 - **Scatterplots illustrating the relationship between patient age and log-transformed upright and supine log-transformed PRA**. Increasing age associated with reduction in log PRA. P-values obtained by linear regression analysis. PRA, plasma renin activity (log-transformed).

3.4.2.4 Plasma Renin Activity and mean 24-hour baseline blood pressure

No statistically significant relationship was seen between baseline 24-hour systolic blood pressure and log-transformed PRA (Figure 3.7). However there were clear positive correlations between baseline 24-hour diastolic BP and baseline upright (p=0.015) and supine (p=0.07) log-transformed PRA (Figure 3.8).

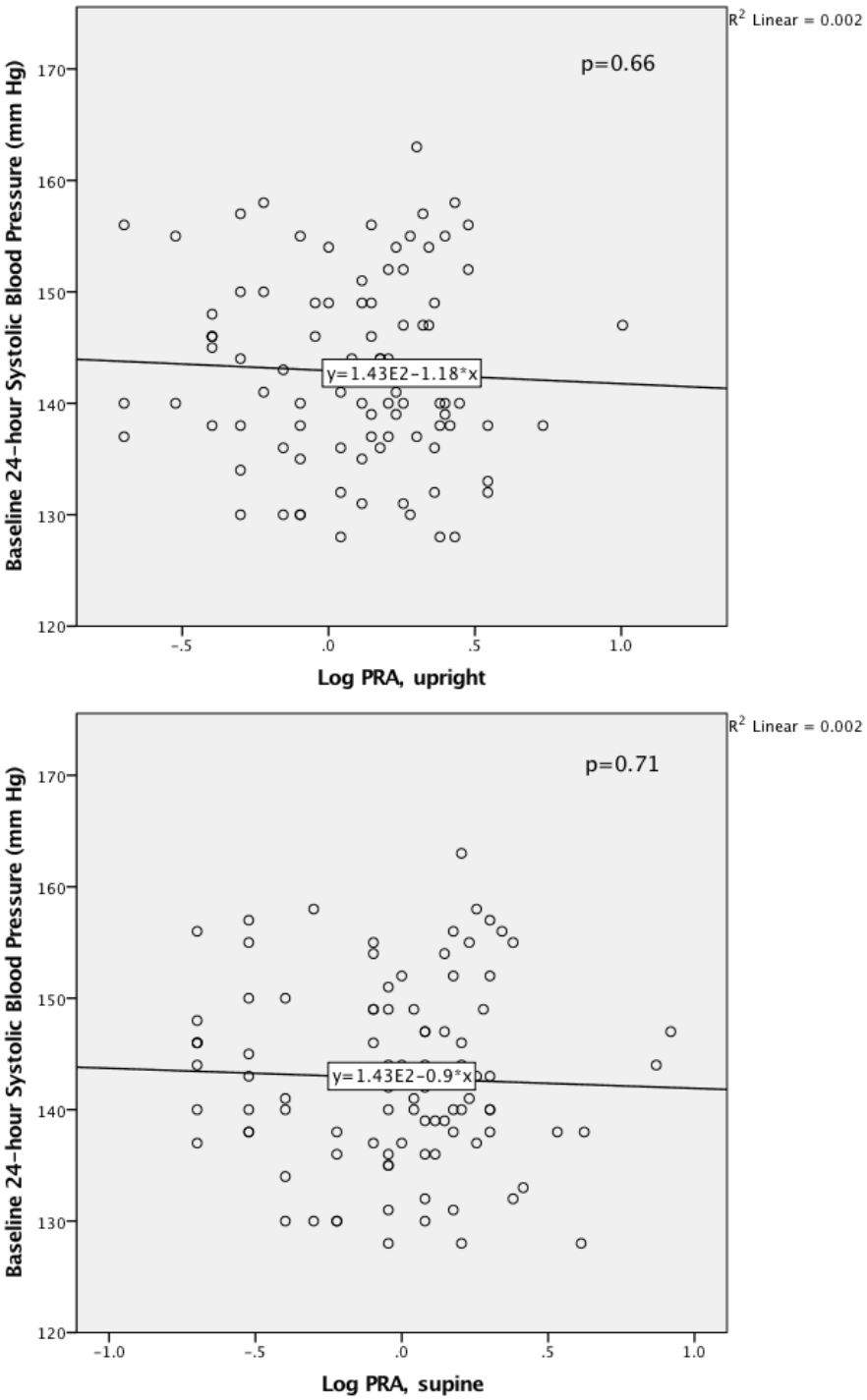


Figure 3.7 - **Scatterplots showing the relationship between log-transformed PRA and baseline mean 24-hour systolic blood pressure**. P-values obtained by linear regression analysis.

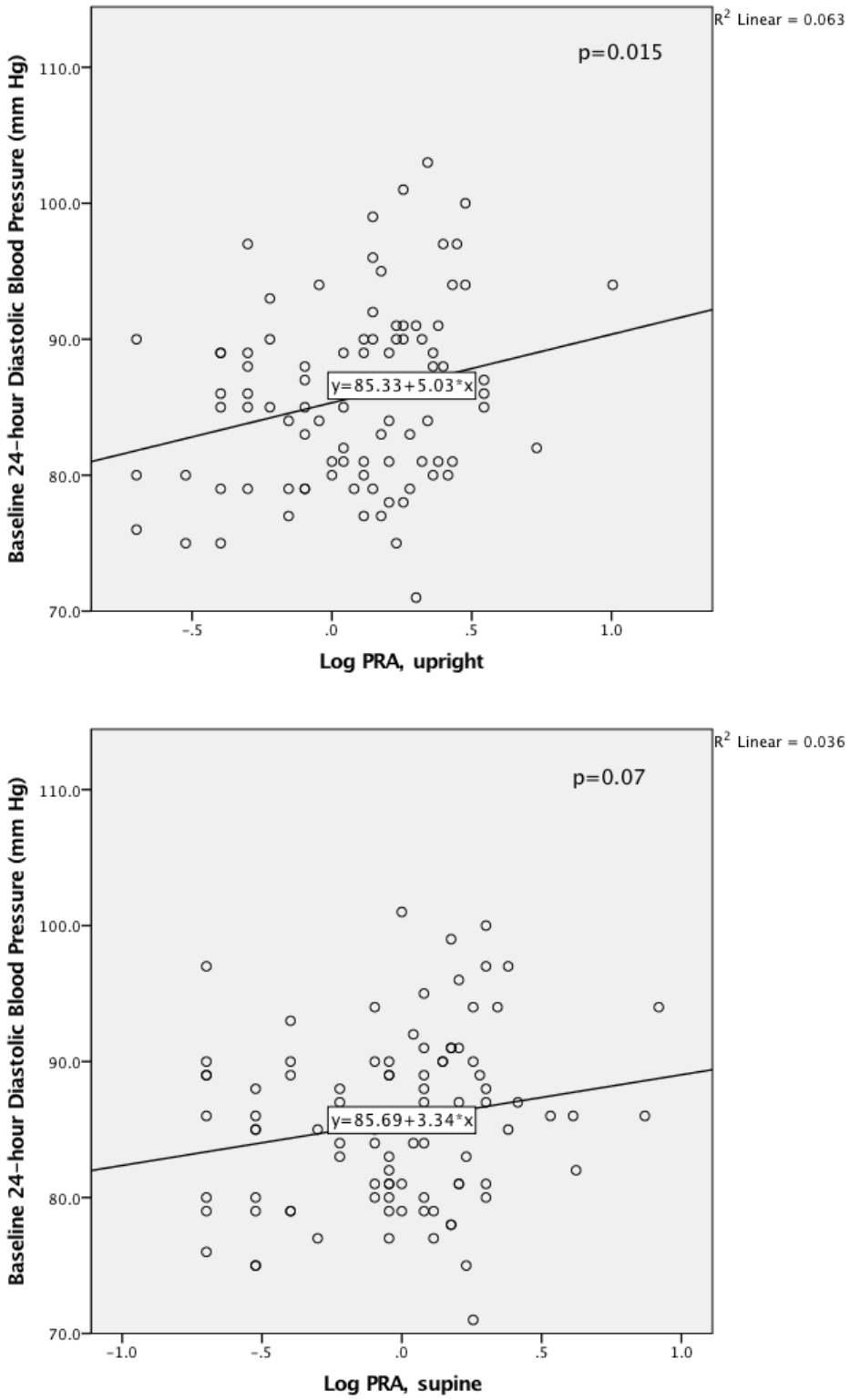


Figure 3.8 - Scatterplots showing the relationship between log-transformed plasma renin activity and baseline mean 24-hour diastolic blood pressure. P-values obtained by linear regression analysis.

3.4.2.5 Mean 24-hour baseline blood pressure and participant age

A statistically significant relationship was observed between baseline 24-hour systolic and diastolic blood pressure and age (Figure 3.9). Physiologically, as age increases, systolic blood pressure increases and diastolic blood pressure decreases.

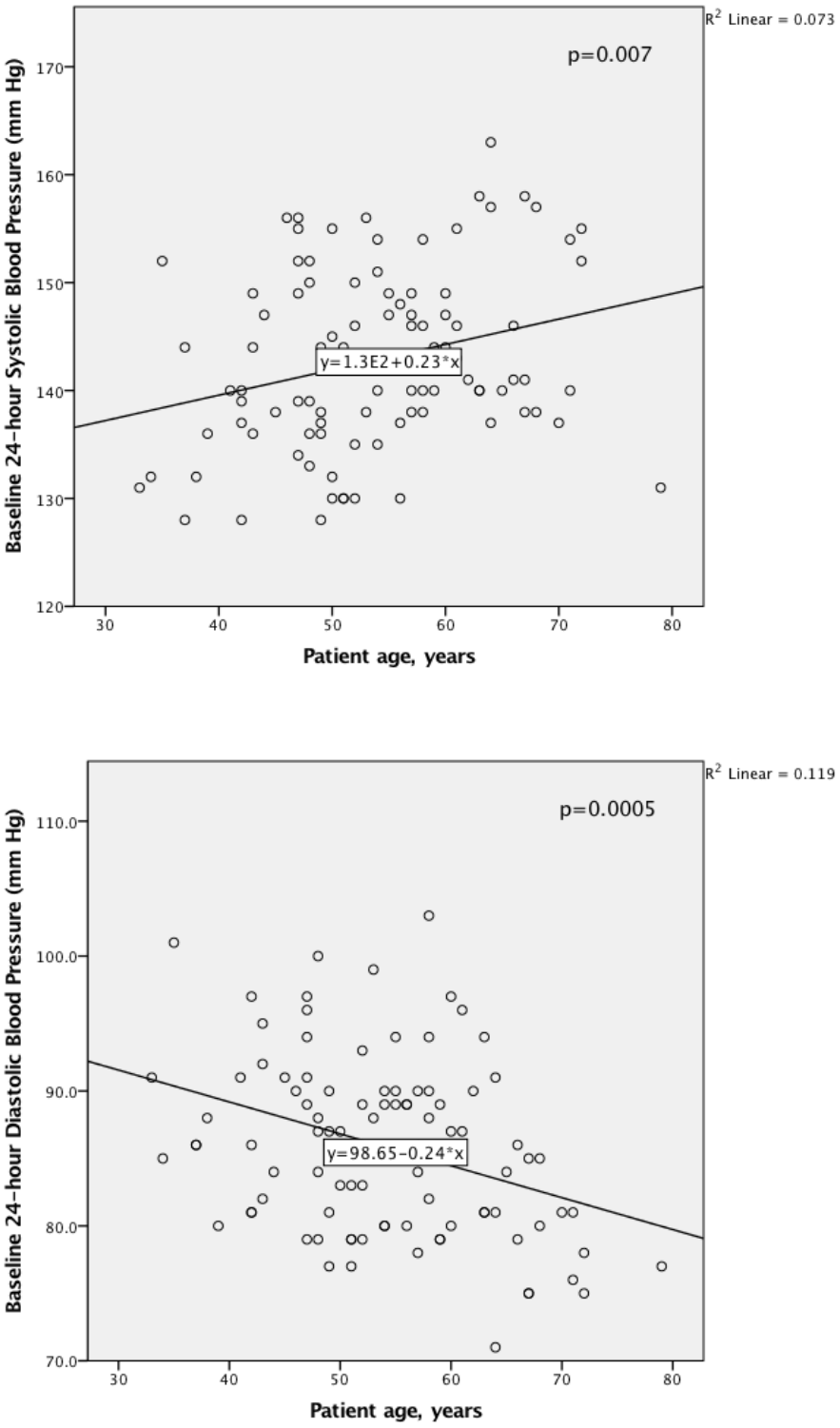


Figure 3.9 - **Scatterplots showing the relationship between advancing age and baseline 24-hour systolic and 24-hour diastolic blood pressure.** P-values obtained by linear regression analysis.

3.4.3 Baseline Characteristics according to REN-5312C/T Genotype

With a minor allele frequency of 0.18, the population was found to be in Hardy-Weinberg Equilibrium, with the HWE equation, $p^2 + 2pq + q^2 = 1$, fulfilled:

0.67 + 0.29 + 0.03 = 0.99.

Most baseline characteristics did not differ between the three REN-5312C/T genotypes. Age, body mass index, waist circumference, smoking habit, alcohol intake, exercise habit, blood pressure, lipid profile and glycaemia were similar. These data are presented in the table overleaf (Table 3.4).

However PRA and serum potassium did differ significantly according to REN-5312C/T genotype. These data are illustrated overleaf (Table 3.4 and Figures 3.10, 3.11).

Table 3.4 – Population characteristics by REN-5312C/T genotype. Data expressed as mean and standard deviation, mean (SD), for continuous variables; count and percentage, %, for categorical variables. N, number; mmHg, millimeters of Mercury; mmol/L, millimoles per litre; mL/kg/hr, milliliter per kilogram per hour; ng/mL/hr, nanograms per milliliter per hour.

Variable	Subgroup	CC; n=67	CT; n=27	TT; n=4	p-value
Age, years		53.1(8.9)	56.6(10.4)	51(13.2)	0.22
Gender	Male	42	19	2	0.66
	Female	25	8	2	
Weight, kilograms (kg)		87.4(17.6)	85.4(16.5)	88.4(14.2)	0.86
Body Mass Index, BMI	kg/m2	29.4(4.7)	29.2(4.5)	29.3(2.4)	0.97
Waist, centimetres (cm)		103.1(13)	102.4(12)	107.5(9.9)	0.75
Smoking	Current	10(14.9%)	5(18.5%)	1(25%)	0.96
	Ex-smoker	21(31.3%)	9(33.3%)	2(50%)	
	Never smoke	35(52.2%)	13(48.1%)	1(25%)	
Alcohol intake	Units per we	11.1(12.1)	12.8(13.2)	10.5(9)	0.83
Added dietary salt	Yes	25(37.3%)	6(22.2%)	1(25%)	0.36
	No	42(62.6%)	21(77.7%)	3(75%)	
Regular exercise	Yes	38(56.7%)	15(55.5%)	3(75%)	0.76
	No	29(43.2%)	12(44.4%)	1(25%)	
Diabetes, known	Yes	5(7.4%)	2(7.4%)	0(0%)	0.86
	No	62(92.5%)	25(92.5%)	4(100%)	
Baseline 24-hour SBP (mmHg)		142.4(7.7)	144.6(9.5)	138(9.4)	0.26
Baseline 24-hour DBP (mmHg)		85.8(6.2)	86.1(7.5)	85.5(6.9)	0.97
Total cholesterol	(mmol/L)	5(1.2)	5.1(1.1)	5.3(0.8)	0.87
Triglycerides	(mmol/L)	2(1.8)	1.4(0.6)	1.7(0.4)	0.31
HDL cholesterol	(mmol/L)	1.4(0.4)	1.4(0.4)	1.4(0.3)	0.9
LDL cholesterol	(mmol/L)	2.8(1)	3(0.9)	3.1(0.7)	0.59
Fasting glucose	(mmol/L)	5.5(1.2)	5.4(0.9)	4.7(0.9)	0.34
Serum sodium	(mmol/L)	139.6(2.4)	139.4(2)	140(0)	0.87
Serum potassium	(mmol/L)	4(0.3)	4(0.3)	4.4(0.2)	0.02*
Creatinine	(micromol/L)	74.9(14.9)	72.9(10.3)	73.3(14.4)	0.8
GFR	(mL/kg/hr)	89.6(22.6)	88.2(14)	86.5(9.5)	0.92
Baseline PRA, upright	(ng/mL/hr)	1.4(0.9)	2(1.9)	1.6(1.1)	0.12
Baseline PRA, supine	(ng/mL/hr)	1.1(0.8)	1.8(1.9)	1(0.6)	0.03*
Log PRA, upright		0(0.3)	0.2(0.3)	0.1(0.4)	0.14
Log PRA, supine		−0.1(0.3)	0.1(0.4)	−0.1(0.4)	0.04*

3.4.3.1 REN -5312C/T genotype and Plasma Renin Activity

REN-5312C/T genotype was statistically significantly associated with baseline supine PRA when analysed by ANOVA (Bonferroni-adjusted). Baseline PRA levels were statistically significantly greater in CT-heterozygotes than CC-homozygotes. Whilst baseline PRA tended to be higher in TT-homozygotes than CC-homozygotes, levels were not as high as CT-heterozygotes, and did not achieve statistical significance. It is difficult to comment on whether the lack of significance in TT-homozygote baseline PRA levels is a true result as there were only 4 participants in the TT-homozygote group.

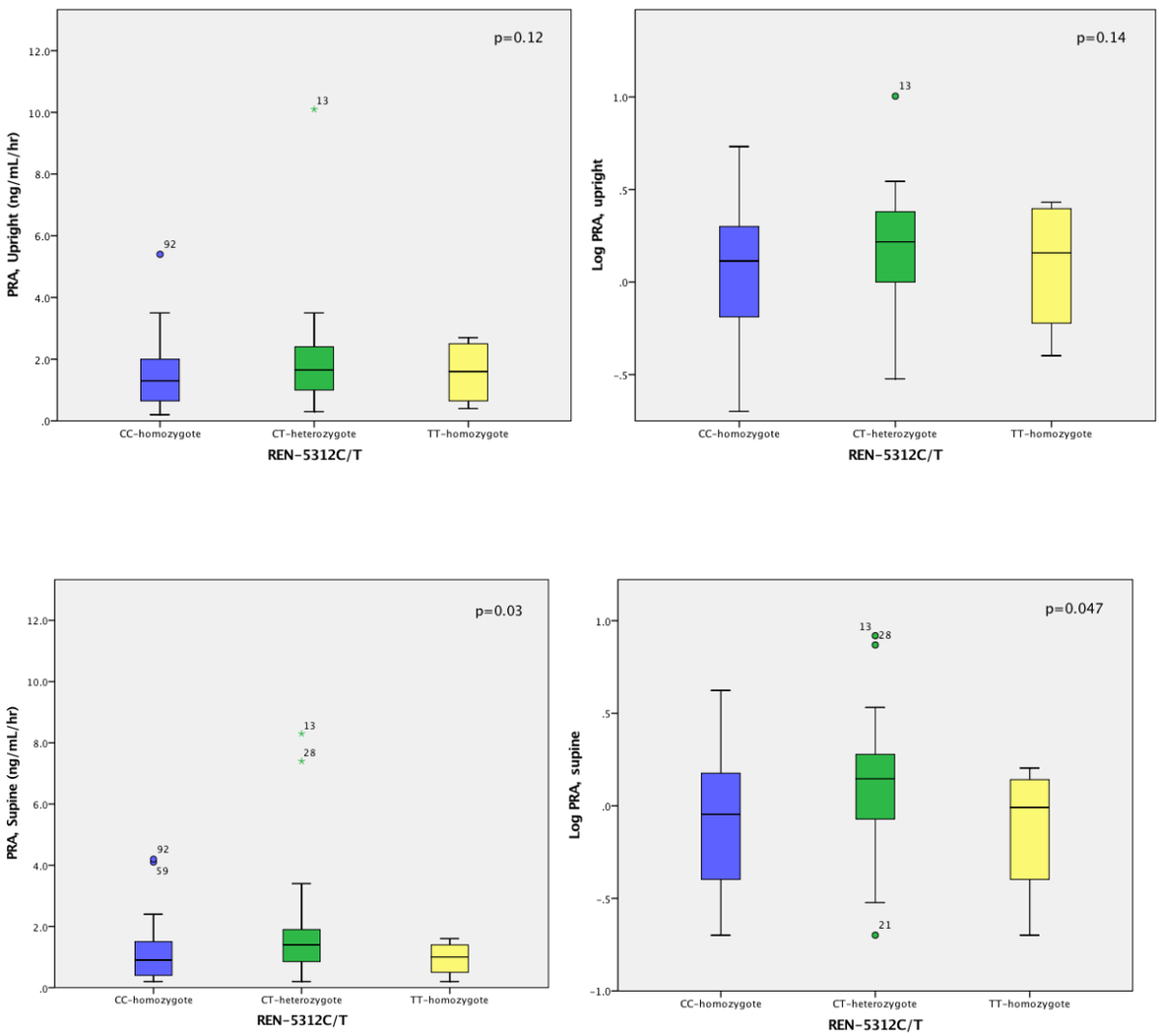


Figure 3.10 – Boxplots illustrating relationship between PRA, upright and supine, and renin -5312C/T genotype. Log-transformed supine and erect PRA are also shown. P-values obtained by one-way ANOVA with Bonferroni adjustment.

3.4.3.2 REN -5312C/T genotype and Potassium

Significantly higher potassium levels were noted in the TT-homozygotes (mean 4.4 mmol/L) *versus* CC-homozygotes and T-allele carriers (mean 4.0 mmol/L) when analysed by one-way ANOVA. This is illustrated in Figure 3.11 (a). It is difficult to comment on or draw any conclusions from this given the small number of participants within the TT-homozygote group (n=4). It is noteworthy however that the mean potassium level observed in TT-homozygotes was still within the normal reference range (3.5 – 5 mmol/L). It would be interesting to see if this would be replicated in a larger cohort, and whether there would be greater variation around the mean value, or a trend for higher mean potassium levels. There was a trend for estimated glomerular filtration rate to be lower amongst TT homozygotes, but this trend did not achieve statistical significance (Figure 3.11 (b)). Therefore, there is no good evidence that the higher potassium levels were due to renal impairment.

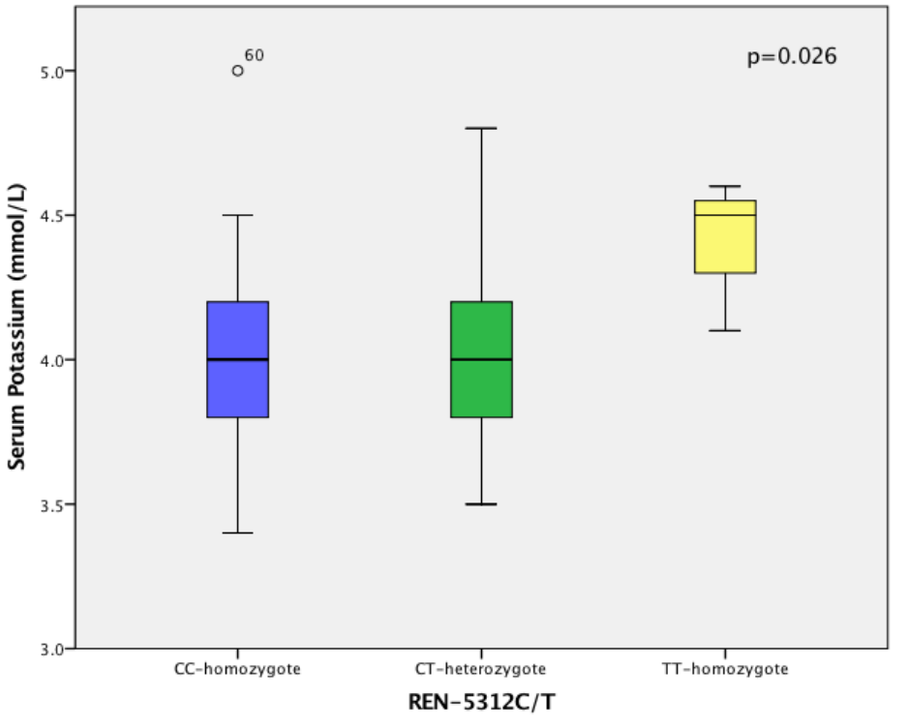


Figure 3.11 (a)

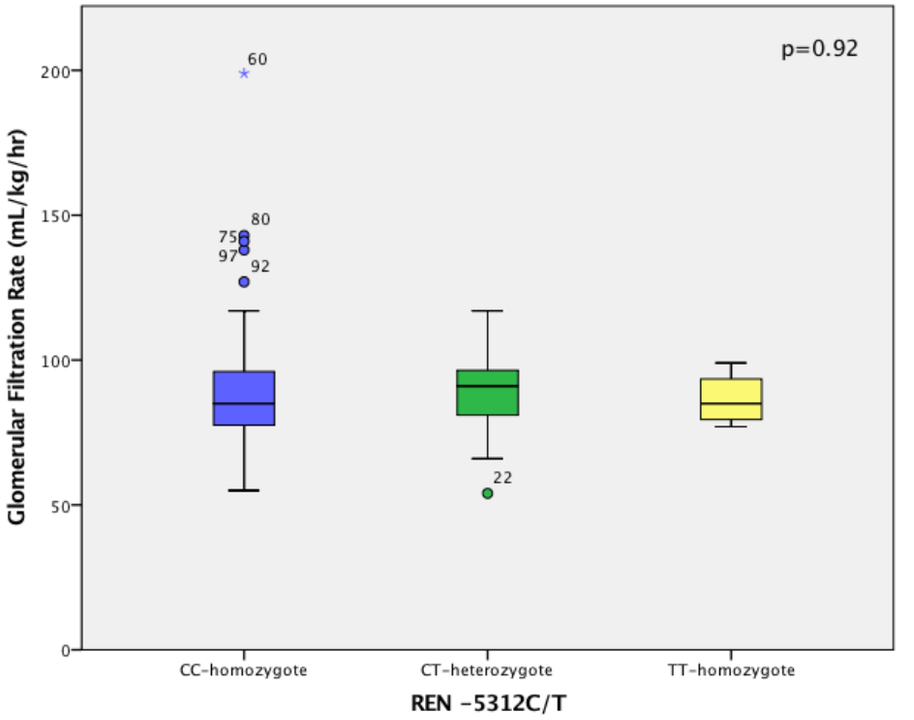


Figure 3.11 (b)

Figure 3.11 (a) and (b) - Boxplots illustrating the relationship between baseline serum potassium levels, glomerular filtration rate and REN-5312C/T genotype. P-values obtained by one-way ANOVA with Bonferroni adjustment.

3.4.4 Adherence to medication

Compliance, quantified by counting of returned capsules, was >95% across all treatment groups.

3.4.5 Study Withdrawals

There were eight study withdrawals during the study period (Figure 2.5). Study withdrawals occurred for the most part with the candesartan use as a result of symptoms related to postural hypotension. Aliskiren was discontinued as a result of GI upset with diarrhoea in one patient. Perindopril caused one subject to withdraw as a result of dry mouth and sore throat. One patient discontinued by choice as a result of the inconvenience and discomfort wearing the 24-hour ABPM machine.

3.4.6 Serious Adverse Events

In addition to the study withdrawals, there were two reported Serious Adverse Events, SAEs, during the study period.

The first was deemed unrelated to study participation, and involved recurrence of an epidermoid cyst on the participant's back that became infected, needing oral antibiotics with inpatient incision and drainage performed. The participant was on aliskiren when this occurred, and study treatment was not affected or interrupted with the patient successfully completing study participation.

The second SAE involved a participant who had completed the clinical trial successfully twenty-one days prior to being admitted to Beaumont Hospital with an ST-segment elevation myocardial infarction (STEMI). This had occurred whilst ascending from a deep-sea dive. The study physician had not been informed of his intention to proceed with deep-sea diving either prior to, during or after study completion. The SAE was not related to his post-trial prescribed antihypertensive medications (amlodipine 10mg once daily and candesartan 32mg once daily). Of note, this participant was of high cardiovascular risk being a smoker, obese and taking statin therapy for dyslipidaemia. His mean 24-hour blood pressure after one month of dual therapy with amlodipine 10mg once daily and candesartan 32mg once daily whilst a study participant had measured 128/75 mmHg, indicating adequate control. Both SAEs were reported to the Beaumont Hospital Ethics Committee and Irish Medicines Board.

3.4.7 Independent Predictors of BP Lowering Responses (Tables 3.5, 3.6, 3.7 and 3.8)

The principal hypothesis tested within this clinical trial was whether REN-5312C/T genotype, alone or in combination with PRA, provided improved prediction of BP lowering responses to RAS blockade. The following generalised linear models (stepwise regression models with backward elimination) were used to test for independent predictors of BP lowering responses. Four linear regression models were tested for each endpoint;

- Model 1 included age, gender, drug treatment, and baseline BP as covariates.
- Model 2 included age, gender, drug treatment, baseline BP, and log supine PRA as covariates.
- Model 3 included age, gender, drug treatment, baseline BP, REN-5312C/T genotype, REN-5312C/T genotype*drug treatment interaction and REN-5312C/T genotype*baseline BP interaction as covariates.
- Model 4 included age, gender, drug treatment, baseline BP, log supine PRA, REN-5312C/T genotype, REN-5312C/T genotype*drug treatment interaction, REN-5312C/T genotype*baseline BP interaction and REN-5312C/T genotype*log supine PRA interaction as covariates.

The results from these models for all 6 endpoints (24-hour, daytime, night-time, systolic and diastolic pressures) are presented in the following four tables (3.5 – 3.8).
It is clear from these tables that blood pressure lowering responses were greater;

- With candesartan treatment compared with aliskiren or perindopril treatments;
- In females compared to males;
- In participants with higher blood pressures;
- In participants with higher PRA levels;
- In REN-5312T allele carriers who also had higher BP levels and/or lower PRA levels.

These associations are discussed in Sections 3.4.7.1 – 3.4.7.7 and illustrated in Figures 3.12 – 3.27.

Table 3.5 Independent Predictors of Blood Pressure Lowering Responses - Model 1: including Age, Gender, Treatment and Baseline BP as covariates. BP, blood pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure; N/S, non-significant.

		24 Hour SBP		Daytime SBP		Night-time SBP		24 Hour DBP		Daytime DBP		Night-time DBP	
Factor	**Level**	**Coeff (Std Err)**	**p-value**	**Coeff (Std Err)**	**p-value**	**Coeff (Std Err)**	**p-value**	**Coeff (Std Err)**	**p-value**	**Coeff (Std Err)**	**p-value**	**Coeff (Std Err)**	**p-value**
Intercept		69.3(12.0)	≤0.0001	75.3(12.3)	≤0.0001	44.7(10.2)	≤0.0001	44.1(7.7)	≤0.0001	35.9(5.9)	≤0.0001	27.9(5.7)	≤0.0001
Age			N/S		N/S		N/S		N/S		N/S		N/S
Gender (female)		-5.95(1.45)	≤0.0001	-5.95(1.56)	0.0002	-6.28(1.71)	0.0003	-3.63(0.95)	0.0002	-3.02(1.02)	0.0035	-4.57(1.18)	≤0.0001
Treatment			0.0009		0.0057		0.0496		0.0004		0.0038		N/S
	Aliskiren	2.14(0.96)	0.0277	2.54(1.03)	0.0145	1.11(1.15)	0.3346	1.43(0.62)	0.0227	1.73(0.69)	0.0124		
	Candesartan	-3.64(0.96)	0.0002	-3.16(1.03)	0.0024	-2.81(1.15)	0.0150	-2.47(0.62)	≤0.0001	-2.20(0.69)	0.0016		
	Perindopril	1.50(0.97)	0.1222	0.62(1.04)	0.5492	1.70(1.15)	0.1410	1.04(0.62)	0.0949	0.47(0.69)	0.4987		
Baseline BP		-0.58(0.08)	≤0.0001	-0.61(0.08)	≤0.0001	-0.44(0.08)	≤0.0001	-0.55(0.07)	≤0.0001	-0.49(0.06)	≤0.0001	-0.46(0.07)	≤0.0001
r squared value		**0.22**		**0.21**		**0.16**		**0.23**		**0.22**		**0.16**	

Table 3.6 Independent Predictors of Blood Pressure Lowering Responses - Model 2: including age, gender, drug treatment, baseline BP, and log supine PRA as covariates. BP, blood pressure; PRA, plasma renin activity; SBP, systolic blood pressure; DBP, diastolic blood pressure; N/S, non-significant.

	24 Hour SBP		Daytime SBP		Night-time SBP		24 Hour DBP		Daytime DBP		Night-time DBP	
Factor Level	**Coeff (Std Err)**	**p-value**	**Coeff (Std Err)**	**p-value**	**Coeff (Std Err)**	**p-value**	**Coeff (Std Err)**	**p-value**	**Coeff (Std Err)**	**p-value**	**Coeff (Std Err)**	**p-value**
Intercept	69.2(11.8)	≤0.0001	75.9(12.0)	≤0.0001	44.0(10.1)	≤0.0001	43.6(7.3)	≤0.0001	43.9(7.5)	≤0.0001	35.2(7.0)	≤0.0001
Age		N/S		N/S		N/S	-0.16(0.05)	0.0014	-0.14(0.06)	0.0114	-0.13(0.06)	0.0349
Gender (female)	-6.08(1.42)	≤0.0001	-6.11(1.56)	≤0.0001	-6.22(1.69)	0.0002	-3.91(0.91)	≤0.0001	-3.66(1.00)	0.0003	-5.12(1.17)	≤0.0001
Treatment		0.0007		0.0046		0.0433		0.0002		0.0026		0.0479
Aliskiren	2.09(0.95)	0.0280	2.49(1.01)	0.0145	1.07(1.13)	0.3447	1.37(0.59)	0.0213	1.65(0.66)	0.0125	0.74(0.77)	0.3343
Candesartan	-3.66(0.95)	0.0001	-3.19(1.01)	0.0018	-2.84(1.14)	0.0131	-2.49(0.59)	≤0.0001	-2.20(0.66)	0.0009	-1.89(0.77)	0.0144
Perindopril	1.57(0.95)	0.0997	0.70(1.02)	0.4934	1.77(1.14)	0.1234	1.12(0.59)	0.0611	0.55(0.66)	0.4082	1.15(0.77)	0.1377
Baseline BP	-0.59(0.08)	≤0.0001	-0.61(0.08)	≤0.0001	-0.43(0.08)	≤0.0001	-0.51(0.07)	≤0.0001	-0.49(0.07)	≤0.0001	-0.46(0.07)	≤0.0001
Ln PRA	-6.42(1.89)	0.0008	-7.07(2.02)	0.0005	-5.83(2.25)	0.0103	-6.57(1.23)	≤0.0001	-6.83(1.37)	≤0.0001	'-4.86(1.59)	0.0025
r squared value	**0.25**		**0.25**		**0.18**		**0.31**		**0.29**		**0.22**	

Table 3.7 - Independent Predictors of Blood Pressure Lowering Responses - Model 3: including age, gender, drug treatment, baseline BP, REN-5312C/T genotype, REN-5312C/T genotype*drug treatment interaction and REN-5312C/T genotype*baseline BP interaction as covariates. BP, blood pressure; PRA, plasma renin activity; SBP, systolic blood pressure; DBP, diastolic blood pressure; N/S, non-significant.

		24 Hour SBP		Daytime SBP		Night-time SBP		24 Hour DBP		Daytime DBP		Night-time DBP	
Factor	**Level**	**Coeff (Std Err)**	**p-value**	**Coeff (Std Err)**	**p-value**	**Coeff (Std Err)**	**p-value**	**Coeff (Std Err)**	**p-value**	**Coeff (Std Err)**	**p-value**	**Coeff (Std Err)**	**p-value**
Intercept		110.3(20.2)	≤0.0001	116.9(22.0)	≤0.0001	99.3(36.2)	≤0.0001	44.1(7.7)	≤0.0001	35.9(5.9)	≤0.0001	27.9(5.7)	≤0.0001
Age			N/S		N/S		N/S		N/S		N/S		N/S
Gender (female)		-6.11(1.47)	≤0.0001	-5.70(1.59)	0.0004	-6.66(1.71)	0.0001	-3.63(0.95)	0.0002	-3.02(1.02)	0.0035	-4.57(1.18)	≤0.0001
Treatment			0.0009		0.0058		0.0500		0.0004		0.0038		N/S
	Aliskiren	2.11(0.95)	0.0278	2.51(1.03)	0.0150	1.05(1.13)	0.3545	1.43(0.62)	0.0227	1.73(0.69)	0.0124		
	Candesartan	-3.61(0.95)	0.0002	-3.14(1.03)	0.0024	-2.75(1.14)	0.0160	-2.47(0.62)	≤0.0001	-2.20(0.69)	0.0016		
	Perindopril	1.50(0.96)	0.1188	0.63(1.03)	0.5407	1.70(1.14)	0.1375	1.04(0.62)	0.0949	0.47(0.69)	0.4987		
Baseline BP		-0.88(0.14)	≤0.0001	-0.90(0.15)	≤0.0001	-0.85(0.27)	0.0021	-0.55(0.07)	≤0.0001	-0.49(0.06)	≤0.0001	-0.46(0.07)	≤0.0001
REN-5312C/T Genotype			0.0262		0.0446		0.0323		N/S		N/S		N/S
	TT	95.1(37.6)	0.0119	103.0(41.1)	0.0129	96.6(71.3)	0.1765						
	CT	-37.3(23.7)	0.1168	-57.0(25.8)	0.0277	-24.3(37.7)	0.5208						
	CC	-57.9(22.1)	0.0092	-45.9(23.6)	0.0532	-72.4(36.8)	0.0506						
REN-5312C/T*Baseline BP Interaction			0.0250		0.0452		0.0244		N/S		N/S		N/S
	TT	-0.68(0.27)	0.0123	-0.71(0.29)	0.0130	-0.73(0.54)	0.1807						
	CT	0.26(0.17)	0.1214	0.39(0.18)	0.0283	0.17(0.29)	0.5505						
	CC	0.42(0.27)	0.0080	0.33(0.16)	0.0453	0.56(0.28)	0.0480						
r squared value		**0.24**		**0.23**		**0.19**		**0.23**		**0.22**		**0.16**	

Table 3.8 - Independent Predictors of Blood Pressure Lowering Responses – Model 4: including age, gender, drug treatment, baseline BP, log supine PRA, REN-5312C/T genotype, REN-5312C/T genotype*drug treatment interaction, REN-5312C/T genotype*baseline BP interaction and REN-5312C/T genotype*log supine PRA interaction as covariates. BP, blood pressure; PRA, plasma renin activity; SBP, systolic blood pressure; DBP, diastolic blood pressure; N/S, non-significant.

		24 Hour SBP		Daytime SBP		Night-time SBP		24 Hour DBP		Daytime DBP		Night-time DBP	
Factor	**Level**	**Coeff (Std Err)**	**p-value**	**Coeff (Std Err)**	**p-value**	**Coeff (Std Err)**	**p-value**	**Coeff (Std Err)**	**p-value**	**Coeff (Std Err)**	**p-value**	**Coeff (Std Err)**	**p-value**
Intercept		121.2(20.0)	≤0.0001	130.8(21.7)	≤0.0001	110.9(36.2)	≤0.0001	65.6(11.4)	≤0.0001	66.1(16.1)	≤0.0001	27.5(5.60)	≤0.0001
Age			N/S		N/S		N/S	-0.12(0.05)	0.0206		N/S		N/S
Gender (female)		-6.11(1.43)	≤0.0001	-5.74(1.55)	0.0002	-6.65(1.70)	0.0001	-3.53(0.90)	0.0001	-2.80(0.97)	0.0046	-4.38(1.16)	≤0.0001
Treatment			0.0006		0.0041		0.0450		0.0001		0.0022		0.0482
	Aliskiren	2.08(0.93)	0.0268	2.47(1.00)	0.0145	1.03(1.12)	0.3578	1.37(0.58)	0.0194	1.67(0.65)	0.0109	0.77(0.77)	0.3218
	Candesartan	-3.65(0.93)	0.0001	-3.19(1.00)	0.0018	-2.79(1.13)	0.0137	-2.49(0.58)	≤0.0001	-2.20(0.65)	0.0008	-1.91(0.77)	0.0144
	Perindopril	1.57(0.94)	0.0943	0.72(1.00)	0.4934	1.76(1.13)	0.1216	1.12(0.59)	0.0569	0.53(0.65)	0.4132	1.14(0.78)	0.1442
Baseline BP		-0.96(0.14)	≤0.0001	-1.00(0.15)	≤0.0001	-0.94(0.27)	0.0007	-0.79(0.12)	≤0.0001	-0.82(0.18)	≤0.0001	-0.45(0.07)	≤0.0001
Ln PRA		-7.8(1.96)	0.0004	-8.11(2.10)	0.0005	-5.68(2.34)	0.0160		N/S		N/S		N/S
REN-5312C/T Genotype			0.0048		0.0073		0.0198		0.0151		0.0341		N/S
	TT	116.2(37.2)	0.0018	128.7(40.6)	0.0017	121.7(71.4)	0.0894	57.2(19.7)	0.0040	75.9(31.6)	0.0169		
	CT	-47.4(23.3)	0.0432	-69.6(25.3)	0.0064	-37.4(37.8)	0.3234	-27.5(11.7)	0.0200	-44.2(16.9)	0.0095		
	CC	-68.8(21.8)	0.0018	-59.1(23.3)	0.0118	-84.3(36.8)	0.0228	-29.7(11.3)	0.0090	-31.7(16.8)	0.0602		
REN-5312C/T*Baseline BP Interaction			0.0250		0.0069		0.0166		0.0331		0.0394		N/S
	TT	-0.84(0.27)	0.0019	-0.90(0.28)	0.0016	-0.92(0.54)	0.0904	-0.65(0.22)	0.0051	-0.82(0.35)	0.0196		
	CT	0.34(0.17)	0.0400	0.48(0.17)	0.0056	0.28(0.28)	0.3346	0.31(0.14)	0.0244	0.48(0.19)	0.0112		
	CC	0.50(0.16)	0.0015	0.42(0.16)	0.0098	0.65(0.28)	0.0219	0.34(0.13)	0.0100	0.34(0.19)	0.0646		
REN-5312C/T*Ln PRA Interaction			N/S		N/S		N/S		≤0.0001		≤0.0001		0.0270
	TT							13.57(2.53)	≤0.0001	13.58(2.78)	≤0.0001	8.17(3.08)	0.0084
	CT							-7.01(2.18)	0.0034	-6.39(2.41)	0.0084	-4.89(2.71)	0.0720
	CC							-7.08(1.51)	≤0.0001	-7.19(1.62)	≤0.0001	-3.28(1.87)	0.0797
r squared value		**0.28**		**0.28**		**0.21**		**0.34**		**0.32**		**0.20**	

3.4.7.1 Mean change in 24-hour blood pressure with each treatment

The greatest blood pressure lowering was achieved with the angiotensin receptor blocker candesartan at the highest maximum dose, 32mg once daily. This was seen for both mean 24-hour systolic and diastolic blood pressure (Figure 3.12). Blood pressure lowering with candesartan remained significant across all 4 linear models, with patients treated with candesartan achieving approximately 3.65 mmHg greater 24-hour systolic blood pressure lowering than average blood pressure reduction for all treatments (Model 4).

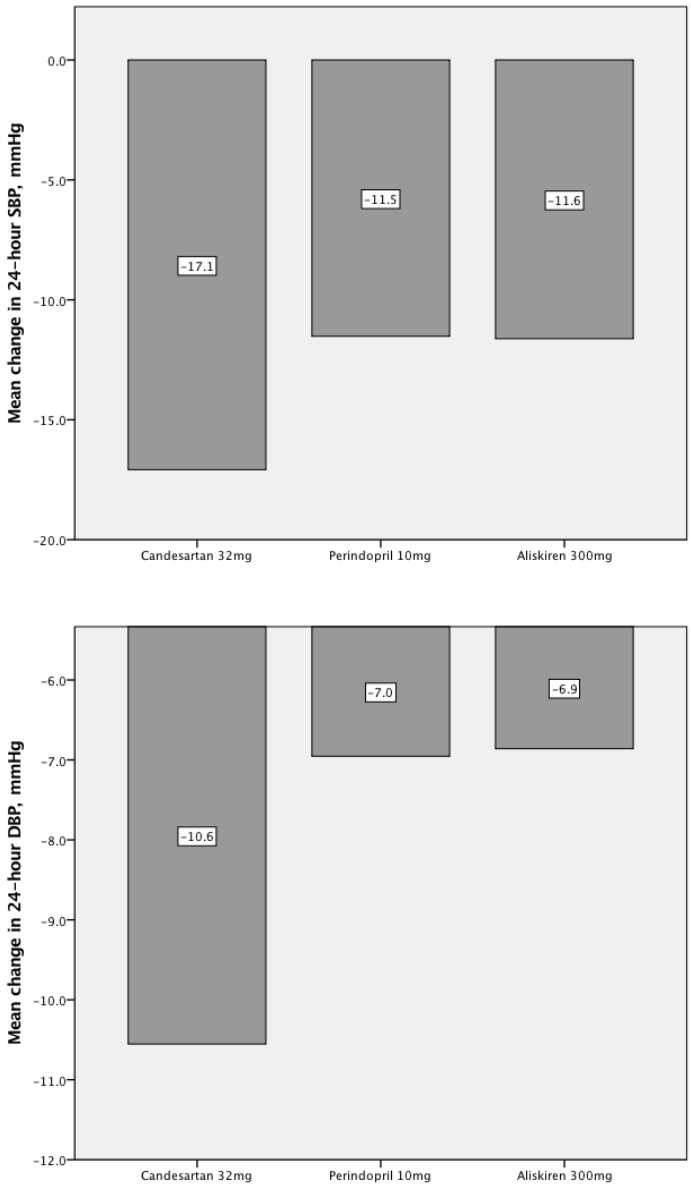


Figure 3.12 – Mean change in 24-hour systolic and diastolic blood pressure with each of the three blockers of the renin-angiotensin system. mmHg, millimetres of Mercury. Data-labels identify mean blood pressure reduction achieved with each treatment.

3.4.7.2 Gender as a predictor of blood pressure lowering response

A statistically significant difference between females and males was observed for blood pressure lowering with RAS blockade. Female participants achieved greater blood pressure lowering when compared with male participants.

This difference was statistically significant and consistent across all measures of blood pressure in the linear model for 24-hour systolic and diastolic blood pressure, with an approximate 6.11 mmHg greater 24-hour systolic blood pressure and 3.53 mmHg greater 24-hour diastolic blood pressure reduction for female participants.

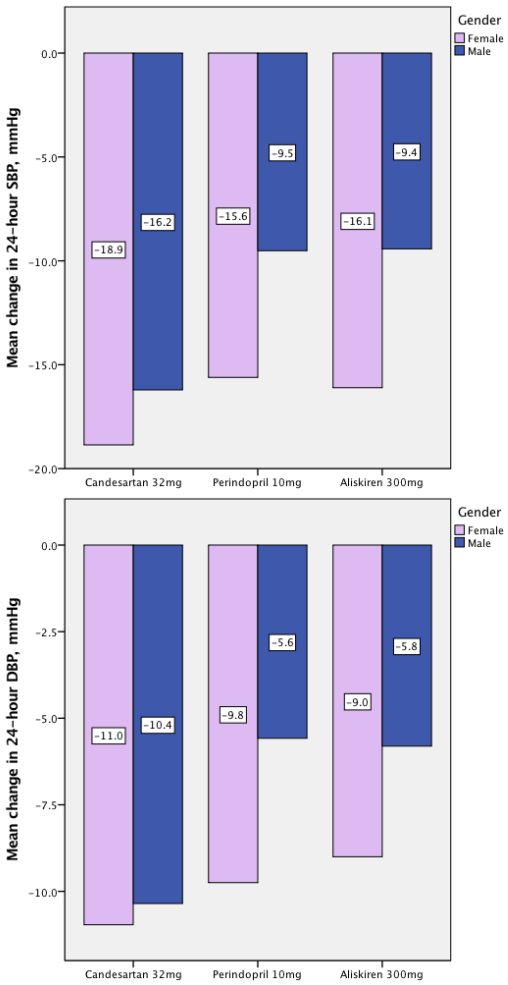


Figure 3.13 – **Relationship between response to antihypertensive treatment and gender for SBP and DBP**. mmHg, millimetres of Mercury; SBP, systolic blood pressure; DBP, diastolic blood pressure. Data-labels identify mean blood pressure reduction with each treatment for females and males for both 24-hour systolic and diastolic blood pressure.

In determining a potential explanation for this difference by gender, it was

notable that both weight and waist circumference were significantly different between males and females (Table 3.3). Females weighed on average 75.6kg with a waist circumference of 95.3cm, and men weighed on average 93kg with a waist circumference of 107cm ($P<0.0005$). The association between increased weight and cardiovascular risk factors including hypertension, insulin resistance, type II diabetes and dyslipidaemia is known (Alshehri 2010).

The differences observed are illustrated by Figure 3.14. Weight was observed as being significantly correlated with waist circumference (Figure 3.15, overleaf).

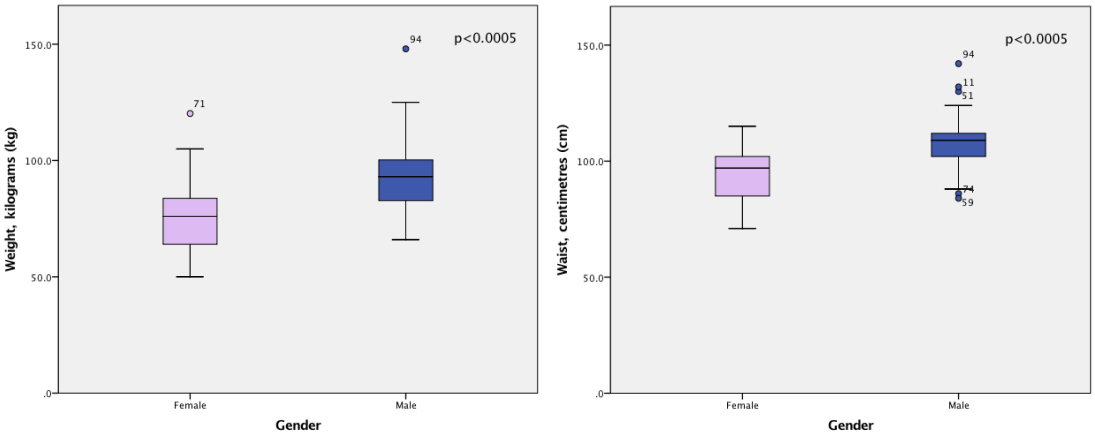


Figure 3.14 (a) **Figure 3.14 (b)**

Figure 3.14 – Relationship between gender and weight (a) and waist circumference (b). Male participants weighed significantly more than female participants. Weight in kilograms, kg; waist circumference in centimetres, cm. P-values obtained by linear regression analysis.

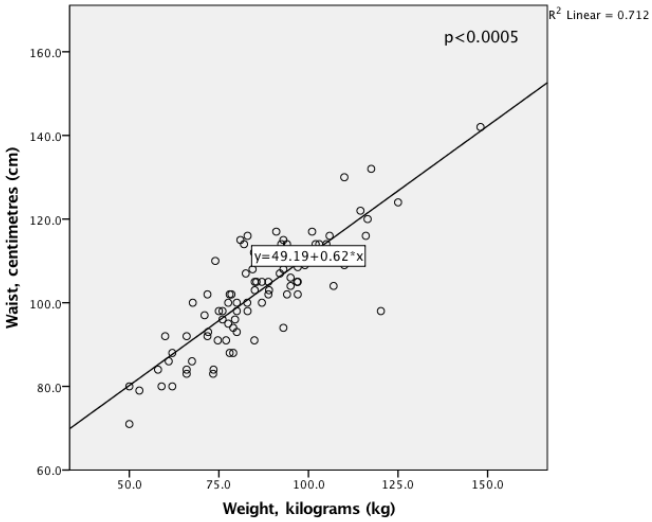
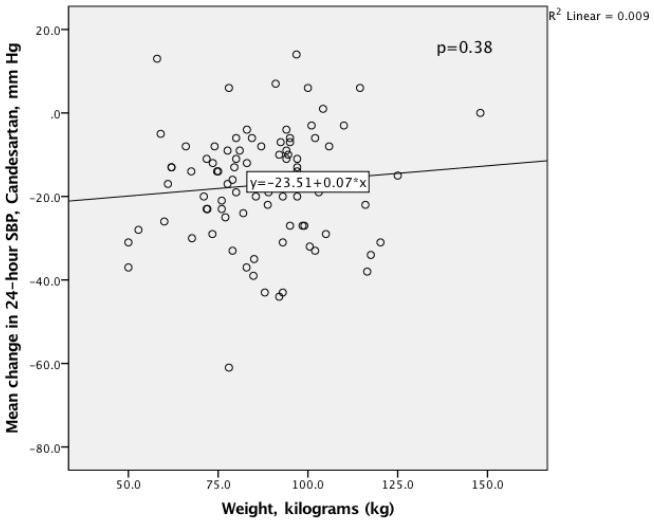
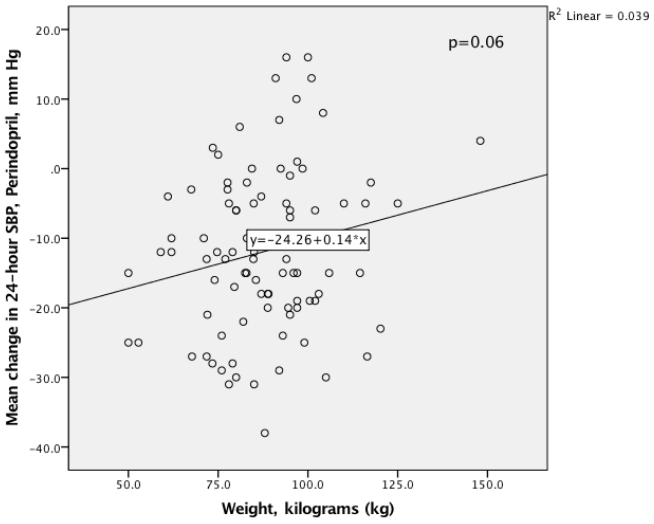


Figure 3.15 – Correlation between weight and waist circumference in the study population. Weight and waist were significantly correlated, as expected. Weight in kilograms, kg; waist circumference in centimetres, cm. P-value obtained by linear regression analysis.

No statistically significant linear dependence of mean change in 24-hour systolic blood pressure with weight was detected. As weight increased however, a trend was observed for a reduction in blood pressure achieved with each treatment. This was most notable for perindopril, where a 1kg increase in weight accounted for an approximate 0.14 mmHg reduction in blood pressure achieved with the drug. These relationships are illustrated by the following figure (Figure 3.16).





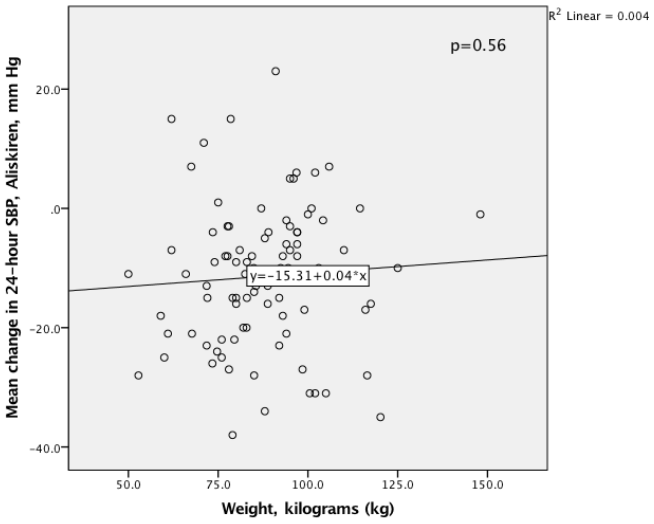


Figure 3.16 – Relationship between weight in kilograms, and 24-hour systolic blood pressure lowering with each treatment. mmHg, millimetres of Mercury; SBP, systolic blood pressure

Waist circumference was significantly associated with a reduction in 24-hour systolic blood pressure achieved with perindopril, p=0.02. For 24-hour diastolic blood pressure, a similar trend was observed with perindopril only in relation to waist circumference, p=0.10. Mean 24-hour blood pressure lowering with each treatment by waist circumference is illustrated in the following figure (Figure 3.17).

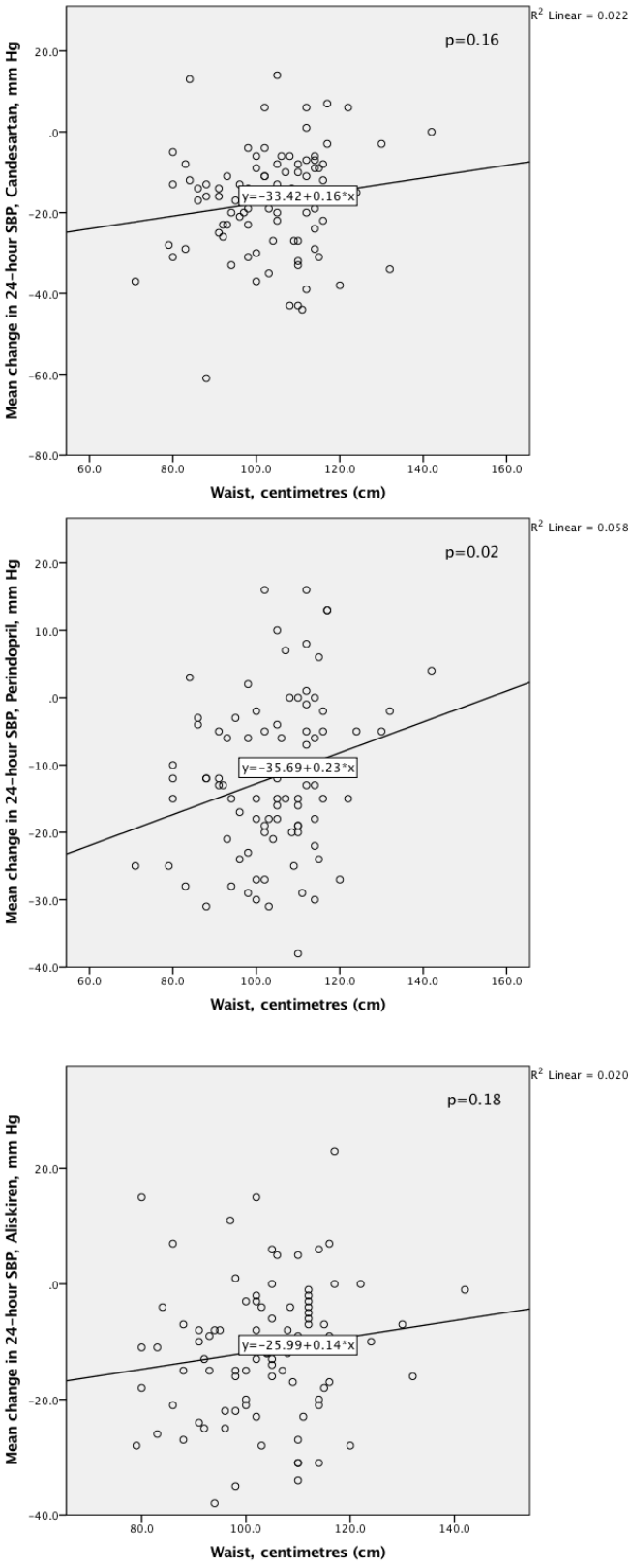


Figure 3.17 – Relationship between waist circumference in centimetres, and 24-hour systolic blood pressure lowering with each treatment. mmHg, millimetres of Mercury; SBP, systolic blood pressure

3.4.7.3 Baseline blood pressure as a predictor of blood pressure lowering response

Baseline blood pressure was significantly associated with antihypertensive effect achieved with each of the three blockers of the renin angiotensin system when analysed by simple linear regression analysis. P-values obtained for each of the three drugs by linear regression for 24-hour systolic blood pressure are illustrated in the following Figure, 3.19. P-values obtained for blood pressure reduction by linear regression for 24-hour diastolic blood pressure were significant for candesartan and perindopril only (Figure 3.19).

Across all linear models 1-4, baseline blood pressure was a significant predictor of response to antihypertensive treatment for both 24-hour systolic and diastolic blood pressure.

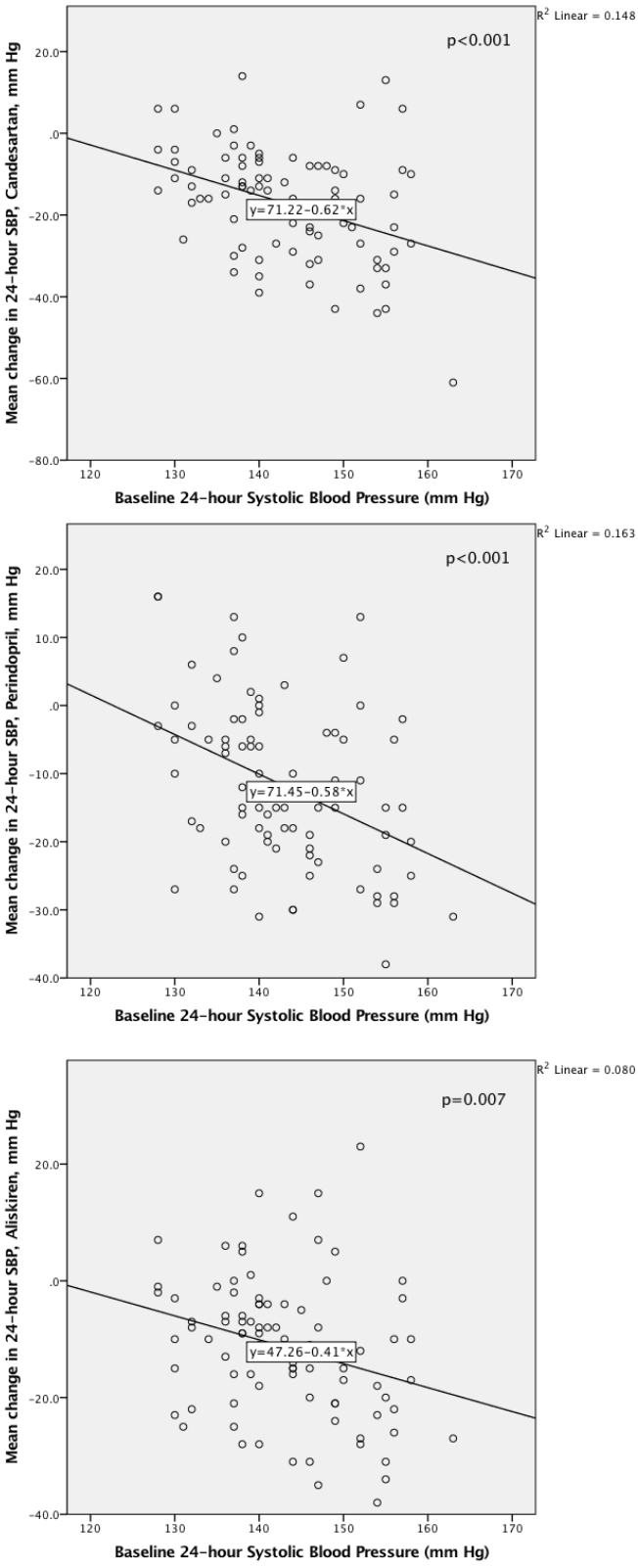


Figure 3.18 – Scatterplots illustrating the associations between mean change in 24-hour systolic blood pressure, SBP, with each treatment and baseline BP for each of the three antihypertensive treatments. mmHg, millimetres of Mercury; SBP, systolic blood pressure

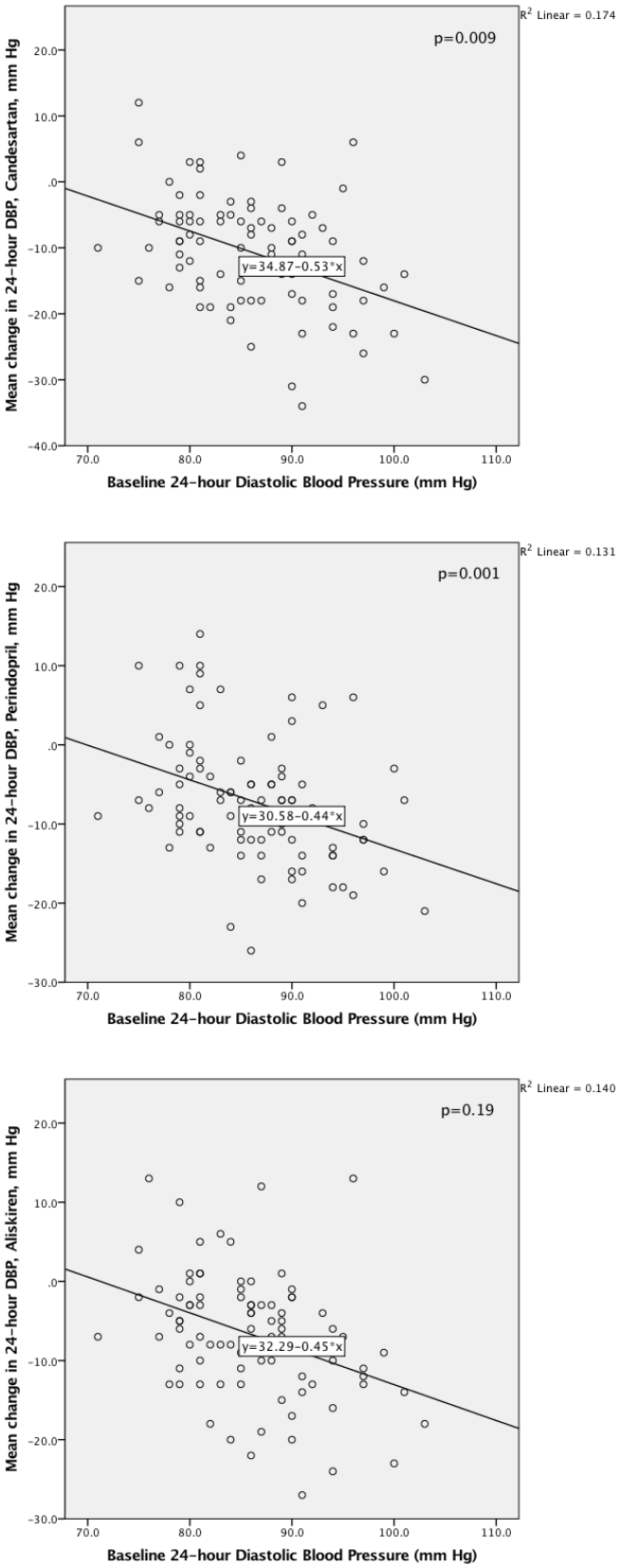


Figure 3.19 – Scatterplots illustrating the association between mean change in 24-hour diastolic blood pressure, DBP, with each treatment and baseline BP for each of the three antihypertensive treatments. mmHg, millimetres of Mercury; DBP, diastolic blood pressure.

3.4.7.4 Mean change in 24-hour systolic blood pressure by plasma renin activity with each treatment

"Low PRA" was defined as those with a supine PRA of ≤ 1ng/mL/hour, and "high PRA" as those with a supine PRA of ≥ 1ng/mL/hour. On review of mean values across all drug treatments, high supine PRA participants achieved greatest blood pressure lowering.

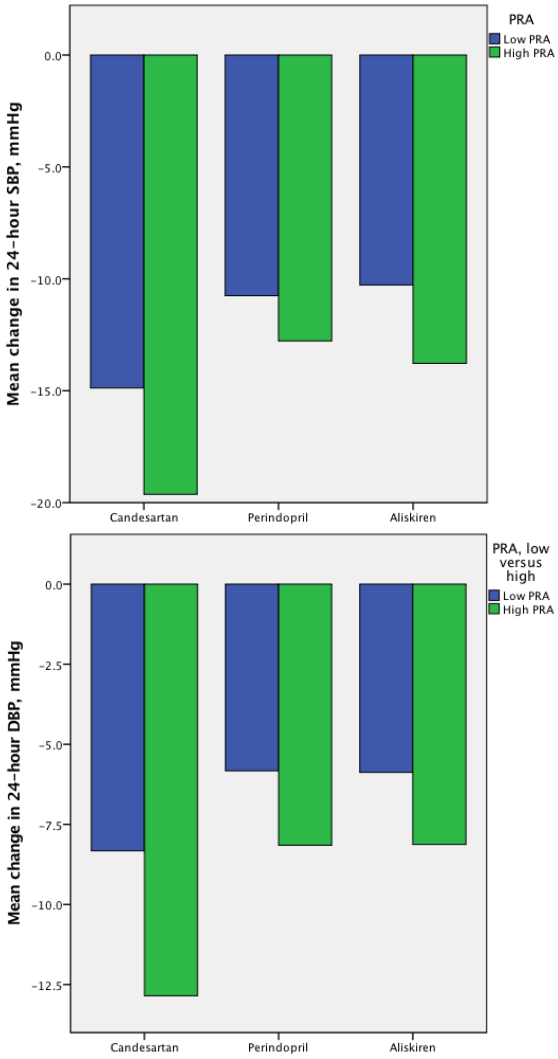


Figure 3.20 – Relationship between antihypertensive effect of each drug and plasma renin activity, PRA. mmHg, millimetres of Mercury; SBP, systolic blood pressure; DBP, diastolic blood pressure

The addition of Log PRA in model 2 was significantly associated with blood pressure lowering response for 24-hour systolic blood pressure, improving the ability of the model to predict blood pressure lowering with R^2 increase from 0.22 in model 1 (Table 3.5) to 0.25 in model 2 (Table 3.6). Similar increases in R^2 were observed on addition of Log PRA to the model as described for all measures of blood pressure.

3.4.7.5 Mean change in 24-hour systolic blood pressure by renin -5312C/T genotype with each treatment

With each drug treatment, CT-heterozygotes achieved greater blood pressure lowering than did CC-homozygotes. BP lowering amongst TT-homozygotes was more variable – these apparent variable responses may be due to the low number of TT homozygotes (Figure 3.21 overleaf).

Addition of genotype to model 1 increased the overall predictive ability, with an increase in R^2 from 0.22 in model 1 (Table 3.5) to 0.24 in model 3 (Table 3.7) for 24-hour systolic blood pressure. Furthermore, the addition of genotype to model 2 increased R^2 from 0.25 in model 2 (Table 3.6) to 0.28 in model 4 (Table 3.8) for 24-hour systolic blood pressure. Similar increases in R^2 values were observed in model 4 for all systolic blood pressure when genotype was added to model 2.

However, addition of genotype to model 1 had little impact on R^2 values for all measures of diastolic blood pressure, as seen in model 3.

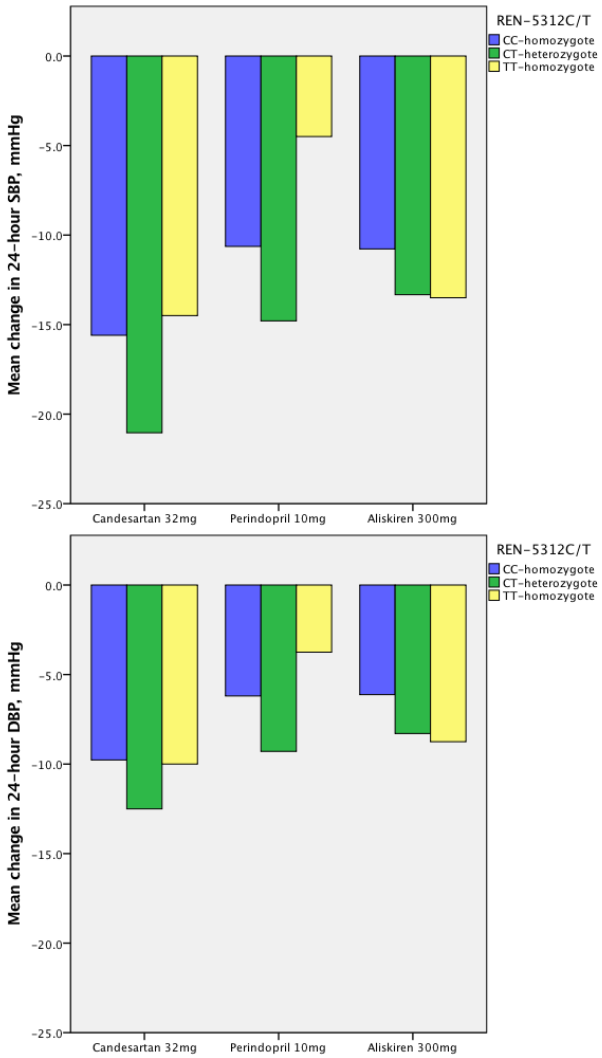


Figure 3.21 – Figure illustrating the relationship between antihypertensive effect of each drug and renin -5312C/T genotype. mmHg, millimetres of Mercury; SBP, systolic blood pressure; DBP, diastolic blood pressure

3.4.7.6 Mean change in 24-hour systolic and diastolic blood pressure with each treatment by low or high baseline blood pressure and genotype

Baseline 24-hour systolic blood pressure measured minimum 128 to maximum 163 mm Hg, and baseline 24-hour diastolic from minimum 71 to maximum 103 mmHg. To stratify the population into two groups of either low or high baseline blood pressure, the median was calculated and the study population divided into two groups for both 24-hour systolic and diastolic blood pressure. Those below the median were considered “low” baseline blood pressure, and those above considered “high” baseline blood pressure (Figure 3.22).

For 24-hour systolic blood pressure, CT-heterozygotes and TT-homozygotes with high baseline BP levels received greater mean blood pressure lowering when compared to CC-homozygotes (Figure 3.23). In Model 3, TT-homozygotes were observed to have a greater reduction in 24-hour systolic blood pressure at -1.56 mmHg greater than the average for every 1mm Hg increase in baseline blood pressure. CT-heterozygotes achieved a -0.62 mmHg greater reduction and CC-homozygotes a -0.46 mmHg greater reduction for every 1 mmHg increase in baseline blood pressure (model 3). This indicated that blood pressure reduction by genotype was greatest in TT-homozygotes with higher baseline BP values, followed by CT-heterozygotes and least in CC-homozygotes with higher baseline BP values.

For 24-hour diastolic blood pressure, similar patterns were observed for those with higher baseline BP values (Figure 3.24 overleaf, lower panel).

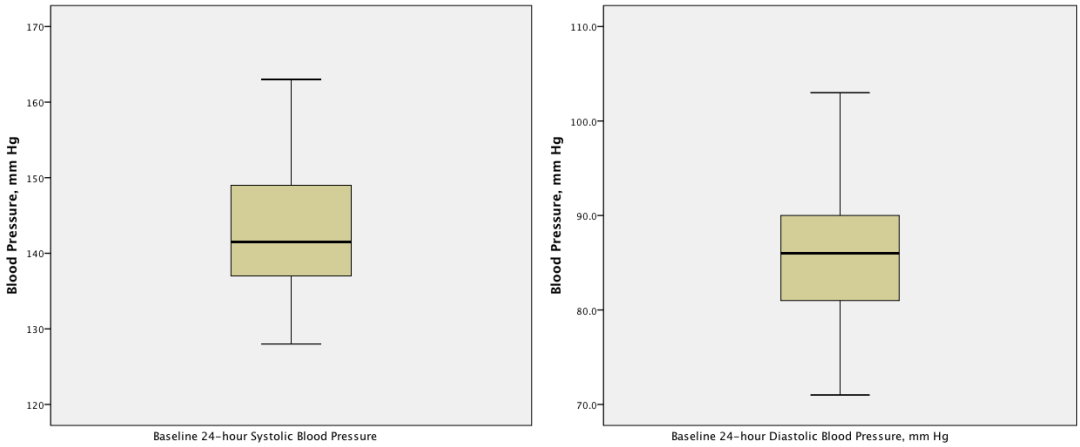


Figure 3.22 – Boxplots of baseline 24-hour systolic and diastolic blood pressures measured in the study population. Maximum, 3rd quartile, median, 1st quartile and minimum are represented. mmHg, millimetres of Mercury.

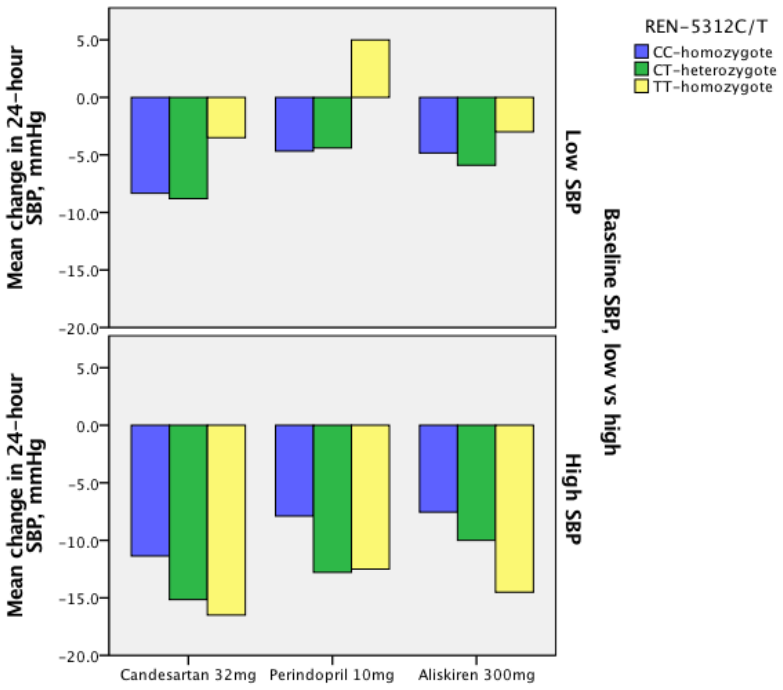


Figure 3.23 - **Figure illustrating mean change in 24-hour systolic blood pressure with each treatment by low or high baseline blood pressure and genotype.** TT-homozygotes with high baseline SBP levels achieved greater blood pressure lowering than CC-homozygotes or CT-heterozygotes. mmHg, millimetres of Mercury; SBP, systolic blood pressure; DBP, diastolic blood pressure.

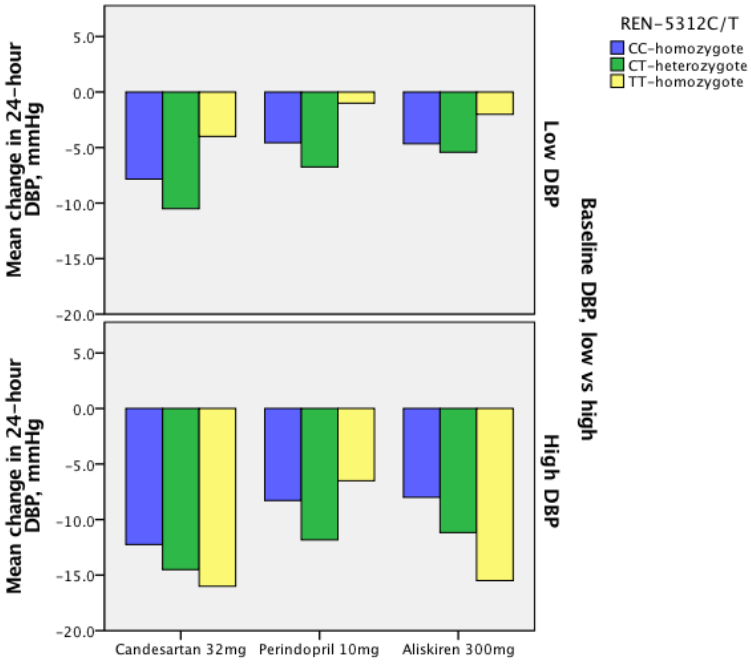


Figure 3.24 - **Figure illustrating mean change in 24-hour diastolic blood pressure with each treatment by low or high baseline blood pressure and renin genotype.** mmHg, millimetres of Mercury; SBP, systolic blood pressure; DBP, diastolic blood pressure.

3.4.7.7 Mean Change in 24-hour Systolic and Diastolic Blood Pressure by REN -5312 C/T Genotype and Plasma Renin Activity

The relationship between renin genotype and Log PRA was evaluated by model 4 (Table 3.8). For systolic blood pressure, log PRA and renin genotype were found to be independent predictors of blood pressure lowering. For diastolic blood pressure, renin genotype and renin genotype*Log PRA interactions were found to be independent predictors of blood pressure lowering.

These complex relationships are illustrated in Figures 3.25 and 3.26. On review of Figure 3.25, low PRA participants achieved greater blood pressure lowering with carriage of the T-allele. Specifically, low PRA TT-homozygotes achieved greater blood pressure lowering than CT-heterozygotes. CC-homozygotes received the least blood pressure lowering. When considering high PRA participants, CT-heterozygotes received greater blood pressure lowering than CC-homozygotes. However, high PRA TT-homozygotes had an increase in blood pressure with perindopril, and achieved less blood pressure lowering with candesartan and aliskiren when compared to the CT-heterozygotes and CC-homozygotes.

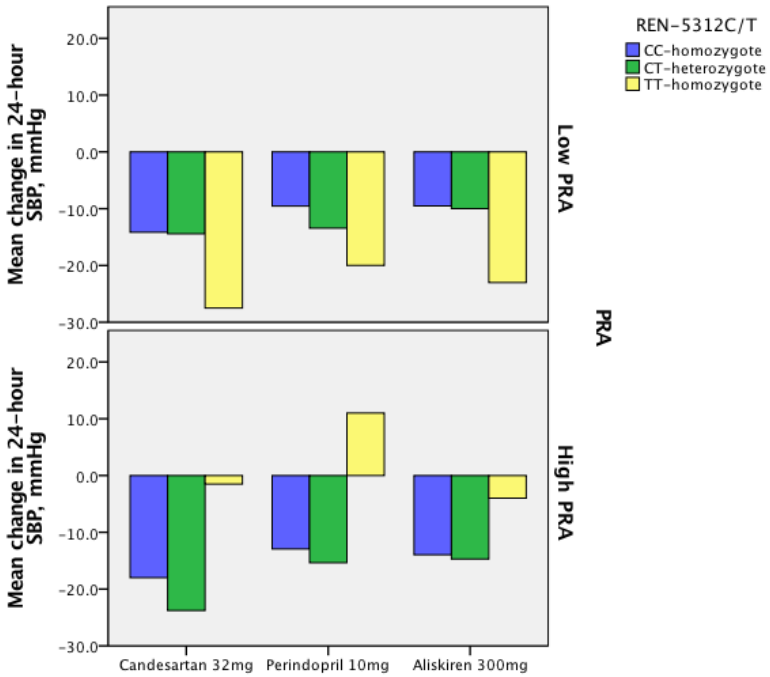


Figure 3.25 – Mean change in 24-hour systolic blood pressure with each blocker of the renin angiotensin system by low or high PRA. Low PRA participants achieved greater blood pressure lowering with carriage of REN-5312T. PRA, plasma renin activity, CC, CC-homozygotes, renin -5312 C/T; CT, CT-heterozygotes, renin -5312 C/T; TT, TT-homozygotes, renin -5312 C/T; PRA, plasma renin activity.

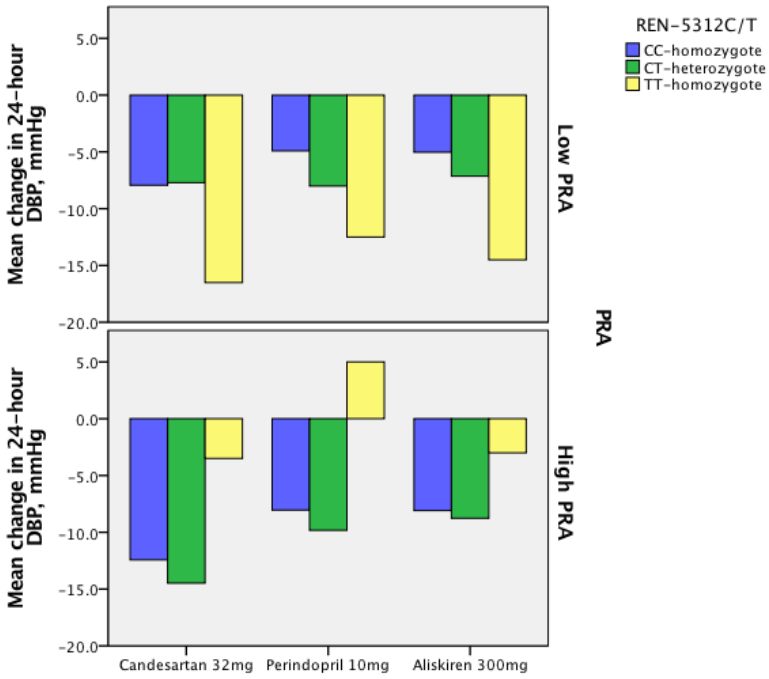


Figure 3.26 - Mean change in 24-hour diastolic blood pressure with each blocker of the renin angiotensin system by low or high PRA. Low PRA participants achieved greater blood pressure lowering with carriage of REN-5312T, and this effect was greatest for TT-homozygotes. High PRA CT-heterozygotes achieved greater blood pressure lowering than CC-homozygotes, with less blood pressure lowering observed in TT-homozygotes. CC, CC-homozygotes, renin -5312 C/T; CT, CT-heterozygotes, renin -5312 C/T; TT, TT-homozygotes, renin -5312 C/T; PRA, plasma renin activity**.**

3.4.8.1 Effect of REN-5312C/T genotype on response to amlodipine monotherapy

10 participants received dual-therapy with the calcium channel blocker amlodipine and each of the three blockers of the renin-angiotensin system. In these participants, baseline ambulatory systolic blood pressure measured >160 mmHg and they received amlodipine monotherapy prior to randomization in order to satisfy the criteria for study inclusion. This allowed the study of response to amlodipine monotherapy by genotype in a small population of very hypertensive individuals.

We sought to evaluate whether there may be a potential relationship between non-RAS blockade and blood pressure lowering by genotype. Specifically, if a patient was to have a reduced response to RAS-blockade, would they have a greater response to a calcium channel blocker instead. These data are illustrated by the figure below (Figure 3.27).

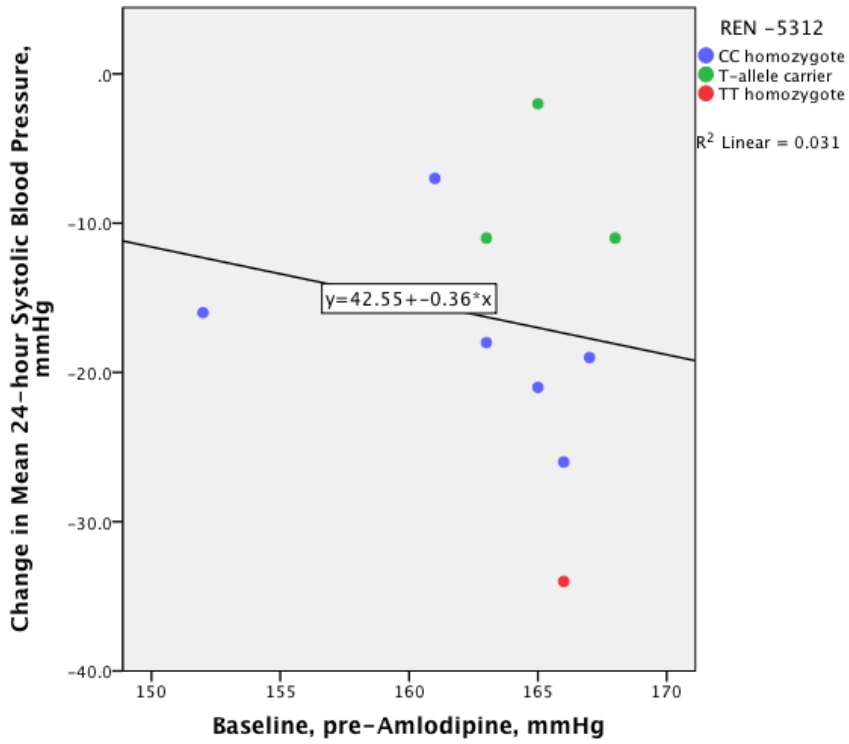


Figure 3.27 – 24-hour systolic blood pressure lowering achieved with amlodipine 10mg monotherapy in ten participants. This graph plots 24-hour systolic blood pressure prior to treatment with amlodipine against the 24-hour systolic blood pressure measurement obtained after treatment with amlodipine 10mg.

Blood pressure lowering was greatest in those with higher baseline blood pressure. CC-homozygotes achieved on average -18 mmHg reduction in 24-

hour systolic blood pressure with amlodipine monotherapy, whilst CT-heterozygotes only achieved -8 mmHg reduction in 24-hour systolic blood pressure. The single TT-homozygote achieved a 24-hour systolic blood pressure reduction of -34 mmHg, but it is difficult to interpret this with only one TT-homozygote participant.

In a longer term and larger study, we would seek to evaluate which of RAS-blockers or calcium channel blockers (CCBs) lower blood pressure to a greater extent for each REN -5312C/T genotype. Theoretically, if a patient is identified as CC-homozygote, the question that needs to be answered is whether they would have maximal gain from commencing a CCB as their main antihypertensive rather than a RAS-blocker.

3.5 Discussion

3.5.1 Results summary

In this study, blood pressure lowering responses were observed to be greater:

- With candesartan treatment compared with aliskiren or perindopril treatments;
- In females compared to males;
- In participants with higher blood pressures;
- In participants with higher PRA levels (supine PRA of ≥ 1ng/mL/hour);
- In REN-5312T allele carriers who also had higher BP levels and/or lower PRA levels (supine PRA of ≤ 1ng/mL/hour).

Additional findings included:

- A novel finding of an association between REN-5312C/T genotype and PRA;
- An association between REN-5312C/T genotype and serum potassium levels;
- A novel finding suggestive of greater blood pressure lowering response with amlodipine monotherapy in REN-5312CC homozygotes versus CT-heterozygotes.

Regarding the dose of the antihypertensives used in this current study, the question has been asked whether or not additional blood pressure lowering would have been achieved with higher doses. Perindopril erbumine has been shown to significantly reduce systolic and diastolic blood pressure in patients with mild to moderate hypertension (P. A. P. Todd & Fitton 1991). There appears to be a flattening of the dose response curve at 8mg (Chrysant et al. 1993). Work published in 1996 determined no significant additional blood pressure lowering when the dose of perindopril erbumine was increased from 8mg to 16mg (Myers 1996). Similar changes in systolic and diastolic pressures were observed during the 12-week study period. The analysis included 260 eligible participants randomized in a parallel group design to either placebo or perindopril up to a maximum dose of 16mg daily. We used a slightly different perindopril salt preparation, perindopril arginine, licensed for use in Ireland at the 10mg maximum daily dose. The newer salt preparation has been used preferentially as it possesses greater stability in higher humidities. The change

in salt has not been shown to affect the efficacy of perindopril as an antihypertensive with similar bioequivalences, and the lipophilic perindopril has favourable tissue penetration (Fox 2007; Fox & Investigators 2003).

As a class, angiotensin receptor blockers (ARBs) are also better tolerated than ACE inhibitors, with cough and rare but potentially life-threatening angio-oedema experienced by individuals taking the latter (Lacourcière & Asmar 1999). ARBs are highly selective for the angiotensin II receptor, AT_1R. Interesting work examining the binding of valsartan, candesartan and losartan suggested differing binding affinities resulting from differences in the degree of their binding interactions with the AT_1R. Candesartan showed high affinity, valsartan moderate affinity and losartan low affinity binding with the receptor through hydrogen bonds (Bhuiyan et al. 2009).

The candesartan affinity was explained by potentially greater number of binding sites available to the drug given its design. In healthy human volunteers the *in vivo* AT_1R blocking effect of candesartan was shown to be approximately twofold greater than that of losartan with longer duration of action (Belz et al. 1997). Such high affinity for the receptor without doubt has an important influence on the potency of candesartan as an antihypertensive. In this work, it is absolutely possible, with such high affinity binding of candesartan to the AT_1R receptor and subsequent slow dissociation, that candesartan is a better antihypertensive drug pharmacologically than perindopril and aliskiren.

Objective evidence for daily doses greater than 32mg daily is lacking. Supra-maximal doses of candesartan have been previously evaluated in proteinuric renal disease, however not formally assessed in terms of hypertension control. In one 2009 study, 269 diabetic patients with proteinuria were randomized to receive 30 weeks of either candesartan 16mg, 64mg or 128mg once daily. The primary endpoint was reduction in proteinuria and the study achieved statistical significance with candesartan 128mg daily ($P<0.0001$) (Burgess et al. 2009). With blood pressure measurement as a secondary endpoint, the authors stated no statistical significance in blood pressure measured across the three treatment groups. As the effect of the higher dosages of candesartan on blood

pressure was a secondary endpoint and not what the study was primarily designed to evaluate this is difficult to interpret.

A significant dose-dependent effect is seen in terms of the effect of aliskiren on systolic and diastolic blood pressure up to the 300mg dose of aliskiren ($P<0.001$) (Gradman et al. 2005). However, studies have shown that there is a negligible increase in antihypertensive effect of aliskiren when doses exceed 300mg and are doubled to 600mg (Gradman et al. 2005). In comparison studies, aliskiren 150mg has been compared to the ARBs irbesartan 150mg and losartan 100mg (Gradman et al. 2005; Stanton et al. 2003). The former study determined the antihypertensive effect of aliskiren 150mg to be comparable to that of irbesartan 150mg and indeed significantly superior when aliskiren was increased to 300mg, the dose of irbesartan remaining at 150mg ($p<0.05$). The latter study demonstrated a comparable blood pressure lowering effect when aliskiren 75mg, 150mg and 300mg were assessed against losartan 100mg (Stanton et al. 2003). An additional factor in terms of dose uptitration to a 600mg dose is that side-effects, in particular gastro-intestinal, are seen to increase when the dose increases from 300mg to 600mg (Weir et al. 2007). Such a significant side-effect would have the potential to impact on quality of life with a subsequent effect on patient compliance.

Gender differences are well acknowledged in both hypertension and cardiovascular disease in general. The way in which the arterial tree ages has been shown to be different between the sexes (Smulyan et al. 2001). Gender differences in various components of the renin-angiotensin system are known to contribute to control of blood pressure, and it is recognised that plasma renin activity is higher in men than women (Kobori et al. 2007; Fischer & Baessler 2002).

In terms of the effect of gender on response to antihypertensive therapy, a 2001 meta-analysis analysed the role of gender differences when controlled-onset extended release verapamil was administered in three prospective, randomized double-blind, placebo-controlled trials. The authors found a greater reduction in 24-hour systolic and diastolic blood pressure for women compared with men ($p<0.001$ 24-hour systolic BP and $p=0.003$ 24-hour diastolic BP) (W. B. White et

al. 2001). A further study evaluated the effect of race and gender in a mixed population of participants between 30 and 59 years of age on response to treatment with hydrochlorthiazide. 225 African Americans and 280 Caucasians were randomized to receive hydrochlorthiazide 25mg once daily. Black race and female gender were both associated with significantly greater systolic and diastolic blood pressure lowering with the diuretic treatment. Women of both races achieved greater systolic and diastolic blood pressure lowering than men, P ≤0.01 for both systolic and blood pressures (Chapman et al. 2002). Further still, the Pharmacogenomic Evaluation of Antihypertensive Responses (PEAR) Study showed that greater systolic and diastolic blood pressure response was seen in females treated with atenolol as monotherapy and as an add-on when compared with response to hydrochlorthiazide (-4.21/0.79 mmHg and -3.73/-2.39 mmHg respectively).

This current study identified the importance of gender in prediction of blood pressure lowering response, with female achieving greater blood pressure than males. It is plausible that significant differences in weight between female and male participants may have contributed to the gender difference observed in this study (Figures 3.14, 3.16). An implication of this may be that drug doses need to be aligned with increase in weight, as higher drug doses may be required for individuals of heavier weight.

Higher baseline blood pressure resulted in greater blood pressure lowering achieved. Baseline blood pressure is a known predictor of response to antihypertensive treatment (Campo et al. 2002). In a study of 225 subjects, a significant correlation was observed between the initial blood pressure and change in blood pressure achieved (Sumner et al. 1988). Patients received either placebo or one of an ACE inhibitor, calcium antagonist, direct vasodilator, α-adrenoceptor blocker or β–adrenoceptor blocker. The change in blood pressure with all treatments including placebo was significantly correlated with initial blood pressure.

Pre-treatment PRA has been advocated as a guide to treatment in hypertensive patients since the 1970s. Patients with higher baseline PRA should theoretically have a correspondingly greater blood pressure reduction when treated with a

RAS blocker. This is reflected in how we manage blood pressure according to age, knowing that younger individuals have more active renin angiotensin systems than older individuals, advancing age associated with a decline in PRA. As such, our first-line drugs in the management of hypertension in individuals in particular under the age of 55 years are RAS blockers. Drugs anticipated as having less of a blood pressure lowering effect in terms of RAS blockade such as amlodipine and diuretics are held in reserve as add-on treatments in those under the age of 55 years. Conversely, hypertensive patients with low pre-treatment PRA will benefit mainly from amlodipine and diuretics, versus specific RAS blockers.

A previous study performed in over 1000 hypertensive individuals assessed baseline PRA and then response to one of six antihypertensive drug classes or placebo. A borderline contribution was seen to the prediction of blood pressure response by PRA, P=0.05 (Preston et al. 1998). Later work performed by Canzanello and colleagues evaluated the validity of measuring pre-treatment PRA in a population of African-American and non-Hispanic white subjects (Canzanello et al. 2008). A higher PRA, >0.65 ng/mL/hr, at the time of enrolment contributed to predicting a greater antihypertensive response to candesartan 32mg daily. After adjusting for other variables, higher PRA was associated with a doubling of the odds of achieving optimum systolic and diastolic BP control with candesartan 32mg. This was most notable in those individuals with higher baseline blood pressure.

In this current study, a threshold of 1ng/mL/hr was assigned to PRA. Above this level patients were considered to have a status of high PRA, and below considered low PRA. Log PRA was consistently statistically significant across all measures of blood pressure as an independent predictor of blood pressure lowering response. We confirmed that high baseline PRA was associated with greater blood pressure lowering.

An interaction was identified between REN-5312C/T genotype and PRA in those with higher baseline blood pressure. These results suggest that this interaction effect may be relevant in guiding antihypertensive therapy. This interaction will potentially allow for stratification of patients into those who will

obtain greater blood pressure lowering with RAS blockade. The greatest advantage of genotyping for REN-5312C/T in the future will likely be for moderate to severe hypertensive individuals with low PRA. We would expect that CT-heterozygotes and CC-homozygotes with moderate to severe hypertension and low PRA will achieve greater blood pressure lowering with RAS blockade, particularly with potent angiotensin receptor blockers such as candesartan. The mechanism for the interaction effect between PRA and the renin genotype is unclear, but perhaps related to varying levels of tissue or circulating RAS components.

We acknowledge the limited predictive effect of one SNP on blood pressure. The interaction observed here could therefore in future constitute one of a wider panel of SNPs tested to determine the most appropriate antihypertensive therapy for an individual. This is of relevance as we continue to move forward in an era of personalised medicine, with less trial and error approach applied and a focus on the “right treatment, for the right person, at the right time” (Scholz 2015).

In a previous study performed by our group, 257 individuals were recruited to a randomised, double-blind, active-comparator, parallel group blood pressure lowering trial comparing response to aliskiren or losartan by REN-5312C/T genotype (Moore et al. 2007). In their study, T-allele carriers on losartan achieved greater blood pressure lowering than CC-homozygotes. Their results are confirmed in this current work, where we have demonstrated greater blood pressure lowering with candesartan over both aliskiren and perindopril. In addition, in this present study, a novel finding was also observed where REN-5312C/T genotype was seen to influence supine PRA (p=0.03). The mechanism by which the SNP did this is unclear, but supine rather than upright PRA is acknowledged to be a more accurate measure of circulating renin when sampling (Lonati et al. 2014).

Significantly higher baseline potassium levels were observed in TT-homozygotes (mean 4.4 mmol/L) when compared with CC-homozygotes and CT-heterozygotes (mean 4.0 mmol/L) (p=0.02, Table3.4). Importantly however, the results observed were still within normal range (3.5 – 5 mmol/L), and so the

clinical significance of this is not clear, warranting further evaluation. There was no convincing evidence of renal impairment in TT-homozygotes, and the mechanism for this is unclear. Possible theories include an effect of REN-5312C/T on kidney sodium and potassium co-transporters in the thick ascending limb of the loop of Henle (Na^+-K^+-$2CL^-$), or an effect on serum aldosterone levels. No significant differences in serum sodium or chloride were observed amongst the genotypes however. It could be considered to measure aldosterone alongside renin in a future study to explore this further.

It was observed in a small hypertensive population in this study that treatment with amlodipine monotherapy resulted in greater blood pressure lowering response for CC-homozygotes (n=6) over CT-heterozygotes (n=3). CC-homozygotes achieved the least blood pressure lowering response with RAS blockade. Therefore, this finding warrants further study, as CC-homozygotes may gain better blood pressure lowering with calcium channel blockade rather than RAS blockade. A larger study would also allow further information on the TT-homozygotes within the population in terms of their response. A study evaluating this response to amlodipine monotherapy in the context of low or high PRA status should be considered.

A significant limitation was that only 4 TT-homozygotes were identified in the study population (n=98). Only one of these was exposed to amlodipine monotherapy prior to the initiation of RAS blockade. The responses observed for 24-hour systolic blood pressure in TT-homozygotes were quite different when compared to CT-heterozygotes and CC-homozygotes. In particular, high PRA TT-homozygotes were observed to have little reduction in 24-hour systolic blood pressure with RAS blockade, and in fact an apparent increase in blood pressure was observed with the ACE inhibitor perindopril for this group. In addition, the one TT-homozygote had greater blood pressure lowering than CC-homozygote and CT-heterozygotes, with a -34 mmHg reduction in 24-hour systolic blood pressure achieved. The study would need to be replicated in a population with a large number of TT-homozygotes in order to be able to draw any meaningful conclusion from either of these observations.

The study was not double-blind, and open label medication was used. However, there was only one clinical observer during the time of the study, which helped to limit any significant effect of observer bias. As a result, study subjects underwent uniform measurement of blood pressures clinically and by 24-hour ABPM with the same routinely calibrated machines and same person assessing them.

3.6 Summary and Conclusions

The data from this pharmacogenetic clinical trial observed that blood pressure lowering responses were greater:

- With candesartan treatment compared with aliskiren or perindopril treatments;
- In females compared to males;
- In participants with higher blood pressures;
- In participants with higher PRA levels;
- In REN-5312T allele carriers who also had higher BP levels and/or lower PRA levels.

Additional findings included an association between REN-5312C/T genotype and PRA; an association between REN-5312C/T genotype and serum potassium levels, and greater blood pressure lowering response with amlodipine monotherapy in REN-5312CC homozygotes versus CT-heterozygotes.

The findings illustrate the potential of REN-5312C/T and PRA in determining optimal antihypertensive treatment for individual patients. It also suggests that REN-5312C/T may have a role in determining those individuals in whom treatment with amlodipine monotherapy would have greater antihypertensive effect than RAS blockade.

The tissue RAS plays an important role in target organ damage sustained as a result of hypertensive disease and diabetes, among other conditions (Bader et al. 2008; Dzau et al. 2001; Skov et al. 2014). It would be useful to define the relationship between carriage of specific renin or (P)RR SNPs with varying degrees of tissue RAS activity. Defining such a relationship would have the potential to allow those to be identified earlier that may benefit from use of RAS blockade in their chronic disease management.

Genetics is undoubtedly an important factor in the development of hypertension. To date, no one individual SNP has been identified by GWAS as having significant blood pressure effects. A number of polymorphisms in the future may form a panel allowing genetic scoring of individuals in terms of risk of

end organ damage. Carriage of specific polymorphisms may also aid in making recommendations for drugs with the greatest antihypertensive effect. Determining which RAS blocker gives the maximal reduction in tissue RAS activity will be important in an attempt to limit end-organ damage related to RAS activity. It would be our conclusion that genotyping could be used in combination with other parameters such as plasma renin activity to help guide choice of antihypertensive agent.

REN-5312C/T may yet become a genotype used in practice to identify patients who will gain maximal benefit from RAS blockade both in terms of blood pressure lowering and protection from end-organ damage. It is doubtful that the REN-5312C/T genotype alone will be an independent predictor of antihypertensive response. It is much more likely that REN-5312C/T will direct treatment as one of a panel of genes identified as having an impact on prediction of response to antihypertensive therapy.

Chapter 4 – Hypertension Genetics and REN-5312C/T: Future Considerations

4.1 Hypertension as an important cardiovascular risk factor

Cardiovascular disease is a major cause of morbidity and mortality world-wide, with hypertension an important risk factor for premature cardiovascular disease. Hypertension was identified as the most important risk factor for stroke in two recent publications. Findings from the INTERSTROKE Study and the Global Burden of Disease Study 2013 (GBD 2013) support the fact that a significant proportion of stroke results from hypertension (O'Donnell et al. 2016; Feigin et al. 2016). INTERSTROKE, an international case-control study in 32 countries, recruited 26,919 participants with blood pressure >140/90 mmHg. They attributed 47.9% of stroke to hypertension in their population, identifying hypertension as a significant risk factor for haemorrhagic over ischaemic stroke. In GBD 2013, stroke burden was evaluated as stroke-related disability-adjusted life years (DALYS) in 188 countries between 1990 and 2013, in those with systolic blood pressure >120 mmHg. They attributed 64.1% of stroke to hypertension. Hypertension is also associated with an increased lifetime risk of coronary heart disease, for both men and women (Turin et al. 2016). Now, as the World Health Organisation pledges to focus on hypertension as one of the major nine non-communicable diseases, hypertension as a disease entity and its management continues to gain importance (World Health Organization 2011; World Health Organization 2013).

4.2 Future renin expression studies

In Chapter 2, we observed greater renin mRNA expression in REN-5312T allele carriers in normal cadaveric human adrenal glands. A future study could be undertaken to evaluate the REN-5312C/T polymorphism and other renin gene polymorphisms in healthy tissue donors. This would be valuable in improving our understanding of the functionality of REN-5312C/T and other renin gene polymorphisms in key tissue-based renin-angiotensin systems (RAS). There would also be the opportunity to collect detailed phenotypic information in living tissue donors. This could allow for greater interpretation and understanding of the effect of REN-5312C/T and renin gene polymorphisms in tissue. A study could be designed to evaluate relationships between renin genotype and blood pressure, serum levels of biochemical markers such as circulating plasma renin

activity or aldosterone and tissue RAS activity through measurement of RAS component mRNA expression levels. In identifying patients with a potential for greater tissue RAS activity, earlier or more potent RAS-blockade could be recommended to limit the potential for end-organ damage.

4.3 Hypertension Genetics

It is acknowledged that genetic factors contribute 30% to 50% to blood pressure variation among individuals (Kunes & Zicha 2009). Candidate gene studies, genome-wide linkage analysis and association studies have allowed for the identification of several genetic loci of interest (X. Wang et al. 2011). The search is still ongoing, and at this time approximately 100 genetic variants are thought to contribute to hypertension.

In a recent publication, 30 new hypertension associated single nucleotide variants (SNVs) were identified (Surendran et al. 2016). The majority of these were found to have small effects on blood pressure. Three of these however were associated with larger effects, >1.5 mmHg/allele. The first of these, *RBM47,* is a protein-coding gene responsible for modifying RNA. Next, *RRAS* encodes a protein involved in processes including angiogenesis and vascular homeostasis (GeneCards WebRef). It has been implicated in the autosomal dominant Noonan's syndrome, whose clinical features include heart abnormalities. Finally, *COL21A1* encodes Type XXI collagen. Type XXI collagen is a component of blood vessel walls, and is found in many tissues including the heart and aorta (Chou & H.-C. Li 2002).

A second recent publication performed genotyping of 128,272 SNPs in 201,529 individuals of European ancestry (Ehret et al. 2016). These were subsequently validated in a further 140,886 individuals. 66 SNPs for systolic and diastolic blood pressure were identified, and 17 of these were new. The variants were observed to have similar effects on blood pressure in South Asians, East Asians and African. This effect was observed in smaller sample sizes. The group also determined a 66-SNP risk score that was found to significantly predict target organ damage in the heart (coronary artery disease risk, left ventricular wall thickness), cerebral vessels (stroke), carotid artery (carotid intima medial

thickness) and eye (central retinal artery caliber). The score significantly predicted urinary albumin/creatinine ratio, but not eGFR, creatinine, microalbuminuria or chronic kidney disease.

It is noteworthy and unfortunate that the REN-5312C/T polymorphism is not included on either of the chips used in these two studies (Exome chip and Cardio-MetaboChip respectively). Hence additional genotyping of REN-5312C/T genetic variant in these populations would be of interest.
More modern sequencing technologies including next–generation sequencing allow for quicker and cheaper DNA and RNA sequencing than Sanger sequencing. An entire genome can be sequenced in one day using next-generation sequencing. In the future, this will significantly and positively affect the ability to screen for causative genes in essential hypertension (Behjati & Tarpey 2013). Data from future sequencing could be merged with studies that have been completed encouraging collaboration between Investigators, private and public healthcare and private industry (Horani et al. 2015). Such “big data” analysis could allow for identification of novel causative genes.

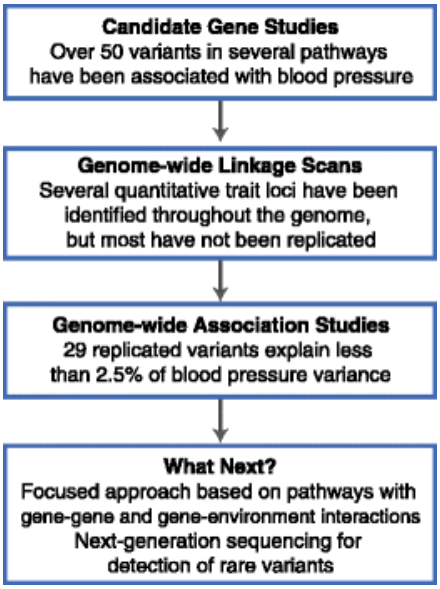


Figure 4.1 - From candidate genes to linkage to genome-wide association studies: What next for hypertension genetics? Reproduced from Basson et al., 2012.

4.4 Hypertension Pharmacogenetics

In Chapter 3 we observed greater blood pressure lowering with RAS-blockade in REN-5312T allele carriers with low PRA and high baseline blood pressure. An exploratory result observed greater blood pressure lowering with amlodipine monotherapy in REN-5312C/T CC-homozygotes than CT-heterozygotes. A larger clinical study comparing RAS-blockade with calcium channel blockers by REN-5312C/T genotype would be valuable. Such a study could help to determine whether or not REN-5312C/T may guide antihypertensive therapy by identifying those in whom one drug class yields greater blood pressure lowering than the other.

In a future clinical study, integrating a dose-escalation with measurement of on-treatment PRA could be considered. Not all patients follow the same pattern of blood pressure lowering with any one particular drug with inter-individual variation in responses seen. Studying the effect of lower and higher doses of each of the renin-angiotensin system blockers on blood pressure and PRA in a dose-escalating fashion may give additional information as to what is happening at each level of drug dose by REN-5312C/T genotype and PRA. In order to quantify and remove placebo effect, it could be considered to have a placebo arm. Ambulatory blood pressure monitoring does reduce the placebo effect, not as effectively however as a placebo arm. Care would need to be taken in establishing appropriate clinical cut-offs for study inclusion with a study including a placebo arm in its design.

Studying the effects of other RAS genes and genes considered of interest currently in hypertension in this population would be worthwhile, with the potential to result in valuable information in a new population. Most genetic variants are responsible for small changes in blood pressure. Therefore it is likely that an array or panel of SNPs when analysed together in a particular patient will predict target organ damage associated with hypertension. The score outlined above is one example. In addition, an array or panel of SNPs including REN-5312C/T when analysed together will be much more likely to assist with choice of optimal antihypertensive agent as opposed to a single genetic variant.

4.5 Dual RAS blockade, renin genotype and blood pressure lowering

We have discussed that binding of (pro)renin to the (P)RR allows for the direct activation of (pro)renin through conformational change. Binding to the (P)RR also initiates a cascade of intracellular signalling events that are thought to contribute to profibrotic and proliferative actions independent of angiotensin II generation. This is referred to as non-proteolytic activation. The “handle-region peptide” is a peptide derived from the prosegment of prorenin. It functions as an inhibitor of the (P)RR through blockade of non-proteolytic activation of prorenin.

Handle-region peptide, HRP, was administered subcutaneously to a streptozotocin-induced diabetic rat population for a 24-week treatment period (Ichihara et al. 2004). Renal tissue angiotensin I and II were reduced, and progression to diabetic nephropathy was inhibited. HRP did not affect renal tissue renin protein or renin mRNA expression in diabetic or control rats. In addition, HRP did not affect the already reduced circulating renin levels in the diabetic rats compared to controls, and circulating prorenin levels remained unaltered. It was suggested that the HRP affected tissue-based RAS, but not circulating RAS activity. Through inhibition of the non-proteolytic activation of prorenin at the (pro)renin receptor, nephropathy was avoided.

Dual-blockade of the renin angiotensin system with angiotensin converting enzyme inhibitors (ACEi) and angiotensin receptor blockers (ARBs) initially showed great promise. Reduction in proteinuria and prevention of structural lesions were observed in experimental diabetic and non-diabetic chronic kidney disease (Gentile et al. 2015). Unfortunately, dual-blockade of the renin angiotensin system in individuals of increased vascular risk with minimal proteinuria did not benefit renal function loss when compared with losartan monotherapy (ONTARGET Investigators Yusuf et al. 2008). In addition, the study of aliskiren in combination with either placebo or an ACEi or ARB was terminated early because of an excess of non-fatal strokes (Parving et al. 2009).

Perhaps then dual-blockade could be achieved instead by blocking the hormonal renin angiotensin system with a single RAS-blocker, in combination with an agent such as a HRP. In addition to RAS-blockade, the HRP would inhibit the intracellular signaling that occurs on (pro)renin receptor activation.

Unfortunately, HRP in combination with the direct renin inhibitor has not shown increased benefit in terms of renin-angiotensin system blockade from a vascular function viewpoint when used in diabetic rats (Batenburg et al. 2013). The same group also found that the use of aliskiren on top of HRP did not provide additional protection against retinal damage in diabetic rats (Batenburg et al. 2014). Thus the effects of HRP in combination with an ACEi or ARB could be studied.

Renin genotype may have a role to play in influencing the selection of patients who would be deemed to have the greatest benefit from dual blockade of the RAS and (pro)renin receptor activated intracellular pathways. A renin gene polymorphism has been reported as contributing to plasma prorenin levels in individuals with diabetic nephropathy (Deinum, Tarnow, et al. 1999; Deinum et al. 1999). A *bgl*I restriction fragment length polymorphism was identified in the renin gene in individuals with diabetic nephropathy. In bb-homozygotes a trend was identified for higher prorenin levels, p=0.07. Thus, renin gene polymorphisms may be affecting plasma prorenin levels. Studying renin gene polymorphisms in the context of plasma renin and prorenin status would be of interest in determining the relationship to target organ damage in diseases such as hypertension. This may then identify individuals who would gain from RAS-blockade and HRP.

4.6 Hypertension Epigenetics

Environmental factors are also important. Epigenetic regulation of DNA is thought in part to result from environment-gene interactions (Millis 2011). Epigenetics is broadly thought of as the modification of gene expression rather than the alteration of genetic code (J. Wang et al. 2015). The result of this is a change in phenotype without a change in genotype. Epigenetics is increasingly regarded as having a role in the pathogenesis of essential hypertension. Epigenetic alterations may also affect response to antihypertensives, and may need to be considered in large clinical trials.

3 biochemical processes can epigenetically modify DNA (Wise & Charchar 2016). These are outlined in brief:

1. DNA methylation – methyl group binding to the 5' carbon of the cytosine ring. This can result in the inhibition of gene transcription, particularly if binding occurs in the gene promoter region. Conversely, this may promote gene transcription if located at gene exon sites. Aberrant methylation has been observed in angiotensin Ia receptors *(Atgr1a)* in spontaneously hypertensive rats (SHR) when compared to the control Wistar-Kyoto rat (WKR) (Pei et al. 2015). These changes were seen in aortic and mesenteric artery endothelial cells, with significantly increased expression of *Atgr1a* by week 20 in the SHR. This occurred as a result of progressive aberrant methylation with age in the *Atgr1a* promoter region in the SHR compared to the WKR. It was suggested by the authors that increased expression of *Atgr1a* in the SHR as a result of aberrant methylation in the promoter may be responsible in part for the hypertension observed in the rat model.
2. Post-translational histone modifications – post-translational N-terminal tail modification that results in changes to dynamics of chromatin. There are potentially 60 modifications that can occur. For example, histone tail acetylation relaxes the structure of chromatin, allowing access of transcription factors to DNA. Histone tail methylation however results in gene silencing. Riviere et al. observed greater H3 hyperacetylation (H3Ac) and tri-methylation at lysine 4 (H3K4me3) in the *Ace1* promoter regions of spontaneously hypertensive rat (SHR) tissues compared to Wistar-Kyoto rats (WKR) (Riviere et al. 2011). Higher tissue expression levels of Ace1 mRNA protein were observed in the same SHR tissue when compared to WKR controls.
3. RNA-based mechanism involving non-coding microRNAs – single stranded segments of non-coding RNA or microRNA bind to mRNA forming double-stranded RNA complexes. microRNAs (miRNAs) influence mRNA degradation or can repress mRNA translation. Two miRNAs, miR181a and miR663, are reported to regulate renin gene expression (Friso et al. 2015).

The effects of epigenetic changes in renin-angiotensin system (RAS) genes have been evaluated in animal models of hypertension. For example, maternal protein malnutrition during pregnancy is known to affect renin-angiotensin

system development in the foetus. Studies in several species suggest that a 50% dietary protein reduction during the antenatal period may contribute to diseases such as hypertension and behavioural abnormalities later in life (Goyal et al. 2010).

Goyal et al. sought to evaluate the potential epigenetic impact of maternal protein deficiency on brain tissue RAS in mice. A 50% antenatal maternal low protein diet resulted in increased mRNA expression of angiotensinogen and ACE 1, and reduced mRNA expression of angiotensin II type-2 (AT2) receptors. In addition, protein levels of ACE1 and AT2 receptor genes were significantly reduced in foetal brain tissue. Aberrant methylation was demonstrated in the promoter region of ACE1 gene, with upregulation of miRNAs involved in the regulation of ACE1 mRNA translation. Reduced expression of a further miRNA thought to regulate AT2 translation, mmu-mir-330, was also observed. The brain RAS is functionally important in the regulation of blood pressure, hence changes in brain RAS have the potential to contribute to hypertension. The same group also demonstrated changes in the expression of systemic RAS in the same offspring of maternal protein restricted mice. Alterations in expression of various components of the RAS were observed in the liver, kidneys, lungs and heart. These changes are illustrated overleaf (Figure 4.1).

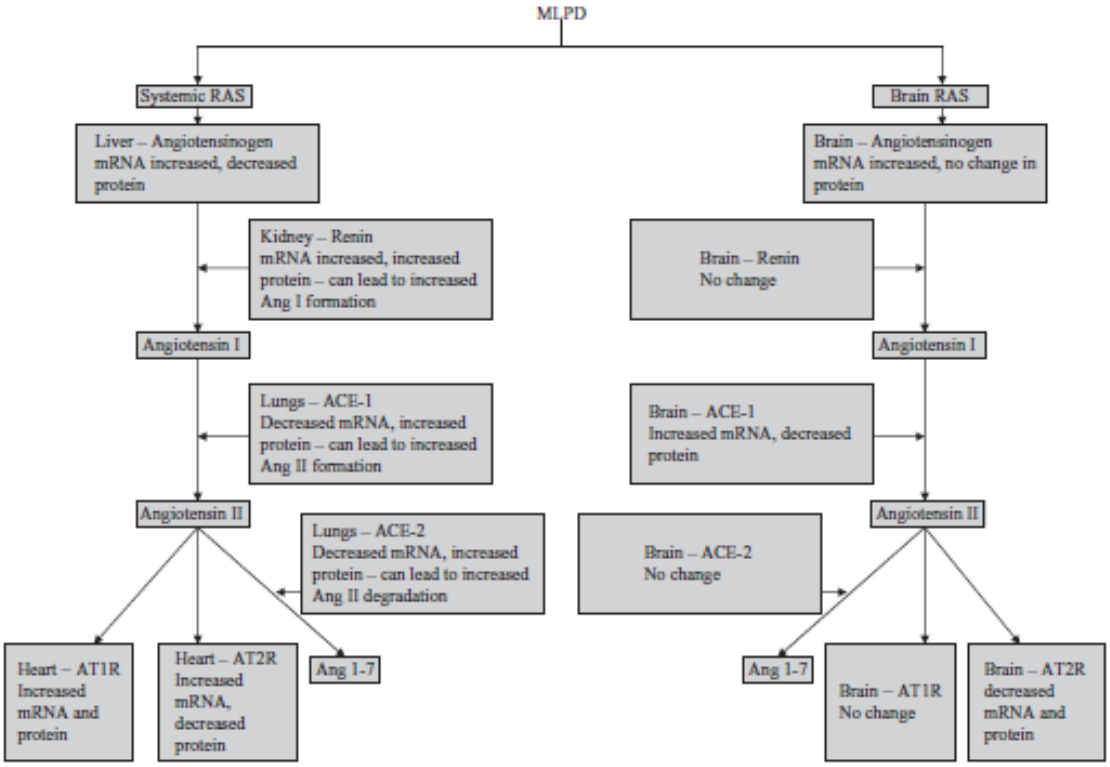


Figure 4.1 – Comparison of the effects of maternal low-protein diet on the systemic and brain renin angiotensin systems (RAS) of mice off-spring. Reproduced from **(Goyal et al. 2010)**.

The study of epigenetics has the potential to allow us to understand the effect of the gene-environment interaction on essential hypertension, and on response to antihypertensive agents.

4.7 Conclusion

Hypertension is a significant risk factor for cardiovascular disease. Pharmacogenetics of hypertension remains an area of significant interest. The identification of the genes that contribute to the significant inter individual differences in response to treatment has been slow. Determining the effect of various RAS gene polymorphisms may assist us in understanding inter individual differences in response to RAS blockade. Genetic influences on circulating RAS components and tissue RAS components may be different however, and need to be studied further. Progress in the future will depend upon our ability to undertake large studies using high-fidelity phenotyping with multiple drugs and multiple ethnic groups utilising advanced bioinformatics and novel genetic techniques. Genetic information will likely be evaluated alongside

biochemical markers, such as PRA, in order to select an appropriate antihypertensive regime.

REN-5312C/T may yet become a genotype used in practice to identify patients who will gain maximal benefit from RAS blockade both in terms of blood pressure lowering and protection from end-organ damage. It is doubtful that the REN-5312C/T genotype alone will be an independent predictor of antihypertensive response. It is much more likely that REN-5312C/T will direct treatment as one of a panel of genes identified as having an impact on prediction of response to antihypertensive therapy.

When a panel of genes with effect on antihypertensive treatment response is identified, further statistical modelling will be required to validate such a panel in addition to traditional predictors of blood pressure lowering response. Such modelling could include the assessment of net reclassification improvement (NRI), or integrated discrimination improvement (IDI) (Pencina et al., 2008). NRI and IDI tables provide an important increase in the power to detect an improvement in risk stratification associated with the use of a new biomarker.

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